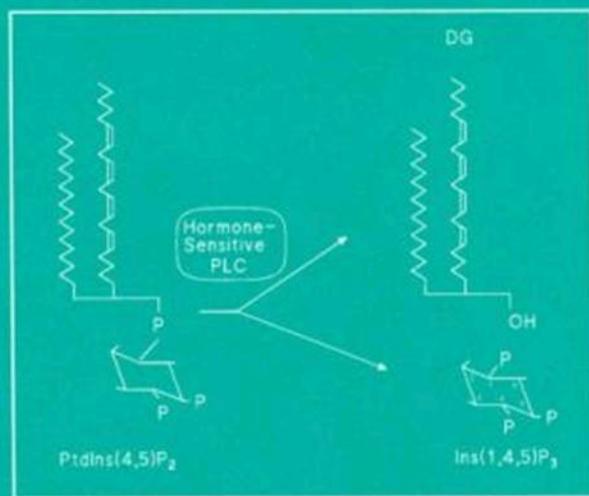


Phospholipid Signaling Protocols

Edited by
Ian M. Bird



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Phosphoinositidase C Activation Assay I

Cell Labeling, Stimulation, and Recovery of Cellular [3 H]Phosphoinositides and [3 H]Phosphoinositols

Ian M. Bird

1. Introduction

1.1. Background

The minor inositol-containing membrane phospholipids, the phosphoinositides, play a central role in cell signal transduction. Activation of a hormone-sensitive phospholipase C (phosphoinositidase C) at the plasma membrane results in the rapid catabolism of the polyphosphoinositides to form the two second messengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃), a water soluble phosphoinositol that promotes the release of Ca²⁺ from intracellular stores, and diacylglycerol (DG), which remains in the plasma membrane and activates protein kinase C (1-3). The metabolic pathways involved in the synthesis of phosphatidylinositol 4,5-bisphosphate, and the metabolic fate of the DG and Ins(1,4,5)P₃ formed on activation of phosphoinositidase C, are summarized in Fig 1.

1.2. Experimental Strategy

Hormone stimulation of phosphoinositidase C causes a rapid (within seconds) loss of PIP₂ and PIP, but slower loss of PI, together with a correspondingly rapid (within seconds) formation of IP₃ and IP₂ (and possibly IP₄), but delayed rise in IP₁. A complication in monitoring changes in the phosphoinositides alone is the ability of cells to resynthesize PI rapidly, and therefore PIP and PIP₂ (see Fig. 1). However, inositol monophosphate phosphatases are inhibited by Li⁺; thus if cells are preincubated in medium containing 10 mM

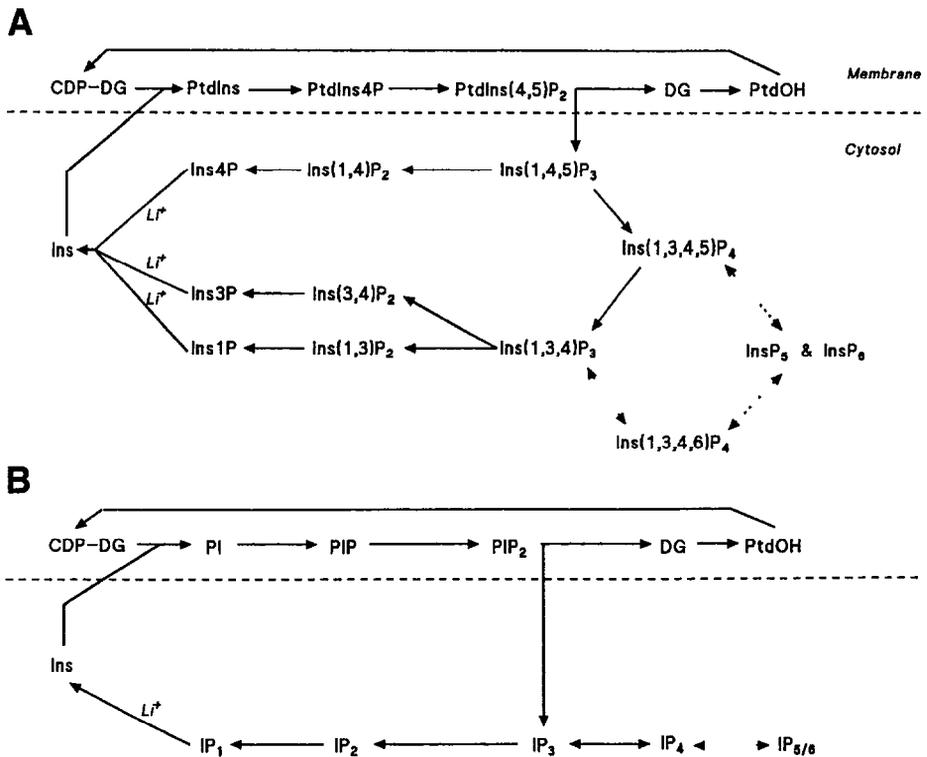


Fig. 1. Metabolic pathways activated as a consequence of phosphoinositidase C action (A) Major metabolic pathways activated by phosphoinositidase C action on PtdIns(4,5)P₂ are shown with solid arrows. Some of the additional pathways that may be activated are shown by broken arrows. Abbreviations: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; DG, diacylglycerol, PtdOH, phosphatidic acid; CDP-DG, CDP-diacylglycerol; Ins, Inositol. For phosphoinositols, abbreviations are in the form Ins(x,y,z)P_n, where x, y, and z refer to the positions of the phosphate groups on the myo-inositol ring and n refers to the total number of phosphates (B) A simplified outline of the metabolic pathways in (A) also showing alternative abbreviations. PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP₂, phosphatidylinositol bis-phosphate; DG, PtdOH, CDP-DG and Ins as above. Phosphoinositols are referred to as IP_n, where n refers to the number of phosphates on the inositol ring. In both panels, sites of Li⁺ inhibition are also shown.

LiCl, the water-soluble phosphoinositol products can accumulate over a longer stimulation time (minutes), predominantly in the form of IP₁ and IP₂. Such accumulation is a highly sensitive indicator of phosphoinositidase C activation.

1.2.1. Cell Prelabeling

Phosphoinositides (with the exception of PI) and phosphoinositols in the small numbers of cells usually available are barely detectable by conventional means. Therefore most studies use radiolabels for "quantification." Radio-labeled glycerol or fatty acids label all phospholipids, including phosphoinositides, but not phosphoinositols; ^{32}P , on the other hand, labels not only all phospholipids and phosphoinositols, but also nucleotide and sugar phosphates. An alternative and widely used approach is to prelabel cells with *myo*-[^3H]inositol. Both phosphoinositides and phosphoinositols become labeled so all metabolites can be monitored and, since *myo*-inositol is not rapidly metabolized through other pathways, a labeled product indicates an inositol-based structure. The only disadvantage is that it takes several days to label phosphoinositides to isotopic equilibrium, or at least a steady state; only under these conditions can changes in radioactivity be interpreted as changes in mass. Nevertheless, detection of phosphoinositidase C activation by increased formation of phosphoinositols can be successful with prelabeling for several hours. However, the attendant problems of increased phosphoinositide labeling due to increased specific activity during stimulation and the nonlinear increase in labeling of phosphoinositols that results means that long-term labeling is the method of choice.

1.2.2. Cell Stimulation Conditions

The Li^+ block technique requires preincubation of cells in a physiological medium containing Li^+ for at least 15 min prior to stimulation, and Li^+ should remain present for the stimulation period. It is also preferable to use medium free of any pH indicators, since phenol red binds to anion exchange resins. The volume of incubation medium should be small (<1 mL if possible), as salts present in the medium are recovered in the final extracts and may interfere with the subsequent chromatographic analysis (*see* Chapters 2–4).

1.2.3. Extraction of Labeled Products from Cells

Three extraction procedures are commonly used for maximum recovery of highly charged radiolabeled products, namely the Bligh and Dyer acidified solvent extraction procedure (4,5), and the perchloric acid (PCA) and trichloroacetic acid (TCA) procedures. An advantage of the Bligh and Dyer procedure (4,5) is that it allows simultaneous and efficient recovery of both phosphoinositols and phosphoinositides. However, if the extraction is to be carried out on plastic culture dishes, or if samples are required for high-performance liquid chromatography (HPLC) analysis, the PCA or TCA extraction methods should be used. In these cases, the phosphoinositides can be recovered from the protein/membrane pellets of PCA (or TCA) lysates by the acidified Bligh and Dyer method (*see* Subheadings 2.3. and 3.3.).

1.2.4. Stability and Storage of Recovered Samples

Products are reasonably resistant to acid degradation, but only when kept at 0–4°C, so all samples should be processed immediately and kept on ice during the extraction procedures. Phosphoinositides in membrane pellets from PCA or TCA precipitation are only stable for several hours at –20°C, because of the presence of residual acid. Provided the aqueous extracts are neutralized, however, they can be stored frozen at –20°C for several weeks. Phosphoinositides extracted using the acidified Bligh and Dyer method can be stored for several hours (overnight) at –20°C, provided they have been dried down (so removing acid) and redissolved in chloroform. To minimize oxidation of the unsaturated fatty acids, samples should be stored in stoppered tubes with a minimum air space above flushed with nitrogen gas. If the phosphoinositides are deacylated (*see* Chapter 2) the neutral glycerophosphoinositol products can be stored frozen at –20°C for several months.

2. Materials

General note: Purchase all solvents to analytical grade. Wear eye protection and use a fume hood when performing extraction procedures. Use standard radioactivity containment and disposal procedures.

2.1. Prelabeling of Cells in Culture

1. *myo*-[³H]Inositol: Aqueous solution (~20 Ci/mmol, 1 mCi/mL, Amersham, Arlington Heights, IL) with anion exchange bead (to adsorb radiolytic degradation products) (*see* Note 1). Store and use under sterile conditions.
2. Cells: Prepare using appropriate conditions for cells, and preferably plate in 12- or 24-well plates at near confluence in growth medium (*see* Note 2).
3. Cell labeling medium: Cell “growth” medium supplemented with 10 μCi/mL *myo*-[³H]inositol (*see* Notes 1 and 2).
4. Sterile tissue culture supplies including pipet tips and 12- or 24-well culture plates.

2.2. Preparation of Labeled Cells for Stimulation

1. M199 (basic physiologic medium or equivalent; *see* Note 3), 0.2% bovine serum albumin (BSA).
2. M199 or equivalent, 0.2% BSA, 10 mM Ins, 10 mM LiCl (*see* Note 8).
3. Agonist stocks prepared to at least 100X conc., and diluted to 10X conc. in M199 or equivalent, 0.2% BSA, 10 mM Ins, 10 mM LiCl (*see* Notes 4 and 8).

2.3. Acidified Bligh and Dyer Extraction

1. Chloroform:methanol:concentrated HCl (CHCl₃:MeOH:HCl), (100:200:1 [v/v/v]).
2. Chloroform.

3. 0.1 M Hydrochloric acid.
4. 1 M Sodium hydroxide.
5. Solvent resistant tubes (5 and 10 mL).
6. Oxygen-free nitrogen gas.
7. Bench centrifuge.
8. Positive displacement or air displacement pipets (*see Note 5*)

2.4. PCA Extraction

1. 10 or 15% (w/v) Perchloric acid, as appropriate (*see Subheading 3.4.*).
2. Distilled water
3. 1,1,2-Trichlorotrifluoroethane (freon):tri-*n*-octylamine (1:1 [v/v]) (*see Note 6*).
4. Microcentrifuge tubes (1.5 mL).
5. Glass tubes (13 × 100 mm).
6. 1-mL Syringe.
7. Microcentrifuge
8. Vortex mixer

2.5. TCA Extraction

1. 10 or 15% (w/v) Trichloroacetic acid, as appropriate (*see Subheading 3.5.*)
2. Distilled water
3. Water saturated diethyl ether.
4. Sodium hydrogen carbonate (100 mM).
5. Microcentrifuge tubes.
6. Glass tubes (13 × 100 mm).
7. 1-mL Syringe.

2.6. Recovery of Phosphoinositides from Acid-Insoluble Pellet

1. Chloroform:methanol:concentrated HCl (CHCl₃:MeOH:HCl), (100:200:1 [v/v/v]).
2. Chloroform.
3. 0.1 M Hydrochloric acid.
4. Distilled water.
5. Solvent resistant tubes.
6. Oxygen-free nitrogen gas
7. Vortex mixer.
8. Bench centrifuge.

3. Methods

Procedures are described for the extraction of 0.5 mL of a cell suspension or for extraction of a cell monolayer. These can be scaled up or down as appropriate. Alternative procedures scaled for extraction of whole tissue are also described in detail in Chapters 4 and 6 (*see also Note 5*).

3.1. Labeling of Cells in Culture

- 1 Cells prepared and plated in 12- or 24-well plates are incubated for 24 h to allow attachment.
- 2 Growth medium is removed and replaced with 0.5 mL of the same medium with added *myo*-[³H]inositol (10 μ Ci/mL). Cells are preferably left to incorporate label for 48 h before use (see Notes 1 and 2).

3.2. Preparation of Labeled Cells for Stimulation

3.2.1. Cells in Culture

1. Remove the labeled medium from each well (to a container in which it can be stored safely for disposal) and wash once and replace with 0.5 mL of M199/BSA. Incubate the cells for 15 min. This washes away extracellular inositol.
2. Remove the medium from each well and replace it with 0.45 mL of M199/BSA with added inositol (unlabeled, 10 mM) and LiCl (10 mM). (Overfill the 1-mL tip and dispense to resistance point only to eliminate large air bubbles in wells.) Incubate the cells for a further 15 min (to allow the cold inositol to enter the cells and start to chase out the labeled inositol, and to allow the Li⁺ to inhibit the inositol phosphate phosphatases).
3. At the end of the 15-min incubation period, make additions as required in a volume of 50 μ L and incubate as required.
4. Terminate stimulation as described in **Subheadings 3.3.–3.6.**)

3.2.2. Cells In Suspension

1. Label *en mass* as for plated cells (i.e., 10 μ Ci/mL medium).
2. Spin cells at 400g for 5 min and resuspend in M199/BSA. Incubate for 15 min.
3. Spin as in **step 2** and resuspend in M199/BSA/LiCl/Ins
4. Spin as in **step 2** and resuspend in M199/BSA/LiCl/Ins at a density of 200,000–250,000 cells per 0.45 mL. Dispense to microfuge tubes or glass tubes (0.45 mL/tube) as appropriate to extraction procedure (see **Subheadings 3.3.–3.6.**)
5. Incubate for 10 min before adding agonists (50 μ L)
6. Incubate as required and extract as described (see **Subheadings 3.3.–3.6.**)

3.3. Acidified Bligh and Dyer Extraction

- 1 Add 1.88 mL of CHCl₃:MeOH:HCl to 0.5 mL of cell suspension; mix and allow to stand for 5–10 min. The sample should form a single clear phase (see **Note 7**).
- 2 Add a further 0.625 mL CHCl₃ followed by 0.625 mL 0.1 M HCl, and mix gently. Two phases will form and any protein will precipitate.
3. Centrifuge the samples for 10 min in a bench centrifuge (160g) to complete phase separation. Both upper and lower phases should be clear, with protein at the interface.
4. Remove 1.8 mL (of approx 2.25 mL total) of the upper aqueous phase (containing inositol and phosphoinositols) and neutralize to pH 7.0 using 1 M NaOH (approx 70 μ L). Store frozen at –20°C.

5. Transfer 1 mL (of approx 1.3 mL total) of the lower organic phase (containing phosphoinositides) to a solvent-resistant tube (5-mL tube if deacylation is to be carried out; *see* Chapter 2) and dry under a stream of nitrogen gas (warming the tube to 35–40°C if necessary). Redissolve the dried material in chloroform as required

3.4. PCA Extraction

1. To cells (0.5 mL) incubated in solvent resistant (microcentrifuge) tubes, add 0.5 mL of 10% PCA (*ice cold*).
2. Alternatively, if cells are adherent to culture dishes/multiwell plates during stimulation, add 0.25 mL of 15% PCA, and scrape the substratum with a syringe plunger. Transfer all material to a solvent-resistant (preferably microcentrifuge) tube; rinse each well with a further 0.5 mL H₂O and transfer these washings to the same (microcentrifuge) tube.
3. Pellet the precipitate by centrifugation (3 min at 3300g) and transfer all the supernatant to a separate tube for neutralization. Complete transfer can be carried out by decanting, provided the pellet is firm (*see* Notes 8 and 9).
4. Add 1.5 mL of freshly prepared freon:octylamine mixture (*see* Note 6) to the aqueous extracts and mix thoroughly by vortexing for 10 s, until the mixture takes on a milky appearance. Centrifuge samples for 2–3 min at 1300g. Three phases should form: water (top), octylamine perchlorate (middle), and freon:octylamine (bottom).
5. Remove 0.7 mL of the top phase, or 0.9 mL for samples from multiwell plates. Check the sample pH; it should be neutral. Store samples frozen at –20°C

3.5. TCA Extraction

1. Carry out steps 1–3 of Subheading 3.4., substituting TCA for PCA.
2. Mix the aqueous extracts with 2 mL water-saturated diethyl ether (*see* Note 10). After phase separation (using brief centrifugation if necessary to obtain a clean interface), discard the bulk of the ether and repeat the extraction four times.
3. Evaporate the remaining ether by standing samples in a stream of air in a fume cupboard. Neutralize each sample to pH 6.0–7.0 by addition of 100 mM NaHCO₃ (approx 50 µL/sample). Store samples frozen at –20°C.

3.6. Recovery of Phosphoinositides from Acid-Insoluble Pellet (*see* Notes 9 and 11)

1. Add 200 µL H₂O to each pellet from 0.5 mL of cell suspension prepared as in Subheadings 3.2. or 3.3. and freeze at –20°C (this softens the pellet). Thaw samples to room temperature.
2. Break up the pellet by vortexing.
3. Add 750 µL of CHCl₃:MeOH:HCl to each tube. Allow samples to stand for 5–10 min. A single clear phase should form.
4. Add 250 µL CHCl₃, and 250 µL 0.1 M HCl to each tube. Centrifuge samples at 75g for 5 min to separate the phases completely.

5. Carefully remove and discard 600 μL of the upper aqueous phase
6. Carefully transfer 400 μL (83%) of the lower organic phase to a solvent-resistant tube (5-mL tube if deacylation is to be carried out, *see* Chapter 2). Remove solvent and residual acid under a stream of nitrogen and redissolve in chloroform as required.

4. Notes

1. As a general rule, for any phosphoinositidase C assay based on *myo*-[^3H]inositol labeling to be sensitive, cell labeling of the phosphoinositides after 48 h should achieve $\sim 100,000$ dpm/well in a 12-well dish (200,000–250,000 cells). This is because, at basal level, the phosphoinositols are usually labeled to ~ 0.1 –1% of the total phosphoinositide (lipid) labeling, and stimulation may only liberate a small percentage of lipid label in a weakly responding tissue. Thus, if poor labeling is achieved, more radioactive tracer can be added to the labeling medium and/or the 20-Ci/mmol preparation can be replaced with a higher specific activity form (45–80 Ci/mmol, NEN DuPont) with labeling at 100 $\mu\text{Ci/mL}$ in growth medium
2. Other factors that influence labeling efficiency are the “cold” inositol concentration of the basic medium, as well as the percent serum present, since serum also contains inositol. Generally 10% serum in a balanced salt-nutrient medium with ~ 10 μM or less inositol will give good results (*see also* Note 1).
3. Indicator-free medium should be used as a rule, since phenol red binds to anion-exchange resins.
4. Many agonists/pharmacological agents are poorly soluble in water and so must be made up in solvents such as ethanol or DMSO. However these agents can also have effects, at least in part, through changes in membrane fluidity. As a general rule, such vehicle effects are minimized or absent by making agents up to at least 100 times the final desired concentration in vehicle, and then diluting to 10 times in M199, 0.2% BSA, 10 mM inositol, 10 mM LiCl. The diluted agent is then added as a 50 μL volume in a final total of 500 μL to give 1X concentration.
5. If air-displacement pipets are being used to dispense volatile solvents or recover the lower organic phase, then they should first be well-primed with the organic solvent so the air inside the pipet becomes saturated with the vapor; otherwise the first few samples will be short-measured.
6. Freon/octylamine should be prepared immediately before use. This mixture will react slowly on standing for more than 30 min.
7. If cells are attached to culture plates, the CHCl_3 :MeOH:HCl can be added directly and then rapidly transferred to a solvent-resistant tube for subsequent phase separation. This procedure, however, is not recommended since it may dissolve some of the plastic (6, 7).
8. A firmer membrane pellet is obtained on centrifugation of the acid lysate if the cell incubation medium contains protein. If the incubation medium lacks protein, it may be added (50 μL of 2% [w/v] BSA) after the acid.

9. When PCA or TCA lysates are pelleted and the acid supernatant decanted from the membrane-protein pellet, it is most important to remove as much of the supernatant as possible. Otherwise, too much water will remain to allow a single phase to form on subsequent phosphoinositide extraction (*see Subheading 3.6., step 3; see also Note 8*).
10. An alternative to using diethyl ether to extract TCA is to carry out the freon/octylamine procedure described for PCA extraction. Samples should be made 2 mM with respect to ethylene diamine tetra-acetic acid (EDTA) before neutralization is carried out.
11. If large numbers of samples are extracted by the PCA (or TCA) method, there can be a considerable delay between decanting the supernatant from the acid lysate and extracting the phosphoinositides from the pellet. Under such circumstances, 200 μ L H₂O should be added to each pellet (**Subheading 3.6, step 1**) after the supernatant is removed and the pellets frozen immediately. The phosphoinositides are stable under these conditions for several hours only, allowing time to complete neutralization of the aqueous extracts; but they should be processed as soon as possible.

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Phosphoinositidase C Activation Assay II

Simple Analysis of Recovered Cellular Phosphoinositides and Phosphoinositols

Ian M. Bird

1. Introduction

In Chapter 1, procedures for cell labeling with [^3H]inositol, stimulation with agonists, and extraction of the phosphoinositols and phosphoinositides are described. In this chapter, simple low-resolution chromatography techniques capable of separating phosphoinositols and phosphoinositides into their general classes using inexpensive apparatus are described in detail. For higher resolution chromatographic techniques capable of separating isomeric forms, see Chapters 3 and 4.

1.1. Separation of Phosphoinositols by Anion Exchange Chromatography

Traditionally, separation of phosphoinositols has been carried out by descending-paper chromatography or high-voltage iontophoresis (see Note 1). However, paper chromatography methods can be very slow (taking up to 10 d), and neither procedure readily allows detection of the trace quantities of material or radioactive material at the levels usually recovered from cells. Nevertheless, they can separate different isomeric forms of phosphoinositols and provide a cheap and simple means of establishing the identity of phosphoinositols. Currently, the simplest and most widely used method to analyze phosphoinositols is anion-exchange column chromatography. The individual classes of phosphoinositols (IP_1 – $\text{IP}_{5/6}$), but not their isomeric forms, can be separated and quantified as described by Ellis et al. (1), and subsequently modified as by Berridge et al. (2) and Batty et al. (3) (see Fig. 1). Although separa-

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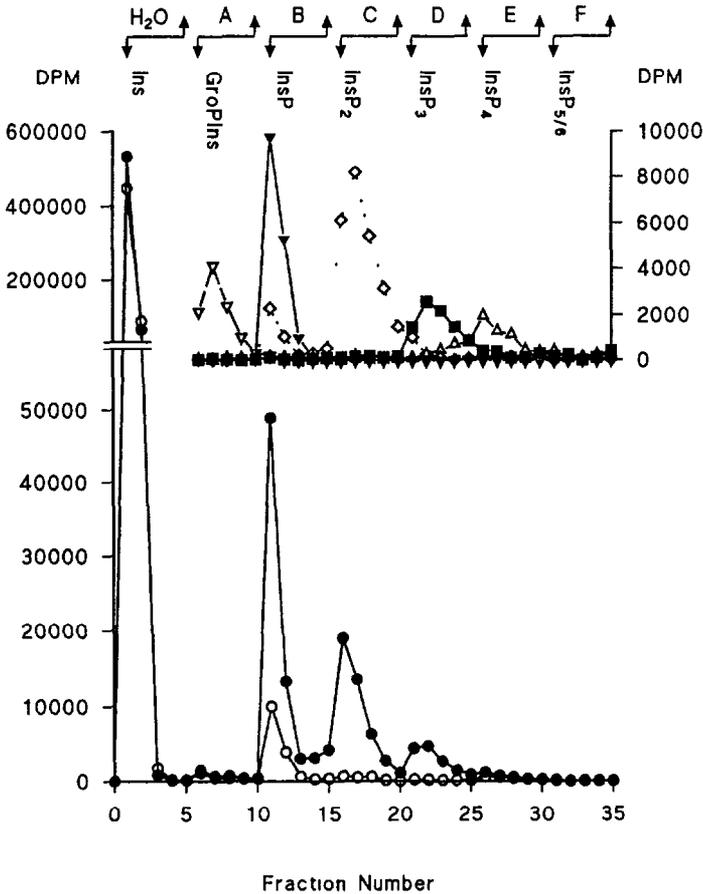


Fig 1 Separation of phosphoinositols by anion-exchange chromatography. The separation of [^3H]phosphoinositols standards (top) and [^3H]phosphoinositols from tissue extracts (bottom) with 100–200-mesh AG1X8 resin are shown. Arrows indicate the buffers used for each fraction. Standards (prepared as in Chapter 5): (open triangle down) GroPIIns (*see also Subheading 3.2.2.*); (closed triangle down) Ins1P; (open diamond) Ins(1,4) P_2 (this is contaminated with some Ins1P); (closed square) Ins(1,4,5) P_3 , and (open triangle up) Ins(1,3,4,5) P_4 . All standards were run in parallel on separate columns. Tissue extracts were prepared from cultured bovine adrenocortical (zfr) cells prelabeled for 42 h with [^3H]inositol and incubated in Li^+ -containing buffer with (closed circles) or without (open circles) angiotensin II (for further details, *see ref. 23*). Phosphoinositols were recovered by PCA precipitation (*see Chapter 1*).

tion of standards is generally good, that of IP_3 and IP_4 may not be complete using this method if conditions are not optimized. To measure total phospholipase C activation alone, it is not always necessary to separate the phospho-

inositols into individual classes, and a simplified procedure can be used (*see Subheading 3.1.1.*).

1.2. Deacylation of Phospholipids and Separation of Products by Anion-Exchange Chromatography

A widely used method to separate phosphoinositides is to deacylate the water-insoluble PI, PIP, and PIP₂ to water-soluble glycerophosphoinositols (GroPIIns, GroPIInsP, and GroPIInsP₂, respectively), which are then separated by a variation of the anion-exchange chromatography procedure (*see Fig. 2*) described in **Subheading 3.1. (4)**. The deacylation procedure should give good recoveries, should not produce free inositol, should be reproducible, and most important of all, should yield a glycerophosphoinositol product with the same isomeric structure as in the parent lipid. A mild and highly specific alkaline hydrolysis procedure, capable of quantitatively deacylating trace amounts of phosphoinositides without isomerization, has been developed by Clarke and Dawson (**5**). It involves transacylation of the fatty acids from the phospholipids to monomethylamine. The reagent is volatile and so can be removed easily by evaporation. The organic products of this reaction are subsequently separated from the aqueous products by solvent extraction (for full details, *see ref. 5*). A modification of their procedure is described below. Although deacylation/anion exchange chromatography is the most widely used approach, only separation of intact lipids (by thin-layer chromatography [TLC]) allows separation and quantification of LysoPI from PI. This may be important in some experimental systems where activation of phosphoinositidase A2 may occur.

1.3. Separation of Phosphoinositides by TLC

If ³²Pi-prelabeled cells are used, it is necessary to identify the phosphoinositides owing to the presence of other labeled phospholipids. Several methods have been described using thin-layer chromatography. The methods described below give clear separation of PIP and PIP₂ in one dimension (*see Fig. 3*). (For more information on phosphoinositide separations, *see refs. 6 and 7 and Note 12.*)

2. Materials

2.1. Separation of Phosphoinositols by Anion Exchange Chromatography

1. AG1X8 anion-exchange resin (formate form, 200–400 mesh) (*see Note 2*)
2. 10 mM Na₂EDTA, pH 7.0.
3. Polypropylene columns (containing 70- μ m frits)
4. Scintillation vials (7-mL) and fluid with high salt/aqueous capacity (e.g., Instagel XF-Packard, Downers Grove, IL).

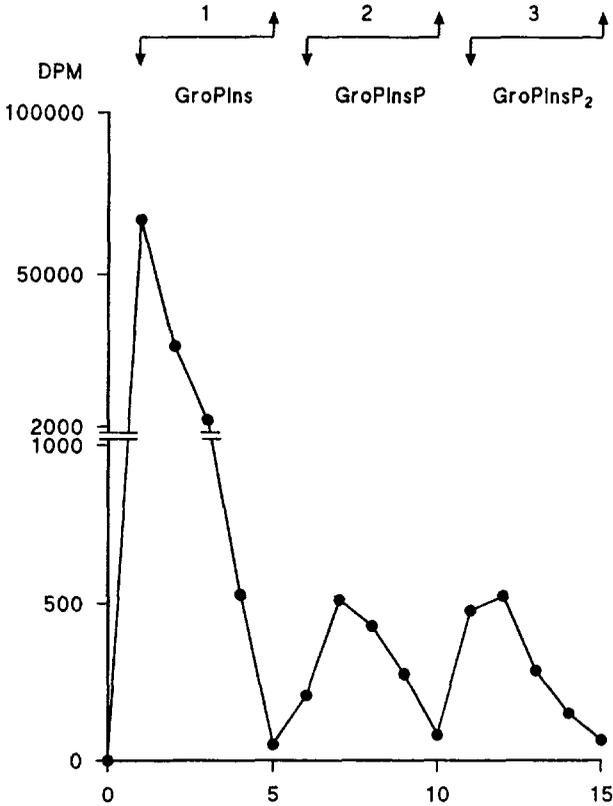


Fig. 2. Separation of phosphoinositide deacylation products (glycerophosphoinositols) by anion-exchange chromatography on 100 to 200-mesh AG1X8 resin. Arrows indicate the buffers used for each fraction. The [^3H]glycerophosphoinositols were prepared from bovine adrenocortical cells labeled for 42 h with [^3H]inositol (23). Labeled products were recovered from cells using the PCA method and phosphoinositides recovered by the acidified Bligh and Dyer extraction procedure (see Chapter 1). [^3H]Phosphoinositides were deacylated in the presence of unlabeled lipid carrier using methylamine deacylation.

5. Racks for columns and vials (see Note 3).
6. Buffer A: 60 mM ammonium formate and 5 mM disodium tetraborate.
7. Buffer B: 200 mM ammonium formate and 100 mM formic acid.
8. Buffer C: 400 mM ammonium formate and 100 mM formic acid.
9. Buffer D: 800 mM ammonium formate and 100 mM formic acid.
10. Buffer E: 1.2 M ammonium formate and 100 mM formic acid.
11. Buffer F: 2.0 M ammonium formate and 100 mM formic acid (see Note 4).

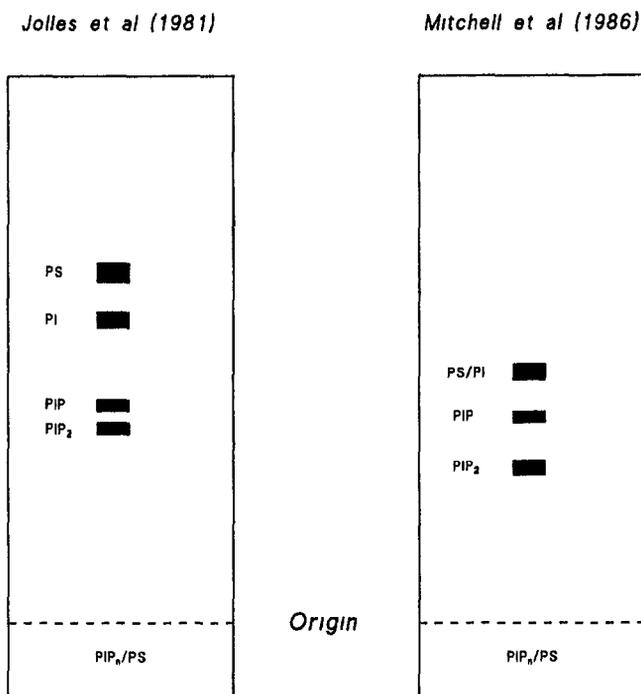


Fig. 3. Separation of the phosphoinositides by thin-layer chromatography. The separation of PI, PIP, and PIP₂ (mixed phosphoinositides preparation, also contains phosphatidylserine [PS]) by the methods of Jolles et al. (6) (left) and Mitchell et al. (7) (right) are shown diagrammatically. For location of Lyso PI, see Note 16

2.2. Deacylation of Phospholipids and Separation of Products by Anion-Exchange Chromatography

General note: All solvents to at least analytical grade.

2.2.1. Deacylation of Phosphoinositides

1. Monomethylamine:water:butanol (50:15:5 [v/v/v]) (see Note 6).
2. *n*-Butanol:light petroleum ether (BP 40–60°C):ethyl formate, 20:4:1 (v/v/v).
3. Mixed phosphoinositides (Sigma, St. Louis, MO) as carriers dissolved in chloroform (1 mg/mL).
4. Water bath at 53°C.
5. 5-mL Tubes and glass marbles.
6. Oxygen-free nitrogen gas.

2.2.2. Separation of Glycerophosphoinositol Products

1. AG1X8 anion exchange resin (formate form, 200–400 mesh).
2. Polypropylene columns (70- μ m frits).

3. Scintillation vials (7-mL) and fluid with high salt/aqueous capacity (e.g., Instagel XF-Packard).
4. Racks for columns and vials.
5. Buffer 1: 180 mM ammonium formate, 5 mM disodium tetraborate.
6. Buffer 2: 300 mM ammonium formate, 100 mM formic acid.
7. Buffer 3: 750 mM ammonium formate, 100 mM formic acid.

2.3. Separation of Phosphoinositides by TLC

General note: All solvents should be to chromatography grade if possible, or otherwise analytical grade.

1. Silica gel 60 TLC plates. glass backed, with concentration zone, without fluorescent indicator, 20 × 20 cm, 0.25-mm thickness (Merck, through EM Science, Gibbstown, NJ)
2. Unlabeled lipid carrier Use phosphoinositides mix (Sigma)
3. Chloroform
4. Filter-paper-lined chromatography tank, with air-tight lid.
5. Second tank containing resublimed iodine (for staining phospholipids).
6. Glass capillary tubes or Hamilton syringe for application of samples.
7. Hair drier
8. Single-edged razor blades
9. Water (in an aerosol dispenser).
10. 1% Potassium oxalate, 1 mM EDTA dissolved in methanol:H₂O (2:3 [v/v]) in an aerosol dispenser (for solvent system 1).
11. Solvent system 1: chloroform:acetone:methanol:glacial acetic acid:H₂O (40:15:13:12:8 [v/v/v/v/v])
12. Solvent system 2: chloroform:methanol:H₂O:concentrated ammonia (48:40:7.5 [v/v/v/v]).

3. Methods

3.1. Separation by Anion-Exchange Chromatography

1. Prepare a slurry of AG1X8 anion-exchange resin in an equal volume of water (*see Note 2*)
2. With the slurry constantly mixing (using a magnetic stirrer), dispense 1.2 mL of slurry (i.e., 0.6 mL of resin) into each column. Add 2 mL of water to each column and allow to drain. Check each column for possible air locks at this stage.
3. Thaw samples (if frozen) and add 1/10 vol of EDTA (to a final concentration of 1 mM).

If collection of [³H]inositol is required:

4. Place a scintillation vial under each column. Load each sample onto a separate column, then rinse the sample tube with water (1 mL) and transfer this to the same column. Allow all columns to drain.

- 5 Remove vials to storage racks and place a fresh vial under each column. Add 2 mL H₂O to each column and allow to drain. Repeat this step three more times.

If collection of [³H]inositol is not required: Place the columns over a tray for **steps 4 and 5** and discard the eluate and washings as radioactive waste.

- 6 Place a fresh vial under each column and elute each column with 2 mL buffer A. Remove vials to storage racks. Repeat this process four more times (*see Note 4*).
- 7 Repeat the procedure in step 6 but elute sequentially with 5 × 2 mL of buffers B, C, D, E, and F. To each 2-mL fraction, add scintillation fluid and count in a liquid-scintillation counter (*see Note 4*).

3.1.1. Modified Procedure for Assessing Total Phospholipase C Activation

- 1 Load samples as above onto 0.25-mL columns of resin (i.e., dispensing 0.5 mL slurry), and elute unbound inositol with 2 × 4 mL H₂O (without collecting [³H]inositol to vials).
- 2 Elute columns with 2 × 2 mL buffer E, collecting both 2-mL fractions. Add scintillation fluid and count in a liquid scintillation counter. The total radioactivity eluted in these fractions reflects total (>98%) breakdown of labeled phosphoinositide (but *see Note 5*).

3.2. Deacylation of Phospholipids and Separation of Products by Anion-Exchange Chromatography

3.2.1. Deacylation of Phosphoinositides

Carry out work in a fume cupboard.

- 1 If assessment of radioactivity, but not mass, of individual lipids is required, add 25 µg (25 µL) mixed phosphoinositides to each sample tube (*see Note 7*).
- 2 Dry all samples under a stream of nitrogen gas.
- 3 Add 0.5 mL of methylamine, water:butanol reagent (freshly prepared—*see Note 6*) to each tube and place in the water bath (53°C). To minimize evaporation of reagent, place a glass marble on each tube.
- 4 After 30 min, transfer the tubes to ice. Remove the marbles, and immediately dry down each sample under a stream of nitrogen gas (*see Note 8*).
- 5 Add 1 mL H₂O to each tube, followed by 1.2 mL of the butanol/petroleum ether/ethyl formate reagent (freshly prepared), and mix thoroughly.
- 6 Separate the aqueous and organic phases by centrifuging tubes in a bench centrifuge for 1–2 min at 1300g. Remove and discard 0.75 mL of the upper (organic) phase and add a further 0.75 mL butanol/light petroleum/ethyl formate reagent to each tube.
- 7 Mix the two phases thoroughly and centrifuge the tubes as in **step 6**. Remove and discard 0.75 mL of the upper organic phase.

- 8 To recover all the lower (aqueous) phase, transfer the bulk (0.75 mL) of the lower phase to a separate tube. Then, carefully add a further 0.75 mL of water to the remaining lower aqueous phase; remove immediately, and combine the recovered lower phases
- 9 Check the pH of the recovered material and neutralize if necessary (*see Note 9*)
Store samples at -20°C .

3.2.2. Separation of Glycerophosphoinositol Products

1. Prepare a slurry of AG1X8 anion-exchange resin in an equal volume of water (*see Note 10*).
2. With the slurry constantly mixing (using a magnetic stirrer) dispense 1.2 mL of slurry (i.e., 0.6 mL of resin) to each column. Add 2 mL of water to each column and allow to drain (check each column for possible air locks at this stage)
- 3 Thaw samples (if frozen).
- 4 Place the columns over a large tray. Load each sample, in turn, onto a column. Rinse the sample tube with water (1 mL) and transfer this to the column. Allow all columns to drain.
- 5 Elute the columns with 2×4 mL of H_2O , allowing the columns to drain each time.
6. Place a vial under each column and elute each column with 2 mL buffer 1 (*see Note 11*). Remove vials to storage racks. Repeat this process four times.
7. Repeat the procedure in **step 6**, eluting sequentially with 5×2 mL of buffer 2 followed by 5×2 mL of buffer 3. To determine radioactivity in each sample, add scintillation fluid to each 2-mL fraction and count in a liquid-scintillation counter (*see Note 12*)

3.3. Separation of Phosphoinositides by TLC

1. Add the chosen solvent (system 1 or 2) to the chromatography tank lined with absorbent paper to give a depth of 0.5–1.0 cm. Place the lid on the tank and leave to equilibrate (*see Note 13*).
2. For separation of phosphoinositides by system 1 only, evenly spray a TLC plate with the potassium oxalate reagent until the gel is completely wet but without excess surface liquid (*see Note 14*). Blot each plate gel-side down on tissue or filter paper to remove excess surface liquid and then lay the plates flat (gel upward) in a stream of air (in a fume cupboard) to dry. Activate the dry plates by heating in an oven (115°C for 10 min). Allow the plates to cool
3. For separation of trace amounts of radiolabeled lipid, add mixed phosphoinositides (50 μg per sample) as carrier to samples as required. Dry down the phosphoinositide samples under a gentle stream of nitrogen gas and redissolve in 20 μL chloroform.
4. Gently draw a pencil line across the plate 1.5 cm from the bottom edge of the concentration zone (2.5 cm deep). Be sure not to press through the silica. Mark crosses on this line at 2-cm intervals

5. Apply the first sample to the plate (on a cross, using a fine glass capillary or using a Hamilton syringe). Allow the applied sample to dry. Rinse the tube with 20 μ L chloroform and apply to the same cross.
6. Repeat **step 5** for the other samples, using a fresh capillary or rinsing the Hamilton syringe between each sample. Load standards in the same way. Dry the plates using a hair drier (cool)
7. Place the plate(s) in the solvent (gel sides facing each other if in pairs).
8. When the solvent has reached the top of each plate, remove, and allow to air-dry in a fume cupboard
9. When the plates are free of all traces of solvent, expose to resublimed iodine until the phospholipid spots and standards are visible. Mark the positions of these spots before they fade.
10. To measure radioactivity or assay phosphate in each spot, lightly spray the area to be scraped with water.
11. Lift away the gel around the spot first, then lift the gel containing the spot from the glass plate and transfer to a tube for phosphorus assay (*see* Chapter 20 of this volume), or a scintillation vial as appropriate (*see* **Note 15**).

4. Notes

1. For more details on paper chromatographic separation of isomers of IP₁, up to IP₅, *see* **refs. 8–14**. For more details on iontophoretic separation of phosphoinositols on paper, *see* **refs. 14–16** and for separation on cellulose-backed TLC plates, *see* **ref. 17**.
2. Although this method is best performed using 200–400-mesh AG1X8 resin (Bio-Rad Laboratories, Richmond, VA), it is possible to use other mesh sizes (100- to 200-mesh resin gives columns that flow faster but give less sharp separations) or less-expensive Dowex anion exchange resin. However, Dowex resin requires washing in bulk before use (5 vol 1 M NaOH; water to neutrality; 2 vol 1 M formic acid; water to neutrality) and results can be more variable.
3. This procedure is simple to carry out in principle, but it can become difficult if large numbers of samples are processed simultaneously. It will be necessary to have storage racks for several hundred collected fractions (each column produces up to 35 fractions), and a racking system that allows the columns to stand directly over the vials. Suitable racks (and columns) can be obtained from most suppliers of anion-exchange resins.
4. Buffer F elutes a combined IP₅/IP₆ fraction. It has not been possible to separate IP₅ and IP₆ using this method. However, all the other phosphoinositol classes can be separated completely if the system is first fully optimized; even with AG1X8 resin there can be variations in the performance of each batch of resin, so it may be necessary to adjust the buffer strengths used. To do this, first prepare unlabeled cell extracts by the method of choice and then spike each blank sample with an appropriate radiolabeled standard (*see* Chapter 5). Load each “sample” onto a separate column and elute sequentially as described. If a buffer not only

brings off the desired standard but also the next standard, then reduce the buffer's ammonium formate strength (try 50-mM steps). Alternatively, if a buffer fails to elute a standard completely within 5×2 -mL vol, increase the buffer strength accordingly. There are additional advantages to carrying out this optimization procedure, as it is possible to collect the 5×2 -mL fractions from each buffer straight into single (20-mL) vials, rather than as individual 2-mL fractions. Thus only seven vials (one for each buffer) are produced from each column, instead of 35, making counting and data processing easier.

- 5 The accuracy with which such an assay procedure reflects the true dose-dependency of activation of phosphoinositidase C will depend on the linearity of the measured response with respect to time. If the true response is linear (i.e., does not rapidly desensitize), nonlinearity of the measured response may still be observed if the cells are not prelabeled to a steady state.
- 6 The original method (5) used methylamine gas to prepare the methylamine reagent. The procedure is potentially hazardous and the reagent is noxious, volatile, and dangerous. An alternate means of preparing the reagent uses 33% methylamine in ethanol (BDH, Poole, UK), mixed 10:3 (v/v) with water. The procedure described here includes butanol to increase phospholipid solubility. The methylamine in ethanol reagent is stable for several months at room temperature, and working reagent can be prepared fresh as required.
7. Carrying out this procedure on [³H]inositol-prelabeled phosphoinositides recovered from labeled cells results in transfer of >95% of radioactivity to the aqueous phase. High-performance liquid chromatography (HPLC) analysis of the products prepared without unlabeled lipid carrier added (**Subheading 3.2.1., step 1**) shows that the products include 1% Ins1P and 1.5% Ins as well as the expected glycerophosphoinositols. If unlabeled lipid carrier is added, however, these figures change to 0.03% Ins1P and 1.5% Ins, respectively (*see also Note 11*).
8. Rapid cooling of samples on ice (in **Subheading 3.2.1., step 4**) after 30 min with methylamine (**Subheading 3.2.1., step 3**) is particularly important if there is to be a delay in drying down the samples (because of sample numbers). Also, when removing the methylamine reagent under nitrogen, the tubes should not be warmed to accelerate the process until at least the bulk of the reagent (and therefore the methylamine) has evaporated. Even then, tubes should not be warmed to above 40°C.
9. If the final aqueous products are not neutral, but alkaline (**Subheading 3.2.1., step 9**), this is either caused by incomplete removal of the methylamine reagent (**Subheading 3.2.1., step 4**) or incompletely mixing of the aqueous/organic phases (**Subheading 3.2.1., steps 5 and 7**) during the extraction of organic products.
10. For comments on the relative merits of alternative choices of anion exchange resin *see Note 2*.
11. In this procedure, the buffer used to elute the GroPIIns fraction contains 180 mM ammonium formate, whereas in the method described for separation of the phosphoinositols, a buffer containing only 60 mM ammonium formate is used. The reason for the higher buffer strength in this application is to elute both

GroPIIns and any additional Ins1P (produced by overhydrolysis of PI—see Notes for deacylation procedure), but not GroPIInsP or GroPIInsP₂. This precaution is necessary because although only 1% of PI may be overhydrolyzed to Ins1P, the relative proportions of the original phosphoinositides are >95:1.1 (PI PIP:PIP₂). Therefore, the quantity of material produced by a 1% formation of Ins1P from PI may equal or exceed the quantity of GroPIInsP formed from PIP

12. The analytical procedures described here can be carried out quickly and reproducibly using relatively simple and inexpensive apparatus. However, to separate and quantify trace amounts of individual isomeric forms of the phosphoinositides and phosphoinositols accurately, high-performance liquid chromatography (HPLC) is used (see Chapters 3 and 4). Several sensitive methods for quantifying unlabeled phosphoinositols have also been recently developed. Of these methods, the most reliable are the competitive-binding assays for Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄. These assays exploit the existence of naturally occurring microsomal binding sites (prepared from bovine adrenal cortex or rat cerebellum) to give assays of both high sensitivity and selectivity (18–22). Such high selectivity also makes it possible to assay samples without chromatographic pre-separation. These assays are now available in kit form. Unfortunately, specific binding sites for other phosphoinositols are unknown at present. An alternative approach to determine the mass of individual phosphoinositols is to separate them using HPLC and then apply a sensitive, but isomer-nonspecific assay to the recovered fractions. Several such spectrophotometric/fluorometric assay procedures have been developed, which generally measure phosphorus or inositol content (22).
13. As for any TLC method, poor results will be obtained if the tank is not properly pre-equilibrated with the solvent or the tank is not air tight. If necessary, seal the lid with a thin layer of silicone grease and/or place a weight on the lid.
14. Prespraying plates with potassium oxalate/EDTA removes any divalent cations from PIP and PIP₂ so their migration is not retarded. However, the quality of the results obtained by this method in particular may be reduced by overwetting or unevenly spraying the plate in **Subheading 3.3., step 1**. The gel coat should be made wet, but not to the point where it starts to detach from the plate. Blotting should also be carried out without delay.
15. Whether the intention is to carry out phosphate assays (see Chapter 20) or scintillation counting on the samples, it is advisable to include among the assay samples some blanks consisting of gel with no visible phospholipid bands. Also, the area scraped for each band should be kept as uniform as practical. If scintillation counting is to be carried out, add 1 mL water to each vial and sufficient scintillant to form a stable gel phase. Mix the sample into the gel thoroughly so it is suspended evenly during counting.
16. Using solvent system 1 (6), LysoPI (a product of phosphoinositidase A2 activation) migrates between PIP and PIP₂. Using solvent system 2 (7), LysoPI migrates slightly below PIP, on silica gel 60 TLC plates. However, Mitchell et al. (7) used silica HL plates (Anartech, Newark, DE) and reported that LysoPI migrates above PIP. Mitchell et al. have also described a further solvent system (chloroform:

methanol:formic acid, 55:25 5 [v/v/v]) used in the second dimension. This cleanly separates LysoPI from PI, PIP, and PIP₂ (as well as other phospholipids) and can be used to confirm more rigorously the identity of phospholipids.

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Phosphoinositidase C Activation Assay III

HPLC Analysis of Cellular Phosphoinositides and Phosphoinositols

Ian M. Bird

1. Introduction

In Chapters 1 and 2, the extraction procedures for recovery of the phosphoinositols and phosphoinositides from cells in suspension or culture are described, together with simple separation procedures to resolve them into their general classes (InsP_1 , InsP_2 , and so on, and PtdIns , PtdInsP , and PtdInsP_2). However, in reality, the metabolism of the phosphoinositols is complex (1) leading to the formation of several isomeric forms in each class. Furthermore, since the discovery of a phosphoinositide 3-kinase (in addition to the previously known 4- and 5-kinases) (2), it is clear that the phosphoinositides also exist in different isomeric forms.

The initial unambiguous identification of the structure of these compounds has required a combination of both chemical and high-powered chromatographic techniques. However, in recent years, high-performance liquid chromatography (HPLC) methods have been developed to resolve most of the known naturally occurring phosphoinositol isomers on anion-exchange columns (like all standard anion-exchange methods, the only limitation is that enantiomeric pairs of phosphoinositols [*see* Chapter 5; **Note 1**] cannot be separated). Such methods are now routinely used for the identification of phosphoinositol products from previously uncharacterized tissues.

The ability of HPLC techniques to separate a complex mixture of phosphoinositols into individual isomers now plays a central role in monitoring changes in the radiolabeling and/or mass of the different phosphoinositols on agonist stimulation. This chapter describes three simple chromatographic procedures

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for the separation of the glycerophosphoinositols and phosphoinositols. For more rigorous identification of unknown peaks, methods for preparation of further commercially unavailable standards, as well as conversion of glycerophosphoinositols to phosphoinositols, are described (*see* Chapter 5). Guidelines for optimization of an HPLC procedure are also described (*see* Subheadings 4.1.–4.5.).

1.1. Sample Preparation

For HPLC/fast-protein liquid chromatography (FPLC) analysis, it is important that the sample is neutral, has a low salt content, and is free of particulate matter. To prevent isomerization of the phosphoinositols and phosphoinositides (*see* Chapter 5, Note 2), it is most important to work on ice and neutralize samples as quickly as practical. Samples should be prepared to a volume of approx. 1 mL, to be loaded, with additions, into an injection loop of 2 mL (or for FPLC, 4 mL).

1.1.1. Phosphoinositols

Full details of methods for the extraction of the phosphoinositols from cell preparations are described in Chapter 1, and from tissues in Chapters 4 and 6. The acidified Bligh and Dyer solvent extraction is not the method of choice for two reasons. First, the aqueous phase containing the phosphoinositols also contains some water-insoluble material and organic solvents. Freeze-drying the samples (in the presence of 1 mg mannitol to act as a carrier) and reconstitution in water, followed by either centrifugation in a microfuge (at 12,000g, for 5 min), or preferably filtration (0.5- μ m filters), can overcome the solvent and particulate contamination. A second and more serious problem, however, is that in extracting the phosphoinositides into acidified medium containing methanol, some aqueous methylphosphoinositol byproducts can be formed through acid hydrolysis of the corresponding phosphoinositide. Although formed in small amounts, it is still sufficient for these products to give rise to additional but artifactual peaks, complicating the interpretation of results (3).

The TCA precipitation method (*see* Chapter 1) is widely used for preparation of HPLC/FPLC samples and produces samples free of methylphosphoinositol byproducts. Where diethyl ether extraction of the TCA is carried out, traces of diethyl ether can be removed by lyophilization (in the presence of 1 mg mannitol), and reconstitution in water. Samples prepared in this way should be free of particulate matter but centrifugation in a microfuge (at 12,000g for 5 min) or filtration (0.5- μ m filter) is advisable.

The PCA precipitation method (*see* Chapter 1) is the most appropriate method for preparation of samples for HPLC/FPLC analysis. Samples prepared in this way contain little particulate matter (however, sample centrifugation [at

12,000g for 5 min] or filtration [0.5- μ m filter] is still recommended as a precaution). Also, in carrying out the freon/octylamine neutralisation procedure, no addition of salt is necessary to achieve neutralisation, and the samples contain little or no organic solvent. Therefore, lyophilisation is not required.

1.1.2. Phosphoinositides

Phosphoinositides are best recovered from the acid insoluble pellet of the TCA or PCA extraction procedure as described in Subheadings 3.4.–3.5. of Chapter 1. Whereas the phosphoinositides cannot be analyzed directly by the HPLC methods described below, because of insolubility in water, they can be analyzed by HPLC following deacylation to their glycerophosphoinositol counterparts (*see* Subheading 3.2., Chapter 2). However, obtaining standards for isomeric forms of the glycerophosphoinositols (e.g., the 3-phosphate and 4-phosphate isoforms) is difficult, so for rigorous identification of individual isomeric forms, the glycerophosphoinositols should be further deglycerated to the corresponding phosphoinositols using limited periodate oxidation (*see* Subheading 3.3., Chapter 5). Once again, the final product ready for HPLC should be solvent free, have neutral pH, and be free of particulate matter.

1.2. HPLC Analytical Methods

The HPLC methods described below are intended as a starting point for separation and identification of unknown phosphoinositols. The HPLC methods quoted are general methods for separation of phosphoinositol isomers from InsP to InsP₄. The separations of individual isomers in a phosphoinositol class (e.g., isomers of InsP₃) are by no means fully optimized because such optimization (choice of buffer strength/pH and of gradient shape) will depend on the needs of the user. The optimization of HPLC methods, however, is also discussed (**Subheadings 4.1.–4.5.**).

To date, most published HPLC methods have used traditional silica-based anion-exchange HPLC columns that must be pumped at high pressure. To obtain good resolution of phosphoinositol isomers on such columns, it has been necessary to have phosphate present in the elution buffer. However this presents practical difficulties in that samples cannot be assayed for mass by phosphate content, so these HPLC methods cannot be readily applied to determination of phosphoinositol mass or to checking the purity of unlabeled compounds. Also, because phosphate is not volatile, it is necessary to desalt any HPLC-purified products/standards by further chromatographic means, so increasing losses.

In recent years, an alternative form of HPLC has evolved from the development of columns based on organic resin beads. This has allowed the development of columns with comparable performance to silica-based columns, but that can run at higher flow rates and lower operating pressure. Because these

columns were originally developed for separation of proteins, this system is referred to as fast-protein liquid chromatography (FPLC). As such columns do not require phosphate to obtain sharp peak profiles, elution buffers that are phosphate-free (and so allow mass determination through assay of phosphorous) can be used. Furthermore, if the buffer is made up of volatile components, they can be completely removed by lyophilization. Such a method for separation of phosphoinositols on an anion-exchange FPLC column is also described.

Most applications of HPLC to date have been to separate radiolabeled phosphoinositols. Unfortunately, phosphoinositols do not absorb at UV wavelengths, and, whereas in-line scintillation counting is possible (*see* Chapter 4), it is relatively insensitive and costly both in capital outlay and running costs. Therefore, most workers collect fractions throughout the run and carry out scintillation counting afterwards. However, this means samples must be run blind, and, if column performance deteriorates, then samples could be wasted. Therefore, to give an instant record of column performance and a measure of the reproducibility of sample separation, most workers apply internal nucleotide standards to the samples before injection, and then monitor the column outlet at 254 nm (or 280 nm if monitoring at 254 nm is not possible). The nucleotides AMP, ADP, and ATP migrate in the regions of inositol mono-, bis-, and trisphosphates, respectively in most systems. It must be remembered, however, that such nucleotide markers should not be added where mass determinations are to be carried out by assay of collected fractions for phosphorous, or where HPLC is being used to prepare purified phosphoinositol standards/substrates for experimental use.

2. Materials

2.1. Radioactive Standards

Standards can be purchased or prepared as described in Chapter 5. In general, standards should be applied to the HPLC as approx 5000–10,000 dpm each to allow easy detection with short counting times.

2.2. HPLC Columns and Equipment

2.2.1. HPLC Methods 1 and 2: Separation on SAX10 Using 0–1.7 Ammonium Formate/Phosphoric Acid, pH 3.7, or 0–1.4 Ammonium Phosphate/Phosphoric Acid, pH 3.7

1. Main column: Partisil or Partisphere SAX10 (25 cm × 4-mm cartridge).
2. Guard column: Partisil or Partisphere SAX10 (1 cm × 4-mm cartridge).
3. HPLC equipment. binary-gradient HPLC equipment with UV monitor, chart recorder, and fraction collector.

2.2.2. HPLC Method 3: Separation on MonoQ Using 0–1.0 Ammonium Formate/Formic Acid, pH 4.5

1. Main column: MonoQ HR5/5 (5 cm × 5 mm) (Pharmacia, Uppsala, Sweden).
2. Guard column: none.
3. HPLC equipment. binary-gradient FPLC equipment (or HPLC equipment capable of operating with low back pressure of approx 300 psi) with in-line UV monitor, chart recorder, and fraction collector.

2.3. Chemicals

1. Deionized double-distilled water (solvent A).
2. Salt buffers (buffer B). 1.7 M ammonium formate adjusted to pH 3.7 with orthophosphoric acid (*method 1*); 1.4 M monobasic ammonium phosphate (*see Note 1*) adjusted to pH 3.7 with orthophosphoric acid (*method 2*); 1.0 M ammonium formate adjusted to pH 4.5 with formic acid (*method 3*).
3. AMP, ADP, ATP (sodium salts; make up a mix of nucleotides at 0.1 mg/mL each, pH 7.0 in water).
4. Methanol and ethanol
5. Scintillation fluid (*see Note 3*)
6. 50% v/v Acetic acid
7. 1.0 M NaCl.
8. 2.0 M NaOH.

2.4. Ancillary Equipment

1. Scintillation vials.
2. Liquid scintillation counter.
3. pH meter (calibrated)

3. Methods

Because there are many different types of HPLC pumps and pump controllers, it is not possible to give step-by-step instructions for carrying out HPLC analysis of samples. However, there are several practical points that should be noted:

3.1. Preparation of Solvents/Buffers

To ensure maximum reproducibility of results between analytical runs:

1. Always use double-distilled, deionized water and high-purity reagents to prepare elution solvents/buffers.
2. Calibrate the pH meter, and adjust the pH of solvent B to within 0.05 pH units to ensure absolute reproducibility of results from a given column.
3. Filter solvent A (water) and solvent B (buffer) through a 0.2- μ m filter before use on the HPLC.
4. Degas solvents A and B before use, or displace gas by bubbling oxygen-free nitrogen through the liquid for a few minutes.

3.2. HPLC Method 1 and 2: Separation on SAX10

- 1 Filter and degas water and appropriate buffer solutions (1.7 M ammonium formate/orthophosphoric acid, pH 3.7 for method 1; or 1.4 M ammonium phosphate/orthophosphoric acid pH 3.7 for method 2) as described in **Subheading 3.1**.
2. Set up the appropriate gradient program, using a flow rate of 1.25 mL/min (operating pressure: 600 psi for new column) and a loop volume of 2.0 mL. Set the in line UV monitor to 254 nm.

Method 1: 0–1.7 M ammonium formate/orthophosphoric acid, pH 3.7

Time (min)	0	10	20	55	70	75	75.5	85
% Buffer	0	0	8	60	100	100	0	0

Method 2: 0–1.4 M ammonium phosphate/orthophosphoric acid, pH 3.7

Time (min)	0	10	55	70	75	75.5	85
% Buffer	0	0	35	100	100	0	0

- 3 Set up the fraction collector program (for both method 1 and 2) as follows.

Time Interval (min)	0–10	10–75	75–85
Time/Fraction (min)	1.0	0.5	1.0

3.3. HPLC Method 3: Separation on MonoQ

1. Filter and degas water and solvent B (1.0 M ammonium formate/formic acid, pH 4.5).
- 2 Set up the appropriate gradient program, using a flow rate of 2.0 mL/min (operating pressure: 300 psi for new column) and a loop volume of 4.0 mL. Set the in-line UV monitor to 280 nm

Method 3: 0–1.0 M ammonium formate/formic acid, pH 4.5

Time (min)	0	5	25	65	85	90	90	95
% Buffer	0	0	4	50	100	100	0	0

3. Set up the fraction collector program for method 3 as follows:

Time Interval (min)	0–10	10–70	70–90
Time/Fraction (min)	1.0	0.5	1.0

3.4. Start-Up

HPLC and FPLC columns are stored long term in alcohol/water mixtures to prevent microbial action and, where relevant, to stabilize the silica base. On start-up prior to analysis:

1. Prime both the HPLC pumps with solvent A (water, degassed!).
2. Pump the column at half the flow rate used in the method of choice until normal operating pressure is achieved.

3. Prepare and degas solvent B. With the column isolated, prime pump B with this buffer. Bring the column back in line.
4. For method 3 only, carry out the following column-precleaning procedure (**Subheading 4., Note 4**):
 - a. Reverse the column, and elute with water at 1 mL/min.
 - b. At 5-min intervals, consecutively apply 4-mL injections of 50% acetic acid, methanol, 1 M NaCl, and 2 M NaOH.
 - c. Return column to its normal flow direction
5. For all methods, run the analytical gradient (without injection of a sample) to prerun the column. The column is now ready for sample analysis.

3.5. Sample Application

1. Make up a mixture of AMP, ADP, and ATP (sodium salt preparations to 0.1 mg/mL each, pH 7.0).
2. Load the sample collector with empty vials.
3. Either spin the sample at 12,000g for 5 min in a microfuge, or pass through a filter (0.5- μ m) to remove particulate matter.
4. Mix the sample with 0.25 mL of nucleotide mix as internal markers. Make up sample plus nucleotide mix to 2.0 mL (methods 1 and 2) or 4.0 mL (method 3) with water.
5. Load the sample into the injection loop.
6. Turn the injection valve to bring the sample loop in line with the column, and start the fraction collector and chart recorder as necessary. At the end of a run, remove vials from fraction collector and add scintillation fluid (*see Subheading 4., Note 3*). Repeat from **step 2** for further samples.

3.6. Shut-Down

At the end of a day of analysis:

1. If necessary, perform any column-cleaning procedure (*see Subheading 3.8.*).
2. Isolate the column and reprime pump B with water
3. Place the column back in line and pump for 10–15 min with water at the same flow rate used in the analytical gradient.
4. Isolate the column once more, and reprime both pumps with alcohol/water storage mix (70% methanol for silica columns, 24% ethanol for MonoQ, filtered and degassed!).
5. Switch the column back in line and pump for 20 min at half the flow rate used in the analytical gradient.
6. Remove the column and seal the column ends before storage

3.7. Overnight Shut-Down

For an overnight shut-down, it is still advisable to flush the column and pumps with water but not necessary to transfer the column to alcohol/water mix. Therefore, carry out the procedure in **Subheading 3.4., steps 1–3**.

3.8. Column Cleaning

Column cleaning becomes necessary either when the column becomes blocked through particulate matter, or when a loss of performance occurs through functional groups being occupied by molecules not removed by the elution buffer. This can be caused by tight binding of higher classes of phosphoinositols (such as InsP₅ and InsP₆) not eluted by moderately weak buffers. If the standard elution procedure removes classes up to InsP₄, then clean the column with high-salt buffers at the end of each day. If weaker elution buffers are used such that InsP₃ or InsP₄ also remain, then clean the column between samples using a higher salt strength buffer than solvent B, or prefractionate samples into individual phosphoinositol classes (see Chapter 2) and desalt by extensive lyophilization before injection.

3.8.1. For Silica Based HPLC Columns

1. Protection from blockage by particulate matter is through sample and solvent filtration. If pressure is elevated to more than twice that of a new column, change the disposable guard cartridge.
2. For removal of higher phosphoinositols, inject 2.0 mL of 10 mM acetic acid or a salt buffer with a higher salt content than solvent B, buffered to pH 3.5.

3.8.2. For the MonoQ FPLC Column

1. If pressure is elevated to twice normal, reverse the column and thoroughly clean using the protocol described in HPLC Method 3 (**Subheading 3.4.**) to remove particulate matter.
2. If blockage is not overcome, then the filter should be replaced.
3. If blockage is still not overcome, some packing material (1–2 mm) should be removed from the top of the column.
4. For removal of higher phosphoinositols between sample injections, inject 1 M salt buffered to low pH (for further details, see the instructions supplied with the column).

3.9. Representative Separations of Phosphoinositols/ Glycerophosphoinositols Using Methods 1, 2, and 3

Standards used in the figures shown were purchased (Ins(1,4)P₂, Ins(1,4,5)P₃, and Ins(1,3,4,5)P₄) or prepared by the methods described as follows: a mixture of Ins1P and Ins4P by alkaline hydrolysis of Ins(1,4)P₂; Ins(1,3)P₂ and Ins(3,4)P₂ by incubation of Ins(1,3,4)P₃ or Ins(1,3,4,5)P₄ with rat brain homogenate in the presence or absence of EDTA, respectively; Ins(1,3,4)P₃ prepared by incubation of Ins(1,3,4,5)P₄ with erythrocyte ghosts (4, 5). In addition to these standards, aqueous-tissue extracts containing a mixture of phospho-

inositols were prepared from cultured bovine adrenocortical (zfr) cells that had first been prelabeled for 42 h with [^3H]inositol and incubated in Li^+ containing buffer with or without angiotensin II (10^{-7} M , 15 min) before extraction with perchloric acid and neutralization with freon/octylamine (6). The [^3H]phosphoinositides from the membranes of prelabeled zfr cells were also recovered by acidified Bligh and Dyer extraction of the PCA pellets, and immediately converted to a glycerophosphoinositol mixture by methylamine deacylation (see Chapter 2 for further details).

3.9.1. Method 1

The separation of phosphoinositol standards and tissue extracts by method 1 are shown in **Fig. 1**. Retention times for adenine nucleotides on this system are 24.0 min (AMP), 36.4 min (ADP), and 55.0 min (ATP). The method resolves $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$, but separation of the monophosphate isomers $\text{Ins}1\text{P}$ and $\text{Ins}4\text{P}$ is poor and, whereas the bisphosphate isomers $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(3,4)\text{P}_2$ resolve, the bisphosphate isomer $\text{Ins}(1,3)\text{P}_2$ comigrates with $\text{Ins}(1,4)\text{P}_2$ (see **Subheading 4., Note 2**).

3.9.2. Method 2

This type of separation procedure is widely used because of its ability to achieve separation of the inositol bisphosphate isomers $\text{Ins}(1,3)\text{P}_2$, $\text{Ins}(1,4)\text{P}_2$, and $\text{Ins}(3,4)\text{P}_2$, as well as the inositol trisphosphate isomers $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ (**Fig. 2A**), and the glycerophosphoinositols (**Fig. 2B**). In the method described here, resolution of the monophosphates $\text{Ins}1\text{P}$ and $\text{Ins}4\text{P}$ is not to baseline (**Fig. 2A**). However, this can be achieved, and separation of the other peaks further improved with this buffer system by modifying the gradient shape further (e.g., see **refs. 7 and 8**) (see **Table 1**). Retention times for adenine nucleotides on this system are 22.5 min (AMP), 37.6 min (ADP), and 62.0 min (ATP).

3.9.3. Method 3

This method successfully resolves both the $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ forms of inositol trisphosphate and gives good separation of $\text{Ins}1\text{P}$ and $\text{Ins}4\text{P}$ (**Fig. 3A**). This procedure also resolves the glycerophosphoinositols from the phosphoinositols (**Fig. 3B**). A limitation of this method is its inability to resolve $\text{Ins}(3,4)\text{P}_2$ and $\text{Ins}(1,4)\text{P}_2$, although separation of $\text{Ins}(1,3)\text{P}_2$ and $\text{Ins}(1,4)\text{P}_2$ is good (**Fig. 4**). Retention times for adenine nucleotides on this system are 17.2 min (AMP), 36.7 min (ADP), and 52.5 min (ATP).

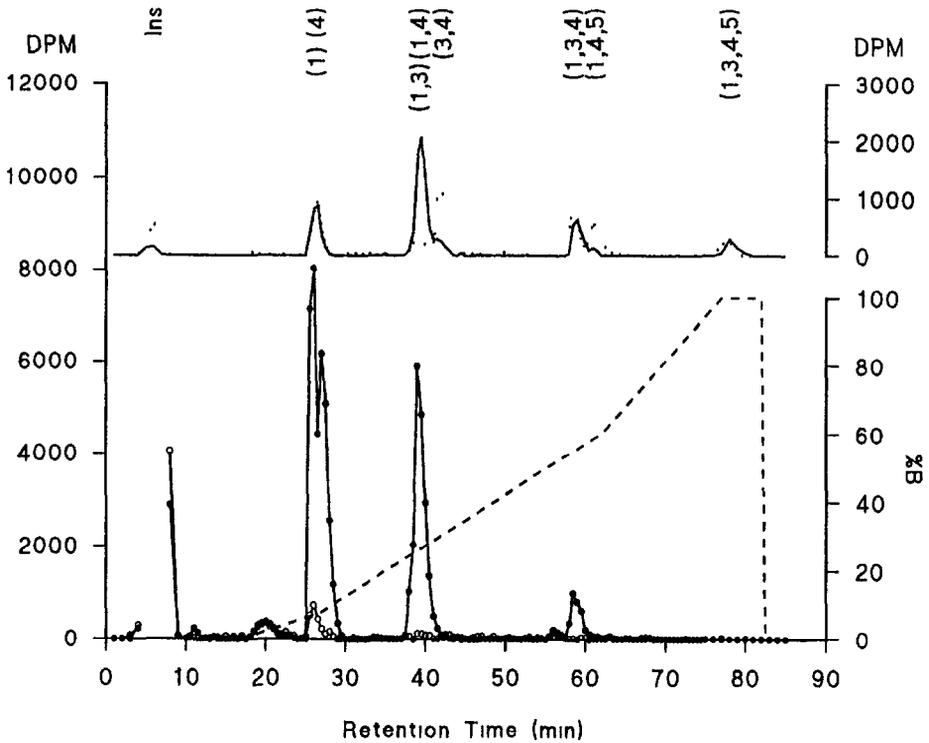


Fig. 1 HPLC method 1: separation of standards and tissue extracts. The separation of [^3H]inositol-labeled standards and tissue extracts by HPLC method 1 are shown. The isomeric identity of each standard is given by numbers in brackets where the numbers refer to the location of the phosphate groups on the inositol ring. Hence (1,4,5) refers to $\text{Ins}(1,4,5)\text{P}_3$. Standards were applied in two separate runs [run 1, solid line; (1),(4),(1,3),(1,4),(1,3,4), (1,3,4,5); run 2, dotted line; (1),(4),(1,3),(3,4)(1,3,4),(1,4,5), (1,3,4,5)] and the results are shown superimposed (top profiles). The lower profiles show the results for [^3H]phosphoinositol-containing cell extracts recovered from zfr cells that had been incubated with (●) or without (○) angiotensin II ($10^{-7} M$, 15 min; see refs. 5 and 6). The programmed elution gradient is also drawn as a broken line, and is corrected for the dead time of the system.

4. Notes

1. Attention should be paid to the fact that both monobasic and dibasic forms of ammonium phosphate exist. There are several similar methods in the literature using one or other of the ammonium phosphate salt forms to prepare solvent B. These buffer-preparation methods should not be confused because if the dibasic salt is mistakenly used in place of the monobasic salt, then an elution buffer of double salt strength will result. At high concentrations, the ammonium phosphate becomes insoluble at room temperature, and any precipitation of salt in the HPLC equipment will lead to serious damage.

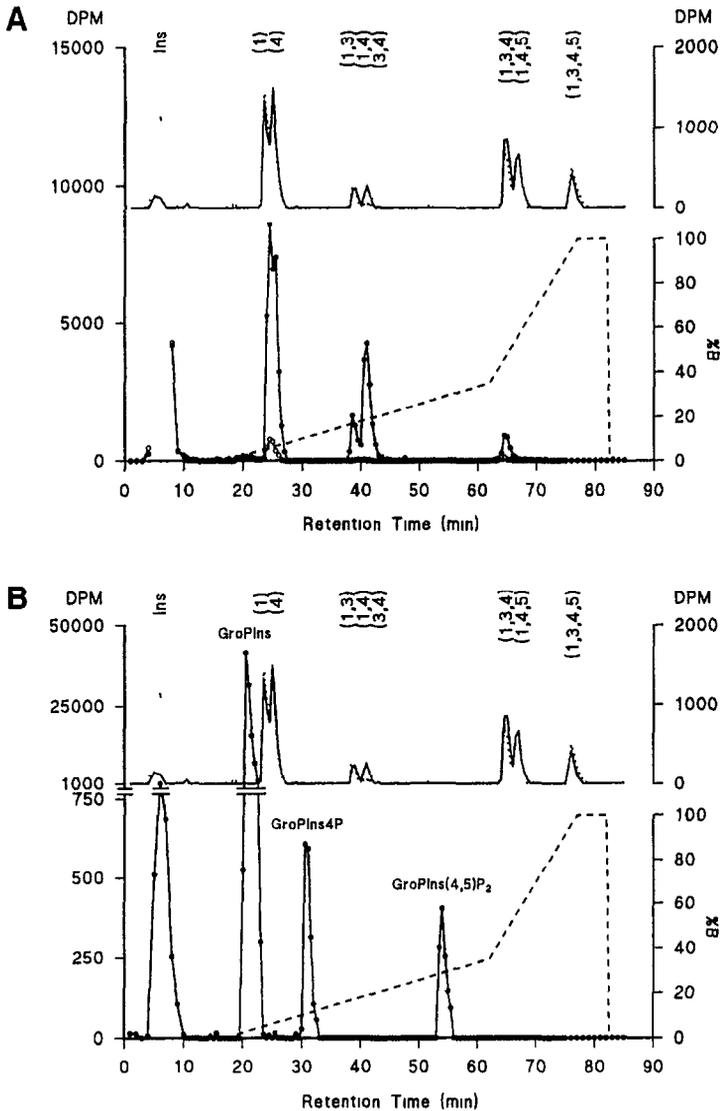


Fig. 2. HPLC method 2: separation of standards and tissue extracts. (A) The separation of [³H]inositol-labeled standards [top superimposed: run 1, solid line, (1),(4), (1,3),(1,4),(1,3,4),(1,4,5),(1,3,4,5): run 2, dotted line; (1),(4),(1,3),(3,4),(1,3,4), (1,3,4,5)] and [³H]phosphoinositol-containing tissue extracts (lower profiles, [○] unstimulated; [●] stimulated) are shown (see Fig. 1), together with the gradient program (broken line) corrected for dead time. (B) The separation of [³H]inositol-labeled standards (top profiles—for details, see [A]) and glycerophosphoinositols ([●], lower profile) are shown.

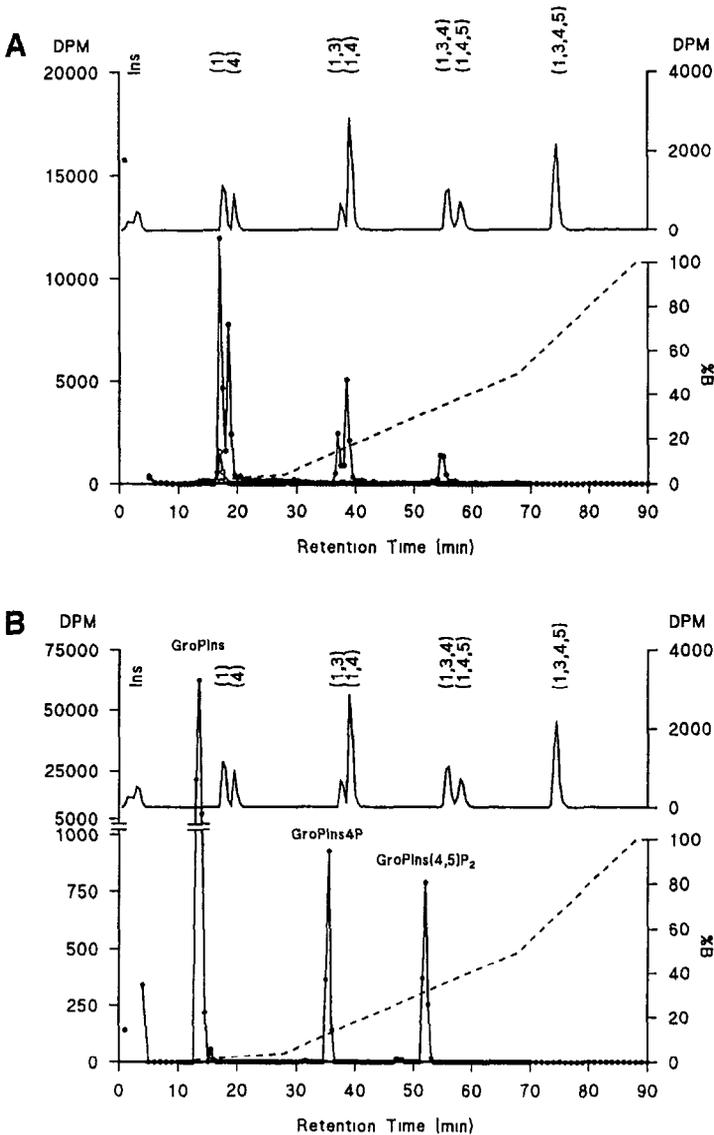


Fig. 3. HPLC method 3: separation of standards and tissue extracts. (A) The separation of [³H]inositol-labeled standards [top profile, solid line; (1),(4),(1,3),(1,4),(1,3,4),(1,4,5),(1,3,4,5)] and [³H]phosphoinositol-containing tissue extracts (lower profiles, [○] unstimulated; [●] stimulated) are shown (see Fig. 1), together with the gradient program (broken line) corrected for dead time. (B) The separation of [³H]inositol-labeled standards (top profiles—for details, see [A]) and glycerophosphoinositols ([●], lower profile) are shown.

Table 1
Optimized HPLC Methods for the Separation of Phosphoinositols Within a Single Class^a

Class	Isomers resolved	Column	Solvent	Ref
InsP	(1), (2), (4)	LiChrosorb NH ₂	Ammonium acetate/- acetic acid pH 4.0	14
	(1), (2), (4)	Partisil SAX10	Ammonium formate/- phosphoric acid pH 5.0	13
	(1), (4)	Partisil SAX10	Ammonium phosphate/- phosphoric acid pH 4.6	15
InsP ₂	(1,3), (1,4), (1,2), (3,4), (4,5)	Partisphere SAX10	Ammonium phosphate/- phosphoric acid pH 3.8	4
InsP ₃	(1,3,4), (1,4,5), (1,5,6), (4,5,6)	Partisphere WAX5	Ammonium phosphate/- phosphoric acid pH 3.2	16
InsP ₄	(1,3,4,6), (1,3,4,5), (3,4,5,6)	Partisphere WAX5	Ammonium phosphate/- phosphoric acid pH 3.2	17
	(1,3,4,5), (1,3,4,6)	Partisil SAX10	Ammonium phosphate/- ammonia pH 4.75	7
GroPinsP _n	GPI, GPI(3)P, GPI(4)P, GPI(4,5)P ₂	Partisphere SAX10	Ammonium phosphate/- phosphoric acid pH 3.8	4

^aReferences for fully optimized HPLC methods for the separation of glycerophosphoinositols and of the phosphoinositols within a single class are listed. The isomeric forms successfully resolved by the authors, and the column/elution buffer system used are also shown. Note that the isomers are listed in the order of elution

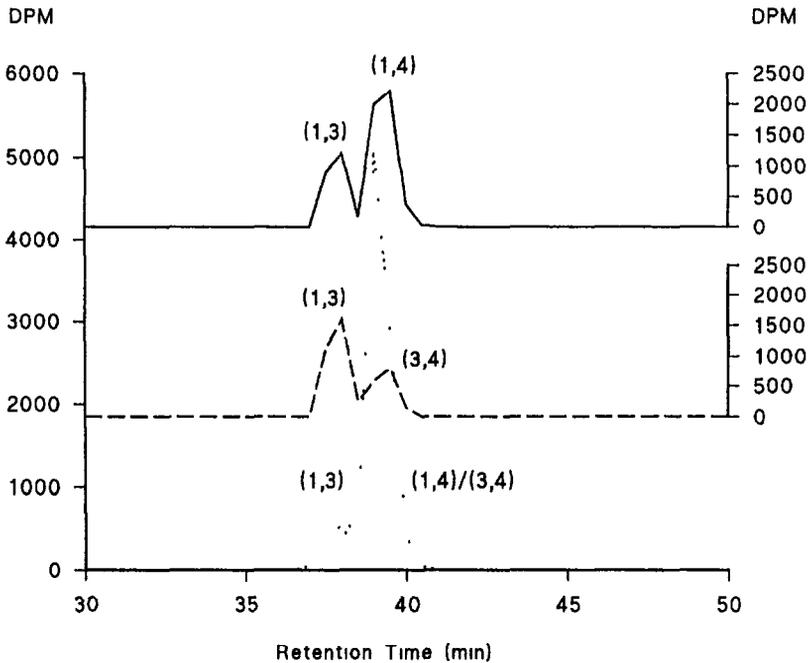


Fig. 4 HPLC method 3: separation of inositol bisphosphate standards. The separation of standards for the inositol bisphosphates $\text{Ins}(1,3)\text{P}_2$, $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(3,4)\text{P}_2$ by method 3 are shown. Samples were run as different mixtures in three separate runs: top profile, solid line, (1,3) and (1,4) mix; middle profile, broken line, (1,3) and (3,4) mix; lower profile, dotted line, (1,3), (1,4), and (3,4) mix. Fraction collection was at 0.5-min intervals in each case

- HPLC method 1 is a variation of the method originally developed for separation of $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ (9), and later modified to allow the further elution of $\text{Ins}(1,3,4,5)\text{P}_4$ (10–12). Separation of $\text{Ins}1\text{P}$ and $\text{Ins}4\text{P}$ has been achieved under modified conditions (13)
- HPLC method 2 uses ammonium phosphate/phosphoric acid for solvent B, in place of the ammonium formate/phosphoric acid buffer used in method 1. A problem that arises with this method in particular is that the ammonium phosphate precipitates out when collected fractions are mixed with scintillation fluid, and an unstable gel can form. To overcome this, fractions can be diluted 1.1 (v/v) with 50% methanol (12) before addition of scintillant
- Unlike silica-based HPLC columns, the MonoQ column used in HPLC method 3 is extremely resistant to strong acid and alkaline attack, and so can be thoroughly cleaned using strong reagents. The column performance can therefore be fully optimized on each day by carrying out the cleaning procedure described. This ensures that consistent performance is observed even over a large number of successive sample runs over consecutive days.

4.1. HPLC Method Development Strategy

In general, inositol phosphates are loaded onto anion-exchange columns in water, as all phosphoinositols are charged at approx. pH 7.0. The phosphoinositols are then eluted using a gradient from water alone (solvent A) to 100% solvent B (a salt buffer). The development or further optimization of any anion-exchange HPLC procedure will require optimization of both the salt concentration and pH of solvent B, as well as the gradient shape/flow rate applied to a given column. To ensure the reproducibility of a method, it may also be necessary to design a column cleaning procedure.

The choice of salt strength and pH of solvent B, and the final gradient shape used will be dependent on the column and the needs of the user. This section is simply intended as a general outline of the order in which to optimize each parameter, and is illustrated by examples from the development of the general separation method described below (HPLC method 3; *see Subheading 3.3.*).

Over the past few years, many HPLC methods have been published that are fully optimized for the separation of individual isomers within a single class of phosphoinositols. Some examples of such methods are listed in **Table 1**.

4.2. Choice of Column

The choice of the column may be influenced by how much information is already available. Separations on a Partisil or Partisphere SAX10 column are well documented, so a published method may provide a suitable starting point. However, it is worth remembering that the more phosphate groups on the inositol ring, the more strongly these groups ionize and bind to the positively charged anion-exchange sites on the column. This binding may become excessive for InsP₄, InsP₅, and InsP₆ on a highly positively charged, strong anion-exchange (SAX) column, necessitating the use of high-salt concentrations and a low pH (3.5 or below) for successful elution. Unfortunately silica columns are easily damaged by acidic buffers of high-salt strength, particularly at a pH below 3.5. In these cases, it is worth considering the use of a weak anion-exchange (WAX) column, to which the phosphoinositols bind less strongly.

In the development of method 3, the MonoQ column was chosen because it was a chemically robust quaternary methyl ammonium anion-exchange column that could withstand regular cleaning by the procedures described, and with anion-exchange properties somewhere between that of silica-based SAX and WAX columns. Also, this column is stable to elution buffers with high-salt content even at a pH as low as pH 2.0. Therefore, any general method developed on such a column could be modified in the future for more detailed separation of any of the phosphoinositol classes.

4.3. Initial Choice of Solvent B Composition

Once a negatively charged phosphate has bound to a positively charged anion-exchange group, then the disruption of binding can be brought about in two ways. The first is to suppress the ionization of the phosphate itself by reducing the pH. The second is to introduce salts of strong acids and bases that will dissociate into anions and cations in solution. These anions and cations can then compete for the phosphate and anion exchange groups, respectively. Optimization of any HPLC procedure will therefore require optimization of both salt concentration and pH of solvent (buffer) B.

The concentration of salt required and the choice of pH for solvent B will be interdependent. Because of the suppression of phosphate ionization at low pH, acidic buffers will generally elute any given phosphoinositol with a lower corresponding salt concentration than more neutral buffers. The aim is to establish a solvent B with pH low enough so binding of the phosphoinositols is not excessively strong and very high salt concentrations are not required for elution, and yet high enough to ensure binding is not too weak and all phosphoinositols within a single class are not eluted together at the first trace of salt. As a rule of thumb on Partisil or Partisphere SAX columns, a pH of 5.0–4.0 will be appropriate for separation of inositol monophosphates, whereas a pH of 4.0–3.5 is generally appropriate for separations of the more strongly ionized trisphosphates and tetrakisphosphates (*see Table 1*). For a general method aimed at the separation of isomers within several phosphoinositol classes, such as those methods described below, a compromise pH must be chosen.

In the event that no information is available on the salt strength necessary to elute the different classes of phosphoinositols from a given column, then for the inositol mono-, bis-, and trisphosphates at least, this can be estimated by attempting to elute the corresponding adenine nucleotides from the column using solvent B preparations of the same pH, but different salt strengths. The elution program should include a 10-min water wash (to allow sample loading), followed by a 30-min linear gradient from 0 to 100% B at approx 1 mL/min. This approach also has the advantage that UV monitoring can be used to give an immediate indication of results from several HPLC runs at different salt strengths. From this information it should be possible to at least estimate an appropriate salt strength for the elution of the phosphoinositols of interest.

Remember that, if samples extracted from tissues are to be analyzed without prior subfractionation, then phosphoinositols of all classes will be present on the column. If a solvent B salt strength and pH are chosen, which is only sufficient to elute the lower phosphoinositol classes (e.g., InsP₁ or InsP₂), then a column-cleaning procedure may be required between sample runs (*see below*) to remove the remaining phosphoinositols.

At the start of the development of method 3, it was found that the adenine nucleotides could be clearly separated on the MonoQ HR5/5 by a linear gradient of 0–100% 1 M ammonium formate/0.1 M formic acid (which has a pH of approx. 4.7). This solvent B was subsequently found to be able to elute InsP, InsP₂, InsP₃, and InsP₄ standards with good baseline separation (Fig. 5).

4.4. Optimization of pH of Solvent B

Having established an initial salt concentration and pH, the next stage is to fully optimize the pH of solvent B to be used in the final method. To do this, a standard mixture of isomeric forms of phosphoinositols in the classes of interest (or if these are not available, tissue extracts known to contain mixtures of isomers in each class of the phosphoinositols) should be separated on a linear gradient of 0–100% B (30 min for initial investigations but 60 min for fine tuning of methods), where solvent B always has the same salt concentration, but the pH is adjusted to different values in each run. A pH is then chosen from the results given, according to the need of the user.

In development of a general separation method (method 3), the salt concentration was fixed at 1 M ammonium formate, but pH was varied in each case between pH 5.5 and 3.5. The samples used in each run were extracts of angiotensin II-stimulated zfr cells because, at this stage, standards for the inositol bis- and monophosphate isomers were not all available, and previous analysis of such tissue extracts on HPLC had suggested the presence of multiple inositol mono-, bis-, and trisphosphate isomers. The samples resolved into major peaks consistent with InsP, InsP₂, and InsP₃ and a minor peak of InsP₄ at all pH values from 5.5 to 3.5 (Fig. 6).

There was no evidence at this stage that InsP isomers could be separated, but the salt concentration used was high, so this was not too surprising. However, there was some evidence of the partial separation of bisphosphate isomers at pH 5.5–4.5, but not below pH 4.5. At higher pH (5.5–4.5), a peak also eluted immediately before InsP, which was consistent with GroPIIns (also known to be present in these cells). At lower pH, this small peak was seen to merge with InsP, and the split InsP₂ peaks merged into a single peak under these elution conditions. Thus, for a method investigating inositol monophosphates or InsP₂ alone, a pH in the range of 5.5–4.5, but not below 4.5, would be chosen.

The effect of pH on resolution of peaks in the inositol trisphosphate region was the opposite of that described for the mono- and bisphosphates. The inositol trisphosphate region only showed a single peak at pH 5.5–4.5, but at the lower values from pH 4.0–3.5, a second minor peak became resolved, eluting before the major InsP₃ peak. This could have been GroPIIns(4,5)P₂ or an unidentified InsP₃ isomer (in fact on analysis with a more optimized system and HPLC methods 1 and 2, it turned out not to be GroPIInsP₂, authors unpublished data); but,

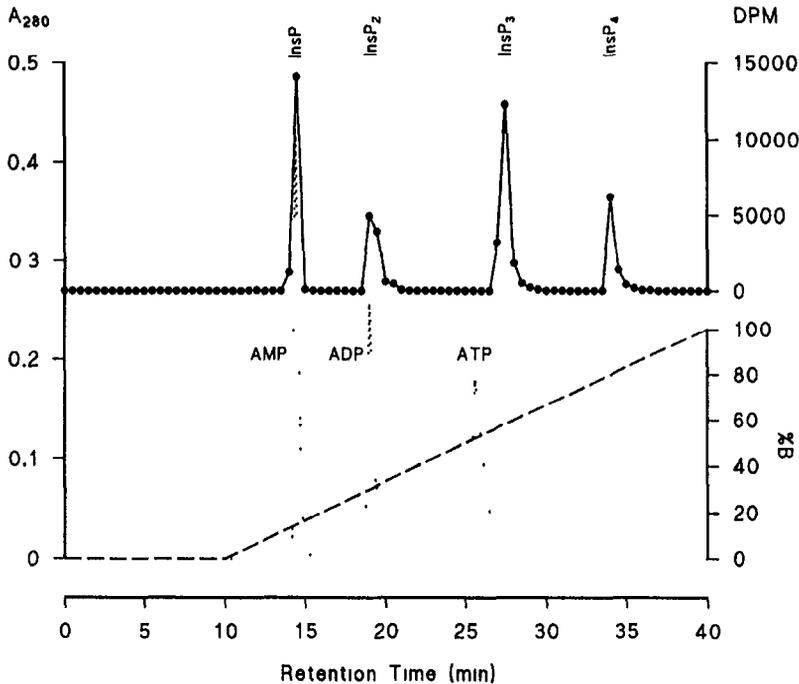


Fig. 5. Initial separation of adenine nucleotides and phosphoinositol standards on MonoQ HR5/5. The separation of [³H]phosphoinositol standards (top profile, [●]) and the adenine nucleotides (bottom profile, dotted line, detected by absorbance at 280 nm) on a MonoQ HR5/5 eluted with a 30 min linear gradient of 0–1 M ammonium formate/0.1 M formic acid (pH 4.7) are shown. The gradient program used is indicated by the broken line (corrected for dead time). AMP, ADP, and ATP and radiolabeled Ins1P, Ins(1,4)P₂, Ins(1,4,5)P₃, and Ins(1,3,4,5)P₄ were coinjected and the column eluted at 1 mL/min throughout while the column outlet was monitored at 280 nm and fractions were collected at 0.5-min intervals.

in either case, if identification of individual isomers of InsP₃ was to be achieved, it would be important to fully resolve this minor peak from the major InsP₃ peaks.

The final pH chosen for further method optimization was pH 4.5. This was based on the argument that, for a general method, good separation of all classes is required, and that at pH 4.0 or below, good separation could be achieved for the triphosphate classes and above, but not the inositol bisphosphates, and certainly not the monophosphates. At a pH of 4.5, the resolution of isomers of the mono- and bisphosphates should be possible by optimization of gradient shape, and it may still be possible to resolve the triphosphates and any tetrakisphosphates.

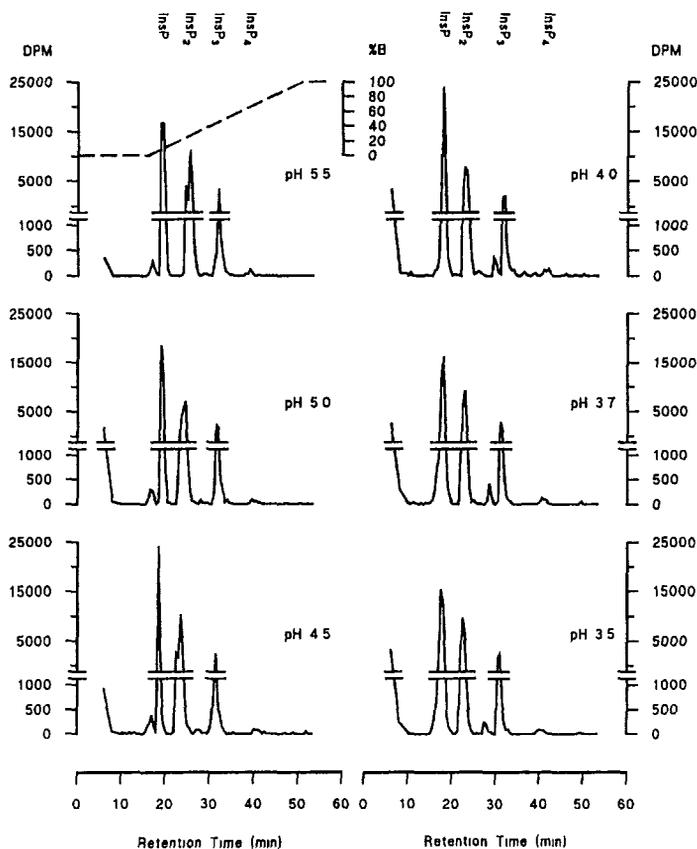


Fig. 6. Effect of pH on separation of phosphoinositols on the MonoQ HR5/5 column. [³H]Phosphoinositol-containing extracts from angiotensin II-stimulated zfr cells were chromatographically separated on a Mono Q HR5/5 with a 35 min linear gradient of 0–1 M ammonium formate buffered to the pH values shown. The gradient program used is shown as a broken line (corrected for dead time). Flow rate was held at 1 mL/min throughout and fractions were collected at 0.5-min intervals. When solvent B was changed after a sample run, the column was subjected to a blank run with the new buffer before the next sample application.

4.5. Optimization of Gradient Program

Having established the composition of solvent B, the final stage is to optimize the gradient conditions through development of the gradient program. It is important to remember that the actual gradient experienced on the column will not be that programmed into the controller as the column must equilibrate with the elution buffer. The volume of the pumping/mixing system and the

column itself will cause the actual gradient experienced to lag behind the programmed gradient. Tighter control of the gradient experienced by the column can be achieved by increasing flow rates and extending the program time.

As a starting point for any method the best approach is to apply a long (90 min), linear gradient (or smooth, concave gradient with no sudden steps) to establish both what the maximum number of resolvable peaks actually is, and at what percent of solvent B they are eluted. It is important to use known standards as well as tissue extracts for testing the method, so that the location of all possible peaks of interest can be determined and any possible adverse effects of sample preparation can be observed. Where the effect of flow rate on peak resolution is to be investigated, it is also best carried out at this stage, using a long, linear gradient.

Having established which peaks can be resolved at what percent of solvent B, the final gradient program can be developed to give optimum separation but in a shorter total time if required. This stage of method development is the most laborious as it is simply a matter of trying different shaped gradients until optimum separation is achieved. It is best to be methodical in the approach used, and to optimize early stages of the program first. Where sudden steps are included, the column and system must be given time to equilibrate. Also, remember that, when a method is highly tuned with shallow gradients separated by sudden steps (such as the kind developed by Dean and Moyer, *ref. 8*), the system may need constant readjustment as column performance degrades. Thus, whereas simple, long, and unstepped gradients may take longer to elute the phosphoinositols, these methods are more robust.

In developing method 3, a flow rate of 2 mL/min was found to slightly improve peak shape, and the long (80 min), concave gradient program shown in **Fig. 3** was found to give baseline separation of the inositol monophosphates as well as the bis- and trisphosphate isomers shown. The minor unidentified trisphosphate was also successfully resolved from the Ins(1,4,5)P₃ and Ins(1,3,4)P₃ peaks found in tissue extracts (**Fig. 3** and unpublished data).

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Measurement of Phosphoinositols and Phosphoinositides Using Radio High-Performance Liquid Chromatography Flow Detection

Lubo Zhang and Iain L. O. Buxton

1. Introduction

1.1. Background

Individual phosphoinositols and phosphoinositides in tissues and cells are commonly analyzed by thin-layer chromatography (TLC), liquid chromatography, and high-performance liquid chromatography (HPLC) after labeling the phospholipid with a radionucleotide such as [γ ³²P]ATP and/or [³H]myo-inositol (1,2). Whereas the TLC method is simple and rapid, it may not be suitable for the routine analysis of inositol phosphates (InsP) in small quantities of tissue or cell samples because of its low sensitivity and poor resolution. Indeed, the analysis of replicate samples in most experiments does not lend itself well to TLC analysis. The inability to separate isomers of the individual InsPs is one of the major disadvantages of low-pressure, ion-exchange chromatography and thus, limits its use. Furthermore, when one employs the ion-exchange approach, it is possible to see increased counts in the high-salt fraction in stimulated samples that are the result of spillover of radioactivity from a lower-order InsP. HPLC separation of InsPs on the other hand, provides high sensitivity and excellent separation of isomers, and thus, is the preferred method if one's goal is to study InsP metabolism and calcium mobilization by inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and/or inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) (3,4).

A disadvantage of the chromatographic methods employing [³H]myo-inositol-radiolabeled samples is the difficulty of quantification of the InsP mass. This is the result of difficulty in labeling all pools of the phospholipid

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to isotopic equilibrium and thus, the inability to determine the specific radioactivity of InsPs being measured. In Chapter 6, an Ins(1,4,5)P₃ radioreceptor assay is described that permits a reasonable approximation of Ins(1,4,5)P₃ mass in cells and tissues. The radioreceptor method, however, only measures Ins(1,4,5)P₃ and thus, InsP metabolism and knowledge of the relative abundance of the inositol lipids is not determined. In Chapter 3, HPLC separation techniques are also described in detail using the conventional approach of fraction collection and counting after separation is complete. In-line monitoring of column performance is achieved by adding adenine nucleotides to samples with UV detection at 254 or 280 nm. In this chapter, we describe a radio-HPLC method for separation of both labeled InsPs and their deacylated lipid precursors using in-line, real-time radioactivity monitoring. This technique has obvious advantages but also requires specific modifications of the HPLC conditions to achieve compatibility with the detector. In addition, whereas procedures for extraction and recovery of phosphoinositols and phosphoinositides from cells are described in detail in Chapter 1, we also describe modified procedures suitable for recovery of phosphoinositols and phosphoinositides from whole tissues.

1.2. Sample Preparation

Depending on one's experimental goal, two different strategies of sample preparation have been employed. If one is only interested in InsPs, cell or tissue samples are lysed and homogenized in either trichloroacetic acid (TCA) or perchloric acid (PCA), which serves to terminate metabolic reactions and precipitate protein, and liberate InsPs in the supernatant. After incubation on ice for 15 min, the homogenate is centrifuged, and the supernatant collected. The acid is removed with either freon/tri-*n*-octylamine for PCA-treated samples or water-saturated diethyl ether for TCA-treated samples prior to analysis (described in more detail in Chapters 1 and 6). The use of PCA precipitation may be a preferred method for preparation of samples for HPLC as the supernatant tends to contain less particulate matter following centrifugation (2).

If one is interested in both phosphoinositides and InsPs, a different sample preparation strategy can be used. Most commonly, the extraction of total lipids from tissue or cell samples is performed by homogenization/solubilization with chloroform-methanol-HCl solution (4,5). After centrifugation of the homogenate, both the organic and aqueous layers are recovered separately. The aqueous upper layer contains the InsPs and can be directly used for HPLC separation. The lipids in the organic layer are subsequently deacylated by chemical removal of the fatty acid chains (4,6,7) and analyzed as glycerophosphoinositol phosphate species on HPLC.

1.3. Radio-HPLC Flow Detection

There are many HPLC methods developed using different columns, solvent mixtures, and modes of detection for the analysis of InsPs (2,4,8). In general, the separation of InsPs depends on the type and size of the column, the composition of the solvent mixture, and the gradient of the solvents. Among different columns used, a Whatman (Clifton, NJ) Partisil strong-anion-exchange (SAX) column or its equivalent is recommended. Because short columns of high plate number provide a rapid and adequate separation of InsPs, one is advised to choose a Partisil SAX 5 (4.6 × 100 mm) over the longer SAX 5 (4.6 × 250 mm) column for this application.

Linear and/or stepwise gradients of aqueous mobile phases consisting of HPLC-grade water and ammonium salts are used to separate InsPs. Ammonium salts commonly employed are ammonium formate, ammonium acetate, and ammonium phosphate. Acidic solvents (pH 3.5–4.0) generally produce sharp separation of the higher-order InsPs. For separation of InsP isomers at low pH, some have employed a stepwise gradient and claimed superior separation (8).

In general, there are two ways to determine radioactivity in each glycerophosphoinositol phosphate and/or InsP after their separation by HPLC. Whereas many studies involve collection of HPLC fractions followed by liquid scintillation counting, in part, the resolution achieved by HPLC separation is often lost using this counting method. On the other hand, a continuous and real-time measurement of radioactivity in each InsP following HPLC separation can be achieved by a radio-HPLC flow detection using an on-line liquid scintillation counter although counting efficiency is reduced under such conditions (see Notes 4 and 14). The authors have used both a Radiomatic® radiochromatography flow detector (Packard, Meridian, CA) and the β-Ram® flow detector from INUS Systems (Tampa, FL). These are essentially liquid scintillation counters modified to count flowing, instead of static samples. When connected to an HPLC, the radioactivity from the HPLC effluent enters a flow cell within the detector and is counted, displayed, and quantified while the sample continues to flow.

2. Materials

2.1. Radiolabeling with [³H]myo-Inositol

1. [³H]myo-inositol (45–80 Ci/mmol) DuPont NEN (Wilmington, DE) NET-906 or equivalent.
2. A suitable labeling buffer: 118 mM NaCl, 4.7 mM KCl, 0.6 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 1.2 mM MgCl₂, 5.0 mM dextrose, 0.5 mM CaCl₂, 10 mM HEPES, 5 mM NaHCO₃ (see Note 1).

3. A suitable stimulation buffer. 115.2 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4 , 1.16 mM MgSO_4 , 20 mM dextrose, 1.8 mM CaCl_2 , 22.14 mM NaHCO_3 , 0.03 mM EDTA. (Li^+ as LiCl may be added at 10 mM; adjust NaCl accordingly, *see Note 6*)
4. Shaking water bath (35°C).
5. Cell incubator if cells are used.
6. Dissection scissors and forceps
7. Low-speed centrifuge suitable for separating cells to tissue pieces from labeling and washing buffers.

2.2. Sample Preparation

1. Extraction solution: chloroform:methanol:concentrated HCl ($\text{CHCl}_3 \cdot \text{CH}_3\text{OH} \cdot \text{HCl}$) 66:33:1 (v/v/v).
2. Methylamine solution: methylamine (40% [wt/v] with water; Aldrich, Milwaukee, WI). water:methanol:*n*-butanol ($\text{CH}_3\text{NH}_2 \cdot \text{H}_2\text{O} \cdot \text{CH}_3\text{OH} \cdot \text{CH}_3[\text{CH}_2]_3\text{OH}$) 24:16:40:10 (v/v/v/v) (*see Note 2*).
3. Butanol reagent: butanol ($\text{CH}_3[\text{CH}_2]_3\text{OH}$) petroleum ether:ethyl formate ($\text{HCO}_2\text{C}_2\text{H}_5$) 20:4:1 (v/v/v).
4. Glass-glass homogenizer (Kontes, Vineland, NJ).
5. Glass or chloroform-resistant test tubes.
6. Refrigerated, low-speed centrifuge.
7. Vortex mixer.
8. Shaking water bath capable of 53°C
9. Liquid nitrogen.
10. Distilled water
11. Freeze-dryer; centrifugal concentrator/dryer (SpeedVac, SAVANT, Farmingdale, NY).
12. 1-mL Syringes.

2.3. Radio-HPLC Flow Detection

1. [^3H] InsP standard mix (2 μCi): [^3H]Ins(1)P, [^3H]Ins(1,4)P₂, [^3H]Ins(1,4,5)P₃, [^3H]Ins(1,3,4)P₃, and [^3H]Ins(1,3,4,5)P₄ (DuPont NEN).
2. HPLC-grade water.
3. Salt buffer for HPLC: 0.05 M ammonium phosphate, 2 M ammonium phosphate, pH 3.8 (*see Note 9*).
4. Whatman Partisil SAX 5 analytic column (10 cm) or equivalent.
5. 1-mL Syringes.
6. HPLC-sample filters (cellulose acetate) with pore size of 0.45 μm .
7. High-ionic-strength compatible scintillation fluid: IN-FLOW[®] BD (INUS Systems) or FLO-SCINT IV from Radiomatic (Meridian, CA; *see Note 3*)
8. HPLC-sample vials.
9. HPLC equipment: gradient HPLC equipment, preferably with autosampler
10. Computer-controlled, on-line radiochromatography flow detector: Radiomatic flow detector A-500 (Packard) or β -Ram flow detector from INUS Systems.

3. Methods

3.1. Radiolabeling with [^3H]myo-Inositol

In general, a higher specific activity of precursor [^3H]myo-inositol will result in a higher signal in inositol lipids and thus, their corresponding InsPs formed following stimulation. There are two [^3H]myo-inositol preparations routinely available (10–25 Ci/mmol; 45–80 Ci/mmol). Choosing the higher specific activity is preferable for most studies. The higher specific activity preparation (containing two rather than one labeled hydrogen atom) is not simply more radiochemical per unit mass, rather it means that, for every [^3H]myo-inositol molecule incorporated, one has introduced twice the radioactivity. The use of a higher radioactive concentration for labeling ($\mu\text{Ci}/\text{mL}$) also increases incorporation of [^3H]myo-inositol into [^3H]phosphoinositides, as it is apparent that free inositol in many cells exists at concentrations well below the K_m (1–2 mM) of the inositol phospholipid synthase (9). The disadvantage of labeling freshly isolated cells and tissues with [^3H]myo-inositol is that it may require long incubation periods that could effect agonist responsiveness of the preparation. For labeling cells in culture, long-term (overnight to 3 d) labeling using relatively low radioactive concentrations (5–20 $\mu\text{Ci}/\text{mL}$) of [^3H]myo-inositol can easily be accomplished. For labeling tissues, short-term (h) labeling using high radioactive concentration (e.g., 200 $\mu\text{Ci}/\text{mL}$) of [^3H]myo-inositol is advisable (*see Note 7*), but the data from such studies may be compromised if near steady-state labeling is not achieved.

Because different phospholipids require different incubation periods of [^3H]myo-inositol to reach isotopic equilibrium (8), it is important to measure the time-course of radioisotope incorporation in individual phospholipids to ensure that all pools of the phospholipid have been labeled to steady state. For many cells and tissues, where agonist stimulated InsP accumulation has been studied, control experiments such as these may not have been performed. Incorporation of [^3H]myo-inositol (200 $\mu\text{Ci}/\text{mL}$) into the inositol-containing phospholipids in gastrointestinal smooth muscle; phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP₂) reaches apparent steady state by 3 h (Fig. 1).

1. Dissect tissues into 5- to 20-mg pieces (it is suggested that tissue wet weight be used to normalize radioactivity).
2. Incubate tissues with [^3H]myo-inositol in labeling buffer at 35°C for 3 h (different tissues may require different labeling times to reach apparent steady-state labeling). The labeling buffer should be adequately oxygenated with H₂O-saturated gas.

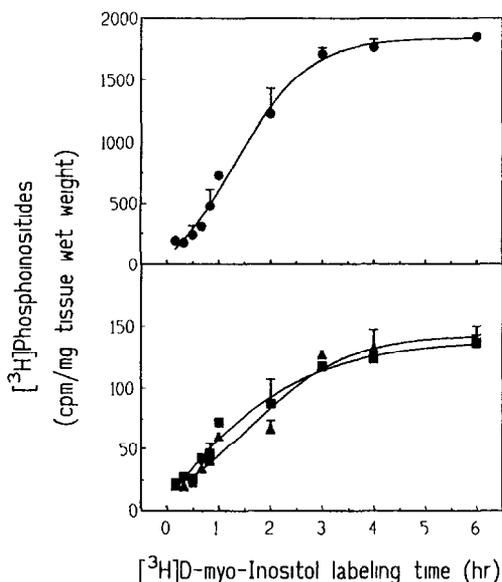


Fig. 1 Time-course of [^3H]myo-inositol labeling of phosphoinositides in canine colonic circular smooth muscle. Labeled phosphoinositides were extracted and separated by HPLC on Partisil SAX column, as described in the text. Areas under the HPLC peaks, in cpm, were quantitated by an on-line scintillation flow-detector and expressed in terms of tissue wet weight. The identity of each lipid was verified by deacylation of lipid standards. Samples were incubated with [^3H]myo-inositol (200 $\mu\text{Ci}/\text{mL}$) for various times as shown. Values for PI (●), PIP (■), and PIP₂ (▲) are the mean \pm SE of two experiments performed in triplicate. (Reprinted with permission from *Molecular Pharmacology*.)

3. After labeling, wash tissues with six changes of fresh Krebs' buffer over 30 min to remove unincorporated radioactivity. Subject tissues to appropriate stimulation in physiological buffer (*see Note 6*).
4. Terminating reactions can be achieved several ways from snap-freezing tissues in liquid N₂ (preferred when further manipulation is desired prior to extraction), to direct addition of the extraction solution for measurement of both lipid and InsPs (*see Subheading 2.2., step 1*) or TCA/PCA when measurement of InsPs alone are desired.

3.2. HPLC Sample Preparation

Lipids and water-soluble inositol phosphates are extracted simultaneously from tissue samples following stimulation using chloroform:methanol:HCl reagent. Keep the samples frozen in liquid nitrogen before the extraction and keep them on ice throughout the procedure (*see Note 8*).

3.2.1. Extraction and Separation of Inositol Phosphates and Phosphoinositides

1. For approx 20 mg tissue (wet weight), add 1 mL ice-cold $\text{CHCl}_3 \cdot \text{CH}_3\text{OH} : \text{HCl}$ (66:33:1, v/v/v)
2. Homogenize at 0–4°C for 1 min.
3. Rinse the homogenizer two times with 1 mL H_2O each time.
4. Combine the H_2O (2 mL) with the homogenate and vortex for 30 s.
5. Add 1 mL $\text{CHCl}_3 \cdot \text{CH}_3\text{OH} \cdot \text{HCl}$ and 1 mL H_2O to the homogenate and vortex again for 30 s.
6. Incubate samples on ice for 3 h. Vortex for 10 s every 30 min.
7. Centrifuge for 10 min at 400g (4°C).
8. Following centrifugation, the sample is comprised of three layers, an aqueous layer on the top containing water-soluble inositol phosphates, an organic layer on the bottom containing lipids, and a middle layer of insoluble material
9. Collect the top, aqueous phase (care should be taken to avoid disturbing the phases) Retain the lower phase for recovery of phosphoinositides (**Subheading 3.2.3.**).
10. Lyophilize the sample taken from the aqueous phase to dryness using SpeedVac centrifuge (requires approx. 4–5 h).
11. If samples are not analyzed by HPLC immediately, do not resuspend samples with H_2O . Keep dried samples covered in the freezer (–20°C) until use within 2–3 d
12. Add 0.2–1.0 mL HPLC-grade H_2O to dried samples and vortex for 30 s.
13. Withdraw samples using a 1-mL syringe
14. Filter samples using cellulose syringe filters with pore size of 0.45 μm into clear glass vials used for HPLC
15. Samples are now ready for radio-HPLC analysis

3.2.2. Monitoring Recovery of Inositol Phosphate Extraction

1. Dilute [^3H]inositol phosphate mix (DuPont NEN), containing Ins(1)P, Ins(4)P, Ins(1,4)P₂, Ins(1,4,5)P₃, Ins(1,3,4)P₃, Ins(1,3,4,5)P₄, InsP₆, from 2 to 100 μL using H_2O .
2. Add 2 μL of the diluted [^3H]inositol phosphate mix to unlabeled control tissue samples to be processed alongside experimental samples
3. Repeat steps 1–15 in the **Subheading 3.2.1.**
4. Add 2 μL of the diluted [^3H]inositol phosphate mix directly into 0.2–1.0 mL HPLC-grade H_2O , and filter as with experimental samples into a glass HPLC-sample vial.
5. Spiked samples and standards can now be separated by HPLC
6. After radio-HPLC analysis, determine the percent recovery of each inositol phosphate as (spike sample CPM/standard CPM) \times 100 (*see Note 4*).

2. Reconstitute dried lipids in 1 mL methylamine solution (*see Subheading 2.2., item 2*).
3. Incubate the samples at 53°C in shaking water bath for 45 min.
4. Lyophilize the sample to dryness using a SpeedVac concentrator (requires approx 2–3 h).
5. Add 1 mL H₂O to the sample and vortex for 30 s.
6. To remove acyl-moieties, add 1 mL butanol reagent (*see Subheading 2.2., item 3*) and vortex for 30 s.
7. Centrifuge for 10 min at 400g at 4°C.
8. Following centrifugation, the sample is comprised of two layers, an aqueous layer on the bottom containing water-soluble glycerophosphoinositol phosphate species and an organic layer on the top containing acyl-moieties
9. Collect and save the aqueous phase.
10. Collect the organic phase and lyophilize it to dryness.
11. Repeat **steps 2–8**.
12. Collect the aqueous phase and combine it with the aqueous sample obtained in **step 9**. Save the organic phase.
13. Lyophilize the combined aqueous sample to dryness (approx 4 h).
14. Repeat **Subheading 3.2.1., steps 11–15**
15. Identify each deacylated lipid by repeating steps 1–14 using standards [³H]phosphatidylinositol (PI) (DuPont NEN), [³H]phosphatidylinositol 4-phosphate (PIP) (DuPont NEN), and [³H]phosphatidylinositol 4,5-bisphosphate (PIP₂) (DuPont NEN).

3.2.4. Determine Efficiency of Deacylation

1. Dry the organic phase obtained in **Subheading 3.2.3., step 12** under a stream of N₂
2. Reconstitute in scintillation fluid.
3. Check for radioactivity by counting in a scintillation counter

If the deacylation is complete, the radioactivity in the organic phase obtained in **Subheading 3.2.3., step 12** should be minimal (*see Scheme 1*). If there is a substantial amount of radioactivity remaining in the organic phase, the deacylation should be repeated. One can determine the approximate deacylation efficiency by checking remaining radioactivity in the organic phase from **Subheading 3.2.3., step 15**.

3.3. Radio-HPLC Flow Detection

The inositol phosphates and deacylated phosphatidylinositols (glycerophosphoinositols) are separated by HPLC over a Whatman Partisil SAX 5 column at a flow rate of 0.5–1.0 mL/min. The molecules of interest are identified by the recorded elution times of radiolabeled standards. Quantification of radioactivity of each inositol phosphate is achieved using an on-line liquid-scintillation detector to determine HPLC peak height/area as recorded in counts per minute (CPM).

Time (min)	Inj	2	25	30	35
H ₂ O	100%			100%	100%
NH ₄ H ₂ PO ₄ (0.05 M)		100%			
NH ₄ H ₂ PO ₄ (2.0 M)			100%		

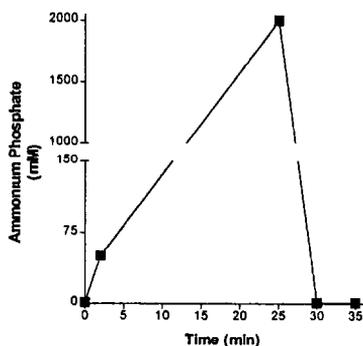


Diagram 1

3.3.1. HPLC Separation

- 1 Use only HPLC-grade H₂O.
- 2 Filter ammonium phosphate buffer using a 0.2- μ m filter before use
3. Degas all solutions (H₂O and ammonium phosphate buffer) before use. This also can be accomplished by vigorously purging solutions with helium for 10 min before use and maintaining a low flow of helium throughout the HPLC procedure as needed (some HPLC systems do not require continual flow for effective purging)
4. Prime HPLC pumps with degassed H₂O and appropriate salt solutions.
5. Equilibrate the column with H₂O at the chosen flow rate for 20 min.
6. Without sample injection, condition the column by running the analytic gradient of the mobile phase used.
7. Inject 90% of sample volume from **Subheading 3.2.1., step 15** and **Subheading 3.2.3., step 14** as needed.
8. Separate the sample using the gradient program in **Diagram 1** (or a similar program) at a suitable HPLC flow rate (the example in **Diagram 1** was accomplished at 0.6 mL/min).
9. The separation of phosphoinositol standards and tissue extracts are shown in **Figs. 2** and **3**. Retention times for phosphoinositols under these conditions are: inositol, 3.88 min; Ins(4)P₁, 12.06 min; Ins(1,4)P₂, 17.06 min; Ins(1,3,4)P₃, 22.43 min; Ins(1,4,5)P₃, 23.4 min; and Ins(1,3,4,5)P₄, 29.12 min. The method resolves Ins(1,3,4)P₃ and Ins(1,4,5)P₃ as shown, and can distinguish Ins(1)P₁ and Ins(4)P₁ as well.
10. The separation of deacylated [³H]myo-inositol-labeled PI, PIP, and PIP₂ in tissue samples (**Fig. 4**) yielded the following retention times under these conditions: PI, 13.25 min; PIP, 15.92 min; and PIP₂, 18.67 min.
11. If the HPLC is heavily used, it is advisable to keep the system running overnight using H₂O at a flow rate of 0.2 mL/min (*see Notes 5 and 10*).
12. If the system must be shut down for several days or longer, isolate the column and prime pumps with H₂O for 10 min. Place the column back in line and pump

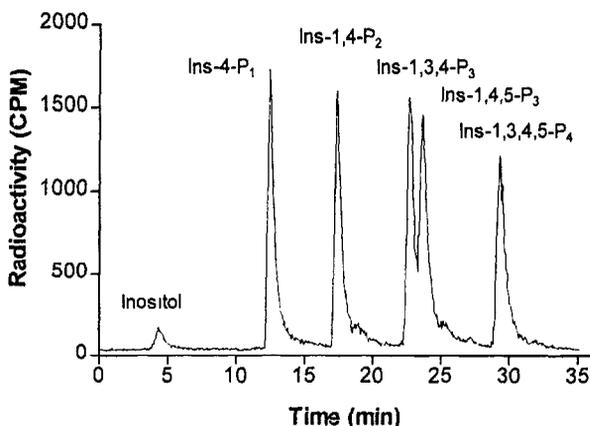


Fig 2. Separation of phosphoinositol standards on a Partisil SAX 5 column. The radioactivity of each [³H]phosphoinositol standard was quantitated as cpm by an on-line scintillation flow detector (INUS β-RAM). Using the gradient program listed in the text, [³H]phosphoinositols standards are well separated and the retention times for phosphoinositols on this system are 3.88 min (inositol), 12.06 min (*Ins*(4)P), 17.87 min (*Ins*(1,4)P₂), 22.43 min (*Ins*(1,3,4)P₃), 23.40 min (*Ins*(1,4,5)P₃), 29.12 min (*Ins*(1,3,4,5)P₄).

for 20 min with H₂O at a flow rate of 0.6 mL/min, followed by 70% (v/v) methanol for 20 min at a flow rate of 0.6 mL/min.

3.3.2. Radioactive Flow Detection

The radioactive-flow detector should be the first detector in line if the HPLC system employs multiple detectors. The radioactive flow detector is essentially an on-line liquid-scintillation counter. The liquid sample, after leaving the HPLC column, flows through tubing into the flow detector where, in the presence of a scintillator, the radioactive emissions are counted and then available to be displayed and quantified. All this is done while the sample continues to flow through the detector cell and finally to waste or recollection (*see Note 11*).

Current radioactive-flow detectors provide a diverter-valve option that allows one to manage the delivery of HPLC flow away from the flow cell if desired. This can be useful during washing and equilibration of the HPLC column. A reservoir of liquid-scintillation cocktail (LS) is connected to a programmable liquid scintillation (LS) pump. The flow rate of the LS pump is digitally programmed by the host computer. A built-in stream splitter, controlled by computer, can precisely split the HPLC sample stream in any desired ratio if only a portion of the sample stream is to be used for radioactive analysis and fraction collection is performed.

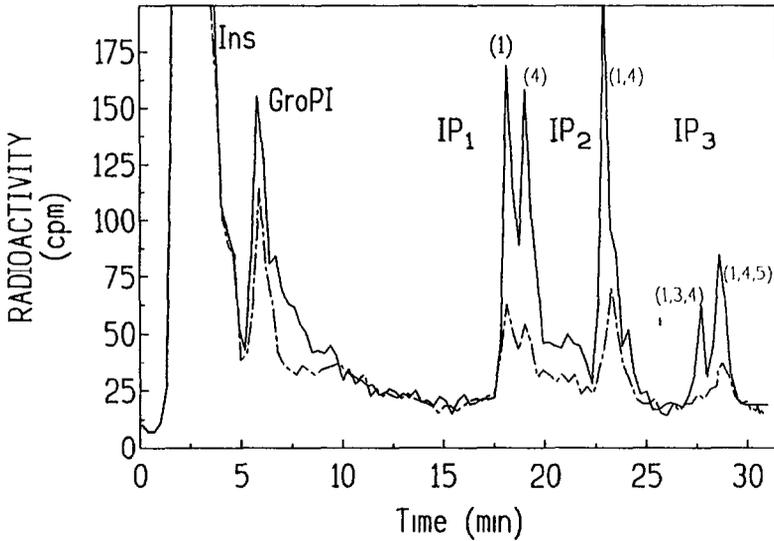


Fig 3. HPLC separation of [^3H] phosphoinositols from a vascular tissue extracts. The radioactivity of each [^3H]phosphoinositol was quantitated as cpm by an on-line scintillation flow detector. The dashed line represents an unstimulated tissue sample, whereas the solid trace is a vessel segment stimulated with catecholamine to activate PLC. Inositol and glycerophosphoinositol are eluted first followed by both isomers of InsP_1 , $\text{Ins}(1,4)\text{P}_2$, $\text{Ins}(1,3,4)\text{P}_3$, and $\text{Ins}(1,4,5)\text{P}_3$. These peaks show different retention times compared to Fig. 2 as a 10-min isocratic step employing water was used to ensure removal of inositol species other than IP_{1-3} (Reprinted with permission from *Life Sciences*)

The internal microprocessor with a large memory buffer capable of storing several hours of data is used to accumulate the radioactive data in real time, and the chromatograms are built, displayed, analyzed, and stored on disk.

1. Calibrate the liquid-scintillation-cocktail pump. This needs to be done upon set-up of the use of the system and every 3 mo if the system is heavily used.
2. Detector setup on the host computer will require:

Isotope.	^3H
Radio update:	3 or 6 s (see Note 12)
Cell type/size:	Liquid/1.5–2.5 mL (see Note 14)
HPLC flow rate.	0.6–1.0 mL/min
LS flow rate.	2.4–4.0 mL/min
LS/HPLC ratio:	4:1 (see Note 13)
Splitter control:	Internal
Split ratio:	100%

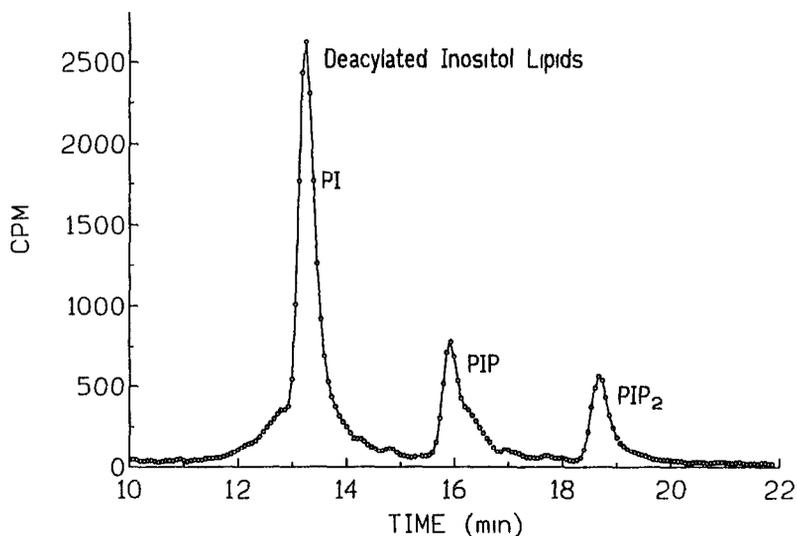


Fig 4 HPLC separation of deacylated [^3H]PI, [^3H]PIP, and [^3H]PIP₂ samples from [^3H]myo-inositol-labeled smooth muscle tissues on a Partisil SAX 5 column. Tissues labeled with [^3H]myo-inositol were processed for detection of inositol-containing lipids as described in the text. The radioactivity of each correspondent [^3H]glycerophospho derivative was quantitated as cpm by an on-line scintillation-flow detector. The example data presented were captured from the HPLC data program as an ASCII file and imported in the data presentation program GraphPad Inplot[®] (San Diego, CA). The first 10 min of HPLC flow data were subtracted to remove the large fraction of nonphosphate-containing counts

- 3 Set the sample run conditions such as run file name(s), run length, and set the event table on the host computer as follows:

Time (min)	Action
0.00	Diverter off
0.00	LS pump on
0.00	Start acquisition
35.0	LS pump off

4. Load the sample on HPLC and start the gradient.

4. Notes

1. In many tissues or cells, the transport system responsible for inositol movement across the plasma membrane also transports glucose into the cell. Thus, in these cases, glucose and inositol compete for the same transporter. Therefore, using a low concentration of glucose (5 mM instead of 20 mM) in labeling buffer containing [^3H]myo-inositol significantly increases inositol transport into the cell.

Additionally, one may wish to evaluate the potential use of insulin (often contained in animal sera and required for glucose entry into most mammalian cells) to stimulate hexose transport. Other media can be used for cell or tissue labeling, but it should be noted that, if inositol enters the cell via the hexose transporter, glucose should be kept low. Furthermore, the presence of inositol in complex media such as tissue culture medium or animal sera will result in competition for uptake of radioactive inositol, effectively reducing the specific activity of labeling. It may also be favorable to lower the $[Ca^{2+}]$ in the labeling buffer as this may increase the pool of labeled phosphatidylinositol bisphosphate in the cell (Buxton, unpublished observation).

2. Methylamine (CH_3NH_2) is a flammable gas at ordinary temperature and pressure, and is a fuming liquid when cooled in ice and salt mixture. Handle it in a fume hood with care. Avoid overexposure because it may cause irritation of the eyes and respiratory system, coughing, skin and mucous membrane burns, dermatitis, and conjunctivitis. However, methylamine is stable in methanol (CH_3OH) with *n*-butanol ($CH_3[CH_2]_3OH$) (see **Subheading 2.2., item 2**). See *NIOSH Pocket Guide to Chemical Hazards* (DHHS/NIOSH 90-117, 1990) p. 144.
3. As a practical matter, the investigator is cautioned that not all scintillation fluors are created equal. For example, we have compared the high-salt compatible INFLOW BD scintillant from INUS System to Ecolume[®] (ICN Research Products, Costa Mesa, CA) and found that the counting efficiency at high salt is markedly different between the two. Indeed, whereas there was an improvement in the detection of all radioactive inositol phosphates with INFLOW BD, the greatest improvement was seen in the recovery of InsP species eluting at high salt. A comparison of the recovery of InsP₄ counts using Ecolume to that injected on the HPLC column and 18%, while in an identical sample detected using INFLOW BD, the recovery of counts was 41%. This is a crucial difference since the amount of InsP₄ expected in biological samples is quite low.
4. The recovery of radioactivity through sample preparation and separation by HPLC will include the efficiency of InsP detection that is characteristic of the flow counter and the particular flow conditions employed. Whereas knowledge of the flow-counting efficiency is important and can be determined separately using spiked samples directly separated by HPLC, determination of recovery following sample preparation steps is crucial for success. Thus, one can count an aliquot of the spiked control tissue sample in the scintillation counter to get an accurate measurement of recovery. The recovery of counts determined in this way should not fall significantly below 80%.
5. The ammonium salts, particularly acidic ammonium phosphate, are hard on HPLC systems, and it is important to flush the system including the column adequately with water between samples (a 15-min posttime is advisable) and following a day's use for at least 2 h. It is important to note that the silica material that constitutes the matrix of the column packing is eroded under the conditions described herein and thus, use of a silica saturator (a silica column used in-line upstream from the point of injection) is recommended.

6. Many investigators have employed Li^+ (as 10 mM LiCl) pretreatment of tissues prior to stimulation on the assumption that it will enhance the accumulation of inositol polyphosphates. One should note that there is little evidence in smooth muscle tissues that blocking the inositol phosphomonoesterases with Li^+ has any effect on the breakdown of InsP_3 . Indeed, one of us has shown that Li^+ does not block the phosphomonoesterase in myometrial smooth muscle (11). Thus, the investigator is encouraged to determine the effect of Li^+ rather than employing it blindly. This notwithstanding, when employed, lithium can be added at a concentration of 10 mM and included for 20 min prior to stimulation
7. It is advisable to use high radioactive concentration of $[^3\text{H}]\text{myo}$ -inositol to label tissue samples. To improve cost-effectiveness, the volume of labeling buffer should be kept as small as possible. The time-course for labeling the phosphatidylinositols is quite different in a given tissue as well as in different tissues. Thus, to ensure that all pools of the phospholipid have been labeled to isotopic equilibrium, it is recommended that the time-course be determined for radioisotope incorporation in individual phospholipids such as PI, PIP, and PIP_2 . Although TLC can be employed for this, details presented here for the analysis of the phosphatidyl inositols by HPLC is preferred.
8. Repetitive freezing and thawing of the samples should be avoided because of a potential significant loss of inositol phosphates. After extraction of the tissues, freeze-drying should be used for reduction of sample size. If the samples are not immediately used in radio-HPLC analysis, it is advisable that they are kept dry and saved at -70°C until use
9. On Partisil SAX columns, the HPLC mobile phase with $\text{pH} \leq 4.0$ will generally provide sharp separation of higher inositol phosphates with a lower corresponding salt concentration than more neutral buffers. However, the pH of the salt buffer should be kept above pH 3.0 because of potential hydrolysis of inositol phosphates.
10. The use of H_2O in the separation of inositol phosphates is particularly useful in cleaning and re-equilibration of the column as the phosphate buffers are highly corrosive and thus, hard on the HPLC system. Furthermore, since water will tend to erode the silica column over time, it is useful to install a saturator (available from column suppliers) in order to presaturate the HPLC mobile phase with silica prior to entry onto the column.
11. Inside the on-line scintillation-flow detector, the sample is counted while it resides in the flow cell. Unlike a static liquid-scintillation counter, where the counting time is a selectable period in which a sample resides in a counting chamber, in a radiochromatography-flow detector, the counting time for the sample is actually the amount of the time that the sample flows into and out of the cell. The time is determined by the size (volume) of the flow cell and the total flow rate of the system (the combined HPLC flow rate and the cocktail rate).
12. The selection of the update time used in calculating the current CPM in the flow cell is determined by the separation of inositol phosphates by the HPLC. For the method given in this chapter, it is recommended that 6 s be used as the update

time (3-s updates will provide better resolution when sample counts are adequate). When a shorter run time is used for HPLC gradients and inositol phosphate peaks are very close to each other, less than 6 s should be chosen for the update time. On the other hand, if a longer run time is used for HPLC gradients and inositol phosphate peaks are well-resolved, use of a update time of 6 s or greater will give greater sensitivity.

13. Because ammonium phosphate tends to precipitate when it mixes with regular scintillation fluid and can thus cause a blockage and system failure of the flow detector, it is very important to choose an efficient liquid scintillation cocktail that mixes well with high concentration of salt solution and produces a homogeneous mixture. It is recommended that FLO-SCINT IV or IN-FLOW BD (described in **Subheading 2.**) be used because they are specifically designed for use with ammonium-phosphate and ammonium-formate gradients up to 2 M in concentration. It should be kept in mind that the total flow rate through the system should be minimized to increase the residence time of the sample in the flow cell. Therefore, it is desirable to choose the lowest ratio of scintillation cocktail to HPLC solvent that gives a stable clear mixture and a good counting efficiency (we have had good success with a 4:1 ratio)
14. The detecting sensitivity and resolution of inositol phosphate peaks after HPLC are determined by the detector flow cell size, the total flow rate, and the update time. The use of a large flow cell, a slow flow rate, and a long update time increases sensitivity, but decreases peak resolution. If the maximum sensitivity is desirable for low level counting, use shallow HPLC gradients, which provide better separation but longer chromatographic run times than steep gradients, a larger flow cell, and a slow total flow rate.

Acknowledgments

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Preparation of [³H]Phosphoinositol Standards and Conversion of [³H]Phosphoinositides to [³H]Phosphoinositols

Ian M. Bird

1. Introduction

Any chromatographic procedure for the separation of phosphoinositols will require a supply of standards, both for validation of the technique and identification of the products. Tritium-radiolabeled standards are commercially available in 1–2 μCi quantities for Ins(1,3,4,5)P₄, Ins(1,4,5)P₃, Ins(1,3,4)P₃, Ins(1,4)P₂, Ins4P, and Ins1P. Although such standards can often be purchased as a standard mixture, it is preferable to purchase them unmixed so they can be used as starting materials for other standards. At present, other standards that are unavailable commercially must be prepared as required using chemical or enzymological methods.

1.1. Enzymatic Preparation of Ins(1,3)P₂, Ins(3,4)P₂, and Ins4P Standards

In rat brain homogenates, Ins(1,3,4)P₃ can only be dephosphorylated through 1-phosphatase or 4-phosphatase activity. The studies of Batty and coworkers (1) have shown that, in the presence of excess EDTA and absence of free Mg²⁺, the 4-phosphatase activity remains, but 1-phosphatase activity is abolished and Ins(1,3,4)P₃ is metabolized exclusively to Ins(1,3)P₂. In the presence of Mg²⁺ (2 mM) 1-phosphatase activity exceeds that of 4-phosphatase activity, and Ins(1,3,4)P₃ is predominantly converted to Ins(3,4)P₂. Therefore, to prepare the inositol *bis*-phosphates Ins(3,4)P₂ and Ins(1,3)P₂, the simplest approach is to degrade commercially available Ins(1,3,4)P₃ by incubation with a high-speed

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supernatant prepared from homogenized rat brain, both with and without free Mg^{2+} present.

The studies of Batty and coworkers (1) have also shown that in the same high-speed supernatants of rat brain homogenates, $\text{Ins}(1,4)\text{P}_2$ is metabolized exclusively by 1-phosphatase action to $\text{Ins}4\text{P}$ when 2 mM free Mg^{2+} is present. Thus, the same procedure can also be used to prepare $\text{Ins}4\text{P}$ from $\text{Ins}(1,4)\text{P}_2$.

Rat brain supernatant also contains a 5-phosphatase that is extremely active and will rapidly remove the 5-phosphate from $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$. Thus it is also possible to prepare $\text{Ins}4\text{P}$ and $\text{Ins}(3,4)\text{P}_2$ indirectly from $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, respectively using exactly the same procedure as above. However, in the absence of Mg^{2+} , the activity of the 5-phosphatase is severely inhibited, so it is not possible to prepare $\text{Ins}(1,3)\text{P}_2$ indirectly from $\text{Ins}(1,3,4,5)\text{P}_4$ by this method.

1.2. Preparation of Phosphoinositol Standards by Chemical Modification

There are two basic strategies for the preparation of phosphoinositol standards. The first is to exploit the ease of phosphate migration around positions 1, 2, and 3 under acidic conditions because of their mutually *cis*-orientated hydroxyl groups; the second is to carry out partial alkaline hydrolysis of inositol polyphosphates that contain phosphate groups in the position of interest (see Notes 1 and 2).

Limited alkaline hydrolysis can be used to partially dephosphorylate inositol polyphosphates right down to their monophosphate equivalents, without isomerization (see Note 2). The degradation products so formed will be a mixture containing phosphate groups in the same positions as were present before hydrolysis. Thus hydrolysis of *D-myo*- $\text{Ins}(1,4)\text{P}_2$ will give a mixture of *D-myo*- $\text{Ins}1\text{P}$ and *D-myo*- $\text{Ins}4\text{P}$, whereas hydrolysis of *D-myo*- $\text{Ins}(1,4,5)\text{P}_3$ will give a mixture of three inositol bisphosphates (*D-myo*- $\text{Ins}[1,4]\text{P}_2$, *D-myo*- $\text{Ins}[4,5]\text{P}_2$, and *D-myo*- $\text{Ins}[1,5]\text{P}_2$) and three inositol monophosphates (*D-myo*- $\text{Ins}1\text{P}$, *D-myo*- $\text{Ins}4\text{P}$, and *myo*- $\text{Ins}5\text{P}$). Remember, however, that phosphoinositols may exist in enantiomeric pairs. Thus, alkaline hydrolysis of $\text{Ins}(1,3,4,5)\text{P}_4$ would yield a mix including the monophosphates *D-myo*- $\text{Ins}1\text{P}$, *D-myo*- $\text{Ins}3\text{P}$, *D-myo*- $\text{Ins}4\text{P}$, and *myo*- $\text{Ins}5\text{P}$, but *D-myo*- $\text{Ins}1\text{P}$ and *D-myo*- $\text{Ins}3\text{P}$ are an enantiomeric pair, so as far as HPLC analysis is concerned, the monophosphate products are $\text{Ins}1\text{P}$, $\text{Ins}4\text{P}$, and $\text{Ins}5\text{P}$. Note also that the inositol monophosphate products are not always formed in equimolar amounts because of the differing orientation of the phosphates in each position and the effects of possible neighboring phosphates on reaction rates.

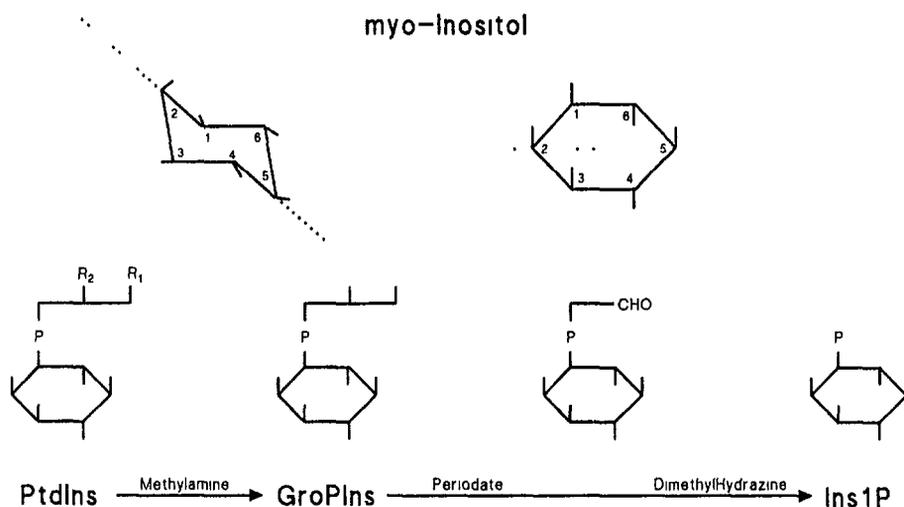


Fig. 1. Basic chemistry of *myo*-inositol compounds: The three-dimensional structure (top left) and Haworth projection of *myo*-inositol (top right) is shown, together with the numbering of the carbon atoms by the D-numbering system. Note that only C-C and C-OH bonds are shown, and the line of symmetry is indicated by a dotted line. (For further details on the stereochemistry of inositol and the phosphoinositols *see* Parthasarathy and Eisenberg [10]). The reaction sequence for the conversion of the phosphoinositides (PtdIns) to phosphoinositols (Ins1P) via glycerophosphoinositol (GroPIIns) and the glycolaldehyde intermediate is also shown (bottom).

1.3. Conversion of Unknown Phosphoinositides into Corresponding Phosphoinositols

The phosphoinositides are extracted from samples of interest by the acidified Bligh and Dyer extraction either directly from the cells or preferably from the insoluble pellet formed by PCA precipitation (*see* Chapter 1). The phosphoinositides recovered in this way cannot be analyzed intact by the HPLC/FPLC methods described in Chapters 3 and 4 because of their insolubility in water. However, they can be converted to their water-soluble glycerophosphoinositol counterparts if the fatty acyl groups are removed (Fig. 1).

Deacylation can be achieved by a variety of methods, including strong acid or strong alkaline hydrolysis; but, in view of the formation of cyclic phosphate intermediates on the inositol ring, and so isomerization in each case (Note 2), these methods are not appropriate here. Instead, complete deacylation without isomerization can be achieved using the methylamine transacylation procedure of Clarke and Dawson (2), exactly as described in Chapter 2. This method

also gives excellent recoveries and so quantification of the glycerophosphoinositols gives an accurate assessment of the phosphoinositide starting materials. Once formed, the glycerophosphoinositols can be separated by HPLC/FPLC (*see below*), but obtaining standards to help identify the products is difficult, and most published methods are optimized to separate the phosphoinositols. Thus, when unambiguous identification of products is required, most workers choose to carry out further conversion of the glycerophosphoinositols to their phosphoinositol counterparts through limited periodate oxidation (**Note 3**) followed by reaction of the glycolaldehyde product with dimethylhydrazine (3,4) (*see Fig. 1*). The phosphoinositol products are then identified using established HPLC procedures. It should be kept in mind that this strategy is suitable for the identification but not quantification of the phosphoinositides, as recovery of the products of the deglyceration procedure described below is only 50–60%.

2. Materials

All chemicals should be HPLC grade or, where not available, analytical grade.

2.1. Enzymatic Preparation of *Ins(1,3)P₂*, *Ins(3,4)P₂*, and *Ins4P* Standards

2.1.1. Preparation of High-Speed Supernatant

- 1 Sodium chloride, normal Saline (0.9% w/v).
- 2 Homogenization buffer. 100 mM KCl, 20 mM NaCl, 2 mM MgCl₂, 25 mM HEPES, adjust pH to 7.4 with KOH.
- 3 Dissection scissors and forceps.
- 4 Polytron tissue homogenizer
- 5 Ultracentrifuge and 10-mL tubes.
- 6 Ice bucket with ice.

2.1.2. Preparation of *Ins(3,4)P₂* and *Ins4P* Standards Using the High-Speed Supernatant

- 1 [³H]Phosphoinositol substrates
- 2 Perchloric acid (PCA), 15% (w/v)
- 3 Bovine serum albumin (BSA); 0.2% (w/v).
- 4 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine (1:1, v/v).
- 5 Incubation medium (composition as for homogenization buffer, *see Subheading 2.1.1., item 2*).
- 6 Microfuge.
- 7 Microfuge tubes (1.5 mL) and floating rack.
- 8 5-mL Glass tubes.
9. Water bath set to 37°C.

2.1.3. Preparation of Ins(1,3)P₂ Standard Using the High-Speed Supernatant

Follow the procedure for preparation of Ins(3,4)P₂, but add 5 mM EDTA to the incubation buffer (see Subheading 3.1.3.).

2.2. Preparation of Phosphoinositol Standards by Chemical Modification

2.2.1. Acid-Catalyzed Formation of Ins2P and D/L-Ins4P from Ins1P

1. Ins1P (cyclohexylammonium salt).
2. Radiolabeled Ins1P standard
3. 2 M Hydrochloric acid.
4. 10 M Sodium hydroxide.
5. Water bath, with lid, set to 80°C.
6. Small glass tubes with nonsealing caps.

2.2.2. Limited Alkaline Hydrolysis of Inositol Polyphosphates

1. 880 Ammonia.
2. Radiolabeled inositol polyphosphate.
3. Liquid nitrogen
4. Mannitol.
5. Pyrex glass ampules (capable of withstanding very high pressure).
6. Ampule-sealing torch and equipment.
7. Freeze-dryer.
8. Oven (capable of 110°C)

2.3. Conversion of Glycerophosphoinositols to Corresponding Phosphoinositols by Limited Periodate Oxidation

1. AG 50W cation-exchange resin (200–400 mesh, Bio-Rad, Hercules, CA)
2. C18 reverse-phase Sep Pak cartridge (Waters Chromatography, Milford, MA).
3. Sodium periodate; 100 mM, see Note 4.
4. Ethane diol; 1% (w/v)
5. Dimethylhydrazine: 1% (w/v) to pH 4.5 with concentrated formic acid.
6. Methanol.
7. 2 M Sodium hydroxide.
8. 1 M Hydrochloric acid.
9. Formic acid
10. Nitrogen gas.
11. Mannitol.
12. Water bath (25°C).
13. Freeze-dryer.
14. Small (6-mL) chromatography column with 70- μ m frit.
15. 10-mL Syringe.
16. Microfuge tubes (1.5 mL) and floating rack.

Important: Prepare the periodate, ethane diol, and dimethylhydrazine reagents fresh.

3. Methods

3.1. Enzymatic Preparation of $Ins(1,3)P_2$, $Ins(3,4)P_2$, and $Ins4P$ Standards

3.1.1. Preparation of High-Speed Supernatant

- 1 Stun and decapitate a rat and remove the cerebral cortex to normal saline (on ice in a small beaker). Chop with scissors and wash free of excess blood by repeated changes of ice-cold normal saline solution.
- 2 Decant off saline solution, transfer fragments to a 25-mL tube, rinse fragments briefly in homogenization buffer, and drain.
- 3 Add 10 mL of homogenization buffer (ice-cold) and homogenize with three \times 15-s bursts of a polytron. Make up to 20 mL with further buffer and transfer to 2×10 -mL centrifuge tubes
- 4 Spin homogenate at 60,000g for 30 min at 4°C. Recover 5 mL of supernatant and keep on ice

3.1.2. Preparation of $Ins(3,4)P_2$ and $Ins4P$ Standards Using the High-Speed Supernatant

- 1 Place 450 μ L incubation medium into a microfuge tube and add substrate (0.1 μ Ci, 0.1 nmol; see **Subheading 1.1.** and **Note 5**) Prewarm to 37°C.
- 2 Add 50 μ L of high-speed supernatant to each tube and incubate at 37°C for 10 min
- 3 Add 250 μ L of ice-cold PCA to terminate phosphatase activity, followed by 500 μ L 0.2% BSA Pellet precipitate material by centrifugation (12,000g for 5 min in microfuge).
- 4 Transfer supernatant to a separate tube (5 mL). Neutralize by addition of 1.5 mL freshly prepared freon/octylamine and mix thoroughly for 10 s. Separate phases by spinning at 1300g for 2–3 min. Recover the upper aqueous phase (containing the products) to a microfuge tube.
- 5 To maximize recovery, wash the freon/octylamine:water interface by adding a further 0.5 mL H₂O, mixing, and separating the phases once more by centrifugation. Recover the upper aqueous phase and combine with material recovered in **step 4**
- 6 The final products can be semipurified into $InsP_1$, $InsP_2$ and $InsP_3$ fractions on AG1X8 columns using the method described in Chapter 2, or purified to single isomers if so desired using HPLC method 3 (described in Chapter 3) Desalting of purified material can then be achieved by lyophilization of recovered fractions.

3.1.3. Preparation of $Ins(1,3)P_2$ Standard Using the High-Speed Supernatant

The above procedure can be repeated exactly but with 5 mM EDTA added to the incubation medium. Alternatively, a simpler incubation buffer has been used (5) consisting of 80 mM KCl, 50 mM HEPES, 2 mM EDTA, pH 7.4.

3.2. Preparation of Phosphoinositol Standards by Chemical Modification

3.2.1. Acid-Catalyzed Formation of Ins2P and D/L-Ins4P from Ins1P

Note: This procedure involves the use of hot acids; work behind a transparent protective shield in a fume cupboard, and wear protective clothing (*see Note 6*).

1. Dissolve 1 mg cold carrier Ins1P in 0.3 mL H₂O in a small tube and add radio-labeled standard (0.5 μ Ci; **Note 7**).
2. Place 1 mL of 2 M HCl in a separate tube and place a loose-fitting cap on each tube. Warm both tubes in a water bath to 80°C (or 100°C, as required; **Note 6**).
3. Transfer 0.3 mL of hot acid to the sample tube and continue to heat for 10 min, or 30 min as required (*see Note 6*).
4. Remove both tubes from the water bath and cool. Neutralize the sample tube by addition of NaOH solution (approx 60 μ L; *see Note 8*). Store products at -20°C

3.2.2. Limited Alkaline Hydrolysis of Inositol Polyphosphates

Note: This procedure involves the heating of alkaline in sealed ampules; work behind a transparent protective shield and *see Note 9* for precautionary measures.

1. Dry the phosphoinositol starting material down in the bottom of a glass ampule (under nitrogen gas or by lyophilization).
2. Add 1 mL of 880 ammonia and freeze the liquid at the bottom of the ampule using liquid nitrogen (keep cooling until the ice cracks). Immediately seal the ampule.
3. Check the ampule for leaks (as the ampule warms to room temperature and the liquid thaws, an ammonia odor will escape from the vial if a leak is present).
4. Place the ampule in an oven and switch the oven on. Heat the ampule in the oven at 110°C for 48 h.
5. Switch the oven off and allow it to cool to room temperature before opening. Remove the ampule and refreeze the contents using liquid nitrogen behind a shield (again, continue cooling until the ice cracks) Immediately open the ampule.
6. Remove the ammonia by lyophilization. Resuspend the final products in H₂O and adjust the pH to 7.0 if necessary. Store the products frozen at -20°C.
7. The mixed products can be separated on AG1X8 columns as described in Chapter 2, or by HPLC method 3 (Chapter 3). Ammonium formate present in the recovered fractions can then be removed by lyophilization.

3.3. Conversion of Glycerophosphoinositols to Corresponding Phosphoinositols by Limited Periodate Oxidation

Carry out all incubations in **steps 2–4** in the dark and in a sealed tube with any air space above the sample flushed with nitrogen gas at each stage.

1. Phosphoinositides should first be converted to their glycerophosphoinositol counterparts using the methylamine deacylation procedure described in Chapter 2 (*see Notes 10 and 11*). The neutralized aqueous products should then be lyophilized, resuspended in H₂O (900 μ L) in a 1.5-mL microfuge tube (**Note 12**), and, if necessary, neutralized to pH 6.5–7.0 using concentrated formic acid.
2. Add 100 μ L of 100 mM sodium periodate and incubate for 20 min (in the dark at 25°C).
3. Add 300 μ L of 1% (w/v) ethane diol and continue incubation for 20 min (in the dark, 25°C). (This step consumes the remaining periodate.)
4. Add 60 μ L of 1% (w/v) dimethylhydrazine reagent and incubate for 4 h (in the dark, 25°C). During this time, flush the tube with nitrogen gas periodically. (Over this 4-h period, the mixture should turn orange.)
5. Prepare a 0.5-mL column of Bio-Rad AG 50W resin (prewashed, *see Note 13*) during the 4-h incubation in **step 4**.
6. Place a clean container below the AG50W column. Load the reaction mix onto the AG50W column and elute with 2 \times 4 mL water. Collect all eluates and neutralize with ammonium hydroxide (concentrated).
7. To remove organic material from the phosphoinositol products, pass the neutralized product mixture through a C18 Sep Pak Cartridge (prewashed, *see Note 14*), collecting all washings. To maximize sample recovery, wash the cartridge with a few milliliters of water and then air-purge. Lyophilize the combined washings (to reduce volume) with added mannitol to act as a carrier if desired.
8. Redissolve sample in water and neutralize as necessary. Analyze recovered material on HPLC.

4. Notes

1. *myo*-Inositol is a six-membered cyclohexane ring with a hydroxyl on each carbon atom. All the hydroxyls except for that in position 2 are arranged equatorially, which means that there is a line of symmetry between carbons 2 and 5 (**Fig. 1**). Thus, enantiomeric pairs may exist if phosphates are placed on positions 1 or 6 (enantiomeric pairs being those on 3 and 4, respectively). This orientation of the hydroxyl groups on *myo*-inositol also means that those on carbons 1, 2, and 3 are mutually *cis* to each other, whereas those on carbons 3, 4, 5, 6, and 1 are all mutually *trans*. This latter point is shown most clearly in a Haworth projection of *myo*-inositol (**Fig. 1**).
2. If a phosphate occupies any one position under conditions where it can momentarily form a cyclic phosphate group across two adjacent hydroxyls, then the phosphate group can migrate to new positions around the ring. This cyclization

reaction occurs extremely slowly across *trans*-orientated hydroxyls, but relatively quickly across mutually *cis*-oriented hydroxyls. Thus it is relatively easy to promote phosphate migration around the 1, 2, and 3 positions but not so easy around the 4, 5, and 6 positions (Fig. 1).

For the phosphoinositols, cyclic phosphate intermediates are only promoted by acidic media and so migration may occur in the extraction procedures described (Chapter 1 and *see below*) if samples are not kept on ice. Under alkaline conditions, cyclic intermediates do not occur, but hydrolysis of the phosphate groups may occur (6, 7).

For the phosphoinositides or glycerophosphoinositols, cyclic phosphate intermediates occur in acid media but may also be formed under strong alkaline conditions from the phosphate of the phosphodiester bond (8). The formation of cyclic phosphate intermediates does not occur, however, in mild alkaline conditions.

3. The relative orientations of the hydroxyl groups around the *myo*-inositol ring and on the glycerol portion of the glycerophosphoinositols is the reason that certain C-C bonds are more susceptible to cleavage through limited periodate oxidation than others. Attack by periodate on vicinal C-OH groups can result in C-C bond cleavage and the ultimate conversion of each C-OH to a CHO (aldehyde) structure. This reaction occurs most readily when the hydroxyls on the vicinal carbon atoms are closest in alignment, i.e., mutually *cis*. Thus, in the glycerol structure where the carbon chain is open and C-C bonds can undergo free rotation, very close alignment can be achieved and rapid attack occurs. In the *myo*-inositol ring, there is no free rotation of C-C bonds, so the mutually *cis*-orientated hydroxyls on positions 1, 2, and 3 are close but not ideally aligned and are attacked more slowly, whereas the mutually *trans*-orientated hydroxyls on positions 3, 4, 5, 6, and 1 are completely resistant to attack. Thus, it is the glycerol portion of the glycerophosphoinositols that is attacked most rapidly by limited periodate oxidation, whereas the inositol ring structure is relatively stable (9).
4. If using periodate that has been standing for a long time, mix the contents before weighing, as that which is on the surface will have degraded.
5. The procedure described here is only a slight modification of that of Batty et al. (1). An important consideration is the quantity and form of substrate added. Most commercially available preparations of phosphoinositol standards are supplied in 10% (v/v) ethanol at approx 1 μCi /nmol/100 μL . In the method described above, addition of 0.1 μCi (i.e., 10 μL) of material results in a final concentration of 200 nM substrate and 0.2% ethanol. This quantity of ethanol has no adverse effect on the reaction, but if larger quantities of standard (e.g., 1 μCi) are to be used as starting material, then it is advisable to dry the substrate under nitrogen gas or by lyophilization, and then redissolve in incubation buffer.
6. This procedure carried out at 80°C will generate a mixture of D-*myo*-Ins1P, Ins2P and D-*myo*-Ins3P from Ins1P starting material (6, 7). As D-*myo*-Ins1P and D-*myo*-Ins3P are enantiomers, then the standards will migrate on HPLC as Ins1P and Ins2P markers. Of the recovered material, approx 30% will be Ins2P. If a marker

for Ins4P is also required, the same procedure can be carried out, but heating is for 30 min in a water bath set to just below 100°C (7). The water bath should not be boiling. The products from this modified procedure give markers for Ins1P, Ins2P and Ins4P (45, 40, and 15%, respectively, the Ins1P marker being a mix of the enantiomers *D-myo*-Ins1P and *D-myo*-Ins3P, and the marker for Ins4P being a mix of the enantiomers *D-myo*-Ins4P and *D-myo*-Ins6P).

Whichever temperature is chosen, it is essential to work behind a shield and wear protective clothing. Also place a loose fitting cap or a cap with a needle through it on each tube (to prevent build-up of pressure while minimizing evaporation and to avoid loss of material if sudden boiling occurs) and place a lid over the water bath as a precaution against splashing by boiling acid and radioactive material.

7. The conditions chosen here give approx 2 μmol cold Ins1P carrier. Added radiolabeled standard may be [^{14}C]Ins1P or [^3H]Ins1P, but the advantage of using the former is that standards so formed can be used as internal standards when analyzing [^3H]inositol-labeled samples
8. The final product formed by this procedure will contain approx 1 *M* NaCl. Provided the starting material is of high enough activity (i.e., use 0.5 μCi of radiolabeled starting material) such that only small volumes (approx 10–20 μL) of the recovered product are required as a standard marker per HPLC run, then this will not adversely influence the results obtained. If a salt-free preparation is required, then, after heating the Ins1P in acid for the required time (**Subheading 3.2.1., step 3**), remove the tubes from the water bath and freeze immediately without neutralization. The hydrochloric acid can then be removed by lyophilization, the dried material resuspended in water, and the pH adjusted to 7.0 as necessary
9. Because the method involves heating and cooling a sealed ampule, and therefore some risk of explosion, it is absolutely essential that protective clothing/glasses are worn, a suitable high-pressure (Pyrex) ampule is used, and heating is carried out in a closed vessel. Always work behind a transparent protective shield and wear protective gloves when cooling, sealing, or opening the ampule.
10. A potential problem with the deglyceration method described is that, with only trace quantities of material, overoxidation can occur, resulting in destruction of the inositol ring. Ideally, for complete deglyceration without destruction of the inositol ring, the sample concentration should be 1–5 *mM* (4). If the initial phosphoinositide sample is a radiolabeled preparation of trace quantity, then the simplest way to avoid overoxidation is to first add cold lipid carrier (1 mg PtdIns or mixed phosphoinositides) and then deacylate exactly as described in Chapter 2 using 1 mL of the methylamine reagent. When the glycerophosphoinositol products are finally resuspended in a volume of 900 μL (**Subheading 3.3., step 1**) the cold carrier will be approx 1 *mM*. Alternatively, if mass measurements are required, so cold carrier cannot be added to the trace quantities of the material of interest, then it is still possible to carry out deglyceration as described, but it may be necessary to reduce the incubation time in **Subheading 3.3., step 2** (*see also ref. 4*)

11. Do not use mannitol for freeze-drying in any steps before the periodate oxidation reaction, as mannitol is a polyol and so may interfere with the reaction.
12. A 1.5-mL Eppendorf microfuge tube is sufficiently large to carry out **Subheading 3.3., steps 2–4**, but small enough to have a limited air space above the solution, and can be sealed after gently flushing with nitrogen. This is an important consideration, as oxygen can attack the polyol during the reactions described and cause formation of byproducts. If a larger tube is used, then flush well with nitrogen gas and seal at each stage.
13. The AG50W resin is a cation-exchange resin and so will not bind phosphoinositols. Its purpose here is to remove unreacted dimethylhydrazine (which is positively charged). The 0.5-mL column is best used after prewashing with 2 M NaOH (4 mL), water (to pH 7.0), 1 M HCl (4 mL), and finally water (to pH 7.0).
14. The C18 Sep Pak cartridge should be washed with 2 mL methanol and then 5 mL water (by syringe) before use. This is necessary because, being a hydrophobic column, it cannot be wetted with water alone.

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Inositol 1,4,5-Trisphosphate Mass Assay

Lubo Zhang

1. Introduction

1.1. Background

The phosphoinositide cascade plays a central role in the transduction of many extracellular stimuli. Activation of plasma membrane-associated phosphoinositide-specific phospholipase C stimulates the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdInsP₂) and generates inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃), which in turn releases calcium from intracellular stores (1).

There are many methods for the determination of Ins(1,4,5)P₃. In Chapters 3 and 4, measurement of Ins(1,4,5)P₃ using high-performance liquid chromatography (HPLC) has been described. This method depends on the labeling of cells or tissues with [³H]myo-inositol and the separation of inositol phosphates isomers by HPLC. Whereas the obvious advantage of the method is that it can assess the complete profile of inositol phosphates and their metabolism in stimulated cells, the difficulty of determining specific radioactivity of Ins(1,4,5)P₃ inside cells limits its ability to quantify the Ins(1,4,5)P₃ mass in most experiments. Other methods for determination of Ins(1,4,5)P₃ mass require prior extensive purification of Ins(1,4,5)P₃ from other inositol phosphates. The purified Ins(1,4,5)P₃ is then hydrolyzed chemically or enzymatically followed by different detection systems (2–5). These methods are relatively slow, expensive, and laborious to perform.

The recently developed Ins(1,4,5)P₃ radioreceptor assays (6,7) provide simple, sensitive, and specific methods for the rapid determination of Ins(1,4,5)P₃ mass. The major advantage of these methods is that they can quantify endogenous Ins(1,4,5)P₃ mass in tissue or cell extracts with little purification. The range and sensitivity of the assays will ultimately depend on the

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Ins(1,4,5)P₃ receptor used. Commercially marketed Ins(1,4,5)P₃ radioreceptor assay kits utilize either bovine adrenals (Amersham [Arlington Heights, IL], cat. no. TRK 1000) or calf cerebellum (DuPont NEN [Boston, MA], cat. no. NEK-064) as a source of the Ins(1,4,5)P₃ receptor. The author has used both kits and found that the kit from DuPont NEN is more economic and easier to perform, and it is discussed in detail in this chapter.

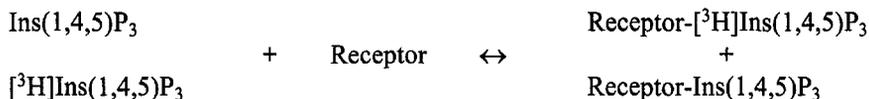
1.2. Sample Preparation

Although inclusion of 10 mM lithium in the medium used to incubate the tissues or cells has been employed for many studies to increase agonist-stimulated accumulation of inositol monophosphate, the precise effects of this cation on the metabolism of both phosphoinositides and their water-soluble products, especially Ins(1,4,5)P₃, remain unclear. It is therefore, suggested that lithium be excluded from the incubation medium.

For extraction of Ins(1,4,5)P₃ from the samples, the acid is usually employed to separate the highly charged Ins(1,4,5)P₃ from membrane proteins. Both trichloroacetic acid (TCA) and perchloric acid (PCA) extraction methods have been used. Although PCA precipitation is a better method for preparation of samples for HPLC because of its little particulate matter produced, for Ins(1,4,5)P₃ radioreceptor assay, TCA precipitation method may be a better choice for its speed and easy usage. Both methods will be given below. Because Ins(1,4,5)P₃ binding to its receptors is optimized at pH 8.5 (8, 9), acid-extracted samples should be neutralized before the assay.

1.3. Ins(1,4,5)P₃ Radioreceptor Assay

As for cyclic AMP assay developed in 1970 (10), the Ins(1,4,5)P₃ radioreceptor assay is based on competitive binding of unlabeled and radiolabeled ligand to endogenous proteins. As illustrated in the diagram, unlabeled Ins(1,4,5)P₃ from samples or standards competes with a fixed quantity of tritium-labeled Ins(1,4,5)P₃ for a constant and limiting amount of the Ins(1,4,5)P₃ receptor. Amount of Ins(1,4,5)P₃ in samples can be determined from the standard curve derived from known quantities of Ins(1,4,5)P₃.



The key component for the assay is the preparation of the Ins(1,4,5)P₃ receptor. Individual investigators can prepare the membranes containing the Ins(1,4,5)P₃ receptor from different tissues in the laboratories without extreme difficulties (6, 7). The Ins(1,4,5)P₃-receptor membrane preparation used in the DuPont Ins(1,4,5)P₃-radioreceptor assay kit (NEK-064) is derived

from calf cerebellum, which contains higher levels of $\text{Ins}(1,4,5)\text{P}_3$ receptors compared with bovine adrenal membranes. The preparation has a very high specificity and sensitivity for $\text{Ins}(1,4,5)\text{P}_3$, and can be used for 192 assay tubes with a hands-on time less than 2 h.

2. Materials

2.1. Sample Preparation

Certain laboratory equipment and supplies such as homogenizer, refrigerated low-speed centrifuge, vortex mixer, polypropylene centrifuge tubes, or microfuge tubes are required.

2.1.1. TCA Extraction

1. Trichloroacetic acid (TCA), 16.7% (w/v) in water.
2. Diethyl ether
3. Tris-HCl, 500 mM, pH 8.5
4. 3-L Separatory funnel.

2.1.2. PCA Extraction

1. Perchloric acid (PCA), 100% (w/v) in water.
2. 10 mM EDTA, pH 7.0
3. Freon(1,1,2-trichlorotrifluoroethane) (Aldrich).
4. Tri-*n*-octylamine (Aldrich, Milwaukee, WI).

2.2. Radioreceptor Assay

2.2.1. Preparation of Membranes Containing the $\text{Ins}(1,4,5)\text{P}_3$ Receptor

1. Buffer A: 20 mM Tris-HCl, 20 mM NaCl, 100 mM KCl, 1 mM EDTA, 5 mM DTT, 1 mg/mL bovine serum albumin (BSA), 0.02% sodium azide (w/v), pH 7.7
2. Buffer B: 50 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide (w/v), pH 8.5.
3. Dissection scissors and forceps.
4. Polypropylene centrifuge tubes and microfuge tubes.
5. Polytron tissue homogenizer.
6. Ultracentrifuge with fixed-angle rotor for 10-mL tubes.
7. Tissues (fresh is recommended).

2.2.2. Assay Protocol

The DuPont kit contains most reagents needed for $\text{Ins}(1,4,5)\text{P}_3$ -radioreceptor assay. These and others not included are listed below.

1. $\text{Ins}(1,4,5)\text{P}_3$ -receptor preparation (DuPont cat no. NEK 064).
2. $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (DuPont cat no. NET-911).
3. $\text{Ins}(1,4,5)\text{P}_3$ (DuPont cat no. NEK 064).

4. Blanking solution: 20 mM EDTA, 20 mM EGTA, 2% inositol hexaphosphate (w/v), 0.05% sodium azide (w/v), pH 7.5 (NEK 064).
5. Assay buffer: 5 mM EDTA, 5 mM EGTA, 50 mM sodium TAPS, pH 8.6. An alternative assay buffer is 50 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide (w/v), pH 8.5 (NEK 064).
6. 0.15 N Sodium hydroxide solution.
7. 1-mL Polypropylene minitubes.
8. Refrigerated low-speed centrifuge with swinging bucket rotor (capable of 2500g).
9. Liquid scintillation counter.
10. 7-mL Counting vials and caps.
11. Liquid scintillation cocktail for aqueous samples

2.3. Bradford Protein Assay

1. 1 mg/mL BSA solution.
2. Bio-Rad (Hercules, CA) protein assay dye reagent (cat no. 500-0006).
3. 5 N Sodium hydroxide solution.
4. 12 × 75-mm Test tubes.
5. Shaking water bath (30°C)
6. Semi microcuvets.
7. Spectrophotometer (595 nm)

3. Methods

3.1. Sample Preparation

The following acid-extraction procedures are given using blood vessel tissue as an example (*see Note 1*). The same procedures could also apply to cell suspensions except that the homogenization step may not be required. Tissue samples are snap-frozen in liquid nitrogen after treatments, and kept at -80°C before analysis. Depending on type of tissues and/or cells, approx 2–10 mg tissue or 1–5 × 10⁵ cells are needed to get on the standard curve.

3.1.1. TCA Extraction

1. For 10 mg tissue (wet weight), add 0.25 mL ice-cold 16.7% TCA.
2. Homogenize at 0–4°C for 1 min.
3. Rinse the homogenizer twice with each of 0.25 mL 16.7% TCA.
4. Combine three fractions (0.75 mL) and centrifuge for 10 min at 1500g at 4°C
5. Collect supernatant and incubate at room temperature for 20 min.
6. Save pellet for protein assay (*see Subheading 3.3.*)
7. During the 20-min incubation in **step 5**, prepare water-saturated diethyl ether by mixing vigorously 1 L H₂O and 1 L diethyl ether in separatory funnel (*see Note 5*). Care should be taken because ether is a hazardous material and can release pressure when used in separating funnel.

- 8 Add 4 vol water-saturated diethyl ether to 1 vol supernatant from **step 5** and vortex
- 9 Remove top layer with vacuumed pipet.
- 10 Repeat **steps 8** and **9** two more times.
- 11 Remove the residual ether by standing samples in a stream of air in a ventilation hood for 5 min.
- 12 Samples are neutralized with 500 mM Tris-HCl (pH 8.5), and kept on ice before assayed (*see Note 4*).

3.1.2. PCA Extraction (11,12)

- 1 Perform **Subheading 3.1.1.**, **steps 1–6** substituting 10% PCA for TCA.
2. Add 0.25 mL 10 mM EDTA (pH 7.0) to the supernatant (0.75 mL).
- 3 Add 0.75 mL of freshly prepared 1:1 (v/v) freon (1,1,2-trichlorotrifluoroethane) and tri-*n*-octylamine to the samples (total volume: 1.75 mL) (*see Note 6*)
- 4 Mix thoroughly by vortexing for 30 s, followed by centrifugation at 1000g for 5 min. This gives three phases: water containing neutralized sample (top), tri-*n*-octylamine perchlorate (middle), and freon plus unreacted tri-*n*-octylamine (bottom)
- 5 Carefully collect the top layer using transfer pipet, and keep it on ice until assayed

3.1.3. Recovery of $\text{Ins}(1,4,5)\text{P}_3$ from Acid-Extraction

1. Add approx 10,000 cpm [^3H]Ins(1,4,5) P_3 to samples before homogenization.
2. After acid-extraction, transfer 0.1 vol samples to scintillation vials. Add 5 mL of scintillation cocktail and count for 5 min.
- 3 Determine the percent recovery as $(\text{count} [\text{cpm}] \times 10/10,000) \times 100$.

3.2. Radioreceptor Assay

The kit from DuPont (cat. no. NEK-064) supplies the Ins(1,4,5) P_3 receptor prepared from calf cerebellum. Alternatively, individual investigators can prepare membranes containing the Ins(1,4,5) P_3 receptor in the laboratories.

3.2.1. Preparation of Membranes Containing the $\text{Ins}(1,4,5)\text{P}_3$ Receptor (7,9)

- 1 Isolated rat cerebella is minced and suspended in 10 vol ice-cold buffer A.
2. Homogenize the tissue with a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) at setting 3.5 in two bursts of 15 s each. Keep the sample cold all the time.
3. Centrifuge at 100,000g for 30 min at 4°C.
4. Discard the supernatant. Resuspend the pellet in half the original volume of buffer A at setting 3.5 for 15 s.
- 5 Repeat **step 3**
6. Discard the supernatant. The pellet is resuspended in buffer B at a protein concentration of 10 mg/mL and saved in 1-mL aliquots at -70°C for subsequent use (*see Note 2*).

Table 1
Preparation of Ins(1,4,5)P₃ Standards

Tube		(pmol/0.1 mL)
a	Undiluted std	12.0
b	0.20 mL std a + 0.30 mL distilled water	4.8
c	0.25 mL std b + 0.25 mL distilled water	2.4
d	0.25 mL std c + 0.25 mL distilled water	1.2
e	0.25 mL std d + 0.25 mL distilled water	0.6
f	0.25 mL std e + 0.25 mL distilled water	0.3
g	0.20 mL std c + 0.30 mL distilled water	0.12
h	Distilled water	0

3.2.2. Assay Protocol

3.2.2.1. PREPARE A SERIES OF Ins(1,4,5)P₃ STANDARDS USING DISTILLED WATER AS DILUENT (SEE TABLE 1)

3.2.2.2. PREPARE Ins(1,4,5)P₃-RECEPTOR PREPARATION/[³H]Ins(1,4,5)P₃ TRACER SOLUTION

Although individual investigators can prepare the receptor preparation/tracer solution in the laboratory, the use of assay kit from DuPont NEN is recommended for its simplicity and economy.

1. To make the receptor preparation/tracer solution in the laboratory for 100 assay tubes, dilute 0.6 mL membrane solution containing the Ins(1,4,5)P₃ receptor (approx 6 mg of protein, *see Subheading 3.2.1.*) into 40 mL with assay buffer. Add 50 pmols [³H]Ins(1,4,5)P₃. Keep the solution on the ice until use.
2. For the DuPont kit, resuspend the receptor preparation/tracer vial with the addition of 2.5 mL of distilled water. Before the assay, dilute the concentrated receptor preparation/tracer 1:15 (v/v) with assay buffer.

3.2.2.3. ASSAY PROTOCOL

1. Follow the assay protocol schematic (in Table 2) (*see Note 3*).
2. Vortex mix the tubes for 3–4 s except the two total counts tubes, which are directly transferred into counting vials.
3. Incubate at 2–8°C for 1 h.
4. Centrifuge at 4°C for 10 min at 2400g, and decant all tubes by inverting and shaking sharply downward. Allow the tubes to remain upside down on absorbent paper for 10 s (*see Note 9*).
5. Solubilize pellets in 50 μL of 0.15 N sodium hydroxide and vortex for 5 s. Incubate the tubes at room temperature for 10 min.
6. Place tubes in counting vials and add 5 mL scintillation cocktail. Shake the vials vigorously for 5 s.

Table 2
Assay Protocol Scheme

	Tube no.	Blanking solution, μL	Distilled water, μL	Standard, μL	Sample, μL	Receptor/tracer, μL
Total counts	1–2	—	—	—	—	400
Nonspecific binding	3–4	100	—	—	—	400
“0” Standards	5–6	—	100	—	—	400
Standards	7–20	—	—	100	—	400
Samples	21	—	—	—	100	400

- Place the vials in liquid scintillation counter and wait for 30 min. Count for 5 min (*see Note 8*).

3.2.2.4. SAMPLE CALCULATION

The amount of $\text{Ins}(1,4,5)\text{P}_3$ in the samples is determined from the standard curve (*see Note 7*). Typical results for the standard curve are shown in **Table 3** and **Fig. 1**.

3.3. Protein Assay

- Add 0.5 mL of 5 N NaOH to the pellets obtained from **Subheadings 3.1.1.** and/or **3.1.2.** Vortex for 10 s
- Incubate for 30 min at 30°C in a shaking water bath.
- During the 30-min incubation in **step 2**, prepare BSA standards by adding 0, 5, 10, 15, 20, 25, 30, 35, and 40 μL of 1 mg/mL BSA solution in test tubes.
- Dilute Bio-Rad protein assay dye reagent 1:4 (v/v) with distilled water before use.
- Depend on protein concentration, add 20–70 μL of samples to test tube.
- Add 1-mL diluted protein assay dye reagent to each test tube, and incubate at room temperature for 15 min.
- Read OD values at 595-nm wavelength by spectrophotometer
- Determine the amount of protein in each sample by interpolation from the BSA standard curve.
- Sample $\text{Ins}(1,4,5)\text{P}_3$ mass determined from **Subheading 3.2.2.4.** can then be normalized as pmol/mg protein.

4. Notes

- Acid extraction of samples converts inositol cyclic phosphates to the corresponding noncyclic phosphates. Therefore, if there is appreciable amount of cyclic inositol 1:2,4,5-trisphosphate in the sample it will cause overestimation of noncyclic inositol 1,4,5-trisphosphate. Because cyclic inositol 1:2,4,5-trisphosphate is stable at neutral pH, validation of a neutral extraction procedure is advisable

Table 3
Representative Data of Standard Curve

	Tube no.	cpm	Average cpm	Net cpm (-blank)	% B/B ₀ ^a	% Recovery ^b
Total counts	1	7932	7950	—	—	
	2	7968				
Blank	3	254	213	0	—	
	4	171				
“0” Standard (B ₀)	5	3091	3072	2859	100.0	38.6
	6	3052				
0.12 pmol	7	2745	2707	2494	87.2	
	8	2668				
0.3 pmol	9	2318	2320	2107	73.7	
	10	2322				
0.6 pmol	11	1977	1971	1758	61.5	
	12	1964				
1.2 pmol	13	1459	1458	1245	43.5	
	14	1457				
2.4 pmol	15	1077	1061	848	29.7	
	16	1046				
4.8 pmol	17	762	782	569	19.9	
	18	802				
12.0 pmol	19	483	500	287	10.0	
	20	516				

^a% B/B₀ = (net CPM of standards/net CPM of “0” standard) × 100

^b% Recovery = (CPM of “0” standard/CPM of total counts) × 100

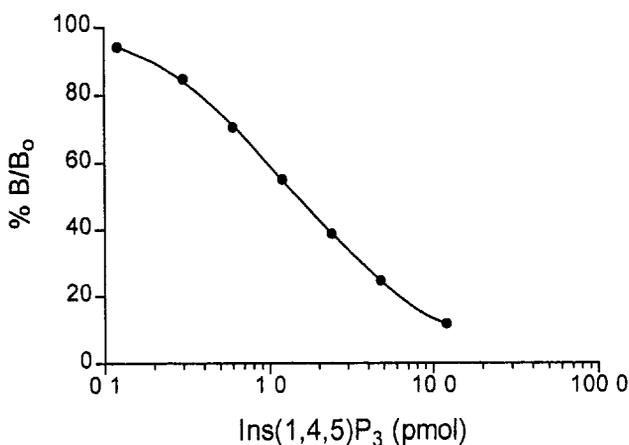


Fig. 1. Ins(1,4,5)P₃ standard curve.

- Constant freezing and thawing of membrane solution containing the Ins(1,4,5)P₃ receptor should be avoided. The diluted working receptor/tracer and Ins(1,4,5)P₃ standard solutions should be freshly prepared before each assay. Do not store and re-use the working solutions. During preparation of working receptor/tracer solution, care should be taken to avoid vigorous vortex and temperature in excess of 4°C.
- Because the Ins(1,4,5)P₃ receptor tends to precipitate in the solution, it is advisable to provide a constant slow stirring of the working receptor/tracer solution while adding it to all tubes.
- Binding of Ins(1,4,5)P₃ to its receptor is pH-dependent and the optimal pH is 8.5. If TCA is chosen to extract samples, neutralization of the extracts should be performed immediately after the acid extraction.
- The freon/octylamine procedure described for PCA extraction could also be used to extract TCA (13). EDTA (2 mM) should be included in samples before neutralization is carried out
- If PCA is chosen to extract samples, a mix solution of freon (1,1,2-trichlorotrifluoroethane) and tri-*n*-octylamine (1:1, v/v) should be freshly prepared before the extraction. Because tri-*n*-octylamine sticks to glass, use polypropylene or polystyrene pipets or pipet tips to transfer solutions containing this compound. A second freon (1,1,2-trichlorotrifluoroethane) and tri-*n*-octylamine extraction on the original extracted samples should be avoided because of a potential significant loss of Ins(1,4,5)P₃.
- It is recommended that a standard curve and extraction recovery be run for each assay. If tissue culture medium is involved in the assay, it is desirable to run standard curve using the medium rather than distilled water.
- To obtain accurate results, any samples with an amount of Ins(1,4,5)P₃ above the upper limit of the standard curve must be diluted with distilled water and reassayed.

- 9 A key step of the assay is the separation of free unbound [^3H]Ins(1,4,5) P_3 from that bound to the receptor. It is very important to remove all liquid from the tubes after centrifugation (**Subheading 3.2.2.3.**) Residual liquid containing unbound counts will cause a loss of sensitivity at the low concentration end of the standard curve. On the other hand, caution should be taken to avoid prolonged inversion of tubes after decanting, because it may cause the pellets to become dislodged from the bottom of the tubes, resulting in incomplete retention of bound counts. The mini sample tubes and foam centrifuge racks provided in the DuPont NEN kit are very easy to use and are highly recommended

Acknowledgments

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Measurement of Cellular Diacylglycerol Content

Wendy Bollinger Bollag and Richard D. Griner

1. Introduction and Theory

With the discovery of the role of polyphosphoinositide hydrolysis in mediating the action of calcium-mobilizing hormones, growth factors, and neurotransmitters (reviewed in **ref. 1**), the development of methods for measuring the formation of the resultant second messengers, inositol 1,4,5-trisphosphate and diacylglycerol (DAG), became essential. The additional discovery of an effector enzyme, protein kinase C, for transducing the changes in DAG levels (reviewed in **ref. 2**) underlined the importance of this second messenger. A sensitive and reliable protocol currently in use in many laboratories is described below for the measurement of DAG levels in stimulated cells.

In 1987 the laboratory of Robert Bell (**3**) developed a technique utilizing a bacterial (*Escherichia coli*) DAG kinase to phosphorylate the DAG contained in lipid extracts of cell samples to yield phosphatidic acid (PA), which could then be separated by thin-layer chromatography (TLC). If this reaction was performed in the presence of ^{32}P -labeled ATP, it became possible to calculate the DAG present in the original cell sample, using the radioactivity determined in [^{32}P]PA and a standard curve generated at the same time using known amounts of DAG. One clear advantage of this method is that changes in DAG content, rather than radiolabeled DAG levels, are measured, with a sensitivity that makes the technique useful for cellular studies (in a range of 100 pmol to 10 nmol) (**ref. 3** and **Fig. 1**). In the case of DAG measurements using radiolabeling methods, it is often difficult to determine the specific activity of the phospholipid hydrolyzed in response to hormone treatment to produce DAG (particularly since the source may be unknown or may, in fact, change **ref. 2**; see Chapter 12 on the Measurement of Phospholipase D Activity). Moreover, the specific activity may be altered by the treatment itself, making a determina-

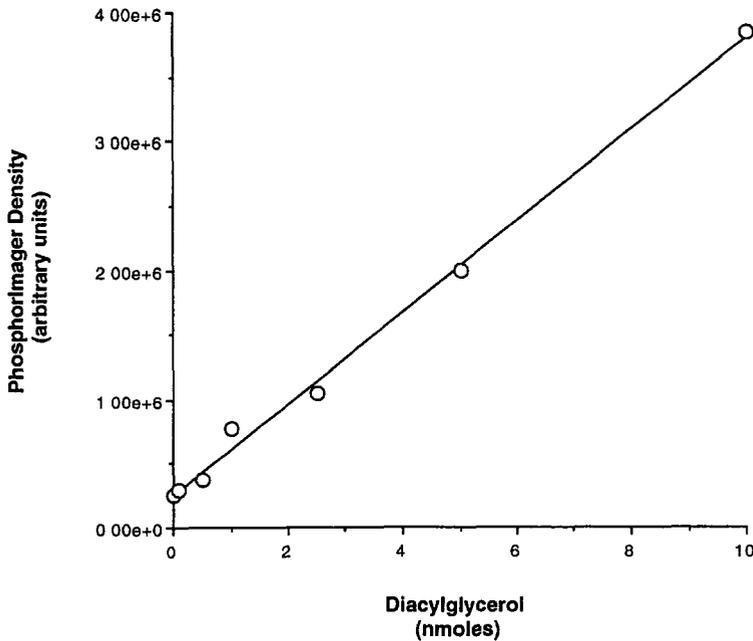


Fig. 1. Diacylglycerol standard curve: Duplicate samples of varying amounts of *sn*-1,2-diacylglycerol (dioleoylglycerol) were subjected to the diacylglycerol kinase assay as described. After separation by thin-layer chromatography, the radioactivity found in [^{32}P]PA was quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). These values were averaged and plotted vs the amount of diacylglycerol in the original sample to give a correlation coefficient of 0.996.

tion of the changes in the absolute mass of the DAG impossible. Thus, the DAG kinase method for the measurement of hormone-induced changes in DAG content yields information on the actual mass of this important second messenger.

2. Materials

The following equipment and supplies are needed for the assay of diacylglycerol mass using the DAG kinase method. Note that, when a reagent must be acquired from a specific source, that source is noted.

1. 25°C Water bath (preferably lucite).
2. Vortex mixer and/or bath sonicator.
3. Nitrogen tank and manifold.
4. Lucite screens for ^{32}P work.
5. TLC chambers
6. Oven.

- 7 Pasteur pipets and bulb.
8. Test tubes and racks.
- 9 2X reaction buffer 100 mM imidazole HCl + 100 mM NaCl + 25 mM MgCl₂ + 2 mM EGTA, pH 6.6 (store at 4°C).
- 10 Diethylene-triamine-pentaacetic acid (DETAPAC) stock solution 100 mM DETAPAC, pH 7.0 (store at 4°C).
- 11 DETAPAC working solution: 1 mM DETAPAC, pH 7.0 (store at 4°C)
12. ATP stock solution: 20 mM ATP in 100 mM imidazole + 1 mM DETAPAC, pH 6.6 (aliquot and store at -70°C). The concentration of this stock solution should be verified by measuring absorbance at 259 nm, using a molar extinction coefficient of 15.4 (3)
13. DAG kinase dilution buffer: 10 mM imidazole + 1 mM DETAPAC, pH 6.6 (store at 4°C).
- 14 *E. coli* DAG kinase (Calbiochem [La Jolla, CA] or Biomol [Plymouth Meeting, PA]; store frozen in aliquots at -70°C).
15. Octyl- β -glucoside/cardiophilin solution: 7.5% D-octyl- β -glucoside + 5 mM cardiophilin in 1 mM DETAPAC, pH 7.0 (store at -20°C under an atmosphere of nitrogen or argon). For preparation of this reagent, 25 mg cardiophilin should first be dried under nitrogen in a glass test tube. 250 mg of Calbiochem's Ultrol D-octyl- β -glucoside and 3.37 mL of 1 mM DETAPAC, pH 7.0 are then added (alternatively, a less pure D-octyl- β -glucoside can be used following recrystallization, see Note 1). The mixture is then sonicated to solubilize the cardiophilin and detergent (frothing of the mixture typically indicates solubilization).
- 16 0.2% SDS in distilled water (store at room temperature)
17. [γ -³²P]ATP (approx 6,000 Ci/mmol).
18. 20 mM Dithiothreitol in 1 mM DETAPAC, pH 7.0 (prepared fresh each time).
19. 0.2 M Sodium chloride (store at room temperature).
20. Chloroform.
- 21 Methanol
- 22 Acetone.
23. Acetic acid.
24. 20 × 20-cm TLC plates (silica gel 60 with concentrating zone, available with glass or aluminum backing from EM Science [Gibbstown, NJ] or Merck [Darmstadt, Germany]) TLC plates are prepared by scoring lanes (the back of a razor blade works well) and marking an origin in pencil (approx 1.3 cm from the bottom of the plate within the concentrating zone). Note that inhalation of silica is hazardous and should be avoided. The plates are then "heat-activated" by baking at 110°C for 30–60 min (however, see Note 2 for an alternative method for plate activation). Heat-activated plates should be stored in a desiccating chamber (containing Drierite) and can be used for up to 24 h after heat activation.
25. Chromatography paper (Whatman 1MM works well)
26. Microcapillary pipets (5 or 10 μ L).
27. Iodine.
28. Phosphatidic acid (dioleoylphosphatidic acid).

29. Diacylglycerol (dioleoylglycerol).
- 30 Film (Kodak XAR-5).

3. Methods

3.1. Preparation of Samples for Assay

The samples of cellular DAG for quantitation are prepared for the assay as described below. Note that chloroform and methanol are hazardous and, as much as possible, should be dealt with in a chemical fume hood. Note also that both are quite volatile (particularly chloroform) and mixtures containing these agents should be made immediately prior to use and capped tightly to prevent alteration of the relative proportions. As a final note, chloroform will dissolve polystyrene, the plastic of which tissue culture vessels and disposable pipets are made. Therefore, glass pipets and test tubes should be used, and care taken to prevent chloroform coming into contact with plastics. Tips used with Pipetmen and Repipetmen are, for the most part resistant to chloroform, and are adequate for this assay. However, the Teflon seal and o-ring within the Pipetmen will be damaged upon repeated use of chloroform and should be periodically replaced. When Pipetmen are used with any organic solvent, it is important to pre-equilibrate the air space in the tip by aspirating and ejecting the solvent prior to distributing the desired volume. Nevertheless, volumes are not precise and any manipulation requiring extreme precision, such as aliquoting a chloroform solution of a DAG standard, requires the use of Hamilton syringes.

1. Cells are stimulated with the desired agents for the appropriate period of time.
2. Reactions are terminated by the addition of room-temperature 0.2% SDS (for a 35-mm dish, 0.4 mL 0.2% SDS should be used, for a 60-mm dish, 0.8 mL) and swirling for several seconds (alternatively, reactions may be terminated by the addition of ice-cold methanol; *see Note 3*). After incubating at room temperature for a few minutes, the SDS extracts are repeatedly aspirated with a Pipetman to minimize viscosity and transferred to test tubes (16 × 100 mm). At this point, a percentage of the SDS extract may be reserved for determination of protein content and normalization. Alternatively, phospholipid phosphate content can be used for normalization purposes (*see step 7*).
3. Chloroform and methanol are added to the test tubes, which are vortexed thoroughly. The ratio desired for optimal extraction of the cellular lipids, as described by Bligh and Dyer (4), is 1:2:0.8 (volume to volume to volume [v:v:v]) of chloroform to methanol to aqueous solution (SDS extract), which should produce a single phase. Thus, for a 60-mm dish, volumes of 0.8 mL SDS, 1 mL chloroform, and 2 mL methanol would yield the appropriate ratio. The cellular lipids are then extracted by incubating for 1–2 h on ice.

4. Chloroform and 0.2 M NaCl are then added to the test tubes, with vortexing, to “break phase,” that is, the chloroform separates from the methanol/water and two phases become readily apparent. The final desired ratio for this step is 1:1:0.9 (v:v:v) of chloroform to methanol to aqueous solution. As an example, for the above 60-mm dish of cells terminated with 0.8 mL 0.2% SDS and extracted with 1 mL chloroform and 2 mL methanol, 1 mL chloroform, and 1 mL 0.2 M NaCl would be added. The samples are then centrifuged briefly to promote complete phase separation (Phase separation can also be achieved, with time, courtesy of gravity.)
5. The entire lower chloroform phase is transferred to a clean test tube using Pasteur pipets. Note that care should be taken to prevent transfer of the aqueous phase. Also note that this technique requires practice (one hint: the chloroform and aqueous phases will separate in the Pasteur pipet to allow transfer of the final few drops of chloroform).
6. The chloroform phase is “washed” with additional volumes of methanol and 0.2 M NaCl, in a ratio of 1:0.5:0.5 chloroform to methanol to NaCl solution (v:v:v). For a 1-mL chloroform phase, 0.5-mL volumes of methanol and 0.2 M NaCl are used. This wash is particularly important with the described SDS termination protocol, as residual SDS in the chloroform phase may inhibit DAG kinase. The samples are vortexed and centrifuged, and the entire lower chloroform phase transferred to clean test tubes.
7. The lower chloroform phases are then dried under nitrogen. After solubilization of the lipid extract in a small volume of chloroform/methanol at a ratio of 2 volumes to 1, a percentage of the samples may be removed for determination of phospholipid phosphate content, as described by Van Veldhoven and Mannaerts (5), for normalization purposes. Samples can be stored dry at -20°C for up to 3 d.
8. DAG standard(s) (in dry chloroform or chloroform/methanol at a ratio of 2 volumes to 1) should be aliquoted (using Hamilton syringes) and dried under nitrogen. The standard curve is linear in the range of 0.1 to 10 nmol of DAG (see Note 6)

3.2. Assay of DAG Content

The DAG contained in the samples prepared as above is then determined using the DAG kinase assay: the lipids are solubilized and incubated at 25°C for 30 min with *E. coli* DAG kinase in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of known specific activity (3). The assay requires several procedures, including solubilization of the sample lipids, performance of the reaction and extraction and separation by TLC of the radiolabeled PA. Initially, however, a few initial actions must be taken to prepare for the assay.

3.2.1. Assay Preparations

Preparations to be made for beginning the assay are as follows:

1. The water bath must be adjusted to 25°C and allowed to come to temperature.

- 2 The octyl- β -glucoside/cardiolipin solution should also be removed from storage to thaw
3. 20 mM Dithiothreitol in 1 mM DETAPAC (pH 7.0) should be prepared and stored on ice until use
- 4 The DAG kinase solution should be prepared by diluting the commercially available DAG kinase 1.1 (volume:volume) with DAG kinase dilution buffer before use. Note that repeated freeze-thawing of DAG kinase should be avoided: DAG kinase should be stored frozen in aliquots at -70°C . Diluted DAG kinase can be frozen once before discarding or may be stored at 4°C for several days (3).
5. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ must be removed from storage at -20°C and allowed to thaw (because of the thick lucite in which this reagent is normally packaged, thawing may take some time) Note that this ATP should be no more than 3 wk beyond its reference date (3) The final ATP solution used to initiate the reaction is prepared by diluting the 20 mM cold ATP stock solution 1:1 (volume:volume) inclusive of the radioactivity, using approx $2\ \mu\text{Ci}$ of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ per sample. As an example, 100 μL of the ATP stock is diluted with 95 μL distilled water and 5 μL (equal to approx $50\ \mu\text{Ci}$) of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ This dilution would represent enough radiolabel for approx 20 samples (although extra volume should always be prepared to ensure adequate quantities) The ATP solution should be stored on ice until use. Note that all manipulations involving $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (or $^{32}\text{P}]\text{PA}$) should be performed behind a lucite shield to minimize radiation exposure

3.2.2. Lipid Solubilization

The lipid samples are solubilized by the addition of 20 μL of the octyl- β -glucoside/cardiolipin solution and sonication for 15 s (3), if a bath sonicator is available. Alternatively, a full 20 s of vigorous vortexing can be used. The samples are incubated at room temperature for 5–15 min. Note that a blank (octyl- β -glucoside/cardiolipin in a clean test tube with the additions described below) should always be performed (preferably in duplicate), since the *E. coli* DAG kinase membrane preparation itself contains DAG (notice that the y -intercept for the standard curve in Fig. 1 is not zero). This blank value is then subtracted from all sample values to yield the DAG contributed by the sample (for an additional caveat concerning *E. coli* DAG kinase, see Note 4).

3.2.3. Reaction Mixture and Initiation

The following reagents are then added to the solubilized lipid samples:

- 1 50 μL 2X reaction buffer.
2. 10 μL 20 mM DTT in 1 mM DETAPAC (prepared fresh each time).
3. 10 μL diluted *E. coli* DAG kinase.

The reaction is initiated by the addition of 10 μL $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ solution, the samples are vortexed and incubated for 30 min at 25°C . The specific activity of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ may be determined by diluting an aliquot 1 to 10 with distilled water (i.e., 10 μL $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ + 90 μL distilled water) and counting 10 μL of

this diluted [γ - ^{32}P]ATP in a liquid scintillation spectrometer. While the samples are incubating, the termination/extraction solution of chloroform/methanol (1 volume to 2 volumes) should be prepared, tightly capped and placed on ice.

3.2.4. Reaction Termination and Extraction

After 30 min, the reactions are terminated by the addition of 2 mL of ice-cold chloroform/methanol termination/extraction solution. 1 mL of 0.2 M NaCl is then added with vortexing, followed by an additional 2 mL of chloroform. The samples are vortexed and centrifuged to promote phase separation (centrifugation is optional since the phases will separate well from the effects of gravity within a short time). The lower phases are removed to clean test tubes (as above in **Subheading 3.1., step 5**, except that the transfer should be performed behind a lucite shield), and the organic solvent is evaporated under nitrogen. The lipid residue can then be separated by TLC immediately or stored (in an appropriate container to minimize radiation exposure) at -20°C overnight.

3.2.5. Separation and Quantitation of [^{32}P]PA

For separation of [^{32}P]PA by TLC, the mobile phase must first be prepared by mixing chloroform, acetone, methanol, acetic acid, and distilled water in a ratio of 50:20:10:10:5 (v:v:v:v:v), respectively. Approximately 100 mL of this mixture is placed in a TLC chamber lined with chromatography paper to aid in equilibration of the chamber with solvent vapor. The lipid samples are then solubilized in a small volume (40–50 μL followed by a 20- μL “rinse”) of chloroform/methanol at a ratio of 2:1 (v:v) and spotted at the origin of the heat-activated TLC plate using microcapillary pipets. Cold PA (approx 25 μg) is also spotted to serve as a standard. The plate is placed in the equilibrated TLC chamber and allowed to develop to within approx 2 cm of the top (development requires 1–2 h). Following removal of the plate from the chamber and evaporation of the solvent mixture, the standard is visualized by placing the plate in a TLC chamber equilibrated with iodine vapor for several minutes. (Note that because iodine vapor is hazardous, this TLC chamber should be maintained in a chemical fume hood; inhalation of iodine should be avoided.) The cold PA will appear as a yellowish-brown spot at an R_F value of approx 0.68. (Note that R_F value is defined as the distance a spot migrates from the origin divided by the distance migrated by the solvent front.) The plate is wrapped in cellophane and exposed to film overnight (less time may be required depending on the amount of DAG in the particular cell type as well as the specific activity of the [γ - ^{32}P]ATP). The autoradiogram is developed and can be used for densitometry or to identify the location of the [^{32}P]PA for an alternative method of quantitation (*see Note 5*). In most cell types a doublet with an R_F value of approx 0.43 (as measured to the middle of the doublet) can also be

observed. This doublet represents phosphorylated ceramide, as will be discussed later (*see Note 7*). In addition, the origins will exhibit spots of varying intensities, depending on the quality of the transfer (i.e., the amount of aqueous phase containing unused [γ - 32 P]ATP that contaminates the chloroform phase).

4. Notes

1. If purified D-octyl- β -glucoside (Calbiochem's Ultrol grade) is unavailable, lower purity D-octyl- β -glucoside may be recrystallized for use. The protocol for recrystallization is as follows
 - a. Add 4.2 mL dry acetone to 1 g D-octyl- β -glucoside and warm to dissolve
 - b. Filter through Whatman no. 1 filter paper and wash vial and filter with a small (measured) volume of dry diethyl ether.
 - c. Add a total of 16.7 mL diethyl ether to acetone/D-octyl- β -glucoside solution and incubate at -20°C for 2 d to allow crystal formation.
 - d. Crystals are collected by filtration, washed with cold, dry diethyl ether, and allowed to air-dry prior to use in the DAG kinase assay.
2. If an oven is not available for heat activation of TLC plates, plates may be activated by placing them in a TLC chamber containing dry acetone. After the solvent has migrated to the top of the plate, the plate should be briefly air-dried and used immediately or stored in a desiccating chamber.
3. As an alternative to 0.2% SDS, reactions may be terminated by adding ice-cold methanol. The cells must then be scraped from the dish and transferred to test tubes as above. The advantage of using methanol is greater speed with which the reactions are halted; the disadvantage is that the scraping of the cells for transfer is more labor- and time-intensive and complete transfer is difficult to ensure, although washing the dishes with an additional volume of methanol minimizes loss in transfer. In addition, removal of samples for normalizing using protein content is not possible; phospholipid phosphate content may be used instead (5). Alternatively, a combination of the two termination procedures can be used: reactions are terminated with ice-cold methanol that is transferred to a clean test tube; cells are then solubilized with 0.2% SDS and transferred. In either case, the final desired ratio is 1:2:0.8 (v:v:v) of chloroform:methanol:aqueous solution (when the methanol termination is used alone, add water as the aqueous solution). Note that, if methanol is used to terminate reactions, some evaporation of the solvent may occur, such that the addition of small amounts of methanol may be required to obtain a single phase.
4. Some bacterial DAG kinase preparations contain low levels of phosphatidate phosphohydrolase activity, which hydrolyzes PA to yield DAG. Such activity is obviously a potential difficulty both in terms of hydrolysis of [32 P]PA formed from DAG and of cold PA contained in the original sample with potential rephosphorylation to [32 P]PA by the bacterial DAG kinase. The activity of phosphatidate phosphohydrolase in the DAG kinase preparation can be estimated by subjecting a PA standard (of 5–10 nmol) to the protocol. Little or no production of [32 P]PA indicates minimal phosphatidate phosphohydrolase activity. If

significant activity is detected, a new lot of DAG kinase should be ordered and tested as above.

- 5 The [^{32}P]PA formed in the assay can be quantified in several ways. Densitometry (or quantitation using a PhosphorImager [Molecular Dynamics, Sunnyvale, CA]) can be performed on the autoradiogram to yield OD values. However, these OD values can be converted to absolute moles of DAG if a standard curve is assayed at the same time (*see Note 6*). Alternatively, the autoradiogram can be used to locate the [^{32}P]PA, which can then be scraped from the plate (or cut if aluminum-backed TLC plates are used) and counted in a liquid scintillation counter. If complete conversion of the DAG in the sample to [^{32}P]PA is assumed, the original DAG content can be calculated using the radioactivity in [^{32}P]PA and the specific activity of ATP: division of the counts measured in [^{32}P]PA by the specific activity of [γ - ^{32}P]ATP provides the quantity of DAG contained in the original sample (Note that the final concentration of ATP in the reaction mixture is 1 mM in 100 μL or 10^{-7} mol; thus, the specific activity is the number of counts in the sample divided by 100 nmol to yield the counts per nmol.) To obtain an estimate of the efficiency of the conversion, a DAG standard containing approximately the amount of DAG in the cell samples may be run at the same time to determine the percentage of the DAG in the original sample likely converted to [^{32}P]PA. Ideally, however, a DAG standard curve should be performed with each experiment. Note that in all cases, the blank values (a measure of the DAG present in the DAG kinase membrane preparation) should be subtracted from the sample values to give the DAG present in the original sample.
- 6 Standard curves are performed using known amounts of *sn*-1,2-DAG (dioleoylglycerol works well) in the range of 100 pmol to 10 nmol (*ref. 3* and *Fig. 1*). DAG should be diluted in cold chloroform and aliquoted rapidly (to prevent excessive solvent evaporation) using Hamilton syringes. Note that upon storage the acyl group in the 2 position of 1,2-DAG will migrate over time to the 3 position. However, standards can be stored for up to 1 mo in dry (water-free) chloroform without significant acyl migration (*3*) (Note also that many commercial 1,2-DAGs contain 1,3-DAG [*3*]), which will result in a lower apparent conversion efficiency and/or greater calculated DAG contents in cell samples.) The DAG standard curve can then be used to calculate directly the DAG content of the original sample.
7. Recent developments in the cellular signaling field indicate that sphingomyelin hydrolysis, like phosphoinositide hydrolysis, may be regulated by hormones, cytokines, and stress stimuli and that a product of this hydrolysis, ceramide, may serve as a second messenger in response to these agents (reviewed in *refs. 6* and *7*). Thus, it became of great interest to develop a method for examining the modulation of ceramide levels by extracellular signaling molecules. Prior studies had indicated that the *E. coli* DAG kinase utilized by Preiss et al. (*3*) also phosphorylated ceramide to yield ceramide phosphate (*8*), and the DAG kinase assay method was, therefore, examined for its feasibility in quantifying ceramide content. With minor or no modifications, this method has proven useful for

the quantitation not only of DAG mass but also of ceramide content (*see*, for example, **ref. 9**) Studies investigating the phosphorylation of ceramide by the *E. coli* DAG kinase have indicated the optimal pH for activity on this compound to be slightly higher than that for activity on DAG (**10**). Accordingly, some investigators have modified the buffer solutions to perform the DAG kinase assay at a pH of 7.0 (**10**) or 7.4 (**8**). However, using the exact protocol described above, we have obtained good results with linear conversion of a ceramide standard (ceramide type III) in the range of 10 pmol to 10 nmol. Note, however, that complete conversion cannot be assumed, making the performance of a standard curve critical for absolute quantitation. On the other hand, the linearity of the response suggests that comparisons relative to an appropriate control should yield quantitative, although perhaps not absolute, values in terms of changes in mass quantities of ceramide Thus, the assay is performed as described above, on lipid extracts of cells in which reactions are terminated with ice-cold methanol Radio-labeled PA and ceramide are then separated by TLC. As previously indicated, ceramide phosphate displays an R_F value of approx 0.43 (measured to the middle of the doublet) Ceramide phosphate can be quantified as described above and compared with a standard curve to yield the absolute mass of ceramide contained in the original sample

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Phosphatidylinositol 4-Kinases

Assays and Product Analysis

Rachel E. Meyers and Lewis C. Cantley

1. Introduction

1.1. Background

Phosphoinositide metabolism plays a central role in the transduction of signals triggered by a wide variety of growth factors and hormones. Phosphoinositides have been implicated in cellular processes as diverse as proliferation and differentiation, cytoskeletal rearrangement, cell survival, and vesicle trafficking (reviewed refs. 1 and 2). An ever-increasing number of lipid kinases are being discovered, each with its own substrate specificity, cellular localization, and unique biological function.

These lipid kinases catalyze the phosphorylation of phosphatidylinositol (PtdIns) at a variety of positions around the inositol ring. PtdIns 4-kinases catalyze phosphorylation at the D4 position of the inositol ring to yield PtdIns 4-P. In a second step, PtdIns (4)P 5-kinases catalyze phosphorylation of PtdIns 4-P at the D5 position of the inositol ring to generate PtdIns 4,5-P₂. These enzymes are part of the canonical PtdIns turnover pathway that generates inositol 1,4,5-trisphosphate and diacylglycerol (3). In a second pathway, phosphoinositide 3-kinase (PI 3-kinase) catalyzes phosphorylation of all of these lipids at the D3 position of the ring, to generate an array of 3-phosphorylated lipids (4).

This chapter focuses on the PtdIns 4-kinases and outlines protocols for assaying the PtdIns 4-kinases and analyzing the lipid products generated.

1.2. Distinguishing Between Phosphatidylinositol 4-Kinases

The PtdIns 4-kinases were divided into two types (II and III) based on their size (*see Note 1*) and sensitivity to various substances (5). The type II enzymes are inhibited by adenosine and the monoclonal antibody 4C5G (6)

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(see **Note 2**) and are stimulated by detergent. The type III enzymes are relatively insensitive to adenosine, are only modestly stimulated by detergent, and are resistant to the 4C5G antibody. **Subheading 3.2.** describes the use of these reagents to type the PtdIns 4-kinase being studied. In addition to the reagents mentioned above, the PI 3-kinase inhibitor wortmannin has also been used to characterize a novel, recently described PtdIns 4-kinase (**7,8**). **Subheading 3.3.** outlines assays to test for inhibition by this fungal metabolite.

1.3. Choice of Substrate

The PtdIns 4-kinases catalyze the conversion of PtdIns to PtdIns 4-P. The PtdIns substrate can be obtained in purified form from Avanti or as a crude mixture with the other phospholipids in a preparation purified from bovine brain (Sigma, St Louis, MO). The crude mixture is cheaper and may be satisfactory for most purposes. The crude brain mixture contains, among other lipids, PtdIns 4,5-P₂, which is phosphorylated exclusively by PI 3-kinase to yield PtdIns 3,4,5-P₃. By running the thin-layer chromatography (TLC) plate using the "long solvent system" (see **Subheading 1.4.**), one can determine whether the enzyme preparation being assayed contains PI 3-kinase activity.

1.4. Lipid Product Analysis

Subheading 3.2. describes the analysis of the lipid products generated in the kinase assay. For many applications, TLC separation will be sufficient to confirm the production of the various lipids. Several solvent systems can be used for this purpose. The first is referred to as the "short system" because it runs quickly because of the high content of organic solvents, uses only one half of a normal TLC plate, and is useful for separating PtdIns 4-P from PtdIns 4,5-P₂. A second solvent system, the "long system," has a lower percent organic solvent and therefore runs over 6–7 h, requires a full TLC plate, and is useful for separating mono- from di- and triphosphorylated lipids. As previously mentioned, triply phosphorylated lipids are the exclusive product of PI 3-kinase and their generation would confirm the presence of PI 3-kinase in the enzyme preparation being assayed.

TLC procedures allow the identification of PtdIns-P, PtdIns-P₂, and PtdIns-P₃ but are relatively poor at separating the different isoforms of lipids (**9**). For example, PtdIns 3-P and PtdIns 4-P do not resolve on the TLC systems described here. For this reason, high-performance lipid chromatography (HPLC) analysis is preferable to distinguish between the various isoforms. **Subheading 3.5.** outlines procedures for deacylating lipids and **Subheading 3.6.** briefly describes their analysis by HPLC. The protocol employs methylamine to deacylate the lipids, yielding glycerophosphoinositols, followed by strong anion-exchange HPLC using a very shallow ammonium phosphate gradient.

2. Materials

2.1. Assay Reagents

1. [γ - ^{32}P] ATP (3000Ci/mmol) (NEN).
2. Phosphatidylinositol (Avanti, Alabaster, AL): 2 mg/mL in chloroform (*see Note 3*).
3. Crude brain phosphoinositides (Sigma, St Louis, MO): dry powder resuspended in chloroform:methanol:1 M HCl (2:1:0.1) at 2 mg/mL and stored at -70°C .
4. Triton X-100.
5. 10 mM ATP
6. 4 M HCl
7. 1 M HEPES.
8. Chloroform:methanol (1:1).
9. 1 M MgCl_2
10. 10 mM Adenosine
11. 10 mM Wortmannin in DMSO.
12. 4C5G antibody.
13. Bath sonicator (*see Note 4*).

2.2. TLC Reagents

1. Foil backed silica gel 60 plates (Merck, Darmstadt, Germany), coated with 1% potassium oxalate (*see Note 5*).
2. 100°C oven.
3. Short solvent system: chloroform:methanol:water:ammonium hydroxide (60:47:11:2).
4. Long solvent system: *n*-propanol:2 M acetic acid (13:7).
5. Nitrogen and air lines.
6. TLC tank.
7. TLC tank with iodine vapor (*see Note 6*)

2.3. Deacylation and HPLC Reagents

1. Methylamine reagent: 26.8 mL 40% methylamine in water (Aldrich, Milwaukee, WI), 16 mL water, 43.7 mL methanol, 11.4 mL *n*-butanol (*see Notes 7 and 18*).
2. 53°C Heating block
3. Speed-vac.
4. Extraction solution: butanol:petroleum ether:ethyl formate (25:5:1.75)
5. Sulfuric acid trap (*see Note 8*).
6. 1 M Ammonium phosphate (dibasic), titrated with phosphoric acid to pH 3.8, degassed and filtered.
7. [^3H] PtdIns 4-P and [^3H] PtdIns 4,5- P_2 (NEN).
8. 0.22- μ filter Eppendorf (Costar, Cambridge, MA).
9. Partisphere SAX anion exchange column (Whatman, Clifton, NJ).
10. Nitrogen line

3. Methods

3.1. Standard PtdIns 4-Kinase Assay

Assays are routinely performed in 100 μL final volume and should contain 20 mM HEPES, 50 μM ATP, 10 μCi [γ - ^{32}P] ATP, 10 mM MgCl_2 , and 0.2 mg/mL sonicated lipids. Both the lipids and the ATP mix should be separately prepared in amounts sufficient for the entire number of assays to be performed (see Note 9). The volumes given below reflect requirements for a single assay and should be multiplied accordingly to assay multiple samples.

3.1.1. Lipid Preparation

1. Aliquot 10 μL of either PtdIns or crude brain phospholipids into an Eppendorf tube and dry under a nitrogen stream (see Note 10)
2. Resuspend lipids in 50 μL of 2X detergent buffer (40 mM HEPES, 2 mM EDTA, 0.3% Triton X-100).
3. Sonicate at 50% duty in water bath sonicator for 5 min (see Note 4).

3.1.2. ATP Mix

1. Combine 1 μL [γ - ^{32}P] ATP (10 μCi), 0.5 μL 10 mM ATP, 1 μL 1 M MgCl_2 , and 37.5 μL H_2O for a total volume of 40 μL . Store on ice until ready to use.

3.1.3. Reaction Set-Up

1. Add protein sample in a final volume of 10 μL to an Eppendorf tube at 4°C (see Note 11).
2. Add 50 μL of lipid mix
3. Add 40 μL of ATP mix and place reaction at 25°C for 10–15 min.
4. Add 25 μL of 4 N HCl to stop the reaction.
5. Extract with 160 μL of chloroform:methanol (1:1) and save the lower chloroform phase (see Notes 10 and 12).

3.2. Typing PtdIns 4-Kinases

This assay is a variation of that described above and allows for the addition of reagents designed to discriminate between the different types of PtdIns 4-kinases.

3.2.1. Lipid Preparation

1. Dry down lipids as in **Subheading 3.1.1.**, but resuspend in 50 μL of 2X buffer (40 mM HEPES, 2 mM EDTA).
2. Sonicate as in **Subheading 3.1.1.**

3.2.2. ATP Mix

Add the reagents as in **Subheading 3.1.2.**, but alter the amount of water added such that the final reaction volume is 100 μL . That is, if the other reagents added to the reaction total a volume of 10 μL , then decrease the water volume

in the ATP mix to 27.5 μL , yielding 30 μL of the ATP mix for each assay. Thus the final reaction contains 50 μL of lipid mix, 10 μL of protein sample, 10 μL of additives and 30 μL of ATP mix for a total volume of 100 μL .

3.2.3. Reaction Set-Up

3.2.3.1. ADENOSINE INHIBITION

Assemble the assay as in **Subheading 3.1.3.**, but titrate in adenosine from 10 μM to 1 mM prior to the addition of the ATP mix (*see Note 13*).

3.2.3.2. DETERGENT ACTIVATION

Assay as in **Subheading 3.1.3.**, but add triton X-100 into the reaction at a final concentration of 0.15%, prior to adding the lipids (*see Note 14*).

3.2.3.3. 4C5G INHIBITION

Preincubate this antibody at 20 $\mu\text{g}/\text{mL}$ with an enzyme preparation for 20 min at 4°C, prior to assembling reaction as in **Subheading 3.1.3.**

3.3. Testing Wortmannin Sensitivity

Assays to test for wortmannin sensitivity are set up with a modified protocol to ensure that the concentration of wortmannin is well-controlled (*see Note 15*).

3.3.1. Lipid Preparation

Dried lipids are resuspended in 40 μL of 2.5X detergent buffer (20 mM HEPES, 1 mM EDTA, 0.375% Triton X-100) and sonicated as in **Subheading 3.1.1.**

3.3.2. ATP Mix

Combine 1 μL of [γ - ^{32}P] ATP, 0.5 μL 10 mM ATP, 1 μL 1 M MgCl_2 , and 2.5 μL of water for a final volume of 5 μL .

3.3.3. Reaction Set-Up

1. Add protein to Eppendorf tube at 4°C.
2. Titrate in wortmannin from 10 nM to 1 μM and adjust volume to 50 μL with 20 mM HEPES (*see Note 16*)
3. Incubate 10–20 min at 4°C.
4. Add 40 μL lipid mix.
5. Titrate in wortmannin in the same amounts as in **step 2**, and adjust the total volume added to 5 μL with water
6. Add 5 μL of ATP mix.
7. Place reactions at 25°C for 15 min then proceed with the remainder of the assay as in **Subheading 3.1.3.**

3.4. TLC Analysis

The lipid products in chloroform are spotted directly onto a coated TLC plate (*see Subheading 2.2.* and *Note 5*) developed using one of two solvent systems. If PtdIns was the assayed substrate then the short solvent system is preferred. If crude brain lipids were used then the long system may be desired.

3.4.1. TLC Plate Preparation

1. Using a pencil, draw a line across the plate approx 1.5 cm from the bottom. At equally spaced intervals along the line, draw a cross bar to mark the place where spotting will occur. Make one cross bar for each sample to be spotted and these should not exceed 15 samples on a given plate.
2. Place plate in 100°C oven for approx 30 min

3.4.2. Spotting and Running

1. Prepare fresh solvents for the TLC tank and add to a tank that is lined with solvent saturated Whatman paper on one side
2. Spot each sample on to the TLC plate by slowly pipeting small volumes of liquid and drying with a stream of air.
3. When samples are dry, place the plate in the TLC tank and allow the solvent to run to the top of the plate (30 min for the short system and approx 6 h for the long system)
4. Remove from tank, dry completely, and expose to film or phosphorimager (*see Note 17*).

3.5. Deacylation of Phosphoinositides

This step leads to the cleavage of the fatty acid chains from the phosphorylated lipids generated in the lipid kinase assay. The resulting glycerophosphoinositol phosphates (GroPIPs), which contain only the inositol head group and the glycerol side chain, can be easily separated by HPLC. The deacylation procedure can be performed on either the lipids extracted in the chloroform phase after lipid kinase assay, or on lipids excised from a TLC plate (*see Note 17*).

3.5.1. Deacylation of Lipid Samples from Chloroform Extraction

1. Dry chloroform-extracted lipids under a nitrogen stream.
2. Add 1 mL of methylamine reagent (*Subheading 2.3.*) and mix well (*see Note 18*).
3. Incubate at 53°C for 50 min.
4. Dry samples down in a speed vac using a cold sulfuric-acid trap (*see Note 8*)
5. Resuspend in 1 mL of water and vortex well.
6. Transfer to a 5-mL glass test tube.
7. Add 1 mL extraction solution (*Subheading 2.3.*) and vortex gently.
8. Remove and discard the upper phase and repeat the extraction.
9. Transfer the lower aqueous phase to an Eppendorf tube and dry in a speed vac (sulfuric acid trap is no longer necessary).

10. Resuspend the samples in 120 μL water and mix with ^3H deacylated standards (**Subheading 3.5.3.**).
11. Transfer to a filter Eppendorf and filter samples by centrifugation.

3.5.2. Deacylation of Lipids Excised from a TLC Plate

1. Place the excised piece of TLC plate directly into an Eppendorf tube and crush slightly (*see Note 17*).
2. Add 1 mL of methylamine reagent (**Subheading 2.3.**) and mix well.
3. Incubate at 53°C for 50 min
4. Spin out the silica and transfer the solution to a new Eppendorf tube
5. Proceed with speed vac and the remainder of the protocol as indicated in **Subheading 3.5.1.**

3.5.3. Deacylation of [^3H]Standards

The deacylated lipid standards can be prepared ahead of time and stored at -70°C . An aliquot (approx 10,000 cpm total) is then added to the samples prepared above, just before filtering.

1. Add an equal number of cpm of [^3H] PtdIns 4-P and [^3H] PtdIns 4,5- P_2 to an Eppendorf
2. Dry down in speed vac
3. Resuspend in methylamine reagent and proceed as in **Subheading 3.5.1.**

3.6. HPLC Analysis

A more complete description of the HPLC analysis of the deacylated lipids can be found in Chapter 3. Our remarks focus on the specific elution program used and the elution profiles expected. The Partisphere SAX anion exchange HPLC column is loaded with deacylated lipids, washed with water and then the bound material is eluted with a gradient of $(\text{NH}_4)_2\text{HPO}_4$, pH 3.8 over 125 min according to the following program: After loading, wash the column for 5 min with water and then initiate a shallow gradient from 0 to 150 mM $(\text{NH}_4)_2\text{HPO}_4$, developed over 55 min. Next, do an isocratic wash at 150 mM $(\text{NH}_4)_2\text{HPO}_4$ for 15 min and then begin a steeper gradient from 250 to 650 mM $(\text{NH}_4)_2\text{HPO}_4$, developed over 25 min. Finally, wash the column for 15 min with 1 M $(\text{NH}_4)_2\text{HPO}_4$, followed by a water wash for 10 min. The eluate from the HPLC is connected to an online continuous flow liquid scintillation detector that can monitor and quantify two different radioisotopes simultaneously. Using this gradient, the following elution profiles are expected: GroPIns 3-P, approx 24 min, GroPIns 4-P, approx 26 min, GroPIns 3,4- P_2 , approx 60 min, GroPIns 4,5- P_2 , approx 62 min. Elution times may vary over 2–3 min from run to run, but alterations in mobility will usually effect all of the lipids in a given sample equally. That is, PtdIns 3-P will always elute approx 1–2 min before PtdIns 4-P and likewise for PtdIns 3,4- P_2 and PtdIns 4,5- P_2 .

4. Notes

1. The PtdIns 4-kinases were originally described as membrane-bound enzymes with apparent molecular weight by gel filtration of either 55 kDa (type II) or >200 kDa (type III). Type I PtdIns kinase was shown to be a PtdIns 3-kinase (9). Recently, a novel PtdIns 4-kinase that is cytosolic, has a molecular weight of approx 125 kDa and has enzymatic properties of a type III enzyme has been described (8,10).
2. 4C5G is a monoclonal antibody raised against a partially purified preparation of bovine brain type II PtdIns 4-kinase. It recognizes all type II PtdIns 4-kinases and inhibits their activity by approx 80–90% (6).
3. PtdIns is stored at -70°C as a 2-mg/mL solution in chloroform. This and all lipids should be thawed before use and returned to -70°C as soon as possible to avoid evaporation.
4. The bath sonicator is used at room temperature and should be cooled with ice if the water gets hot (usually following multiple uses).
5. The oxalate treatment is performed to chelate the divalent cations that could act as counter ions for the inositol phosphates and decrease the mobility of the lipids on the TLC plate. To oxalate-coat the TLC plates, fill a TLC tank with the dry plates in a TLC holder. Slowly fill the tank from the bottom up, with the 1% potassium oxalate solution (in 50% methanol), such that the solvent front heading up the TLC is always ahead of the buffer filling the tank. When the plates are completely submerged, remove them from the tank and allow them to air-dry.
6. This chamber can be used to stain the bulk phospholipids on a TLC plate and confirm proper running of the system. To do so, the TLC plate is placed into the iodine tank (with a gloved hand) for 1–5 min as the color develops. When the lipids can be easily visualized, the plate is removed and can be exposed to film as usual.
7. This reagent can be made up to 6 mo in advance and stored in a dark bottle at 4°C .
8. To prevent methylamine vapor from damaging the vacuum pump, a flask containing concentrated sulfuric acid is placed on dry ice and attached to the centrifuge to serve as an acid trap. Ideally, the acid is cooled until it becomes a slurry that is not completely frozen, prior to pumping. Replace with fresh sulfuric acid when the solution becomes brown.
9. It is helpful to make enough mix for an extra one to two reactions to ensure that, upon repeated pipeting, enough solution remains.
10. The lipids are in organic solvent and will drip out of the pipet tip readily—so pipet quickly. To avoid this problem, the tip can be prewashed with chloroform by pipeting approx 150 μL of chloroform up and down two to three times before using the tip. Additionally, once the lipids are dry, they are stable and should be resuspended and sonicated just before use.
11. The protein sample can be a crude protein lysate or a sample that has been isolated on beads such as GST or protein A. If the protein is recovered and immobilized on beads, then it should receive a final wash in 20 mM HEPES, 1 mM EDTA, with the appropriate protease inhibitors before assay. If the protein is on beads, then one should also frequently tap the tube during the 10- to 15-min reaction to maintain a slurry.

12. The methanol will partition into the aqueous phase and yield a final organic (chloroform) phase that is approx 60–70 μL . This can be directly spotted onto a TLC plate (**Subheading 3.4.**) or stored at -70°C . If the reaction is scaled up and yields a large amount of chloroform, then the volume should be reduced to $<50 \mu\text{L}$ by evaporation under a nitrogen stream prior to spotting on the TLC.
13. The type II enzyme has a K_i for adenosine of approx $20 \mu\text{M}$, whereas the type III enzyme has a K_i of approx $1500 \mu\text{M}$ (**5**).
14. Both the type II and the type III enzymes are maximally active in detergent. However, the type II enzyme can be stimulated as much as 50-fold in the presence of Triton X-100 (**11**), whereas the type III enzyme is only slightly stimulated by detergent.
15. Although wortmannin is known to inhibit PI 3-kinase irreversibly (**12**), it may not covalently modify the PtdIns 4-kinase and therefore must be present throughout the preincubation and during the assay (REM and LCC, unpublished results). To do so, add a second aliquot of wortmannin into the reaction prior to the addition of the ATP mix. The reaction will be diluted twofold by the addition of lipids and the ATP mix, therefore equal amounts of wortmannin are added to both the preincubation and the final reaction. For example, if the assay is to contain 500 nM wortmannin, then add $2.5 \mu\text{L}$ of a $10\text{-}\mu\text{M}$ stock of wortmannin to the preincubation (**Subheading 3.3.3., step 2**) and then add $2.5 \mu\text{L}$ of $10 \mu\text{M}$ wortmannin and $2.5 \mu\text{L}$ of water to the reaction just prior to the addition of the ATP mix (**Subheading 3.3.3., step 5**).
16. Wortmannin is not stable in aqueous solution and should be diluted in water just prior to use and stored on ice.
17. If the TLC spot is to be deacylated then leave the plate wet, wrap it in plastic wrap, and briefly expose to film. Using the film to align, and a hypodermic needle to poke holes, mark the four corners of the spot by piercing through the film onto the TLC plate. Now, using the same needle, lift or scrape the silica within the square and transfer to an Eppendorf tube.
18. Methylamine is volatile and toxic. Use it only in a hood.

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Phosphatidylinositol(3,4,5)Trisphosphate (PtdIns(3,4,5)P₃) Mass Measurement Using a Radioligand Displacement Assay

Jeroen van der Kaay, Pete J. Cullen, and C. Peter Downes

1. Introduction

1.1. Background

The phosphatidylinositol 3-kinases (PI 3-kinases), which play a pivotal role in cellular signaling, represent a family of enzymes that phosphorylate phosphoinositides on the 3-position of the inositol ring (1). PtdIns(3,4,5)P₃ (phosphatidylinositol(3,4,5)trisphosphate) is one of the principle products of this reaction and has been suggested to be a second messenger involved in many physiological phenomena.

The traditional techniques employed to measure changes in this signaling molecule involve radiolabeling with high levels of [³²P]PO₄ or [³H]inositol and the subsequently extracted phosphoinositides are analyzed by multiple chromatography steps. Another more simple approach is to assay PI 3-kinase activity in cell extracts and/or appropriate immunoprecipitates. The latter procedure, however, is an indirect measurement that detects the association of PI 3-kinases in molecular complexes and does not necessarily correlate with the enzyme's activity state.

Therefore, a straightforward, highly specific, and sensitive method, avoiding costly, time-consuming, and hazardous procedures, has been developed to allow detection of subpicomolar quantities of PtdIns(3,4,5)P₃ in samples from either cell cultures or whole tissues (2).

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1.2. Sample Preparation

Agonist-provoked increases in PtdIns(3,4,5)P₃ are usually transient because of phosphatase action; therefore an instant quenching of cells or tissue is required in order to avoid enzymic degradation of PtdIns(3,4,5)P₃ during the extraction process. Cells are preferably directly quenched with chloroform:methanol:conc. hydrochloric acid (40:80:1 by vol). Plastic culture dishes however dissolve in chloroform and in these circumstances ice-cold 10% trichloric acid (TCA) can be used to kill the cells. Whole tissue samples are best snap frozen in liquid nitrogen and can be stored at -80°C. The tissue can then be finely powdered under liquid nitrogen using a pestle and mortar. After transferring the samples to a polypropylene tube the lipids are extracted as described above. Cell suspensions can be also be snap frozen and be stored at -80°C.

1.3. Assay of PtdIns(3,4,5)P₃

1.3.1. Principle of the Assay

The assay is based on radioligand displacement of radiolabeled inositol (1,3,4,5)tetrakisphosphate (Ins[1,3,4,5]P₄) from binding sites present in cerebellum or platelets that display high affinity and specificity for Ins(1,3,4,5)P₄ (3,4). Following an ordinary Folch extraction of the phospholipids from cell or tissue samples, Ins(1,3,4,5)P₄, the polar headgroup of PtdIns(3,4,5)P₃, is released by alkaline cleavage and its mass can be measured by radioligand displacement using a calibration curve obtained using unlabeled Ins(1,3,4,5)P₄ standards. The sensitivity limit is approx 0.3 pmol and, in general, lipid extracts from samples containing 1 mg of cellular protein are sufficient for determination of the basal level of PtdIns(3,4,5)P₃.

1.3.2. The Use of Ins(1,3,4,5)P₄ Binding Protein from Cerebellum vs the Use of GAP1^{IP4BP} from Platelets

The isolation procedure of both Ins(1,3,4,5)P₄-binding proteins are described in **Subheading 3.3**. Ins(1,3,4,5)P₄-binding protein can be isolated from the crude membrane fraction from cerebella of rat, sheep, and pig. Since sheep (and bovine) cerebella are banned in the United Kingdom and rat cerebella give a low yield of protein, pig cerebellum is recommended as a convenient source (six pig cerebella yield enough material for approx 4000 assays).

The use of cerebellar protein in the displacement assay requires [³²P]Ins(1,3,4,5)P₄ of high specific radioactivity (3000 Ci/mmol), which is not commercially available but can be prepared as described in **Subheading 3.2**.

The gene encoding the Ins(1,3,4,5)P₄-binding protein in pig platelet membranes has been cloned and is classified as GAP1^{IP4BP} (4). The protein can be expressed in a truncated form as a glutathione-S-transferase (GST)-fusion pro-

tein in *Escherichia coli* under an IPTG-inducible promoter. A 500-mL culture in LB medium gives enough Ins(1,3,4,5)P₄-binding protein for approx 750 assays. The recombinant GAP1^{IP4BP} protein can be used with [³²P]Ins(1,3,4,5)P₄, but also with the commercially available [³H]Ins(1,3,4,5)P₄ (21 Ci/mmol) because of the low nonspecific binding of this protein which is easily purified from the *E. coli* lysate using GST immobilized on agarose beads.

Separation of bound from unbound labeled Ins(1,3,4,5)P₄ in both cases is achieved by filtration (**Subheading 3.4.1.**), although the GAP1^{IP4BP} leaves the opportunity for separation by centrifugation after precipitation with γ -globulins and polyethylene glycol (PEG) as described in **Subheading 3.4.2.2.**

1.3.3. Required Components for the PtdIns(3,4,5)P₃ Mass Assay

The PtdIns(3,4,5)P₃ mass assay requires some components that are not commercially available but can be prepared as described in this chapter.

1. Recombinant or partially purified Ins(1,4,5)P₃ 3-kinase for the preparation of [³²P]Ins(1,3,4,5)P₄ of sufficiently high specific activity (3000 Ci/mmol) to be used with the cerebellar-binding protein.
2. Ins(1,3,4,5)P₄-binding protein can be readily isolated from cerebellum freshly collected from a local slaughterhouse
3. The availability of the recombinant GAP1^{IP4BP} allows the use of commercially available [³H]Ins(1,3,4,5)P₄ (specific activity of 21 Ci/mmol) and obviates the need to prepare [³²P]Ins(1,3,4,5)P₄ and the precautions needed when processing the samples.

2. Materials

2.1. Preparation of Ins(1,4,5)P₃ 3-Kinase

2.1.1. Partial Purification of Recombinant Rat Brain Ins(1,4,5)P₃ 3-Kinase

- 1 *E. coli* containing a vector with DNA insert coding for ratbrain Ins(1,4,5)P₃ 3-kinase (5)
- 2 200 mL LB medium
3. Ampicillin (50 μ g/mL final conc.).
4. Isopropyl- β -D-thiogalactopyranoside (IPTG, at 1 mM final conc.).
- 5 Centrifuge.
- 6 10 mL of ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.4 mM benzamidine, 0.4 mM phenylmethylsulfonyl fluoride, 5 μ M leupeptin, 5 μ M pepstatin, and calpain inhibitors I and II at 5 μ g/mL).
7. Sonicator probe (Jencons Scientific [Bedfordshire, UK] ultrasonic processor)
8. Triton X-100.
9. Ultracentrifuge and a rotor for 15,000g.
10. Calmodulin (CaM) affinity column (1.25 \times 3 cm; Sigma, St. Louis, MO).
11. Elution buffer (20 mM Tris-HCl, pH 7.5, protease inhibitors [see item 6] and 12 mM β -mercaptoethanol) with the following additions:

12. 100 mL Elution buffer supplemented with 0.2 mM CaCl₂, 0.4 M NaCl, and 0.5% Triton X-100.
13. 100 mL Elution buffer supplemented with 0.2 mM CaCl₂, 0.4 M NaCl.
14. 150 mL Elution buffer supplemented with 2 mM EGTA, 0.4 M NaCl.
15. Amicon Centrifrep 30 filter (Naperville, IL).

2.1.2. Partial Purification of Ratbrain Ins(1,4,5)P₃ 3-Kinase

If recombinant Ins(1,4,5)P₃ 3-kinase is not accessible, then the enzyme can be partially purified relatively easily from rat brain, which is a good source for this enzyme. The reader is referred to two papers describing this purification in detail (6, 7).

2.1.3. Ins(1,4,5)P₃ 3-Kinase Activity Screen

1. Dowex anion exchange resin. AG 1 × 8, 200–400 mesh in the formate form (BioRad, Hercules, CA).
2. Column holders (or 10-mL syringe plugged with glass wool).
3. Freon (1,1,2 Trichlorotrifluoroethane).
4. Trioctylamine.
5. [³H]Inositol(1,4,5)trisphosphate ([³H]Ins(1,4,5)P₃ from Amersham).
6. 80 μL of assay buffer, which in 0.1 mL gives final concentrations of 50 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 1 mM ATP, 1 mg/mL BSA, and 0.005 μCi [³H]Ins(1,4,5)P₃ for each fraction/sample to be assayed
7. 10% TCA (w/v).
8. 0.8 M Ammonium formate/0.1 M formic acid.
9. 1.2 M Ammonium formate/0.1 M formic acid
10. FloScint IV, scintillant vials, liquid scintillation counter

2.2. Preparation of [³⁻³²P]Ins(1,3,4,5)P₄

1. 60 μL of assay buffer, which in 0.2 mL gives final concentrations of 50 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 1 mM EGTA, 1.018 mM CaCl₂, 0.1 mM Ins(1,4,5)P₃ (Cell Signals, Lexington, KY), 1 μM CaM (Sigma), and 20 mg/mL BSA
2. 100 μL of 10 mCi/mL [³²P]ATP (3000 Ci/mmol)
3. 40 μL of recombinant Ins(1,4,5)P₃ 3-kinase (0.1 mg/mL).
4. 10 mM EDTA.
5. Screw-cap tube (1.5 mL, Sarstedt, Leicester, UK).
6. Boiling bath or thermoblock at 100°C.
7. High-performance liquid chromatography (HPLC) system.
8. HPLC partisphere-SAX column.
9. 500 mL of water and 500 mL of 1.0 M NH₄H₂PO₄, pH 3.8 (both 0.2 μM filtered).
10. Liquid scintillation counter
11. Scintillant vials.
12. Dialysis tubing (molecular weight cutoff 12–14 kDa).
13. 3 L Distilled water

2.3. Preparation of *Ins(1,3,4,5)P₄*-Binding Protein

2.3.1. Preparation of Cerebellar *Ins(1,3,4,5)P₄*-Binding Protein

1. Six pig cerebella (approx 100 g) freshly collected from the slaughterhouse.
2. 2.5 L Ice-cold homogenization buffer (20 mM NaHCO₃, 1 mM dithiothreitol, 2 mM EDTA, pH 8.0).
3. Motor-driven homogenizer consisting of glass mortar tube and a pestle with a steel shaft (minimum 75-mL capacity).
4. Ultracentrifuge with 50-mL tubes to be spun at 5000g and 100,000g.

2.3.2. Preparation of Recombinant GAP1^{IP4BP}

1. *E. coli* (strain BL21) containing plasmid with insert coding for the truncated GST-tagged GAP1^{IP4BP} (GST-sC2GAP1^{IP4BP}) and cotransformed with a plasmid coding for GroES/GroEl chaperones.
2. IPTG (to induce expression of the proteins).
3. Ampicillin and kanamycin
4. 500 mL LB medium
5. Centrifuge.
6. 100 mL Ice-cold lysis buffer (53 mM Na₂HPO₄, 3 mM NaH₂PO₄, 45 mM NaCl [= PBS], 1 mM EDTA, 1 mM EGTA, 1 mM β-mercaptoethanol).
7. Lysozyme
8. Sonicator with probe (Jencons ultrasonic processor)
9. Triton X-100.
10. Ultracentrifuge (100,000g)
11. 1 mL 50% Glutathione agarose (Sigma).
12. Rotary shaker (4°C)
13. 50 mL High salt wash buffer (75 mM Tris-HCl (pH 8.0), 0.3 M NaCl).
14. Glycerol
15. Elution buffer if you choose to elute the GAP1^{IP4BP} from the glutathione- agarose beads: 75 mM Tris-HCl, pH 8.0, 1 M NaCl, 0.1% Triton X-100, 20 mM glutathione (Subheading 3.4.2.2.). Readjust the pH after addition of glutathione to 8.0.
16. Polyethylene glycol (PEG) of average mol wt 3500 Dalton
17. γ-Globulin.

2.4. *Ins(1,3,4,5)P₄* Isotope Dilution Assay

1. Unlabelled *Ins(1,3,4,5)P₄* (CellSignals).
2. Radiolabeled *Ins(1,3,4,5)P₄*.
3. *Ins(1,3,4,5)P₄*-binding protein (cerebellar membranes or recombinant GAP1^{IP4BP}).
4. Assay buffer (0.1 M sodium acetate, 0.1 M KH₂PO₄, 4 mM EDTA, pH 5.0 with acetic acid).
5. Samples to be analyzed.
6. Glass microfiber filters (Whatman, Clifton, NJ, GF/C, 25 mm).
7. Vacuum manifold for 12 samples (Millipore, Bedford, MA).
8. Ice-cold wash buffer (25 mM sodium acetate, 25 mM KH₂PO₄, 1 mM EDTA, 5 mM NaHCO₃, pH 5.0 with acetic acid).

9. Scintillant (FloScint IV).
10. Scintillation vials (5 mL).
11. Liquid scintillation counter.
12. Open Eppendorf racks allowing Eppendorf tubes to be placed in ice
13. Vortex mixer
14. 1 M KOH/acetic acid, pH 5.0, at 4°C. (Make up 0.5 L of 2 M KOH and add acetic acid to give a pH of 5.0, then adjust the volume to 1 L.)

2.5. Sample Preparation

1. Trichloroacetic acid (TCA, 10% w/v).
2. Chloroform:methanol:conc. HCl (20:40:1 by vol).
3. Liquid nitrogen
4. Pestle and mortar
5. 5% w/v TCA/1 mM EDTA
6. Chloroform.
7. 0.1 M HCl.
8. Synthetic lower phase (prepared by mixing chloroform, methanol and 0.1 M HCl at ratio of 1:1:0.9 by volume and collecting the lower phase).
9. Polypropylene Eppendorf tubes (1.5 mL)
10. Polypropylene screw-capped tubes (1.5 mL)
11. Microcentrifuge
12. Vacuum concentrator.
13. 1 M KOH.
14. Boiling water-bath or hot plate at 100°C.
15. 1 M acetic acid.
16. Butanol:petroleum ether (bp 40–60°C):ethylacetate (20:4:1 by vol).
17. Dilute acetic acid (**Subheading 3.5.1., step 15**).

3. Methods

3.1. Preparation of *Ins(1,4,5)P₃* 3-Kinase

3.1.1. Partial Purification of Recombinant *Ins(1,4,5)P₃* 3-Kinase (2,5)

1. Grow *E. coli* containing bluescript plasmid with the cloned DNA insert overnight in 50 mL LB medium supplemented with 50 µg/mL ampicillin at 37°C.
2. Dilute culture to an A_{600} of 0.5 with fresh prewarmed medium (30°C).
3. Induce expression by addition of 1 mM IPTG.
4. Grow for 2 h at 30°C.
5. Harvest bacteria by centrifugation for 20 min at 10,000g.
6. Resuspend in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.4 mM benzamidine, 0.4 mM phenylmethylsulfonyl fluoride, 5 µM leupeptin, 5 µM pepstatin, and calpain inhibitors I and II at 5 µg/mL) (*see Note 1*).
7. Sonicate on ice (5 × 30 s and cool on ice in between, output is set at 80% of the maximum)
8. Add triton X-100 to a final concentration of 1% by vol.

9. Shake the lysate on a rotary shaker in a cold room for 1 h
10. Centrifuge the lysate for 30 min at 100,000g.
11. Apply the supernatant to a CaM affinity column (1.25 × 3 cm).
12. Elution is performed in elution buffer (20 mM Tris-HCl, pH 7.5, protease inhibitors [above-mentioned] and 12 mM β-mercaptoethanol) with the following additions.
13. First elution step: 3 × 20-mL elution buffer supplemented with 0.2 mM CaCl₂, 0.4 M NaCl, and 0.5% Triton X-100.
14. Second elution step 3 × 20-mL elution buffer supplemented with 0.2 mM CaCl₂, 0.4 M NaCl.
15. Third elution step. 5 × 20-mL elution buffer supplemented with 2 mM EGTA, 0.4 M NaCl.
16. The fractions are screened for Ins(1,4,5)P₃ 3-kinase activity (**Subheading 3.1.2.**).
17. The active fractions are pooled and concentrated using an Amicon Centriprep 30 filter (protein concentration is approx 0.1 mg/mL).
18. Aliquots of 50 μL are stored at -80°C

3.1.2. *Ins(1,4,5)P₃ 3-Kinase Activity Screen*

1. Reaction mixtures of 0.1 mL contain 50 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 1 mM ATP, 1 mg/mL BSA, 0.005 μCi [³H]Ins(1,4,5)P₃, and 0.02 mL of enzyme fraction
2. Incubate at 37°C for 20 min and quench the reaction with 0.1 mL 10% TCA (w/v) Assay the samples in duplicate.
3. The protein precipitates are removed by centrifugation, and the supernatants are neutralized by addition of 0.5 mL freon:trioctylamine (1:1 by vol).
4. Vortex and spin for 1 min at full speed in a microcentrifuge.
5. Apply supernatant onto a 1-mL Dowex column (Hercules, CA) (2 mL of 50% slurry of Dowex)
6. First elution is with 10 mL of 0.8 M ammonium formate/0.1 M formic acid This "Ins(1,4,5)P₃"-fraction will contain unconverted [³H]Ins(1,4,5)P₃ (and eventual degradation products, i.e., [³H]InsP₂, [³H]InsP, [³H]inositol).
7. Second elution is with 10 mL of 1.2 M ammonium formate/0.1 M formic acid This "Ins(1,3,4,5)P₄"-fraction will contain the [³H]Ins(1,3,4,5)P₄ produced from [³H]Ins(1,4,5)P₃ by the Ins(1,4,5)P₃ 3-kinase Since the separation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ is not 100% absolute, control samples of [³H]Ins(1,4,5)P₃ and [³H]Ins(1,3,4,5)P₄ without added enzyme/or with boiled enzyme are analyzed in parallel. If the separation is poor—i.e., more than 25% overflow of [³H]Ins(1,4,5)P₃ in the "Ins(1,3,4,5)P₄"-fraction or vice versa—the first elution should be adapted by increasing or decreasing the molarity of the ammonium formate, respectively
8. Add 10 mL of FloScint IV to both fractions, mix well, and count the radioactivity.
9. Calculate the total radioactivity of the samples—i.e., the sum of the "Ins(1,4,5)P₃" and the "Ins(1,3,4,5)P₄" fraction Screen for an increased percentage of the total radioactivity in the "Ins(1,3,4,5)P₄" fraction.

3.2. Preparation of [³⁻³²P]Ins(1,3,4,5)P₄

1. Add 60 μL assay buffer, which in 0.2 mL gives a final concentration of: 50 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 1 mM EGTA, 1.018 mM CaCl₂, 0.1 mM

Ins(1,4,5)P₃, 1 μ M CaM, and 1 mg/mL BSA, to 100 μ L of 10 mCi/mL [³²P]ATP (3000 Ci/mmol) in a screw-cap microtube.

- 2 Start reaction by adding 40 μ L of recombinant Ins(1,4,5)P₃ 3-kinase (from preparation described in **Subheading 3.1.**).
- 3 Incubate for 30 min at 37°C.
- 4 Terminate reaction by addition of 0.8 mL 10 mM EDTA and subsequent boiling for 2 min.
5. Cool on ice and centrifuge for 2 min at 13,000g.
- 6 Apply the supernatant via a 2-mL sample loop to an HPLC Partisphere-SAX column (4.6 \times 250 mm) eluted with a gradient made of water and 1.0 M NH₄H₂PO₄, pH 3.8
- 7 The gradient used is 0–2 min 0%, 2–5 min 50%, 5–65 min 100%, 65–75 min 100%, 75–76 min 0%, and 76–90 min 0% at a flow rate of 1 mL/min.
- 8 After the start of the gradient, Ins(1,3,4,5)P₄ elutes at approx 42 min. Therefore collect [³²P]Ins(1,3,4,5)P₄ in a 50-mL Falcon tube (Los Angeles, CA) from 40–48 min and collect the rest of column eluant in a conical flask. Always calibrate the column when this is done for the first time—use either commercial [³H]Ins(1,3,4,5)P₄ standard or 1% of the sample (*see Note 2*).
9. Dilute [³²P]Ins(1,3,4,5)P₄ in 20 mL distilled water.
- 10 Dialyze the sample, using dialysis tubing with a molecular-weight cutoff of 12–14 kDa, three times against 1000 mL distilled water for 45 min. The dialysates will contain radioactivity and should be handled with care (*see Note 3*).
11. Collect retentate, which contains approx 0.5 mCi [³²P]Ins(1,3,4,5)P₄ and dilute in distilled water to 40 mL final volume.
- 12 Aliquot [³²P]Ins(1,3,4,5)P₄ in 20 \times 1 mL and 1 \times 20-mL portions (approx 10 μ Ci/mL) (*see Note 4*).
- 13 Store at –20°C.

3.3. Preparation of Ins(1,3,4,5)P₄-Binding Protein

3.3.1. Preparation of Cerebellar Ins(1,3,4,5)P₄-Binding Protein

- 1 Collect six fresh whole pig brains from the slaughterhouse and keep them on ice (*see Note 5*).
- 2 Cut the cerebella loose with a scalpel or scissors and collect them in a beaker on ice. The cerebellum is a clearly distinct tissue with a fine reticular structured motif
- 3 Transfer three pig cerebella to a precooled 75-mL mortar tube and add 50 mL ice-cold homogenization buffer (20 mM NaHCO₃, pH 8.0, 2 mM EDTA, and 1 mM dithiothreitol)
4. The steel shaft of the pestle is compatible with an electric drill and 5–10 strokes are given to grind the (soft) tissue.
5. Transfer the homogenate to a centrifuge tube and homogenize the next three cerebella.
- 6 Pool the homogenates and centrifuge for 10 min at 5000g.
7. The supernatant is transferred to 50-mL ultracentrifuge tubes and the pellet is re-extracted once with the same volume of homogenization buffer.
8. The pooled supernatants are centrifuged for 20 min at 100,000g.

9. The pellet is washed twice and resuspended in homogenisation buffer at a final concentration of 10–20 mg/mL.
10. Aliquots of 4 mL are stored at –80°C.

3.3.2. Preparation of Recombinant Ins(1,3,4,5)P₄ Binding Protein (GAP1^{IP4BP})

1. Grow an overnight 100 mL culture of *E. coli* transformed with plasmids encoding GST-σC2GAP1^{IP4BP} and the chaperones GroEL and GroES in LB medium supplemented with ampicillin and kanamycin (50 µg/mL of each) at 29°C (see Note 6)
2. Dilute culture 1:10 with fresh LB medium (plus antibiotics).
3. Grow up to A₆₀₀ and then induce with 1 mM IPTG for 5 h
4. Harvest bacteria by centrifugation for 20 min at 10,000g.
5. Resuspend bacteria in 20 mL ice-cold lysis buffer (PBS, 1 mM EDTA, 1 mM EGTA, 1 mM β-mercaptoethanol), transfer to a 50-mL Falcon tube and keep on ice
6. Add 2 mg/mL (final concentration) lysozyme and keep on ice for 30 min.
7. Sonicate on ice (5 × 30 s with cooling on ice in between; output is set at 80% of the maximum).
8. Add 2% (by volume) Triton X-100 and keep on ice for 30 min.
9. Centrifuge the lysate for 30 min at 100,000g.
10. Transfer the supernatant to a 50-mL Falcon tube and incubate with 1 mL of 50% glutathione immobilized on agarose beads overnight on a rotary shaker at 4°C
11. To remove proteins that bind nonspecifically to the beads, wash twice with 20 mL of cold lysis buffer and then twice with 20 mL of cold high salt buffer (75 mM Tris-HCl, pH 8.0, 0.3 M NaCl; centrifuge for 1 min at 1000g).
12. Resuspend the beads in 10 mL high-salt buffer with added glycerol (50% by volume)
13. Store at –20°C.

3.4. Ins(1,3,4,5)P₄ Isotope Dilution Assay

The assay involves measuring the quantity of [³²P] or [³H]Ins(1,3,4,5)P₄ bound to a highly specific binding protein(s) in the presence of known amounts of unlabeled Ins(1,3,4,5)P₄ to generate a standard curve and unknown amounts in the case of the samples to be analyzed. A typical calibration curve using the cerebellar Ins(1,3,4,5)P₄-binding protein and the GAP1^{IP4BP} is shown in Fig. 1. Ins(1,3,4,5)P₄ is cleaved from PtdIns(3,4,5)P₃ by alkaline hydrolysis, the yield of Ins(1,3,4,5)P₄ by this method is 62%. So for absolute amounts of PtdIns(3,4,5)P₃, the final values read from the calibration curve need to be corrected with this factor (see Notes 18 and 19). Figure 2 shows the amounts of the side products formed in a schematic representation of the alkaline hydrolysis. In ref. 2, it is shown that these products do not interfere with or contribute to the measured signal.

A very important feature of the assay is its selectivity for Ins(1,3,4,5)P₄ over Ins(1,4,5)P₃. The latter compound is formed by alkaline cleavage of PtdIns(4,5)P₂, which is usually present at 1000-fold higher levels in resting cells. The K_d for Ins(1,3,4,5)P₄ is approx 5000-fold lower than the K_d for

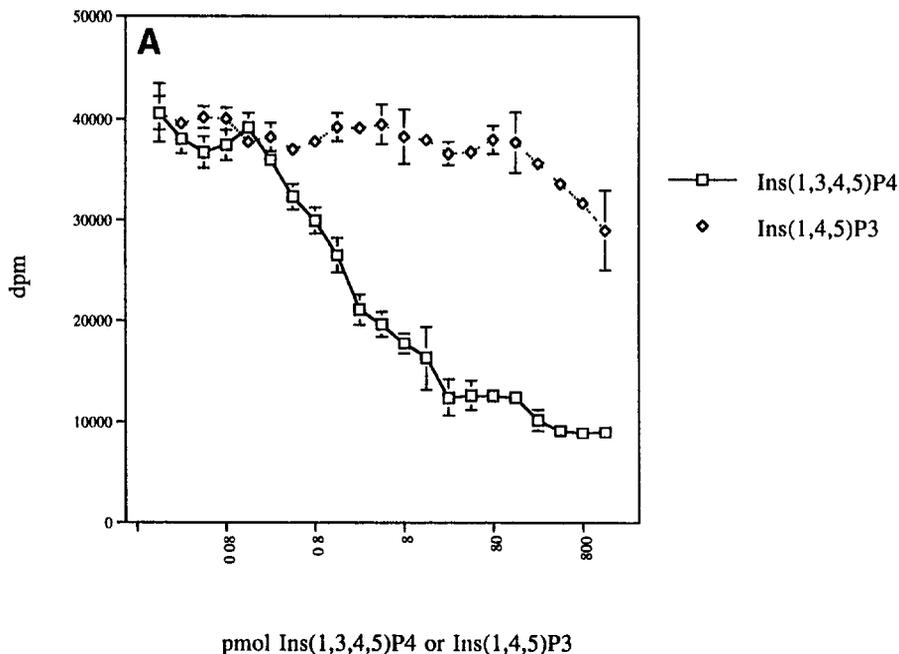


Fig 1. (A) Displacement of [³²P]Ins(1,3,4,5)P₄ by authentic Ins(1,3,4,5)P₄ and Ins(1,4,5)P₃ using Ins(1,3,4,5)P₄-binding protein isolated from sheep cerebellum. The displacement of [³²P]Ins(1,3,4,5)P₄ (3×10^5 dpm) by Ins(1,3,4,5)P₄ (□) and Ins(1,4,5)P₃ (◇) at indicated concentrations was measured in a final assay volume of 0.32 mL after a 30-min incubation on ice. Each data point represents a determination in triplicate \pm SD obtained from five independent experiments.

Ins(1,4,5)P₃, so there will be no “noise” arising from PtdIns(4,5)P₂ also present in the lipid extracts of cells.

3.4.1. Assay Procedure Using the Cerebellar Ins(1,3,4,5)P₄ Binding Protein

1. Make Ins(1,3,4,5)P₄ stock of 10 mM in H₂O and store at -80°C .
2. Prepare 1-mL aliquots of unlabeled Ins(1,3,4,5)P₄ standards of the following concentrations: 0.67, 1, 2, and 3×10^{-9} ; 0.67, 1, 2, and 3×10^{-8} ; 0.67, 1, 2, and 3×10^{-7} ; 0.67, 1, 2, and 3×10^{-6} M (see Note 7).
3. The calibration curve consists of 16 samples—use the above prepared Ins(1,3,4,5)P₄ concentrations—assayed in triplicate (giving 48 samples in total). The samples contain 50 μL of Ins(1,3,4,5)P₄ and 50 μL of 1 M KOH/acetic acid, pH 5.0 at 4°C .
4. The samples to be analyzed are 100 μL of 0.5 M KOH/acetic acid (pH 5.0) obtained after alkaline cleavage of cellular (or synthetic) lipids (Subheading 3.5.) and are preferably assayed in triplicate, but at least in duplicate.

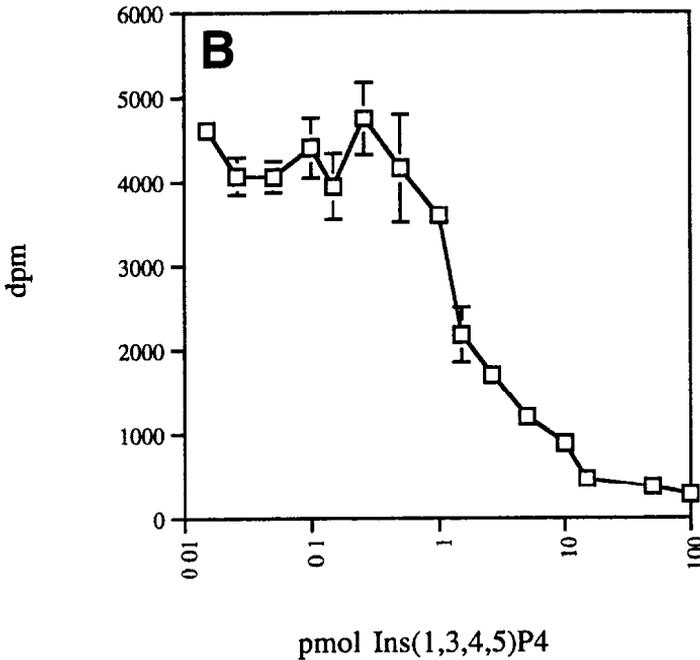


Fig 1 (B) Displacement of [³H]Ins(1,3,4,5)P₄ by authentic Ins(1,3,4,5)P₄ using recombinant GAP1^{IP4BP}. The displacement of [³H]Ins(1,3,4,5)P₄ (4 × 10⁴ dpm) by Ins(1,3,4,5)P₄ at indicated concentrations was measured in a final assay volume of 0.40 mL after a 30-min incubation on ice. Each data point represents a determination in triplicate ± SD obtained from two independent experiments

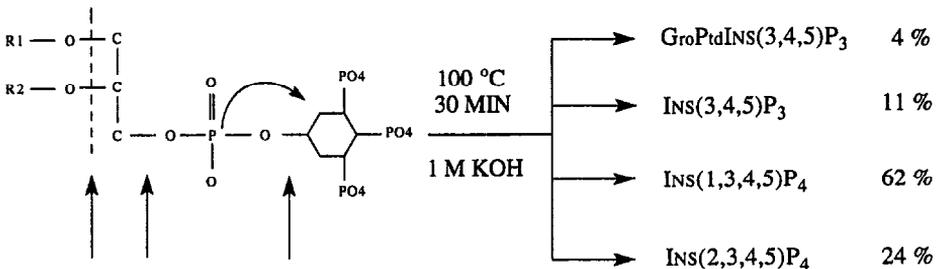


Fig. 2. Schematic representation of the products from PtdIns(3,4,5)P₃ subjected to alkaline hydrolysis.

5. All steps are on ice.
6. Each sample for the Ins(1,3,4,5)P₄ assay contains 400 μL final volume: 100 μL of sample (Ins(1,3,4,5)P₄ standard or actual sample), assay buffer (**Subheading**

2.5.), [^{32}P]Ins(1,3,4,5) P_4 (0.05 μCi in H_2O) and Ins(1,3,4,5) P_4 -binding protein (**Subheading 3.4.1.**) (see **Notes 8–11**).

7. The incubation is started by addition of the binding protein
8. The samples are vortexed and put on ice for 30 min
9. Meanwhile, set up the vacuum manifold with GF/C filters.
10. Just before transfer of the samples onto the filters, prewet the filters with 1 mL ice-cold wash buffer (**Subheading 2.5.**).
11. Pipet the samples onto the prewetted filters and immediately rinse twice with 2×5 mL ice-cold wash buffer. Filtration of the unbound from bound needs to be rapid (within seconds) Leave the vacuum long enough to dry the filters thoroughly
12. The filters are transferred to scintillation vials and 4 mL of FloScint V or any other suitable liquid scintillation cocktail is added.
13. Let the filters extract >12 h and determine the radioactivity in a liquid scintillation counter

3.4.2. Assay Procedure Using the Recombinant GAP1^{IP4BP}

3.4.2.1. SEPARATION OF BOUND FROM UNBOUND RADIOACTIVITY BY FILTRATION

1. All steps are essentially as described in **Subheading 3.4.1.** except that instead of cerebellar Ins(1,3,4,5) P_4 -binding protein, GAP1^{IP4BP} is used
2. The GAP1^{IP4BP} coupled to agarose beads is stored in high salt buffer with 50% glycerol. Just before the start of the assay, an appropriate amount of beads is washed two times in Ins(1,3,4,5) P_4 wash buffer and resuspended in this buffer. Use an appropriate amount of GAP1^{IP4BP} in 100 mL wash buffer per assay (see **step 4**)
3. Although [^{32}P]Ins(1,3,4,5) P_4 can still be used it can now be replaced by [^3H]Ins(1,3,4,5) P_4 (commercially available from NEN Dupont [Stevenage, UK] at 21 Ci/mmol). Use 0.02 μCi of [^3H]Ins(1,3,4,5) P_4 in 100 μL H_2O per assay.
4. It is recommended that the amount of GAP1^{IP4BP} to be used for each assay is determined for every new preparation. Maximal and nonspecific binding is determined in 0.5 M KOH/acetic acid pH 5.0 in the absence or presence of 1 mM Ins(1,3,4,5) P_4 (or 0.1 mM Ins P_6 [phytate], which is cheap), respectively. Aim at a maximal binding of approx 20% of the total input when you use [^3H]Ins(1,3,4,5) P_4 or 10% when [^{32}P]Ins(1,3,4,5) P_4 is used. A maximal binding of 20% is acceptable with respect to influence on the free-ligand concentration and, since [^3H]Ins(1,3,4,5) P_4 is quite expensive, this approach is most economical
5. Since the agarose beads settle rather quick, it is recommended to revortex the samples halfway through the 30-min incubation on ice

3.4.2.2. SEPARATION OF BOUND FROM UNBOUND RADIOACTIVITY BY CENTRIFUGATION

1. All steps are essentially as in **Subheadings 3.4.1.** (up to **step 7**) and **Subheading 3.4.2.1.** The assay starts by addition of the GAP1^{IP4BP}, either coupled to the agarose beads or eluted from the beads by four washes of 30 min at room temperature with 0.5 mL of elution buffer (**Subheading 2.3.2., step 15**) (see **Notes 12–14**).
2. After 20 min equilibrium binding on ice, add 0.1 mg of γ -globulin (as carrier protein) and 1 mL of 25% PEG (avg mol wt 3350 Dalton).

3. Leave for 5 min on ice and centrifuge at 12,000g for 5 min at 4°C
4. Aspirate the supernatant and count the radioactivity in the pellet

3.5. Preparation of Samples

3.5.1. Adhesive Cells

1. Cells grown on tissue culture plasticware, are fixed with 10% TCA (w/v) and left on ice for 10 min.
2. Cells are scraped off and transferred to Eppendorf tubes and the well/dish is washed once with 0.5 vol 10% TCA.
3. Spin for 2 min at full speed in a microfuge, aspirate the supernatant, and wash the pellet once with 5% TCA/1 mM EDTA. This step removes inositol phosphates and all other polar compounds.
4. Folch extraction of the pellet ensures an efficient recovery of the phospholipids. Add 0.75 mL of CHCl₃:MeOH:conc HCl (40:80:1 by volume) to the pellet and leave for ≥20 min on ice (*see Note 15*)
5. Add 0.25 mL of CHCl₃ and 0.45 mL of 0.1 M HCl.
6. Vortex thoroughly and spin 1 min at full speed in a microfuge.
7. Transfer the lowerphase to a 1.5 mL screw-capped eppendorf tube and re-extract the upper phase and the protein interphase with 0.45 mL of synthetic lower phase (**Subheading 2.5., item 7**). Be careful not to transfer any of the protein interphase since protein contaminants will affect the efficiency of the alkaline hydrolysis (*see Note 17*)
8. The pooled lower phases are dried down in a vacuum concentrator (*see Note 16*).
9. Add an appropriate amount of 1 M KOH to the dried lipids (50 μL/1 mg of cellular protein; *see Notes 18 and 19*) and vortex thoroughly to wash the lipid pellet from the bottom of the tube
10. Incubate for 30 min at 100°C (*see Note 20*). The polar headgroups (and fatty acids) from the phospholipids are cleaved from the glycerol backbone. Releasing Ins(1,3,4,5)P₄ from PtdIns(3,4,5)P₃, Ins(1,4,5)P₃ from PtdIns(4,5)P₂, and so on
11. Neutralize with an equal amount of 1 M acetic acid.
12. Add 5 vol water saturated butanol/petroleum ether (40–60°C)/ethyl acetate, vortex, spin for 1 min at full speed in a microfuge, and aspirate the supernatant. This step removes the fatty acids
13. Repeat **step 12**
14. The lower phase is dried down in a vacuum concentrator to remove any traces of butanol.
15. Resuspend in 110 μL of dilute acetic acid to a final pH of 5.0. This step is very important because the sample, which now contains approx 0.5 M KAc, dictates the pH of the subsequent Ins(1,3,4,5)P₄-binding assay. Use 40 mM acetic acid and check the pH (using accurate indicator strips, if necessary redry the sample and use appropriately rediluted acetic acid).
16. Use 100 μL of the sample in the binding assay. The samples should register in the sensitive range of the calibration curve. Therefore it is advisable to run pilot samples, so that samples can be diluted if necessary.

3.5.2. Suspensions of Cells

1. Cells grown in suspension can be processed as described in **Subheading 3.5.1.** Alternatively, the cells can be killed by addition of 1.1 l vol of CHCl_3 and MeOH and 0.01 vol of concentrated HCl
2. Leave for ≥ 20 min on ice and vortex regularly.
3. Process further as **Subheading 3.5.1., step 4**

3.5.3. Tissue

1. The Folch extraction of the phospholipids is as described in **Subheading 3.5.1.**
2. The tissue of interest needs to be snap frozen in liquid nitrogen and can be stored at -80°C . Isolation of the tissue needs to be achieved as quickly as possible
3. Use a liquid nitrogen cooled pestle and mortar to powder the tissue finely. Residual pieces of bone can be removed at this stage since bone is not pulverized and clearly distinguishable from powdered tissue.
4. Add an appropriate volume of CHCl_3 :MeOH:conc. HCl (40:80:1 by vol) to the powdered tissue and induce phase separation by addition of CHCl_3 and 0.1 M HCl to final ratios of 1.1:0.9 (by volume).
5. Standardize for cellular protein and use equally weighed amounts of powdered tissue. The latter is important because the efficiency of the alkaline cleavage is dependent on the amount of lipids present at the start. The protein determination is necessary to estimate the volume of 1 M KOH to be used for the hydrolysis (*see Notes 17–19*).
6. Process further as **Subheading 3.5.1., step 4**

4. Notes

4.1. Preparation of *Ins(1,4,5)P₃* 3-Kinase

4.1.1. Partial Purification of Recombinant *Ins(1,4,5)P₃* 3-Kinase (2,5)

1. It is essential to include the calpain inhibitors I and II since the *Ins(1,4,5)P₃* 3-kinase is extremely sensitive to enzymic cleavage by these proteases. Make up 2-mg/mL stocks in ethanol

4.2. Preparation of [$3\text{-}^{32}\text{P}$]*Ins(1,3,4,5)P₄*

2. Designate one column for the [^{32}P]*Ins(1,3,4,5)P₄* purification and if possible an injection needle, injection port, and sample loop. These items tend to be very hard to clean and may trouble other users.
3. For more detail on desalting of inositol phosphates by dialysis, *see ref. 8*. Do not dialyze the sample too long (e.g., overnight) because [^{32}P]*Ins(1,3,4,5)P₄* dialyzes slowly but significantly.
4. The [^{32}P]*Ins(1,3,4,5)P₄* sample should be stored diluted at approx 10 $\mu\text{Ci/mL}$ to avoid radiolysis at -20°C

4.3. Preparation of Ins(1,3,4,5)P₄ Binding Protein

4.3.1. Preparation of Cerebellar Ins(1,3,4,5)P₄ Binding Protein

5. The cerebella can be snap frozen in liquid nitrogen and stored at -80°C for at least 3 mo. A cooled pestle/hammer and mortar is used to powder the cerebella, which are then processed as in **Subheading 3.3.1.**

4.3.2. Preparation of Recombinant Ins(1,3,4,5)P₄ Binding Protein (GAP1^{IP4BP})

6. The *E. coli* strain expressing the truncated GST- $\sigma\text{C2GAP1}^{\text{IP4BP}}$ can also be grown at 37°C .

4.4. Ins(1,3,4,5)P₄ Isotope Dilution Assay

4.4.1. Assay Procedure Using the Cerebellar Ins(1,3,4,5)P₄-Binding Protein

7. The Ins(1,3,4,5)P₄ solutions used for the calibration curve can be kept at 20°C for months.
8. Premix the Ins(1,3,4,5)P₄ assay buffer and the [³H] or [³²P]Ins(1,3,4,5)P₄ for the calculated number plus five samples to be assayed (i.e., standard curve plus unknowns).
9. The unused remainder of the cerebellar Ins(1,3,4,5)P₄-binding protein can be refrozen at -80°C . Unused GAP1^{IP4BP} however can not be reused.
10. The binding assay is facilitated by the use of two vacuum manifolds and a "repeating" pipet for both the pipetting of the assay component and the washing of the filters.
11. The 30-min incubation on ice comfortably allows the processing of three sets of 24 samples.

4.4.2. Assay Procedure Using the Recombinant GAP1^{IP4BP}

4.4.2.1. SEPARATION OF BOUND FROM UNBOUND RADIOACTIVITY BY CENTRIFUGATION

12. The eluted GAP1^{IP4BP} can be dialyzed or kept in its elution buffer and should be kept at -20°C with 50% glycerol added.
13. Elution of the GAP1^{IP4BP} from the beads is probably not necessary.
14. If the background in the samples is too high, the pellets can be washed with ice-cold water or wash buffer. Carefully decant the supernatant into a small waste beaker (relatively high radioactive) and subsequently immerse the eppendorf tubes in a 5-L beaker, horizontally. Decant the wash, into a bigger waste beaker (low radioactive). Perform the wash procedure as quickly as possible to avoid dissociation of the radiolabeled Ins(1,3,4,5)P₄.

4.5. Preparation of Samples

4.5.1. Adhesive Cells

15. Folch extraction of the (phospho)lipids can also be done at room temperature but if the extraction is longer than 1 h, maintain on ice or at 4°C .

16. Samples can be stored at -20 or -80°C as dried lipids for at least 2 mo. Alternatively the samples can be stored dry after the butanol extraction (**Subheading 3.5.1., steps 8 and 14**, respectively)
17. Do not contaminate the organic phase with particulate material (mostly denatured protein) from the interphase.
18. In a series of samples, start with approximately the same amount of material to ensure equal efficiency of alkaline hydrolysis between these samples. When the extracted amount of lipid is too high for the amount $50\ \mu\text{L}$ of $1\ \text{M}$ KOH, the recovery of $\text{Ins}(1,3,4,5)\text{P}_4$ from $\text{PtdIns}(3,4,5)\text{P}_3$ can drop from 62 to 20% (each mole of ester bond consumes a mole of hydroxyl ions). Although the $\text{Ins}(1,3,4,5)\text{P}_4$ assay will tolerate $125\ \text{mM}$ of KAc (pH 5.0), increasing the amount of KOH for more efficient recovery of $\text{Ins}(1,3,4,5)\text{P}_4$ is therefore limited. In many cells, 1 mg of cellular protein will correlate with a total lipid content of 0.6 mg, but adipocytes, for instance, will contain far more lipid per mg of cellular protein. Do not exceed 5 mg of lipid (5–10 mg cellular protein) per sample.
19. Considering **Note 18**, it is advisable to check the efficiency of alkaline hydrolysis of $\text{PtdIns}(3,4,5)\text{P}_3$ if absolute values are to be obtained. This can be done by spiking $0.05\ \mu\text{Ci}$ of $[^{32}\text{P}]\text{PtdIns}(3,4,5)\text{P}_3$ into the lipid extract prepared as in **Subheading 3.5.1., step 8**, and processing further to **step 14**. Resuspend in 1 mL H_2O , add $0.01\ \mu\text{Ci}$ of each $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ and $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ as internal standard, and analyze on an HPLC partisphere SAX column ($4.6 \times 250\ \text{mm}$) eluted with a gradient made of water and $1.0\ \text{M}$ $\text{NH}_4\text{H}_2\text{PO}_4$, pH 3.8. The gradient used is: 0–5 min 0%, 5–105 min 100%, 105–110 min 100%, 110–111 min 100%, and 111–120 min, 0% at a flow rate of 1 mL/min, and 1-min fractions are collected.
20. The time for efficient alkaline hydrolysis is at least 30 min but can be safely extended to 2 h.

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Detection of Phosphatidylinositol-4-Phosphate 5-Kinase Activity Using Thin-Layer Chromatography

Gregory J. Parker, Joost C. Loijens, and Richard A. Anderson

1. Introduction

Phosphatidylinositol-4-phosphate 5-kinases (PIP5Ks; EC 2.7.1.68) synthesize phosphatidylinositol 4,5-bisphosphate (PI 4,5-P₂) from phosphatidylinositol 4-phosphate (PI 4-P). The hydrolysis of PI 4,5-P₂ by phosphoinositide-specific phospholipase C generates the second messengers 1,2-diacylglycerol, which activates several protein kinase C isoforms, and inositol 1,4,5-trisphosphate, which stimulates the release of calcium from intracellular stores (1). PI 4,5-P₂ can also be phosphorylated by the PI 3-kinase, generating phosphatidylinositol 3,4,5-trisphosphate (PI 3,4,5-P₃), a second messenger of generally unknown function; however, some isoforms of protein kinase C may be targets (2) as well as proteins that contain pleckstrin homology (PH) domains (3). Furthermore, PI 4,5-P₂ regulates multiple enzymes and several actin-binding proteins (4), is bound by PH domains found in some signaling proteins (5), and appears to play a role in the secretory vesicle cycle (6,7).

PIP5Ks have been isolated from brain, erythrocytes, adrenal medulla, liver, and other sources (8–13). In cells, PIP5K activity is found on the plasma membrane (8,9,13), associated with the cytoskeleton (14), on the endoplasmic reticulum (15), and in nuclei (16). There is also a cytosolic population of PIP5Ks (8). The substrate, PI 4-P, and product of this kinase, PI 4,5-P₂, are primarily found on the plasma membrane, but can also be detected on isolated endoplasmic reticulum and within nuclei (15,17).

The cloning of PIP5Ks established a new family of kinases because their predicted amino acid sequence lacked homology to all known lipid, protein, and sugar kinases (18–20). The regulation and cellular function of these kinases is currently being addressed in a number of laboratories. Areas being examined

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include yeast homologs (21), vesicular trafficking and secretion (6), the regulation of these kinases by G proteins and their subsequent involvement in cytoskeleton assembly (22–26), the role of phosphatidylinositol transfer protein (27–29), the involvement of PIP5Ks in a nuclear phosphoinositide cycle (14,16,30), alternative substrate use (31), the association (32) and tyrosine phosphorylation (14,32) of PIP5Ks by the EGF receptor, and the role of PIP5Ks in p55 TNF-receptor signaling (18). A more complete review of PIP5Ks is available (33).

1.1. Preparation of Assay Reagents

The *in vitro* PIP5K assay described in **Subheading 3.2.** has three critical components: the kinase, the lipid substrate, and ATP.

There are four primary sources for PIP5Ks: purified recombinant proteins; cell or tissue extracts; column fractions from a biochemical purification; and immunoprecipitates, either with anti-PIP5K antibodies or as part of a larger immune complex. Testing purified *Escherichia coli* or mammalian-expressed PIP5Ks is probably the easiest assay. Because *E. coli* does not possess a phosphoinositide cycle, crude bacterial lysates (usually 10–20 μ L) may also be tested directly. Typically, recombinant PIP5Ks will need to be dialyzed into an appropriate buffer, such as Tris- or phosphate-buffered saline. The presence of DTT and 20% glycerol appears to stabilize the kinase during dialysis, but not affect the activity of the enzyme (unpublished observations). A relatively large sample of purified enzyme or crude bacterial lysate (20 μ L) may be easily tested in the system described in **Subheading 3.2.** A representative PIP5K assay, using recombinant human enzymes expressed and purified from *E. coli*, appears in **Fig. 1.**

Cell or tissue extracts can be assayed in the same manner as *E. coli* lysates or recombinant enzymes simply by adding a convenient volume of an extract to a single kinase reaction. Whereas there are no known specific inhibitors of PIP5Ks, it has been shown that high concentrations of detergents, like Triton X-100, can nonspecifically inhibit the *in vitro* PIP5K assay. It also has been shown that most components of a standard, nondenaturing lysis buffer (such as RIPA without SDS) do not substantially inhibit the PIP5K assay, if at all (unpublished observations).

For column fractions, it is typical to test 5–10 μ L in a single-kinase reaction. For quantification of PIP5K activity from a column fraction, it is important to keep the ionic strength of the assay constant by adding iso-KCl or water.

When the PIP5Ks to be tested are from immunoprecipitates, there are no obvious pitfalls, except for those already mentioned, such as the presence of detergents. This problem can be alleviated by washing the pelleted PIP5Ks extensively in 1X kinase reaction buffer (**Subheading 3.1.2.**). Another trick is the handling of the bead volume. Typically, this is done by resuspending the

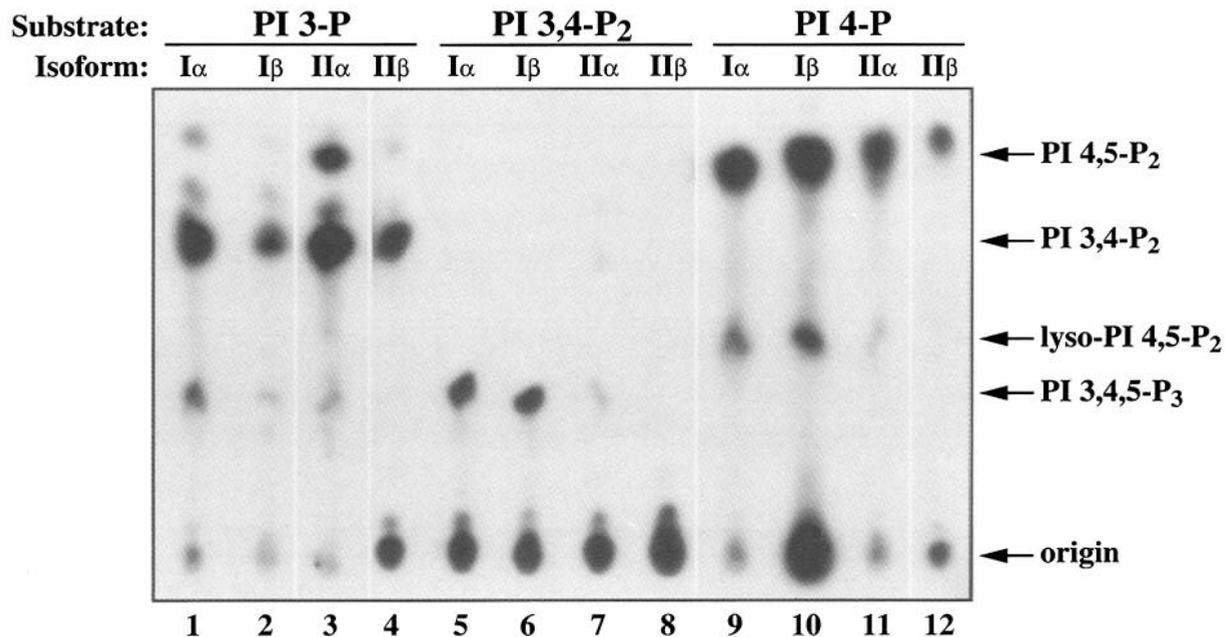


Fig. 1. Recombinant human PIP5K isoforms phosphorylate PI 3-P, PI 4-P, and PI 3,4-P₂ in vitro: *E. coli*-expressed enzymes (0.2 μ g PIP5KI α , 0.7 μ g PIP5KI β , 2 μ g PIP5KII α , and 180 μ g PIP5KII β) were assayed using 80 μ M of each lipid substrate. The reaction was run for 10.5 min at room temperature (22°C). The positions of the reaction products are marked with labeled arrows. All lanes were from the same TLC plate. Exposures to X-ray film were for 5 min (lane 3) or 15 min (lanes 1, 2, and 9–11) at room temperature or 1.5 h at -80°C (lanes 4–8 and 12).

immunoprecipitation support (for example, Protein-A Sepharose) in a small (20 μL) volume of 1X kinase reaction buffer or 50 mM Tris (pH 7.4) and then adding half (10 μL) to 40 μL of reaction mix, as described in **Subheading 3.2.1**. It has been shown that some polyclonal antibodies raised against PIP5Ks are inhibitory (**ref. 8**; unpublished observations).

The substrate, PI 4-P, can be presented to the PIP5K in a number of ways, including mixed detergent micelles, artificial liposomes, and membranes (that can be prepared from red blood cells by sonication). The protocol described here, however, deals strictly with the use of micelles to present lipid to the enzyme (**Subheading 3.1.1**). The K_m for PI 4-P for recombinant PIP5Ks is 50–80 μM (whereas the K_m for native enzymes is generally much less), so adding 50–80 μM PI 4-P exogenously allows the *in vitro* reaction to proceed unhindered. Similarly, the K_m for ATP for the different PIP5Ks has been reported to be 5–25 μM *in vitro* (**8**). Therefore, by adding excess cold ATP (50 μM), the kinase reaction can proceed at its maximum rate.

Recently, it has been reported that PIP5Ks can utilize synthetic PI 3-P and PI 3,4- P_2 (in addition to natural PI 4-P) as substrates, generating PI 3,4- P_2 , PI 3,4,5- P_3 , and PI 4,5- P_2 , respectively (**31**). These substrates are now commercially available (Matreya [Pleasant Gap, PA] phosphatidylinositol 3-phosphate, dipalmitoyl; cat. no. 1773; phosphatidylinositol *bis*-3,4-phosphate, dipalmitoyl; cat. no. 1774) and may be tested in the protocol described in **Subheading 3.2**. For an initial assay, simply substitute an equal amount of these substrates (*see Note 2*) for PI 4-P when preparing the kinase reaction mix (**Subheading 3.2.1**, **step 2**). It is important to note that synthetically prepared PI 3-P, when phosphorylated by a PIP5K to produce PI 3,4- P_2 , will have a slower TLC mobility than PI 4,5- P_2 because of differences in the lengths of the acyl chains (the synthetic lipids are di- C_{16} esters). This can be seen by comparing **Fig. 1**, lane 1–4 to lanes 9–12. It is likely that natural PI 3,4- P_2 and PI 4,5- P_2 will have nearly identical TLC mobilities under the conditions described in **Subheading 3.2.2**.

The regulation and stimulation of PIP5Ks are relatively unexplored areas. Assays to address these issues will not be directly dealt with here, but there are two significant biochemical differences between PIP5Ks worth mentioning. Initially, two distinct PIP5Ks, called type I (PIP5KI) and type II (PIP5KII), were purified from erythrocytes (**8**). Type I PIP5Ks were stimulated by phosphatidic acid, whereas type II PIP5Ks were insensitive to phosphatidic acid treatment (**34**). The other major difference between the activities of PIP5KI and PIP5KII was the inability of PIP5KII to phosphorylate PI 4-P in native membranes, whereas the activity of PIP5KI toward PI 4-P in membranes and liposomes was similar. Although PIP5KII could not phosphorylate PI 4-P in native membranes, it could utilize this substrate when it was presented in liposomes (**8**).

1.2. Assay of Phosphatidylinositol 4,5-Bis-phosphate Production

This introductory section is designed to cover some of the more subtle aspects of the PIP5K assays described in **Subheading 3.2.1**. The easiest way to perform this assay will be to purchase all of the necessary reagents, in particular, the substrate PI 4-P and a standard for PI 4,5-P₂. However, these two lipids can also be prepared from bovine brain using neomycin-affinity chromatography (35).

It has been noted by a number of investigators that type I PIP5Ks can be stimulated by phosphatidic acid (26,34). Although the details of these experiments will not be described here, it is important to note that detergent is required to properly present phosphatidic acid to the PIP5K. In many cases, detergent inhibits PIP5K activity (unpublished observations), so, in testing phosphatidic-acid stimulation, the detergent that is present will inhibit some of the activity, but phosphatidic acid should stimulate activity above the detergent-inhibited level.

Depending on the source of the kinase being analyzed, separating the organic and aqueous layers during the lipid-extraction steps can be a difficult manipulation. Ultimately, the decision to remove the top or bottom layer is up to the individual investigator; however, it is important to note that as the amount of protein (or immunoprecipitation support, such as protein-A Sepharose) present increases, the more difficult it becomes to cleanly separate the two layers. Denatured proteins will be found at the interface between the organic and aqueous layers as a thin, white or yellowish film. These proteins must be separated from the organic layer. Very often, this protein film will become stuck to the side of the tube when removing either of the layers. In this case, using a pipet tip to scrape these proteins out of the tube and into a radioactive waste container is probably the best solution.

In the final analysis of the reaction products (**Subheading 3.2.3**), it is common to find labeled PI 4,5-P₂ in two spots on the TLC plate (**Fig. 1**, lanes 9–12). The spot with the faster mobility is authentic PI 4,5-P₂, whereas the slower-moving spot is lyso-PI 4,5-P₂. This product arises via the removal of one acyl chain of the phospholipid via a chemical deacylation. When attempting to quantify reaction products (**Subheading 3.2.3**), both of these spots should be removed from the TLC plate for analysis.

2. Materials

2.1. Preparation of Assay Reagents

2.1.1. Phosphatidylinositol 4-Phosphate

1. L- α -Phosphatidylinositol 4-monophosphate (Sigma, St. Louis, MO, cat no P9638).
2. 50 mM Tris (pH 8.0).

- 3 Bath sonicator
4. Argon or N₂ gas
- 5 0.5-mL Eppendorf tubes.

2.1.2. 10X Reaction Buffer(s)

1. 1 M Tris (pH 7.0-7.5)
- 2 0.5 M EGTA (pH 8.0).
- 3 1 M MgCl₂

2.1.3. Iso-KCl

1. 1 M KCl.
2. 1 M NaCl
3. 1 M Tris-HCl (pH 7.4)
- 4 1 M NaN₃

2.1.4. [³²P]-γ-ATP

- 1 150 mCi/mL [³²P]-γ-ATP (DuPont NEN [Boston, MA]; cat. no. NEG-035C).
2. 95% Ethanol.
3. iso-KCl (*see Subheadings 2.1.3. and 3.1.3.*)

2.2. Assay of Phosphatidylinositol 4,5-Bis-phosphate Production

2.2.1. The Assay

1. 10X Reaction Buffer (*see Subheadings 2.1.2. and 3.1.2.*).
- 2 5 mM ATP in dH₂O or iso-KCl
3. [³²P]-γ-ATP solution (*see Subheadings 2.1.4. and 3.1.4.*).
- 4 2.5 mM phosphatidylinositol 4-phosphate solution (*see Subheadings 2.1.1. and 3.1.1.*).
5. 1 N HCl.
6. Chloroform:methanol (1:1).
7. Methanol:1 N HCl (1:1)
8. Radioactive waste container for organic liquids
9. Radioactive waste container for solids
10. Eppendorf tubes (1.5 mL)
11. Vortexer
12. Microcentrifuge.

2.2.2. Separation of Reaction Products by Thin-Layer Chromatography

- 1 TLC plate pretreatment solution 2% sodium tartrate, 60 mM EDTA (pH 8.0), 50% ethanol; dissolve 80 g sodium tartrate and 70.13 g EDTA in 1.5 L of dH₂O. Adjust the pH to 8.0 and bring up the volume to 2 L. While stirring, add 2 L 95% ethanol (*see Note 1*).
2. Thin-layer chromatography (TLC) plates (Whatman, Clifton, NJ K6 silica gel 60, 20 × 20 cm, 250 μM thick; cat. no. 4860-820).

3. Chromatography tank lined with filter paper.
4. Razor blade (with a notch cut in the cardboard shield, exposing 0.5–1.0 cm of the razor's edge).
5. Soft pencil.
- 6 Ruler.
7. PI 4,5-P₂ standard (L- α -phosphatidylinositol 4,5-diphosphate; Sigma P9763). Alternatively, if a recombinant source of PIP5K is available, that can be run in a control kinase reaction and used to identify the location of PI 4,5-P₂ on the TLC plate.
8. Iodine vapor or a solution of 3% cupric acetate and 8% phosphoric acid (w/v) to visualize unlabeled lipid standards.
9. Chloroform:methanol:15 N NH₄OH:dH₂O (90:90:7:22).
10. 50- μ L Capillary pipets.
11. X-ray film cassette.
- 12 X-ray film
13. Autoradiography orientation markers.
14. Organic solvent waste container

2.2.3. Quantification of Reaction Products

- 1 Light box
2. Soft pencil.
3. Razor blade.
- 4 Scintillation vials
5. Scintillation cocktail (e.g., BioSafe II; RPI [Mount Prospect, IL], cat no. 111195)
6. Scintillation counter.

3. Methods

3.1. Preparation of Assay Reagents

3.1.1. Phosphatidylinositol 4-Phosphate (see **Note 2**)

- 1 Dissolve 1 mg of L- α -phosphatidylinositol 4-monophosphate in 305 μ L 50 mM Tris (pH 8.0). The final concentration of PI 4-P will be 2.5 mM.
2. Vortex until dissolved
3. Form PI 4-P micelles using bath sonication for 15 s.
4. Place 20- μ L aliquots into 0.5-mL Eppendorf tubes
5. Store under argon or N₂ at -20°C.

3.1.2. 10X Reaction Buffer

1. For a typical 10X reaction buffer, mix 2.5 mL 1 M Tris (pH 7.5), 0.5 mL 1 M MgCl₂, and 0.1 mL 0.5 M EGTA (pH 8.0)
2. Adjust to pH 7.5
3. Bring up the volume to 50 mL with dH₂O (see **Note 3**).

3.1.3. Iso-KCl

- 1 Mix 130 mL 1 M KCl, 20 mL 1 M NaCl, 10 mL 1 M Tris-HCl (pH 7.4), and 250 μ L 1 M NaN₃
2. Bring up the volume to 1 L with dH₂O and store at room temperature.

3.1.4. [³²P]- γ -ATP

- 1 For every 67 μ L of 150 mCi/mL [³²P]- γ -ATP (DuPont NEN; cat. no. NEG-035C), add 500 μ L 95% ethanol and 434 μ L iso-KCl. At the reference date, this solution will have a specific activity of 10 μ Ci/ μ L (see Note 4)
- 2 Store at -20°C.

3.2. Assay of Phosphatidylinositol 4,5-Bis-phosphate Production

3.2.1. The Assay (see Note 5)

1. Place each kinase sample to be assayed into a 1.5-mL Eppendorf tube
- 2 Prepare the reaction mix (see Note 6).

Stock solution	Volume/tube	(Final)
10X Reaction buffer	5 μ L	1X
5 mM ATP	0.5 μ L	50 μ M
[³² P]- γ -ATP solution	x μ L (see Note 7)	10 μ Ci/nM
2.5 mM PI 4-P	1.6 μ L	80 μ M
dH ₂ O	x μ L (see Note 7)	—

3. At room temperature, start the reaction by adding the appropriate volume of the reaction mixture to each tube prepared in step 1 (save the extra reaction mix for quantification, Subheading 3.2.3.).
- 4 To stop the reaction, add 100 μ L of 1 N HCl (see Note 8)
5. Immediately after stopping the reaction, add 200 μ L of chloroform:methanol (1:1) and vortex (see Note 9)
6. Centrifuge at maximum speed in a microcentrifuge for 1 min. Remove and discard the aqueous (upper) layer in a liquid-radioactive-waste container.
- 7 To further remove reaction byproducts (such as ATP), add 80 μ L of methanol:1 N HCl and vortex.
8. Centrifuge at high speed for 1 min; remove and discard the aqueous (upper) layer in a liquid-radioactive-waste container.
- 9 If desired, the lipids may be rewashed with another 80 μ L of methanol:1 N HCl (followed by vortexing, spinning for 1 min, and removing the aqueous phase) (see Note 10)

3.2.2. Separation of Reaction Products by TLC

1. Well in advance of their need, pretreat the silica 60 TLC plates with 60 mM EDTA, 2% sodium tartrate, and 50% EtOH (pH 8.0). Using a chromatography tank, treat an entire box of plates by submerging them in 4 L of this solution (Subheading 2.2.2., item 1) for 10 min. Allow the plates to air dry and then bake them in an oven for 10 min and store in a cool, dry place until needed (see Note 1).

2. When reaction products are going to be analyzed, take a pretreated TLC plate and using the notched razor blade, scrape the silica gel off each edge of the plate (using the notch as a guide)
3. With a soft pencil, draw a line across the plate 2.5 cm from the bottom edge. Place marks no closer than at 1.0-cm intervals along the line of origin to indicate where each sample will be spotted. A maximum of 14 reactions can be loaded onto a single plate.
4. Bake the TLC plate again in an oven for 10 min and allow it to cool (*see Note 11*).
5. Prepare the chromatography tank by lining it with filter paper and adding 209 mL of chloroform:methanol:15 N NH₄OH:dH₂O (90:90:7:22). Allow it to equilibrate for at least 30 min.
6. Using a 50- μ L capillary pipet, spot only the amount of lipid sample that can be drawn into the pipet by capillary action onto a pretreated TLC plate. Move onto the next spot, allowing the previous spot(s) to dry. Repeat this stepwise spotting until the entire sample has been loaded onto the plate (*see Note 12*).
7. Develop the TLC plate with chloroform:methanol:15 N NH₄OH:dH₂O (90:90:7:22) until the solvent front reaches the top of the TLC plate. This takes approx 2 h, but can be left longer to increase the separation between the labeled spots.
8. After running the plate, remove it from the chromatography tank and allow it to air-dry for 5–10 min. Wrap the plate in plastic wrap and attach autoradiography orientation markers. Overlay with X-ray film and place it in a cassette. Expose the film at -70°C or room temperature for at least several hours or overnight.
9. Phospholipids are visualized with iodine vapor or by spraying the TLC plate with 3% cupric acetate and 8% phosphoric acid (w/v) and baking for 40 min at 120°C . Labeled reaction products are visualized by autoradiography and identified by comparing them to unlabeled phospholipid standards. Radioactivity in each spot is determined by scraping the silica gel corresponding to the [³²P]-labeled phospholipid and quantifying by scintillation counting (**Subheading 3.2.3**).

3.2.3. Quantification of Reaction Products

1. Using the orientation markers, overlay the TLC plate on the developed film.
2. With a soft pencil, mark the areas containing PI 4,5-P₂ and lyso-PI 4,5-P₂ in each lane based on the location of the lipid standards.
3. Using a razor blade, scrape both the PI 4,5-P₂ and lyso-PI 4,5-P₂ regions from a single lane into a scintillation vial.
4. Add scintillation cocktail and count each vial using the [³²P] channel of a scintillation counter.
5. Count 1 μ L of the reaction mixture so that the specific activity of labeling can be calculated (*see Note 13*).

4. Notes

1. Sometimes, the sodium tartrate precipitates out of the TLC pretreatment solution while it is being made or during the pretreatment of the plates (most likely because the solution becomes saturated). However, it appears that plates pretreated under

these conditions are still acceptable for the separation of labeled phospholipids using this protocol.

2. If an alternative PIP5K substrate is being tested (such as PI 3-P or PI 3,4-P₂), prepare its stock solution in the same manner as described in **Subheading 3.1.1**.
3. Depending on the system being tested, the 10X reaction buffers that have been published typically are made so that the final (1X) concentrations are: 50 mM Tris (pH 7.4–7.6), 5–10 mM MgCl₂, and 0.5–1 mM EGTA (pH 7.5).
4. To calculate the specific activity of the [³²P]-γ-ATP stock solution after the reference date has passed, use the following equation:

$$e^{[-(1 \times \text{number of days decayed} \times 0.693)/14.3]} \times 10 \mu\text{Ci}/\mu\text{L}$$

where 0.693 is the decay constant (λ) for [³²P], 14.3 is the half-life of [³²P], and 10 $\mu\text{Ci}/\mu\text{L}$ is the reference activity of the solution (as made in **Subheading 3.1.4**) on its reference date.

5. The assay described in **Subheading 3.2.1** is based on a final volume of 50 μL . The reaction can be scaled up or down, although the effects of changing the reaction volume by more than twofold are not known.
6. Be sure to make enough reaction mix for at least one more assay than the number you will actually do. For example, if you have five samples to assay, make up enough mix for six reactions.
7. The volume of [³²P]-γ-ATP added depends on the solution's specific activity (**Subheading 3.1.4**; **Note 4**). The final volume of the reaction mix should be brought up to 50 μL (less the volume of sample to be assayed) with dH₂O.
8. A typical reaction time for a type I PIP5K is 5.5 min. Type II PIP5K reactions are generally 20 min long. These reaction times can be varied depending on the application. While these assays are usually done for short periods of time at room temperature, it is also possible to run the reaction for much longer times (2 h) at 4°C. Reactions can also be run at 30 or 37°C. It is important to stop the reactions at specific times, especially if the reaction products are to be quantified. If necessary, stagger the start and stop times when a large number of reactions are being processed.
9. It is critical that the 200 μL of chloroform:methanol (1:1) be added to the stopped kinase reaction immediately. This is achieved by using two pipetmen (one for each hand). Deliver 100 μL 1 N HCl with one pipetman, followed by 200 μL of chloroform:methanol (1:1) with the other. Vortex.
10. A second methanol:1 N HCl extraction is recommended when the samples being analyzed are from immunoprecipitates or contain a large amount of protein at the organic/aqueous interface.
11. Alternatively, you may microwave the TLC plate for 10 min on the highest setting. As with baking, allow the plate to cool before use.
12. If the volume of sample to be spotted on the TLC plate is large (greater than 80 μL), it may be desirable to dry the lipids down by vacuum centrifugation for 30 min (without heat). Resuspend them in 40 μL chloroform:methanol.

12 N HCl (200:200:1), vortex, and quickly spin the tubes in a microcentrifuge to collect the sample from the wall of the tube. Some investigators feel that a portion of the labeled product may be lost during these additional steps and that experiments become less reproducible. In general, spotting samples onto the TLC plate can be a time-consuming process if multiple samples are being tested, so adding this step is one way to save time.

- 13 To determine the specific activity (SA) of PI 4,5-P₂ labeling by a PIP5K, one must first determine the specific activity of the reaction mix. Start by counting 1 μL of the reaction mix (or, to avoid pipeting errors, a dilution of the reaction mix equal to 1 μL) that was left over from the assay (**Subheading 3.2.1., step 3**). Multiply the Cpm of the reaction mix by the total volume of the reaction mix added to each reaction (in the sample protocol listed, this value is 40 μL) and divide this by the product of the volume of the mix counted (in this case, 1 μL) times the total ATP concentration (in **Subheading 3.2.1., step 2**, you added 50 μM ATP to a 50-μL reaction, so the [ATP] is 2.5 nmol) times the constant 2.2×10^6 . The equation looks like this (and assumes PI 4-P + ATP → PI 4,5-P₂ + ADP):

$$SA = \frac{[(Cpm) (total\ mix\ volume\ per\ reaction)]}{[(mix\ volume\ counted) ([ATP]) (2.2 \times 10^6)]}$$

The final units for SA are μCi/nmol ATP.

To determine pmol of PI 4,5-P₂ produced per minute, use the following equation:

$$[(Cpm\ of\ scraped\ TLC\ spot) (1000)] / [(SA) (2.2 \times 10^6) (reaction\ time)]$$

Please note that the factor of 1000 is used to convert from nmol to pmol. The incorporated counts can be divided by the volume of lysate tested (giving the units pmol PI 4,5-P₂/min/μL) or if the protein concentration of a purified enzyme is known, this value can be divided by mg of protein (giving a value with the final units pmol PI 4,5-P₂/min/mg).

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Determination of Phospholipase C- or Phospholipase D-Catalyzed Phosphatidylcholine Hydrolysis

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1. Introduction

Signal-activated phospholipase-catalyzed hydrolysis of phosphatidylcholine involves three distinct enzymes: phospholipase A₂, phospholipase C (PLC), and phospholipase D (PLD) (1). PLC-catalyzed hydrolysis generates *sn*-1,2-diacylglycerol (DAG) and choline phosphate, whereas PLD stimulates the generation of phosphatidate (PA) and choline. Choline and choline phosphate are probably not messengers, although there have been some claims of a signaling role for the latter. DAG is the physiological activator of protein kinase C, whereas PA has an incompletely defined messenger function but has been demonstrated to activate a number of serine/threonine kinases and to play a role in secretion and rho-dependent actin stress-fiber formation (2). PA and DAG are apparently interconvertible through the action of phosphatidate phosphohydrolase and diacylglycerol kinase. However, work from this laboratory has recently demonstrated that the acyl structure of PLD-derived PA and PLC-derived DAG is distinct, the latter being polyunsaturated, whereas the former is more saturated/monounsaturated suggesting a specificity between the two signaling pathways (3,4). Indeed, the DAG generated from PLD-derived PA does not activate protein kinase C *in vivo*. It is thus of importance to be clear of the source of the DAG and PA when attempting to define the signaling of an agonist-stimulated cell. Polyunsaturated DAG is generally derived from phospholipase C-catalyzed phosphatidylinositol 4,5-*bis*-phosphate hydrolysis; however, there are examples of agonist-stimulated phospholipase C-catalyzed phosphatidylcholine hydrolysis (5-7). Whereas this would suggest that determining the production of choline or choline phosphate in parallel with DAG

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would be sufficient to define the activated phospholipase, cells also contain choline kinase, which rapidly converts choline to choline phosphate. Therefore, although determining changes in cellular choline and choline phosphate levels in response to agonist stimulation will suggest whether a PLC or a PLD has been activated, it is essential to perform such an analysis together with an assessment of the lipid products. An indication of PLD activation can be gained by use of the transphosphatidylation assay (8), although it is not certain if the "alcohol trap" is complete, so it is unwise to rely fully on this approach to distinguish between the pathways. In this chapter, we present methods to determine the production of choline and choline phosphate (9) and also to determine the acyl structure of DAG and PA (3,4). Together, these assays define if a PLC or a PLD pathway, or both, are being stimulated.

2. Materials

Note: All solvents should be chromatography grade or higher.

2.1. Determination of Choline and Choline-Phosphate Generation

1. [³H]Choline chloride (86 Ci/mMol, Amersham, Arlington Heights, IL).
2. DMBGH (DMEM medium containing 10 mM glucose, 20 mM HEPES pH 7.4, and 1% [w/v] BSA).
3. Dowex-H⁺, 50 × 8, 200 mesh (Sigma, St. Louis, MO)

2.2. Characterization of Dowex 50 H⁺ Ion-Exchange Resin

1. [³H]Choline (86 Ci/mMol, Amersham).
2. [¹⁴C]Choline phosphate (50 Ci/mMol, Amersham)
3. [³H]Glycerophosphocholine (prepared by N → O transacylation as described in ref. 9) (*see also* Chapter 2).
4. KCl.

2.3. Diradylglycerol and Phosphatidate Analysis

2.3.1. Extraction of Lipids

1. Methanol.
2. Chloroform.
3. KCl

2.3.2. Separation of Phosphatidic Acid

1. Chloroform.
2. Methanol.
3. KCl.
4. Ammonia.
5. Plastic-backed, silica-gel 60 thin-layer chromatography (TLC) plates (1.05748, Merck, Darmstadt, Mannheim, Germany).

- 6 Iodine crystals.
- 7 Kromasil 5- μm silica high-performance liquid chromatography (HPLC) column, 2.1 \times 250 mm (Hichrom, Reading, UK).

2.3.3. Phosphatidate Acyl-Chain Analysis

- 1 17:0/17:0 phosphatidate (Avanti Polar Lipids, Alabaster, AL).
- 2 Methanolic 3 *N* hydrochloric acid (Supelco, Bellefonte, PA).
- 3 DB-23 gas chromatography column; 30 \times 0.25 mm id \times 0.25- μm film thickness (J & W Scientific, Folsom, CA).

2.3.4. Derivatization Diacylglycerol Species

- 1 1,2-12:0/12:0 Diacylglycerol (DAG) (Avanti Polar Lipids).
- 2 3,5-dinitrobenzoylchloride (Aldrich, Milwaukee, WI).
- 3 Pyridine (AR grade, Fisher, Pittsburgh, PA; stored/dried over KOH pellets).
- 4 Sep-Pak Vac 3-cc 200-mg cartridges (Waters, Milford, MA, cat. no. WAT054945)
- 5 Diethyl ether (HPLC grade, Fisher, Loughborough, UK).

2.3.5. HPLC Separation of Derivatized DAG Species

- 1 Cyclohexane (HPLC grade, Fisher)
- 2 2,2,4-trimethylpentane (HPLC grade, Fisher).
- 3 Propan-2-ol (HPLC grade, Fisher).
- 4 Acetonitrile (HPLC grade, Fisher).
- 5 Kromasil 5 μm , 2.1 \times 250-mm silica HPLC column (Hichrom).
- 6 Spherisorb 5- μm , 3.2 \times 250-mm S50DS2 HPLC column (Hichrom)

3. Methods

3.1. Determination of Choline and Choline Phosphate Generation

Methods exist in the literature for separating choline metabolites by thin-layer chromatography, however ion-exchange separation (9) is quick, simple, and reproducible.

1. Cells, in suspension or cultured in 24-well plates, are labeled for 48 h with 2 $\mu\text{Ci/mL}$ [^3H]choline chloride in DMEM (or other medium) containing 2% serum. The radiolabel concentration and the length of labeling may require variation depending on cell type.
2. 2 h prior to the experiment, the radiolabeled medium is replaced with 0.5 mL of fresh, unlabeled serum-free medium and cells returned to the incubator.
3. After the 2 h preincubation, the cells are washed for 15 min in DMEM containing 10 mM glucose, 20 mM HEPES pH 7.4, and 1% (w/v) BSA (DMBGH). This can also be done in other physiological buffers containing BSA and glucose.
4. The cells are then stimulated by aspirating the buffer and adding 150 μL agonist in DMBGH.
5. The incubations are terminated by the direct addition of 0.5 mL ice-cold methanol to the well. This permits the determination of both the choline metabolites

associated with the cell and those released into the medium. If only cell-associated metabolites are to be analyzed, the medium is aspirated prior to methanol addition and fresh buffer added to maintain the methanol:water ratio.

6. The contents of each well are scraped into an insert vial and the well is washed with a further 0.2 mL methanol
7. 310 μL CHCl_3 is added and the tubes are vortex mixed and stood for 20 min at room temperature or overnight at 4°C .
8. 390 μL CHCl_3 and 480 μL H_2O are added to the tubes, which are mixed and centrifuged at 1200g for 5 min to split the phases
9. 0.8 mL of the upper aqueous phase are taken and made to 5 mL with H_2O . This is then loaded onto a 1-mL Dowex-50 H^+ column. The run-through is collected together with a water wash of 5–8 mL (volume determined to elute a glycerophosphocholine standard) as the glycerophosphocholine fraction. A 2-mL aliquot of this fraction is transferred to a 20-mL scintillation vial, scintillant is added, and the radioactivity is determined.
10. A further volume of water (usually a further 10–15 mL for a 1-mL column, again as determined from the characterization profile) is added to the column and collected as the choline-phosphate fraction. A 2-mL aliquot of this fraction is transferred into a 20-mL scintillation vial, then scintillant is added and counted
11. The choline fraction is then eluted with 5–7 mL of 1 M KCl (depending on column characteristics). Scintillant is added to the whole sample and this is counted

3.2. Characterization of Dowex 50 H^+ Ion-Exchange Resin

1. Nonradiolabeled cellular extracts are prepared as above to which are added 1 μCi each of [^3H]choline, [^{14}C]choline phosphate, or [^3H]glycerophosphocholine
2. A 1-mL Dowex-50 H^+ column is prepared, the extract is loaded, and the column is eluted with 25×1 mL H_2O , each of which is collected and its associated radioactivity is determined by scintillation counting.
3. The column is then eluted with 15×1 -mL additions of 1 M KCl, each fraction is collected, and the radioactivity is determined. Each sample is counted using a dual-label counting program ($^3\text{H}/^{14}\text{C}$) and, because the choline is only eluted following the addition of KCl, it is possible to characterize the elution profiles of each metabolite on the one column (See Fig. 1 for a typical column profile)

3.3. Diradylglycerol and Phosphatidate Analysis

3.3.1. Extraction of Lipids

1–2 nmol (0.5–1 μg) of total diradylglycerol or phosphatidate per sample is required for molecular species analysis. This is usually obtainable from one 75-cm² flask of confluent fibroblasts (approx 1×10^7 cells).

1. Wash cells twice with phosphate-buffered saline, removing as much liquid as possible
2. Add 2 mL methanol (this kills cells and starts extraction) followed by internal standard(s) as required (see Subheading 3.3.3. and 3.3.4.).

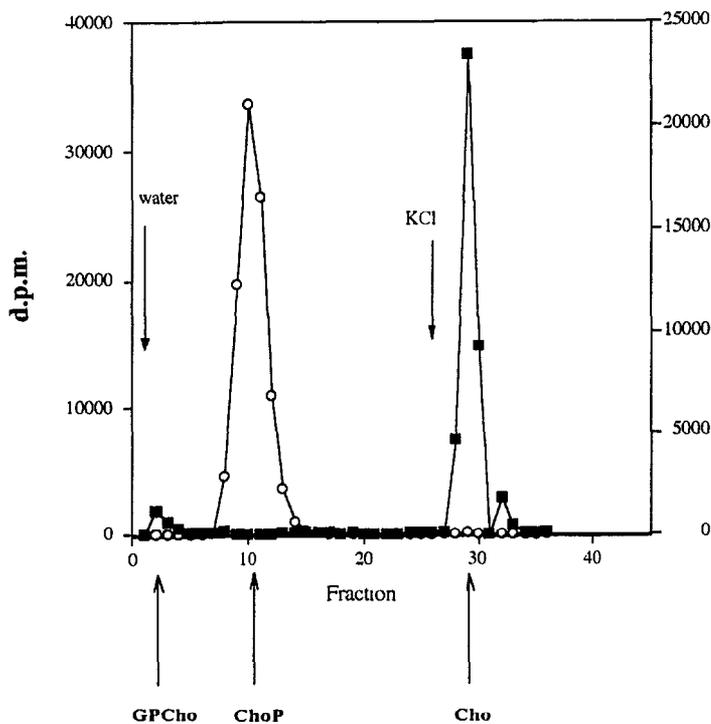


Fig 1 Separation of labeled choline metabolites by ion-exchange chromatography.

3. If using adherent cells, scrape and transfer to a 12-mL glass tube with PTFE-lined screw-cap.
4. Rinse any remaining cells from container with 1 mL methanol and combine in tube.
5. Add 6 mL chloroform to sample.
6. Mix (if two phases appear, add a little more methanol until a single phase is achieved)
7. Leave to stand for 10 min
8. Add 0.88% KCl (3 mL or to top of tube whichever is the lesser, this should give a chloroform:methanol:water ratio of approx 2:1:1, v/v/v).
9. Cap tightly and shake vigorously.
10. Allow the two phases to separate.
11. Carefully remove and discard upper aqueous phase (contains proteins, carbohydrates, and so on) together with any interfacial material.
12. Wash lower organic phase containing the lipids with 3 mL synthetic upper phase (methanol:0.88% KCl, 1:1, v/v)
13. Transfer organic phase to a clean glass tube and dry at room temperature under a stream of nitrogen.
14. Resuspend in chloroform:methanol (1:1, v/v) and store at -20°C in a small, screw-capped tube

3.3.2. Separation of Phosphatidic Acid

- 1 Spot the lipid samples in 20 μL chloroform:methanol (2:1, v/v) as 5-mm bands in a line along the middle of a plastic-backed silica TLC plate together with a spot of authentic unsaturated phosphatidate (10 μg) as a marker.
2. Run to top of plate in chloroform:methanol:ammonia solution (65:35:7, v/v/v), phosphatidate will remain at the origin, whereas most other, nonacidic lipids migrate away
3. Cut plate 1 cm above the origin, discarding the upper portion (this contains most of the lipids).
- 4 Take the lower portion, turn through 180° and run in chloroform:methanol:acetic acid (90:10:10, v/v/v) to top.
5. Allow to dry and detect lipids with iodine vapor by placing for several minutes in a tank containing iodine crystals. (*Note*: Iodine will only detect lipids containing carbon-carbon double bonds, saturated lipid will not be detected. Minimum detection limit is approx 2 μg .)
6. Mark spots with a pencil and then remove iodine stain by exposure to water vapor from a hot water bath
7. Scrape phosphatidate spots (R_f of approx 0.5 in the second solvent system) into a sintered funnel (pore size 3) and elute from silica with 10 mL chloroform:methanol:water (5:5:1, v/v/v).
8. Dry under a stream of nitrogen.
- 9 Resuspend in chloroform:methanol (2:1, v/v) and store at -20°C in screw-capped tubes

3.3.2.1. ALTERNATIVELY USE HPLC

1. Resuspend dried sample in 20 μL chloroform:methanol (2:1, v/v).
2. Separate total lipid sample on a silica column (Kromasil, 5 μm , 2.1 \times 250 mm), using a solvent gradient of 100% chloroform:methanol:ammonia solution (70:28:2, v/v/v) and changing to 100% chloroform:methanol:ammonia solution (50:48:2, v/v/v) over 30 min at 0.5 mL/min, collecting the components of interest
- 3 Dry under a stream of nitrogen.
- 4 Resuspend in chloroform:methanol (2:1, v/v) and store at -20°C in screw-capped tubes

UV detection is often unsuitable since few lipids contain usable chromophores without prior derivatization, hence we use evaporative light-scattering detection (ELSD), as this can be quite sensitive without the requirement for any lipid modification. Whereas this detection is essentially destructive, careful calibration of the HPLC separation allows diversion and collection of the lipid before it reaches the detector by using a switching valve.

With minor solvent modifications this can be used to purify most phospholipids.

3.3.3. Phosphatidate Acyl-Chain Analysis

For fatty acid methyl ester analysis add, as an internal standard, 1 μg of a fatty acid not normally found at significant levels in the sample and that does

not coelute with another fatty acid—e.g., heptadecanoic acid (17:0), nonadecanoic acid (19:0), heneicosanoic acid (21:0), or a suitable glycerolipid containing these fatty acids, e.g., 17:0/17:0-phosphatidate.

- 1 Dry sample together with an internal standard in a small, screw-capped tube with PTFE seal.
- 2 Add 100 μL 3 *N* methanolic hydrochloric acid.
- 3 Transmethylate sample at 100°C for 2 h.
- 4 Remove from heat and allow to cool
- 5 Dry under a stream of nitrogen and resuspend in a small volume of hexane
- 6 Separate fatty acid methyl esters (and any dimethyl acetals derived from alkenyl lipids) on a polar, capillary gas-chromatography column (e.g., DB-23; 30 m \times 0.25 mm id \times 0.25 μm film thickness [J&W Scientific] using splitless injection at 220°C with purging at 1 min, 12 psi head pressure, and a temperature program starting at 55°C for 2 min, then rising to 140°C at 70°C/min, then to 205°C at 1°C/min).

3.3.4. Derivatization of Diacylglycerol Species

See Pettitt and Wakelam (3) for further details and examples of use of this method. For 1,2-DAG species analysis, we add 1 μg 1,2-12:0/12:0 DAG as internal standard and 1 μg 1,2 + 1,3-20:0/20:0 DAG as marker to each sample prior to lipid extraction and then derivatization with 3,5-dinitrobenzoylchloride.

- 1 Dry total lipid extract in 12-mL screw-capped Pyrex glass tubes
- 2 Add 0.5 mL 3,5-dinitrobenzoylchloride (50 mg/mL in dry pyridine)
- 3 Derivatize for 15 min at 60°C.
- 4 Stop reaction by addition of 2 mL methanol-water (3:1, v/v), followed by 2 mL water.
- 5 Pass onto C18 solid-phase extraction cartridge (e.g., Sep Pak Vac 3-cc 200-mg cartridges) prewashed sequentially with 5 mL diethyl ether, 5 mL methanol, and 5 mL methanol-water (3:1, v/v).
- 6 Wash sample with 15 mL methanol water (3:1, v/v)
- 7 Elute sample with 10 mL diethyl ether (use a high-purity grade or redistill)
- 8 Dry, resuspend in diethyl ether, and store at -20°C in small screw-capped tubes

3.3.5. HPLC Separation of Derivatized DAG Species

1. Dry crude dinitrobenzoylated samples under a stream of nitrogen. Resuspend in cyclohexane (20 μL)
- 2 Separate the dinitrobenzoylated material into lipid classes on a silica column (e.g., Kromasil; 5 μm , 2.1 \times 250 mm), using a solvent gradient of 100% 2,2,4-trimethylpentane.cyclohexane.diethyl ether:propan-2-ol (49:49:2.0:1, v/v/v/v) and changing to 100% cyclohexane.diethyl ether:propan-2-ol (85:15:0.1, v/v/v) over 45 min at 0.5 mL/min with detection at 254 nm, collecting the relevant peaks
- 3 Dry the purified diacylglycerol derivatives and resuspend in acetonitrile propan-2-ol (1:1, v/v).

- 4 Separate into the individual diacylglycerol molecular species on a C18 reverse-phase column (e.g., Spherisorb S50DS2; 5 μ m, 3.2 \times 250 mm), using a gradient of 100% acetonitrile:propan-2-ol (9:1, v/v) and changing to 50% acetonitrile:propan-2-ol (1:1, v/v) over 45 min at 0.5 mL/min with detection at 254 nm
5. Identify individual peaks by cochromatography of authentic, derivatized standards. Gas chromatographic analysis of the component fatty acids in a particular peak can be used to confirm identity.

In the first HPLC clean-up stage, 1,3-diacylglycerol elutes with a retention time of approx 16–18 min and 1,2-diacylglycerol at 19–21 min. 1-alkyl,2-acylglycerol and 1-alkenyl,2-acylglycerol coelute with a retention time of 14–15 min, but once collected from HPLC they can be separated on silica TLC using a solvent system of hexane:diethylether (65:35, v/v), which causes the alkyl forms to run slightly ahead of the alkenyl forms. Once eluted from the silica with 10 mL diethyl ether, they can be dried and further separated into individual species by C18 reverse-phase HPLC. Partial resolution of the molecular species on the silica column results in the longest acyl chain, most saturated species eluting first with the shortest chain, most saturated species eluting last. By including 1,2-12:0/12:0 and 1,2-20:0/20:0 diacylglycerols as markers/internal standards, the sample 1,2-diacylglycerols should normally elute between these two peaks.

4. Notes

- 1 A number of short-chain primary aliphatic alcohols can be utilized in the transphosphatidyl transfer assay, i.e., methanol (1%), ethanol (1%), propanol (0.75%), and butanol (0.3%). We use butanol since it can be used at a lower concentration and is thus less toxic.
2. It is advisable to include an internal positive control in each experiment, e.g., 100 nM TPA in Swiss 3T3 cells induces a large increase in choline and choline phosphate.
- 3 Whereas the level of glycerophosphocholine rarely changes in response to acute stimulation, there is a high level of radioactivity associated with this fraction in some cell types, therefore its separation from the choline phosphate fraction is advisable.
4. The use of Dowex 50 \times 8–200 resin is recommended, this gives a good balance between binding capacity and speed of use.
5. All lipid work should be performed in glass, not plastic, since many of the solvents used will attack plastic, often leaching out plasticizers, dyes, and other components.
6. All drying should be performed under a stream of nitrogen to minimize lipid oxidation.
- 7 Wear gloves when handling TLC plates to prevent contamination from finger lipids.

- 8 In separating phospholipids, we have found that all silicas continuously release small amounts of esterified fatty acid, in particular palmitate and stearate, which can cause problems with subsequent fatty acid analyses. This problem is minimized using HPLC since the amount of this contamination is related to the volume of solvent to which the silica is exposed. For small samples (<1 µg of separated lipid), where contamination can be a serious problem, we recommend HPLC separation although this will take longer than TLC.

Acknowledgments

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Measurement of Phospholipase D Activity

Wendy Bollinger Bollag

1. Introduction and Theory

The importance of diacylglycerol (DAG) as a second messenger for calcium-mobilizing hormones, neurotransmitters, and cytokines was underscored by the discovery of an effector enzyme, protein kinase C, which was also a cellular receptor for phorbol ester tumor promoters (reviewed in **ref. 1**). However, with continued study it became apparent that hydrolysis of the polyphosphoinositides by phosphoinositide-specific phospholipase C was not the only pathway through which DAG could be generated (reviewed in **ref. 2**). In addition to phospholipases C that utilize other phospholipids as substrates (e.g., phosphatidylcholine-specific phospholipase C), a mechanism for DAG production involving the combined action of phospholipase D (PLD) and phosphatidate phosphohydrolase has been demonstrated (reviewed in **ref. 3**). Thus, phospholipid hydrolysis by PLD yields phosphatidic acid (PA), which can be dephosphorylated by phosphatidate phosphohydrolase to produce DAG. Furthermore, it appears that, whereas hormone-induced phosphoinositide hydrolysis by phospholipase C is an important pathway of initial DAG formation, sustained DAG generation often arises through this second PLD mechanism (reviewed in **ref. 1**).

The potential activation of PLD in response to hormones can be monitored by utilizing a unique property of PLD: In aqueous solution this enzyme can catalyze not only the hydrolysis of phospholipids to yield PA, but, in the presence of small amounts of ethanol, phospholipids can be ethanolized by PLD to generate the novel phospholipid, phosphatidylethanol (PEt) (**Fig. 1**) (**4**). Whereas PA can be formed both by the action of PLD and the phosphorylation of phospholipase C-derived DAG by DAG kinase (*see Fig. 2*), PEt is thought to represent a specific marker of PLD activity (**5**). Furthermore, this unusual

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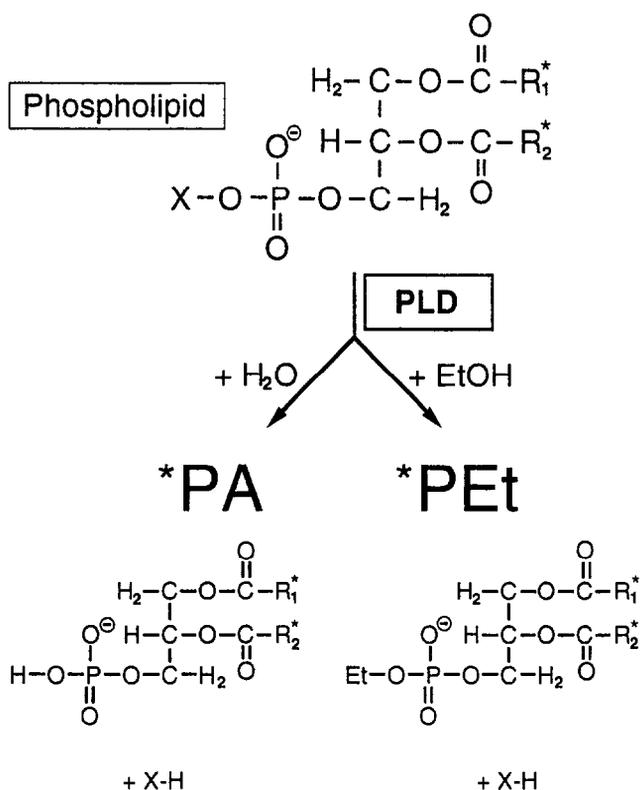


Fig 1 Phospholipase D activity in aqueous solution in the presence of ethanol generates phosphatidic acid and phosphatidylethanol. In aqueous solution, phospholipase D (PLD) catalyzes not only the hydrolysis of phospholipid to yield phosphatidic acid (PA), but, in the presence of small amounts of ethanol (EtOH), PLD can effect the ethanolysis of a phospholipid to generate phosphatidylethanol (PEt). Whereas PA can also be produced via the phosphorylation of phospholipase C-derived diacylglycerol kinase (see Fig. 2), PEt is a novel phospholipid that appears to originate solely as a result of PLD activity. In cells in which phospholipids are prelabeled with radioactive fatty acid (R_1^* or R_2^*), the activation of PLD in response to a hormone results in increases in the levels of radiolabeled PA and PEt. PLD activity also results in the release of the polar head group (X-H) from the phospholipid.

phospholipid is metabolized slowly (6) or not at all (5), so even transient PLD activation may be detected by this method. Thus, cells are labeled with radioactive fatty acids and stimulated with hormones in the presence of a small amount of ethanol. The lipids are then extracted with chloroform/methanol, separated by thin-layer chromatography (TLC) and quantified as described in **Sub-heading 3.3.**

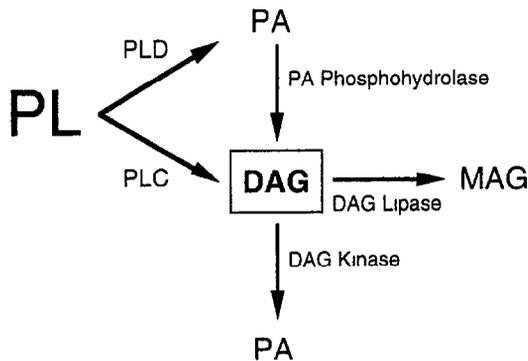


Fig. 2 The interconversion of phosphatidic acid and diacylglycerol. The hydrolysis of a phospholipid (PL) by phospholipase C (PLC) yields diacylglycerol (DAG), hydrolysis by phospholipase D (PLD) produces phosphatidic acid (PA). Whereas PA can be converted to DAG by the actions of PA phosphohydrolase, DAG can be metabolized by DAG kinase to generate PA or by DAG lipase to form monoacylglycerol (MAG). Thus, note that DAG can arise either directly via the activity of PLC or indirectly through the combined actions of PLD and PA phosphohydrolase. Similarly, PA can be generated directly by PLD-mediated phospholipase hydrolysis or indirectly via the activities of PLC and DAG kinase. Therefore, because of the rapidity of these interconversions, neither DAG nor PA can serve as a marker of the activity of a particular phospholipase (PLC or PLD).

2. Materials

The following equipment and supplies are needed for assay of phospholipase D activity after labeling cells with a radiolabeled fatty acid. When a reagent must be acquired from a specific source, that source is noted.

1. Vortex mixer.
2. Nitrogen tank and manifold.
3. TLC chambers
4. Oven.
5. Pasteur pipets and bulb.
6. Test tubes and racks.
7. (9,10-³H[N])oleic acid (2–10 Ci/mmol) (or (9,10-³H[N])myristic acid [10–60 Ci/mmol]; *see Note 1*)
8. Ethanol (absolute).
9. 0.2 M Sodium chloride (store at room temperature).
10. 0.2% SDS containing 5 mM EDTA (pH approx 7.0) (store at room temperature)
11. Chloroform
12. Methanol
13. Ethyl acetate.
14. Iso-octane.

15. Acetic acid (glacial).
16. Microcapillary pipets (5 or 10 μL)
17. 20 \times 20-cm TLC plates (silica gel 60 with concentrating zone, available with glass or aluminum backing from EM Science (Gibbstown, NJ) or Merck (Darmstadt, Germany) TLC plates are prepared for use by marking lanes and an origin in pencil (the origin should be approx 1.3 cm from the bottom of the plate within the concentrating zone). The plates are then "heat-activated" by baking at 110°C for 30–60 min (however, *see Note 4* for an alternative method for plate activation) Heat-activated plates should be stored in a desiccating chamber (containing Drierite) and can be used for up to 24 h after heat activation
18. Iodine
19. Phosphatidic acid.
20. Phosphatidylethanol (available from Biomol, Plymouth Meeting, PA or Avanti Polar Lipids, Alabaster, AL)
21. En³Hance spray (Dupont/NEN, Wilmington, DE)
22. Film (Kodak XAR-5)

3. Method

3.1. Labeling of Cells

Typically, cells are "downregulated" in a serum-free medium containing bovine serum albumin (fatty acid-free or RIA grade) as a carrier for the fatty acid. Thus, using sterile technique, cells are labeled for approx 24 h (but *see Note 1*) with approx 5 μCi per sample of [³H]fatty acid. An alternative method for determining potential PLD activation in response to an agent of interest involves labeling of cellular phosphatidylcholine with [³H]choline and examining agent-induced release of the radiolabeled head group (*see Note 2*).

3.2. Preparation of Samples for Assay

Samples are prepared by stimulating radiolabeled cells with the hormone of interest for the desired time period in the presence of small amounts of ethanol. Lipids are then extracted with chloroform/methanol and separated by TLC. Note that chloroform and methanol are hazardous and, as much as possible, should be dealt with in a chemical fume hood. Note also that both are quite volatile (particularly chloroform) and mixtures containing these agents should be made immediately prior to use and capped tightly to prevent alteration of the relative proportions. As a final note, chloroform will dissolve polystyrene, the plastic of which tissue culture vessels and disposable pipets are made. Therefore, glass pipets and test tubes should be used, and care should be taken to prevent chloroform from coming into contact with plastics. Tips used with Pipetmen and Repipetmen are, for the most part resistant to chloroform, and are adequate for this assay. However, the Teflon seal and o-ring within the Pipetmen will be damaged on repeated use of chloroform and should be peri-

odically replaced. When Pipetmen are used with any organic solvent, it is important to pre-equilibrate the air space in the tip by aspirating and ejecting the solvent prior to distributing the desired volume. Nevertheless, volumes are not precise and any manipulation requiring extreme precision, such as reserving a portion for determination of phospholipid phosphate, requires the use of Hamilton syringes.

1. Cells are stimulated with the desired agents for the appropriate period of time in the presence of 0.5% (volume to volume [v:v]) ethanol
2. Reactions are terminated by the addition of room-temperature 0.2% SDS containing 5 mM EDTA (for a 35-mm dish, 0.4 mL SDS solution should be used; for a 60-mm dish, 0.8 mL) and swirling for several seconds (alternatively, reactions may be terminated by the addition of ice-cold methanol; *see Note 3*). After incubating at room temperature for a few minutes, the SDS extracts are repeatedly aspirated with a Pipetman to minimize viscosity and transferred to test tubes (16 × 100 mm). At this point, a percentage of the SDS extract may be reserved for determination of protein content and normalization. Alternatively, phospholipid phosphate content (7) can be used for normalization purposes (*see Note 3*).
3. Chloroform and methanol and acetic acid are added to the test tubes, which are vortexed thoroughly. The ratio desired for optimal extraction of the cellular lipids is 1.2:0.8:0.08 (v:v:v) of chloroform to methanol to aqueous solution (SDS extract) to acetic acid, a ratio which should produce a single phase. (Note that both the EDTA and acetic acid are added to maximize extraction of PA into chloroform. PA can bind calcium, thereby increasing its hydrophilicity. EDTA prevents this association with calcium, and the acetic acid increases the protonation of PA, both of which should increase the hydrophobicity of the PA and promote partitioning into chloroform.) Thus, for a 60-mm dish, volumes of 0.8 mL SDS, 1 mL chloroform, and 2 mL methanol would yield the appropriate ratio. The cellular lipids are then extracted by incubating for 1–2 h on ice.
4. Chloroform and 0.2 M NaCl are then added to the test tubes, with vortexing, to “break phase,” that is, the chloroform separates from the methanol/water and two phases become readily apparent. The final desired ratio for this step is 1:1.0:0.04 (v:v:v) of chloroform to methanol to aqueous solution to acetic acid. As an example—for the above 60-mm dish of cells terminated with 0.8 mL 0.2% SDS and extracted with 1 mL chloroform, 2 mL methanol, and 0.08 mL acetic acid—1 mL chloroform and 1 mL 0.2 M NaCl would be added. The samples are then centrifuged briefly to promote complete phase separation. (Phase separation can also be achieved, with time, courtesy of gravity.)
5. The complete lower chloroform phase is transferred to a clean test tube using Pasteur pipets. Note that care should be taken to prevent transfer of the aqueous phase. Also note that this technique requires practice (one hint: the chloroform and aqueous phases will separate in the Pasteur pipet to allow transfer of the final few drops of chloroform).

- 6 The lower chloroform phases are then dried under nitrogen. After solubilization of the lipid extract in a small volume of chloroform/methanol at a ratio of 2 vol to 1, a percentage of the samples may be removed for determination of phospholipid phosphate content, as described by Van Veldhoven and Mannaerts (7), for normalization purposes. Samples can be stored at -20°C for up to 3 d

3.3. Separation and Quantitation of Radiolabeled PA and/or PEt

For separation of radiolabeled PA and PEt by TLC, the mobile phase is first prepared by mixing ethyl acetate, iso-octane, acetic acid, and distilled water in a ratio of 65:10:15:50 (v:v:v:v), respectively, in a glass bottle. Note that this mixture will appear as two phases. After capping tightly, the mixture should be shaken well to thoroughly saturate the organic phase with water. Approximately 100 mL of the upper, organic phase is placed in a TLC chamber. (Note that lining the TLC chambers with chromatography paper is not necessary for optimum separation.) The lipid samples are then solubilized in a small volume (40–50 μL followed by a 20- μL “rinse”) of chloroform/methanol (at a ratio of 2:1 vol:vol) to which is added approx 25 μg of cold PA and PEt (the cold phospholipids aid in optimal separation of the radiolabeled samples). The samples are spotted at the origin of the heat-activated TLC plate, using microcapillary pipets. Cold PA and PEt (approx 25 μg) are also spotted to serve as standards. The plate is placed in the TLC chamber and allowed to develop to within approx 2 cm of the top (development requires 1–2 h). Following removal of the plate from the chamber and evaporation of the solvent mixture, the standard is visualized by placing the plate in a TLC chamber equilibrated with iodine vapor for several minutes. (Note that, because iodine vapor is hazardous, this TLC chamber should be maintained in a chemical fume hood; inhalation of iodine should be avoided.) The cold PA and PEt will appear as yellowish-brown spots at R_F values of approx 0.36 and 0.48, respectively. (Note that R_F value is defined as the distance a spot migrates from the origin divided by the distance migrated by the solvent front.) The PA and PEt spots are marked with pencil and after evaporation of the iodine, the plates are sprayed with En^3Hance , according to the manufacturer’s recommendations, and exposed to film for several days. The spots corresponding to PA and PEt are then scraped into scintillation fluid and quantitated by liquid scintillation spectrometry (*see Note 5*).

4. Notes

1. Typically [^3H]oleic acid or myristic acid are used to radiolabel cells for determination of PLD activation, although [^3H]stearic, arachidonic (6) and palmitic (8) acids have also been utilized. [^3H]Oleate has the advantage that it is a major constituent, and thus labels the phosphatidyl moiety of all phospholipids. Therefore, using this fatty acid, PLD activity can be monitored regardless of the iden-

tity of the phospholipid that serves as the substrate of the enzyme. On the other hand, in some cell types myristic acid is incorporated primarily into phosphatidylcholine (e.g., ref. 9). If, using other methods (e.g., by examining production of [^3H]choline metabolites, as discussed in Note 2), it is determined that phosphatidylcholine is the likely substrate of hormone-induced phospholipid hydrolytic enzymes, myristic acid may be a better choice for prelabeling. Prelabeling periods of 2 h (Wendy Bollinger Bollag and EunMi Jung, unpublished observation) to 3 d (10) have been successfully employed for monitoring PLD activation in response to hormones. A typical prelabeling time is 20–24 h with [^3H]oleic or myristic acid (e.g., ref. 11). Under certain circumstances, prelabeling of cells may be impossible or ill-advised (see Note 6); in such cases postlabeling with [^{14}C]ethanol may be warranted, as described in Note 6.

2. Since PLD often utilizes phosphatidylcholine as a substrate, another method for examining the hormone-induced activation of this enzyme is to monitor the formation of [^3H]choline and phosphorylcholine in [^3H]choline-prelabeled cells. This method is described in detail in Chapter 11 of this book. The disadvantage of this method is the rapidity of the interconversion of the various metabolites. Therefore, time-courses must be performed to determine which metabolite appears at the earliest time-point: the appearance of choline first suggests the activity of PLD, whereas initial production of phosphorylcholine indicates the action of a phospholipase C.
3. As an alternative to 0.2% SDS containing 5 mM EDTA, reactions may be terminated by adding ice-cold methanol. The cells must then be scraped from the dish and transferred to test tubes as above. The advantage of using methanol is the greater speed with which the reactions are halted; the disadvantage is that the scraping of the cells for transfer is more labor- and time-intensive and complete transfer is difficult to ensure, although washing the dishes with an additional volume of methanol minimizes loss in transfer. In addition, removal of samples for normalizing using protein content is not possible; phospholipid phosphate content (7) may be used instead (see Subheading 3.2., step 2). Alternatively, a combination of the two termination procedures can be used: reactions are terminated with ice-cold methanol that is transferred to a clean test tube; cells are then solubilized with 0.2% SDS containing 5 mM EDTA and transferred. In either case, the final desired ratio is 1:2:0.8:0.08 (v:v:v) of chloroform:methanol aqueous solution:acetic acid. Note that, when the methanol termination is used alone, add 5 mM EDTA (pH approx 7.0) as the aqueous solution. Note also that, if methanol is used to terminate reactions, some evaporation of the solvent may occur, such that addition of small amounts of methanol may be required to obtain a single phase.
4. If an oven is not available for heat activation of TLC plates, plates may be activated by placing them in a TLC chamber containing dry acetone. After the solvent has migrated to the top of the plate, the plate should be briefly air-dried and used immediately or stored in a desiccating chamber.
5. For quantitation of the radioactivity contained in PA and PEt, the autoradiogram is aligned with the TLC plate to locate the [^3H]PA and PEt, which can then be

scraped from the plate (or cut if aluminum-backed TLC plates are used) and counted in a liquid scintillation counter. Theoretically, the spots visualized using iodine can be scraped and quantified; however, our laboratory has observed some incongruities between iodine-visualized cold standards and the radiolabeled phospholipids. This disparity may reflect heterogeneity between the PA and PEt species found in cells vs in the standards. Therefore, ideally the autofluorogram should be utilized to identify the regions for scraping and quantitation. The use of densitometry is also not recommended, since the fluorescence, and thus the quantitation, of the radiolabeled phospholipids is critically dependent on the application of the En^3 Hance, the homogeneity of which is difficult to ensure.

Note that if aluminum-backed TLC plates are used to separate and quantify PA and PEt by liquid scintillation spectrometry, the pieces of the plate should be cut so as to ensure that they lie flat at the bottom of the scintillation vials (large vials should be used). This precaution will ensure minimal quenching of the counts by the pieces. When the TLC plates are cut, silica may flake from the aluminum backing. To prevent possible loss of radiolabeled phospholipid imbedded in the silica, spots should be cut out over an appropriate surface so that the flakes can be retrieved. Weighing paper works well in that it can be shaped into a funnel to allow easy transfer of silica flakes to scintillation vials. Because solubilization of the radiolabeled PA and PEt into scintillation fluid requires time, vials should not be subjected to liquid scintillation spectrometry immediately after the addition of scintillation fluid. Instead, the vials containing the TLC plate pieces should be allowed to sit at room temperature for several days before counting to achieve maximum efficiency. On the other hand, if the silica is scraped from the TLC plate into the scintillation fluid, solubilization appears to be more rapid. It is also possible to extract the radiolabeled phospholipids from the silica using chloroform; however, chloroform quenches luminescence of the scintillant and must be completely evaporated before addition of the scintillation fluid and subsequent spectrometry.

Another potential problem with quantitation of the data is the fact that variations in background radioactivity have been observed from TLC plate to TLC plate after separation of radiolabeled phospholipids. Thus, differences between samples separated on various TLC plates may be either minimized or enhanced relative to the control values, depending on whether the background for that plate was greater or less than that of the plate on which the control samples were separated. This variability is more of a problem with PEt since the absolute levels of radioactivity found in this phospholipid are only approx one-half to one-third or less than that found in PA.

The plate-to-plate variability can be controlled for in several ways. First, if possible, all samples can be separated on the same TLC plate. Obviously, this is impractical in many cases; however, if cell number permits, additional control samples can be performed such that control lipid extracts can be separated on each TLC plate and the treated samples compared to the appropriate control values for that plate. Alternatively, experiments can be repeated many times.

Because the background fluctuates randomly between TLC plates, with repetition the differences should compensate for one another. Of course, this strategy will tend to increase variability in the data, but the net result should be accurate. Finally, it should theoretically be possible to scrape or cut out small sections of each TLC plate on which no sample was separated and subject these sections to liquid scintillation spectrometry. The resulting background counts could then be subtracted from the measured radioactivity in the PA and PEt separated on that particular plate. With respect to which section of the TLC plate will yield background counts most representative of the entire plate, the most likely candidate appears to be the section of plate on which the nonradioactive phospholipid (PA or PEt) standard was separated. However, it is not clear that this section will be entirely representative, since no radiolabeled lipids have traversed the lane, as occurs for sample lanes, in which radioactive neutral lipids migrate with the solvent front. If this option is selected, it is suggested that the individual investigator determine the section of the TLC plate most appropriate for each cell system.

- 6 Under certain circumstances, radiolabeling of cellular phospholipids prior to hormonal stimulation is either not feasible or presents other problems. As an example, if PLD activation is thought to occur with long-term exposure (e.g., many hours to days), radiolabeling in the presence of the hormone might result in differences in specific activity of potential phospholipid substrates with this prolonged hormone treatment. Subsequent measurement of PLD activity might then yield erroneous results, because of production of PEt from substrates of different specific activities in control vs treated samples. In this case, it may be desired to stimulate unlabeled cells for long time periods prior to acute labeling with [^{14}C]ethanol (1–5 mCi/mmol). Using this method, PLD activation in response to prolonged hormone exposure can be monitored without the concern that the treatment may be altering specific activity of the substrate and yielding inaccurate results.

The critical point to remember when handling [^{14}C]ethanol is the extreme volatility of the reagent. [^{14}C]Ethanol is purchased in special ampules that must be opened, according to the manufacturer's directions, after cooling in liquid nitrogen. Our laboratory mixes the small volume of radiolabel with a small amount of a 1:1 (v:v) mixture of absolute ethanol to water. Thus, 500 μL of cold ethanol/water solution is added to 100 μCi of [^{14}C]ethanol. All manipulations should be performed in a chemical fume hood to prevent inhalation of the radiolabel. Unused [^{14}C]ethanol should be stored in Wheaton reactivials with Teflon-lined screw caps (Aldrich, Milwaukee, WI). Labeling and storage of the [^{14}C]ethanol should be performed in containers lined with activated charcoal paper to absorb volatilized radiolabel. Charcoal paper can be purchased from Schleicher and Schuell (Keene, NH) and should be periodically disposed of and replaced to ensure maximum absorbancy.

Unlabeled cells are stimulated with the desired agents for the appropriate period of time prior to addition of 0.5% (v.v) [^{14}C]ethanol/ethanol (approx 5 μCi per cell sample). As an example, 20 μL of the 1:1 [^{14}C]ethanol/water solution described above is added to 2 mL of medium in a 35-mm dish. Alternatively,

the [^{14}C]ethanol can be added simultaneously with the agent of interest, much as described with the [^3H]oleate-prelabeling method. The cells are then incubated for 30–60 min (in a container lined with activated charcoal paper). Reactions are terminated as described in **Subheading 3.2., step 2**, for the [^3H]oleate-prelabeling technique, and the radiolabeled PEt extracted with chloroform, also as described in **Subheading 3.2., steps 3–6**.

[^{14}C]PEt is separated by TLC, basically as described in **Subheading 3.3.**, except that samples are solubilized in a small volume of chloroform/methanol to which is added only cold PEt. After development of the TLC plate in the solvent system described above, autofluorography is performed using En^3Hance , also as described. (We have found too little incorporation of the radiolabel into PEt to observe exposure of the film in the absence of the scintillant, although theoretically this radioisotope should allow autoradiography.) The autofluorogram is then used to locate the PEt spot for excision and quantitation by liquid scintillation spectrometry.

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Monitoring of Phospholipase A₂ Activation in Cultured Cells Using Tritiated Arachidonic Acid

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1. Introduction

1.1. Background

Phospholipase A₂ (PLA₂, EC 3.1.1.4, phosphatide *sn*-2 acyl hydrolase) is a ubiquitous enzyme that plays a role in the response of a wide variety of cells to specific stimuli. PLA₂ is an important component of the cellular machinery that is activated by inflammatory stimuli and it maintains cell homeostasis by membrane remodeling. The role of PLA₂ in the production of proinflammatory lipid mediators makes this enzyme an interesting therapeutic target for the treatment of inflammatory disorders (1).

PLA₂ catalyses the hydrolysis of the *sn*-2 fatty acyl bond of membrane phospholipids to liberate free arachidonic acid (AA) and lysophospholipids (2). AA is a C₂₀ polyunsaturated fatty acid containing four *cis* double bonds ($\Delta^{5,8,11,14}$ eicosatetraenoide). Even if the major pathway of AA release is via PLA₂, AA can also be released from sequential cleavage of phosphatidylinositols by phospholipase C (PLC) to generate diacylglycerol (DAG), that is subsequently acted on by DAG lipase to liberate AA (3).

The release of AA from membrane phospholipids is the rate-limiting step for the synthesis of eicosanoids (1). The eicosanoids family is made up of three clans: the prostanoids (prostaglandins and thromboxanes), which are synthesized via the cyclooxygenase pathway; the leukotrienes, lipoxins, and hydroxy-eicosatetraenoic acids (HETEs), which are formed via the lipoxygenase pathway; and the epoxides, which are formed by a cytochrome P-450 epoxigenase pathway (3).

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This chapter focuses on PLA₂ activation in cultured cells. It describes a quick and simple method to label cells with [³H]AA and/or its metabolites and to measure the release of [³H]AA upon activation of these cells with a Ca²⁺-mobilizing hormone.

1.2. Experimental Strategy

Since two major pathways of AA release are generally proposed, it is important to make sure that AA release is the result of PLA₂ activation. A simple control to verify involvement of PLA₂, is to incubate cells with quinacrine, a potent blocker of PLA₂, prior to their stimulation (4,5). Pretreatment with 300 μM quinacrine should completely inhibited the release of AA, thus indicating that the release phenomenon is principally mediated by the action of PLA₂. If the release of AA is not inhibited in the presence of quinacrine (Sigma, St. Louis, MO), it suggests that the phenomenon is mediated by an alternative pathway, most likely through the sequential cleavage of phosphatidylinositols by PLC and DAG lipase. The involvement of this pathway can be further verified with a specific inhibitor of DAG lipase, RHC-80267 (Calbiochem, La Jolla, CA) (6). Pretreatment with 10 μM RHC-80267 will completely inhibit the release of AA if the phenomenon is principally mediated via DAG lipase. If AA release is not inhibited in the presence of RHC-80267, the pathway through DAG lipase is not contributing importantly to the generation of AA, thus suggesting that AA is mostly released via the PLA₂ pathway. Both pathways can contribute in concert to the generation of AA. Since, at the present time, the mechanisms of PLA₂ activation are not clearly defined and appear to vary in a cell-specific and hormone-specific fashion, it is important to determine the contribution of each pathway in a particular system.

One important thing to consider when using inhibitors is to make sure that these compounds do not affect the basal release of AA (*see Note 3*).

This chapter describes the methods used for labeling phospholipids with [³H]AA and for measuring the release of [³H]AA and/or its metabolites from cells cultured into 24-multiwell plates. Obviously, depending on the system to be studied, cells can also be cultured into 6- or 12-well plates. Also, the incubation medium and the culture medium used in the assays may vary depending on the different cell types and their optimal culture requirements. Subtle refinements may be needed to optimize the release of AA in a desired system.

2. Materials

2.1. Cell Labeling

1. [5,6,8,9,11,12,14,15-³H]Arachidonic acid (1 mCi/mL) (Amersham, Arlington Heights, IL), stored at -20°C Use caution, this is radioactive!
2. Culture medium DMEM or as appropriate (*see Note 4*).

2.2. Release of [³H]Arachidonic Acid

- 1 Incubation medium: 25 mM HEPES pH 7.2, 150 mM NaCl, 5 mM KCl, 5.5 mM dextrose, 0.8 mM MgSO₄, 1 mM CaCl₂, 0.1% bovine serum albumin (BSA). Adjust to pH 7.2 with NaOH, prepared fresh (*see Note 5*).
2. Water bath (37°C) (*see Note 6*)
- 3 Scintillation vials
- 4 0.1 N NaOH
5. Scintillation fluid.
6. Liquid scintillation counter (β-counter)

3. Methods

3.1. Cell Labeling

1. Grow cells to confluence (or required density) into 24-multiwell plates
2. Replace the medium with 1 mL of culture medium (DMEM without serum or as appropriate, *see Note 4*) containing 0.5 μCi/mL of [³H]AA (*see Note 7*)
3. Incubate the cells for 16 h at 37°C

3.2. Release of [³H]Arachidonic Acid

- 1 Preincubate the incubation medium at 37°C in a shallow water bath (*see Note 8*)
- 2 Put cells plates in a shallow water bath and remove the medium (*see Note 9*).
- 3 Wash the cells four times at 37°C with 1 mL of incubation medium (*see Note 10*).
- 4 Add 400 μL of incubation medium at 37°C (or 300 μL incubation medium if you add 100 μL specific inhibitor, *see Note 3*)
5. Incubate the cells for 15 min at 37°C (*see Note 11*)
- 6 Using start–stop time-course of 15 s between each stimulation, add 100 μL of test agents (hormones) to stimulate the cells at 37°C (Vt = 500 μL) (*see Note 12*).
7. Incubate the cells for another 15 min (for dose–response relationships) or for different periods of time (for time-course relationships) (*see Note 13*)
8. Remove the medium containing released [³H]AA and transfer it into a scintillation vial.
9. Solubilize the cells by adding 300 μL NaOH 0.1 N in each well (*see Note 14*).
10. Transfer the content of each well into a scintillation vial.
- 11 Add about 4 mL of scintillation fluid in each vial.
12. Vigorously shake the vials.
13. Determine the radioactive content by scintillation counting, using a β-counter.
14. Express the data as shown in **Fig. 1** (*see Note 15*)

4. Notes

1. To minimize decomposition, it is recommended that stocks of [³H]AA be stored at –20°C. Under these conditions decomposition is not expected to exceed 3.5% per month in the first 2 mo, but thereafter may accelerate. This increase in the rate of decomposition during storage is characteristic of many tritium-labeled compounds. So make sure that the [³H]AA that you use is not degraded.

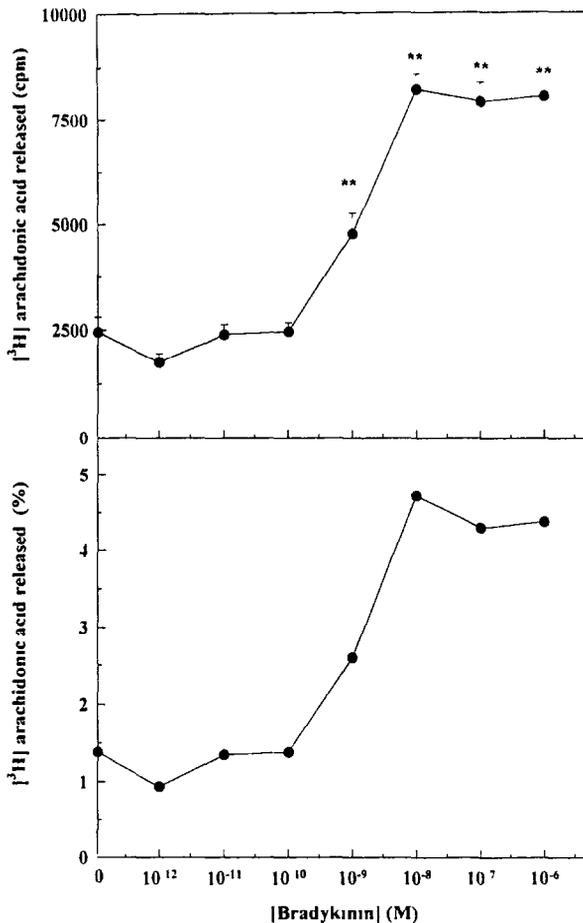


Fig 1 Typical dose-dependent effect of bradykinin on the release of [³H]arachidonic acid by bovine aortic endothelial cells: The [³H]AA-labeled cells were stimulated for 15 min at 37°C with the indicated concentration of bradykinin. Radioactivity released into the medium was then determined. (A) Results expressed in cpm. Each value represents the means ± SD of triplicate determinations (B) Results expressed as ratio of total [³H]AA content

2. All experiments should be performed at least in duplicate and more preferably in triplicate.
3. The control sample is stimulated with the incubation medium only. You can also stimulate with a specific inhibitor only to make sure that the inhibitor does not affect the basal level of [³H]AA release.
4. Although we use DMEM in our own studies, there is no reason to believe that it cannot be replaced with other basic media (Krebs, M199, Eagles, and so on),

- depending on the different cell type requirements. It is important to remove serum 18 h before evaluating PLA₂ activity in order to avoid the effects of growth factors or other compounds that could interfere in the assay.
5. For a 24-multiwell plate, you need approx 200 mL incubation medium. Although we use HEPES buffer in our own studies, there is no reason to believe that it can not be substituted for another depending on the different cell type requirements. However, Ca²⁺ and BSA are needed. Also, it is important to maintain a neutral pH with HEPES, HBS, or PBS.
 6. You need a shallow bath in which the plate sits 2–5 mm deep. The experiments can also be carried in a culture incubator; however it is not very convenient and the CO₂ content in the incubator's atmosphere must be taken into account to maintain neutral pH.
 7. Prepare a stock solution (25 mL for a 24-multiwell plate) of 0.5 μCi/mL of [³H]AA and add 1 mL to each well. To quantify the radioactivity loaded into your cells, keep a portion of the culture medium containing the [³H]AA and count it
 8. All the experiments are done in a shallow water bath at 37°C
 9. To save time in removing the medium, use a suction apparatus. Use caution. The medium is radioactive!
 10. To assure that your washing steps are efficient, count the medium that you remove during washes. You have to wash until the radioactive content of the medium reaches a stable and low value. Four washing steps are usually enough to bring the basal level approx 1000 cpm/mL. Under these conditions, the expected release of AA will be at least twofold above basal level.
 11. This step is to allow equilibration of the cells. If you need to use specific inhibitors, some require longer incubation periods to produce their effect. So, make sure that the exposure to the inhibitor is long enough
 12. When adding hormones and inhibitors, make sure to rock the culture plate delicately to get a homogeneous mixture.
 13. Depending on the system to be studied, cells may need to be stimulated for longer periods of time. A time-course relationship experiment may be needed
 14. The radioactive content of the cells is an indication of [³H]AA incorporation into the cells: It can allow the expression of data in percent of cellular [³H]AA released ($\frac{[\text{}^3\text{H}]\text{AA release}}{[\text{}^3\text{H}]\text{AA incorporated} + [\text{}^3\text{H}]\text{AA release}}$).
 15. The usual method for data analysis is a dose–response curve, in which the [³H]AA release (expressed in cpm) is plotted (*y*-axis) against the concentration of activating ligand (*x*-axis). The *x*-axis is normally drawn on a logarithmic scale (7)

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Assay of Cellular Diacylglycerol and Monoacylglycerol Lipases

Bryan A. Wolf

1. Introduction

Arachidonic acid has been implicated as a second messenger in numerous systems, and many agonists stimulate the intracellular accumulation of arachidonic acid (1,2). Arachidonic acid may be generated by phospholipase A₂ hydrolysis of phospholipids, or by hydrolysis of diacylglycerol through the sequential action of diacylglycerol lipase and monoacylglycerol lipase (3–6). These activities can be easily measured using sensitive radiometric assays as described herein.

In brief, diacylglycerol lipase activity is measured as the hydrolysis of a commercially available *sn*-2-labeled diacylglycerol substrate followed by thin-layer chromatography (TLC) separation of the end-products (Subheadings 2.3. and 3.3.). Monoacylglycerol lipase is measured in a similar fashion, except that the substrate, 2-arachidonyl-*sn*-glycerol has to be synthesized by hydrolysis of 1-stearoyl-2-arachidonyl-*sn*-glycerol with *Rhizopus arrhizus* lipase (Subheadings 2.1.2., 2.1.3., 3.1.2., and 3.1.3.). In order to minimize loss of lipids by adsorption on glassware surfaces, silanized glassware should be used (Subheadings 2.1.1. and 3.1.1.).

2. Materials

2.1. Preparation of Assay Reagents

2.1.1. Silanization of Glassware

1. Glassware to be silanized.
2. Dimethyldichlorosilane (Sigma, St. Louis, MO, flammable and toxic; see Note 1): 10% (v.v) in toluene. Store in glass bottle at room temperature in chemical fume hood or ventilated flame cabinet. Solution can be re-used 5–10 times.

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3. Toluene.
- 4 Methanol.
- 5 Large beakers (150–500 mL) to hold the glassware to be silanized.

2.1.2. Preparation of 2-Arachidonyl-*sn*-Glycerol and 2-[¹⁴C]-Arachidonyl-*sn*-Glycerol

1. Round-bottomed, silanized 13 × 100-mm borosilicate tubes with Teflon-lined screw caps (Fisher, Pittsburgh, PA).
2. 1-stearoyl-2-arachidonyl-*sn*-glycerol (Sigma or Avanti Polar Lipids, Pelham, AL). Store at –20°C under nitrogen
- 3 Chloroform (HPLC grade or better).
- 4 1-stearoyl-2-[¹⁴C]arachidonyl-*sn*-glycerol (0.55 mCi/mmol from Amersham). Store at –20°C under nitrogen
- 5 50 mM TES buffer pH 7.40 supplemented with 2.5 mM CaCl₂ and 1 mM MgCl₂. Store at 4°C.
- 6 *Rhizopus arrhizus* lipase (Boehringer Mannheim, Indianapolis, IN).
7. Shaking water bath (37°C).
8. Chloroform/methanol (1.2, v.v), at 4°C to stop reaction.
9. Refrigerated table-top centrifuge
- 10 Conical 13 × 100-mm silanized borosilicate glass tubes with Teflon-lined screw-cap tubes.
11. Silica gel G 20 × 20-cm channeled TLC plates with preadsorbent zone, (Analtech, Newark, DE), preactivated 30 min at 110°C
- 12 Solvent system (high-performance liquid chromatography [HPLC] grade or better) for **item 11**; petroleum ether:diethyl ether:acetic acid (140:60:2, v:v:v), made fresh
- 13 TLC tank
14. Whatman chromatography paper, 20 × 20 cm.
- 15 Disposable 50-μL micropipets.
16. Flat-edged spatula for scraping silica.
- 17 Weighing paper, 3 × 3 in.
- 18 Silica gel G plate with 10% boric acid
- 19 Solvent system (HPLC grade or better) for **item 18**, chloroform acetone:methanol (96:4:2, v:v:v), made fresh.

2.1.3. Ester Assay

1. 12 × 75-mm Borosilicate disposable tubes.
2. Ethanol:ether (3:1, v.v).
3. 2 M Hydroxylamine hydrochloride.
- 4 3.5 N NaOH.
- 5 4 N HCl.
6. 0.37 M Ferric chloride in 0.1 N HCl.
- 7 1 mM Cholesteryl acetate in ethanol. Store at –20°C.
- 8 Spectrophotometer or plate-reader (525-nm filter).

2.2. Sample Preparation

1. Potter-Elvehjem homogenizer.
2. 50 mM TES pH 7.40 supplemented with 1 mM EGTA. Store at 4°C.

2.3. Assay of Diacylglycerol Lipase and Monoacylglycerol Lipase

1. Silanized 13 × 100-mm borosilicate glass tubes (round-bottom and conical) with Teflon-lined screw-caps.
2. 50 mM TES pH 7.40, 1 mM EGTA, 2.5 mM CaCl₂, 1 mM MgCl₂, made fresh.
3. 1-stearoyl-2-arachidonyl-*sn*-glycerol (Sigma or Avanti Polar Lipids) or 2-arachidonyl-*sn*-glycerol (**Subheading 3.1.2.**). Store at -20°C under nitrogen
4. 1-stearoyl-2-[¹⁴C]arachidonyl-*sn*-glycerol (0.55 mCi/mmol from Amersham, Arlington Heights, IL). Store at -20°C under nitrogen.
5. 2-[¹⁴C]-Arachidonyl-*sn*-glycerol (**Subheading 3.1.2.**). Store at -20°C under nitrogen
6. Multitube vortex mixer
7. Water bath sonicator.
8. Shaking water bath (37°C)
9. Chloroform:methanol (1:2, v:v) supplemented with 0.25 mg/mL butylated hydroxytoluene
10. Carrier lipids for extraction steps (monoacylglycerol, diacylglycerol, arachidonic acid, triacylglycerol)
11. Table-top refrigerated centrifuge.
12. Silanized Pasteur pipets.
13. Chloroform
14. Distilled water.
15. Nitrogen evaporator.
16. Channeled silica gel G 20 × 20-cm TLC plate with preadsorbent zone, activated for 30 min at 110°C
17. Solvent system for item 16, petroleum ether:diethyl ether:acetic acid (140:60:2, v.v.v), made fresh
18. Disposable 50-μL micropipets.
19. Thin-layer chromatography tank
20. Whatman chromatography paper, 20 × 20 cm.
21. Radioactivity scanner or liquid-scintillation spectrometer

3. Method

3.1. Preparation of Assay Reagents

3.1.1. Silanization of Glassware (**Note 2**)

1. In a chemical fume hood, add the dimethylchlorosilane solution (10% in toluene) to the glassware. Completely cover all the glassware with the solution and check that there are no air bubbles trapped in the vials, pipets, or tubes
2. Incubate for 30 min at room temperature in the fume hood.
3. Remove the silanizing solution.
4. Rinse the glassware with toluene twice.

- 5 Rinse the glassware with methanol twice.
- 6 Dry glassware in oven at 110°C

3.1.2. Preparation of 2-Arachidonyl-*sn*-Glycerol and 2-[¹⁴C]-Arachidonyl-*sn*-Glycerol (Note 3)

- 1 To prepare 2-arachidonyl-*sn*-glycerol, resuspend 10 mg of 1-stearoyl-2-arachidonyl-*sn*-glycerol in 10 mL of chloroform and aliquot into 10 silanized tubes. To prepare 2-[¹⁴C]-arachidonyl-*sn*-glycerol, resuspend 10 μCi of 1-stearoyl-2-[¹⁴C]arachidonyl-*sn*-glycerol in 1 mL of chloroform in one silanized tube.
2. To each tube of cold material, add a trace amount (0.01 μCi) of 1-stearoyl-2-[¹⁴C]arachidonyl-*sn*-glycerol
3. Evaporate under nitrogen and resuspend in 100 μL of 50 mM TES (pH 7.4) supplemented with 2.5 mM CaCl₂ and 1 mM MgCl₂.
4. To each tube, add 2500 U of *Rhizopus arrhizus* lipase.
5. Incubate 15 min at 37°C in a shaking water bath.
- 6 Add 2 mL ice-cold chloroform:methanol (1:2, v.v)
- 7 Vortex for 1 min
8. Add 1 mL of chloroform, and 1 mL of water.
9. Vortex for 1 min.
10. Centrifuge for 15 min (4°C at 800g).
11. Remove the lower phase with a silanized Pasteur pipet and transfer to a clean, silanized, conical tube
12. Evaporate under nitrogen.
13. Add 500 μL chloroform, vortex for 1 min, evaporate under nitrogen, and reconstitute in 25 μL chloroform
- 14 Working in a fume hood, use disposable micropipets to spot the content of each tube onto a separate lane of the silica gel G plate. Let the plate air-dry at room temperature
15. Develop the plate in the petroleum ether/diethyl ether/acetic acid solvent system (Note 4).
16. With a clean flat-edge spatula, scrape the silica spot corresponding to monoacylglycerol (*R_f* 0.20). Use the weighing paper to transfer to a clean silanized round-bottom tube (Note 5).
17. To elute the monoacylglycerol from the silica, add 1 mL of chloroform:methanol (1:2, v:v), vortex for 1 min, centrifuge for 15 min (4°C at 800g), and carefully remove supernatant to a clean silanized tube, without disturbing the silica pellet.
- 18 Repeat step 17 two more times, and combine the eluates. Evaporate under nitrogen and reconstitute in chloroform
19. The purity of monoacylglycerol can be checked by spotting a small aliquot of the preparation on the 10% boric acid silica gel G plate, which is developed in chloroform:acetone:methanol. In this system, 2-arachidonyl-*sn*-glycerol has an *R_f* of 0.22 (Note 6).
20. The mass of 2-arachidonyl-*sn*-glycerol obtained is quantitated by ester assay using cholesteryl acetate as a standard (Subheading 3.1.3.) Store at -70°C under nitrogen at a concentration of 0.1 mg/mL in chloroform.

3.1.3 Ester Assay (Note 7)

- 1 Evaporate the lipid to be assayed and the standard curve of cholesteryl acetate (0, 100 nmol to 2 μ mol) in 12 \times 75-mm disposable glass tubes
- 2 Add 0.6 mL of ethanol ether.
- 3 Add 0.1 mL hydroxylamine hydrochloride.
- 4 Add 0.1 mL NaOH.
- 5 Vortex-mix and incubate 20 min at room temperature
- 6 Add 0.12 mL of 4 N HCl. Vortex-mix
- 7 Add 0.1 mL of ferric chloride solution. Vortex-mix.
- 8 Measure absorbance at 525 nm

3.2. Sample Preparation (Note 8)

- 1 Cells (1–2 mg protein) are homogenized with 10–20 manual strokes in a homogenizer in 0.1–1 mL of TES buffer with 1 mM EGTA
2. Homogenates are used fresh, or stored at -20°C prior to use.

3.3. Assay of Diacylglycerol Lipase and Monoacylglycerol Lipase

- 1 To a silanized round-bottom glass tube, add 85 μ L of 50 mM TES buffer pH 7.4 (supplemented with 1 mM EGTA or 2.5 mM CaCl_2 and 1 mM MgCl_2 , Note 9)
- 2 Add 10 μ L of cell homogenate (10–100 μ g protein)
- 3 Add 5 μ L of radioactive substrate dissolved in ethanol (for diacylglycerol lipase 2 mM final of 1-stearoyl-2-arachidonyl-*sn*-glycerol [0.025 μCi], for monoacylglycerol lipase 10 μ M final of 2-arachidonyl-*sn*-glycerol [0.025 μCi], Note 10)
4. Vortex for 1 min
- 5 Sonicate in a water bath sonicator (4°C) for 5 min (Note 11)
6. Incubate for 5 min in shaking water bath (37°C , Note 12)
- 7 Add 2 mL of ice-cold chloroform/methanol.
- 8 Incubate for 15 min in a dry-ice/alcohol bath
9. Add 5 μ g of monoacylglycerol, diacylglycerol, arachidonic acid, and triacylglycerol to each tube (Note 13)
- 10 Add 1 mL of chloroform and 1 mL of water
11. Vortex for 1 min
12. Sonicate for 30 min at 4°C in water sonicator
- 13 Vortex for 1 min
- 14 Centrifuge for 15 min (4°C at 800g).
- 15 With a silanized Pasteur pipet, remove the lower organic phase and transfer to a clean silanized conical tube (Note 14).
- 16 Add 1 mL of chloroform to the remaining upper aqueous phase and vortex for 1 min.
17. Centrifuge for 15 min (4°C at 800g)
18. Remove the lower organic phase and combine to the previously removed organic phase (step 15)
19. Repeat steps 16–18.

- 20 Add 1 mL of water to the combined organic phase, vortex for 1 min, and centrifuge for 15 min (4°C at 800g).
21. Discard the upper aqueous phase by aspiration
22. Evaporate the organic phase under nitrogen
- 23 Add 500 μL of chloroform, vortex for 1 min, and evaporate under nitrogen.
- 24 Add 25 μL of chloroform.
- 25 Working in a fume hood, use the disposable micropipets to spot the samples onto the preadsorbent zone of the channeled silica gel G TLC, plate (**Note 15**) Let the plate air-dry at room temperature
- 26 Develop the plate for 30–45 min in petroleum ether/diethylether/acetic acid (**Note 4**)
27. Locate the spots corresponding to monoacylglycerol (R_f 0.19), diacylglycerol (R_f 0.45), arachidonic acid (R_f 0.63), and triacylglycerol (R_f 0.88) by comparison with iodine-stained cold standards (**Note 16**)
28. Quantitate the radioactivity in monoacylglycerol, diacylglycerol, arachidonic acid, and triacylglycerol with the radioactivity scanner or by scraping the corresponding silica fractions that are measured by liquid scintillation spectrometry (**Note 17**)

4. Notes

- 1 Dimethyldichlorosilane is flammable, highly toxic (by inhalation, contact, and ingestion), and lachrymator. Handling must be in a chemical fume hood with suitable protective clothing, gloves, and eye/face protection.
2. Dimethyldichlorosilane (10% in toluene) can be reused 5–10 times if the glassware to be silanized is disposable. Rinsing should be complete to remove any trace of dimethyldichlorosilane. In particular, vials and tubes should be overturned and allowed to drain. Silanized glassware should be stored in appropriately marked containers, separate from general glassware.
- 3 The starting material 1-stearoyl-2-arachidonoyl-*sn*-glycerol should be as pure as possible. Splitting the starting material into 10 aliquots is useful to allow separation of the end products (**Subheading 3.1.2., step 14**) on analytical (rather than preparative) TLC plates. Solvents should be HPLC grade or better, and clean, freshly silanized glassware should be used throughout. To avoid contamination from hand soaps, gloves are worn. To avoid oxidation of the unsaturated bonds of arachidonic acid, samples should be stored under nitrogen. The addition of radioactive material in **Subheading 3.1.2., step 2** is useful to follow the yield of the reaction as well as to identify the end products on the TLC plate.
- 4 Prior to use, the TLC tank should be extensively rinsed in methanol/chloroform, and air-dried in a fume hood. The tank should be lined with chromatography paper, and the freshly made solvent system carefully poured into the tank approx 30 min prior to its intended use. The tank is covered with a glass pane that should be tight-fitting (put a weight on), and allowed to equilibrate for 30 min. The plate is then introduced, and allowed to develop 30–40 min or until the solvent front has reached the top of the plate. The separation can be affected by temperature and ambient humidity, thus it is important to use a freshly activated plate, and to perform the development in a chemical fumehood.

5. The retention time of the various lipids in this chromatography system should be worked out in a prior test run using cold standard phospholipid (does not migrate), monoacylglycerol (R_f 0.2), diacylglycerol (R_f 0.45), arachidonic acid (R_f 0.63), and triacylglycerol (R_f 0.88). These cold lipids can be easily visualized by placing the dried plate in a tank with a few crystals of iodine. The iodine vapors will react with unsaturated bonds, and should only be used for the test run with standards. Alternatively, radioactive standards can be used and the lanes scraped into 1-cm fractions that are counted for radioactivity by liquid scintillation spectrometry. If a radioactivity scanner is available, the position of the radioactive standards and of the end products of the lipase-digested samples can be quickly and accurately determined (6).
6. Typically, greater than 90% of the monoacylglycerol formed is 2-arachidonyl-*sn*-glycerol.
7. The ester assay is linear between 100 nmol and 2 μ mol. The standard curve is fitted by linear regression analysis that is used to determine the ester content of the sample. Since each molecule of 2-arachidonyl-*sn*-glycerol has only one ester bond, the moles of ester measured reflect the mass of the sample. In practice, approx, 0.5–1 mg of monoacylglycerol are used to detect its ester content in this assay.
8. Whole-cell homogenates can be used as well as plasma membrane and cytosolic fractions obtained by subcellular fractionation (6).
9. Standard conditions for measuring diacylglycerol lipase and monoacylglycerol lipase are: pH 7.4, 2.5 mM CaCl_2 , and 1 mM MgCl_2 . In a new cell system, it is important to characterize the pH dependency to determine the optimal pH for measuring lipase activity. TES buffer is used to achieve a pH between 6.0 and 8.0. At pH 8.0 and above, Tris buffer is used. At pH 6.0 and below, MES buffer is used. The calcium dependency of the lipase activities should be measured. The addition to the incubation buffer of 1 mM EGTA coupled with the omission of CaCl_2 and MgCl_2 will generate a calcium- and magnesium-free media. The calcium-dependency of the lipase activities can be tested with a detailed calcium dose curve (nM to mM) obtained by varying the calcium:EGTA ratio (7).
10. Preparing the radioactive substrate in ethanol enables complete dissolution. The concentration of the substrate in ethanol is adjusted to achieve the desired final concentration in 100 μ L of the reaction mix. The final concentration of substrate should be several-fold higher than the apparent K_m . The apparent K_m of islet diacylglycerol lipase is 0.86 mM and of islet monoacylglycerol lipase 0.14 μ M (6).
11. Vortexing and sonication are important to achieve complete mixing of the substrate. A water-bath sonicator is used filled with 1–2 in. of ice-cold water.
12. A time-course should be performed to determine the linear portion of the activity as a function of time.
13. The addition of carrier lipids drastically increases the recovery of the radioactive material to >95%. Typically, relatively cheap neutral lipids can be used such as monoolein, diolein, triolein, and arachidonic acid (Nu-Check-Prep, Elysian, MN).
14. The multiple extraction steps with chloroform increase the final recovery of radioactive material. The purpose of the water wash (**Subheading 3.3., step 20**)

is to remove any aqueous material that contaminates the organic phase and interferes in the chromatography step **Subheading 3.3., step 23** is a concentration step designed to recuperate any dried lipid from the inner sides of the tube

15. The use of channeled plates with preadsorbent zones is highly recommended. Using the disposable micropipets, the sample is streaked onto the upper half of the preadsorbent zone (make sure that this is higher than the top of the solvent when the plate is placed in the tank). The channels prevent any possibility of crosscontamination during development.
16. *See Note 5.* Since carrier lipids are added routinely during extraction, the easiest approach is to lightly iodine-stain the plate to localize the various fractions of interest. Scrape the stained fractions into scintillation vials, add scintillation cocktail, vortex, and count in a spectrometer. Alternatively, a radioactivity scanner can be used to quantitate the radioactivity in each lane.
17. To calculate enzyme activity, the counts in either monoacylglycerol (for diacylglycerol lipase) or arachidonic acid (for monoacylglycerol lipase) are expressed as a percentage of the total radioactivity on the lane (sum of radioactivity in monoacylglycerol, diacylglycerol, arachidonic acid, and triacylglycerol). This percentage is then converted to the amount of diacylglycerol or monoacylglycerol hydrolyzed by multiplying it by the mass of substrate originally present in each reaction tube. Enzyme activity is then normalized to the amount of protein present, divided by the incubation time, and expressed as the apparent specific activity (mass of substrate hydrolyzed/milligram of protein/min).

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Isotopic Efflux Studies as Indices of Phospholipase Activation

Jacob Vadakekalam and Stewart Metz

1. Introduction

Traditionally, phospholipase activation is monitored in static incubations by the intracellular accumulation of lipidic byproducts of phospholipid hydrolysis. Thus, for phospholipase C (PLC), diacylglycerol accumulation can be assessed, as can intracellular accumulation of phosphorylated derivatives of inositol. For PLD, phosphatidic acid is often assessed, or one of its transphosphatidylaton products (e.g., phosphatidylethanol) is used. For PLA₂, the intracellular accumulation of arachidonic acid (and/or lysophospholipid) is quantified. However, these approaches have several limitations in terms of both specificity and sensitivity (Table 1).

Data from such traditional approaches can profitably be complemented by radioactivity efflux studies in which cells are prelabeled with choline, inositol, or arachidonate, and then perfused with medium into which efflux of label (initially screened merely as total radioactivity) can be assessed every 1–2 min. This approach takes advantage of the facts that PLD (which is often directed at phosphatidylcholine) will directly release choline (or inositol if directed against PI). PLC will yield inositol phosphates in the aqueous fraction of the cell; in the absence of Li⁺, these will be dephosphorylated to inositol, much of which effluxes into the medium. PLA₂ will yield free arachidonate (AA), a portion of which will exit the cell as unmetabolized AA or as its more polar metabolites (1,2). (Conceivably, polar lysophospholipids could also exit the cell, especially in the presence of albumin in the perfusate, although this has not been formally assessed.) This method offers several advantages over traditional static incubations that assess accumulation of lipidic metabolites intracellularly, but

Table 1
Caveats in Use of Static Incubations to Assess Intracellular Accumulation of Lipidic Metabolites of Phospholipid Hydrolysis

-
- 1 Metabolites can be rapidly metabolized, interconverted, or re-esterified (*10*).
 - 2 Radiolabeling of lipid pools with lipid-soluble precursors (e.g., fatty acids) has many interpretive problems. Also, metabolically "inactive" pools of phospholipid substrates may produce high "basal levels" of lipid byproducts, and obscure acute changes induced by agonists
 3. Accumulation in the medium of certain constituents (e.g., H⁺, released hormones) may perturb physiologic changes (*see Table 2*, item 7)
 4. Important rapid and/or phasic changes can be easily missed (*11*).
-

that have several significant limitations (**Table 1**). However, it requires careful standardization and rigorous attention to methodologic and interpretive caveats (**Table 2**). Furthermore, one must beware of the existence of distinct metabolic pools of the relevant metabolite; for example, disparate changes may be seen for intracellular vs extracellular pools of metabolite (*see ref. 3*).

In this chapter we describe in detail the study of [³H]inositol efflux from prelabeled islets in response to agonist stimulation, as an example of this technique. Additional comments and illustrative data are also included, however, on the study of [³H]choline- or [³H]arachidonic acid-prelabeled islets to indicate the flexibility of this approach.

2. Materials

2.1. Labeling

- 1 [2-³H]myo-Inositol (10–25 Ci/mmol; aqueous; Dupont NEN, Boston, MA).
- 2 RPM-1640 medium (Gibco-BRL, Grand Island, NY)
3. HEPES
4. Dextrose.
- 5 Penicillin, streptomycin.
6. Fetal bovine serum (FBS).
7. 95% O₂ and 5% CO₂ mixture
8. 60 × 15-mm Petri dish (Falcon, Los Angeles, CA, cat. no. 1007)
9. Desiccator

2.2. Perfusion

1. Krebs-Ringer bicarbonate buffer (pH 7.4).
- 2 Myo-inositol (unlabeled).
- 3 95% O₂ and 5% CO₂ mixture.
- 4 Dextrose.
5. Bovine serum albumin (BSA) (fraction V, RIA grade).

Table 2
Caveats in Using Isotopic Efflux Studies as Indices of Phospholipid Hydrolysis

Problem	Solutions
1 Exchange mechanisms (e.g., base exchange) can also lead to efflux of label.	<ul style="list-style-type: none"> a. Document reduction in substrate (phospholipid) by <i>mass</i> measurements b. Demonstrate that qualitatively similar patterns of efflux occur in the absence of "chase" (but <i>see</i> item 4, <i>below</i>).
2 Lack of isotopic equilibrium (i.e., short labeling period) may limit conclusions, especially if "compartmentation" exists, provision of chase (to prevent reincorporation of label) can disturb isotopic equilibrium.	<ul style="list-style-type: none"> a. Document isotopic steady state, ideally in both lipidic and aqueous compartments. b. <i>See</i> solution 1b, <i>above</i> c. Provide mass measurements of metabolite.
3. Labeling of endogenous substrate may be perturbed by experimental conditions or test agents	<ul style="list-style-type: none"> a. Document that labeling is unaltered by test agents. b. Confirm results using exogenous substrate c. Express results as <i>fractional</i> efflux rates (i.e., in terms of total labeling in lipid extracts)
4 Released label may be reincorporated into substrate, or adhere to tubing	<ul style="list-style-type: none"> a. Provide "chase" (but <i>see</i> items 1 and 2, <i>above</i>). b. Concomitantly assess intracellular metabolites prior to their metabolism or reincorporation (e.g., IPs). c. Block reincorporation (10). d. Block adherence by adding albumin to perfusate (including during preincubation period), or flushing line with chase
5. Leakiness of cell membrane (or adherence to cells of dpm from the labeling period) can cause physiologically irrelevant efflux.	<ul style="list-style-type: none"> a. Document a concurrent <i>rise</i> (rather than a <i>decline</i>) in intracellular levels of the metabolite (i.e., in the aqueous phase of cell extracts) (12) b. Document integrity of cell membranes c. Eliminate bubbles in perfusion line d. Wash cells extensively after labeling (until basal efflux rates are stable).
6. Unanticipated metabolites may contribute to efflux of radioactivity (<i>see also</i> item 5, <i>above</i>).	<ul style="list-style-type: none"> a. Identify metabolites in medium (anion or cation exchange chromatography; TLC system for aqueous samples) (2). b. Block interconversion or breakdown of metabolites (e.g., using Li^+) (4,5).

(continued)

Table 2 (continued)

Problem	Solutions
	c. Document stoichiometric intracellular generation of lipidic byproduct (e.g., diglycerides, phosphatidic acid, lysophospholipid).
7. Constituents of perfusion medium (e.g., albumin [3], fatty acids) may influence results. In static incubations, compounds which might influence results (e.g., H ⁺ , insulin [11], phospholipid metabolites) may accumulate in the medium.	a Maximize test conditions for all variables. b. Confirm results from static incubations with dynamic incubations (perfusions)
8 In neurosecretory cells, unincorporated label might accumulate in secretory granules or vesicles, and be cosecreted passively.	a Verify findings in the presence of a secretory inhibitor (one not directly altering phospholipase activation)

- 6 Syringe holder (e.g., cat. no. 09 753 10A, Fisher, Pittsburgh, PA)
7. Solvent debubblers (Alltech, Deerfield, IL)
8. Nylon filter, pore size to depend on the cells studied, e.g., 62- μ mesh is used for perfusing islets (Tetko, Briarcliff, NY).
9. Perfusion pump (e.g., Monostat, New York) and tubing (1/16 in. id)
10. Fraction collector (e.g., Gilson fractionator, Middleton, WI)
11. Water bath at 37°C

2.3. Phospholipid Extraction from Pancreatic Islets

- 1 Chloroform (high-performance liquid chromatography [HPLC] grade)
2. Methanol (HPLC grade)
- 3 12 N HCl
4. Argon gas
- 5 500 mM KCl/50 mM EDTA mixture.
- 6 Polypropylene centrifuge tubes (15 mL; solvent resistant).
7. Sonifier (Branson 450, Danbury, CO).

3. Methods

3.1. Labeling the Cells

Islets are routinely cultured in Petri dishes for 18 h (see Notes 1 and 2) in batches of 100, in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, 11.1 mM glucose, 5 mM HEPES, and [³H]myo-

inositol (25 $\mu\text{Ci}/\text{mL}$; *see Note 3*) under 95% O_2 . For other cells/tissue preparations, media and labeling conditions will require optimization along the lines indicated elsewhere in this volume.

3.2. Perifusion

- 1 After completion of the labeling period, groups of 100 islets are transferred into each of the syringe holders, which are already fitted with the nylon mesh (*see Note 4* and *Fig. 3* inset).
2. The islets are perifused (*see Note 5*) at 1 mL/min for 30 min at 3.3 mM glucose to wash off any superficially adherent label and to establish a stable basal rate of inositol efflux and insulin secretion
- 3 The preincubation media is then replaced by the incubation media (*see Note 6*), islets are routinely incubated for 30–60 min.
- 4 The perifusion medium consists of Krebs-Ringer bicarbonate buffer containing 0.2% BSA (*see Note 7*), 1 mM unlabeled *myo*-inositol (*see Note 8*), gassed with 95% O_2 , 5% CO_2 gas mixture and maintained at 37°C.
5. Samples of the effluent are collected every 1–2 min using the fractionator
6. The perfusate is analyzed for the content of [^3H]inositol using 4 mL of the scintillation cocktail (Ultimagold, Packard, Meriden, CT) and 0.5 mL of the perfusate (*see Note 9*). The rest of the medium is stored at -20°C for subsequent measurement of insulin (*see Note 10*)

3.3. Phospholipid Extraction

1. At the end of the incubation (*see Note 11*), the syringe holders are taken apart and the islets on their polyethylene filters are transferred to polypropylene centrifuge tubes
- 2 1 mL absolute methanol, 2 mL chloroform, and 10 μL of concentrated hydrochloric acid (*see Note 12*) are added
3. Islets are sonified for 2 X 10 s (constant pulse) and are left overnight at 4°C under argon gas
- 4 The next day, phase separation is induced by addition of 750 μL of KCl/EDTA mix (*see Note 12*), followed by centrifugation at 1200 rpm for 6 min.
- 5 The upper (aqueous) and the lower (organic) phases are separated and an aliquot (100 μL) of both phases is counted for content of [^3H]inositol. The organic phase is stored under argon gas at -20°C until sample can be analyzed by TLC for phosphoinositides within 1 wk (*see Note 13*).

3.4. Data Presentation

- 1 Efflux of [^3H] is expressed as fractional efflux/min per 100 islets, calculated using the DPMs in each perfusate sample as the numerator. The denominator is the (initial) total [^3H]phospholipid content (determined by adding the total DPMs in the perfusate samples to the final [^3H]-containing phospholipid fraction of the islets, obtained by phospholipid extraction of the islets at the end of the experimental incubation). At each time-point, the DPMs released during the previous 2

min are subtracted from the initial total DPMs to yield the corrected denominator for the next sample

2. Data are corrected for the dead space of the perfusion apparatus.
- 3 The incremental responses for [^3H] release can be calculated by subtracting the mean of the last four basal values during the preincubation period from each of the values during the incubation period. Areas under the curve can be calculated from total incremental DPM (i.e., after subtracting basal values) and can be expressed as a percentage of the [^3H]-containing phospholipid fraction

4. Notes

1. The labeling compound used depends on the phospholipase activity to be studied. For example, [^3H]inositol is used to study phosphoinositide-directed lipases, [^3H]choline to study phosphatidylcholine (and sphingomyelin)-directed lipases, [^3H]arachidonic acid to study either phospholipase A_2 activity (directly) or phospholipase C activity (which generates arachidonoyl-enriched diglyceride, which in turn is hydrolyzed sequentially by diacyl- and monoglycerol lipases). The enzymatic source of the released arachidonic acid must be determined for each cell type (*see ref. 3*). The labeled islets are perfused and efflux of [^3H] is monitored (*see Fig. 1 and 2*)
2. The cells should be labeled with inositol to isotopic steady state. Although this has been shown to take many hours, some investigators have studied islets after labeling for a shorter period, even as short as 2 h (4) However, even when islets were studied after labeling for 18 h, only PIP2 and PIP had reached apparent steady-state labeling, 50 h were required for PI (which composes 85% of total islet inositides) to be labeled to steady state (5) Hence, observations using shorter periods of labeling need to be confirmed with cells labeled to isotopic steady state Similar considerations should be made for studies based on labeling with [^3H] fatty acids or choline
3. The final specific activity of the labeled inositol depends on the medium used For example, RPMI-1640 contains 35 mg/L of inositol, whereas CMRL 1066 contains only 0.05 mg/L of inositol. Thus, the appropriate amount of label required for the system needs to be established for each medium and the type of cells
4. The nylon mesh is fitted between the metal mesh and the "O" ring (*see Fig. 3 inset*). The perfusion apparatus is set up as outlined in Fig. 3 Islets are perfused with the chamber lying on its side or held vertically If the islets are perfused with holder held vertically, then chambers should not be positioned into the water bath until the medium has started to flow in order to prevent the cells falling off the mesh Note that cells can also be retained if suspended on columns of Sephadex G10 (for cells $>20\ \mu$) (6). Alternatively, cells may be grown on bio-beads (7,8).
- 5 The tubing size and length should be manipulated to optimize the dead space for the system We routinely use tubing of 1/16 in id The tubes can be primed with BSA (e.g., 0.5%) to prevent released insulin from adhering to the tubing.
- 6 Switching of the media permits air bubbles to enter the perfusion system. To prevent the bubble-induced agitation of the cells, debubblers are connected to the

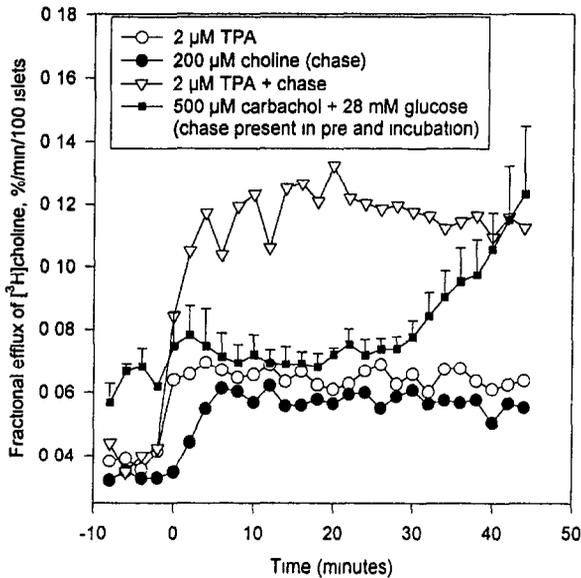


Fig 1. Fractional efflux of [^3H]choline. illustrative responses. Islets were prelabeled with [^3H]choline ($10 \mu\text{Ci}/\text{mL}$) in batches of 100. Preincubation media contained 3.3 mM glucose. The incubation medium for first group (\circ) contained $2 \mu\text{M}$ TPA (without any chase in the medium), the second group (\bullet) contained $200 \mu\text{M}$ choline (as the chase alone); the third group (∇) contained $2 \mu\text{M}$ TPA plus $200 \mu\text{M}$ choline, starting at 0 min. The fourth group (\blacksquare ; $n = 3$) was perfused with media containing $200 \mu\text{M}$ choline (during preincubation and incubation periods) and then was incubated with $500 \mu\text{M}$ carbachol plus 28 mM glucose starting at 0 min; the result is shown as mean \pm SE. The efflux of [^3H]choline induced by cold choline alone presumably represents base exchange

system as close as possible to the chambers. They are filled with adequate amount of the perfusion media (approx $300 \mu\text{L}$). Instead of debubblers, a sample valve (Fisher, three port) may also be used

7. Although the cell labeling for our studies has been performed using RPMI-1640 medium, other basic serum-free media (e.g., TCM 199 or Eagle's medium) may be used for other cells/tissues

During the actual perfusions, we observed a dose-dependent inhibition of PLC by BSA (fraction V, RIA grade, Sigma, Fig. 4), confirming a previous observation (9). Since some BSA is required for optimal islet function and to maximally recover secreted insulin (see Note 5), 0.2% BSA was chosen as the best concentration to study the efflux of [^3H]inositol when insulin secretion is studied concurrently. BSA is also recommended as a general component of perfusion media for other cell types, particularly in studies of fatty acid efflux.

8. Presence of cold inositol in the perfusate (chase) helps to augment the response by inhibiting reincorporation of released label (a similar approach can be taken to

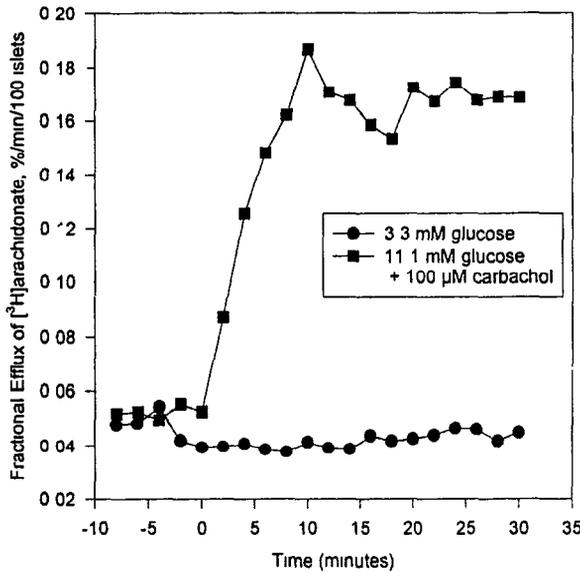


Fig. 2 Fractional efflux of $[^3\text{H}]$ arachidonate: representative response. Islets were prelabeled with $[^3\text{H}]$ arachidonic acid ($10 \mu\text{Ci}/\text{mL}$) in batches of 100. One group (●) was perfused at 3.3 mM glucose. The second group (■) was incubated with 11.1 mM glucose plus 100 μM carbachol, starting at 0 min following the preincubation period at 3.3 mM glucose. The media (preincubation and incubation) contained 0.5% BSA.

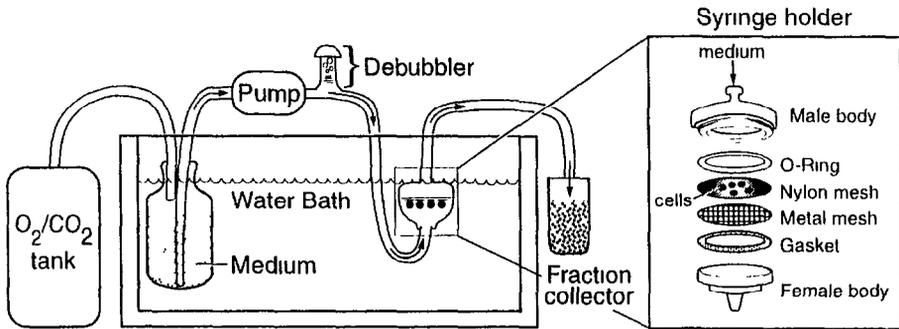


Fig. 3. Perfusion apparatus. The components of the perfusion apparatus are connected serially as indicated in the diagram. The syringe holder's components are shown in the inset.

study choline efflux; see Fig. 1) We have routinely used 1 mM inositol chase in the preincubation and incubation media. Further increases in the concentration of cold inositol augmented the basal efflux, but the incremental response to the agonist was unchanged (Fig. 5).

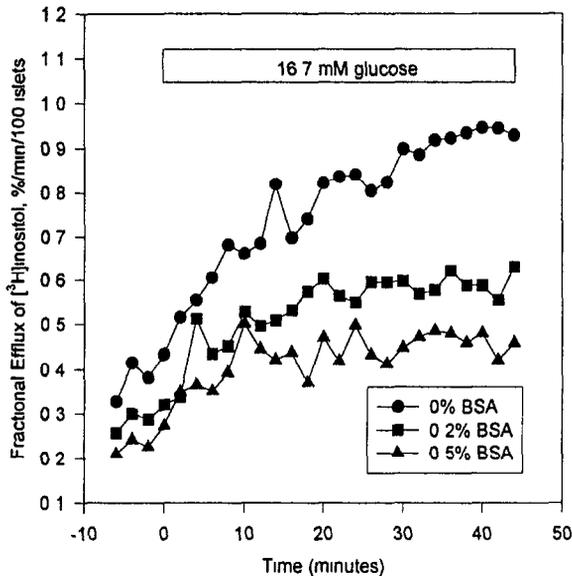


Fig. 4. Effect of BSA on fractional efflux of inositol. The islets were prelabeled with [^3H]inositol in batches of 100 as described. The incubation media (following preincubation at 3.3 mM glucose) contained 16.7 mM glucose, starting at time 0, in the presence of 0% BSA (●), 0.2% BSA (■) or 0.5% BSA (▲).

9. In order to establish the identity of the [^3H]metabolites in the medium, the medium can be analyzed by column chromatography (anion-exchange chromatography for inositol phosphates and cation-exchange chromatography for choline metabolites), as described elsewhere in this volume. Analysis of the [^3H] content of the perfusate of the islets labeled with [^3H]inositol revealed that it consisted almost entirely of [^3H]inositol. The increase in inositol efflux in response to agonist could be further abolished by introduction of lithium into the media (to impede the catabolism of IP to inositol), indicating that the augmentation of inositol efflux reflects phospholipase C activation (5).
10. Depending on the BSA concentration routinely used for insulin measurement (i.e., in the RIA standard curve), the perfusate concentration of BSA should be brought up (to a maximum of 0.5%) to prevent insulin adhering to the glass tubes in the assay. If less than the concentration of BSA needed for RIA is present in the perfusate, additional BSA should be added to each sample immediately after collection.
11. When the pump is turned off, cells may be washed back into the tubing. In order to prevent this, the chambers are removed from the inverted position in the water bath before the pump is turned off.
12. For studies of [^3H]fatty acid-prelabeled cells, similar extractions are carried out without any modification. For studies of [^3H]choline-prelabeled cells, similar

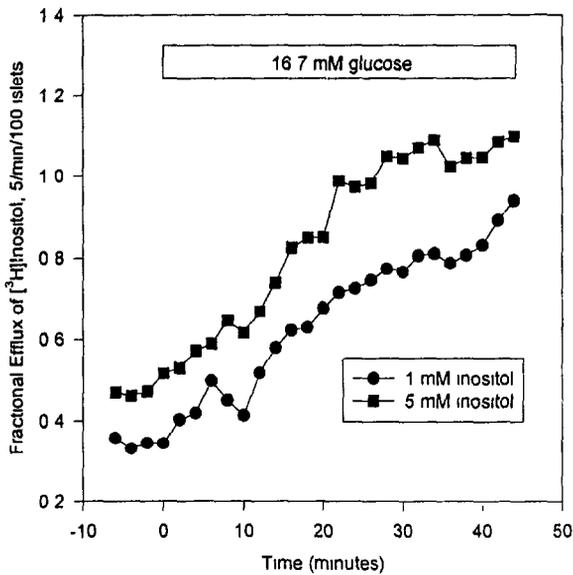


Fig. 5 Effect of chase at two concentrations on fractional efflux of inositol. The islets were pre-labeled with $[^3\text{H}]$ inositol in batches of 100 as described. The incubation media (following preincubation at 3.3 mM glucose) contained 16.7 mM glucose in presence of 1 mM inositol chase (●) or 5 mM inositol chase (■)

extractions are carried out except that HCl, KCl, and EDTA are omitted and phase separation is induced with 750 μL of water.

- For thin-layer chromatography to separate the phosphoinositides, 250- μm thick SL60 silica gels (Whatman, Clifton, NJ) are treated with oxalate by running the plates overnight in 1.2% potassium oxalate, dissolved in methanol and water (2:3). The plates are then preactivated by heating at 110°C for 30 min prior to loading the samples. The organic phase of the islet extract is dried down on ice under argon gas and is resuspended in 20 μL chloroform:methanol (2:1) \times 2 and loaded onto the plates. The plates are then developed in a solvent system consisting of methanol, chloroform, ammonium hydroxide, and water (100:70:15:25). The relevant areas (PtdIns, PtdIns(4)P and PtdIns(4,5)P₂) of the plates are subsequently scraped after autoradiographic exposure for 2 d at -70°C using EN³HANCE spray (Dupont). The scrapings are counted for $[^3\text{H}]$ content after adding 1 mL of methanol and 4 mL scintillation fluid. For separation of $[^3\text{H}]$ choline-containing moieties or identification of $[^3\text{H}]$ arachidonate metabolites, *see elsewhere* this volume

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Extraction and Measurements of Prostanoids and Leukotrienes by Radioimmunoassays

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1. Introduction

1.1. Background

Recent advances in the mechanisms of regulation of leukotriene and prostaglandin biosynthesis, as well as their mechanisms of action, have generated a renewed interest in this field. One can cite the cloning of enzymes involved in the lipoxygenase (LOX) and cyclo-oxygenase (COX or PGHS) pathways, such as the leukotriene C₄ synthase and the novel inducible COX enzyme named COX-2, and the cloning of receptors to eicosanoids. The discovery of the inducible prostaglandin synthase, COX-2 or PGHS-2, distinct from the constitutive enzyme, renamed COX-1 (1-4), opened a new area of research toward the development of novel and selective nonsteroidal anti-inflammatory compounds (NSAIDs) for the improved treatment of inflammatory diseases. Indeed, the specific inhibition of the COX-2 isoenzyme is expected to reduce inflammation without the side effects (gastric and renal toxicity) associated to classical nonselective NSAIDs.

1.2. Pathways

Eicosanoid synthesis is initiated by the activation of an ubiquitous enzyme, the phospholipase A₂ (PLA₂) (EC 3.1.1.4), a phosphatide *sn*-2 acyl hydrolase (Fig. 1; ref. 5). Several types of PLA₂ have been reported: the 85-kDa cytosolic PLA₂ and the 14-kDa secreted PLA₂ have been most investigated in regard to their potential role in eicosanoid synthesis. The PLA₂ catalyzes the hydrolysis of *sn*-2 fatty acyl of membrane phospholipids generating free arachidonic acid (AA) and lysophospholipids (6). The release of AA is the rate-limiting step for the synthesis of eicosanoids (5). A second pathway of AA release

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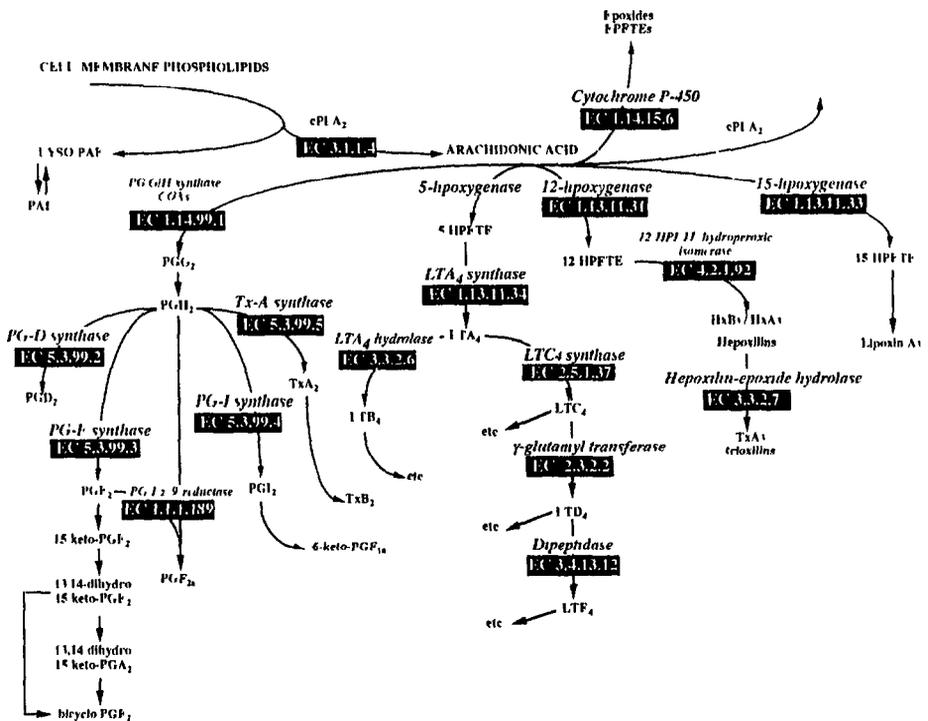


Fig. 1. Lipoxygenase (LOX), cyclo-oxygenase (COX or PGHS), and other metabolic pathways of arachidonic acid

involves the sequential cleavage of phosphatidylinositols by PLC and diacylglyceride lipase. AA is a C₂₀-unsaturated fatty acid containing four *cis* double bonds. Once released, AA is rapidly metabolized via enzymatic and nonenzymatic pathways. The main products of AA metabolism are the prostanoids (prostaglandins and thromboxanes), which are synthesized via the COX/PGHS pathways (EC 1.14.99.1), the leukotrienes formed via the 5-LOX pathway, the lipoxins formed by the 5- and 12- or 15-LOX, the heptoxilins formed by the 12-LOX, and a number of hydroperoxy- or hydroxy- or epoxy-derivatives formed by the action of either LOX or epoxygenases (cytochrome P-450 pathway). In the COX pathway, other enzymes are involved in the formation of prostanoids, for instance the thromboxane synthetase and the prostacyclin synthetase (Fig. 1). In the 5-lipoxygenase pathway, a cascade of enzymes such as the LTA₄ hydrolase and the LTC₄ synthase are involved in LT synthesis (Fig. 1).

The present chapter focuses on the measurement of prostanoids and leukotrienes. It describes tested methods such as radioimmunoassays (RIA) using ³H- or ¹²⁵I-labeled tracers and charcoal or Amerlex-M magnetic separation (AM) with a brief outlook of scintillation proximity assay (SPA).

1.3. Mediators

1.3.1. From the Cyclo-oxygenase Pathway

Prostaglandins were discovered in the 1930s and chemically identified in the early 1960s (7). Prostaglandins are classified in several types (mainly D, E, and I) according to the nature of functional groups on the prostanoid acid skeleton, whereas thromboxanes form a different class of compounds. Prostaglandins are not stored but synthesized on cell stimulation. PGI₂, also named prostacyclin, is the main product of the arachidonic acid metabolism in endothelial cells of arteries and veins and is chemically unstable, being spontaneously converted into 6-keto-PGF_{1α}. It inhibits platelet aggregation and promotes vasodilatation acting via the stimulation of adenylate cyclase in underlying smooth muscle cells. PGE₂, another primary prostaglandin, evokes vasodilatation, and increases blood flow and cardiac outflow. In vivo, PGE₂ is very rapidly converted to an inactive metabolite (13,14-dihydro-15-keto PGE₂) via the prostaglandin 15-dehydrogenase and 13-reductase and excreted into the urine after further ω-oxidation (Fig. 1). PGF_{2α} is a weak vasoconstrictor but a potent bronchoconstrictor. TxA₂, initially isolated from human platelets, is another unstable compound derived from the endoperoxides, and spontaneously hydrolyzes to yield TXB₂. TxA₂ induces platelet aggregation and contracts numerous vascular and nonvascular smooth muscles. It opposes the action of PGI₂ on platelets.

1.3.2. From the Lipoxygenase Pathway

The lipoxygenases lead to the formation of other series of arachidonic acid metabolites. Three lipoxygenase isozymes, the 5-, 12-, and 15-LOXs, have been described in mammalian cells; the initial product of a LOX reaction with AA is an hydroperoxide (HpETE). In the 5-LO pathway, 5-HpETE is the precursor of the leukotrienes. Leukotriene B₄ (LTB₄) is a potent chemotactic, chemokinetic, and leukocyte-aggregating agent. It plays a role in inflammation and tissue damage. LTC₄, D₄, and E₄ are the biologically active constituents of the slow-reacting substance of anaphylaxis (SRS-A). They are potent vasoconstrictors and bronchoconstrictors, causing also mucus secretion, vascular wall permeability, plasma leakage, and edema. They play a major role in airway inflammation and asthma (8–10).

2. Materials

2.1. RIAs of Prostaglandins and Thromboxane

- 1 Polyclonal antibodies (PAb) (1 bottle of lyophilized rabbit antiserum of anti-6-keto-PGF_{1α}, anti-PGE₂, anti-PGF_{2α}, anti-TxB₂) from Sigma (St. Louis, MO). Store at –80°C for up to 1 yr or use as soon as possible once reconstituted at 4°C.

2. Tritiated tracers (925 KBq [25 μ Ci] of [5,8,9,11,12,14,15(n)- 3 H] 6-keto-PGF_{1 α} , 1 85 KBq (50 μ Ci) of [5,6,8,11,12,14,15(n)- 3 H] PGE₂, 1.85 KBq (50 μ Ci) of [5,6,8,9,11, 12,14,15(n)- 3 H] PGF_{2 α} , 925 KBq (25 μ Ci) of [5,6,8,9,11,12,14,15(n)- 3 H] TxB₂) from Amersham Store at -20° C and use within 1 yr. Use caution, this is radioactive
3. Prostanoid standards (1 mg of PGE₂, PGF_{2 α} , 6-keto-PGF_{1 α} , TxB₂) from Sigma. Store at -80° C and use within 1 yr.
4. Other chemicals (50–500 g of TRIZMA-HCl, TRIZMA base, sodium azide, gelatin type A from porcine skin [300 bloom], activated charcoal untreated 100–400 mesh, dextran T70) from Sigma Store at 25° C
5. Scintillation fluid (4 L of PicoFluor 40) and disposable polypropylene tubes (500 tubes of 12 \times 75 mm) from Packard (Downers Grove, IL), ICN (High Wycombe, UK), Amersham, Hughes & Hughes (Wellington, Somerset, UK), and/or Sarstedt (A. Laurent, QC, Canada). Store at 25° C.

2.2. RIAs of Leukotrienes

1. The antiserum (anti-LTB₄, anti-LTC₄ [monoclonal], anti-LTC₄/D₄/E₄) from Amersham Store at -15 to -30° C and use within 1 mo.
2. Tritiated tracers (18 5 kBq [0 5 μ Ci] of [5,6,8,9,11,12,14,15(n)- 3 H] LTB₄ and [14,15(n)- 3 H] LTC₄ [also use for the LTC₄/D₄/E₄ assay]) from Amersham Store at -15 to -30° C once diluted and use within 1 mo
3. Leukotriene standards (10 ng, 50 ng of LTB₄, LTC₄ [also for the LTC₄/D₄/E₄ assay]) from Amersham Store at -15 to -30° C once reconstituted and use within 1 mo.
4. Other chemicals (assay buffer, charcoal suspension) from Amersham Store at -15 to -30° C once reconstituted and use within 1 mo.
5. Scintillation fluid and tubes as **Subheading 2.1., item 5.**

2.3. Extraction and Purification Procedures of Prostanoids or Leukotrienes

1. Cartridges (1 box [Sep-Pak or Amprep] of 50 columns of C18 reverse phase, 360 mg or C2, 100 mg) from Millipore (Bedford, MA), Amersham, Bond Elut, or Bakerbond (Phillipsburg, NJ) Store at 25° C.
2. Buffers (4 L of phosphate buffer, ethanol, ultrapure water, hexane, ethyl acetate, methanol, methyl formate, and chloroform) from BDH (Toronto, ON, Canada) or other suppliers. Store at 25° C under a fume hood.

3. Methods

3.1. RIAs for Prostaglandins and Thromboxane

1. The antibodies are prepared by diluting one bottle of the stock antibody in 13 mL of mixed TRIS buffer. This preparation will be enough to run an assay of 130

Table 1
Range of Concentrations for the Standard Curves of Four Prostanoids

Standards	Curve range, ng/mL												
	1 ^a	2	3	4	5	6	7	8	9	10	11	12	13
6-keto-PGF _{1α}			20	10	5	2.5	1.25	0.625	0.3125	0.156	0.078		
PGF ₂	80	40	20	10	5	2.5	1.25	0.625	0.3125	0.156	0.078		
PGF _{2α}				10	5	2.5	1.25	0.625	0.3125	0.156	0.078		
TxB ₂					5	2.5	1.25	0.625	0.3125	0.156	0.078	0.038	0.019

^aTubes

tubes The mixed TRIS buffer is prepared as follows. 25 mL of TRIZMA-HCl (0.05 M, i.e., 3.3 g in 500 mL, adjust pH with HCl at 7.4 at 25°C) plus 25 mL of a mixture of TRIZMA-base (0.05 M, i.e., 3.3 g in 500 mL), sodium azide (0.1%, i.e., 0.5 g in 500 mL), and gelatin (0.2%, i.e., 1.0 g in 500 mL)

- The [³H] tracers are prepared by diluting a certain volume (μL) of radiolabeled [³H] tracer in 13 mL of mixed TRIS buffer to obtain approx 5000 cpm/100 μL
- The standard curves are prepared by adding 1 mL of TRIZMA-HCl buffer to 1 mg of each standard to obtain stock solutions of 1 mg/mL. Take 10 μL and add 9.990 mL of buffer to obtain stock solutions of 1 μg/mL. Further dilute with the same buffer to obtain the indicated (*see Table 1*) concentrations by twofold dilutions. For instance, to obtain the first concentration (80 ng/mL), add 80 μL of 1 μg/mL solution to 1 mL buffer, then take 500 μL and add it to the next tube containing 500 μL buffer to obtain 40 ng/mL, and so on.
- The overall RIA protocol is summarized in **Table 2**
- Add the various components (add first the buffer, standards, or samples, then the tracer, and complete with the antiserum) in 5-mL plastic tubes, mix the samples vigorously, cover the tubes with aluminium foil, and incubate at 4°C for 12–24 h (overnight). The next day, add 200 μL of activated charcoal suspension (0.5 g of activated charcoal plus 0.1 g of Dextran T70 in 25 mL of 0.05 M of TRIZMA-HCl, pH 7.4) per tube on ice, except tubes 1 and 2 (i.e., corresponding to the total counts). It is important that the dextran be in solution before the addition of the charcoal. The suspension should be stirred and kept at 4°C on ice for at least 30 min before use. It should be vortexed once more just prior to use. Vortex and keep on ice for 10 min. Centrifuge for 10 min at 3000 rpm at 4°C. Transfer the supernatant in scintillation vials. Add 4.0 mL of scintillation fluid. Radioactivity count for 60 s/sample (cpm)
- Calculate the average counts per minute (cpm) for each set of duplicate tubes. Subtract the NSB from all specific binding tubes (B₀, standards and samples) Calculate the %B/B₀ for each standard and sample = (std or sample cpm - NSB cpm) / (B₀ cpm - NSB cpm) × 100. Generate the standard curve by plotting % B/B₀ as a function of the log [prostanoids] in pg/mL.

Table 2
Summarized RIA Protocol^a for Prostaglandins and Thromboxane

Tubes	Volume, μL						Charcoal	Total
	Buffer	Standards	Samples	[^3H] Tracer	Antiserum			
1, 2 TC	200	0	0	100	0		200 buffer	500
3, 4 NSB	200	0	0	100	0		200	500
5, 6 B ₀	100	0	0	100	100		200	500
Standards*	0	100	0	100	100		200	500
Samples ^{††}	0	0	100	100	100		200	500

^aThe standard curves (*) require 8–11 tubes in duplicate. The amount of buffer, tracer, etc. is calculated to safely run 120 tubes (^{††}) TC, total counts (no charcoal added), NSB, nonspecific binding; B₀, zero standard

3.2. RIAs for Leukotrienes

1. Reconstitute the bottle of antiserum by adding 12.5 mL of assay buffer. The LTB₄ assay buffer is made of 50 mL UltraPure water, Tris-HCl (0.05 M, pH 8.6, at 25°C) and gelatin (0.1% w/v). The LTC₄ assay buffer is made of 50 mL of UltraPure water, phosphate (0.05 M, pH 7.4), NaCl (0.14 M), and gelatin (0.01% w/v).
2. The tracer is in 250 μL of methanol:water:acetic acid (60:40:0.1), pH 5.6. Add 250 μL to 12.5 mL of assay buffer. It gives approx 2700–2900 cpm/100 μL (total counts).
3. The standard curve for LTB₄ is prepared by adding 2.5 mL of assay buffer to the lyophilized vial (10 ng). Pipet 500 μL of the stock (4 ng/mL) and add to 500 μL of assay buffer (tube 1: 2 ng/mL). Further dilute with the same buffer to obtain the indicated (*see Table 3*) concentrations by twofold dilutions.
 The standard curve for LTC₄/D₄/E₄ (include LTC₄) is prepared by diluting 50 μL of the stock (50 ng in 250 μL of 0.05 M phosphate buffer:ethanol [2:1] pH 6.9, i.e., 200 ng/mL) with 1.2 mL of assay buffer (resulting: 8 ng/mL). Pipet 500 μL of the stock (8 ng/mL) and add to 500 μL of assay buffer (tube 1: 4 ng/mL). Further dilute with the same buffer to obtain the indicated (*see Table 3*) concentrations by twofold dilutions.
4. The overall RIA protocols are summarized in **Tables 4 and 5**.
5. Add first the buffer, standards or samples, then the tracer and complete with the antiserum. After the addition of the various components in polypropylene tubes, mix the samples vigorously and incubate for 2 h at 25°C (for LTB₄) or 1 h at 37°C (for LTC₄/D₄/E₄). Alternatively, the tubes may be incubated for both LTB₄ and LTC₄/D₄/E₄ assays overnight at 2–8°C. Cover the tubes with aluminium foil. The next day, prepare the charcoal suspension; gently mix and keep at 4°C on ice for at least 30 min before use. It should be vortexed once more just prior to use. Add 200 μL of activated charcoal suspension (50 mL UltraPure water, 2.0% w/v charcoal, 0.4% w/v of Dextran T70, 0.05 M Tris-HCl, pH 8.6, and 0.1% w/v

Table 3
Range of Concentrations for the Standard Curves of Leukotrienes

Standards	Curve range, ng/mL							
	1 ^a	2	3	4	5	6	7	8
LTB ₄	2	1	0.5	0.25	0.125	0.062	0.031	0.016
LTC ₄ /D ₄ /E ₄	4	2	1	0.50	0.25	0.125		

^aTubes

Table 4
Summarized RIA Protocol for LTB₄

Tubes	Volume, μ L						
	Buffer	Standards	Samples	[³ H] Tracer	Anti- serum	Charcoal	Total
1, 2 TC	200	0	0	100	0	200 buffer	500
3, 4 NSB	200	0	0	100	0	200	500
5, 6 B ₀	100	0	0	100	100	200	500
Standards*	0	100	0	100	100	200	500
Samples ^{††}	0	0	100	100	100	200	500

TC, total counts (no charcoal added), NSB, nonspecific binding, B₀, zero standard

The standard curve (*) requires 8 tubes in duplicates. The amount of buffer, tracer, and so on is calculated to safely run 125 assay tubes (††)

Table 5
Summarized RIA Protocol for LTC₄/D₄/E₄

Tubes	Volume, μ L						
	Buffer	Standards	Samples	[³ H] Tracer	Anti- serum	Charcoal	Total
1, 2 TC	300	0	0	100	0	250 buffer	650
3, 4 NSB	300	0	0	100	0	250	650
5, 6 B ₀	200	0	0	100	100	250	650
Standards*	100	100	0	100	100	250	650
Samples ^{††}	100	0	100	100	100	250	650

TC, total counts (no charcoal added), NSB, nonspecific binding, B₀, zero standard

The standard curve (*) requires 6 tubes in duplicates. The amount of buffer, tracer, and so on is calculated to safely run 125 assay tubes (††)

gelatin) per tube on ice, except tubes 1 and 2 (i.e., corresponding to the total counts). Vortex and keep on ice for 10 min. Centrifuge for 10 min at 3000 rpm at 4°C. Transfer the supernatant in scintillation vials. Add 10 mL of scintillant. Radioactivity count for 4-min samples in a β -scintillation counter.

- 6 To calculate the average cpm, average the absorbance readings of NSB wells and Bo wells. $Bo - NSB = \text{corrected } Bo$. $\%B/Bo = ([\text{samples and standards cpm} - NSB \text{ cpm}] / \text{corrected } Bo) \times 100$. Plot %B/Bo (*y*-axis) against the Log of the LT concentration (in pg/mL) (*x*-axis). Values >80% and <20% should be reassayed.

3.3. Extraction and Purification Procedures of Prostanoids or Leukotrienes

3.3.1. Solid Phase Extraction of Prostaglandins and Thromboxane

3.3.1.1. ACCORDING TO CAYMAN CHEMICAL (ANN ARBOR, MI)

1. Add 4 mL of 0.1 M phosphate buffer, pH 4.0, to 250 μ L of plasma, urine, or other fluids and vortex.
2. Activate the C18 reverse-phase cartridge (360 mg) by rinsing with 2.5 mL of ethanol and then 2.5 mL of UltraPure water. Do not allow the cartridge to dry.
3. Run the sample through the column.
4. Rinse with 2.5 mL of water and then 2.5 mL of HPLC-grade hexane.
5. Elute with 2.5 mL of ethyl acetate containing 1% methanol and keep the eluant.
6. Evaporate under reduced pressure (for example, using a Speed-Vac evaporator) or under a stream of nitrogen.
7. Reconstitute with \pm 250 μ L of EIA buffer, vortex, and run the assay.

3.3.1.2 ACCORDING TO AMERSHAM (ALSO INCLUDE LEUKOTRIENES)

1. Add 125 μ L of 2 M HCl, to acidify at pH 3.0, 500 μ L of plasma, urine, or other fluids and vortex.
2. Activate the C2 cartridge (100 mg) by rinsing with 2 mL ethanol and then 2 mL of UltraPure water. Do not allow the cartridge to dry.
3. Run the sample through the column.
4. Rinse with 5 mL water, then 5 mL 10% ethanol and finally 5 mL HPLC-grade hexane.
5. Elute with 5 mL methyl formate and keep the eluant.
6. Evaporate under reduced pressure (for example, using a Speed-Vac evaporator) or under a stream of nitrogen.
7. Reconstitute with \pm 250 μ L EIA buffer, vortex, and run the assay.

3.3.2. Solid Phase Extraction of Leukotrienes

3.3.2.1. ACCORDING TO CAYMAN CHEMICAL

1. Add 4 mL of 0.1 M phosphate buffer, pH 4.0, to 250 μ L of plasma, urine, or other fluids and vortex.
2. Activate the C18 cartridge (360 mg) by rinsing with 2.5 mL ethanol and then 2.5 mL UltraPure water.

3. Run the sample through the column.
4. Rinse with 2.5 mL water and then 2.5 mL HPLC-grade hexane.
5. Elute with 2.5 mL 90 ethanol:10 water and keep the eluant.
6. Evaporate under reduced pressure (for example, using a Speed-Vac evaporator) or under a stream of nitrogen.
7. Reconstitute with 250 μ L EIA buffer, vortex, and run the assay

3.3.3. Liquid Phase Extraction for Prostaglandins, Thromboxane, and Leukotrienes

3.3.3.1. ACCORDING TO AMERSHAM

1. Add 1 mL acetone to 500 μ L of sample and shake for 2 min.
2. Centrifuge at 10,000g at 4°C for 5 min.
3. Add 2 mL HPLC-grade hexane to the supernatant.
4. Shake for 2 min and centrifuge at 10,000g at 4°C for 5 min.
5. Discard the upper layer of hexane.
6. Adjust pH of the lower layer to 3.0–4.0 with 1 M citric acid.
7. Add 2 mL chloroform and shake for 2 min
8. Centrifuge at 10,000g at 4°C for 5 min.
9. Keep the lower layer and re-extract the top layer with 2 mL chloroform.
10. Combine the two chloroform extracts
11. Evaporate under reduced pressure (for example, using a Speed-Vac vacuum evaporator) or under a stream of nitrogen.
12. Reconstitute with 250 μ L EIA buffer, vortex, and run the assay.

4. Notes

4.1. RIAs of Prostaglandins and Thromboxane

1. The RIAs are dextran-coated charcoal [^3H] RIAs, using charcoal adsorption as a mean of separation. The assay is based on the competition between unlabeled prostanoids (either standards or samples of PGE₂, 6-keto-PGF_{1 α} , PGF_{2 α} , or TxB₂) and a fixed quantity of the respective tracer ([^3H] or [^{125}I] prostanoids) for a limited number of binding sites on a specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added nonradioactive ligand (either a standard or a sample)
2. The proposed methods can be used without any extraction procedures with samples containing culture media with less than 10% serum, or superfusion buffers (i.e., perfusates from lungs or kidney). Important: These assays are not suitable for direct use with plasma. For plasma, urine, or other biological fluids, it is necessary to extract before assaying (see Subheadings 2.3., 3.3., and 4.3.). Blood samples should be centrifuged immediately after collection in sodium citrate or EDTA to obtain plasma samples
3. Antisera have been developed in rabbit using a given prostanoid linked to BSA as the immunogen. Antisera are provided as prediluted and lyophilized with either

- no preservative added or 0.02% sodium azide. Prior to reconstitution, they can be stored for months at 2–8°C (see **Subheading 2.** for stability and **Subheading 3.** for reconstitution)
4. The specificity of each antisera, as evaluated by the manufacturer (in the present cases, Sigma), is presented in **Table 6.**
 5. The sensitivity of each RIA, as evaluated by the manufacturer (Sigma), is presented in **Table 7**
 6. Again, use caution while handling, storing, and disposing of radioactive material. All chemicals should be considered as potentially hazardous. For instance, sodium azide is classified as toxic when undiluted.
 7. Antibodies, tracers and/or standards are available separately (from Sigma, Amersham, NEN Du Pont [Wilmington, DE], Cascade-Assay Designs [Ann Arbor, MI], Cayman, or ICN [Costa Mesa, CA]) for the RIAs of PGE₂, 6-keto-PGF_{1α}, PGF_{2α}, TxB₂, and some other prostanoids. Prostanoid RIA kits (using either [³H] prostanoids or [¹²⁵I] prostanoids with various separation techniques such as charcoal adsorption and second antibody/protein precipitation, Kodak Amerlex-M (antibody-coated magnetic particles), or the Scintillation Proximity Assay (SPA system) containing all necessary reagents are available from Amersham (Biotrak) or NEN DuPont.
 8. The Amerlex-M second antibody reagent is a donkey antirabbit serum coated on to magnetizable polymer particles. A specific antibody bound to a given prostanoid is reacted with the Amerlex-M second antibody. Separation of the antibody-bound fraction is made by either magnetic separation or centrifugation (for 10 min at 1500g) and decantation of the supernatant. Radioactivity is measured in the pellet (*Note:* In the charcoal adsorption, it is measured in the supernatant).
 9. The Amersham SPA assay use specific antibodies (for example PGE₂ antiserum) that are coupled onto fluomicrospheres (beads containing scintillant). It eliminates the need to separate antibody bound from free ligand, and the use of scintillant. It is rapid, simple, and as sensitive as common RIAs. In the case of PGE₂, the assay is based on the conversion of PGE₂ to methyl oximate PGE₂ deriviate (more stable) by methoxamine hydrochloride. (Contact Amersham for more details)

4.2. RIAs of Leukotrienes

10. The RIAs are charcoal [³H] RIAs. The assay is based on the competition between unlabeled leukotrienes (LTB₄, LTC₄, or LTC₄/D₄/E₄) and a fixed quantity of the respective tracer ([³H] leukotrienes) for binding to a limited quantity of an antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added nonradioactive ligand (either a standard or a sample). Measurement of the protein-bound radioactivity enables the amount of unlabeled LTs in the sample to be determined. Separation is achieved by adsorption of the free LTs on to charcoal followed by centrifugation (*see above*). Measurement is done in the supernatant

Table 6
Percent Crossreactivity (50% B/B₀ displacement to)

Crossreactant	6-keto-PGF _{1α}	PGE ₂	PGF _{2α}	TxB ₂
6-keto-PGF _{1α}	100			<0.1
13-14-dihydro-15-keto-PGE ₂ or PGF _{2α}	0.1		<0.1	
PGA ₁	0.8	1.5	<0.01	<0.1
PGA ₂	0.2	2.0	<0.01	<0.1
PGB ₁	0.6	0.6	<0.01	<0.1
PGB ₂	0.6	1.4	<0.01	<0.1
PGE ₁	23.0	270	<0.1	<0.1
PGE ₂	4.0	100	<0.1	<0.1
PGF _{1α}	17.5	3.2	60.0	<0.1
PGF _{2α}	7.0	3.4	100	0.5
TxA ₂	0.6			100

Table 7
Maximal Sensitivity of Prostanoid RIAs Using the Charcoal Adsorption Method and Sigma's Antisera

Prostanoids	Detection limit (at 90% B/B ₀)
6-keto-PGF _{1α}	10 pg/tube
PGE ₂	15 pg/tube
PGF _{2α}	5 pg/tube
TxB ₂	4 pg/tube

11. See Note 2
12. The specificity of each antisera, as evaluated by the manufacturer (in the present cases, Amersham), is presented in **Table 8**.
13. The sensitivity of each RIA, as evaluated by the manufacturer (Amersham), is presented in **Table 9**.
14. Again, use caution while handling, storing, and disposing of radioactive material.
15. Antibodies, tracers and/or standards are available separately (from Amersham, NEN Du Pont, Cascade-Assay Designs, Cayman, and ICN) for the RIAs of LTB₄, LTC₄, and LTC₄/D₄/E₄. A number of RIA kits (using [³H] leukotrienes) are available for the determination of these leukotrienes, one of which using the SPA system (Amersham or NEN DuPont). An SPA kit assay is also available for LTB₄ from Amersham

Table 8
Percent Crossreactivity (50% B/B₀ displacement to)

Crossreactant	LTB ₄	LTC ₄	LTC ₄ /D ₄ /E ₄
5(S)-HETE	<0.043		0.355
6-trans-LTB ₄	16.6		
11-trans-LTD ₄		<0.001	
11-trans-LTE ₄		<0.001	
12(S)-HETE	<0.07		
15(S)-HETE	<0.04		
20-hydroxyl-LTB ₄		<0.001	
20-OH-LTB ₄	3.9		
LTB ₄	100	<0.001	0.455
LTC ₄	<0.025	100	100
LTD ₄	<0.025	5.4	181.8
LTE ₄	<0.025	0.5	92.9
LTF ₄			121.3
TxB ₂	<0.035		<0.018
PGF _{2α}	<0.047		<0.018
6-keto-PGF _{1α}	<0.039		<0.018
AA	<0.039		0.218

Table 9
Selectivity and Maximal Sensitivity of Leukotriene RIAs According to Amersham Assay Kits

Leukotrienes	Nonspecific binding	Specific binding of tracer	Detection limit (at 90% B/B ₀)
LTB ₄	2.3–2.6%	39–44%	15 pg/mL (1.5 pg/tube)
LTC ₄			80 pg/mL (8 pg/tube)
LTC ₄ /D ₄ /E ₄	46–53%	2.8–3.0%	50 pg/mL (5 pg/tube)

4.3. Extraction and Purification Procedures of Prostanoids or Leukotrienes

1. Solid-phase extraction procedures as modified from Cayman or Amersham and liquid phase extraction from Amersham.
2. Although many assays (*see* Table 1 in Chapter 18) have been validated for urine, plasma, or other biological fluids, interferences may lead to erroneous results. It is prudent to purify the sample for consistency. It is very important that all the organic solvent be removed as traces interfere with the assays. These extraction procedures can be validated by using an exogenous sample of radiolabeled metabolite (spike) run through the same procedure to assess the recovery rate.

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Measurements of Prostanoids, Leukotrienes, and Isoprostanes by Enzyme Immunoassays

Bruno Battistini, Serge Picard, Pierre Borgeat, and Pierre Sirois

1. Introduction

We have summarized in Chapter 16 the recent advances in the mechanisms of regulation of leukotriene and prostaglandin biosynthesis and their pathways of synthesis. We have outlined a detailed summary for measuring prostaglandins, thromboxane, and leukotrienes via radioimmunoassays, and evoked the new scintillation proximity assays (SPAs). This chapter also focuses on the measurement of prostaglandins, thromboxane, leukotrienes, and isoprostanes, but via enzyme immunoassays (EIAs).

We are adding one group of mediators that we have not discussed until now: The isoprostanes. Lipid peroxidation (free-radical catalyzed) leads to the formation of this unique series of nonenzymic (non-COX-derived) arachidonic acid metabolites dubbed isoprostanes. They are potential mediators of oxidant injury (1). F2-isoprostane (or 8-epi PGF_{2α}) is a potent renal vasoconstrictor (2); it also constricts guinea pig and human airway smooth muscles (3). The measurement of isoprostanes may have a diagnostic utility in the assessment of lipid peroxidation in humans.

2. Materials

2.1. EIAs of Prostaglandins and Thromboxane

1. Antibodies (anti-6-keto-PGF_{1α}, anti-PGE₂, anti-PGF_{2α}, and anti-TxB₂) from Cayman (Ann Arbor, MI). Store at -20°C for up to 1 yr or use within 4 wk once reconstituted at 4°C.
2. Tracers (linked to acetylcholinesterase; AChE:6-keto-PGF_{1α}, AChE:PGE₂, AChE:PGF_{2α}, AChE:TxB₂) from Cayman. Store at -20°C for up to 1 yr or use within 2 wk once reconstituted at 4°C.

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3. Buffers (EIA buffer, wash buffer, Tween-20) from Cayman Store at -20°C for up to 1 yr.
4. Prostanoid standards (5 ng/mL of PGE_2 , $\text{PGF}_{2\alpha}$, 6-keto- $\text{PGF}_{1\alpha}$, and TxB_2) from Cayman. Store at -20°C for up to 3 mo or use within 6 wk once reconstituted at 4°C
5. 96-Well plates are precoated with mouse antirabbit monoclonal IgG antibodies for 6-keto- $\text{PGF}_{1\alpha}$, $\text{PGF}_{2\alpha}$, and TxB_2 or with goat antimouse polyclonal antibodies for PGE_2 , from Cayman. Store at -20°C for up to 3 mo.

2.2. EIAs of Leukotrienes

- 1 These Cayman EIA kits can be stored at -80°C for more than 6 mo.
2. Antibodies (rabbit anti- LTB_4 , anti- LTC_4 , and anti- LTE_4)
- 3 Tracers (linked to acetylcholinesterase; AChE.LTB_4 , AChE.LTC_4 , and AChE.LTE_4).
- 4 Buffers (EIA buffer pack, wash buffer pack, Tween-20)
- 5 Leukotriene standards (5 ng/mL of LTB_4 , LTC_4 , and LTE_4)
- 6 96-Well plates are precoated with mouse antirabbit monoclonal IgG antibodies.

2.3. EIA of 8-isoprostane (8-iso $\text{PGF}_{2\alpha}$)

1. This Cayman EIA kit can be stored at -20°C for more than 6 mo.
2. Antibodies (anti-8-*iso* $\text{PGF}_{2\alpha}$).
3. Tracers ($\text{AChE.8-iso PGF}_{2\alpha}$)
4. Buffers (EIA buffer packet, wash buffer packet, Tween-20).
5. Standards (5 ng/mL of 8-*iso* $\text{PGF}_{2\alpha}$)
6. 96-well plates are precoated with mouse antirabbit monoclonal IgG antibodies.

3. Methods

3.1. EIAs of Prostaglandins and Thromboxane

1. The antibodies are prepared by reconstituting the antiserum (one bottle) with 30 mL EIA buffer. The EIA buffer is prepared by dissolving one EIA buffer packet in 500 mL UltraPure water. The EIA-mixed buffer is a 1-L phosphate buffer preparation (to prepare, add 500 mL of 1 M NaH_2PO_4 [59.99 g in 500 mL] to 500 mL of 1 M Na_2HPO_4 [70.98 g in 500 mL] until reaching pH 7.4) to which you add 100 mg sodium azide, 23.4 g sodium chloride, 370 mg tetrasodium EDTA, and 1 g bovine serum albumin (BSA). Stir at 25°C until dissolved.
2. The AChE tracer (bottle) are reconstituted with 30 mL of EIA buffer.
3. The standard curves are prepared by reconstituting the standard vial with 1 mL EIA buffer. Further dilute with EIA buffer to obtain the following concentrations by doubling dilutions. Take 100 μL of the first tube (5 ng/mL), add 900 μL of EIA buffer to give 500 pg/mL, take 500 μL and add it to the next tube containing 500 μL of EIA buffer to obtain 250 pg/mL, and so on (*see Table 1*)
4. The overall EIA protocol is summarized in **Table 2**.
5. Use plates precoated with mouse monoclonal antibodies (which are antirabbit IgG) blocked with a proprietary formulation of blocking agents. After the addition of various components in wells (*see Table 2*), cover the plate with a plastic

Table 1
Range of Concentrations for the Standard Curves of Four Prostanoids

Standards	Curve range, ng/mL							
	1 ^a	2	3	4	5	6	7	8
6-keto-PGF _{1α}	1000	0 500	0 250	0 125	0 0625	0.0313	0 0156	0 0078
PGE ₂	1000	0 500	0 250	0.125	0 0625	0.0313	0 0156	0 0078
PGF _{2α}	1000	0.500	0 250	0 125	0.0625	0.0313	0 0156	0 0078
TxB ₂	1000	0 500	0.250	0.125	0 0625	0.0313	0 0156	0 0078

^aTubes**Table 2**
Summarized EIA Protocol for Prostaglandins and Thromboxane

Tubes	Volume, μL					Total
	Buffer	Standards	Samples	AChE Tracer	Antibody	
1,2 B	0	0	0	0	0	0
3,4 NSB	100	0	0	50	0	150
5,6 B ₀	50	0	0	50	50	150
7,8 TA	0	0	0	0	0	0
Standards* 9–24	0	50	0	50	50	150
Samples 25–98	0	0	50	50	50	150

The standard curves (*) require eight wells in duplicate.

B, blank, NSB, nonspecific binding, B₀, maximum binding; TA, total activity

film, and incubate at 4°C for 18 h (overnight) Empty the plate and rinse five times with washing buffer (the washing buffer is prepared by dissolving one washing buffer pack in 500 mL of UltraPure water; the buffer is a 4.0 M phosphate solution at pH 7.4). Add 0.25 mL Tween-20 to the buffer before use. Add 200 μL of Ellman's reagent to each well (one vial reconstituted with 50 mL of UltraPure water; protect from light) Add 5 μL of AChE tracer to the "TA" well Shake with an orbital shaker in the dark for 90–120 min, i e., until B₀ wells equal in the range of 0.3–0.8 AU (absorbance unit). Read at 412 nm (distinct yellow color).

- Calculate the average of the absorbance readings of NSB wells and B₀ wells $B_0 - NSB = \text{corrected } B_0$. % B (for samples and standards)/B₀ = (samples or std absorbance - NSB) × 100 Plot % B/B₀ (y-axis) vs the Log of the eicosanoid concentration (in pg/mL) (x-axis). Values greater than 80% and less than 20% should be reassayed.

Table 3
Range of Concentrations for the Standard Curves of Leukotrienes and 8-Isoprostane

Standards	Curve range, ng/mL								
	1 ^a	2	3	4	5	6	7	8	9
LTB ₄	1000	0.500	0.250	0.125	0.0625	0.0313	0.0156	0.0078	0.0039
LTC ₄	1000	0.500	0.250	0.125	0.0625	0.0313	0.0156	0.0078	0.0039
LTE ₄	1000	0.500	0.250	0.125	0.0625	0.0313	0.0156	0.0078	0.0039
8-Isoprostane	1000	0.500	0.250	0.125	0.0625	0.0313	0.0156	0.0078	0.0039

^aTubes

3.2. EIAs of Leukotrienes and 8-1 Isoprostane

Proceed as described in **Subheading 3.1.**, but with the Standards in **Table 3.**

4. Notes

4.1. EIAs of Prostanoids

- 1 These EIAs are acetylcholinesterase (AChE)-based EIAs available as kits from Cayman. The assay can also be run with your own antibody (or from another source) to a specific prostanoid. Each of the kit components are available separately. The assay is based on a competition between a free prostanoid (the standards or the samples) and the corresponding prostanoid tracer (metabolite linked to an AChE molecule) for a limited number of the prostanoid-specific rabbit antiserum binding sites (*11*). The concentration of the tracer is held constant as the concentration of the standard or sample varies. Thus, the amount of tracer that is able to bind to the antiserum will be inversely proportional to the concentration of the free prostanoid in the well (absorbance α [bound tracer] α [1/prostanoid]).
- 2 It is imperative to have good pipetting technique. If nonspecific binding (NSB) is high, it might have been exposed to the specific antibody or poorly washed. If the maximum binding (B_0) is low, the water may be contaminated with organic solvents (use deionized water that is ultrapure), the plates require more development time, or the Ellman's reagent is degraded.
3. Although many assays (*see Table 1* in Chapter 18) have been validated for urine, plasma or other biological fluids (i.e., culture media), interferences may lead to erroneous results using EIAs. Therefore, it remains prudent to purify the sample for consistency. Solid-phase extraction or liquid phase extraction procedures for prostaglandins, thromboxane, and leukotrienes, as modified from Cayman or Amersham, are described in Chapter 16.
- 4 The antiserum for three of the four prostanoids is isolated from rabbit blood and bound to the mouse monoclonal rabbit antibodies previously attached to the well. The antiserum for PGE₂ is a monoclonal antibody that binds to goat antimouse polyclonal antibodies (PAb).

Table 4
Percent Crossreactivity (50% B/B₀ displacement to)

Crossreactant	6-keto-PGF _{1α}	PGE ₂	PGF _{2α}	TxB ₂	8-isoprostane
6-keto-PGF _{1α}	100	1.0	2	<0.01	0.1
13-14-dihydro -15-keto-PGE ₂ or PGF _{2α}		<0.01		<0.01	
PGA ₁		<0.01	<0.01		
PGA ₂			<0.01		
PGB ₁		<0.01	<0.01		
PGB ₂		<0.01	<0.01		
PGD ₂	<0.01	<0.01	7	0.44	0.1
PGE ₁		18.7	<0.1		0.2
PGE ₂	0.92	100	<0.1	<0.01	0.2
PGF _{1α}	0.8	<0.01	100	0.05	0.2
PGF _{2α}	2.1	<0.01	100	0.22	0.14
TxB ₂	<0.01	<0.01	<0.1	100	0.04

Table 5
**Mid and Maximal Sensitivity of Prostanoid
 and 8-Isoprostane EIAs According to Cayman Assay Kits**

Prostanoids	50% B/B ₀ , pg/mL	Detection limit (at 80% B/B ₀)
6-keto-PGF _{1α}	58.4	15.4
8-isoprostane	31.4	6.0
PGE ₂	112	29
PGF _{2α}	84.6	14.2
TxB ₂	53.9	7.4

- The specificity of each antisera, as evaluated by the manufacturer (in the present cases, Cayman), is presented in **Table 4**.
- The sensitivity of each antisera, as evaluated by the manufacturer (Cayman), is presented in **Table 5**.
- Some of the kits from Cayman (i.e., for PGD₂) require derivation prior to use. For the determination of PGE₂, it may be advisable to use the bicyclo PGE₂ EIA that involves conversion of all major PGE₂ metabolites into one single derivative (i.e., bicyclo PGE₂) since PGE₂ is rapidly metabolized in the circulatory system (half-life: 30 s). The enzyme label is still acetylcholinesterase (AChE). It may offer a kinetic superiority, low background, high sensitivity, precision, and versatility (unlike peroxidase)

Table 6
Percent Crossreactivity (50% B/B₀ displacement to)

Crossreactant	LTB ₄	LTC ₄	LTE ₄	Peptido-LT
5(S)-, 12(S)-DiHETE	<0.01			
5(S)-HETE	0.03			
6-trans LTB ₄	39			
6-trans-12-epi-LTB ₄	0.7			
12(S)-, 15(S)-HETE	<0.01			
20-carboxy-LTB ₄	<0.01			<0.01
20-hydroxy-LTB ₄	0.5			
LTB ₄	100	<0.01	<0.01	<0.01
LTB ₅	100	<0.01	<0.01	
LTC ₄	<0.01	100	10	100
LTD ₄	<0.01	20	9	100
LTE ₄	<0.01	16.1	100	67
LTF ₄	<0.01	NA		

Table 7
Mid and Near Maximal Sensitivity of Leukotrienes
EIAs According to Cayman Assay Kits

Prostanoids	50% B/B ₀ , pg/mL	Detection limit at >80% B/B ₀
LTB ₄	27	7
LTC ₄	93	25
LTE ₄	191.9	28.7

8. EIA kits are also available for the determination of prostanoids from Amersham using horseradish peroxidase (HRP) analytes as tracers instead of AChE. Coating is done with donkey antirabbit.

4.2. EIAs of Leukotrienes

9. Antibodies, tracers, and/or standards are available separately (from Cayman) for the EIAs of LTB₄, LTC₄, and LTE₄ Leukotriene EIA kits (also for a nonselective peptido-leukotrienes, i.e., LTC₄/D₄/E₄ together) containing all necessary reagents are available from Cayman, Amersham, or Cascade-Assay Designs.
10. The specificity of each antiserum, as evaluated by the manufacturer (Cayman), is presented in **Table 6**.
11. The sensitivity of each RIA, as evaluated by the manufacturer (Cayman), is presented in **Table 7**.
12. An EIA kit (only for LTC₄/D₄/E₄) is available from Amersham, using horseradish peroxidase (HRP) analytes as tracers instead of AChE.

4.3. EIA of 8-Isoprostane

- 13 Antibodies, tracers, and/or standards are all available separately (from Cayman) for the EIA of 8-isoprostane (8-*iso* PGF_{2α}).

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Measurements of Arachidonic Acid Metabolites Derived from the Lipoxygenase Pathways by High-Pressure Liquid Chromatography

Pierre Borgeat, Serge Picard, Bruno Battistini, and Pierre Sirois

1. Introduction

We have summarized in Chapter 16 the recent advances in the mechanisms of regulation of leukotriene and prostaglandin biosynthesis and their pathways of synthesis. This chapter focuses on the measurement of the leukotrienes and other LOX products by high-pressure liquid chromatography (HPLC). At this point, we also present a complete summary in **Table 1** comparing the various approaches used for the measurements of arachidonic acid metabolites derived from the prostaglandin endoperoxide synthase and the lipoxygenase pathways, as presented in Chapters 16–18.

2. Materials

1. Two HPLC pumps (Beckman, Fullerton, CA model 110B).
2. Two pumps (Scientific System, State College, PA, model 300).
3. Autosampler allowing injection of 2-mL samples (such as the Waters, Milford, MA, Wisp autosamplers).
4. Photometer allowing dual wavelength (229–235 and 270–280 nm) detection, or two fixed-wavelength photometers.
5. Solvent selection valve (Waters SSV).
6. Two double, three-way, air-actuated high-pressure valves (Rheodyne, Colalt, CA models 7030 and 5701)
7. Dynamic mixing chamber
8. Inline filters (2- μ m pore size).
9. One Lee Visco-Jet Micro-Mixer with 250 μ L internal volume.
10. Computer for the integrated control of the HPLC system and for data acquisition and processing.

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Mediators	Assays	Sources	Mediators	Tracers	Sensitivity (pg/ml)	Range (pg/ml)	Time frame ¹	No of samples (tubes vs wells)	Advantages	Limitations	
Prostanoids	1 RIA (charcoal)	Sigma / Amersham	PGE ₂	[³ H]	15 pg/tube	80 - 80 000	overnight	120 /btle antiserum 6000 / btle tracer	- less expensive	- longer procedure not suitable for biological fluids (unless extracted) radioactivity	
			6-keto-PGF _{1α}	[³ H]	10 pg/tube	80 - 20 000	overnight				
			Amersham	PGI _{2a}	[³ H]	5 pg/tube	90 - 10 000	overnight			
				TxB ₂	[³ H]	4 pg/tube	20 - 5 000	overnight			
				and some more							
				PGI ₂	[³ H]	20	30 - 2 000	overnight	100	- suitable urine/plasma	need pre-purification
				6-keto-PGF _{1α}	[³ H]	140	140 - 5 000	2 days	100		
				PGI _{2a}	[³ H]	30	30 - 2 000	overnight	100		
			TxB ₂	[³ H]	20	50 - 3 000	2 days	100	- suitable plasma		
2 RIA + Amerlex M ²	Amersham	PGI ₂	[¹²⁵ I]	8	12.5 - 1600	2.5 h	100	- suitable urine/plasma	limited tracer's life		
		6-keto-PGF _{1α}	[¹²⁵ I]	<20	30 - 4 000	overnight	100	- suitable plasma	more radioactivity		
		TxB ₂	[¹²⁵ I]	<20	30 - 4 000	overnight	100	- suitable plasma			
3 RIA + SPA ³	Amersham	PGI ₂	[¹²⁵ I]	10	12.6 - 1 600	overnight	100	- suitable urine/plasma	radioactivity		
		6-keto-PGF _{1α}	[¹²⁵ I]	140	140 - 5 000	overnight	100	- suitable urine/plasma			
		TxB ₂	[³ H]	10	50 - 3 000	1 day	100	- suitable plasma			
4 EIA ⁴		Cayman	PGI ₂	ACHe	29	7.8 - 1000	overnight	96/plate	monoclonal	need pre purification	
			6-keto-PGF _{1α}	ACHe	23.6	3.9 - 500	overnight	96/plate			
			PGF _{2α}	ACHe	24	15.6 - 2 000	overnight	96/plate			
			TxB ₂	ACHe	13.3	7.8 - 1000	overnight	96/plate	- non isotopic		
		Amersham	and many more								
			PGE ₂	HRP	16	20 - 640	5 h	96/plate	- fast assay		
			6-keto-PGF _{1α}	HRP	3	10 - 1280	2 h	96/plate	- suitable urine/plasma		
			TxB ₂	HRP	3.6	10 - 1280	2 h	96/plate	- suitable for all samples		
Leukotenes	1 RIA (charcoal)	Sigma / Amersham	LTB ₄	[³ H]							
			LTC ₄ /D ₄ /E ₄	[³ H]							
		Amersham	LTB ₄	[³ H]	15	16 - 2 000	3-4 h or overnight	125	- suitable serum/plasma	need pre purification	
			LTC ₄	[³ H]	88	80 - 5 000	overnight	100			
			LTC ₄ /D ₄ /E ₄	[³ H]	50	125 - 8 000	1 h or overnight	125		need pre-purification	
	2 RIA + SPA			LTB ₄	[³ H]	62	62 - 2 000	overnight	500		need pre-purification
3 EIA	Cayman	LTB ₄	ACHe	2.23	3.9 - 500	overnight	96/plate				
		LTC ₄	ACHe	25		overnight	96/plate				
		LTE ₄	ACHe	28.7		overnight	96/plate				
		LTC ₄ /D ₄ /E ₄	ACHe	11.8		overnight	96/plate				
		Amersham	LTB ₄	HRP	6	6.2 - 800	3.5 h	96/plate	- fast assay		
			LTC ₄ /D ₄ /E ₄	HRP	10	15 - 960	5.5 h	96/plate	- suitable plasma	- suitable plasma	
Isoprostanes	1 FIA	Cayman	8-iso PGF _{2α}	ACHe	6		overnight	96/plate	see above EIA	see above FIA	
Lipoxygenase products	1 HPLC		PGI ₂						- analysis of several products at one time	- expensive equipment required	
			LTB ₄						- minimal sample preparation procedures	- limit of detection ≥ 1 ng	
			LTC ₄ /D ₄ /E ₄						- internal standards		
			HTFES						- high level selectivity		
			Lipoxins								

¹Will vary depending on the temperature of incubation (e.g., 2–8°C, 25°C, or 37°C) ²With the magnetic polymer beads, simple magnetic separation, eliminating centrifugation ³Scintillation proximity assays do not necessitate to separate bound from free ligand, more automation, increase throughout, greater productivity, no solvents, vials, and so on (see Amersham). ⁴Rapid, nonisotopic, one plate, less manipulation, faster reading with EIA plate reader (vs γ -counter), no centrifugation

11. Columns and cartridges: Waters Resolve C₁₈ Radial PAK cartridges (5- μ m particles of 5 \times 100 mm) or Waters Resolve C₁₈ steel column (5- μ m particles of 3.9 \times 150 mm); Beckman Ultrasphere ODS C₁₈ column (5- μ m particles of 4.6 \times 150 mm); Waters analytical Resolve Silica and Resolve C₁₈ Guard-Pak inserts (precolumns).
12. Arachidonic acid metabolite standards (prostaglandin B₂, leukotriene B₄, leukotriene C₄, leukotriene D₄, leukotriene E₄, 5-HETE, and 19-OH prostaglandin B₂) from Cayman (Ann Arbor, MI), Cascade Biochem (Ann Arbor, MI), or Biomol (Plymouth Meeting, PA).

3. Methods

3.1. Mobile Phases (see Table 2)

3.2. HPLC System Program (see Table 3)

Suitable for the HPLC system illustrated in Table 3.

3.3. The HPLC System

- 1 The scheme of the HPLC system used for automated on-line extraction and profiling of lipoxygenase products is shown in Fig. 1.
2. Biological samples and HPLC standards are prepared as follow To 1 mL of cell suspension (containing no serum or plasma; see Note 9 for more complex samples), add 0.5 mL of a mixture of methanol:acetonitrile (1:1, v:v) containing 12.5 ng each of 19-OH-PGB₂ and PGB₂ as internal standards
3. Complete the volume to 2 mL with methanol.
- 4 Store at -20°C overnight
5. Centrifuge at 1000g for 10 min to pellet the precipitated material
6. Pour out the supernatants into autosampler vials (4-mL vials are used with WISP autosampler)
- 7 Prepare a mixture of HPLC standards containing 12.5 ng each of the following products. 19-OH-PGB₂, 20-OH-LTB₄, PGB₂, LTB₄, LTC₄, LTD₄, LTE₄, and 50 ng 15-HETE per mL of methanol:water (1:1) Add 1 mL methanol:water (1:1) to 1 mL of the mixture of HPLC standards, and pour the diluted mixture into a 4-mL vial. Figure 2 shows a chromatogram illustrating the separation of the various lipoxygenase product standards

4. Notes

- 1 The HPLC methodology described offers several *advantages* for analysis of lipoxygenase products. In contrast to EIA or RIA methods that measure a single compound at a time, HPLC allows the profiling of several products present in a biological sample, such as HETEs, various LTs (LTB₄ and cysteinyl-LTs), metabolites of LTB₄ (and cysteinyl-LTs), lipoxins, and even the COX product 12-hydroxy-heptadecatrienoic acid, thus providing more information on the AA metabolite content of a given sample. The on-line extraction not only eliminates time-consuming procedures, but most importantly, minimizes sample losses and

Table 2
Composition of Solvent Mixtures

	Volume, %			
	A	B	C	D
Water	54	5	20	100
Methanol	23	63	80	
Acetonitrile	23	32		
H ₃ PO ₄	0.01	0.01	0.06	0.05
DMSO ^a	0.011		0.003 ^b	

^aDMSO, dimethylsulfoxide.

^bThe pH of solvent mixture C is adjusted to 4.5 (apparent pH) using ammonium hydroxide (2 N).

Table 3
HPLC System

Time	Solvent Mixtures, ^a %			External events
	A	B	C	
0	100	0	0	Pump 3 ON, 1.5 mL/min (solvent A) Pump 4 ON, 3 mL/min (solvent D) Valve 1 in on-line extraction mode Valve 2 in re-equilibration mode
2 min 45 s				Pumps 3 and 4 OFF
2 min 50 s				Valve 1 in elution mode
3 min 00 s				Valve 2 in elution mode
3 min 30 s				Autozero of the UV photometers
5 min 00 s	100	0	0	
5 min 05 s	85	15	0	
7 min 05 s	85	15	0	
7 min 35 s	55	45	0	
10 min 05 s	55	45	0	
12 min 35 s	30	70	0	
15 min 05 s	30	70	0	
15 min 25 s	0	100	0	
22 min 25 s	0	100	0	
22 min 30 s	0	0	100	SSV in position 2
30 min 00 s				Valve 2 in reequilibration mode
30 min 05 s				Valve 1 in on-line extraction mode
36 min 00 s				Pump 4 ON, 3 mL/min, solvent D
37 min 30 s	100	0	0	SSV in position 1
42 min 30 s				Pump 4 OFF
45 min 00 s	100	0	0	Injection of the next sample

^aPump 1 and/or pump 2 deliver the indicated mixtures of solvents A, B, and C at 1.5 mL/min.

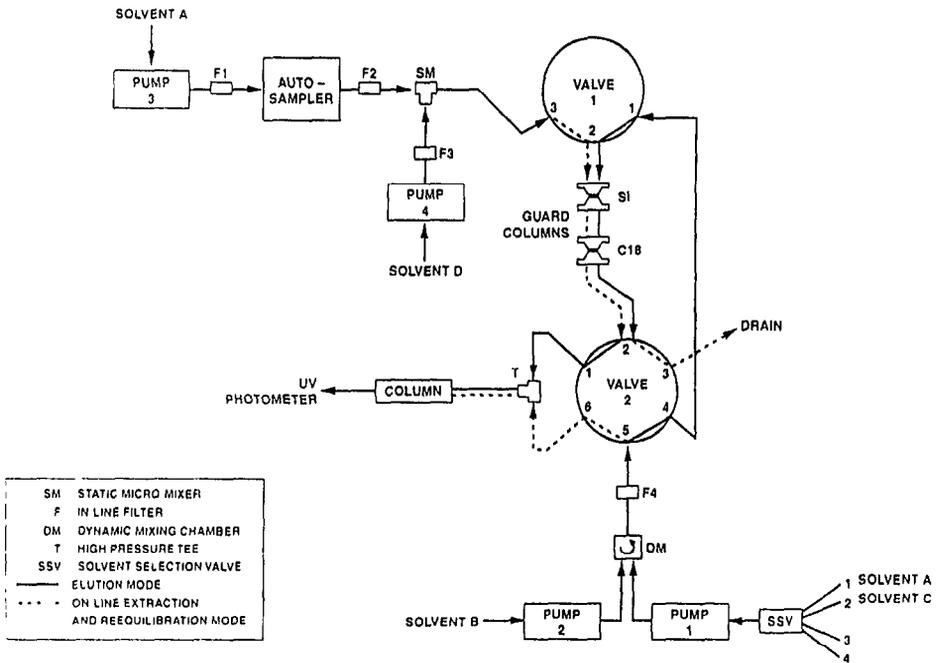


Fig. 1. Scheme of the HPLC system used for automated on-line extraction and profiling of lipoxygenase products

deterioration; the presence of internal standards allows correction for variation in amounts of sample injected. The method provides elution of cysteinyl-LTs with excellent peak shape and complete separation from other eicosanoids (and most contaminants) and thus allows high-sensitivity measurement of these compounds. Finally, the UV monitoring of the solutes at two wavelengths (229–235 nm and 270–280 nm) ensures a high level of selectivity, especially for compounds carrying a conjugated triene or tetraene (leukotrienes and lipoxins). Indeed, these chromophores provide high absorption coefficient at 270–280 nm, whereas absorbance at 229–235 nm is weak.

- The limitation of the HPLC method is at the level of the sensitivity; the method is suitable for samples containing a minimum of 1–5 ng of compounds containing a conjugated triene (or tetraene) and 5–25 ng for compounds containing a conjugated diene, such as HETEs. The limit of detection will vary according to the performance of the column and of the UV photometers; the limit of detection may also be higher in analysis of samples such as tissue homogenates or media containing serum or plasma, and so on, which often yields chromatograms with noisy baseline (numerous contaminants). For analysis of such samples, it is especially important to monitor elution at both wavelengths in order to assess the identity of solutes.

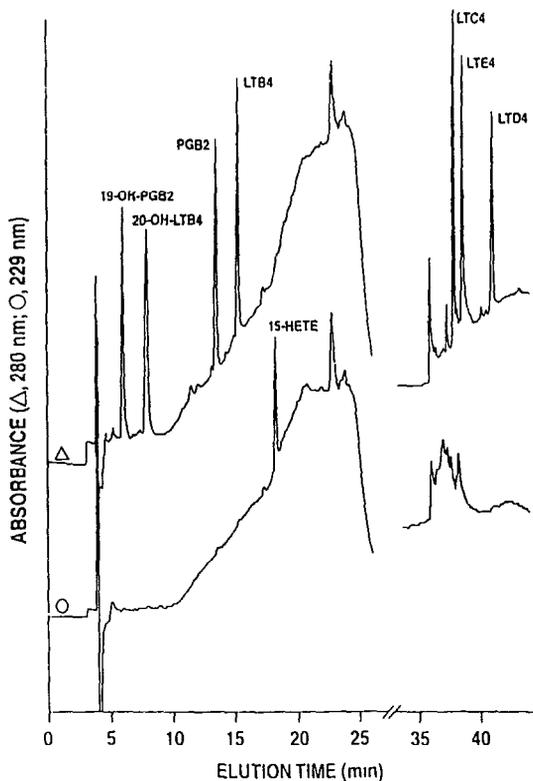


Fig. 2. HPLC chromatogram of various lipoxygenase product standards (12.5 ng each, except 15-HETE, 50 ng) Attenuation settings of the UV photometers were 0.2 AU (absorbance units) at 229 nm, and 0.03 and 0.05 (before and after 35 min of elution time, respectively) AU at 280 nm

3. The Waters Resolve C₁₈ packing is a non-end-capped packing. The selection of this column is required for the separation of cysteinyl-LTs (leukotrienes C₄, D₄, and E₄) as described herein. An ionic interaction between the free silanols in the C₁₈ silica supports and the amino groups of the cysteinyl-LTs enables the retention and separation of these compounds. If analysis of cysteinyl-LTs is not required, the Beckman Ultrasphere ODS (C18) column (or other columns containing end-capped C18 packing) may be used.
4. It is imperative that the mobile phases (i.e., water, methanol, and acetonitrile used in the preparation of solvents) be of the highest purity. H₃PO₄, 85% aqueous solution, and DMSO must be HPLC grade. The composition of solvent mixtures does not take into account the small volumes of DMSO, H₃PO₄, and NH₄OH added. Prepare 4 L of each solvent mixture. Solvents A and B are filtered through a 1 × 10-cm steel column packed with octylsilyl silica particles (37–45 μm). The column is washed with 100 mL methanol and 30 mL acetonitrile:water (75:25)

The first 100 mL of solvent mixture filtered is discarded. The column is washed between each 4-L batch

5. Trace amounts of DMSO have no effect on the separation of the various compounds. DMSO has a high UV cutoff and is added to solvents A and C to counteract the effect of the increasing concentration of organic solvent on the UV absorbance (baseline drift) during the gradient elution. The volume of DMSO added to solvents A and C may be modified as required to control the baseline drift. When solvent mixture C reaches the column, a solvent front is observed on the chromatogram. LTC₄ may elute too close from this front. If it is the case, increase the water content in the solvent mixture C by 5 or 10%. If analysis of cysteinyl-LTs is not required, the running time of the solvent mixture C may be decreased by 50%; this will still allow an adequate washing of the Guard-Pak inserts (precolumns) and column and will shorten the run time per sample.
6. The HPLC system described (**Fig. 1**) includes a high-pressure valve (valve 1) that protects the autosampler from the system back pressure. Depending of the type of autosampler used, valve 1 may or may not be necessary. For example, the Waters WISP 710 or 712 autosamplers can stand the system back pressure and valve 1 is not necessary. With the Waters WISP 715 or 717 autosamplers, the high-pressure valve is necessary.
7. On-line sample dilution (via pump 4 and the static micromixer; see **Fig. 2**) is a useful feature of the system described; it allows direct injection of large volume samples (up to 4 mL with appropriate modification of the sample loop in the autosampler) containing up to 75% organic solvent directly onto the C18 precolumn for sample concentration and extraction. The elevated organic solvent content of the samples (50–75%) prevents adsorption of lipophilic solutes to vials and inner parts of the injection system.
8. PTFE inserts (TTS-313 from Chromatography Sciences) used with the 4-mL vials of a WISP autosampler allow an almost complete aspiration of samples. Without inserts, more than 0.5 mL of samples cannot be aspirated from the vials.
9. For the preparation of samples containing serum or plasma, add 0.5 vol of the organic solvent mixture containing the two internal standards (see **Subheading 3.3., step 2**) to the samples plus 10 vol of acetonitrile and store overnight at –20°C to allow precipitation of the proteins. Centrifuge to eliminate the precipitate. Then concentrate the supernatants with a stream of nitrogen (or other evaporation system) to a final ratio of approx 50:50 organic solvent:water in the samples; it is sometimes necessary to centrifuge the samples again to remove a fine precipitate prior to HPLC analysis. The same procedures can be applied for the preparation of samples such as tissue homogenates, which also contain large amounts of proteins. Denaturation of plasma samples or tissue homogenates with only one or two volumes of organic solvent results in rapid loss of the HPLC column efficiency (and clogging). Various problems specific to HPLC assay of lipoxygenase products in plasma have been addressed previously by Surette et al. (*1*).
10. For the quantitation of lipoxygenase products, the peak heights (or areas) are proportional to the mass of compounds injected. Quantitation is achieved by com-

parison of the peak heights (or areas) of lipoxygenase products in samples with those obtained using a mixture of calibrated standards (*see* Borgeat et al. [2] for more details on quantitation procedures). PGB₂ used as HPLC standard enables corrections for variation in sample recovery, injection volume, and so on. 19-OH-PGB₂ is a relatively polar compound that elutes before the LTB₄ metabolites and lipoxins. Its presence on the chromatograms attests of the efficiency of the on-line extraction.

Acknowledgments

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Measurement of Sphingomyelin and Ceramide Cellular Levels After Sphingomyelinase-Mediated Sphingomyelin Hydrolysis

Pino Santana, Luisa F. Fanjul, and C. M. Ruiz de Galarreta

1. Introduction

Sphingomyelin (SM) is the latest addition to the family of membrane phospholipids that is a source of molecules behaving as intracellular messengers (1). Ceramide is one of the products of sphingomyelin hydrolysis by specific sphingomyelinases (SMases) and appears to be involved in the signaling of apoptosis initiated by regulatory molecules that include cytokines, interferon- γ (IFN- γ), and chemotherapy drugs, as well as UV and ionizing radiations (2-4).

The existence of SMase-dependent sphingomyelin hydrolysis may be easily assessed because, as illustrated in the Fig. 1, serine, fatty acids, and phosphocholine (P-choline) are successively incorporated into the SM biosynthetic pathway, and therefore SM and its products of hydrolysis may be successfully labeled using any of the above molecules. Although the ceramide moiety may also be labeled using serine or a fatty acid (i.e., palmitic), the choice of serine is grounded on the higher specificity that is obtained, since only phosphatidylserine is labeled in addition to SM and ceramide when using [^3H]serine, in contrast to the wide array of other membrane lipids that are labeled with fatty acids. In addition the incorporation of [^{14}C] choline labels the polar moiety, and water-soluble metabolites of choline can be separated and quantified, thus allowing the confirmation of the existence of a SMase-dependent breakdown of sphingomyelin.

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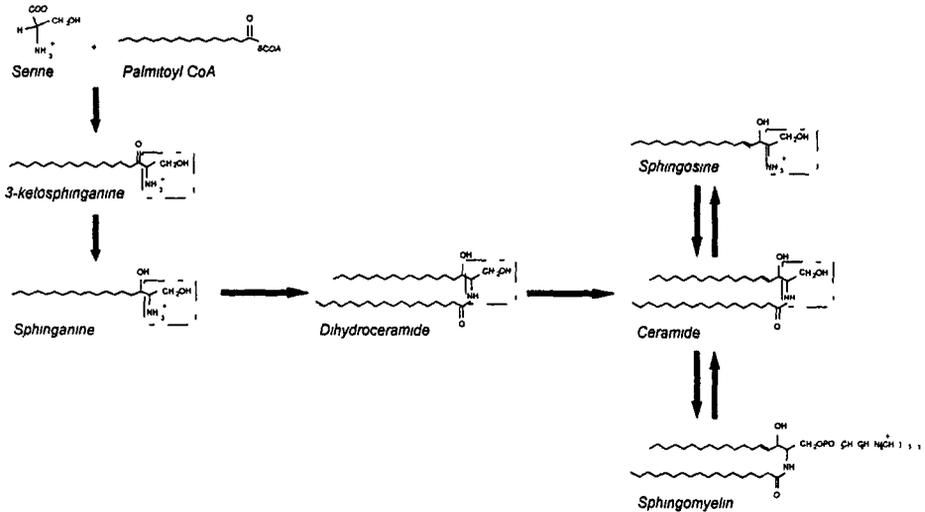


Fig 1. Synthesis of sphingomyelin

2. Materials

2.1. Quantitation of Sphingomyelin and Its Products of Hydrolysis Labeled with [^3H]Serine and [^{14}C -Methyl]Choline

2.1.1. Labeling and Extraction

1. [^3H]Serine (22 Ci/mmol)
2. [^{14}C -Methyl]choline chloride (53 Ci/mmol)
3. Serine and choline (tissue-culture grade, Gibco-BRL, Gaithersburg, MD, or similar)
4. Chloroform (analytical grade).
5. Methanol (99.8% analytical grade).
6. 0.1 N HCl.
7. 0.01 N HCl
8. 18 × 120-mm Glass tubes

2.1.2. Alkaline Hydrolysis

1. 0.1 N KOH in 100% methanol.
2. Chloroform (analytical grade)
3. Balanced salt solution (BSS): 135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl_2 , 0.5 mM MgCl_2 , 5.6 mM glucose, 10 mM HEPES, pH 7.2.
4. 100 mM EDTA sodium salt, pH 7.5.
5. 12 × 75-mm Glass tubes.

2.1.3. Separation and Quantitation of SM and Ceramide

1. TLC plates: 60 Å silica gel, 0.25-mm layer thickness, glass backing (Merck, Darmstadt, Germany, keiselgel 60, Whatman, Clifton, NJ, 4865821 or similar).
2. Sphingomyelin standard (Sigma, St. Louis, MO, cat. no. 7004).
3. Chloroform.benzene.ethanol (thin-layer chromatography [TLC] grade; 80:40:75, v/v)
4. Chloroform.methanol:NH₄OH 28% (TLC grade; 65:25:5, v/v)
5. Vials and scintillation cocktail

2.1.4 Separation and Quantitation of Water Soluble Metabolites

1. TLC plates: 60 Å silica gel, 0.25-mm layer thickness, glass backing (Merck keiselgel 60, Whatman 4865821 or similar)
2. Ethanol 50% (analytical grade).
3. Methanol:NaCl 0.5%.NH₄OH (100:100:4, v/v).
4. Vials and scintillation cocktail.

3. Methods

3.1. Quantitation of Sphingomyelin and Metabolites Labeled with [³H]Serine and [¹⁴C-Methyl]Choline

3.1.1. Labeling and Extraction

1. Label cells (1–2 × 10⁶) for 24/48 h with 1–2 μCi/mL of [³H]serine and/or [¹⁴C-methyl]choline (*see Notes 1–3*).
2. Wash the cells twice with warm, serum-free medium and equilibrate for 30 min in medium supplemented with 25 mM serine and/or 25 mM choline
3. Perform the treatments and stop the experiment by aspirating the medium, add 1 mL ice-cold methanol, and keep 15 min at –4°C.
4. Scrape the cells and transfer to 18 × 120-mm glass tubes. Rinse the wells with an additional 1 mL methanol to maximize recovery
5. Add 4 mL chloroform and 0.5 mL 0.1 N to all the tubes, vortex, and centrifuge for 10 min at 800g
6. If the cells were labeled only with [³H]serine, discard the upper phase, wash the organic phase with 3 × 2 mL 0.01 N HCl, and dry the lipids under nitrogen (*see Note 4*)
7. If the cells were labeled with both [³H]serine and [¹⁴C-methyl]choline, pool the the upper methanolic phases and speed vac evaporate to measure water-soluble metabolites (*see Note 4*)

3.1.2. Alkaline Hydrolysis

Alkaline hydrolysis is performed to remove phosphatidylserine and phosphatidylcholine that are labeled with [³H]serine and [¹⁴C-methyl]choline, respectively, and may interfere with SM separation by TLC.

1. Add 2 mL of 0.1 N KOH in methanol to the lipid film in each tube and incubate at 37°C for 1 h. Vortex the tubes and cover with parafilm.
2. Extract the sphingolipids with 2 mL chloroform and 1.2 mL BSS/EDTA 100 mM (1.08 mL/0.12 mL). Vortex and centrifuge at 800g for 5 min (see Note 5).
3. Transfer 1.75 mL of the lower organic phase to 12 × 75-mm glass tubes (it is sometimes beneficial to transfer 1.75 mL in three steps of 0.75, 0.5, and 0.5 mL with drying after each transfer).
4. Add 50 µg of sphingomyelin standard to each tube to act as a carrier and aid visualization of the lipid after TLC. Dry under nitrogen (see Note 4).

3.1.3. Separation and Quantitation of SM and Ceramide

1. Redissolve the lipids with 50 µL chloroform/methanol (1:1) and spot on a TLC plate.
2. Run with solvent described in **Subheading 2.1.3., item 3**, up to 2 cm from the plate end. Dry the plates with a hair dryer.
3. Run the plates as above but with the solvent system described in **Subheading 2.1.3., item 4**.
4. Scrape the plates in 1-cm fractions starting from the origin (SM R_f :0.05, ceramide R_f :0.75), transfer silica to a scintillation vial and count (see Note 6).

3.1.4. Separation and Quantitation of Water-Soluble Metabolites

1. Redissolve the evaporated samples in 100 µL of 50% ethanol and spot on a TLC plate.
2. Run twice to the end of the plate with the solvent system described in **Subheading 2.1.4., item 3**. Phosphocholine (R_f :0.32) will be separated from choline (R_f :0.088) and CDP-choline (R_f :0.5) (see Note 6).
3. Scrape the plates in fractions of 1 cm from the origin and transfer silica to a scintillation vial to count.

4. Notes

1. The time and amount of isotope needed to label the cells may change from cell to cell, but they will usually be within the limits that are provided here.
2. A higher efficiency in the labeling may be achieved if serine- and/or choline-free media are used. Also, labeling in serum-free conditions (when possible) will increase labeling.
3. The cells may be labeled separately or simultaneously with the two compounds.
4. The lipids and water-soluble metabolite extracts may be stored at this point at -20°C if desired.
5. BSS/EDTA may be premixed since the solution is stable for 1 mo at 4°C.
6. R_f s may change depending on the environmental humidity.
7. See Fig. 2.

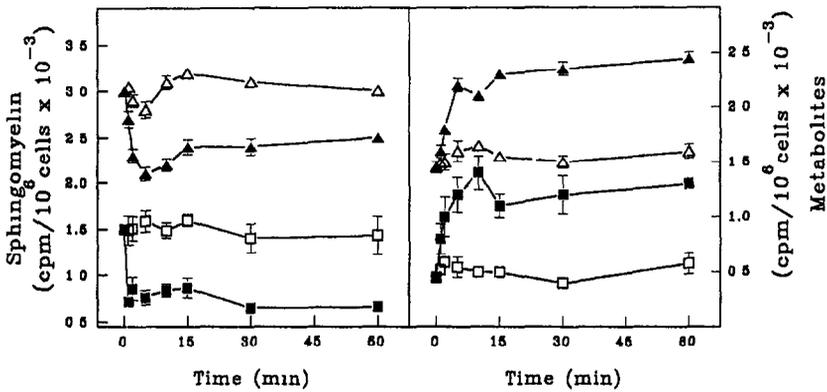


Fig. 2. Spingomyelin (left panel) and ceramide and choline (right panel) levels in HL-60 cells labeled with [³H]serine (□—□ and ■—■) or [¹⁴C]methyl choline (△—△ and ▲—▲), as described above and without (open symbols) or with the addition of bacterial spingomyelinase (Sigma cat. no S8633; 0.3 U/mL; solid symbols)

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Sphingomyelin and Ceramide Mass Assay

Pino Santana, C. M. Ruiz de Galarreta, and Luisa F. Fanjul

1. Introduction

As described in Chapter 19, a demonstration of changes in sphingomyelin (SM) and ceramide cellular contents (in response to regulatory molecules and/or other stimuli) is essential to establish their involvement in the signaling process. The study of variations in SM and ceramide cellular levels, resulting from SMase-mediated SM hydrolysis, may be performed using the method described in Chapter 19. However, increased levels of ceramide and/or SM may occur under circumstances in which the isotopic labeling method would be inefficient in evaluating the changes (i.e., increased rate of synthesis), or simply impossible to use (i.e., when working with whole tissues or organs). The measurement of sphingomyelin and ceramide mass provides the best solution to these problems.

To quantitate SM mass, a method that evaluates SM phosphate content against a standard curve of disodium phosphate may be used (1). Because ceramide is almost as good a substrate for DAG kinase as diacylglycerol itself, ceramide mass may be measured with few changes by the DAG-kinase method (2), as it is described in Chapter 7. A method adapted and modified from an amino acid analysis procedure (3,4) is described below as a nonradioactive alternative to the DAG-kinase method (5).

The method takes advantage of the low basal levels of sphingosine existent in the cells and tissues, and the fact that ceramide can be deacylated to generate the free amino group containing sphingosine. Sphingosine can, in turn, be derivatized to form a fluorescent compound, separated by HPLC from the other sphingoid bases, and quantitated by fluorescence detection. Basal tissue or cel-

ular levels of sphingosine are also measured in each sample and thereafter subtracted from the total sphingosine accumulated as a consequence of ceramide deacylation, to obtain the tissue/cell content in ceramide.

2. Materials

2.1. Sphingomyelin Content by Phospholipid Phosphorus Assay

2.1.1. Lipid Extraction

- 1 Kill solution: chloroform methanol:1 *N* HCl (100:100:1 v/v), analytical grade
2. Balanced salt solution (BBS): 135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, and 10 mM HEPES, pH 7.2.
- 3 100 mM EDTA sodium salt, pH 7.5
4. 50-mL Polypropylene tubes
5. 16 × 100 mm Glass tubes.

2.1.2. Alkaline Hydrolysis

1. Sphingomyelin standard (Sigma, St. Louis, MO, cat. no. 7004)
- 2 0.1 *N* KOH in 100% methanol (99.8%), analytical grade
3. Chloroform, analytical grade.
- 4 Balanced salt solution (BSS). 135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, and 10 mM HEPES, pH 7.2
5. 100 mM EDTA sodium salt, pH 7.5.
6. 12 × 75 mm Glass tubes

2.1.3. Sphingomyelin Separation and Extraction

1. CHCl₃:MeOH(1:1, v/v), analytical grade
2. TLC plates 60 Å silica gel, 0.25-mm thickness, glass backing (Whatman, Clifton, NJ, cat. no 4865821, or similar characteristics).
3. Chloroform:methanol:glacial acetic acid:water (50:30:8:4, v/v), chromatography grade.
4. Iodine crystals.
5. 16 × 100-mm Glass tubes.
6. Chloroform:methanol:concentrated HCl (20:10:0.1, v/v).
7. 12 × 75-mm Glass tubes.

2.1.4. Phosphorus Determination

1. 74.7 mg of Na₂HPO₄ in 50 mL of water.
2. Isopropanol, analytical grade
3. 70% Perchloric acid.
4. Color reagent: Mix 1 vol of 6 *N* H₂SO₄ (4.15 mL concentrated H₂SO₄ in 20.9 mL H₂O) with 1 vol of 2.5% (NH₄)₂MoO₄ (0.125 g in 5 mL H₂O), 4 vol of 2.5% ascorbic acid (0.5 g in 20 mL H₂O), and 4 vol distilled H₂O (*see Note 1*).

2.2. Cell and Tissue Ceramide Content by HPLC

2.2.1. Cell Lipid Extraction

1. Kill solution: chloroform:methanol:1 *N* HCl (100:100:1 v/v/v).
2. Balanced salt solution (BSS): 135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, and 10 mM HEPES, pH 7.2
3. 100 mM EDTA sodium salt, pH 7.5
4. 12 × 75-mm Glass tubes.

2.2.2. Tissue Lipid Extraction

1. Polytron tissue homogenizer
2. 16 × 100-mm Glass tubes.
3. Phosphate-buffered saline (PBS). 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ · 7H₂O, 1.4 mM KH₂PO₄, pH 7.3.
4. Chloroform:methanol (2:1, v/v), chromatography grade.

2.2.3. Alkaline Hydrolysis

1. 0.1 *N* KOH in 100% methanol.
2. Lipid standards: Sphingosine (Sigma cat. no. S 6879), Ceramide (Type III, from bovine brain, Sigma cat. no. C 2137).
3. Chloroform, analytical grade
4. Balanced salt solution (BSS). 135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, and 10 mM HEPES, pH 7.2.
5. 100 mM EDTA sodium salt, pH 7.5.

2.2.4. Deacylation with 1 *N* KOH

1. 1 *N* KOH in 100% methanol.
2. 1 *N* HCl in 100% methanol
3. Chloroform, analytical grade.
4. 1 *M* NaCl

2.2.5. Derivatization with *o*-Phthalaldehyde (OPA)

1. OPA reagent: Mix 99 mL 3% boric acid in water (adjust pH to 10.5 with KOH) and 1 mL ethanol containing 50 mg of OPA (*o*-phthalaldehyde, Sigma cat. no. P 0657) and 50 μL of 2-mercaptoethanol (see Note 2).
2. Methanol (HPLC grade).
3. 5 mM K₂HPO₄ (to pH 7.0 using KOH)
4. HPLC vials.

2.2.6. HPLC Separation and Quantitation

1. Methanol (HPLC grade).
2. Nova Pack C18 column (pore size 4 μm, 3.9 × 150 mm, Waters).
3. Fluorimetric detector (excitation wavelength 340 nm and emission wavelength 455 nm).

3. Methods

3.1. Sphingomyelin Content by Phospholipid Phosphorus Assay

3.1.1. Lipid Extraction

- 1 Add 10 mL kill solution and 3 mL BSS/EDTA 100 mM (2.7 mL/0.3 mL) to 30×10^6 cells, to extract the lipids, vortex and centrifuge 800g, 10 min. This can be done in a 50-mL polypropylene tube
- 2 Transfer 90% of lower organic phase to 16 \times 100-mm glass tubes (i.e., for an extraction with 10 mL of kill, transfer 4.5 mL), and dry down under nitrogen (*see Note 3*).

3.1.2. Alkaline Hydrolysis

- 1 Make up two 30- μ g sphingomyelin standards (ST₁), dissolved in chloroform:methanol (1:1, v:v) to be used in the calculation of the sphingolipid recovery at the end of the experiment Dry down
2. Add 2 mL of 0.1 N KOH in methanol to the lipid film in each standard and samples tubes and incubate at 37°C for 1 h Vortex the tubes and cover with parafilm
3. Extract the sphingolipids with 2 mL Chloroform and 1.2 mL BSS/EDTA 100 mM (1.08 mL/0.12 mL) (*see Note 4*)
4. Vortex and centrifuge 800g for 5 min.
5. Transfer 1.75 mL of the lower organic phase to 12 \times 75-mm glass tubes (it is sometimes beneficial to transfer 1.75 mL in three steps of 0.75, 0.5, and 0.5 mL with drying after each transfer).
6. Dry down under nitrogen.
7. Re-extract the lipid again with 0.5 mL chloroform, 0.5 mL methanol, and 0.3 mL dH₂O. Vortex and centrifuge 800g for 5 min Transfer 0.5 mL of the lower phase to clean glass 12 \times 75-mm glass tubes (*see Note 5*).
8. Dry down under nitrogen (*see Note 3*)

3.1.3. Sphingomyelin Separation and Extraction

1. Resuspend the lipids in 50 μ L of chloroform:methanol (1:1 v/v) and load 40 μ L on a TLC plate.
2. Develop the plate with the solvent system described in **Subheading 2.1.3., item 3**, for 16 cm.
3. Allow the plate to dry and visualize the lipids by iodine staining, mark the SM spots (R_f 0.37), and remove the iodine with a hair dryer (*see Note 6*).
4. Scrape the sphingomyelin from the plate and transfer the silica into 16 \times 100-mm glass tubes. Also scrape two comparable areas of the plate that do not contain any lipids to be used as zero for the calculations
5. Wash the silica 3 \times 500 μ L of the solvent described in **Subheading 2.1.3., item 6** Pool the washes into 12 \times 75-mm glass tubes. Dry the tubes under nitrogen (*see Note 3*)

3.1.4. Phosphorus Determination

1. Prepare two tubes containing the same amount of sphingomyelin as done in **Subheading 3.2.1., step 2** (ST₂), and dry them down. The ratio ST₂/ST₁ allows an estimation of the losses occurring in the procedure.
2. Make up phosphorus standard curve by diluting 74.7 mg of Na₂PO₄ in 50 mL of water, make serial dilutions to yield the following standard tubes: 25, 10, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, and 0 µg phosphorus (PO₄ equivalent) in 25 µL. Add 100 µL isopropanol and dry these tubes down prior to use.
3. To all tubes, add 50 µL 70% perchloric acid. Cap the tubes with glass marbles and heat at 180°C on a heating block for 30 min (*see Note 7*).
4. Centrifuge to pull down any condensation on the walls and then vortex
5. Add 1 mL color reagent described in **Subheading 2.1.4., item 4**, to all the tubes, cover the tubes with parafilm, and heat at 50°C in water bath for 1 h
6. Vortex and centrifuge again and transfer 0.8 mL to fresh tubes, carefully avoiding the silica gel fines.
7. Read standard curve and samples absorbance at 760 nm, and calculate the estimate PO₄ equivalent of the samples against standard curve

3.1.5. Calculations

$$\text{Total SM (pmol)} = [(\mu\text{g } PO_4 \text{ EE}) - \text{zero value}] \times \text{recovery} \times 10530 \text{ pmol SM}$$

where µg PO₄ EE = estimated phosphate equivalent (calculated against the absorbances of the standard curve); zero value = µg PO₄ EE of the blank silica; recovery = [(µg PO₄ EE for the two SM standards put in at **Subheading 3.1.4., step 2**)/(µg PO₄ EE for the two SM standards put in at **Subheading 3.1.1., step 2** - zero value)]; and 10530 pmol SM = picomol of SM that contain 1 µg PO₄.

3.2. Cell and Tissue Ceramide Content by HPLC

Although the method is specially useful to evaluate tissue levels of ceramide, it may equally be used to measure ceramide cellular content. The differences in lipid extraction and calculations for cell and tissue are therefore described.

3.2.1. Cell Lipid Extraction

1. Add 2 mL kill solution and 0.6 mL BSS/EDTA (540 µL/60 µL) to 6 × 10⁶ cells, vortex and centrifuge the tubes.
2. Transfer 90% (500 µL) of the lower organic phase to two different sets of 12 × 75-mm glass tubes, and dry under nitrogen (*see Note 3*).

3.2.2. Tissue Lipid Extraction

1. Weigh the tissue and homogenize in 8 vol (w/v) of ice-cold PBS with a polytron tissue homogenizer.
2. Transfer the homogenate (0.4 mL) to 16 × 100-mm glass tubes.

3. Add 3 mL chloroform:methanol (2:1 v/v) to extract the lipids. Vortex and centrifuge at 800g for 10 min.
- 4 Transfer 250- μ L aliquots of the organic phase in duplicate to two different sets of 13 \times 100-mm glass tubes and dry down under a nitrogen stream.
5. One set of tubes marked "basal" will be used after mild alkaline hydrolysis to evaluate tissue basal content of sphingosine.
- 6 The other set marked "total" will be deacylated to convert ceramide to sphingosine (*see Note 3*).

3.2.3. Alkaline Hydrolysis

- 1 Make up a sphingosine (SP₁) standard curve (1000, 500, 100, and 50 pmol), dissolved in chloroform:methanol (1:1, v/v) in duplicate, and dry under nitrogen
- 2 Add 500 μ L of 0.1 N KOH in methanol to the lipid film of each standard or sample tube and incubate at 37°C for 1 h. Vortex the tubes and cover with parafilm
3. Add 500 μ L CHCl₃, 270 μ L BSS, and 30 μ L 100 mM EDTA, to all tubes to extract the sphingolipids. Vortex and centrifuge 800g for 5 min.
4. Transfer 500 μ L lower phase to new glass tubes.
5. Dry down under nitrogen (*see Notes 3 and 8*)

3.2.4. Deacylation with 1 M KOH

- 1 Make up standard of sphingosine (SP₂) and ceramide (1000, 500, 100, and 50 pmol), dissolved in chloroform:methanol (1:1, v/v) to calculate the recovery of the deacylation procedure and dry under nitrogen (*see Note 9*).
2. Add 500 μ L of 1 M KOH in methanol to the lipid film of each standard and sample tube. Incubate 1–1.5 h at 100°C in a heat block (*see Note 7*).
3. Allow the tubes to cool down and neutralize with 500 μ L of 1 N HCl in methanol
- 4 Extract the sphingoid base adding 1 mL of chloroform and 900 μ L of 1 M NaCl.
5. Vortex, centrifuge and remove the upper phase
7. Dry down under a nitrogen stream the lower phase (*see Note 3*).

3.2.5. Derivatization with o-Phthalaldehyde (OPA)

1. Redissolve the sphingoid base in 50 μ L methanol.
- 2 Mix with 50 μ L OPA reagent described in **Subheading 2.2.4., item 1**.
3. Incubate for at least 5 min at room temperature.
4. Add 500 μ L methanol:5 mM potassium phosphate, pH 7.0 (90:10, v/v)
- 5 Centrifuge for 30 s in a microcentrifuge to clarify.
- 6 Transfer 500 μ L to HPLC vials.

3.2.6. HPLC Separation and Quantitation

- 1 Use the Nova Pack C18 column.
2. Make the injection with a loop size of 20 μ L.

3. Elute isocratically with methanol:5 mM potassium phosphate, pH 7.0 (90:10, v.v) at a flow rate of 0.6 mL/min.
4. Adjust the fluorescence detector to 340 nm excitation and 455 nm emission to quantitate. A profile of the retention time of sphingosine is shown in **Note 10**

3.2.7. Calculations for Ceramide Tissue Content

$$\text{pmol ceramide/g tissue} = [(\text{pmol total} \times \text{recovery1} \times \text{recovery2}) - \text{pmol basal}] \times 4800$$

where *pmol total* = picomol in the total sample (deacylated) tubes read against the standard curve made in **Subheading 3.2.3, step 1**; *recovery1* = mean value of all points (50, 100, 500, and 1000 pmol) calculated as follows: peak area of each point in SM standard curve/peak area of each point of SP₂; *recovery 2* = mean value of all points (50, 100, 500, and 1000 pmol) calculated as follows: peak area of each point of SP₂ standard curve/peak area of each point of SP₁; *pmol basal* = picomol in the basal sample (alkaline hydrolysis) tubes read against the standard curve made in **Subheading 3.1.3., step 1**; and 4800 (30 × 8 × 20) = pmol contained in 20 μL injected × 30 = pmol contained in the 250-μL aliquots of the organic phase after lipid extraction × 8 = pmol in 2 mL chloroform or 0.4 mL homogenate × 20 = pmol in the total 8 vol (8 mL), where 1 g of tissue would have been homogenized.

3.2.8. Calculations for Ceramide Cellular Content

$$\text{pmol ceramide}/10^6 \text{ cells} = [(\text{pmol total} \times \text{recovery1} \times \text{recovery2}) - \text{pmol basal}] \times 1/ \text{millions cells}$$

4. Notes

1. Ascorbic acid has shelf life of 30 d when stored at 4°C.
2. OPA reagent should be prepared fresh daily.
3. At this point, lipids may be stored at -20°C for several days if desired.
4. BSS/EDTA may be premixed and stored for 1 mo at 4°C.
5. Re-extraction is recommended, because the extraction with BSS generates salt and water contamination of the lipid extract, which may, in turn, produce changes in SM mobility on the TLC plates.
6. *R_fs* may experience large changes depending on the room humidity.
7. This procedure as well as deacylation should be performed in a fume hood.
8. Alkaline hydrolysis is performed to remove glycerophospholipids, particularly those containing a free amino group (phosphatidylserine or phosphatidylethanolamine) that might be derivatized, and interfere during the HPLC separation and quantitation of the sphingoid base.
9. Although the efficiency of ceramide deacylation is usually close to 100%, variations may occur from experiment to experiment. Because of this, the use of the

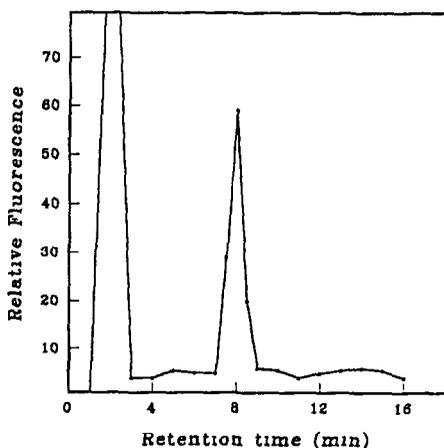


Fig. 1. Profile of OPA derivatized sphingosine. OPA fully eluted after 3 min. OPA derivatized sphingosine is usually eluted at approx 8 min retention time.

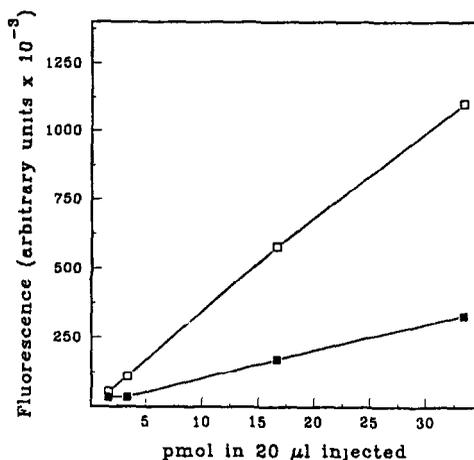


Fig. 2. Fluorescence values (arbitrary units) of the OPA derivatized sphingosine (\square — \square) and deacylated ceramide (\blacksquare — \blacksquare) standard curves.

two different standard curves (sphingosine and ceramide) to calculate the efficiency of the deacylation (ceramide curve) and the extraction procedures (sphingosine curve) is recommended.

10 See Fig. 1.

11. See Fig. 2.

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Sphingosine Kinase

Assay and Product Analysis

Ana Olivera and Sarah Spiegel

1. Introduction

The phosphorylation of long chain sphingoid bases on the primary alcohol group occurs in cells by the action of sphingosine kinase (1–3). Sphingosine kinase is present in the cytosolic fraction of most cells (4–7) and in the membrane fraction of certain tissues and organisms (8,9). The reaction product, sphingosine-1-phosphate (SPP), was considered for more than 20 yr to be merely an intermediate in the catabolism of long-chain sphingoid bases to palmitaldehyde and phosphoethanolamine (3,10). Studies in our lab stimulated new interest in the potential roles of SPP as a second messenger. Initially, we found that exogenous SPP initiated cell division of quiescent Swiss 3T3 fibroblasts (11) and induced inositol trisphosphate-independent release of calcium from intracellular stores (11,12). SPP also has been shown to affect several signal transduction pathways including phospholipase D activation (13), stimulation of the Raf/MEK/ERK signaling pathway (14,15), and inhibition of ceramide-induced activation of stress-activated protein kinase (SAPK/JNK) that leads to apoptotic responses (15). Additional effects of SPP include stimulation of tyrosine phosphorylation of focal adhesion kinase (FAK) and the cytoskeleton-associated protein paxillin (16). These tyrosine phosphorylations are mediated through the activation of the small G protein rho, which also mediates stress fiber formation induced by SPP (16). Most importantly, SPP levels and sphingosine kinase activity can be modulated in cells by external stimuli, including PDGF (17), NGF (18), TPA (15,19), and the B subunit of cholera toxin (20), suggesting that SPP might play an important role as a second messenger in pathways modulated by these growth factors. PDGF-induced

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mitogenesis and cell survival in Swiss 3T3 fibroblasts is mediated, at least in part, by the formation of SPP (17). Similarly, in PC12 cells, activation of sphingosine kinase and generation of SPP is involved in the neurotrophic actions of NGF (18). Moreover, antigen clustering of IgE receptors on mast cells stimulates SPP formation, but not inositol trisphosphate, leading to calcium mobilization (21). Further support for the notion that SPP functions as a second messenger emerged from the use of inhibitors of sphingosine kinase. For example, the competitive inhibitors, dihydrosphingosine and *N,N*-dimethyl sphingosine, not only inhibit cell growth (17) and survival responses (15), but also block MAPK activation, phosphorylation of the SH2/SH3 adaptor protein Crk, and inhibit stimulation of AP-1 DNA-binding activity and cyclin-dependent kinase activation (*cdc2* and *cdk2*) induced by PDGF (22,23).

1.1. Sphingosine Kinase Assay

In view of the importance of SPP in cell growth and survival and the responsiveness of sphingosine kinase to external mediators, we developed a reliable and sensitive method to measure sphingosine-kinase activity. Previously, several types of assays have been used to determine sphingosine-kinase activity utilizing either [³²P]ATP or [³H]sphingosine as a tracer. A major deficiency of these methods has been the separation of the product from the substrate, since SPP is soluble, at least to some extent, in both polar and nonpolar solvents. Complex isolation procedures were developed when [³H]sphingosine was used as substrate (2,5,7), in which either the [³H]SPP product was separated on a Dowex 1 (OH⁻ form) ion exchange column in several large-volume fractions from [³H]sphingosine (7), or separated using TCA precipitation, acetone extraction, basic extraction, saponification, acidification, and finally extraction with chloroform to eliminate most of the contaminating [³H]sphingosine (2). Perhaps the simplest method of separating [³H]sphingosine from [³H]SPP is chloroform:methanol (2:1) extraction in alkaline conditions, in which most of the [³H]SPP has been reported to partition in the aqueous phase (5). However, for samples that do not contain high sphingosine kinase activity, we have found that this assay with [³H]sphingosine is not reliable. Moreover, synthesis of [³H]sphingosine is not a simple task and the commercially available material is very costly.

A quantitative sphingosine kinase assay using [³²P]ATP has been described by Buehrer and Bell (9), in which the product is acylated to form *N*-caproyl-SPP. After alkaline hydrolysis to eliminate glycerophospholipids and excess caproic anhydride, *N*-caproyl-SPP was extracted with a biphasic system and the radiolabeled *N*-caproyl-SPP in the organic phase was resolved by thin-layer chromatography (TLC). We have developed a different method that is simple, sensitive, reproducible, and relatively rapid to measure sphingosine kinase

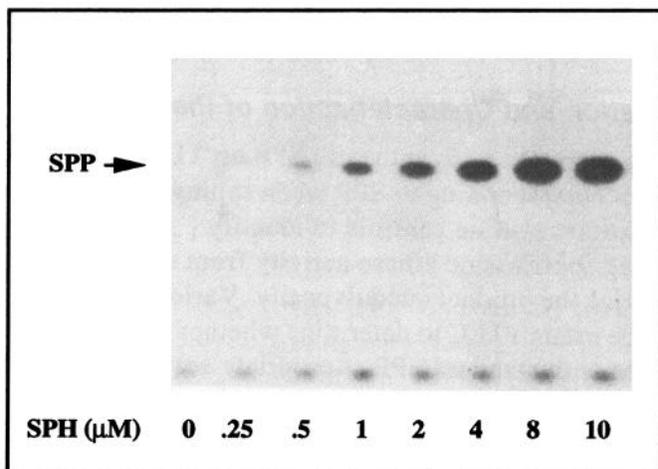


Fig. 1. Autoradiogram from a representative experiment demonstrating increased formation of SPP with increasing amounts of BSA complex added. Arrow indicates the location of standard SPP visualized with ninhydrin spray.

activity using [^{32}P]ATP and sphingosine as substrates. The protocol, described below in detail, includes a description of the preparation of sphingosine kinase from Swiss 3T3 fibroblasts, which can be used for the measurement of sphingosine levels in cells (17). A similar protocol can also be used for preparation of sphingosine kinase from other cell types or tissues. In brief, the samples to be assayed are incubated with sphingosine and [^{32}P]-labeled ATP at 37°C. As specified in the **Subheading 4.**, attention should be paid to the form of delivery of sphingosine and the presence in the reaction mixture of Mg^{2+} , the only ion required for activity. After the incubation period, [^{32}P]-labeled lipids are extracted with a solvent in acidic conditions, in which 70–80% of the labeled SPP partitions in the organic phase, separated from the [^{32}P]ATP. The extracted phospholipids are then separated by thin layer chromatography, visualized by autoradiography, and radioactive spots corresponding to authentic SPP are scraped from the plates and counted in a scintillation counter for quantitation. In some instances, the only radioactive spots detected had the same relative migration as standard SPP (see Fig. 1). In such cases, TLC separation is not necessary.

Determination of sphingosine-kinase activity in crude enzyme preparations by assays described above have demonstrated a high degree of substrate stereospecificity. The naturally occurring D(+)-erythro-isomer of sphingosine is the most favored substrate (9,24,25). The D(+)-threo (9,24) and L(-)-threo forms (9,25) have been found to inhibit sphingosine kinase activity, whereas the

L(-)-erythro isomer has been reported to be either a poor substrate (9) or an inhibitor (24).

1.2. Identification and Characterization of the Product

Although comigration with authentic SPP on TLC and the absence of a radioactive spot corresponding to SPP when sphingosine is not included in assays are important routine controls to identify [^{32}P]-SPP, it is important when measuring sphingosine kinase activity from different sources to initially characterize the product unequivocally. Various solvent system combinations can be used in TLC to determine whether the putative radioactive SPP comigrates with standard SPP. Appropriate solvent systems other than that described below include: chloroform:ethanol:water (65:35:8, v/v) (SPP R_f = 0.31), chloroform:methanol:ammonium hydroxide (13:7:1, v/v) (R_f = 0.0), in which most phospholipids, but not SPP, migrate from the origin; chloroform:methanol:acetic acid (30:30:2:5, v/v) (R_f = 0.35) (11); and chloroform:methanol:ammonium hydroxide (4:1:0.1, v/v).

In addition, the resistance of SPP to alkaline hydrolysis can be used to further identify the product. Thus, the organic phase containing standard SPP together with the radiolabeled lipid is evaporated to near dryness, resuspended in methanolic KOH (0.1 M), and incubated at 37°C for 1 h. After neutralization, lipids are extracted with chloroform/water and the lipids in the organic phase resolved by TLC. SPP can also be cleaved by periodate oxidation followed by borohydride reduction to yield [^{32}P]-ethylene glycol monophosphate, which can then be separated and identified by paper chromatography (6).

2. Materials

2.1. Preparation of Sphingosine Kinase from Swiss 3T3 Fibroblasts

- 1 Culture media for Swiss 3T3 fibroblasts: cells are subcultured at a density of 1.5×10^4 cells/cm² in DMEM supplemented with 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% calf serum
- 2 Phosphate-buffered saline (PBS)
- 3 Ice tray.
- 4 Aspiration system.
- 5 Sphingosine kinase buffer: 20 mM Tris-HCl (pH 7.4) (or 0.1 M potassium phosphate, pH 7.4) containing 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 10 µg/mL leupeptin and aprotinin, 1 mM PMSF, 0.5 mM 4-deoxy pyridoxine, and 40 mM β-glycerophosphate.
- 6 Ice bucket with liquid nitrogen
- 7 Microfuge tubes and floating rack
- 8 Ultracentrifuge and 10-mL ultracentrifuge tubes.

2.2. Substrate Solutions

2.2.1. Sphingosine

- 1 D-erythro-sphingosine: dissolve in ethanol at 50 mM, in a screw-capped glass tube, and store at -70°C . This solution is stable for months
- 2 Bovine serum albumin (BSA), tissue culture grade (4 mg/mL in PBS), or 5% Triton X-100
3. Vortex mixer
4. Bath sonicator.

2.2.2. $[\beta^{32}\text{P}]\text{ATP}/\text{Mg}^{2+}$ mixture

1. ATP: 20 mM, freshly prepared in a solution containing 200 mM MgCl_2 .
- 2 $\gamma[^{32}\text{P}]\text{ATP}$ (10 mCi/mL).

2.3. Reaction Mixture and Incubation

- 1 Ice bucket.
2. Sphingosine kinase buffer (*see* Subheading 2.1.).
3. 15-mL Conical glass centrifuge tubes with screw caps.
- 4 Test tube rack.
5. Vortex mixer
- 6 Water bath at 37°C

2.4. Separation and Analysis of Sphingosine-1-Phosphate

1. 1 N HCl
2. Chloroform:methanol:HCl (100:200:1, v/v).
3. Chloroform
4. 2 N KCl.
5. Vortex mixer.
6. Centrifuge.
7. Aspiration system.
8. TLC glass chamber containing butanol:ethanol:acetic acid:water (80:20:10:20, v/v).
9. TLC silica gel G60 plates (20 \times 20 or 10 \times 20 cm).
10. Ruler and pencil.
11. Standard SPP solution (1 mM in 4 mg/mL BSA)
12. Hamilton syringe or 100- to 200- μL pipet.
13. Hair dryer.
14. Autoradiography film
15. 0.2% (w/v) Ninhydrin spray solution in ethanol
- 16 Silica gel scraper.
17. Scintillation tubes and scintillation fluid.
18. Scintillation counter set to count $[\beta^{32}\text{P}]$

3. Methods

3.1. Preparation of Cell Lysates Containing Sphingosine Kinase

1. Culture Swiss 3T3 fibroblasts to confluency in 100-mm Petri dishes in DMEM containing 10% calf serum, then serum-starve them overnight if the effect of various growth factors on sphingosine kinase is to be investigated.
2. Wash cells twice on ice with 10 mL cold PBS, aspirate, and add 500 μ L of sphingosine kinase buffer
3. Scrape cells, transfer to 1.5-mL tubes on ice, and immediately freeze in liquid nitrogen
4. Disrupt cells by freeze-thawing. This step is performed by successively placing cells in liquid nitrogen, then thawing in 37°C bath. Repeat six times
5. Transfer cell lysates to prechilled ultracentrifuge tubes and spin at 105,000g for 90 min at 4°C.
6. Measure protein concentration of the supernatants, which correspond to cytosolic fractions. Supernatants can be stored at -70°C and sphingosine kinase activity is stable for several months. However, when cells have been treated with different stimuli to determine their effect on sphingosine kinase activity, it is better to perform the assay as soon as possible

3.2. Preparation of Substrates

3.2.1. Preparation of Sphingosine

1. To prepare 1 mM sphingosine complexed with BSA, pipet 1 mL of the BSA solution in a glass tube and vortex while adding 20 μ L of 50 mM sphingosine drop by drop. Vortex for an additional few seconds. Sonicate the solution in a bath sonicator for 1–2 min. This solution may be slightly cloudy, but no particulate matter should be present.
2. To prepare 1 mM sphingosine-Triton X-100 micelles, pipet 1 mL of 5% Triton-X 100 into a glass tube. Add 20 μ L of 50 mM sphingosine, vortex, and sonicate for a few seconds. A clear solution should result
3. Both preparations of sphingosine are stable at -20°C for a few months.

3.2.2. Preparation of Radiolabeled ATP/Mg²⁺ Mixture

1. Calculate the volume of ATP/Mg²⁺ mixture required by multiplying the number of samples times 10 (10 μ L of ATP mixture/sample).
2. Immediately before starting the reaction, mix 9 parts of unlabeled ATP- MgCl₂ and 1 part [γ ³²P]ATP (approx 10 μ Ci/sample), and vortex. Keep on ice

3.3. Reaction Mixture

1. Place glass conical tubes in rack in ice-water tray
2. Add cell extracts. The protein concentration suitable for assays ranges from 40–120 μ g for Swiss 3T3 fibroblast extracts, but this must be determined independently for each type of cell to be analyzed.
3. Add sphingosine kinase buffer to 180 μ L.

4. Add 10 μL of 1 mM sphingosine (the final concentration, 50 μM , is saturating) delivered either as sphingosine-BSA complex or sphingosine-Triton X-100 micelles
- 5 Vortex tubes gently.
6. Start reactions by addition of 10 μL of [$\gamma^{32}\text{P}$]ATP (10 μCi , 20 mM MgCl_2 , 200 mM) and vortex gently.
7. Place rack in water bath and incubate for 30 min at 37°C (linearity with time of incubation must be established for each cell type).
8. Pipet an aliquot (1 μL) of the [$\gamma^{32}\text{P}$]ATP into a scintillation vial and count to determine total radioactivity added

3.4. Separation and Analysis of Sphingosine-1-Phosphate: Quantitation of Sphingosine Kinase Activity

- 1 After the incubation period, place the rack in iced water.
2. Terminate reactions by addition of 20 μL of 1 N HCl followed by 0.8 mL of chloroform:methanol HCl (100:200:1, v/v).
- 3 Vortex vigorously and let stand at room temperature for 5–10 min
4. Add 240 μL of chloroform and 240 μL of 2 N KCl to separate phases.
- 5 Vortex vigorously and let stand for 5–10 min.
- 6 Centrifuge for 5–10 min at 400g.
- 7 Aspirate the aqueous (upper) phase and cap tubes.
8. With a pencil and a ruler, mark the origin on a TLC plate where samples will be applied 2 cm from the bottom of the plate and 0.5 cm apart. Apply standard SPP in lanes at the end of each plate
9. Spot samples of the organic phase (50–100 μL) drop by drop onto the TLC plates, with either a Hamilton syringe or 200- μL pipet with a gel loading tip. For more rapid application of the sample, dry each spot with a hair dryer or heat the TLC plate on a warm hot plate.
- 10 After the sample spots are completely dry, place the TLC plate in a TLC chamber containing 1-butanol:methanol:acetic acid water (80:20:10:20, v/v)
11. When the solvent front reaches the top of the plate, remove the plate from the chamber and allow it to air dry in a fume hood.
12. Expose the plate to autoradiography film for 5–16 h and then develop the film.
13. As shown in **Fig. 1**, the only major radiolabeled phospholipid detected has the same R_f as standard SPP. However, when sphingosine kinase activity is measured using extracts from other types of cells, additional spots may be detected
14. To determine the R_f of standard SPP, spray the end of the plate where SPP standard was spotted with ninhydrin solution, while covering up the rest of the plate. Warm the sprayed area with a hair dryer. After a few seconds, the band corresponding to standard SPP will be stained pink.
15. Mark the areas on the TLC plate that correspond to the radioactive spots and authentic SPP
- 16 Spray these areas lightly with water to wet the plates and scrape the marked areas onto a piece of weighing paper. Transfer to scintillation vials
17. Add scintillation fluid, shake, and count in a scintillation counter

- 18 To determine specific activity, expressed as pmol of SPP formed per minute per mg protein (U), it is assumed that the ratio of radiolabeled ATP to unlabeled ATP is the same as the ratio of radiolabeled SPP to unlabeled SPP generated. First, calculate the specific activity of ATP as cpm/pmol by dividing cpm of [γ - 32 P]ATP added per tube (from the total counts) by 180 nmol of unlabeled ATP. From the specific activity of [γ - 32 P]ATP, convert the cpm of the SPP spots into pmol of SPP.

4. Notes

1. When sphingosine is added as Triton X-100 micelles (final concentration of Triton X-100 in the assay, 0.25%), the apparent activity of the enzyme is two to threefold higher than when sphingosine is added as a BSA complex. In sphingosine-kinase preparations from Swiss 3T3 fibroblasts and some other cell types and tissues, the stimulatory effect of Triton X-100 is detectable at concentrations as low as 0.1%, and is maintained up to 0.5%. However, the effect of Triton X-100 in other cell types should be examined independently.
2. The conditions for the sphingosine-kinase assay described (concentration of protein, time of incubation, saturating concentration of sphingosine) have been optimized for Swiss 3T3 cells. In these experiments, the K_m for sphingosine was found to be 9 μ M and V_{max} was reached at concentrations of 30–50 μ M (26).
3. The specific activity of ATP can be modified, depending on the application. If the sphingosine kinase activity is very low, ATP specific activity can be increased for greater sensitivity.

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Analytical Methods and Steps to Sample Preparation for Determination of Molecular Species of Fatty Acids

Sanda Clejan

1. Introduction

1.1. Background

The generation of the lipid-signal molecules diacylglycerol (DAG) and phosphatidic acid (PA) from phospholipids like phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositols (PIs) has been implicated in the transduction events essential for proliferation and differentiation in many cells. If the DAG or PA produced by cells is being generated by hydrolysis of a given phospholipid, then the fatty acid molecular species (MSFA) profile of the lipid-signal molecule should be nearly identical to that of the hydrolyzed phospholipid. Therefore, it is possible to find the source of stimulated DAG or PA by comparing the molecular species profile of the cellular DAGs or PAs to those of parent phospholipids (1,2). Lipids can be characterized by their fatty acids that differ in chain length, degree of unsaturation, configuration, and position of the double bonds, and the presence of other functionalities (*see Note 1*). High-performance liquid chromatography (HPLC) methods have been developed and are now routinely used to resolve most of the molecular species, either with or without derivatization. The nonderivatized HPLC procedures are fast and simple but relatively large amounts of lipids are required and the resolution is sometimes questionable. The derivatized HPLC procedures are more complex, but have a good resolution and are essential when diacyl, alkylacyl, and alkenylacyl subclasses have to be separated.

This introductory chapter explains how to prepare lipid samples from cells or tissues for subsequent determination of molecular species of fatty acids. Also described here are the separations into neutral and PLs species and meth-

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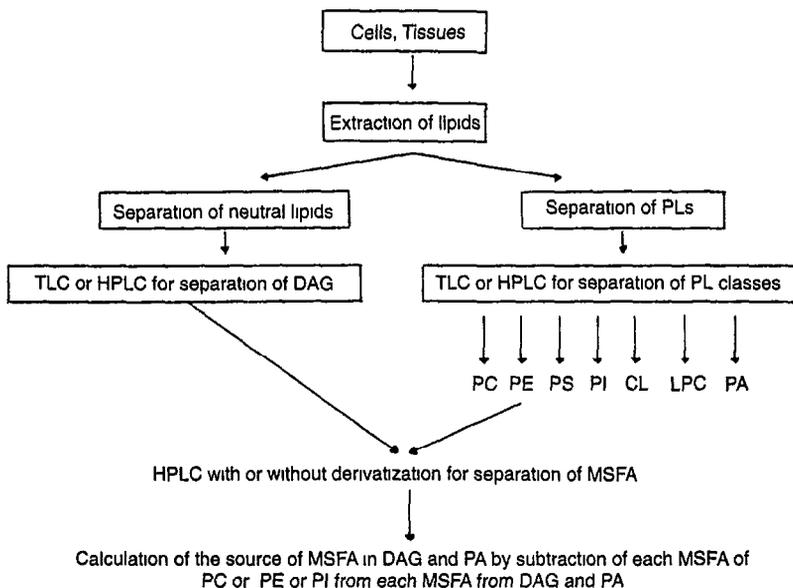


Fig 1 Flowchart of the methods used to separate DAG and PLs for analysis of MSFA and final calculations of the source of DAG and PA

ods for separation of DAG from neutral lipids and of classes of PLs. Chapter 23 describes three simple HPLC procedures for the identification of the molecular species of these extracted and separated lipids, namely DAG and PA and the separated PLs classes. Some methods may require multiple steps, including different procedures of derivatization and lipid hydrolysis, which are also described in detail.

1.2. Experimental Strategy

For subsequent HPLC analysis, it is important that lipids are extracted and separated at the appropriate pH and salt concentration and are free of particulate matter. To protect fatty acids from peroxidation and prevent acyl migration, it is important to keep the samples on ice, gently flush N_2 continuously, and add butylated hydroxy toluene (BHT) and boric acid at key steps in the process (see Notes 2 and 3). A flowchart for the different steps necessary to separate molecular species of fatty acids (MSFA) is presented in Fig. 1.

1.3. Extraction of Lipids

Many methods for extraction are available. The main procedures involve extraction by a combination of organic solvents. The Folch procedure is better suited for subsequent MSFA separation.

The most serious problem with the Folch wash procedure (3) is acyl migration and for extraction from specific cells, like endothelial cells, peroxidation (see Notes 2 and 3). Tocopherol addition, together with BHT will solve this problem. The centrifugation and removal of the organic layer and two additional extractions are precautionary steps to eliminate particulate matter. In general, lyophilization is not required but division of the cell lipid extract into four portions, i.e., triplicates plus a reserve sample is a recommended approach (see Note 4). The polyphosphoinositides are not well-extracted by Folch procedure and require specialized procedures.

1.4. Separation of Neutral Lipids by TLC or HPLC for Quantitative Separation of Diacylglycerol

For DAG analysis, the lipid extracts must be separated in a neutral lipid fraction by a simple silicic acid-column method. Complete separation of DAG may be achieved by thin-layer chromatography (TLC) and scraping off the band with subsequent extraction of DAG from the TLC powder, or by a very simple HPLC method that will also quantitate the DAG. If MSFA of DAG are identified by benzoilation, the TLC method is preferred (4). If DAG MSFA are quantitated by HPLC without derivatization, the second method of separation of DAG offers better results (6, 7).

1.5. Separation of Phospholipid Classes

After separation from neutral lipids, PLs classes can be resolved by a two-step single dimensional HPTLC (6,8) or by a simple, isocratic, silica column HPLC method (9). Again, the TLC method is preferred when used with derivatization methods or separation of ether alkenyl PLs (when necessary), whereas the HPLC method which preserves fatty acid composition is more efficient with MSFA, which does not involve derivatization (see Note 5).

2. Materials

All chemicals should be at least analytical or HPLC grade.

2.1. Extraction of Lipids from Cells or Tissues

1. Chloroform
2. Methanol.
- 3 BHT.
- 4 Tocopherol.
- 5 Potassium chloride.
6. Capped polypropylene microcentrifuge tubes.
7. Glass wool.
8. Pasteur pipets.
9. N₂ Gas.
10. Polypropylene vials (see Note 6)

2.2. Silicic Acid Fractionation into Neutral Lipids and Phospholipids

1. Chloroform
2. Acetone.
3. Methanol.
4. Silicic acid (commercial grade, 100–200 mesh).
5. Glass wool.
6. Pasteur pipet

2.3. Separation of Diacylglycerol by TLC

1. Sonicator.
2. Centrifuge.
3. N₂ Tank.
4. Toluene/ether/methanol.
5. Diethylether
6. Primuline (Merck Darmstadt, Mannheim, Germany) (*10*)
7. UV light viewer.
8. 20 × 20- μ m Silica gel 60, 0.25 mm thick (Merck)
9. Standard DAG. dipalmitoyl-*sn*-glycerol, 1-stearoyl 2-arachidonyl-*sn*-glycerol.

2.4. Separation of Diacylglycerol by HPLC

1. Equipment:
 - a. High-pressure isocratic-solvent delivery system.
 - b. Waters (Milford, MA) M6000 delivery pump
 - c. U 6K injector
 - d. Detector: R-401 differential refractometer.
 - e. Fraction collector
 - f. Recorder + convertor (analog/digital) + integrator (CPLLOT).
 - g. Column and guard column (*see Note 7*) Stainless steel column packaged with silicic acid (5 μ m), microporosil column, 30 cm × 4.0 mm.
2. Standard DAG. dipalmitoyl *sn*-glycerol, 1-stearoyl-2-arachidonyl-*sn*-glycerol.
3. Chemicals.
 - a. Diethylether
 - b. Hexane
 - c. Isopropanol
 - d. Glacial acetic acid
 - e. N₂ Gas

2.5. Separation of Phospholipid Classes by HPLC

1. Equipment.
 - a. High-pressure pump-solvent delivery system with microprocessor control programming and fraction collector (Model 6000 A, Waters), UV wavelength detector (Model 450), operated at 205 nm, with Model U6K injector
 - b. Guard column: silica particles (35–55 μ m) (*see Note 7*).

- c. Main column: Hibar II, 250 × 4.6 mm (EM Laboratories, Gibbstown, NJ).
- d. Column packing: 10- μ silica acid LiChrospher S1-100 (EM Laboratories) (*see Note 8*).
2. Chemicals:
 - a. 2-Propanol.
 - b. Potassium-phosphate, 25 mM (pH 7.0).
 - c. Hexane.
 - d. Absolute ethanol.
 - e. Millipore (Bedford, MA) filter 0.5- μ m FH.
 - f. Glacial acetic acid
 - g. Commercial standards: PC, PE, PI, PS, SM, LPC, PA (Sigma, St. Louis, MO).

2.6. Separation of Phospholipid Classes by HPTLC

1. HPTLC plates (10 × 10 cm) silica gel 60 (Merck).
2. Horizontal TLC chamber (vertical not suitable).
3. Microapplicator with micrometer adjustment (Microapplicator Company).
4. Phospholipid standards from commercial sources (Sigma). A composite standard phospholipid mixture can be prepared in accordance with the composition of the sample (approx 0.3 μ L/mL).
5. Reflectance densitometer, run in the slit-scanning mode with scanning speed at 5-mm/min.
6. Chloroform
7. Methanol.
8. 2-propanol
9. Potassium chloride
10. Ethyl acetate
11. Copper acetate.
12. Phosphoric acid.

3. Methods

3.1. Extraction of Lipids from Cells or Tissues

Carry out all incubations in capped polypropylene tubes (*see Note 6*) and flush with N₂ at each stage.

1. Add to the cells (at least 10⁴ cells or 2–4-mg protein in the cell suspension), sequentially with vortexing, 1 mL of chloroform:methanol (1:2, v/v), 0.66 mL chloroform and 0.6 mL of 0.88% (w/v) potassium chloride. Methanol contains 0.05% boric acid
2. Vigorously mix for 5 min.
3. Allow phases to separate.
4. After a minimum of 5 min, centrifuge the samples at 2000g for 5 min
5. Discard the upper layer
6. Add 1 mL chloroform to the lower organic phase.
7. Filter the solution through glass wool in a Pasteur pipet.

8. The collected eluate is then evaporated under N_2 at room temperature and redissolved in 0.5 mL chloroform

3.2. Silicic Acid Fractionation into Neutral Lipids and Phospholipids

- 1 For lipid loads of maximum 20 mg, use a Pasteur pipet with 1–2 g silicic acid (prepared as a slurry in chloroform) and containing a piece of glass wool at the narrow end of the tube (*see Note 8*)
2. Add the chloroform lipid extract from **Subheading 3.1**
3. Add, twice, 4 mL chloroform; collect the neutral lipids
4. Add, twice, 8 mL acetone, collect glycolipids and cerebrosides (*see Note 9*)
5. Add, twice, 4 mL methanol, collect the phospholipid fraction.

3.3. Separation of Diacylglycerol by TLC

- 1 Spot at the origin (1.5 cm from base of the plate), side by side, the neutral lipid extract and the DAG standard on a 20 × 20-cm silica gel 60 plate.
2. Prepare the TLC chamber, and then introduce the TLC plate in the chamber containing 10 mL of toluene:ether:methanol (80:10:10, v/v/v).
- 3 Develop to 10 cm from the origin
4. Remove the plate from the chamber and, for 15 min, allow the excess solvent to evaporate in a chromatography tank flushed with N_2
- 5 Spray the plate with 0.001% primuline.
- 6 Identify the DAG band by comparison with the standard DAG by viewing under UV light R_f of the DAG is 0.58–0.62. Delineate the band.
- 7 Scrape off the band
8. Extract the DAG from the TLC powder with 2 mL diethylether with vortexing
9. Sonicate for 30 s.
10. Centrifuge at 1000g for 5 min
- 11 Repeat steps 8–10 two more times and pool the extracts
- 12 Remove the ether with N_2 bubbling and use immediately for benzoylation.

3.4. Separation of Diacylglycerol by HPLC

1. Prepare the mobile phase hexane:isopropanol:acetic acid (100:100 0.1, v/v/v); degas.
2. Start-up: Preclean the microporasil column and the guard column (*see Note 7*) Prime HPLC pump with hexane at half the flow rate used in the method (1 mL/min)
3. Load the injection loop with 0.15 mL of the neutral lipid sample, passed prior through a filter (to remove particulate matter) or, separately, load 0.15 mL of 10 mg DAG standard (*see Note 10*)
4. Turn the injection valve to bring the sample loop in line with the column.
5. Adjust flow rate at 2 mL/min.
- 6 DAG elutes, as a bimodal peak at 8.6–12.0 min, following the nonesterified cholesterol that elutes at 7 min (**Fig. 2**).
7. The DAG collected in the fraction collector is flushed with N_2 and used immediately for MSFA determination (*see Note 11*)
8. Proceed with the standard end-of-analysis shut-down or overnight shut-down

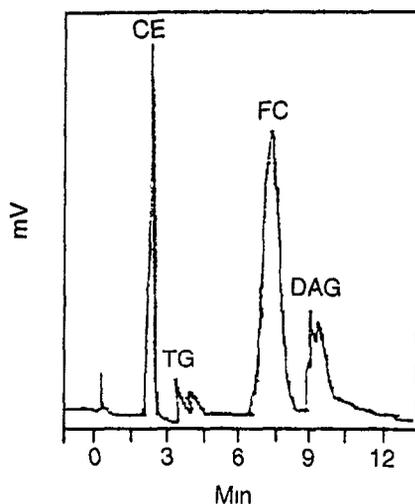


Fig. 2. HPLC separation of DAG after silicic acid separation of neutral lipids (60 μ g of lipid phosphorus from platelet membranes). CE, cholesterol esters; TG, triglycerides; FC, free cholesterol; DAG, diacylglycerol.

3.5. Separation of Phospholipid Classes by HPLC

1. Prepare standards (2 mg/mL) in hexane-2-propanol-water, (40:54.6, v/v/v).
2. Prepare the phospholipids separated as described in **Subheading 3.2., step 5**, in 1 mL hexane solvent.
3. Prepare the eluting solvent hexane:2-propanol:potassium phosphate:25 mM ethanol:acetic acid (367.490:62:100:0.6) as follows: add 490 mL of 2-propanol to 62 mL of 25 mM potassium phosphate (pH 7.0). Mix well. Add 367 mL hexane and 100 mL absolute ethanol. Filter through a 0.5- μ m type FH Millipore filter and only then add 0.6 mL glacial acetic acid. Degas (*see Note 12*).
4. Start up. Prime HPLC pump with the eluting solvent at a flow rate of 0.5 mL/min
5. Load into the injection loop: 0.2 mL of PL sample or 0.2 mL of standards and a mix of each phospholipid separately (PC + PE + PI + PS + SM).
6. Turn the injection valve to bring the sample loop in line with the column.
7. Adjust flow rate at 0.5 mL/min for the first hour (60 min)
8. PE, PA, PI, PS, and CL elute, respectively at 10, 16, 24, 38, and 57 min and are collected
9. Adjust flow rate at 1 mL/min for the next hour. PC, SM, and LPC elute at 82, 96, and 110 min and are collected (**Fig. 3**).

3.6. Separation of Phospholipid Classes by HPTLC

1. Prewash the HPTLC plates by developing them with methanol for 1 h. Dry them for 1 h at 110°C

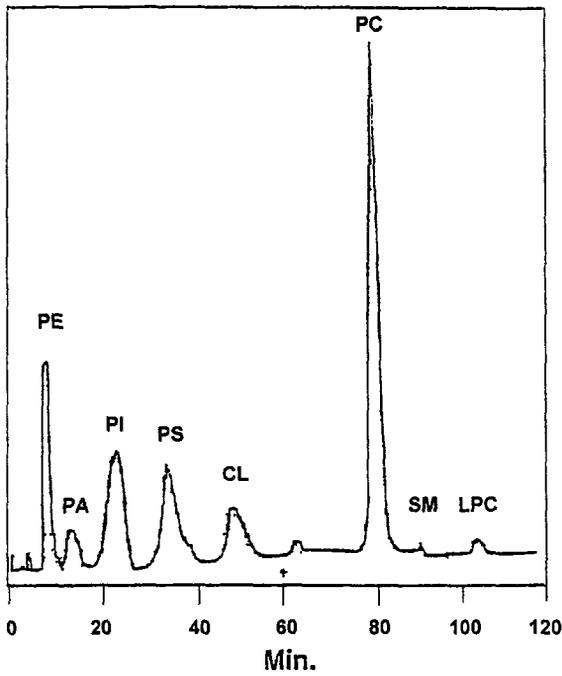


Fig 3 HPLC separation of PLs classes after silicic acid fractionation of PLs (approx 1.5 mg PL from TF1 hematopoietic cells) PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine.

2. Prepare the plate by using a sharp and soft pencil. Number the samples and standards at a distance of 8 mm between samples. Begin the application line at 8 mm from the edge.
3. Prepare the samples from the dry lipid extract by resuspending them with 2 μL chloroform and load the samples on the plate with a Hamilton syringe. Load two samples, one standard and continue in this order. Load two plates with the same samples and standards, in the same order.
4. Wash the syringe 10 times with chloroform.
5. Prepare the running solution: chloroform:methanol:2-propanol:potassium chloride, 0.25%:ethylacetate (30.9:25:6:18, v/v/v/v/v).
6. Prepare the chamber for running, by introducing a Whatman paper in the chamber and wetting it with the running solution.
7. Put the plate in the horizontal chamber, leaning the upper edge against the chamber wall. The solvent should wet the plate, but not above the start line.
8. Let the solvent ascend to the upper line. For optimal separation, the plate may be developed twice, but has to be dried with N_2 gas between the two runnings.

9. Develop one plate after drying it for 15 min at room temperature with 3% copper acetate in 8% aqueous phosphoric acid, spray to transparency, and then char the plate at 180°C for 10 min.
10. Calculate the R_f of the black spots and note the precise location on the second plate, which was not sprayed (see Notes 13 and 14).
11. Scrap the PL spots and extract the samples from the TLC powder with 2 mL chloroform with vortexing.
12. Centrifuge at 1000g for 5 min.
13. Repeat steps 11–12 twice more.
14. Remove the chloroform with N₂ bubbling and use immediately for benzoylation.

4. Notes

1. Phospholipids are characterized by a phosphate group esterified to the *sn*-3 position of glycerol; and a number of acyl residues (1–4 acyl residues) attached through an ester bond at *sn*-2 position of glycerol and either an ester, ether, or vinyl ether bond at the *sn*-1 position. The various head groups of phospholipids, which are the phosphate base, make the separation of different PLs in separate classes possible. The PL classes can then be separated into molecular species based on the acyl composition.
2. The lipids should be in undegraded and uncontaminated state after they underwent all the procedures. The effectiveness of the procedures depends on the chemical nature of the lipid components and the kind of association in which they occur. The isomerization of monoglycerides and of diglycerides by acyl migration can be prevented by adding boric acid, but in large quantities boric acid can interfere with the fractionation procedures.
3. In general, to avoid peroxidation of double bonds, all solvents should be de-aerated by bubbling N₂, and all extractions and subsequent operations should be carried out under N₂. An antioxidant, like BHT or tocopherol, or a combination of both should be used, but high quantities of antioxidants can interfere with subsequent quantitations of fractionated lipids when monitored by absorbance in the 200- to 214-nm range.
4. Because of general lability of lipids (peroxidation and hydrolysis), one should never store them for extended periods of time or in a dry form. Lipids should be stored at –80°C, dissolved in a small volume of benzene or chloroform. With dry samples, there is a high possibility of polymerization reactions of polyunsaturated lipids.
5. The strategy of which method to use for separation of PL classes depends also on the quantity of the desired PL to be separated and, thus, whether enough material is present for subsequent MSFA analysis. For example, cardiolipin (CL), a four-acyl chain PL, whose MSFA composition cannot be analyzed by standard procedures, requires high yields of separated CL. The isolation and quantitation of lysophosphatidyl choline, whose MS determination is very important in study of cardiac tissues, also offer challenges that require high yields.

- 6 Lipid samples are best kept in polypropylene vials that do not release plasticizers when exposed to chloroform:methanol (11).
7. To protect the column against blockage by finely suspended particles in the eluates or in the neutral lipid solution applied to the column, a guard column packed with silica particles, 35–55 μm , is inserted ahead of the analytic column. If the column pressure is more than twice that of a new column, one has to change the disposable guard cartridge
8. Since there are significant differences in the selectivity of silica columns even from the same manufacturer, it is necessary to make adjustments in the concentration of water and acetic acid in the elution solvent with each column to maximize the resolution of phospholipids and to obtain consistently the same retention times
- 9 If not used for separation of MSFA from glycolipids, this fraction can be discarded. When the material originates from brain lipids, the fraction may contain small amounts of cardiolipin that will be lost if analysis of MSFA of CL is intended, and, therefore, must be pooled with the methanol fraction containing phospholipids and most of cardiolipin.
- 10 In using both dipalmityl glycerol and 1-stearyl-2-arachidonyl-*sn*-glycerol to construct standard curves that are equivalent, one confirms that this quantification does not discriminate between lipids with regard to the number and location of double bonds.
11. Recovery of DAG is typically 96–103% with a good reproducibility (SD of 2 mV on a total of 18–35 mV)
12. The order in which PL classes elute depends mostly on the polarity of the head group, whereas the retention time depends mostly on the amount of water in the mobile phase. If the amount of water in the mobile phase increases, the retention time decreases for all PLs but PA, which is not affected. Conversely, an increase in the concentration of acetic acid in the mobile phase does not affect PLs, but only CL, which has an increased retention time.
- 13 Good separation of all major classes of PL is achieved by this method, except for SM, which overlaps with the PC component.
14. The R_f of different PL with this method are SM 0.04; PC. 0.07; PS 0.14; PI 0.19, PA: 0.25; PE: 0.30; and ceramide: 0.58.

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HPLC Analytical Methods for the Separation of Molecular Species of Fatty Acids in Diacylglycerol and Cellular Phospholipids

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1. Introduction

1.1. Background

Separation of PLs or the lipid mediators DAG and PA into individual molecular species is a complex multistep procedure. For example, first, phospholipid classes must be quantitatively isolated in amounts large enough for further analysis. In Chapter 22, we described the multiple steps and analytical methods necessary to achieve this fractionation with good resolution and recoveries. Once pure phospholipid fractions (PC, PE, PI, and CL) or pure DAG or PA are obtained, analyses of their molecular species have customarily been performed in a series of steps that include partial hydrolysis, derivative formation, and/or a combination of several different types of chromatographic procedures (1,2). These methods would ideally be simple enough to be performed routinely, but at the same time, the methods have to be rigorously controlled qualitatively and quantitatively to show that the fractions of fatty acids collected are not changed by the method used. It is also important to differentiate changes in molecular species of fatty acids because of dietary manipulations. These are not easy tasks. Length and unsaturation of fatty acids, the double-bond position in the chain or in the PL molecule, and the proportions and the topographically heterogeneous distribution of lipid constituents inside the cell membrane, are all factors responsible for the structural and dynamic properties of the membrane edifice.

Because of these factors, a variety of techniques have been developed. The HPLC of intact glycerophospholipids (without derivatization), as described

here, may be followed in special circumstances by transmethylation of individual peaks and analysis of fatty acid methylesters by gas chromatography (2). Other examples of such techniques used for separation and analysis of molecular species of fatty acids (MSFA) are gas chromatography-mass spectroscopy (GC-MS); GC-MS of trimethyl silyl derivatives (or *t*-butyl dimethyl silyl derivatives (3,4); HPLC with fluorescent detection, using naproxen derivatives of DAG (5); positive and negative ion electrospray MS (6); fast atomic bombardment (FAB) (7); electron capture negative chemical ionization (CI) MS (8); and HPLC in tandem with CI (9).

1.2. Experimental Strategy

In this chapter, we first describe a relatively simple and sensitive HPLC direct method for separation of MSFA of PC, PE, PS, and PI, which can be applied with few modifications to DAG analysis. We also describe a derivative method (benzoylation) for DAG, which can also be applied to specific phospholipids after their conversion to DAG by hydrolysis with phospholipase C. Finally special cases, like PA and cardiolipin (CL), that need separate methods for quantitation of MSFA are covered.

1.3. Reverse-Phase HPLC Without Derivatization for Separation of Intact Molecular Species of Fatty Acids

As currently performed, purified PC, PE, PI, and PS, or other PL classes are each chromatographed on a C18 reverse-phase column with optimal separations for quantitation by phosphorus analysis for PC or PE in the range of 100–1000 μg and PI or PS in the range of 25–400 μg (see Note 1). Within any class of phospholipid, the order of elution of MS is constant and dependent only on the composition of the component fatty acids. A relative retention time (RRT) is calculated by dividing the retention time of each peak by the retention time of the reference peak. An example of the calculation of the RRT in cultures of a murine stem-cell line responsive to erythropoietin is given in Table 1. Each molecular species is numbered based upon its retention time, as shown in Fig. 1 and identified in Table 1. Based on theoretical considerations, the RRT of a particular molecular species is the same in all the phospholipid classes. Using RRT relationship based on the predictability of the effective carbon number in *sn*-1, as well as the position of the unsaturated fatty acid in *sn*-2, it is possible to calculate the RRT for FA molecular pairs which are not being specifically identified.

The method can be used only when the cell or tissue extracted PLs have no or very minor completely saturated pair species (e.g., 16:0/18:0, 18:0/18:0, or 16:0/16:0) that are not detected by the HPLC online UV-detector response at 205 nm. Also, as the method is based on absorbance of double bonds at 205 nm,

Table 1
Relative Retention Time (RRT)
of Intact Individual Molecular Species of Fatty Acids

Peak no.	<i>sn</i> -1, <i>sn</i> -2 Molecular species
1	14:0,22:6
2	18:2,18:3 and 14:0,20:4
3	16:1,18:2
4	18:2,22:6 and 15:0,22:6
5	16:0,20:5 and 18:2,20:4
6	18:2,18:2
7	16:0,16:1
8	16:1,18:1
9	16:0,22:6
10	16:0,20:4
11	16:0,18:2
12	18:1,18:2
13	16:0,22:5 (<i>n</i> -3)
14	16:0,22:5 (<i>n</i> -6) and 18:0,20:5
15	16:0,20:3 (<i>n</i> -6)
16	17:0,18:2 and 16:0,20:3 (<i>n</i> -9)
17	16:0,18:1
18	18:1,18:1
19	18:0,22:6
20	18:0,20:4
21	18:0,18:2
22	18:0,18:1
23	18:0,22:5 (<i>n</i> -3)
24	18:0,22:5 (<i>n</i> -6)
25	18:0,20:3 (<i>n</i> -6)
26	18:0,20:3 and 18:1,20:4
27	20:0,22:6 and 20:0,20:4

The major fatty acid species were separated as described in **Subheading 3.1**. Peak numbers correspond to the elution sequence shown in **Fig. 1**. RRT was calculated with respect to 16:0,22:6 FA (peak 9). The actual molecular species represented by each peak were determined by comparison with either the RRT of standards determined by gas chromatography or with the predicted RRT calculated as described in **Subheading 1.2**.

where completely saturated species are not detected, a calibration curve—in order to compensate for absorbance differences of more saturated versus polyunsaturated species at 205 nm—is necessary (10). The method may also overestimate MSFA of diacyl species, when alkenyl or alkyl ether linkages

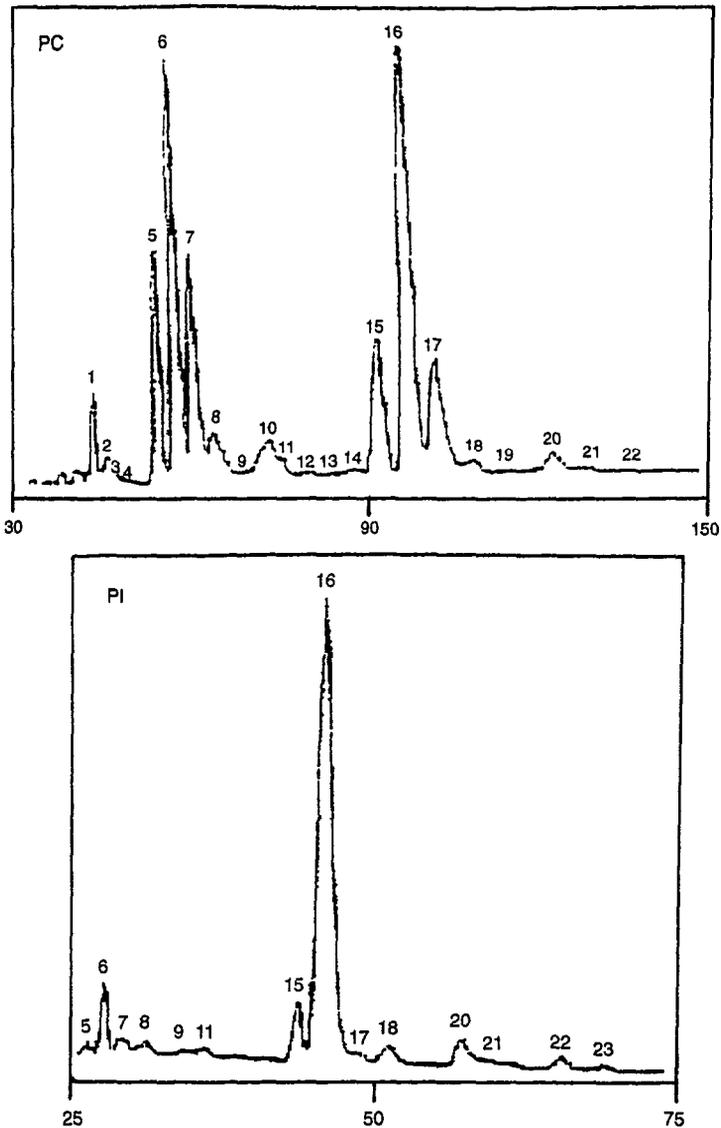


Fig. 1. (continued on opposite page) HPLC separation and MS distribution of PC, PE, and PI in cultures of B6Sut cells performed as shown in B Subheading 3.1. Internal standard 16.0, 22:6 (peak 9). The *sn*-1 and *sn*-2 MS are described in Table 1, each MS was numbered based on its retention time.

are present (see Note 2). But, when most PLs or DAGs species are diester compounds, the separation to resolve ether lipids from diester compounds need not be pursued.

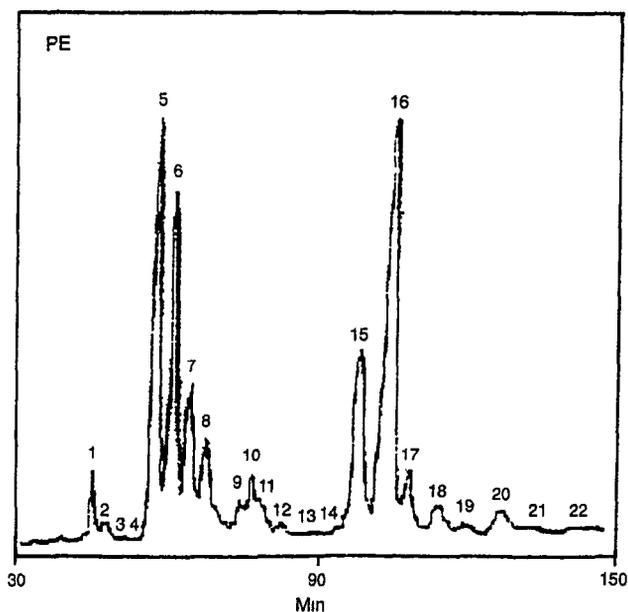


Fig 1 (continued)

1.4. Separation of Molecular Species of Diacylglycerol and Phospholipids Classes with Derivatization by Dinitro-Benzoylation

A summary of the methodology used to determine quantitatively the molecular species present in a phospholipid class is outlined in the flowchart depicted in Fig. 2. Lipids that contain 1-ether linked or alkenyl ether linked FA chains may be eluted differently than ester-(1,2 diacyl) linked lipids, when otherwise identical lipids are separated by HPLC. To determine the chemical linkage between FA moieties and glycerol, a method based on conversion of DAG into ^{32}P -labeled PA, TLC separation of the radiolabeled PA and treatment with acid or acid + base (alkenyl ether linkages are acid labile and ether linkages are base stable) can be used (10).

The step of HPLC separation of subclasses of diacyl classes can thus be avoided when it is proven that cells have little (1%) or no alkenyl 2-acylglycerobenzoates. Thus, two basic strategies can be used: The first is to exploit the ease of separation of diacyl benzoate compounds, the second is to carry out complete analysis of subclasses of phospholipids.

Some steps have to be carried out with all HPLC applications. For example, for in-line monitoring of column performance and reproducibility of sample separation, most workers add internal standards with fatty acid pairs with a

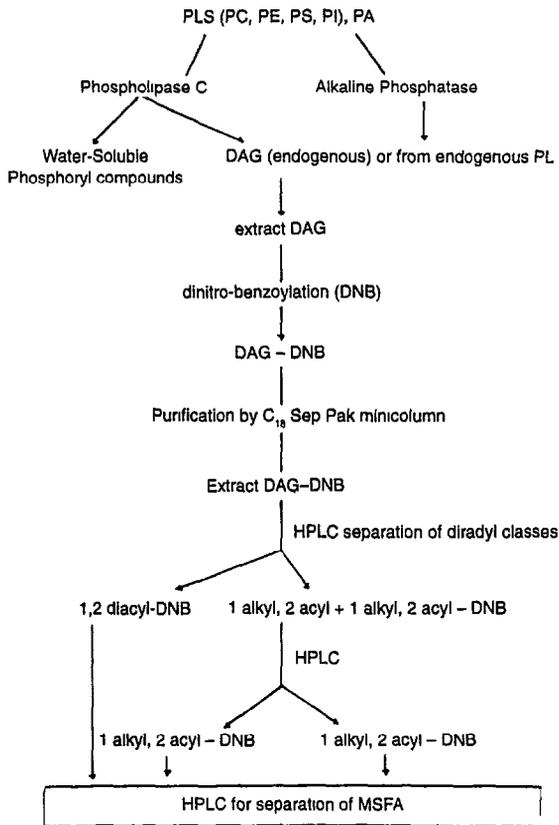


Fig 2. Flowchart of the methods used to quantitate the three subclasses of PLs for HPLC separation of MSFA by hydrolysis and dinitro-benzoylation (DNB)

variety of short *sn*-1 chain (C12) and long *sn*-2 chain (C 20), and long *sn*-1 and short *sn*-2 pairs, and pairs with both a monounsaturated (18:1) or a polyunsaturated (20:4) FA in *sn*-2 position and a medium *sn*-1 chain (18:0). The internal standards are added to the samples before injection and the column eluate is monitored at 254 nm. Next, an optimization of the gradient program is required. Initially, it is better to apply a long linear gradient to establish the elution profile of all components, using known standards as well as tissue extracts to test the method, locate all possible peaks, detect any adverse effects of sample preparation, and analyze the effect of flow rate on peak resolution. All this will eliminate costly repeats later. The early stages must be optimized first and when a change in gradient is necessary, the column must be given time to equilibrate.

1.5. Analysis of Molecular Species of Fatty Acids of Phosphatidic Acid

PA is not hydrolyzed by bacterial phospholipase C. PA can, however, be analyzed after conversion to DAG by hydrolysis with alkaline phosphatase (11). The DAG produced in this way is then purified, benzoylated as described above (see **Subheading 1.4.**), and the MS analyzed as for benzoylated DAG (see **Subheading 3.2.**). As quantitative hydrolysis by alkaline phosphatase is not complete for some more saturated molecular species, the results will overestimate polyunsaturated species, yet underestimate more saturated species. Thus, when plant or bacterial materials are extracted, a different strategy for separation of PA's MSFA is advocated. This is based on a reverse-phase ion-pair HPLC technique, in which PA being a negatively charged compound, requires an external cationic counter-ion, present in the mobile phase to form neutral species. As there are two negative charges in the PA molecule, they require two cationic ions for neutralization. In order to optimize the HPLC system, tetraalkylammonium phosphates (TAAP) are used. The retention characteristics of PA components are affected by both the size and the concentration of quaternary ammonium counter-ions present in the mobile phase. The capacity factors of molecular species increases with increase in the chain length of the alkyl group in alkyltriethylammonium counter-ion, thus the ammonium counter-ion with the largest number of carbon atoms (C18-dodecyltriethylammonium phosphate [DTAP]) tends to offer better selectivity (12). Other HPLC variables to be optimized in addition to types and concentration of ion-pair reagents are stationary-phase specifications and mobile-phase composition (see **Note 3**).

1.6. Analysis of Cardiolipin Molecular Species of Fatty Acids

CL is a phospholipid with two DAG moieties, a fact that provides particular challenge for molecular species analysis (13). Attempts to separate non-derivatized CL (see **Subheading 2.1.**) failed because of the presence of peroxidized CL analoges and impossibility to make molecular species assignment. Preliminary species analysis of CL of its DAG moieties, which can be released by phospholipase C treatment and subsequent benzoylation (see **Subheading 1.4.**), failed because of the extremely high number of peaks, all present in very low amounts.

The method described here involves derivatization by methylation of the phosphate group and benzoylation of the free hydroxyl (**Fig. 3**). Such derivatization gives highly apolar products that are UV-detectable after reverse-phase HPLC separation into well-defined MS.

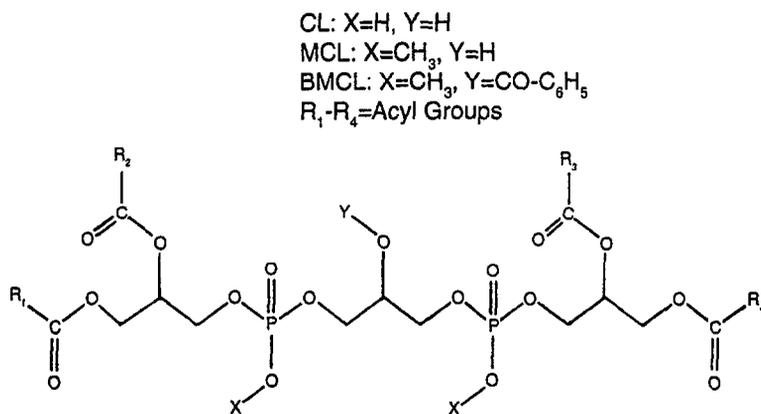


Fig 3. Structure of the cardiolipin CL, the intermediate methyl-cardiolipin 1,3-bisphosphatidyl-2-benzoyl-*sn* glycerol dimethylester (MCL), and the product of the derivatization benzoyl-methyl-cardiolipin (BMCL)

2. Materials

All chemicals should be HPLC grade or, where not available, analytical grade.

2.1. Reverse-Phase HPLC for Separation of Intact Molecular Species of Fatty Acids of Phospholipid Classes

- 1 Test samples and standards:
 - a Prepare PC, PE, PI, and PS extracts from cellular experimental material (Chapter 22). Dissolve PC and PE in 100 μ L ethanol and PI and PS in 20 μ L chloroform.
 - b Standards of most fatty acid molecular pairs (prepared at 2 mg/mL) can be purchased commercially, and for the ones that are not available, the RRT may be deducted.
2. Chemicals:
 - a. Double-distilled water.
 - b. Choline chloride, 20 and 30 mM.
 - c. Dibasic potassium phosphate, 25 mM.
 - d. Methanol.
 - e. Acetonitrile.
 - f. Acetic acid
 - g. Ethanol.
 - h. Chloroform.
3. HPLC Equipment: Standard reverse-phase HPLC equipment with isocratic pump, UV monitor, chart recorder and Fraction collector; main column—Ultrasphere ODS, C18, 25 \times 4 6-mm cartridge (see Note 4). A guard column is not necessary, but a screen-type filter is recommended.

2.2 Methods for Separation of Molecular Species of Fatty Acids with Derivatization

2.2.1. Phospholipase C Hydrolysis

- 1 Diethylether containing 0.01% BHT.
- 2 Incubation buffer: 0.2 M potassium phosphate (pH 7.0).
3. 1 mM 2-Mercaptoethanol.
4. 0.5 mM Zinc chloride
5. 0.001% Boric acid, pH 7.0.
- 6 10 U/mL Phospholipase C (from *Bacillus cereus* and *Bacillus thuringiensis*) (Sigma, St Louis, MO).
7. Sonicator.
- 8 Water bath.

2.2.2. Sample Derivatization

1. Pyridine (dried over NaOH for 30 min before use) (*see Note 5*).
- 2 3,5-Dinitrobenzoyl chloride (DNB).
3. Glass-sealed tubes
- 4 Water bath.
- 5 Methanol.
6. C18 Sep-Pak minicolumn (Waters, Milford, MA).
- 7 Diethylether
- 8 Methanol
9. N₂ tank.

2.2.3. HPLC Separation of Diradyl Classes

- 1 Resuspension solution: cyclohexane:diethylether:ethanol (85:15:0.1, v/v/v)
2. Basic binary gradient HPLC equipment with pump injector, detector, Model 481 LC-Spectrophotometer, data module (Waters), Fraction collector and a μ Porasil main column (5 μ m, 3.9 mm \times 30 cm).
3. Mobile phase: solvent A hexane:cyclohexane:diethylether:ethanol (49:49:2:0.1; v/v/v/v); solvent B cyclohexane:diethylether:ethanol (85:5:0.1; v/v/v/v).

2.2.4. TLC Separation of Alkylacyl and Alkenylacyl Species

1. TLC plates: silica gel G60 containing F254 fluorescent indicator (20 \times 20 cm) (Merck, Darmstadt, Mannheim, Germany).
2. Hexane.
3. Diethylether.

2.2.5. Separation of Molecular Species of Fatty Acids by Reverse-Phase HPLC

1. Internal standard: 1,2-12:0,12:0-DNB, prepared as described in **Subheading 3.2.2**.
2. Resuspension solution: acetonitrile:propane-2-ol (1:1, v/v).

3. Basic isocratic HPLC equipment with reverse-phase C18, LiChrosorb RP-18 column (5 μm , 4 mm \times 25 cm) (*see Note 6*).
4. Mobile phase. A- acetonitrile:propane-2-ol (9:1, v/v), B- acetonitrile:propane-2-ol (1:1, v/v).

2.2.6. Hydrolysis of Phosphatidic Acid with Alkaline Phosphatase

1. Internal standard: distearoyl PA.
2. Sonicator.
3. Incubation buffer: 50 mM Tris-HCl, pH 7.4, and 3 mM deoxycholate
4. Calf intestine alkaline phosphatase, specific activity 3000 IU/mg.

2.2.7. Reverse-Phase Ion-pair HPLC of Phosphatidic Acid—Molecular Species of Fatty Acids Without Derivatization

1. PA standard: distearoyl PA (Sigma, 10 mg/mL in hexane).
2. Tetraalkylammonium phosphate
3. Basic HPLC equipment with injector fitted with a 20- μL loop, solvent delivery system, interfaced with a spectrophotometer variable-wavelength UV detector and stationary phase: Octadecyl silica Column, like high efficiency Nova Pak C18 (4 μm , 30 cm \times 3.9 mm) or LiChrosorb RP-18 (10 μm , 25 cm \times 4.6 mm) (EM Science).
4. Mobile phase. acetonitrile:methanol:water:dodecyltriethylammonium phosphate (DTAP), 3 mM (70:22:8.1, v/v/v/v)

2.3. Analysis of Cardiolipin Molecular Species

1. For derivatization of CL.
 - a. Standards: tetrapalmytoyl CL and bovine heart CL (Sigma).
 - b. Diazomethane (*see Note 7*).
 - c. Chloroform.
 - d. Methanol.
 - e. HCl, 0.1 M
 - f. Anhydrous pyridine.
 - g. Benzoylation reagent: 0.2 mL Pyridine, 10 μmol Benzoic anhydride, and 10 μmol 4-(dimethyl amino) pyridine.
 - h. *n*-Hexane.
2. For purification of BMCL:
 - a. Silica gel G100 column.
 - b. Elution solvent: *n*-hexane:propane 2-ol (9:1, v/v).
 - c. *n*-Hexane.
3. For HPLC separation of BMCL:
 - a. Binary-gradient HPLC instrument with: main pumps, dynamic mixer, system controller, channel-variable wavelength UV detector.
 - b. Stationary phase: Nucleosil 100-5 C18 reverse-phase column (25 cm \times 4 mm) (Rainin)

- c. Mobile phase: Solvent A—acetonitrile:propane-2-ol:methanol:water (50:25:20.5, v/v/v/v) Solvent B—acetonitrile:propane-2-ol (2:1, v/v)

3. Methods

3.1. Reverse-Phase HPLC for Separation of Intact Molecular Species of Fatty Acids of Phospholipids Classes

3.1.1. Preparation of Elution Buffers

1. Calibrate the pH meter and adjust the pH of buffers to within 0.05 pH units to ensure reproducibility of results
2. Filter solutions and buffers through a 0.2- μm filter before use
3. Degas all solutions before use, by bubbling N_2 through the liquid for 5 min
4. For separation of PC, PE, or PI samples, prepare the mobile phase: choline chloride, 20 mM in methanol:water:acetonitrile (90.5:7:2.5, v/v/v)
5. For PS samples prepare the mobile phase: choline chloride, 30 mM, in methanol:25mM dibasic potassium phosphate:acetonitrile:acetic acid (90.5:7:2.5:0.8, v/v/v/v) (see **Notes 8** and **9**).

3.1.2. Start-Up

1. Prime HPLC pump with degassed chloroform for PI and PS species analysis and with ethanol for PC and PE samples
2. Pump the column with chloroform or ethanol at 1.0 mL/min and then with the mobile phase
3. Carry out column precleaning and bring the column back in line

3.1.3. Sample Application

1. Pass each sample or standard through a 0.5- μm filter.
2. Make up the sample: Mix the sample for PC and PE in 100 μL ethanol, for PI and PS in 20 μL chloroform (or mix the samples in the mobile phase)
3. Load the sample into the injection loop.
4. Turn the injection valve to bring the sample loop in line with the column

3.1.4. Flow Rates

1. The elution is kept at a constant flow rate at 2.0 mL/min for 150 min for PC and PE, 120 min for PS, and 75 min for PI.

3.1.5. Collection of Molecular Species

1. Set UV detector at 205 nm, and collect accordingly.
2. As shown in **Table 1**, molecular species separation occurs even on the basis of the position of double bonds.
3. Since many molecular species of PI and PS are present only in trace amounts in samples to be analyzed, it may be necessary to collect them in batches to have sufficient material for analysis (see **Notes 10** and **11**).

3.2. Methods for Separation of Molecular Species of Fatty Acids with Derivatization

3.2.1. Phospholipase C Hydrolysis

1. Dry under N_2 the phospholipid standards and the individual phospholipids collected from HPLC (Chapter 22, Subheading 3.5) or HPTLC (Chapter 22, Subheading 3.6).
2. Resuspend in 25 μ L diethylether.
3. Sonicate with 1.25 mL incubation buffer for 5 min on ice (*see Note 12*).
4. Incubate the sonicated phospholipids with 50 IU phospholipase C from *B. cereus* and 2 IU from *B. thuringiensis* in a shaking water bath at 37°C for 4 h (*see Note 13*).
5. Extract the generated DAGs with 2 mL diethylether at room temperature under N_2 bubbling.
6. Repeat **step 5** three times (*see Note 14*).

3.2.2. Sample Derivatization (*see Note 15*)

1. Before derivatization prepare enough fresh DNB solution of 3,5-dinitrobenzoyl chloride (25 mg) in 0.5 mL dried pyridine, to derivatize all samples and standards. Add 0.5 mL DNB solution to each sample in a glass test tube, top with N_2 gas and seal immediately after the addition of the DNB solution.
2. Leave the sealed test tubes in a shaking water bath at 60°C for 15 min (*see Notes 16 and 17*).
3. Stop the reaction by adding 2 mL methanol:water (4:1, v/v).
4. Prepare a C18 Sep-Pak minicolumn, by washing sequentially with 10 mL diethylether, 15 mL methanol, and twice with 15 mL methanol:water (4:1, v/v).
5. Add each derivatized sample and elute with 25 mL freshly redistilled diethyl ether.
6. Dry under N_2 , resuspend the sample in 1 mL diethylether, and store at -20°C (*see Note 18*).

3.2.3. HPLC Separation of Diradyl Classes

1. Resuspend the DNB materials in 20 μ L of cyclohexane:diethyl ether:ethanol (85:15:0.1, v/v/v).
2. Prime both HPLC pumps with degassed diethylether and run the mobile phase analytical gradient (**step 4**) without injection of the sample to prerun the column.
3. Apply the DNB sample into the injection loop.
4. Set up the following linear gradient program, using a flow rate of 1 mL/min, 0–30 min, 100% solvent A and 30–40 min, 100% solvent B.
5. Set in line the spectrophotometer monitor to 254 nm.
6. Collect the samples (*see Fig. 4; Note 19*).

3.2.4. TLC Separation of Alkylacyl and Alkenylacyl Species

1. Pass the plates through diethylether (containing 0.01% boric acid) and activate them by drying at 60°C for 1 h.

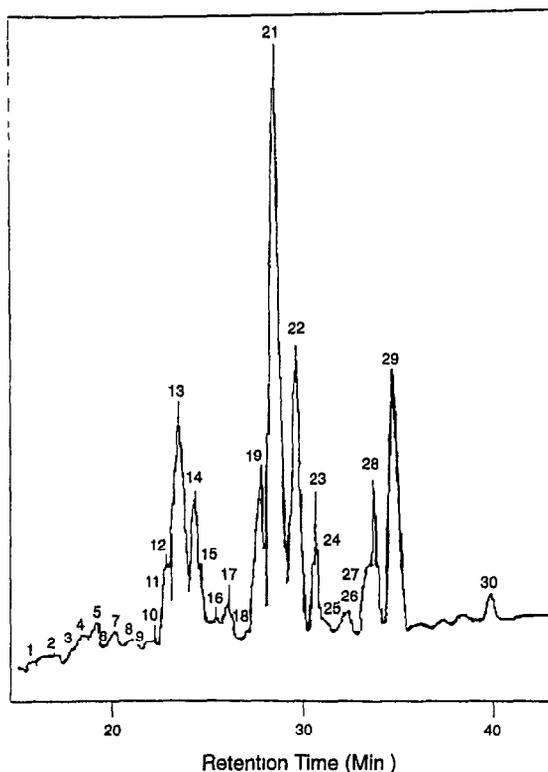


Fig. 4 HPLC separation of DNB—molecular species of DAG in cultures of *Mycoplasma capricolum* cells performed as shown in **Subheading 3.2**. Internal standard 12:0,12 0. The principal peaks are: (11) 18 0/20:4; (12) 18:1/18.2 + 16:1/18.1; (13) 16:0/18 2; (14) 18:1/20:3; (19) 18:1/18:1; (20) 18:0/18:2; (22) 16:0/16:0; (23) 18:0/18:1; (29) 16:0/18:0; (30) 18:0/18:0.

2. Spot at a distance of 1 cm the peaks collected from HPLC (*see Subheading 3.2.3.*).
3. Separate, using hexane:diethyl ether (13:7,v/v).
4. Dry the plate
5. View under UV, 254 nm. The DNB derivatives appear as dark spots on a green background.
6. Scrape off the samples of alkyl and alkenyl glycerols (R_f 1,3 DNB-DAG 0.29; 1,2 DNB-DAG 0.33; 1-alkenyl,2-acylglycerol-DNB 0.37, and 1-alkyl,2-acylglycerol-DNB 0.43) and elute with diethylether (*see Note 20*).

3.2.5. Separation of Molecular Species of Fatty Acids by Reverse-Phase HPLC

1. Resuspend each sample from **Subheading 3.2.4.** in 20 μL of resuspension solution under N_2 .

2. Add 5 μL of the internal standard 1,2-12:0/12:0-DNB.
3. Prime HPLC pump, run the mobile phase, prerun the column as described in **Subheading 3.2.3**.
4. Apply the sample into injection loop.
5. Set up the following linear gradient program using a flow rate of 1 mL/min 0–45 min, 100% solvent A, 45–90 min, 100% solvent B
6. Set in line the UV monitor to 254 nm.
7. Collect the samples. Identify the peaks via retention times in relation to the internal standard (see **Note 21**; **Fig. 4**)

3.2.6. Hydrolysis of Phosphatidic Acid with Alkaline Phosphatase (14)

- 1 Dry the separated PA samples under N_2 . Add as an internal standard 1 nmol/mg total phospholipid of distearoyl PA to 30- μM PA sample.
- 2 Disperse the mixture by sonication for 5 min in the incubation buffer with Tris-HCl and deoxycholate
- 3 Preincubate the dispersed mixture in a water bath for 15 min at 37°C
4. Add 5 μL alkaline phosphatase solution (10 IU) and incubate at 37°C for 30 min.
- 5 Terminate the reaction by addition of 2.5 mL HCl, 0.2 N
- 6 Extract the formed DAG with chloroform:methanol (1.1, v/v) and vortex for 5 min. Discard the upper phase
7. Neutralize the lower organic phase with 2.5 mL sodium bicarbonate, 1 N
- 8 Centrifuge at 3000g for 15 min. Separate the chloroform phase (see **Note 22**)

3.2.7. Reverse-Phase Ion-Pair HPLC of Phosphatidic Acid Molecular Species of Fatty Acids Without Derivatization

- 1 Prime the HPLC pumps, prerun the columns with solvents
2. Inject 10 μL of 1% PA in chloroform with 5 μL internal standard into the guard and main column via the injector fitted with the loop.
3. Pump the degassed and filtered mobile phase under isocratic conditions at a flow rate of 1 mL/min.
- 4 Set in line the UV monitor at 208 nm.
5. Identify the peaks monitored by a recorder via retention times in relation to the internal standard (**Fig. 5**)

3.3. Method for Analysis of Cardiolipin Molecular Species

- 1 Convert CL to the free acid form by adding 2 mL chloroform, 4 mL methanol, and 2 mL 0.1 M HCl.
- 2 Incubate for 5 min at 0°C on ice and extract the acid form of CL into the organic phase by adding 2 mL chloroform and 2 mL 0.1 M HCl
3. Dry under N_2 bubbling and dissolve in 1 mL chloroform, recover organic lower phase
4. Add 1 mL chloroformic diazomethane to CL and incubate for 15 min at 0°C
5. Remove chloroform and excess reagent by bubbling N_2 . The residue is MCL (see **Note 23**).

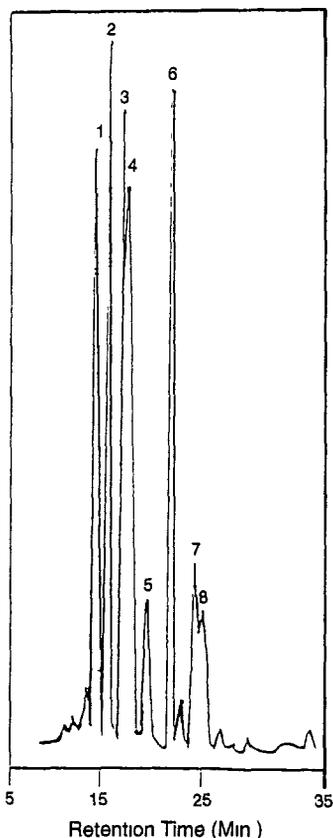


Fig. 5. Reverse-phase ion-pair HPLC of PA in cultures of TF1 cells performed as in **Subheading 3.3.** on Nova-Pak C18 stationary phase and UV detection at 208 nm. Peaks: (1) 18:0/20:4; (2) 16:0/18:3; (3) 16:0/18:2, (4) 16:0/18:1, (5) 18:0/18:2, (6) 18:1/18:1; (7 + 8) 18.0/18.1

6. Dry the MCL by resuspension in anhydrous pyridine and evaporation. Repeat twice
7. Dissolve the final residue in the benzylation reagent in a glass tube with screw-cap, fill with N_2 , and seal with screw-cap.
8. Shake gently in a water bath at room temperature for 1 h.
9. Evaporate the pyridine and add 1 mL water.
10. Extract the BMCL product with 5 mL *n*-hexane.
11. Purify the BMCL by a silica gel column (equilibrated in *n*-hexane) with 10 mL elution solvent (*see Note 24*)
12. Dissolve the BMCL in 0.2 mL ethanol, inject in the 0.5-mL loop
13. Run a linear gradient as follows: 0–150 min solvent A:solvent B (80:20, v/v); 150 min–215 min solvent B 100%
14. Quantitate HPLC chromatograms using a UV detector set at 228 nm (*see Note 25*)

4. Notes

1. The use of large samples for MSFA separation in PS or PI is not possible because it will result in obliteration of the small peaks adjacent to the predominant peaks and/or tailing of the one or two predominant species of PI and PS. If intact molecular species are quantitated by phosphorus analysis, it is necessary to reduce the amount of choline chloride by Folch extraction. This is because large quantities of salt interfere with phosphorus determination.
2. Fatty acid chains at position *sn*-1 of PC or PE are joined to the glycerol backbone via an ester or by an alkenyl or alkyl ether linkage and analysis of PL fatty acid chain linkage is recommended when there are doubts about the presence of a 1-alkenyl-2-acyl glycerol phosphate indicated by the generation of lyso-PA following acid hydrolysis, or about the presence of 1-alkyl-2-acyl glycerol phosphate, indicated similarly by the generation of lyso-PA following both acid and base hydrolysis.
3. When injecting a sample into an HPLC column, it is best to dissolve the sample in a solvent very similar to the mobile phase. With whole lipid extracts, there is still nonlipid material that is insoluble in the mobile phase. If this material is injected into the HPLC column, it will precipitate on the column. This causes a rapid increase in the back pressure of the column and may alter the chromatographic characteristics of the column.
4. All good-quality reverse-phase columns are adequate for reverse phase separation of MSFA, if the carbon load (% carbon) is high and thus free silanols (silica) are in smaller amounts. By increasing the carbon load, there are less problems with tailing. Also, these columns are best stored for longer periods in the mobile phase used in the separation.
5. Careful drying of the pyridine, immediately before use, gives the best recoveries, since even small amounts of moisture decrease the derivatization efficiency.
6. The main characteristic to consider in choosing the reverse-phase column for benzoylated DAG molecular species is the efficiency of the column = number of theoretical plates/column or = plates/meter/length. Thus the column described (25 cm, 5 μ m) will have 15,000 plates (60,000 plates/m \times 0.25 m). A good 15-cm, 3- μ m particle column will be very similar in number of plates (100,000 plates/m \times 0.15 m).
7. Diazomethane is both toxic and explosive. All manipulations have to be carried out under a hood, avoiding the use of rough glass surfaces and grease, and all incubations done at 0°C.
8. The addition of choline chloride to the mobile phase helps to increase the efficiency of chromatography and thus narrow the peaks and decrease the tailing. A decreased efficiency is caused by interactions between the polar groups immobilized in the stationary phase and those dissolved in the mobile phase, and interreactions between polar groups of the PL and exposed silanols on the reverse-phase column. When tailing obliterates the molecular species separation in spite of addition of choline chloride, acidification of the mobile phase with phosphoric acid, 1 mL of mobile phase, is helpful. But if intact molecular species are to be

collected for further analysis, the higher acidic mobile phase may hydrolyze the acyl chains and therefore neutralization of the phosphoric acid with sodium bicarbonate is recommended.

9. The role of acetonitrile is to form a complex with the double bonds of the fatty acid, making the molecular species with double bonds more soluble in the mobile phase and decreasing the retention time of MS in proportion to the number of double bonds in the acyl groups. Thus, by increasing the concentration of acetonitrile in the mobile phase, a repositioning of molecular species is possible. However, increased acetonitrile concentration also increases tailing.
10. Recoveries, as determined by phosphorus measurement, are: for PI, PE, and PC, 93.5–103%; for PS, 78.5–88.8%. The variation of intra and inter assays is below 10% for PE, PC, and PI, but 16.4% for PS.
11. HPLC analysis of 1–50 mg of 16:0/22:6 MS gives a linear standard curve. Calibration of MS can be performed by comparing each MS obtained, based on the absorbance at 205 nm, with analyses by gas chromatography of the same peaks, in order to compensate for absorbance differences of more saturated vs polyunsaturated species.
12. Drying and resuspension of phospholipids are carried out to concentrate the fatty acids for subsequent derivatization and analysis. Nevertheless, this step is also a source of exogenous (e.g., from glass wall, solvents, pipet tips) and endogenous contamination (hydrolysis of covalently bound fatty acids).
13. Incubation with 50 IU phospholipase C from *B. cereus* is sufficient for complete hydrolysis of PC, PE, and PS phospholipid classes. PI is not completely and efficiently hydrolyzed. Addition of 2 IU of PI-specific phospholipase C (from *B. thuringiensis*) is thus indicated.
14. Inadvertently heating the diethylether extract while drying can promote rearrangement of the DAG and yield a mixture of 1,2 and 1,3 DAG.
15. In general, after **step 6 of Subheading 3.2.1.**, DAG can be derivatized directly. However, if relative amounts of DNB subclasses are to be quantitated by UV absorbance, it is necessary to further purify the DAG before derivatization and remove impurities that will interfere with quantitation of alkylacyl classes.
16. The DNB group has a high molar absorption with a maximum at approx 254 nm, where nonderivatized lipids and usual HPLC solvents do not absorb. The benzylation reaction is completed within 15 min at 60°C, without isomerization of polyunsaturated fatty acids. However, because some samples need a longer incubation time to yield internal standard recoveries of >95%, one may increase the incubation time to 30 min.
17. Because the method involves the heating and cooling of a sealed ampule, there is some risk of explosion. It is absolutely essential to work behind a protective shield and wear protective clothing/glasses. A suitable high-pressure test tube or ampule must be used and heating carried out with a lid over the water bath as a precaution against splashing.
18. Reaction mixtures containing the derivatized samples should be kept frozen below –20°C if not analyzed during the next 24 h because storage at 4°C leads to a slow increase of saturated pairs of MSFA.

Table 2
Relative Retention Time of Molecular Species of Cardiolipin

Peak no.	RT, min	RRT	Molecular species R ₁ , R ₂ /R ₃ , R ₄
1	42	0.25	20:4, 18:2/18:2, 18:3
2	51	0.30	18:2, 18:3/18:3, 18:3
3	56	0.32	18:2, 18:3/18:2, 18:3
4	61	0.36	18:2, 18:2/18:3, 18:3
5	68	0.40	?
6	74	0.44	18:2, 18:2/18:2, 18:2
7	82	0.48	?
8	90	0.53	18:2, 18:1/18:2, 18:2
9	106	0.63	18:2, 18:1/18:2, 18:1
10	130	0.78	18:1, 18:1/18:2, 18:1
11	152	Present only in hydrogenated CL	18:1, 18:1/18:1, 18:1
12	176	Present only in hydrogenated CL	18:1, 18:0/18:1, 18:1
13	212	Present only in hydrogenated CL	18:1, 18:0/18:1, 18:0

Separation on HPLC as shown in **Subheading 3.3**. The positional assignment of the R₁, R₂/R₃, R₄ is tentative according to the preferred esterification site of individual FA (other additional positional isomers are possible) RRT calculated relative to synthetic tetra-palmitoyl-CL (16 0,6:0/16.0,16:0)

19. HPLC using a μ silica column (Porasil) with change of the gradient solution at 30 min was found to clearly separate 1,2 and 1,3 DAG, DNBs from other components but only partially separate 1-alkyl,2-acyl glycerol-DNB from 1-alkenyl,2-acyl glycerol-DNB. When there is little or no alkenyl DAG (less than 1% of total DAG), the subsequent TLC step (**Subheading 3.2.4.**) can be avoided, and the HPLC peak can be assumed to be solely 1-alkyl,2-acyl glycerol for all subsequent analyses of endogenous DAGs. The presence of different molecular species within a specific diacyl class has minimal effects on peak mobility on μ Porasil.
20. A large peak just ahead of 1-alkyl,2-acyl glycerol DNB may appear if there are still contaminants from the derivatization procedure.
21. A limitation of this method is that separation of individual DAG classes on a reverse-phase C18 column is unable to fully resolve all the molecular species (i.e., over 27 are present solely in 1,2 DAG from hematopoietic cell lines). As different C18 columns have slightly different retention characteristics, most species can be identified when two columns are used. Identification by reference to authentic standards and collection of peaks, transmethylation, and analysis by capillary GLC can be done if peaks not identified in **Table 1** are present.

22. The maximal activity of alkaline phosphatase occurs at neutral pH with a detergent concentration of 3 mM deoxycholate (with SDS and Triton X-100, the activity is considerably less). Alkaline phosphatase hydrolyzes all phosphomonoesters (ROP) at similar rates, regardless of the size or chemical nature of the R group, because dephosphorylation of the enzyme-product intermediate is rate-limiting.
23. To check the conversion of CL to MCL, use a TLC silica gel 60 F254 and determine phosphate in the iodine-stained spots. CL remains at origin ($R_f = 0$) and MCL is spotted at $R_f = 0.24$ (near solvent front). A yield of minimum 90% is acceptable.
24. The silica column method of purification is fast and removes contaminants, like 4-(dimethylamino)pyridine. If there is also excess of benzoic anhydride, this will remain and appear in the solvent peak of the subsequent HPLC separation.
25. Since CL consists of two DAG moieties, the assignment of CLMS needs precise knowledge of the molecular composition of these moieties. **Table 2** gives some FA pairs with positional distribution of individual acyl groups among *sn*-1 and *sn*-2 ester sites.

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Analysis of Molecular Species of Cellular Sphingomyelins and Ceramides

Sanda Clejan

1. Introduction

1.1. Background

Ceramides (CER) have been proposed to be the intracellular mediators of responses to such agents as interferon- γ (IFN- γ), dexamethasone, tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and vitamin D₃. These agents induce hydrolysis of the plasma membrane sphingomyelin (SM) by a sphingomyelinase, followed by downstream activation of signaling kinases and nuclear translocation of NF- κ B, with the final effect of induction of cell differentiation and/or apoptotic death (*1*). The growing interest in the SM-CER-dependent cell signaling makes an accurate and simultaneous determination of molecular species (MS) of both SM and CER desirable. Numerous MS are found in SM and CER, and these occur in characteristic proportions in different species, organs, subcellular organelles, and developmental stages. More than half of the fatty acids (FA) in CER contain a hydroxyl group at the 2 carbon position, the other half being non-hydroxy FA. In Chapter 22 the methods for extraction and separation of lipids from cellular samples, the separation of classes of phospholipids (PLs), and finally, the molecular species (MS) analysis are described. Separation of SM and ceramides CER follow very similar pathways (e.g., hydrolysis of SM to CER is similar to hydrolysis of PLs to diacylglycerol [DAG]). However, as seen in **Fig. 1**, SM consists of an *N*-acyl-fatty acid linked to a long-chain hydrocarbon and phosphorylcholine. The FA position of the molecule varies as in PLs, but with more major long-chain (C20-C24) saturated and monounsaturated FAs present than in PLs. The sphingoid base is much simpler than the second FA chain (*sn*-2) in PLs, with

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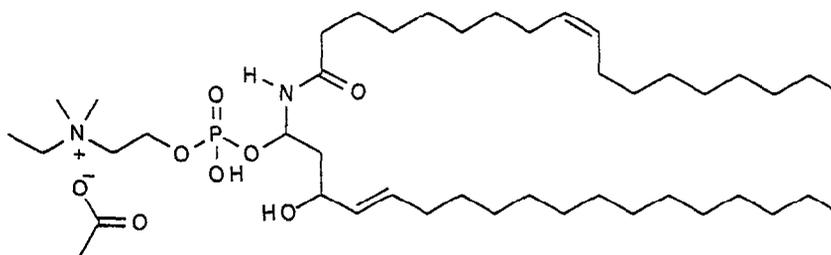


Fig 1 Structure of SM (*N*-oleoyl-[4E]-sphingenine phosphorylcholine).

C18-sphingenine (18:0) as the major component and additional small amounts of C18-sphinganine (18:1) and C20-sphingenine (20:0). Whereas C20-sphingenine is associated mostly with 18:0 FA, the C18-sphingenine and C18-sphinganine are associated with a variety of long-chain FAs. Reverse-phase (RP) HPLC separations (without derivatization) similar to those described in Chapter 23, Subheading 3.1., are not efficient for the separation of intact SM molecular species because “critical pairs” are not completely resolved (2) (e.g., SM with 24:1 and 22:0 FA are eluted together). Still, for specific, very limited purposes, such as to gain approximative knowledge of the MS involved, this method can be used for separation of MS of SM (see Notes 1 and 2).

A method using positive and negative ion-spray mass spectroscopy (3) also does not separate the pairs with C20-sphingenine. Therefore the methods for separation of MS of SM or CER described in this chapter involve multiple steps with hydrolysis, benzylation, gradient elution HPLC for separation of nonhydroxy FA of CER (NFA-CER), and hydroxy FA of CER (HFA-CER) and argentation chromatography for separation of saturated from monounsaturated FA, prior to reverse-phase HPLC separation of MS.

1.2. Experimental Strategy

Two different strategies for separation of MS are presented in Fig. 2. Pathway 1 involves: benzylation of SM that is very similar to the method discussed in Chapter 23, Subheading 3.2.2. (see Note 3); separation of saturated and monoenoic benzyolated SM (BSM) by argentation chromatography; and separation of MS by a reverse-phase HPLC. Pathway 2 involves hydrolysis of SM with sphingomyelinase or hydrofluoric acid (HF) to CER and benzylation to B-CER. Subsequent separation of NFA-CER and HFA-CER will be described in detail in this chapter. The subsequent steps to analysis of MS of CER are similar to the ones in pathway 1 for SM.

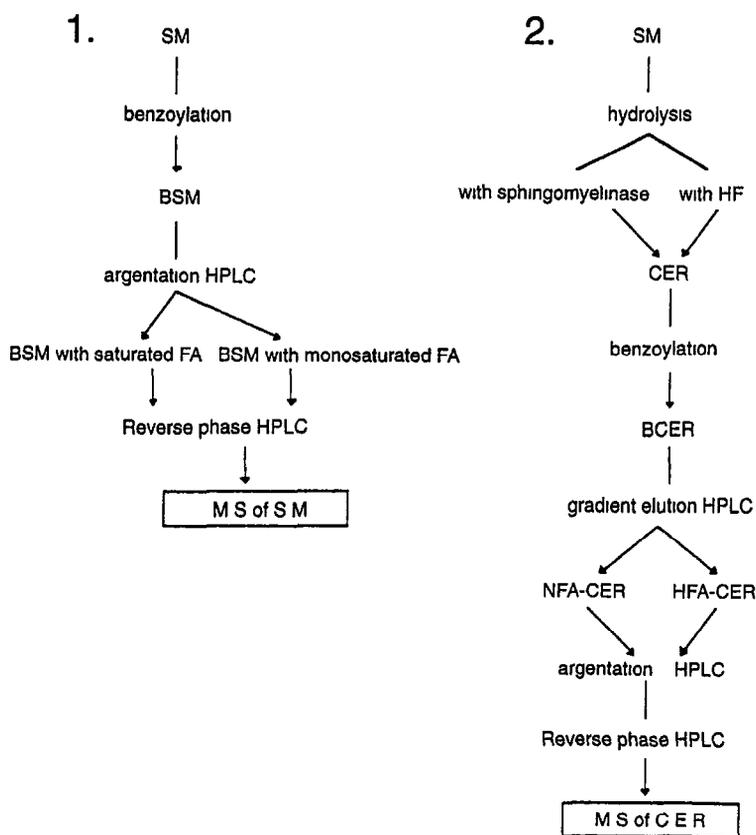


Fig. 2. Flowchart of the pathways for separation of molecular species of SM and CER.

1.2.1. Hydrolysis of Sphingomyelin to Ceramide

The common method of hydrolysis of SM with hydrochloric acid in anhydrous methanol or in methanol/water mixtures (4) leads to incomplete dephosphorylation of the base and formation of byproducts of sphingosine (5). Alkaline hydrolysis also is unsuitable because it produces low yields of sphingosine bases (6). To circumvent these limitations, methods involving enzymatic hydrolysis of SM to CER (7) with bacterial sphingomyelinase and an efficient hydrolysis with HF (8) are described.

1.2.2. Gradient Elution HPLC for Separation of Benzoylated Nonhydroxy Fatty Acid-Ceramide from Benzoylated Hydroxy Fatty Acid-Ceramide

Many different HPLC separations with isocratic elution and detection at 280 nm of benzoylated CER (B-CER) have been described (9). However, the

sensitivity of the assay is increased 15-fold if the detection is performed at 230 nm, at which B-CER have the absorption maximum (10). The solvent system used also is important, because only a few solvents do not absorb at 230 nm. The solvent chosen, dioxane-hexane, has some other drawbacks (see Note 4), but the separation can be achieved in only 10 min with high sensitivity.

1.2.3. Argentation Chromatography for Separation of Saturated Fatty Acid-Ceramide and Monounsaturated Fatty Acid-Ceramide

Argentation HPLC separates the lipids based on the number of double bonds independently of the number of carbon atoms, taking advantage of the capacity of Ag^+ ions to complex with olefinic double bonds in the molecule (11). Thus, separation of B-CER in saturated and monounsaturated FA-CER or B-SM in the same two species makes the next step HPLC reverse-phase separation of molecular species almost 100% complete. The Ag^+ in the column is complexed with sulfonic acid that is bonded to silica particles. Argentation HPLC has the capacity to separate *cis* and *trans* geometric positional isomers with different degrees of unsaturation.

1.2.4. RP-HPLC for Separation of Molecular Species of Benzoylated Sphingomyelin or Benzoylated Ceramide

As seen in Fig. 3, when compared with RP-HPLC analysis without preliminary argentation chromatography (Fig. 3A), the saturated (Fig. 3B) and unsaturated (Fig. 3C) fatty acids, separated by argentation chromatography and subsequent reverse-phase HPLC, showed a near complete separation of all critical molecular species pairs (see Note 5).

2. Materials

All materials should be at least analytical grade or HPLC grade.

2.1. Hydrolysis of Sphingomyelin

For extraction and separation of SM prior to hydrolysis follow the procedures in Chapter 22 (see Note 6).

2.1.1. Hydrolysis of Sphingomyelin with Sphingomyelinase

1. Standard: bovine brain ceramide type IV (Sigma, St. Louis, MO).
2. Sonicator.
3. 0.1 M Tris-HCl, pH 7.4.
4. 6 mM Magnesium chloride
5. 0.1% Triton X-100

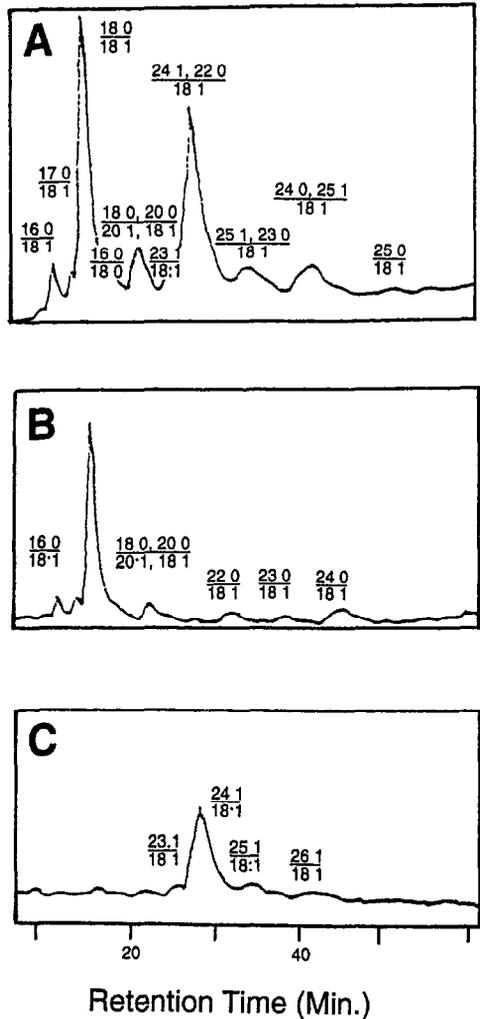


Fig. 3. (A) Analysis of cells of B-SM neuroblastoma N1E 115 by RP-HPLC on Nucleosyl-5 C18 column (solvent: methanol:acetonitrile:phosphate buffer, 5 mM, pH 7.4) (100:20:1, v/v/v) at a flow rate of 2 mL/min. B-SM approx 60 µg (B,C), same RP-HPLC analysis from the two peaks collected from argentation chromatography (B) From saturated SM fraction (C) From monounsaturated SM fraction. Near each peak, the major FA composition is given in the numerator and the long chain base composition in the denominator

6. Sphingomyelinase (ca 2000 IU).
7. Diethylether (HPLC grade).
8. N₂ Gas

2.1.2. Hydrolysis of Sphingomyelin with HF

1. Chloroform.
2. Polypropylene tubes
3. 40% HF.
4. 10% Ammonia

2.2. Gradient Elution HPLC for Separation of Benzoyl Nonhydroxy Fatty Acid-Ceramide from Benzoyl Hydroxy Fatty Acid-Ceramide

1. HPLC equipment: constant-pressure pump with solvent delivery system and solvent programmer, Model U-6K injector, variable UV spectromonitor with pressure resistant cell, fraction collector
2. Stationary phase: stainless-steel column (50 cm × 2.1 mm) packed with Zipax pellicular particles (27 μm) covered with porous silica (Varian, San Fernando, CA).
3. Linear gradient: 2–8–5% dioxane in hexane.
4. Other solvents: carbon tetrachloride, methanol, dichloromethane, and hexane (for the regeneration of the column)

2.3. Argentation Chromatography for Separation of Saturated Fatty Acid-Ceramide and Monounsaturated Fatty Acid-Ceramide

1. HPLC basic equipment: (*see Subheading 2.2.*).
2. Stationary phase: Chromopak silver column (25 cm × 4.6 mm).
3. Mobile phase for argentation HPLC of B-SM: methanol:isopropanol (8:2, v/v); and hexane:isopropanol (9:1, v/v) for B-NFA-CER and/or B-HFA-CER.

2.4. RP-HPLC for Separation of Molecular Species Benzoyl Sphingomyelin or Benzoyl Ceramide

1. HPLC basic equipment (*see Subheading 2.2.*).
2. Stationary phase: Nucleosil 5 C18 column (5 μm, 30 cm × 4 mm).
3. Mobile phase: methanol:acetonitrile:potassium phosphate buffer, 5 mM, pH 7.4 (100:20:1, v/v/v) for B-SM or methanol for B-NFA-CER and/or B-HFA-CER

3. Methods

3.1. Hydrolysis

3.1.1. Hydrolysis of Sphingomyelin with Sphingomyelinase

1. Prepare 1.5 mL 0.1 M Tris-HCl, pH 7.4, 6 mM magnesium chloride, and 0.1% Triton X-100.
2. Immediately resuspend the SM lipid extract (from 10⁴ cells or 2–4 mg protein in cell suspension) and the SM standard (2 mg/mL) (*see Note 7*)
3. Sonicate each sample for 5 min on ice.
4. Add sphingomyelinase, 2 IU
5. Incubate for 2 h at 37°C.

- 6 Re-extract with 3 mL of diethylether, twice.
7. Pool the two diethylether extracts, dry under N₂ and store at -20°C (see Notes 8 and 9)

3.1.2. Hydrolysis of Sphingomyelin with HF

- 1 Dry the chloroform solution of SM cellular extract (1–2 μmol SM) or the SM standard in a polypropylene tube by passing gently N₂ gas so that the SM dries as a thin film at the bottom of the tube.
- 2 Add to the dry film HF 40%, 0.2 mL and allow the reaction to take place at 40°C for 72 h (see Note 9).
- 3 Partition the content of the tube with 1 mL chloroform:methanol (2.1, v/v), collect the lower organic phase (see Note 10)
4. Transfer the tubes to an ice bath and neutralize with ammonia

3.2. Gradient Elution HPLC for Separation of Benzoyl Nonhydroxy Fatty Acid-Ceramide from Benzoyl-Hydroxy Fatty Acid-Ceramide

1. Start up. Preclean the column and prime (equilibrate) with dioxane, 2.8% in hexane at a flow rate of 2 mL/min, followed by a 20-min linear gradient, 0–10%, at 2 mL/min
2. Set up in-line UV monitor at 230 nm.
- 3 Load the injection loop with 10 μL, B-CER (10–50 nmol) dissolved in 100 μL of carbon tetrachloride or, separately, load 10 μL standard B-NFA-CER or B-HFA-CER, prepared in tetrachloride, in the same way as the B-CER samples. The samples are first passed through a filter (0.5-μm) to remove particulate matter.
4. Turn the injection valve to bring the sample loop in line with the column
5. Elute 3 min with a linear gradient of 2.8–5% dioxane in hexane (v/v). The gradient is initiated at the time of injection at a rate of 4 mL/min (see Note 11)
6. B-NFA-CER elutes at 2 min, followed by B-HFA-CER, which elutes at 3 min (Fig. 4).
- 7 Collect the fractions and use immediately for argentation chromatography.
- 8 Regenerate the column to its initial polarity by reversing the gradient for 1 min and equilibrating the column with 2.8% dioxane in hexane (v:v) for 4–5 min.
9. Once the column resolution deteriorates, wash successively with methanol, dichloromethane, and hexane (see Note 12).

3.3. Argentation Chromatography for Separation of Saturated Fatty Acid-Ceramide and Monounsaturated Fatty Acid-Ceramide

1. Start up: Preclean the column and equilibrate with methanol:isopropanol (8.2, v/v). Run the solvent at a flow rate of 0.5 mL/min.
2. Set up in-line UV monitor at 230 nm.
3. Load the injection loop with 10 μL filtered BSM (10–50 nmol) or B-HFA-CER or B-NFA-CER sample, dissolved in methanol-isopropanol solvent.
4. Proceed with standard HPLC procedures.

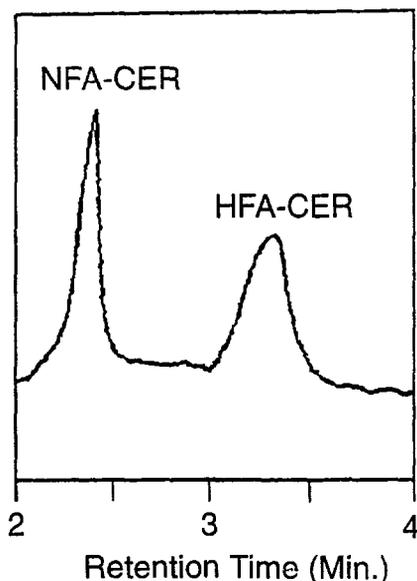


Fig. 4. Gradient-elution HPLC of benzoylated CER (8 nmol) on a Zipax column (elution with a linear gradient of 2.8–5.5% dioxane in hexane [v/v] at 4 mL/min, detection at 230 nm). Separation of NFA-CER from HFA-CER.

5. Elute with methanol:isopropanol (8:2, v/v) pumped isocratically at a flow rate of 1 mL/min for B-SM. Elute with hexane:isopropanol (9:1, v/v) at a flow rate of 0.5 mL/min for B-NFA-CER and at a flow rate of 1.5 mL/min for B-HFA-CER.
6. Two fractions are obtained, one with saturated FA at 10–20 min retention time and one with unsaturated FA at 30–38 min retention time (Fig. 5, shown for B-SM).
7. Collect the fractions.
8. Proceed with regular, end-of-analysis shut-down, carrying all the column cleaning procedures necessary.

3.4. RP-HPLC for Separation of MS of Benzoyl Sphingomyelin or Benzoyl Ceramide

1. Filter and degas the potassium phosphate buffer adjusted at pH 7.4.
2. Prime both the guard and the main column with methanol:acetonitrile:potassium phosphate buffer (100:20:1, v/v/v), and pump the solvent isocratically (without injection of a sample) at 1 mL/min.
3. Add (apply) samples obtained from argentation chromatography (Subheading 3.3.).
4. Turn the injection valve to bring the sample loop in line with the column.
5. Pump the solvent isocratically at a flow rate of 2 mL/min.
6. Set up the UV monitor at 230 nm.
7. Collect the fractions (see Fig. 3 and Note 13).

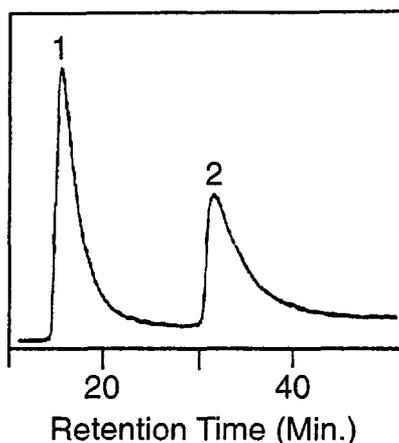


Fig. 5. Argentation chromatography of B-SM (40 μ g) from neuroblastoma N1E 115 cells on Chromopak silver column, solvent: methanol:2-isopropanol (8.2, v/v), flow rate 1 mL/min. Peak 1, as analyzed by GC, contains only saturated FA (16:0, 18:0, 22:0, 23:0, 24:0, 25:0, 26:0). Peak 2 contains only monoenoic FA (23:1, 24:1, 25:1, 26:1)

4. Notes

- 1 The mobile phase methanol:potassium phosphate buffer, 5 mM, pH 7.4 (9:1, v/v) has to be used for separation of intact SM molecular species. Then follow exactly Subheading 3.1.5. in Chapter 23.
- 2 The differences in the hydrophobic interactions between the SM and the ligand of the stationary phase are mostly because of the nature of the double bond (*cis-trans*). The double bond in the sphingoid is *trans*, whereas most of FA side chains have *cis* double bonds. As expected on the basis of their conformational differences, *cis* unsaturated FAs have shorter retention times than the corresponding *trans* unsaturated FAs.
- 3 The benzylation reagent can be simplified to a 0.5 mL benzoic anhydride in tetrahydrofuran and incubation at 20°C for 2 h. For purification of the benzyolated product (B-SM) follow exactly the method described in Subheading 3.2.2. of Chapter 23. After silicic acid fractionation, the methanol fraction contains the 3-*O*-benzyolated SM (B-SM).
- 4 The precautions necessary when working with toxic substances, such as working under an efficient hood with good aeration, behind a shield, and wearing protective clothes have to be taken when using this solvent system.
- 5 **Figure 3A** shows that SM with monounsaturated FA can not be separated from SM with a saturated FA that has two less carbon atoms.
- 6 For the separation of SM from PLs, follow the method described in Subheading 3.5. of Chapter 22. Only by HPLC can SM be separated efficiently from PC.

- 7 Several drawbacks affect the enzymatic conversion and lower the reliability of the procedure, including the presence of contaminating lipases in the crude lipid extract, or even in the sphingomyelinase
- 8 The enzymatic reaction converts only 40–50% of SM into the product CER
- 9 At 0°C only 15% of SM is hydrolyzed in 24 h, whereas under the same conditions complete degradation of PC to DAG is observed. Thus, the reaction of SM with HF is much slower than the reaction of PLs with HF. At 40°C complete hydrolysis (90–95%) occurs in 72 h
- 10 The extent of hydrolysis of SM is determined by TLC on silica gel G and formation of a single lipid-soluble product (CER) with an R_f of 0.45 and with the TLC plates developed in chloroform:methanol (95:5, v/v), and the plate stained with iodine vapors.
11. The viscosity of dioxane at 20°C is high enough to require higher pressure for delivery through the column
- 12 Amounts as low as 10 pmol of B-CER present in the injection sample can be detected and quantitated.
- 13 In general, the B-NFA-CER samples are resolved in four to five peaks. When “shoulders” appear on peaks, repeating the chromatography with a more nonpolar solvent, like changing the ratio of hexane to isopropanol from 9:1 to 9.5:0.5 may help. However, for better resolution of these minor peaks, the flow rate has to also be lowered to 0.5 mL/min and the time required for the latter resolution is twice as long. As the amount of CER in these better-resolved minor peaks is too small for accurate GLC analysis of the fatty acids and long-chain bases, the determination of the composition of these pairs must be based more on the location of the peaks (e.g., peaks in the front of the chromatograms may be caused by sphingenine-containing CER with saturated and monounsaturated FA, whereas peaks further up in the chromatograms could be caused by CER with di- and tri-unsaturated FA). Thus, accurate assignment of these minor peaks remains in doubt.

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Solubilization and Assay of Cellular and Tissue Protein

Cynthia E. Shaw

1. Introduction

Solubilization and accurate quantification of cellular protein are central to analytical methods such as Western analysis. Alternate homogenization buffers can be used according to need, but must match the assay used. The Bio-Rad (Hercules, CA) assay, based on the method of Bradford (1), is faster, but is not able to tolerate SDS although it can tolerate β -mercaptoethanol. In contrast the bicinchoninic acid (BCA) assay (2) has greater linear range, and has greater tolerance for SDS but not β -mercaptoethanol. However, there is also less tolerance for a large sample volume. Salt buffers can also be used with PMSF, leupeptin, or aprotinin added in each assay, as long as the interfering reagents are omitted as appropriate. In this chapter, procedures are described for both assays, scaled for use with a microtiter plate and plate reader. If a plate reader is unavailable, however, the same assays can be run in a visible-wavelength spectrophotometer, but with all assay/sample volumes scaled accordingly to match the cuvet volume used.

2. Materials

2.1. General Equipment

1. Polytron or glass homogenizer
2. Cell scrapers.
3. Sonicator
4. Microfuge.
5. 14-mL Polypropylene tubes.
6. 1.5-mL Microfuge tubes.
7. 96-well Microtiter plates.
8. P2, P20, P200, and P1000 pipets, and tips.

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Table 1
Bio-Rad Protein Assay Standards Preparation

Std #	μg/10 μL	μg/1.0 mL	μL water	μL BSA (2 mg/mL)
S1	0.0	0	2000	0
S2	0.5	50	1950	50
S3	1.0	100	1900	100
S4	2.0	200	1800	200
S5	3.0	300	1700	300
S6	4.0	400	1600	400
S7	5.0	500	1500	500

Freeze standards as 200-μL aliquots at -20°C, sufficient for 20 assays each.

- 9 8-Channel pipet, 12-channel pipet, or repeat dispensing pipet if available
10. Plate reader or spectrophotometer capable of reading absorbance at 570 or 595 nm

2.2. Reagents

2.2.1. Bio-Rad and BCA Assay

1. 1000X Stock PMSF, 0.1 M in EtOH, stored at -20°C
2. 1000X Stock aprotinin, 5 mg/mL in water, stored at -20°C
3. 1000X Stock leupeptin, 5 mg/mL in water, stored at -20°C
4. Prepare a 10-mg/mL stock of BSA for long-term storage (200 mg in 20 mL water as 1 mL aliquots). Dilute 0.4 mL of stock to 2.0 mL volume to use as 2 mg/mL stock for standards preparation (*see also Note 1*).

2.2.2. Bio-Rad Protein Assay Only

1. 1X Salts buffer: Prepare 50 mM Tris base, 10 mM EDTA, 150 mM NaCl, pH to 7.4, and add 0.1% (v/v) Tween-20. Store at 4°C.
2. β-Mercaptoethanol, store at 4°C
3. Bio-Rad protein assay reagent (Bio-Rad cat. no. 500-0006): Dilute with water, 1 vol reagent to 4 vol water. Filter through Whatman (Clifton, NJ) no. 1 filter paper. Store at 4°C.
4. To prepare standards, dilute 2 mg/mL bovine serum albumin (BSA) according to **Table 1** (*see also Note 1*)

2.2.3. BCA Protein Assay Only

1. 5X Salts Buffer: 750 mM NaCl, 250 mM Tris-HCl, 2.5 mM MnCl₂, 2.5 mM MgCl₂, 25mM EGTA, pH to 7.4. Store at 4°C.
2. 10X SDS stock: 10% SDS, pH to 7.4. Store at room temp.
3. BCA reagents A and B (Sigma, cat. no. B9643 and C2284).
4. To prepare standards, dilute 2 mg/mL standard as in **Table 2** (*see also Note 1*)

Table 2
BCA Protein Assay Standards Preparation

Std. #	$\mu\text{g}/10 \mu\text{L}$	$\mu\text{g}/1.0 \text{ mL}$	μL water	μL BSA (2 mg/mL)
S1	0	0	1000	0
S2	0.25	25	987	12.5
S3	0.5	50	975	25
S4	1.0	100	950	50
S5	2.5	250	875	125
S6	5.0	500	750	250
S7	7.5	750	625	375
S8	10.0	1000	500	500

Freeze standards as 200- μL aliquots at -20°C , sufficient for 20 assays each

3. Methods

3.1. Sample Preparation

3.1.1. Lysis Buffers Preparation

Prepare lysis buffer according to the protein assay you intend to use. For BCA assay lysis buffer, all steps involving use of lysis buffer should be carried out at room temperature. For Bio-Rad (Bradford) assay lysis buffer, keep reagents and samples on ice at all times (*see Note 2*).

3.1.1.1. BIO-RAD ASSAY

- 1 Prepare lysis buffer fresh by adding 1 $\mu\text{L}/\text{mL}$ each of stock aprotinin, PMSF, leupeptin, and β -mercaptoethanol to the salt buffer. Keep on ice.

3.1.1.2. BCA ASSAY

- 1 Prepare lysis buffer fresh by mixing 2 vol of salts buffer with 7 vol of water. Then add 1 vol of SDS stock (*see Note 3*).
- 2 Add 1 $\mu\text{L}/\text{mL}$ each of stock aprotinin, PMSF, and leupeptin solutions to the salt buffer. Keep at room temperature (*see Note 2*).

3.1.2. Tissue Homogenization

1. Obtain fresh tissue or allow frozen tissues to partly thaw on ice. If dissection is required, this can be carried out while tissue is still partly frozen if using a sharp scalpel.
2. Cut tissue to approx 3-mm cubes and transfer one cube to a sterile polypropylene tube (14-mL). Add lysis buffer to 1 mL (for approx 100 mg, or as convenient). Immediately polytron tissue for 30 s (repeating if necessary until the tissue is disrupted, but chill on ice between 30 s grinds).
3. Dispense 4 \times 200- μL aliquots to separate tubes and make each up to 1 mL with lysis buffer. Sonicate tissue samples, and immediately spin for 3 min at 12,000g in microfuge (to pellet insoluble tissue fragments).

Table 3
Lysis Buffer Volumes Recommended for
Common Cell Culture Dishes

Plate/dish size	Recommended volume/ well or dish, μL
24 well	25
12 well	50
6 well	100
35 mm	100
60 mm	200
100 mm	500
T75	500

4. Take off 0.9 mL supernatant from each to clean tube, mix, and dispense in 0.5-mL aliquots to microfuge tubes for storage. Retain 50 μL for protein assay (assay 10 μL , both undiluted and diluted 10-fold). Store remaining samples at -20°C , or long term at -70°C .

3.1.3. Cell Lysis

1. Aspirate medium from wells/plates using a fine-point pipet tip or blunt needle attached to a vacuum line. Replace with cold, serum-free balanced salt medium (0.9% NaCl, PBS or equivalent) to remove any residual serum/protein.
2. Aspirate medium completely from wells
3. Add lysis buffer to each well (see **Table 3** for volumes) and scrape cells from plate using a cell scraper, or plunger from a 1-mL syringe
4. Collect all lysate carefully and transfer to storage tube.
5. Sonicate briefly to complete solubilization.
6. Assay 1–2 μL for protein if cells were near-confluent (see **Note 4** and **Table 3**)

3.2. Bio-Rad Protein Assay Procedure

See **Note 4**. For plate layout, see **Fig 1**. Samples should not exceed 5% of total volume (see **Note 5**).

1. Set-Up: Thaw BSA standards and samples. Prepare sheet with sample layout of 96-well plate. Turn on plate reader. Warm-up takes 15 min
2. Add 10 μL (or sample vol if less; see **Note 5**) sample buffer to triplicate standard wells S1 through S7 (there is no S8 in this assay)
3. Add 10 μL water to each triplicate sample well (U1–U24).
4. Add 10 μL of standards to standard wells S1–S7 working from lowest to highest concentration: 0, 0.5, 1, 2, 3, 4, and 5 $\mu\text{g}/\text{well}$
5. Add samples (10 μL , or sample vol if less, see **Note 5**) to triplicate sample wells U1–U24.
6. Add 200 μL Bio-Rad protein reagent to each well using a “repeating” or “multi-channel” pipet.

Standards			Unknowns			Unknowns			Unknowns		
S1	S1	S1	U1	U1	U1	U9	U9	U9	U17	U17	U17
S2	S2	S2	U2	U2	U2	U10	U10	U10	U18	U18	U18
S3	S3	S3	U3	U3	U3	U11	U11	U11	U19	U19	U19
S4	S4	S4	U4	U4	U4	U12	U12	U12	U20	U20	U20
S5	S5	S5	U5	U5	U5	U13	U13	U13	U21	U21	U21
S6	S6	S6	U6	U6	U6	U14	U14	U14	U22	U22	U22
S7	S7	S7	U7	U7	U7	U15	U15	U15	U23	U23	U23
S8	S8	S8	U8	U8	U8	U16	U16	U16	U24	U24	U24

Fig. 1. Plate layout for 96 well microtiter plate assay. S1-S8 denote positions of standards 1-8, and U1-U24 denote layout of unknown samples, all assayed in triplicate

7. Place plate in plate reader Set up Bio-Rad program:
 - a. Single wavelength, 595 nm
 - b. Mix for 2-5 s
 - c. Delay: at least 5 min, no more than 1 h.
8. Save or print results as appropriate, and analyze as in **Subheading 3.4.**
9. It may be necessary to dilute some samples and rerun Always use new std curve if fresh reagent is mixed.

3.3. BCA Protein Assay Procedure

See **Note 4.** For plate layout, see **Fig. 1.** Samples should not exceed 2.5% of total volume (see **Note 5**).

1. Set-up. If available, set oven or incubator at 37°C. Thaw BSA standards and samples. Prepare sheet with sample layout of 96-well plate.
2. Add lysing buffer to each standards well only. The volume used will depend on the sample volume used (see **step 5** and **Note 5**).
3. Add 10 μL deionized water to each sample well (U1-U24).
4. Add 10 μL of BSA standard to appropriate standard wells S1-S8 working from lowest to highest concentration. 0, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 $\mu\text{g}/\text{well}$
5. Remix each sample before dispensing to wells (U1-U24) in triplicate. Use same volume for each as in **step 2**, and no more than 5 μL (see **Note 5**).
6. Calculate the volume of BCA reagent needed to add 200 $\mu\text{L}/\text{well}$. A full plate requires 20 mL. Mix 1 part (0.4 mL) reagent B plus 50 parts (20 mL) reagent A. Dispense 200 $\mu\text{L}/\text{well}$.
7. Incubate for 30 min at 37°C, or 2 h at room temperature. Turn on plate reader 15 min before assay ends
8. If incubated at 37°C, allow samples to cool 5-10 min. Read immediately at 562 nm (for plate reader use 570-nm filter, single wavelength).
9. Analyze data as for the Bradford assay.
10. It may be necessary to dilute some samples and rerun Always use new standard curve if more reagent is mixed

3.4. Calculations

- 1 Calculate the mean absorbance for the zero standards and subtract from all other values.
- 2 Calculate the means of the triplicate determinations for each standard.
- 3 Graph the mean absorbance for each standard against the protein for each standard, and perform a linear regression fit. For a good assay, the line should pass through the origin without being forced. The r^2 value should routinely exceed 0.95 for both assays.
- 4 Calculate.

$$\text{Protein value in sample} = (\text{absorbance} - \text{intercept})/\text{gradient}$$

4. Notes

1. Both Bio-Rad and Sigma sell standardized protein solutions, but check the concentration of the stocks and adjust dilutions accordingly.
2. The SDS in the lysis buffer for BCA protein assay precipitates out of solution when samples or lysis buffer are kept on ice. This is the reason stock salts buffer is stored at 4°C without SDS added. Thus samples are actually more stable at room temperature unless frozen solid for long term storage. In contrast, the Tween-20 in the lysis buffer for the Bio-Rad protein assay remains in solution when cold, and so samples and lysis buffer should be kept on ice.
3. Because of the poor solubility of SDS in the BCA assay lysis buffer, it is important to prepare the lysis buffer by mixing the 2 vol salts buffer and 7 vol water first, then adding the 1 vol of SDS buffer. If SDS solution and 5X salts buffer are mixed first and water added last, solubilization can be achieved by warming to 37°C.
4. Both the accuracy and precision of these protein assays is entirely dependent on accuracy and precision of pipeting. Be sure to use calibrated pipets for all steps, but, when using sample volumes of 1–2 μL , the use of a P2 pipet (Gilson) or equivalent is recommended.
5. For both assays, the accuracy of the values obtained also depends on the premise that each well contains identical amounts of lysis buffer, water, and reagent. This is because lysis buffer in particular can raise the baseline absorbance even without protein present. The limits given (2.5% sample volume for Bio-Rad protein assay and 5% total volume for BCA assay) are the point at which the baseline is raised sufficiently to render the linear range almost unusable. Best results are obtained in both assays if the sample volume is well below these limits, but it is also of vital importance therefore that the same volume of lysis buffer is added to standard wells S1–S8, for blanking purposes, as is used for sample volume in the sample wells U1–U24.

Acknowledgments

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Western Immunoblot Analysis

Cynthia E. Shaw and Jing Zheng

1. Introduction

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1) combined with Western immunoblot analysis (2,3) is a widely used technique for quantitatively and qualitatively evaluating specific protein expression. This chapter describes a Western immunoblot technique routinely used in our laboratories for protein analysis with representative data shown in Fig. 1. The protocols for SDS-PAGE and electrophoretic transfer are based on using the Mini-PROTEAN II system (Bio-Rad, Hercules, CA). Protein detection is based on using the enhanced chemiluminescence (ECL) detection method (Amersham, Arlington Heights, IL). In this technique, mixtures of solubilized proteins under denaturing conditions (in the presence of detergent [SDS], and reducing agent [mercaptoethanol]) are separated by electrophoresis based on their molecular weights, followed by transferring proteins to PVDF membrane and identifying a protein of interest using a specific antibody. It is highly recommended that, before beginning the protocol, the operator read **Subheading 4**.

When using Western blotting techniques to quantify a protein of interest by densitometry from film, it is important to check that the quantification is truly linear for the sample loading and exposure time of interest since film is nonlinear at extremely high or extremely low levels of exposure. We recommend performing a test blot with a serial dilution of a single sample or standard and then checking the correlation between signal and loading to establish the linear range. An optimized Western blot probed for endothelial cell nitric oxide synthase in human endothelial cells is shown (Fig. 2).

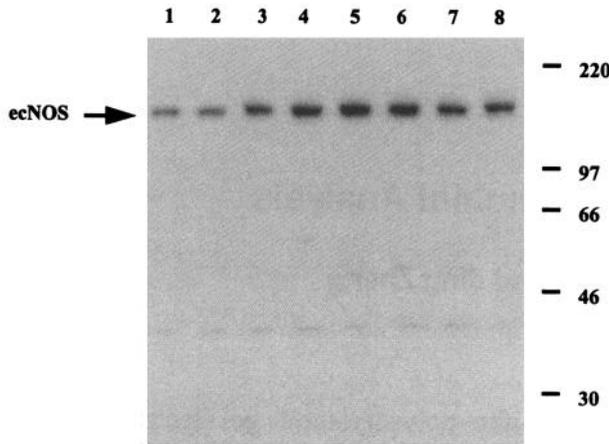


Fig. 1. Western immunoblot analysis of protein expression of eNOS in ovine fetoplacental artery endothelial cells in culture: Proteins ($2 \mu\text{g}$ protein/lane) were separated by electrophoresis (7.5% SDS-PAGE gels). After transfer to the membrane, immunoblotting was performed using a mouse monoclonal ecNOS antibody (1:750 [$0.33 \mu\text{g}/\text{mL}$]; Transduction Laboratories, Lexington, KY) with ECL detection at 5-min exposure, as described. Lanes 1–2, controls; lanes 3–8, cells treated with basic fibroblast growth factor (1 or 10 ng/mL) for 24 h.

2. Materials

All to electrophoresis grade where available:

1. MiniProtein II Electrophoresis Cell and Transfer Apparatus (Bio-Rad).
2. Gel combs 10 or 15 wells ($\times 2$).
3. Power supply (200/2.0 [Bio-Rad]) or equivalent.
4. Rocking platform.
5. Heating block or boiling water bath.
6. Vacuum line.
7. Glass plates and 1.0-mm gel spacers.
8. Immobilon-P (PVDF) membrane (Millipore, Bedford, MA).
9. "Rainbow" molecular-weight markers (Amersham).
10. Filter papers (Bio-Rad).
11. Acrylamide.
12. *Bis*-acrylamide.
13. Tris-base.
14. Ethylene-diamine tetra-acetic acid(EDTA) (disodium salt).
15. SDS.
16. Glycine.
17. Ammonium persulfate.

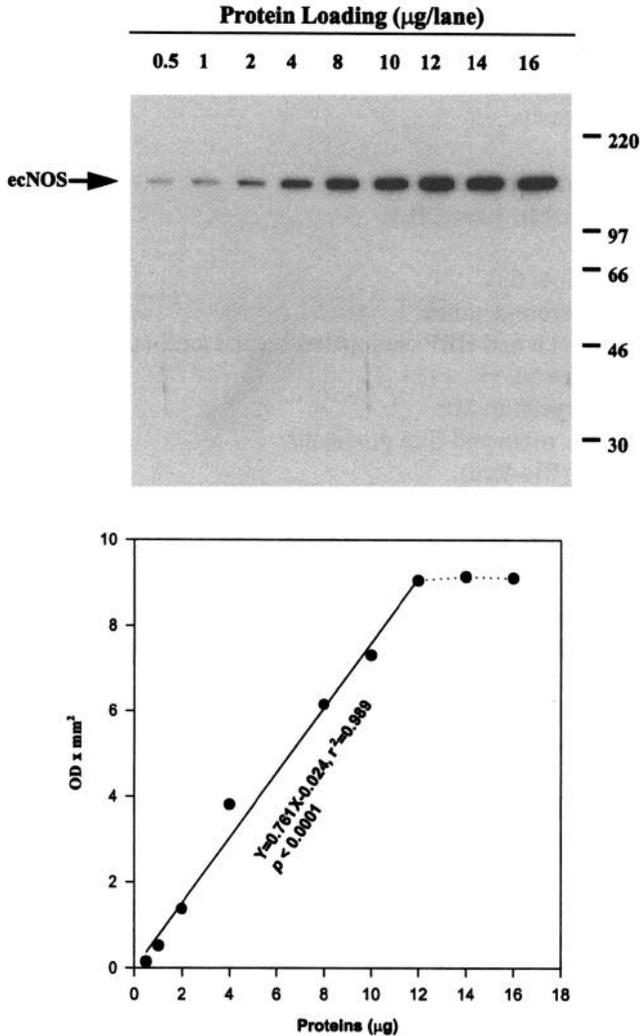


Fig. 2. Validation of quantification of Western analysis for protein expressions of ecNOS in human umbilical vein endothelial cells: Proteins obtained from endothelial cell lysates were serially diluted and separated by electrophoresis (7.5% SDS-PAGE gels). After transfer to the membrane, immunoblotting was performed using ecNOS antibody with ECL detection, as described above. Levels of ecNOS were then quantified by scanning densitometry (Bio-Rad 670 scanning densitometer). *Note:* The linear increase in signal for ecNOS is only in the range of 0.5–12 but not 12–16 μg protein, at 5-min exposure.

18. *N, N', N'*-Tetramethylethylenediamine (TEMED)
19. β -Mercaptoethanol.
20. Glycerol.
21. Coomassie blue dye.
22. Tween-20.
23. Skim/low-fat milk powder.
24. Glass wool (Fisher, Itasca, IL)
25. Parafilm
26. Gel-loading pipet tips.
27. Eppendorf/microfuge tubes.
28. ECL detection kit and HRP conjugated second antibody (Amersham)
29. X-ray film cassette
30. Amersham Hyperfilm MP
31. Access to dark room and film processor
32. Densitometer (Bio-Rad).

2.1. Preparation of Assay Reagents (see Note 1)

2.1.1. Solutions for SDS-PAGE

1. 30.8% *bis*-acrylamide. 30% (w/v) acrylamide, 0.8% (w/v) *bis*-acrylamide. Dissolve 30 g acrylamide and 0.8 g *bis*-acrylamide in 100 mL deionized H₂O (di H₂O). Store at 4°C (see Note 1).
2. Resolving gel buffer: 1.5 M Tris base, 8 mM EDTA, 0.4% (w/v) SDS. Dissolve 90.75 g Tris base and 1.52 g EDTA with 2.0 g SDS in approx 450 mL di H₂O. Adjust pH to 8.8, and make up to 500 mL. Store at 4°C.
3. Stacking gel buffer: 0.5 M Tris base, 8 mM EDTA, 0.4% (w/v) SDS. Dissolve 6.0 g Tris base and 0.3 g EDTA with 0.4 g SDS in approx 75 mL di H₂O. Adjust pH to 6.8 and make up to 100 mL. Store at 4°C.
4. 20% Ammonium persulfate solution. Dissolve 10 mg per 50 μ L di H₂O before use.
5. *N, N, N', N'*-Tetramethylethylenediamine (TEMED). Store at 4°C.
6. 10% (w/v) SDS: 50 g SDS in 500 mL di H₂O, Adjust pH to 7.0.
7. 5X Electrophoresis buffer: 125 mM Tris base, 960 mM glycine, 0.5% (w/v) SDS. Dissolve 15.0 g Tris base with 72.0 g glycine and 50 mL of 10% SDS stock in approx 800 mL H₂O. Make up to 1000 mL. Store at 4°C. There is no need to adjust pH. Dilute to 1X with di H₂O immediately before use.
8. 5X Sample Buffer: 0.25 M Tris base, 10% (w/v) SDS, 50% (v/v) glycerol, 10 mM EDTA. First prepare 0.5 M Tris, pH 6.8 by dissolving 6.0 g Tris base in approx 75 mL di H₂O. Adjust pH to 6.8 and make up to 100 mL. Mix 5 mL 0.5 M Tris, pH 6.8 with 1.0 g SDS, 5 mL glycerol and 0.0372 g EDTA. Combine above in 20-mL glass scintillation vial containing stir bar. Heat briefly to 40°C to dissolve EDTA. Add a small amount of Coomassie blue dye (mg).

2.1.2. Preparation of Reagents for Transfer to PVDF Membrane

1. 10X Transfer buffer: 200 mM Tris base, 1.5 M glycine. Dissolve 48.4 g Tris base and 226.0 g glycine in di H₂O and make up to 2 L (see Note 2). 1X Transfer buffer

is then prepared from 10X stock as needed by diluting 10X stock to 1X working solution containing 20% (v/v) methanol (MeOH). Chill for 1 h at -20°C before use.

2.1.3. Preparation of Reagents for Immunoblotting

1. Buffer A: 20 mM Tris-base, 500 mM NaCl, pH 7.5. Combine 87.66 g NaCl with 7.27 g Tris base in 2.5 L di H₂O. Adjust pH to 7.5 and make up to 3.0 L.
2. Buffer B (wash buffer, normal): Buffer A, 0.1% (v/v) Tween-20 (*see Note 3*). Combine 1 L buffer A with 1 mL Tween-20 (*see Note 3*).
3. Buffer C (blocking buffer): Buffer B, 5% (w/v) powdered milk. Dissolve 10 g powdered milk in 200 mL buffer B. Filter through glass wool, dispense in 50-mL volumes, and store at -20°C .
4. Primary antibody buffer: Buffer B, 1% (w/v) BSA. Dissolve 2 g BSA (Fraction V) in 200 mL buffer B, dispense in 50-mL volumes, and store at -20°C .
5. Secondary antibody buffer: Buffer B, 0.5% (w/v) powdered milk. Prepare by diluting 20 mL buffer C to 200 mL final volume with buffer B. Dispense in 50-mL volumes and store at -20°C .

3. Methods

3.1. SDS-PAGE

3.1.1. Assembly of Glass Plates.

1. Clean glass plates and spacers thoroughly with water, and then 95% ethanol before assembly.
2. Cut a 1-cm wide strip of parafilm. Place rubber pads over red pads of a gel casting stand, and place (stretch) parafilm over gray pads.
3. Position glass plates in a clamp assembly with all screws opened as follows: acrylic block, a larger glass plate, two spacers (one on each side), and a small glass plate. Fasten screws hand-tight.
4. Place the assembled unit into the gel-casting stand, on top of the rubber pads and parafilm, with acrylic block facing inside. Make sure plates and spacers line up at bottom or it will leak! Push down firmly to click into position (*see Note 4*).
5. Number each gel and put mark 2.5 cm from the top of small glass plate.

3.1.2. Pouring the Resolving Gels

1. Prepare the resolving gel solution at a desired percentage of acrylamide as directed in **Note 5**. Add 30.8% bis-acrylamide, the resolving buffer glycerol, and di H₂O in a vacuum flask (*see Note 5*).
2. Clamp the flask securely to a stir plate. Degas by applying vacuum for 15 min while stirring (*see Note 6*).
3. For the volumes given in **Note 5**, add 10 μL TEMED and 45 μL 20% ammonium persulfate. Swirl after each addition.
4. Quickly transfer the mixture between glass plates to the pen mark using a transfer pipet. Immediately and very gently overlay gel with di H₂O.
5. Polymerize for 60 min (*see Notes 7 and 8*).

3.1.3. Pouring the Stacking Gels (3%)

- 1 Invert the gel-casting stand to drain off water overlay. Wash the top of the polymerized resolving gels gently with d H₂O. Invert again and remove excess water using the corner of a paper towel. Position the combs. Make sure combs are completely dry.
- 2 Make up 3% stacking gels. To a small, conical flask add 0.610 mL *bis*-acrylamide (30:0.8), 1.520 mL 0.5 M Tris (stacking gel) buffer (pH 6.8) and 3.870 mL d H₂O. Degas 5 min. This is enough for two gels.
- 3 Add 10 μ L of TEMED and 30 μ L of 20% ammonium persulfate. Swirl after each addition.
- 4 Quickly transfer the mix down the side of the comb on the top of the resolving gel using a fine-tipped transfer pipet. Immediately displace any air bubbles beneath comb.
- 5 Polymerize 45–60 min (see Note 7). Squirt a small amount of electrophoresis buffer on top of the stacking gel and remove combs by lifting vertically.
- 6 Rinse the wells with electrophoresis buffer and fill the wells with electrophoresis buffer.

3.1.4. Preparation and Loading of Samples

- 1 Thaw molecular-weight rainbow markers and samples on ice (both should be stored at -20°C) (see Note 9). Turn on boiling water bath or heat block.
- 2 Prepare sample buffer by prewarming buffer to ensure complete solution, and adding 5 μ L β -mercaptoethanol to 0.5 mL of 5X sample buffer. Mix before use.
- 3 Label microfuge tubes and pipet calculated volume of sample into each tube. Also prepare a tube with molecular-weight markers (10 μ L for 10-well comb, 5 μ L for 15-well comb) and empty tubes for blank lanes.
- 4 Adjust sample volumes with d H₂O such that they are equal in volume, preferably to a total volume of 10–20 μ L (up to 40 μ L for a 10-well comb, and 25 μ L for a 15-well comb). Prepare blank samples with an equal volume of water for all other lanes.
- 5 Add calculated volume of 5X sample buffer (5 μ L for a 20- μ L sample) to make a final concentration of 1X.
- 6 Close caps, punch hole in cap with an 18-gage needle.
- 7 Centrifuge a few seconds to bring sample down in bottom of the tube.
- 8 Place tubes in boiling water for 3 min or heating block (75°C) for 5 min.
- 9 Centrifuge a few seconds to concentrate sample in bottom of the tube.

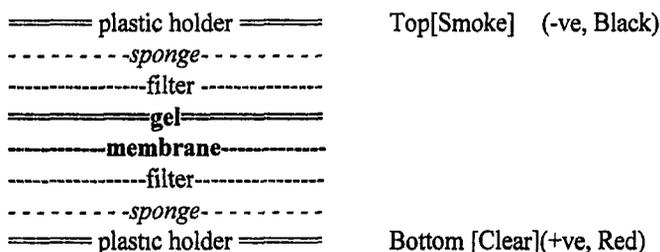
3.1.5. Electrophoresis (see Note 10)

- 1 Fill lower chamber two-thirds full with electrophoresis buffer. Fill gaskets of electrode/plate assembly with d H₂O.
- 2 Take the assembled gel units off the gel-casting stand and snap into position on the electrode assembly, with the smaller glass plate facing in and acrylic block on the outside. Place the electrode assembly into the tank.
- 3 Fill inner reservoir to halfway between the top edges of the small and large glass plates. Check assembly for leaks; in case of leaks, repeat the electrode assembly.

4. Add more electrophoresis buffer to outer chamber, to 1 cm below top of inner/lower plate, to equalize pressure. To prepare a "buffer dam," which is used if you will run only one gel, assemble glass plates in block as above, except omitting the spacers
5. Using gel-loading pipet tips, add sample buffer to any blank lanes first, then add molecular-weight markers. Finally add samples/standards to remaining lanes
6. Align electrode leads, labeled red and black, with red and black power terminals (see **Note 11**). Put top over electrophoresis unit, connect leads to power unit
7. Run at constant 100 volts until blue dye front reaches end of gel (approx 2 h). The resistance for a 7.5% gel should be 30–40 mA at the beginning and should be 12–15 mA at the end of the electrophoresis for one gel apparatus, and double for two run at the same time (**Note 12**).
8. When the gel electrophoresis has begun, prepare the ice pack and 1X transfer buffer for the transfer apparatus. Store transfer buffer in cold room to chill before use.

3.2. Electrophoretic Transfer to PVDF Membrane (see **Note 10**)

1. At the end of the run, turn off power unit and disconnect electrodes. Prepare two dishes containing cold transfer buffer.
2. Disassemble plate assembly. Remove clamps and spacers. Separate glass plate carefully and then remove the stacking gel by scraping upwards with a gel spacer
3. Notch the upper right-hand corner of the gel then carefully pick up at corners/edges (use gloved hands and, if necessary, lift a corner of the gel using a wet gel spacer first) and transfer into dish containing transfer buffer
4. Soak gels in 1X transfer buffer for 15 min at room temp with very slow or no agitation.
5. Wash electrophoresis unit with tap and then distilled H₂O. Clean glass plates with detergent, then rinse by soaking with distilled H₂O.
6. While gels are soaking, prewet sponges (two per gel) and filter papers (two per gel) in transfer buffer. Prewet Immobilon P membrane (cut to size) in methanol (Important!), and then di H₂O
7. Fill half of the electrophoresis tank with the transfer buffer.
8. After 15-min incubation of gels, set up the following layers:



Before each subsequent layer is added, pour a little buffer over the uppermost surface so air is excluded with each addition. If air becomes trapped, remove by rolling a wet 10-mL pipet over the surface.

9. When complete, snap hinge in place and insert cassette into apparatus such that gel (top) is on black cathode (–) side and the membrane (bottom) in on the red anode (+) side
10. Place frozen ice pack into transblot tank.
11. Add more transfer buffer in the tank. Do not overfill the tank. Buffer should cover transfer cassettes, but should not contact the electrodes.
12. Turn on power unit to 30 V, and run at constant voltage overnight (15 h). Beginning resistance should be between 30 and 40 mA, and should be between 50 and 60 mA at the end of the transfer. The resistance will double if two gel apparatus are run at the same time (*see Note 14*).
13. Turn off power unit. Disassemble cassettes one at a time. Rinse briefly in d H₂O, and blot away excess H₂O (*see Notes 15 and 16*)
14. Mark each colored molecular-weight marker band with a pencil. Notch corner of membrane, and store frozen in plastic wrap if required or block overnight in milk buffer C and incubate with antibody immediately
15. Rinse apparatus and components with tap H₂O, and then H₂O.

3.3. Immunoblotting (*see Note 17*)

It is important in Western-blotting applications to optimize both the first and second antibody concentrations. The second antibody is initially screened alone (i.e., without first antibody incubation at all) on a test blot with the protein of interest (loaded with 20 µg protein per lane if no further information is available on loading). This is to ensure that no bands are “nonspecifically” detected near the molecular weight of the protein of interest. If they are, then another source of antibody must be used. Initially this is performed at the dilution recommended by the manufacturer.

The dilution at which the first antibody is used will depend on the antibody preparation. If in doubt, run multiple lanes of a known standard (20 µg each) and cut the resulting membrane into strips. Then for each strip try a range first of 1:250–1:5000. Choose the dilution that gives the strongest signal combined with the lowest background.

Finally, the second antibody dilution is reoptimized. Again a blot of a replicated standard or sample is run and cut in lanes. All strips are incubated at the optimum first-antibody dilution, and the a serial dilution of the second antibody is tested on the strips, initially spanning 1:250–1:1000-fold. The dilution giving the strongest signal with the cleanest background is used for future blotting applications.

3.3.1. Antibody Binding to Membrane

1. Dilute primary antibody specific to the protein of interest, in the primary antibody buffer, according to specific instructions; or, thaw reserved primary antibody (*see Note 18*)

2. Rinse the membrane with buffer A to remove transfer buffer (*see Note 20*)
3. Block the membrane with buffer C for 2 h, or longer, rocking gently at room temperature.
4. Incubate membrane 2 h in the primary antibody at room temperature. If protein is not abundant, incubation can be continued overnight at 4°C.
5. Briefly rinse the membrane with buffer A to remove excess primary antibody
6. Wash the membrane: buffer B, 15 min; buffer B, 3 × 5 min.
7. Choose horseradish peroxidase (HRP)-conjugated antibody specific to species used to raise primary antibody; for example, goat-antimouse, or goat-antirabbit. Dilute secondary antibody in the secondary-antibody buffer, according to supplier's recommendation (and experimental results). Incubate the membrane in the second antibody for 1 h at room temperature.
8. Wash the membrane: buffer A; 5 min, buffer B, 15 min; Buffer B, 3 × 5 min; and buffer A, 3 × 5 min (*see Note 21*).

3.3.2. ECL Detection of Bound Antibody

1. Mix ECL reagents 1:1 immediately prior to use. For one minigel membrane, use 10 mL total volume, or 5 mL each of reagent 1 and reagent 2
2. Drain excess buffer from the membrane by blotting the membrane on clean paper towel.
3. Place the membrane protein-side up in clean ECL-only dish (*see Note 19*) Add mixture of ECL reagents to protein-side of the membrane. Gently rock the membrane by hand to keep surface evenly covered; do not allow surface of membrane to be uncovered
4. Incubate for exactly 1 min
5. Drain excess ECL reagents from the membrane on clean paper towel, but do not allow the membrane to dry.
6. Place the membrane with protein-side down on clean plastic wrap. Carefully wrap membrane and smooth out air pockets.
7. Place membrane in autoradiography cassette with protein-side up. Tape opposite edges to the intensifying screen, to ensure that the blot will not slip, and to ensure that it does not stick to the film.
8. Expose the membrane to ECL hyperfilm for a desired time (generally 1–15 min) in darkroom, as required. It is safe to work under a red safety light. The ECL reaction remains approximately linear for up to 30 min.
9. Cover film cassette with black bag to prevent light leaks. Develop the film in darkroom.
10. Lay the film over the membrane and mark positions of each molecular-weight marker

3.4. Membrane Reprobing

While Northern and Southern blots are easily stripped and reprobed because of the UV crosslinking of RNA or DNA to the membrane at the time of preparation, this is not so for Western blots. Stripping procedures have been

described by Kaufmann et al. (4), but in our experience a significant proportion of bound protein is lost. However, because of the lability of the horseradish-peroxidase enzyme used for the ECL procedure described here, reprobing can be performed following freezing of the membrane overnight, which destroys activity of bound enzyme, and reblocking of the membrane for 1–2 h with the blocking buffer. The best results are obtained if the reprobing uses a new primary antibody raised in a different species, so the appropriate second antibody does not encounter any residual primary antibody from the first probing. However, if probing and reprobing with antibodies raised in the same species, the signal from the first probing will still be diminished by extensive reblocking. It will obviously help if the second probed signal is much stronger than the first. In our experience, membranes can be reprobbed up to three times before signal loss becomes excessive. The time between probings is not apparently relevant as long as membranes are stored wrapped and frozen at -20°C .

4. Notes

1. Electrophoresis-grade chemicals should be used, because impurities may interfere with the gel polymerization and electrophoresis mobility. Acrylamide monomer is neurotoxic, so wear gloves and mask when handling it. Take similar precautions when weighing SDS.
2. For high-molecular-weight proteins (>150 kDa), SDS (0.1%; w/v) may be added to transfer buffer. However, SDS may interfere with protein binding to transfer membrane.
3. Sufficient Tween-20 in buffer B is critical for reducing background. Tween-20 has a density of approx 1.0. Because Tween-20 is very viscous, we recommend taking up in a pipet tip and then ejecting the whole tip into buffer B with mixing for at least 30 min. For a more stringent wash buffer, we recommend buffer A, 0.05% NP 40 (tergitol, w/v), 0.05% SDS (w/v), 0.125 sodium desoxycholate (w/v).
4. To check leakage, you may add dH_2O between glass plates. After checking, drain excess H_2O . If leakage occurs, reassemble glass plate.
5. A recipe for commonly used resolving gels is given in **Table 1**.
6. If using an aspirator, connect vacuum hose first, then turn on water. Remove hose from flask before turning off water. Best results are obtained if gel solution reaches room temperature before degassing.
7. After polymerization, a sharp optical discontinuity will be visible at the interface between the gel and water. Failure of gel polymerization is generally caused by aged AP and/or TEMED. If polymerization failure occurs, reprepare fresh acrylamide mixture with a new batch of ammonium persulfate or/and TEMED.
8. If gels are not used on the same day, cover with parafilm and store for 1–2 d at 4°C .
9. Total sample protein per lane should be between 10 and 50 μg , in less than 40 μL for a 10-well comb (adjust accordingly for other combs). The ideal amount depends on your preferred detection system and the relative abundance of your

Table 1
Recipe for Common Resolving Gels^a

Stock solution	Final acrylamide concentration ^b					
	4.0%	6.5%	7%	7.5%	10%	12%
30.8% bis-acrylamide	2.60	4.22	4.54	4.86	6.49	8.12
Res. gel buffer	5.00	5.00	5.00	5.00	5.00	5.00
d ₁ H ₂ O	9.07	7.45	7.13	6.81	5.18	3.55
Glycerol	3.33	3.33	3.33	3.33	3.33	3.33

^aThe acrylamide-bis is reserved at 4°C. This recipe makes 20 mL, enough for two minigels

^bUnits in the table are mL.

target protein. Coomassie-blue staining is relatively insensitive, and can detect 0.1–1.0 mg of protein per band, whereas ECL can distinguish as little as 1 pg per band (depending on the specificity of the primary antibody). In addition to molecular-weight standards, it is a good idea to run a sample containing the protein of interest as a control.

10. Never handle high-voltage leads unless the voltage is turned down to zero and the power supply is turned off.
11. If you reverse the leads, your samples will migrate out of the wells and will be lost in the electrophoresis buffer.
12. Leakage between upper and lower chamber of the electrophoresis apparatus could change resistance out of these ranges. During electrophoresis, check levels of the electrophoresis buffer in the upper chamber frequently. If leakage occurs, you can add more electrophoresis buffer into upper chamber.
13. Make sure buffer level covers cassettes, and secure top and electrodes.
14. Transfers can also be run at 100 V, 2 h; however, efficiency is greater for higher molecular-weight proteins using the overnight transfer. Alternatively if transferring high molecular-weight proteins, transfer 1.5–2 h at 100 V, with 0.1% SDS in transfer buffer.
15. You may recover used transfer buffer and store at 4°C, which can be used at least once more.
16. If you need to check the efficiency of transfer, you can stain the remaining gel with Coomassie blue, and the membrane prior to the blocking with 0.1% (w/v) Ponceau-S in 5% acetic acid (v/v) (Sigma).
17. All incubations and washes should be done on a rocking platform.
18. You may recover and reuse the primary-antibody solution, however, multiple usage will reduce sensitivity. In our laboratories, we routinely use the recovered antibody once more. Store the recovered antibody at 4°C or –20°C, according to manufacturer's suggestion.
19. HRP-conjugated antibodies may stick to sides of containers and bind to the ECL reagent, which will reduce the sensitivity of detection.

20. If the membrane has been stored in freezer, first wet with MeOH, rinse in d H₂O, then buffer A. If the stored membrane was blocked before storage; reblock membrane 15 min, then proceed with immunoblotting
21. Since Tween-20 in buffer B will interfere with the ECL reaction, wash the membrane thoroughly with buffer A before ECL detection.

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Immunohistochemistry and Immunocytochemistry

Jing Zheng

1. Introduction

Immunohistochemistry and immunocytochemistry are powerful techniques for localizing the molecular expression of proteins in tissues and cells, especially when combined with the *in situ* hybridization technique. Immunohistochemistry and immunocytochemistry techniques are primarily considered to be qualitative measurements, but when used together with a computerized imaging program, staining distribution and intensity can be semiquantified. This chapter describes immunochemical techniques routinely used in our laboratories for protein localization in tissue sections and cultured cells with the representative staining shown in **Figs. 1** and **2**. The protocols are based on an indirect immunoperoxidase detection via the avidin:biotinylated-peroxidase complex method with 3,3'-diaminobenzidine as the chromogen, a popular method suitable for bright-field microscopy. The first immunohistochemical protocol described is intended for paraffin-embedded tissue sections and the second immunocytochemical protocol is intended for cultured cells. It is highly recommended that, before beginning the protocol, the operator read **Subheading 4**.

2. Materials

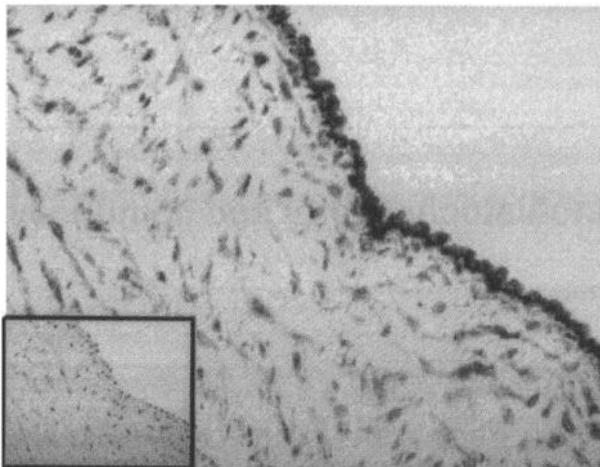
2.1. Preparation of Reagents and Solutions

2.1.1. Buffer

1. Buffer A (10 mM phosphate-buffered saline [PBS], 0.14 M NaCl, pH 7.4): 8 g NaCl, 1.2 g Na₂HPO₄, 0.2 g KCl Dissolve in distilled water (dH₂O). Adjust pH to 7.4. Make up to 1 L with dH₂O. Store at room temperature.
2. Buffer B (wash buffer) Buffer A, 0.3% (v/v) Triton X-100. Store at room temperature
3. Buffer C (antibody-dilution buffer): Buffer A, 1% (w/v) bovine serum albumin (BSA), 0.1% (w/v) sodium azide, pH 7.4. Store at 4°C.

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ecNOS

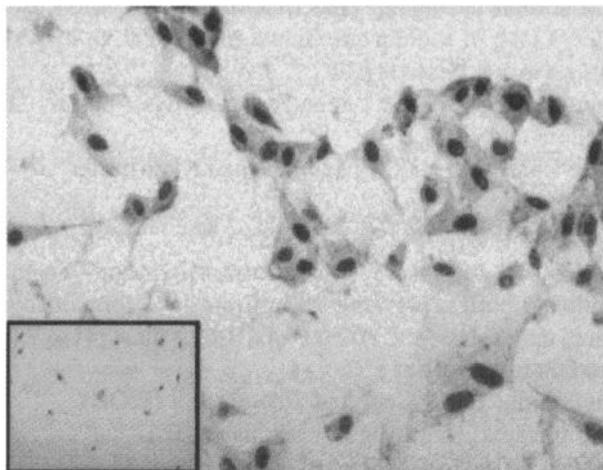


AT₁-R



Fig. 1. Immunohistochemical localization of endothelial cell nitric oxide synthase (ecNOS) and angiotensin II type 1 receptors (AT₁-R) in the ovine placental artery from a representative ewe at 142 days' gestation. Dark cytoplasmic color indicates the positive staining. Dark nuclear color is hematoxylin counterstaining. Tissue sections were incubated with mouse antibody against ecNOS (2.5 µg/mL; upper panel) or rabbit antibody against AT₁-R (2 µg/mL; bottom panel). An adjacent section was used for controls, replacing the primary antibody with an equivalent amount of mouse (for ecNOS) or rabbit (for AT₁-R) IgG protein (*see figure inserts*).

ecNOS



AT₁-R

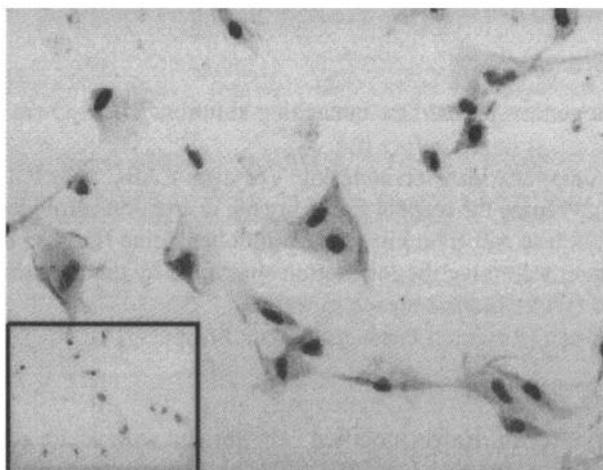


Fig. 2. Immunocytochemical localization of ecNOS and AT₁-R in ovine fetoplacental artery endothelial cells. Dark cytoplasmic color indicates the positive staining. Dark nuclear color is hematoxylin counterstaining. Endothelial cells were incubated with the primary ecNOS (2.5 µg/mL; upper panel) or AT₁-R (2 µg/mL; bottom panel) antibody. The endothelial cells in adjacent chambers were used for controls, replacing the primary antibody with an equivalent amount of mouse (for ecNOS) or rabbit (for AT₁-R) IgG protein (*see figure inserts*).

- 4 0.2 M Sodium cacodylate (Electron Microscopy Sciences, Fort Washington, PA) buffer: 42.8 g of sodium cacodylate and 900 mL of dH₂O. Adjust pH to 7.5, and make up to 1 L. Store at 4°C.
- 5 Tissue-storage buffer: 0.1 M sodium cacodylate buffer, 0.01% sodium azide, pH 7.5. Store at 4°C.
- 6 Tissue-wash buffer: 0.1 M sodium cacodylate buffer, pH 7.5. Store at 4°C.

2.1.2. Tissue Processing

1. Fixative (4% formaldehyde [Electron Microscopy Sciences] in 0.1 M sodium cacodylate buffer, pH 7.5). 200 mL of 16% formaldehyde, 400 mL of 0.2 M sodium cacodylate, and 100 mL of dH₂O. Adjust pH to 7.5. Add dH₂O and make up to 800 mL. Store at 4°C.
2. Dehydration: 100, 95, 85, and 70% ethanol (EtOH).
3. Clearing agent: Hemo-De (Fisher, Itasca, IL)
4. Paraffin: TissuePrep2 with a melting point of 55–57°C (Fisher)
5. HistoPrep pen (Fisher)

2.1.3. Staining

- 1 Endogenous peroxidase-quenching solution: 3% H₂O₂ in 60% methanol. Make up fresh solution before use.
- 2 Immunoperoxidase staining kit: Vectastain ABC elite Kit (Vector, Burlingame, CA). Prepare the reagent following the instructions provided by the manufacturer.
3. Peroxidase substrate kit: 3,3'-diaminobenzidine (DAB) kit (Vector). Prepare the reagent following the instructions provided by the manufacturer.
4. 10% (v/v) Chlorine bleach in water.
5. PAP pen (Research Products, Mount Prospect, IL)

2.1.4. Counterstaining

- 1 Hematoxylin: Harris modified hematoxylin with acetic acid (Fisher)
2. 1% Acid alcohol: 1 L of 70% EtOH and 10 mL concentrated HCl
3. 0.2% Ammonia H₂O: 1 L of tap H₂O and 2 mL of 28% NH₄OH.

3. Methods

3.1. Tissue Preparation (see Note 1)

1. Fix tissues (3–4 mm thick) in 4% formaldehyde in sodium cacodylate buffer, pH 7.5, for 2 d at 4°C (see Note 2)
2. Discard the fixative and immerse tissues in 0.1 M sodium cacodylate buffer overnight at 4°C (see Note 3)
3. Label cassettes with a HistoPrep pen.
4. Remove any excess tissues and cut the tissue with a fresh razor blade so it fits into a cassette
5. Transfer a piece of tissue to a labeled cassette and store cassettes in 70% EtOH overnight.

6. Transfer the cassettes in 85% EtOH, 1 h, 100% EtOH, 2 × 2 h; and the clearing agent, 3 × 1.5 h
7. Transfer cassettes to the melted paraffin in a 57–58°C oven and incubate overnight (*see Note 4*).

3.2. Tissue Embedding (see Note 1)

1. Fill warm embedding molds on warm plate (approx 60°C) with melted paraffin. Remove and discard cassette lid.
2. Use a warm forceps to transfer the tissue to the center of mold bottom in melted paraffin, and orient at desired position (*see Note 5*)
3. Use cassette bottom with flat-side down to cover the mold and add more paraffin until the cassette bottom is submerged. Move the mold to a freezer at –20°C (*see Note 6*)
4. When paraffin is cool (approx 15 min), remove the cassettes with tissue from molds. Store tissue blocks at 4°C (*see Note 7*).

3.3. Tissue Sectioning (see Note 1)

1. With a razor blade, trim excess paraffin surrounding the tissue and leave an equal margin (3–4 mm) of paraffin on all four sides of the tissue
2. Label positively charged glass slides (Fisher) using an HistoPrep pen and set on 37–40°C heating plate or slide warmer. Add several drops of dH₂O onto one end (away from frosted end) of the slide
3. Place a tissue block onto the microtome.
4. Cut cross sections at 5–6 μm thick. Transfer tissue sections with a wet paintbrush to a glass slide and let the sections float on water. Place two adjacent sections on each slide, one for primary antibody staining and one for control (*see Note 8*)
5. After the sections have stretched and wrinkles are removed (approx 10–15 min), drain out excess water
6. Place slides in a slide box and dry the slides in an oven at 35–37°C overnight.

3.4. Immunohistochemical Staining Procedure (see Note 9)

1. Mark the slides with an HistoPrep pen (i.e., the antigen you are staining, concentration of the primary antibody, and date). Put tissue sections in slide rack.
2. Deparaffinize in the clearing agent in a staining dish: 2 × 5 min
3. Rehydrate: 100% EtOH, 2 × 1 min; 95% EtOH, 1 min, 70% EtOH, 1 min.
4. Quench endogenous peroxidase activity. 3% H₂O₂ in 60% methanol for 15–20 min.
5. Wash in dH₂O for 5 min.
6. Lay slides with tissue sections face up in a slide box with wet paper towels on the bottom. Add the buffer B onto the slides to cover whole sections and incubate for 5 min
7. Replace the washing buffer with blocking serum (from Vectastain ABC *elite* kit) in the washing buffer, close the slide box, and incubate for 20 min
8. Use Kimwipes to wipe off excess wetness surrounding the section and encircle each tissue section with the PAP pen (*see Note 10*)

9. Pipet the primary antibody and control (20–60 μL /tissue section, depending on size of the section) in buffer C into the center of the PAP pen circle. Make sure the whole tissue section is covered. Close the slide box and incubate for 1 h (*see Notes 11–13*)
10. Wash in buffer B for 2×5 min.
11. Add biotinylated secondary antibody (from Vectastain ABC *elite* kit), close the box, and incubate for 40 min. While incubating, prepare avidin biotinylated-peroxidase complex solution (from Vectastain ABC *elite* kit) according to the kit instructions and let the solution sit at room temperature for at least 30 min
12. Wash in buffer B for 2×5 min.
13. Add avidin–biotinylate peroxidase complex solution, close the box, and incubate for 40 min
14. Wash in buffer B for 2×5 min
15. Wash in dH_2O for 5 min. While washing, prepare DAB according to the DAB kit instructions.
16. Incubate the sections with DAB at room temperature for 3–7 min or until staining is at desired intensity (*see Note 14*).
17. Wash in buffer B for 5 min.
18. Wash in tap H_2O for 5 min
19. Transfer slides back to the baskets.
20. Counterstain with hematoxylin for 1 min (*see Note 15*).
21. Wash in tap H_2O briefly.
22. Dip the slides in 1% acid alcohol three times
23. Wash in tap H_2O briefly
24. Dip the slide in 0.2% ammonia H_2O 15–20 times
25. Wash in running tap H_2O for 5 min. Check counterstain under microscope. If counterstain is too strong, go back to **step 22** and then repeat **steps 23–25**. If counterstain is too weak, go back to **step 20** and then repeat **steps 21–25**.
26. Wash in gently running tap H_2O for 10–15 min. Dehydrate in following solutions: 70% EtOH, 2 min, 90% EtOH, 2 min; 100% EtOH, 2×2 min.
27. Clear in clearing agent for 3×3 min.
28. Mount: Put a drop or two of mounting reagent (Fisher) onto sections and place a cover slip. Wipe off bottom of slide and let them dry overnight at room temperature (*see Note 16*)

3.5. Immunocytochemical Staining Procedure

This procedure is essentially the same as that described above in The Immunohistochemical Staining Procedure (**Subheading 3.4.**), with slight modification. This procedure is intended for cells plated in appropriate medium at approx 5000 cells/chamber in eight chamber glass slides (Nunc, Naperville, IL) and incubated for 24 h to allow attachment.

1. Rinse subconfluent cells cultured in glass chamber slides with the buffer B
2. Fix cells in 4% formaldehyde in sodium cacodylate buffer, pH 7.5, for 1 h at room temperature.

3. Wash in dH₂O for 5 min
4. Quench endogenous peroxidase in 3% H₂O₂ in 60% methanol for 15–20 min.
5. Wash in dH₂O for 5 min
6. Incubate in buffer B for 5 min.
7. Replace buffer B with blocking serum in buffer B and incubate for 20 min.
8. Pipet the primary antibody and controls (100–200 μL/chamber) in the buffer C into desired chambers of slide, close lid of the slide box, and incubate for 1 h
9. Follow **Subheading 3.4., steps 12–17.**
10. Remove upper structure and gasket of the slide according to the manufacturer's instructions and transfer slides to the slide rack.
11. Follow **Subheading 3.4., steps 19–28.**

4. Notes

1. Tissue fixation, embedding, and sectioning are critical for successful immunostaining and should be processed under the advice of a trained histologist
2. The volume of the fixative should be at least 10 times the tissue volume. The length of time for fixation depends on the size and type of the tissue block. In our experience, 2-d fixation at 4°C is suitable for most tissues such as blood vessels, adrenal, uterus, and placental tissues.
3. For long-term storage, fixed tissues can be kept in 0.1 M sodium cacodylate buffer, pH 7.5 with 0.01% sodium azide at 4°C. Before immunostaining, the tissues should be washed thoroughly with 0.1 M sodium cacodylate buffer (without sodium azide) to avoid azide interfering with peroxidase–substrate interaction.
4. The length of time for impregnation (the process in which hot paraffin penetrates the tissues) depends on the size and type of the tissue block. In our experience, impregnation overnight without vacuum in a 57–58°C oven is suitable for most tissues.
5. Quickly transfer tissue from impregnation container to embedding molds to prevent formation of a thin layer of solidified paraffin around the tissue. If it forms, put the tissue back in hot paraffin until solidified paraffin around the tissues is completely melted
6. When pouring hot paraffin into the mold, avoid trapping air bubbles under the cassette bottom. If this occurs, add more hot paraffin on one side of mold to drive air bubbles out.
7. After the paraffin is cooled, it should be easy to take the tissue block attached to the cassette bottom out of the mold. If not, put the tissue block in mold back at –20°C until it is easily removed. Do not force the tissue block out of the mold, which may separate the tissue block from the cassette bottom. If by chance this occurs, melt the tissue block completely and re-embed it.
8. Leave a space (approx 3 mm) between two tissue sections for PAP pen encirclement.
9. During the immunostaining, never let tissue sections dry (in order to prevent high nonspecific staining). Before introducing new solutions in every step including deparaffinization, dehydration, washing, treatment with antibodies, and counterstaining, drain the old solution and tap the slides on stacked paper towels. Also,

- in all washing steps, add the washing buffer on an area alongside the tissue sections rather than directly on the tissue sections, which may damage or wash away the tissue section.
10. Make sure the PAP pen encirclement surrounding the section is dry to avoid a leak that may cause mixing of the primary antibody with the control solution you use. If by chance a leak forms, wash the section and re-encircle the section.
 11. Use several concentrations of the primary antibody in the preliminary study to determine one optimal concentration for a desired tissue. In our experience, 1–5 $\mu\text{g}/\text{mL}$ of affinity purified primary antibody works most times.
 12. If you plan to compare staining intensity among different tissue sections, stain all tissue sections for comparison in one run.
 13. Controls usually consist of omitting the primary antibody and/or the secondary antibody; staining a tissue section that has been known to contain (positive control) and/or not to contain the antigen (negative control); replacing the primary antibody with the preimmune serum/IgG preparation or unrelated antibody at the same concentration of the primary antibody used; and absorbing the primary antibody with excess amount of the antigen, which may be the most reliable control. It is generally acceptable, however, to only use the the first three controls.
 14. Staining development can be checked under microscope. DAB is carcinogenic! Wear gloves and lab coat when working with it and dispose the waste into the 1:10 chlorine bleach container. When using DAB as a substrate for peroxidase, positive staining will be indicated by brown or black color, depending on whether nickel solution is added (*see* instructions for DAB subtract kit from Vector). Other color chromagens also are available from Vector, which are useful, especially when colocalization of two antigens is needed.
 15. Hematoxylin counterstaining will give blue nuclear staining. Other colors (green or red) of nuclear counterstaining are also available by using methyl green or nuclear fast red solutions (also from Vector).
 16. Make sure no air bubbles are entrapped under the cover slides. If by chance this happens, gently press the cover slide down to force bubbles out or carefully lift the cover slide and remount it.

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Extraction of Cellular and Tissue RNA

Daniel S. Millican and Ian M. Bird

1. Introduction

Total undegraded cellular RNA, largely free of contaminating DNA, can be rapidly and easily isolated from homogenized tissue or cultured cells using acid pH-guanidinium thiocyanate/phenol/chloroform extraction (1). Commercially available acid phenol/guanidinium thiocyanate solutions (2), such as RNAzol™ B (Cinna Scientific, Cleveland, OH) and TRIzol (Gibco-BRL, Gaithersburg, MD), allow one-step, complete solubilization of tissue or cells under conditions that inhibit RNase activity. By subsequent addition of chloroform, phase separation is forced to occur and RNA is extracted into an aqueous phase, separated from lower organic phase by an insoluble protein interphase. Guanidinium thiocyanate remains in the aqueous phase and so continues to act as an RNase inhibitor by disrupting protein–nucleic acid interactions. However, the protein interphase is still rich in RNase and, on accidental recovery with the aqueous phase, may result in degradation of the RNA. To eliminate this contamination, aqueous phase can be re-extracted in fresh phenol/chloroform/isoamyl alcohol solution (see Note 1) such as PCI reagent (5 Prime→3 Prime [Boulder, CO]) and spun through a barrier material that separates phases of greater and lesser density. The lighter aqueous phase does not penetrate the barrier, whereas the organic phase and any residual protein interphase migrate through the barrier to become trapped underneath. Heavy grade phase-lock gel tubes (5 Prime→3 Prime) are suitable for procedures using guanidinium thiocyanate, which imparts higher density to the aqueous phase (3). RNA isolated by this procedure is suitable for use in Northern analysis, slot blotting, and RT/PCR. Commercially available kits can be used to further isolate poly(A+) RNA-enriched fractions for use in detection or ampli-

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fication of rare transcripts. We describe here a procedure for total RNA extraction from homogenized tissues or cultured cells using RNazol™ B, purification by passage through phase-lock gel tubes, and an optional chromatographic poly(A+) RNA enrichment procedure using mini oligo(dT) spin columns (5 Prime→3 Prime), together with the spectrophotometric determination of RNA yield and purity. Ultimately, RNA quality should be further evaluated by ethidium bromide visualization following agarose/formaldehyde gel electrophoresis (*see* Chapter 29). Good quality RNA is indicated not only by clear 18 and 28S subunits, but also by discrete RNA bands, especially of high molecular weight, together with an absence of excess low-molecular-weight (<200 bases, degraded) RNA.

2. Materials

Unless otherwise stated, all reagents are molecular-biology grade.

- 1 Autoclave
2. NanoPure/UltraPure (18Ω) Water.
- 3 Microcentrifuge, 12,000g, Fisher (Pittsburgh, PA) Micro H.
4. Vacuum centrifuge/vacuum pump, Labconco (Kansas City, MO) (78110)
5. Diethyl pyrocarbonate, Sigma (St. Louis, MO, cat. no. D5758).
- 6 Sterile, disposable 100-mm Petri dishes.
- 7 Chloroform.
8. Isopropanol.
9. Ethanol (200 proof).
10. RNazol B, TelTest (Friendswood, TX) (CS105)
11. Heavy grade phase-lock gel (PLG) tubes, 5 Prime→3 Prime (Boulder, CO) (cat. no. p1-183182)
- 12 Phenol chloroform isoamyl alcohol (PCI) reagent, 5 Prime→3 Prime (cat. no. 1-737642).
- 13 Molecular biology grade (MB grade) water, 5 Prime→3 Prime.
- 14 Sterile dissection instruments.
- 15 Liquid nitrogen (N_l).
16. Na₂HPO₄, anhydrous.
- 17 KH₂PO₄, anhydrous.
18. NaCl.
19. Trichloro acetic acid (TCA).
20. Polytron homogenizer with approx 0.25-in. blade, Fisher (cat. no. 15338700P).
21. 2-mL Cryovials, Fisher (cat. no. 033377D).
22. 15-mL Homogenization tubes, Fisher (cat. no. 1495910B).
23. Cell scrapers, Fisher (cat. no. 087732).
24. Mini oligo (dT) spin column kit, 5 Prime→3 Prime (cat. no. 2-600750).
25. Centrifuge, 50-mL conical tube, 200g
26. Oligo dT spin column carrier tubes (1.6-cm inner diameter).
27. Centrifuge rotor adapters which accommodate chosen carrier tubes

2.1. Preparation of Solutions

All glassware should be thoroughly cleaned and autoclaved before use. Wear gloves at all times and use new weighing boats with autoclaved spatulas. Weigh reagents to 1% accuracy. When using a pH meter, calibrate first for pH 4.0–7.0 range, then rinse electrode in distilled water, followed by DEPC water. Store magnetic stir bars in ethanol and rinse in DEPC water before use. Unless otherwise stated, keep solutions at room temperature. Store and handle hazardous items in accordance with safety guidelines.

1. DEPC Water: Add 500 μ L diethyl pyrocarbonate (DEPC) per L to 5 L filtered deionized water in a conical flask and swirl to mix. Stand at room temperature 4 h (to inactivate RNAses), then autoclave (liquid cycle) to deactivate DEPC/ sterilize. Allow to cool overnight before making up other aqueous solutions.
2. Molecular-biology (MB) grade water: MB quality is essential for making solutions coming in direct contact with RNA. Store at 4°C.
3. RNazol B: Store protected from light at 4°C.
4. CHCl_3 : Store working stock in 100-mL bottle at 4°C
5. Phenol/chloroform/isoamyl alcohol (PCI) solution: Store at 4°C.
6. 75% ethanol: Dilute 75 mL of 200-proof ethanol with 25 mL MB grade water. Store at 4°C. Replace after 1 mo.
7. Isopropanol: Store working stock in a 100-mL bottle at 4°C.
8. 12% TCA: Dissolve 60 g trichloroacetic acid in DEPC water to a final volume of 500 mL. Store at 4°C.
9. Phosphate-buffered saline (PBS) pH 7.4: To 4 L DEPC water, add 6.186 g Na_2HPO_4 (mol wt 142), 1.633 g KH_2PO_4 (mol wt 137.99), and 52.60 g NaCl (mol wt 58.44), and adjust pH to 7.4. Adjust volume to 6 L with DEPC water. Dispense in 500-mL volumes to glass bottles and autoclave on liquid cycle (lid loose!) Store at 4°C.

3. Methods

Before performing the procedure, ensure that RNazol B, chloroform, PCI solution, isopropanol, fresh 75% ethanol, and MB-grade water are stored at 4°C. Microcentrifuge temperature should also be equilibrated to 4°C in a cold room. For optional poly(A+) RNA isolation, ensure that absolute ethanol, and appropriate kit reagents (mussel glycogen, 3 M sodium acetate, pH 5.2) are also at 4°C. Clean and store the spectrophotometer cuvet in a 50-mL tube containing DEPC water. During the procedure, keep everything on ice. Wear gloves and work over sterile napkins. Each 100-mg tissue cube processed will require four microcentrifuge rotor wells, so up to three cubes can be processed simultaneously using a 12-well rotor. Alternatively, each cell culture dish processed will require only one rotor well.

3.1. Tissue and Cell solubilization in RNAzol B

3.1.1. Collection and Storage of Tissues

1. Place fresh weigh boats in shallow styrofoam containers and add liquid nitrogen (N_2) into and around weigh boats. Add N_2 to an additional container for transfer of processed samples to a -70°C freezer. Also place sterile Petri dishes on ice and add cold PBS.
2. Transfer freshly obtained tissues to sterile Petri dishes containing cold PBS (to rinse away blood that contains abundant RNase). Working quickly with a sterile scalpel, slice into cubes of approx 3–4 mm size weighing approx 100 mg each. Immediately transfer cubes to weigh boats immersed in N_2 to snap freeze the tissue.
3. Immerse appropriately labeled cryovial into N_2 surrounding the weigh boat. Holding the vial with a hemostat, transfer cubes (using forceps) into the tube, cap, and transfer in N_2 to -70°C freezer or N_2 -dewar. Tissues are stable long term at $\leq -70^\circ\text{C}$.

3.1.2. Tissue Solubilization

1. Clean the polytron blade at 3/4 speed in 12% TCA (30 s), then DEPC water, and finally ethanol. Allow to air-dry.
2. Prepare eight phase-lock gel (PLG) tubes per 100-mg tissue cube. Centrifuge for 3 min at 12,000g to spin the barrier material to the bottom of the tube. Label 4 for first and 4 for second-round extractions and stand at room temperature.
3. Transfer the 100-mg tissue cube directly from the $\leq -70^\circ\text{C}$ storage into 4 mL cold RNAzol B in a 15-mL polypropylene tube (see Note 2). Disrupt the tissue completely with the polytron, (15 s at one-third speed, then 5 s at three-quarter speed). Place the homogenate on ice, and dispense into $4 \times 1.5\text{-mL}$ microcentrifuge tubes at 1 mL per tube. Keep all tubes on ice.
4. Between samples rinse the polytron blade in fresh RNAzol B (30 s at three-quarter speed). Repeat the homogenization process from **step 3** for the remaining tissue cubes, remembering that each cube will require four microcentrifuge rotor wells for processing.
5. Proceed to **Subheading 3.2**. Finally, clean the polytron blade before autoclaving and long-term storage.

3.1.3. Cell Solubilization from 60- to 100-mm Dishes

1. Prepare one phase-lock gel (PLG) tube per cultured dish: Centrifuge (3 min at 12,000g) to spin the barrier material to the bottom of the tube. Also, prepare a suction line (to efficiently and completely remove the culture medium from culture plates/dishes) and place a cell scraper in a 15-mL tube containing 5 mL RNAzol B.
2. Remove the cell-culture dishes from the incubator one at a time (see Note 3). Quickly remove all medium by suction (serum contains RNase).

3. Add 1 mL cold RNazol B to the center of the dish. Scrape the cells into the RNazol B with a scraper by first scraping left to right from top to bottom of dish. Then turn the dish through 90° and repeat the process. Finally, scrape around the outer edge, wipe all liquid to the bottom of the dish and transfer 1.0–1.1 mL of material to a 1.5-mL microcentrifuge tube, on ice.
4. Shear the cells by aspirating 10–15 times with a pipet (1-mL tip; *see Note 4*).
5. Discard the used pipet tip and return the scraper to the storage tube (containing RNazol B). Repeat the scrapping/homogenization process from **step 2** for as many culture dishes as can be processed, remembering that each dish will require one microcentrifuge rotor well for processing.
6. Proceed to **Subheading 3.2**.

3.2. Recovery of Total RNA

3.2.1. Phase Separation

1. Add 0.15 vol cold CHCl_3 (150 μL) to each microcentrifuge tube. Vortex for 10 s and stand on ice for 5 min. Vortex each tube again for 10 s and spin at 12,000g in a microcentrifuge (30 min at 4°C).

3.2.2. Phenol-Chloroform Extraction of Residual Protein from Aqueous Phase Using PLG Tubes

1. Recover up to 550 μL of the upper aqueous phase to a prespun PLG tube (more aqueous solution may be present, but complete recovery increases the risk of RNase contamination from the protein interphase). Add 500 μL of PCI reagent to each tube, and mix thoroughly by inversion (10 times, do not vortex). Place on ice for 10 min, and then spin at 4500g for 5 min at 4°C (5 Prime→3 Prime, also states that it is possible to perform this step at 12,000g).
2. Transfer the upper aqueous phase to a fresh prespun PLG tube and repeat the extraction procedure exactly as described above. (This second extraction step is not so necessary for cell extracts but is for tissue extracts.) Transfer the final aqueous phase to a fresh, sterile 1.5-mL tube and stand on ice.

3.2.3. Total RNA Precipitation

During all stages involving the pelleting and washing of RNA, be careful not to disturb the pellet when discarding the supernatant. Look for a small brownish disk or smear at the bottom of the tube. Preparations for poly(A+) RNA isolation (**Subheading 3.3.**) can also be made while performing the total RNA precipitation (**step 1** below).

1. Add a small excess (600 μL) of cold isopropanol to each tube containing PCI-purified aqueous phase. Invert each tube several times to ensure thorough mixing of the samples and then move the samples from the ice tray to a dry rack (no ice) before transfer to the -20°C freezer. Place on the bottom shelf (coldest) for 1 h to allow RNA precipitation.

- 2 During the incubation, set a water bath or block heater to 65°C. Make preparations for poly(A⁺) mRNA spin column chromatography if required.
3. After 1 h, remove the precipitated RNA from the -20°C freezer and invert twice. Spin at 12,000g at 4°C for 30 min.

For immediate poly(A⁺) RNA purification, proceed to **Subheading 3.3.**, and perform **steps 1–3** during centrifugation. Otherwise continue with total RNA solubilization as below.

- 4 Discard the supernatant (tip swiftly and smoothly in one movement) and add 800 µL of cold 75% ethanol (down the side of the tube so the pellet is not dislodged). Spin the tubes again at 12,000g for 10 min at 4°C.
5. Quickly and smoothly tip off the supernatant, and dry the pellets by inverting the tubes on a clean paper towel for 5 min, followed by spinning for 5 min in a vacuum centrifuge, with microcentrifuge tube lids open. Do not overdry pellets! (*see Note 5*). Remember: Always have the rotor spinning while there is a vacuum applied.

For immediate poly(A⁺) RNA purification, proceed to **Subheading 3.3., step 4**. Otherwise continue with total RNA solubilization as below.

- 6 For cultured cell extracts dedicated to total RNA quantitation add 110 µL MB-grade water to each tube. For tissue extracts, add 27.5 µL MB-grade water to each of the four tubes used to process one tissue cube. To solubilize the RNA completely, transfer the tubes (lids closed) to a water bath at 65°C for 3 min, vortex 10 s, return to the water bath for 2 min, and then place on ice for 3 min. Vortex and centrifuge (300g for 5–10 s) each tube briefly to draw all liquid to the bottom of the tube(s). For tissue extraction, pool contents of the four tubes, mix, and spin briefly (*see Note 6*).
- 7 Transfer 10 µL of the final product to a fresh 1.5-mL tube and add 90 µL MB-grade water. Immediately store the remaining 100 µL undiluted total RNA at ≤ -70°C. Keep the diluted samples on ice for spectrophotometry (*see Subheading 3.4.*)

3.3. Poly(A⁺) RNA Isolation from Total RNA Using Oligo (dT) Spin Columns

1. While pelleting the total RNA samples, remove the required mini oligo (dT) spin column kit (5 Prime→3 Prime) components from storage at 4°C, and allow to come to room temperature. Lay out and label one oligo dT column per tissue cube or culture dish. Perform the entire procedure over sterile napkins.
- 2 Resuspend the column resin/storage buffer evenly by repeated inversion. When initially removing the tip stoppers (to release pressure), hold the column tip upwards so that pressure will not force the liquid out of column. Slowly remove the tip stopper, position the slot of an RNase-free collection tube over the column tip, then invert and remove the “top” stopper (i.e., loading-end stopper). Place the entire column/collection tube assembly into a suitable carrier tube. Spin out the

storage buffer into slotted collection tube at 200g for 10 s using a centrifuge fitted with appropriate carrier-tube adapters (*see Note 7*). Discard the storage buffer from the collection tube, replace the tip stopper, and add 1 mL 0.5 M NaCl buffer (from kit) to the column. Replace the top stopper and suspend the resin evenly by repeatedly inverting the column.

3. Transfer 0.6 mL of elution buffer per set of tubes (four for each tissue, or multiple cell-culture dish extracts) to a corresponding single microcentrifuge tube. Bring a water bath or block heater to 65°C
4. Following vacuum drying of the total RNA, for each set of tubes (four for each tissue, or multiple cell-culture dish extracts) add water to a combined volume of 1 mL. To solubilize the RNA completely, transfer the tubes (lids closed) to a water bath at 65°C for 3 min, vortex for 10 s, return to the water bath for 2 min, and then place on ice for 3 min. Vortex and spin each tube briefly to draw all liquid to the bottom of the tube(s). Pool the tube contents as appropriate to a combined volume of 1 mL, mix, and spin briefly (*see Note 8*).
5. Transfer a 10- μ L aliquot to a separate tube containing 90 μ L MB-grade water and store frozen for later estimation of total RNA yield.
6. Add 250 μ L loading buffer to each tube containing 990 μ L resolubilized RNA, vortex briefly, and place on ice
7. Spin 0.5 M NaCl buffer from each column (200g for 10 s) into the collection tube and discard buffer. Load the RNA samples onto the corresponding columns. Stopper both ends and incubate for 15 min, with inversions every 3 min to suspend the resin. (Excessive resuspension will hinder binding of poly(A⁺) RNA to the oligo-dT resin.)
8. When removing tip stoppers, hold the column tip upwards so that pressure will not force liquid out of column. Slowly remove the tip stopper, position the slotted collection tube over the tip, invert and remove the other (top) stopper. Spin the columns for 10 s at 200g and discard all liquid from the collection tube.
9. Transfer the elution buffer aliquots to a 65°C water bath or heating block
10. Wash each column sequentially with 750 μ L of 0.5 M NaCl buffer once, and then twice with 1 mL of 0.1 M NaCl buffer. Spin the columns for 10 s at 200g and discard liquid from the collection tubes each time
11. Change to fresh carrier and collection tubes. Add 500 μ L of 65°C elution buffer to each column, and immediately spin for 10 s at 200g (*see Note 9*).
12. Split the contents of each collection tube to two fresh microcentrifuge tubes. To each tube add 10 μ L mussel glycogen solution, and 25 μ L 3 M sodium acetate, pH 5.2 solution (both reagents are provided in the spin-column kit). Then add 750 μ L cold absolute ethanol. Mix and chill overnight at -20°C.
13. Spin samples for 30 min (12,000g at 4°C) and discard the supernatant. Add 800 μ L of 75% ethanol to each tube and spin (12,000g at 4°C for 10 min). Discard the supernatant and invert tubes onto sterile napkins to dry. Vacuum centrifuge for 3 min only.
14. Add 55 μ L MB-grade water to the bottom of each tube. Transfer all samples to a 65°C water bath for 3 min, vortex for 10 s, and return to the 65°C water bath for

2 min, then place on ice for 3 min. Vortex each tube and spin briefly to draw the liquid to the bottom of the tubes. Recombine appropriate pairs of samples, vortex, and spin briefly to recover the liquid.

15. Transfer 10 μL from each tube of recombined material to a fresh microcentrifuge tube and add 90 μL MB-grade water for spectrophotometric quantitation. Store the remaining 100 μL of undiluted poly(A+) RNA solution at $\leq -70^\circ\text{C}$.

3.4. Spectrophotometric Quantitation

Measure total RNA and poly(A+) RNA absorbance (A_{260}) against an MB-grade water blank using a 260-nm light source. Purity can be determined by the $A_{260}:A_{280}$ ratio. A ratio of 1.7 is generally acceptable, with improved purity indicated by ratios up to 2.0:1 (see **Note 10**).

- 1 RNA concentration can be calculated using the equation:

$$\mu\text{g}/\mu\text{L} = (0.04)(A_{260}) (1/n) (DF)$$

where n is the cuvet pathlength and DF is the dilution factor (see **Note 11**). RNA aliquots measured by spectrophotometry were generally diluted by a factor of 10. Calculate to three decimal places for yields below 1 $\mu\text{g}/\mu\text{L}$ and to two places for higher yields.

2. Total yield can be calculated by multiplying concentration ($\mu\text{g}/\mu\text{L}$) by the μL volume in which the RNA was resolubilized.
3. Efficiency of poly(A+) recovery can be determined as a percentage of the total RNA initially recovered (calculated from the aliquot taken prior to oligo dT spin column purification).

$$[(\mu\text{g}/\mu\text{L poly(A+) RNA})(110 \mu\text{L vol})]/[(\mu\text{g}/\mu\text{L total RNA aliquot})(990 \mu\text{L vol})]$$

Recovery of 5% is normal for single-pass column purification (see **Note 12**)

4. Do not add diluted spectrophotometry aliquots back into undiluted samples. Store concentrated RNA samples at $\leq -70^\circ\text{C}$.

4. Notes

1. Isoamyl alcohol acts as an antifoaming agent. 5 Prime \rightarrow 3 Prime also adds 8-hydroxyquinoline (an antioxidant) to the PCI reagent that also imparts a yellow color to the organic phase, making it easier to distinguish from the colorless aqueous phase.
2. This volume is sufficient for most tissues, including liver and kidney which contain much endogenous RNase activity. If extracted RNA is consistently degraded, however, reoptimization of this volume may be necessary.
3. Alternatively, all dishes can be removed at once and medium quickly removed by suction before snap freezing in a shallow tray of N_2 and storage at $\leq -70^\circ\text{C}$. This gives slightly lower yields, but can be useful if running tightly time-controlled experiments. If degradation of the final RNA product is consistently seen, try also rinsing the dish with serum-free medium before extraction.

- 4 Aspirate smoothly, allowing a small amount of air into the tip on the first few strokes. On subsequent strokes, foaming will occur. This will cause shearing of the cells, but not the RNA, when drawn back and forth through the tip.
5. It is important not to overdry the pellets since they will be more difficult to resolubilize.
6. We have frequently observed coprecipitation of a water insoluble, transparent pellet using this total RNA isolation procedure on some tissues. As the coprecipitate is water insoluble, RNA can be pipetted away from transparent pellet after heating, into a fresh tube, and spectrophotometric quantitation is not affected. Do not attempt to resolubilize by further mixing or aspiration, as RNA will shear. The coprecipitate is removed during passage through oligo (dT) columns when performing the optional poly(A+) RNA isolation procedure. In other tissues, a whitish, pellet, probably of polysaccharide composition (4), is coprecipitated with RNA and solubilizes after incubation at 65°C.
7. Begin timing of spin after centrifuge has achieved 200g.
- 8 Alternatively, total RNA previously solubilized as in **Subheading 3.2.** can be diluted to 1 mL. However, poly(A) tails of solubilized mRNA may degrade with long-term storage, even at $\leq -70^{\circ}\text{C}$.
- 9 This elution recovers approx 90% of column bound poly(A+) RNA. A second elution will recover all remaining poly(A+) RNA.
10. Nucleic acid and protein absorption spectra overlap, but nucleic acids show a maximum at 260 nm, whereas proteins show a maximum at 280 nm. To allow for this, the purity of RNA recovered by the described techniques is traditionally quoted as a ratio of the A_{260}/A_{280} . The ratio, without correction, should be 1.5–2.0, with a value of 1.7 and above considered good. However it is wise initially to check the RNA by Northern analysis, since these values will not reveal whether RNA is intact (it all absorbs the same—intact or degraded), and values can be thrown by contaminating phenol. If a sophisticated spectrophotometer capable of three-wavelength measurement, perhaps with automatic correction, is available, the value at A_{320} can be used as background correction for the A_{260} and A_{280} values. The ratio will then be lower (typically 1.45–1.9), but more consistent. Remember also, whichever method you use, the reliability of ratios as a measure of purity decreases with decreasing yields of RNA. Thus for small yields of RNA ($<1\ \mu\text{g}$) it may be best to measure undiluted product. The cuvet should be rinsed thoroughly between samples, however, to ensure no cross contamination.
11. Given the absorbance of an analyte in solution, concentration can be determined from the equation (5):

$$A = (\alpha_s) (c) (n)$$

where A is absorbance, or optical density (OD), by analyte at a particular wavelength, α_s is the specific absorbance of the analyte, c is the analyte concentration, and n is the cm length of light pathway through solution. Given the specific

absorbance (ϵ) of RNA is 0.025 mL/cm/ μ g (i.e., 1 OD unit = 40 μ g/mL/cm pathlength), this equation becomes:

$$c = (A) (1/\alpha_s) (1/n) = (A) (40) \mu\text{g/mL/cm} (1/n) \text{ cm} = (40) (A) (1/n) \mu\text{g/mL}$$

Taking further into consideration a dilution factor (DF) for spectrophotometry samples and a unit-correction factor for concentration expression in μ g/ μ L, the equation becomes:

$$c = (40) (A) (1/n) \mu\text{g/mL} (DF) (1/1000) \text{ mL}/\mu\text{L} = (0.04) (A) (1/n) (DF) \mu\text{g}/\mu\text{L}$$

- 12 Percentage poly(A⁺) RNA recovery does not reflect mRNA levels relative to total RNA, as poly(A⁺) RNA is only mRNA enriched with less tRNA and rRNA. Percentage poly(A⁺) RNA is measured instead to ensure the efficiency and consistency of the method. Typically poly(A⁺) RNA should be 2–5% of the total RNA initially applied to the column.

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Size Separation and Quantification of mRNA by Northern Analysis

Ian M. Bird

1. Introduction

Most acute changes in cell activity often involve changes in activity of existing metabolic pathways either as a consequence of cell signaling changing the phosphorylation of rate-limiting proteins, or allosteric control as a consequence of changes in the environment. Long-term changes in cell activity, however, may also relate to changes in expression of key proteins. This is particularly true in changes in gestation/development or in removal of cells from an *in vivo* to an extended *in vitro* culture system. Changes in protein levels may occur through mechanisms that are transcriptional (i.e., altered rates of transcription) or posttranscriptional (changes in mRNA turnover), translational (altered synthesis of protein from existing mRNA) or posttranslational (altered protein stability). Northern analysis is a standard procedure by which abundant levels of mRNA can be characterized by size and quantified so that effects of treatment on message level can be determined. Whereas a change in message level with treatment is an indication that regulation may be occurring at the level of mRNA it is not, however, sufficient alone to demonstrate whether this is transcriptional or posttranscriptional. Results from Northern analysis should always be normalized for loading using an appropriate housekeeping gene product (*see Note 1*) and should also be compared to the results of Western analysis for quantification of the corresponding protein wherever possible.

The procedure described below is based on that of Sambrook et al. (*1*) Recovered RNA is size separated in the presence of formaldehyde, both to maintain denaturation and to inhibit RNase activity. Electrophoresis is also performed in the presence of ethidium bromide, to provide a means of visualization of the RNA under UV light. The principle changes in this procedure

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over that initially described are the more rapid and complete transfer of the RNA to membrane using vacuum blotting, and the improvement in membrane manufacturing technology, which, in our experience, allows the recommended nylon membrane to be reprobbed as many as four to five times.

1.1. Laboratory Practices for Handling RNA

It is vital that good sterile technique is used when working with RNA. Clean spatulas and glasswear and thoroughly rinse with plenty of 18- Ω ultrapure water or DEPC water before air-drying. Place lids loosely on bottles, and cover flask necks/bottle lids with foil. Wrap spatulas in autoclave bags. Autoclave on dry cycle for 40 min. We keep stir bars specifically for preparing solutions in 75% ethanol, which we dry with a clean paper towel before use, and rinse thoroughly in DEPC water before returning. All other disposables such as weigh boats should be kept in appropriate boxes/cabinets/drawers free of dust/contamination. Glass/plastic plates and plastic equipment (gel rigs) that cannot be autoclaved should be rinsed thoroughly in excess water and then DEPC water before air-drying and storage in a closed drawer/cabinet. When using a pH meter, rinse the electrode thoroughly in DEPC water before placing in the solution. Pipet tips and microfuge tubes can be purchased certified as RNase/DNase free, or can be more economically prepared by autoclaving (put tubes in empty pipet-tip boxes). One additional, but often overlooked source of contamination is aspirated material inside pipets. This can be avoided by attention to technique, routine cleaning of pipets, and the use of pipet tips containing air filters.

2. Materials

Purchase all reagents as molecular-biology grade, i.e., RNase- and DNase-free.

- 1 Diethylpyrocarbonate (DEPC) (*see Note 2* concerning safety)
- 2 Sodium chloride.
- 3 Trisodium citrate dihydrate.
- 4 MOPS.
- 5 Tris base.
- 6 EDTA: disodium salt, dihydrate
- 7 Sodium hydroxide: pellets
- 8 Sodium acetate: trihydrate.
- 9 Glacial acetic acid
- 10 Sodium pyrophosphate: decahydrate.
- 11 Polyvinylpyrrolidone (mol wt 360,000).
- 12 Ficoll (mol wt 400,000)
- 13 Sodium dodecyl sulfate (SDS).
- 14 Ethidium bromide: sodium salt.
- 15 Glycerol.

- 16 Bromophenol blue
17. Xylene cyanol FF.
- 18 Formaldehyde.
19. Formamide.
20. Ethanol: 200 proof.
- 21 tRNA: RNase free, purified from *E coli* MRE600 (Boehringer Mannheim, Mannheim, Germany, cat no. 109 550).
22. Bovine serum albumin (BSA, Fraction V) (Sigma, St. Louis, MO).
23. MW ladder. as appropriate (Gibco-BRL, Grand Island, NY).
24. Submarine gel apparatus with 15 × 15-cm gel tray (Bio-Rad, Hercules, CA).
25. Matching multiwell comb (e.g., Bio-Rad 15-well comb).
26. Power supply (e.g., Bio-Rad 200/2.0 or, for low-voltage overnight runs, Bio-Rad Powerpac 300)
- 27 Vacuum transfer apparatus (Bio-Rad) with vacuum/regulator
- 28 Hybridization oven with roller bottles (Techne, Model HB-1D, Princeton, NJ)
- 29 UV crosslinker (Bio-Rad).
30. Vacuum centrifuge
31. Water bath.
32. Microfuge (4°C).
33. Heat-sealable bags and bag sealer (KayPack, Minneapolis, MN)
- 34 Sample heating block (95°C).
35. High-strength, analytical-grade agarose (Bio-Rad)
36. Magna graph hybridization membrane (MSI, Westboro, MA).
37. Sterile 1.5-mL microcentrifuge tubes
- 38 Sterile pipet tips.
39. Filter paper: Whatman no 1 (Clifton, NJ)
- 40 Filters: 0.45 and 0.2 μm

2.1. Stock Solutions

(Most of these are also available commercially.) Wear gloves at all times, since hands are a source of RNase and DNase. Prepare all solutions using autoclaved glassware and work in a clean work area (*see Subheading 1.1.*).

- 1 *DEPC water*: 0.5 mL DEPC (diethyl pyrocarbonate, *see Note 2*) per L. Add DEPC to 5 L filtered, distilled, and deionized water in a conical flask, and swirl to mix. Let stand at room temperature for 30 min, then autoclave (liquid cycle) to deactivate DEPC/sterilize. Allow to cool overnight before making up other aqueous solutions. Alternatively use molecular-biology grade water (5Prime-3Prime, Boulder, CO).

2.1.1. For Preparing and Running the Gel

- 1 *0.5 M EDTA, pH 8.0*: Weigh 16.8 g disodium EDTA into 80 mL DEPC water, and adjust pH to 8.0 with concentrated or pelleted (2 g) NaOH. Adjust volume to 100 mL and autoclave to sterilize.

2. *20X SSC*: 3.0 M NaCl, 0.3 M trisodium citrate, pH 7.0 Weigh out 175.3 g NaCl and 88.2 g Na₃citrate and dissolve in 800 mL DEPC water. Adjust pH to 7.0, adjust volume to 1 L, and autoclave to sterilize
3. *5X MOPS buffer (formaldehyde-gel-running buffer)*: 0.1 M MOPS, 0.03 M sodium acetate, 0.005 M EDTA. In a sterile 1-L container, put 20.6 g MOPS, 4.1 g sodium acetate, and add 800 mL DEPC water. Adjust pH to 7.0 with NaOH (approx 10 pellets), and add 10 mL 0.5 M EDTA (pH 8.0). Bring up to 1 L, and sterilize by filtration (0.2 μm) into preautoclaved 500-mL bottles. Cover bottles in foil (to keep out light) and store in the dark.
4. *Ethidium bromide stock*. **Caution:** Wear gloves and mask!! Add 100 mg of ethidium bromide to 10 mL water, and stir for several hours. Store at 4°C in a dark or foil-wrapped tube (light-sensitive)

2.1.2. For Loading the Samples

1. *3 M Sodium acetate, pH 5.2*: Weigh 204.1 g sodium acetate into 400 mL DEPC water and add glacial acetic acid (>50 mL) until all sodium acetate is dissolved and pH is 5.20 ± 0.01. Make up to 500 mL with DEPC water, dispense into 100-mL bottles, and autoclave to sterilize. Store at 4°C in sterile screw-capped bottle.
2. *75% Ethanol*: 75 mL absolute ethanol, 25 mL DEPC water. Store at 4°C in sterile screw-capped bottle.
3. *Formaldehyde-gel loading buffer*: 50% glycerol, 1 mM EDTA (pH 8.0) bromophenol blue, xylene cyanol FF. Prepare by mixing the following: 5.00 mL glycerol (sterile by autoclave), 20.0 μL 0.5 M EDTA (pH 8.0; in DEPC water), 25.0 mg bromophenol blue, 25.0 mg xylene cyanol FF, 4.98 mL DEPC water. Aliquot as 1-mL volumes and store at 4°C

2.1.3. For Vacuum Blotting to Membrane

1. *Denaturing solution*: 0.15 M NaCl, 0.05 M NaOH. Weigh 8.8 g NaCl and 2.02 g NaOH into 1 L of DEPC water. Autoclave to sterilize. (Alternatively in the absence of any available DEPC water, weigh 2.02 g NaOH into 1 L of sterile normal saline solution and sterilize.)
2. *Neutralizing solution*: 0.15 M NaCl, 0.1 M Tris, pH 7.5. Weigh 8.8 g NaCl and 12.1 g Tris base into 800 mL DEPC water, and adjust pH to 7.5 with concentrated HCl. Make up to 1 L. Autoclave to sterilize. (Alternatively in the absence of any available DEPC water, weigh 12.1 g Tris base into 1 L of sterile normal saline solution, adjust pH to 7.5, and sterilize.)

2.1.4. For Hybridization

1. *tRNA*: 10 mg/mL. Stored frozen in DEPC water at -20°C in 200-μL aliquots.
2. *5XPE buffer*: 0.25 M Tris-HCl pH 7.5, 0.5% w/v sodium pyrophosphate, 5% SDS, 1% polyvinylpyrrolidone (mol wt 40,000), 1% ficoll (mol wt 400,000), 25 mM EDTA. First make the following solutions:
1 M Tris HCl, pH 7.5. weigh 15.8 g Tris HCl into 80 mL DEPC water, and adjust pH to 7.5. Make up to 100 mL in DEPC water and filter (0.2 μm) to sterilize

5% BSA: Weigh 5 g BSA (fraction V) into 100 mL DEPC water and dissolve, with warming to 37°C if necessary. Do not shake! Sterilize by filtration (0.45 μ m). Store in 20-mL aliquots at -20°C for use.

To 45 mL DEPC water in a 100-mL bottle, add 25 mL 1 M Tris-HCl pH 7.5, 0.5 g sodium pyrophosphate, 5.0 g SDS, 1.0 g polyvinylpyrrolidone (360 kDa), 1.0 g ficoll (400 kDa), 5.0 mL 0.5 M EDTA, pH 8.0. Heat to 65°C until completely dissolved (approx 1 h with occasional mixing). Cool to 37°C, then add 20 mL of 5% BSA (fraction V) stock solution (reserved at -20°C), for a final concentration of 1% BSA. (Do not add powdered BSA directly to 5X PE.) Once dissolved, reheat to 65°C for 15 min, and filter immediately (while still hot) through a 0.45- μ m filter. **Note:** This will take at least 45 min to filter 100 mL, and recovery will only be 85–95 mL. Do not use 0.2- μ m filter, as it will take much longer! Store at room temperature

3. *Prehybridization/hybridization buffer* (prepare fresh as needed) In a sterile 50-mL tube mix 10.0 mL formamide, 5.0 mL 20X SSC, 4.0 mL 5X PE, 1.0 mL DEPC water, 100 μ L tRNA stock (tRNA does not have to be boiled) Total volume is 20 mL, sufficient for one to two blots.
4. *10% SDS:* Dissolve 50 g SDS in 500-mL sterile (bottled) water and adjust pH to 7.2 with HCl. This solution does not require further sterilization.
5. For posthybridization washing, make up the following buffers (1 L each prepared from stock 20X SSC and 10% SDS) for membrane washing (*see Note 3*)
 - a. *Low-stringency wash buffer:* 2X SSC/0.1% SDS.
 - b. *High-stringency wash buffer:* 0.1X SSC/0.1% SDS.

2.2. Membrane Stripping

1. *Stripping solution:* Prepare 1 L of 0.1X SSC/0.5% SDS from stock 20X SSC and 10% SDS.

3. Methods

3.1. Northern Analysis

Work under sterile conditions; wear gloves.

1. Turn on vacuum centrifuge refrigeration trap and 65°C water bath
2. Wash gel apparatus, tray, and comb with ethanol, water, and then DEPC water. Dry carefully, and tape open ends of plastic tray with autoclave tape to seal. Put the gel apparatus in the hood (formaldehyde!) and set the unit level. Place tray in the tank, and set the comb/holder such that the comb teeth are approx 2 mm above the gel tray and 1.5–2 cm inside the taped end. Fill with tray one-half full with DEPC water to check for leaks.

3.1.1. Gel Preparation

1. For a 15 \times 15-cm gel tray, mix agarose to desired percentage w/v (typically 1–1.5%). The final vol is 150 mL, so for a 1% gel use 1.5 g agarose with 95 mL DEPC water in flask 1. Mix 25 mL formaldehyde with 30 mL 5X MOPS gel-running

- buffer (to give a final concentration of 2.2 M and 1X, respectively) in flask 2. Add 10 μL stock ethidium bromide solution.
- 2 Allow agarose to stand for 5 min, then heat the agarose solution in a microwave oven (2 min, swirling every 30 s; be careful—it will boil) Then cover both flask necks with foil, and incubate both flasks at 65°C for 15 min (water bath)
 3. Pour the formaldehyde solution (flask 2) into the agarose solution (flask 1) (slowly, down the side of the flask to avoid making bubbles) Incubate at 65°C for another 15 min
 - 4 Tip out the DEPC water from the tray and dry the tray and comb with a paper towel and set in place.
 5. Pour the agarose solution into the tray Wait 45 min to set. Meanwhile prepare 1.6 L 1X gel-running buffer (320 mL 5X MOPS buffer to 1600 mL final volume in DEPC water)
 6. Once the gel is set, pour some buffer over the wells of the gel and wait 10 min before pulling out the comb Pull straight up, not at an angle, or the divisions between wells will tear. Then lift out the gel tray and remove the tape from the gel ends. Place the gel tray back in the tank. Add all of the buffer until the gel is completely submerged (at least 5 mm).

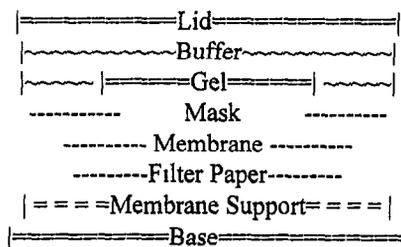
3.1.2 Sample Preparation

Work on ice:

- 1 Take required amount of sample (in molecular-biology grade water) into Eppendorf tube and make up to 100 μL . Add 10 μL 3 M sodium acetate (pH 5.2), and 500 μL ethanol (ice-cold) Stand on ice 5 min and spin 12,000g for 15 min at 4°C
- 2 Tip off supernatant and add 0.5 mL ethanol (75%) Invert but do not mix
- 3 Spin once more for 10 min, and tip off supernatant Add 500 μL more ethanol (75%), and spin for 5 min.
4. Tip off supernatant, drain inverted for 5 min, then dry 5 min in vacuum centrifuge
5. Put BRL RNA molecular-weight markers (3 μL) in tube and process as a sample below (do not worry about 3- μL volume error, as markers are not quantified)
- 6 Premix 100 μL DEPC water, 40 μL 5X MOPS gel-running buffer, 70 μL formaldehyde, and 200 μL formamide, in a sterile tube (sufficient for 20 samples) and add as a single aliquot of 20 5 μL to each sample/standard.
7. Cap samples and incubate at 65°C for 15 min.
8. Add 2 μL formaldehyde gel-loading (dye) buffer to each sample/standard and mix for 10 s.
- 9 Incubate 5 min at 65°C and then transfer to ice
10. During the 5 min incubation, prerun gel at 70 V
- 11 Turn off power, load samples into wells (*see Note 4*). Be careful not to poke through the base of the well or tear the divisions between wells (*see Note 5*) Run samples into gel at 50 V for 30 min. Then lower voltage to 18 V for overnight run (assuming 16 h run time—adjust accordingly) (*see Note 6*)

3.1.3. Vacuum Blotting to Membrane

1. Turn off power and remove gel tray to a glass dish. Photograph the gel as required under UV light (*see Note 7*).
2. Put the gel tray/gel back in the glass dish and cover with NaCl/NaOH denaturing solution (approx 200 mL). *Carefully* slide the gel out of the tray by pushing from the *top* end, with fingers well spread to give even pressure. Incubate for 30 min.
3. Carefully aspirate off the solution using a suction device.
4. Add Tris-NaCl neutralizing solution until gel is submerged, and incubate for 30 min.
5. Remove liquid as above, and then add 250 mL 10X SSC (make by diluting 250 mL 20X SSC with 250 mL DEPC water) to submerge the gel. Incubate for 15 min. During this time prepare the blotting apparatus (**steps 6–8**).
6. Cut a filter paper pad (2.5 cm larger height and width than gel) to fit on the membrane support (*see diagram in step 9*).
7. Use the gel tray as a guide to cut a piece of membrane for blotting. Remove the top piece of protective paper but leave the membrane on the bottom sheet and place on a larger sheet of foil. Turn up the edges of the foil (to act as a shallow dish) and pour sufficient DEPC water on the membrane to just wet it completely.
8. Clean a glass plate with ethanol. (The plate will be used to transfer the gel to the pressure blotter. The plate should be larger than the gel but narrower than the glass dish.)
9. Assemble the blotting apparatus as described below (and manufacturer's diagram).



Wet the porous base with 10X SSC and spread along the gray tubular seal around the edge of the vacuum-blotter base. Put the filter pad on the porous base and completely saturate by pouring 10X SSC over the top surface. Pick up the wet membrane by the left and right edges, and bend *slightly* so the vertical center line is dropped below the edges. Immediately lower the membrane center line down onto the wet filter paper center line, and then slowly lower the left and right membrane edges. Check for air bubbles between the membrane and the paper; there should be none by this procedure. Put the mask in place, with the opening centered on the membrane, and edges of the opening overlapping the membrane on all four sides, while the outer edges are over the tubular gray reservoir seal. Put the upper retainer/reservoir on and press down evenly to secure the latches.

Pour some 10X SSC on the membrane again so that it will eliminate air gaps when the gel is placed on top. Check between the membrane and the mask for air bubbles before transferring the gel. It is important at this stage that all three components are centered relative to each other, and that the mask overlaps the membrane evenly on all sides.

Slide the clean glass plate under the submerged gel (Do not remove the 10X SSC first, leave the gel submerged. Start by placing the plate under the *side* of the gel, rather than the top or bottom, so that the wells do not tear as the gel bends slightly.) Transfer the gel/glass plate onto the membrane/mask so that it is centered. Use a UV transilluminator (*see Note 7*) to check that the outermost lanes containing samples will be directly over membrane, not the mask. Then place fingers evenly spread to support the gel edge, and slide out the glass plate. Check alignment with the transilluminator

- 10 Connect the vacuum regulator and gage between the vacuum line/pump (*see Note 8*) and a solvent trap. With a thumb over the vacuum inlet, check that any vacuum applied will not exceed 5–7 psi. *Only then* connect hose to vacuum blotter and switch on. Immediately set vacuum to 7 psi. As the vacuum is applied, the mask should flatten out and a sharp line should form at the edges of the filter paper below the mask. The gel will pull down except at the edges if a true seal is formed. At this point pour 1–1.5 L 10X SSC over the gel and check the vacuum is 7 psi. Leave for 90 min. Put cover on tank to protect gel during this time. (*Optional* Place the sponge soaked in 10X SSC on top of the gel instead of 1.5 L 10X SSC in tank.)
- 11 At the end of vacuum blotting, turn off the vacuum, and undo the latches on the retainer to release the unused SSC. Remove the lid and sponge. Gently lift one corner of the gel and view both the gel and membrane with the transilluminator (*see Note 7*). At least 90%, if not all of the RNA should have been transferred. If transfer was poor (because of low vacuum), decide whether to continue blotting for longer (although you will now need fresh SSC).
- 12 Once transfer is complete, remove the gel and mask. Then place the membrane in a glass dish and rinse briefly in DEPC water (to remove SSC). Then drain by holding vertically for a few seconds, and lay flat on clean filter paper. Leave until the membrane looks visibly dry. Then place on a clean paper towel or filter paper and transfer to the UV crosslinker. Close door, turn power on, and then crosslink at 120 mJ. Once the crosslinker has finished, and the beeper has sounded, turn off, open the door, and remove the membrane.
- 13 Viewing the membrane under UV (*see Note 7*), use a pencil to mark the positions of the wells that were loaded with samples, and on the right side the levels of the 28S and 18S subunits. Also mark the positions of any molecular weight markers. Cut the top right corner (for future orientation) and mark the blot with experimental date(s) and tissues.
- 14 Place the blot in a clean hybridization bag and seal for future hybridization. Store at -20°C .
- 15 Clean the blotter thoroughly. Salt residues left behind on the mask or seals will interfere with forming a vacuum. Keep the unit clean!

3.1.4. Hybridization Protocol Using tRNA

- 1 The 20-mL vol hybridization buffer is more than sufficient for one large (15 × 15-cm) blot in a hybridization tube. Open one end of the tube, and place the semidry blot into the dry hybridization tube (this is the easiest method for large blots). Tip/pipet the solution directly onto the center line of the Northern blot, and then replace end cap. Place the bottle on a bench and slowly rock left and right until all the membrane is wet. Try to avoid large air bubbles. If they occur, open the bottle again and peel one edge of the membrane back using forceps. (Grip the edge only. Do *not* rub the membrane surface with forceps since this will result in nonspecific binding later on in the signal area.) Do not worry about small air bubbles. Place in the hybridization oven for at least 4 h (overnight is best) at 42°C. **Carry out all remaining steps behind a radioactivity shield**
2. Take sufficient probe (preferably generated by asymmetric PCR [3], see Chapter 30) for 1×10^6 dpm/mL hybridization mix and dilute to minimum of 100 μ L using DEPC water, in a microfuge tube. Heat probe to 95°C (i.e., without boiling) for 5 min and then transfer directly to ice/water for 3 min. Spin briefly to bring condensation down from lid/tube sides.
- 3 Open the small part of the tube containing prehybridized membrane, and add the appropriate volume of boiled cDNA probe to the prehybridization buffer (The same solution can be used for prehybridization and hybridization.)
- 4 Put the tube back in the oven at 42°C (water bath) overnight.
- 5 After the incubation: Remove the tube from 42°C oven. Working behind a shield, remove the port cover and tip all the hybridization buffer to a waste beaker. Discard as hot waste.
6. Add 10 mL of 2X SSC/0.1% SDS, to remove excess unbound probe. Then tip off 2X SSC/0.1% SDS to a beaker and discard as radioactive waste.
- 7 Add 20 mL 2X SSC/0.1% SDS, and incubate for 15–30 min at 42°C. Tip off and discard as hot waste.
- 8 Repeat with 20 mL 0.1X SSC/0.1% SDS for 30 min at 42°C, but discarding waste down radioactive designated sink.
- 9 Repeat with 20 mL 0.1X SSC/0.1% SDS for 30 min at 42°C (see Note 3).

3.1.5. Autoradiography

Wrap the dry blot in plastic wrap, scan on a direct radioimaging scanner (e.g., Phosphorimager) if required, and then tape down on a film cassette. Keep under film (Amersham Hyperfilm MP [Arlington Heights, IL] is best for [32 P] at -70°C). Develop the film after the required time (varies both with probe and tissue mRNA level). Quantify by densitometry.

3.2. Probe Stripping and Rehybridization

1. Prepare 1 L of 0.1X SSC/0.5% SDS
2. Put the membrane in hybridization bottle and set oven to 65°C. Bring 100 mL of 0.1X SSC/0.5% SDS to boil (microwave). Pour over the membrane and return to oven. Repeat three more times over 60 min.

3. Check the membrane by overnight exposure or by Geiger counter
4. Rehybridize as required for other signals and/or housekeeping mRNA (*see Note 1*). MagnaGraph membrane can be reliably reprobbed four times without significant loss of signal

4. Notes

1. Because of the cumulative effect of possible errors in accurate spectrophotometric quantification of RNA, loading errors (sample loss) and variations in transfer efficiency, the quantity of RNA loaded into a lane and transferred to membrane, even by an experienced operator, can vary by 10–20%, and by more for an inexperienced operator. Convention therefore states that data from Northern analysis should be normalized to the level of message for a housekeeping gene in the same lane (i.e., the level of message in the same lane of a gene product expressed at constant and preferably abundant level under all circumstances). This, as many workers in the field will tell you, is easier said than done, because all so called housekeeping genes can change in their expression at one time or another. Popular candidates are β -actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and 28S or 18S subunits, for which probes/sequences are widely available (*see Fig. 1*). In reality other messages are often used by different groups, but this is acceptable provided the cells/tissue in question can be shown to express this housekeeping message at a constant level under the conditions of interest. On a more practical note, the number of replicate experiments necessary to achieve significance in demonstrating a change in level of the message of interest is reduced considerably by normalization procedures.
2. DEPC is both a potent carcinogen and, under rare circumstances, explosive. The material should always be handled carefully, with gloves, and the bottle should always be stored sealed at 4°C.
3. If room temperature is low, salt precipitation may occur, particularly in the 2X SSC/0.1% SDS buffer. To be safe, the temperature must be above 20°C for all washing steps. Use a water bath set to 20°C to preheat the buffer if this is a recurrent problem. These wash buffers were optimized for asymmetric PCR generated probes (*3*), but also be aware that those generated by other methods such as random-primer labeling or end labeling of oligonucleotides may require the use of elevated temperature (up to 65°C) for the 0.1X SSC/0.1% SDS wash in order to remove the higher background radioactivity. Alternatively stringency of washing can be further increased by raising the SDS% or lowering the SSC concentration further. Conversely if initially strong signal is unduly lost, stringency can be reduced by lowering the % SDS or raising the SSC concentration.
4. With a pipet set to approx 22 μ L, this should not be a problem. Try to avoid creating air bubbles at the end of loading as they may disturb the sample and wash it out of the well. In the event that samples actually float out, this usually indicates ethanol was not completely removed at the vacuum-centrifugation step, and is likely to be caused by incomplete tipping of the 75% ethanol from the pelleted RNA. In the event the sample is extremely viscous, this often indicates an RNA preparation contaminated with salt

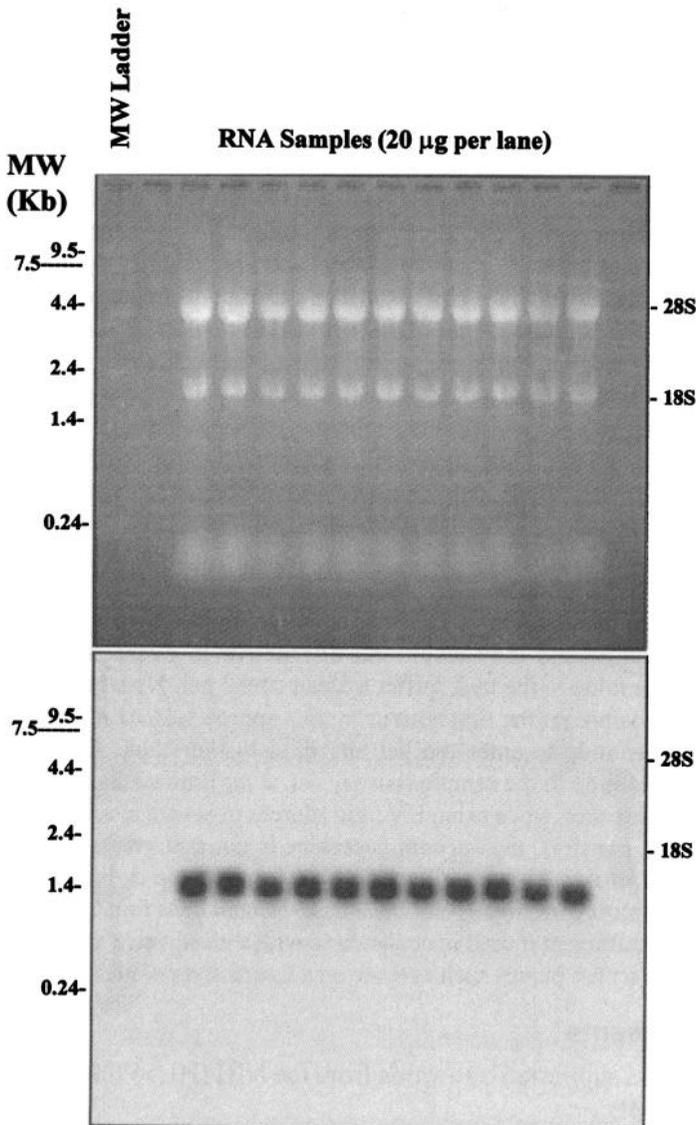


Fig. 1. Size separation and Northern hybridization analysis of total cellular RNA. Total cellular RNA was recovered from cultured cells by the procedures described in Chapter 28. RNA (20 µg per lane) was size separated overnight on a 1.1% formaldehyde/agarose gel as described. Following transfer to hybridization membrane, detection of GAPDH mRNA (approx 1.4 kb) was achieved using an antisense probe generated by asymmetric PCR (3) (Chapter 30). Note the consistency of expression between lanes, consistent with the appearance of 28S and 18S subunits under UV light.

Further RNA precipitation and wash steps, or use of larger volumes of RNAzol B should be considered during preparation of samples.

5. If you are not familiar with this technique, it is a good idea to prepare a practice gel and samples and try it first. In particular, see just how much effort it takes to break the wells and experiment with the pipet position in the well. When loading samples into gels of this type, the sample falls into the well by virtue of its higher density. Thus it is not necessary to place the tip deep in the well, which runs the risk of poking a hole through the bottom with subsequent loss of sample. In my experience, successful loading occurs best when you are comfortably leaning forward over the gel rig and, hold the pipet at an angle and approach the well from one side. Place the tip just into the center of the well and just below its surface. You should be able to do this without touching the walls of the well at all. If you are having trouble with shaking hands, make sure the elbow of your pipeting arm is also down on the bench. This is not a difficult technique once you are used to it.
6. The values given (50 V for 30 min = 25 Vh, and 18 V for 16 h = 290 Vh) are for a Bio-Rad DNA SubCell with 30 cm between electrodes. Thus the total Vh will need to be adjusted if your electrodes are at a different distance. In addition, if you want to run gels more quickly, reduce the time to yield the same total Vh. In the rig described herein, we do not recommend voltages higher than 50 V long term because of excess heating. Note also for shorter runs a higher ethidium bromide background may result. An alternative to overcome this is to add ethidium bromide to the tank buffer instead of the gel. Note however that RNA will not be visible for the first hour using this approach since it takes time for the ethidium bromide to enter the gel and bind to the RNA. Alternatively, add ethidium bromide to the sample (but see ref. 2 for limitations of this approach).
7. Wear eye protection when using UV light sources or severe eye damage can occur.
8. For vacuum transfers, the vacuum necessary is not that great. Manufacturers of vacuum and pressure blotters will provide an electric pump dedicated to the blotter, which is the most convenient solution. House vacuum lines found in many laboratories are also sufficient if used in conjunction with a bleed valve and pressure gauge. Multistage vacuum pumps such as those on a freeze dryer would be too much.

Acknowledgments

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Preparation of Single-Stranded Antisense cDNA Probes by Asymmetric PCR

Daniel S. Millican and Ian M. Bird

1. Introduction

Monitoring the changing levels of mRNA in cells is often used to determine if changes in protein level may relate to changes in mRNA stability or gene transcription. A commonly used technique by which this can be achieved is Northern analysis (*see* Chapter 29), or for low levels of mRNA, reverse transcriptase-polymerase chain reaction (RT-PCR) assay (*see* Chapter 31). In both cases, sensitive detection methods still rely on use of labeled probes. Whereas oligonucleotides have widely been used as probes, their short length and variable GC:AT content contributes to difficulties in consistently achieving sensitivity without high background. Many other improved methods of generating larger probes have been developed over the years, but they can generally be described as three types, namely double-stranded DNA (dsDNA) generated by random primed or symmetric PCR methods, riboprobes by RNA polymerase generation of antisense copy from target cDNA, and, more recently, antisense single-stranded DNA (ssDNA) synthesis by unidirectional or asymmetric PCR. The most commonly used approach is a technique called random priming (*1,2*), which uses random hexomers or nanomers to prime abundant quantities of purified template to extend into short complementary copies. The advantage is flexibility, since any template can be used without knowledge of sequence, and kits are readily available. However a limitation of random priming is that product formed is both sense and antisense, so setting up competition between the target membrane and sense products for antisense probe, and this is exacerbated still further by the yield being less than the starting template. The result is higher backgrounds and poorer linearity of quantification than with other methods (*3*). Whereas symmetric PCR-generated probes offer better results,

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higher background still occurs (4) because of equimolar yield of sense strand. Whereas riboprobes offer the other extreme of detection, i.e., low backgrounds, high sensitivity, and good linearity, their setup (5) is complicated since dedicated constructs are required to set the probe sequence next to an appropriate priming site for RNA polymerase. The more recent development of antisense single-stranded cDNA probe methodologies (unidirectional PCR amplification or asymmetric PCR amplification) has resolved this dilemma, producing probes of high sensitivity and low background with up to 15 times the sensitivity of random primed probes and excellent linearity of detection even at the lowest levels of target RNA (3). Since the methods are PCR-based, however, templates do not need extensive modification since selection of oligonucleotide sequences determines the target amplified. Thus, such methods are flexible, being successfully achieved on a variety of templates including recombinant plasmids or dsDNA from RT-PCR, and the complications of establishing riboprobe methodologies are avoided. In addition, these probes are relatively easily stripped from hybridization membranes, so allowing repeated probing in Northern analysis up to four times.

Of the two methods for single stranded antisense probe generation by PCR, we favor asymmetric PCR over unidirectional PCR since it requires less initial template; by providing forward and reverse primers at a 1:100 ratio, double-stranded amplification in the first half of the reaction generates additional sense strand to act as template for an excess of antisense strand generation in later cycles. In this chapter we describe a general protocol for the development of predominantly antisense cDNA probes labeled with [α - 32 P]dCTP by asymmetric PCR. Using this methodology, probes can be routinely generated in a range of sizes from 200–2000 bases, at high specific activity (3.12×10^8 dpm/ μ g) using a one step reaction. The resultant product is >90% antisense, exceeds initial template by >20-fold, and does not require clean up before use. Although these protocols were developed and optimized for Northern analysis, we have found them equally useful in detection of DNA targets such as in RT-PCR product Southern analysis and in screening of isolated clones.

1.1. Strategy

1.1.1. Recombinant Plasmid Template and Custom Oligonucleotide Primers

Although PCR amplification could be performed using template in the form of a heterogeneous pool of dsDNA, the possibility of spuriously primed products is virtually eliminated by isolation of a cDNA containing the desired probe template within it. Ligation of the dsDNA containing the template region into a plasmid allows improved replication fidelity when cloned in transformed cells

over that achieved by pooling symmetrically amplified product (*see Note 1*). The region of the insert to be amplified as probe should be specific to your message of interest, not common to a family of proteins. Oligonucleotide primers specific to the region of interest can be designed using software such as "Primer" (Sci-ed Software, Durham, NC), which selects primer sequences based on criteria including melting temperature, GC content, secondary structure potential, and paired primer complementary. Alternatively, universal plasmid primers may be used, in which case the entire insert and plasmid sequence flanked by the primer pair sequence will be amplified (*see Note 2*).

1.1.2. Thermal Cycling Profiles for Symmetric and Asymmetric Amplification

Each thermal cycle consists of dsDNA template denaturation, primer annealing, and *Taq* DNA polymerization temperature plateaus. The temperature and/or period of each plateau can be suited to probes dependent on their length and on the melting temperatures of the primers. The denaturation of the dsDNA template is consistently performed at 94°C. Annealing can generally be performed at 45°C, 10–15°C below the desired primer melting temperature, to promote greater yield (*see Note 3*). *Taq* DNA polymerase synthesizes most efficiently at 72°C, incorporating approx 1000 bases per minute. Maximal symmetric yield occurs prior to 30 amplification cycles (6) after which free nucleotides and *Taq* activity become limiting. However, since the later cycles of asymmetric amplification are linear, cycle number should be increased to 40 (7). Products of 500 bases or less consume fewer nucleotides, so asymmetric amplification cycle number should be increased still further to 50.

1.1.3. Evaluating PCR Parameters by Symmetric Amplification

Template is amplified using abundant forward and reverse primers (30 pmol each). Repeated thermal cycles give rise to an exponential increase of both sense and antisense strand copies of template DNA. If the thermal profile is appropriate, minimal overextension or premature termination will occur and electrophoresed product will appear as a sharp band. If conditions are not optimal, target sequence can be restricted from neighboring sequence to improve product specificity (*see Note 2*).

1.1.4. Agarose/TAE Electrophoresis of Symmetric Products

Symmetric products are size separated in agarose/TAE and visualized by ethidium bromide staining to confirm amplification efficiency and fidelity. **Figure 1A** shows, left to right, discretely resolved 936-, 1083-, 957-, 1092-bp bands amplified from different recombinant plasmids, p1–p4, using different custom primer pairs (3). All reactions were performed using the same cycling

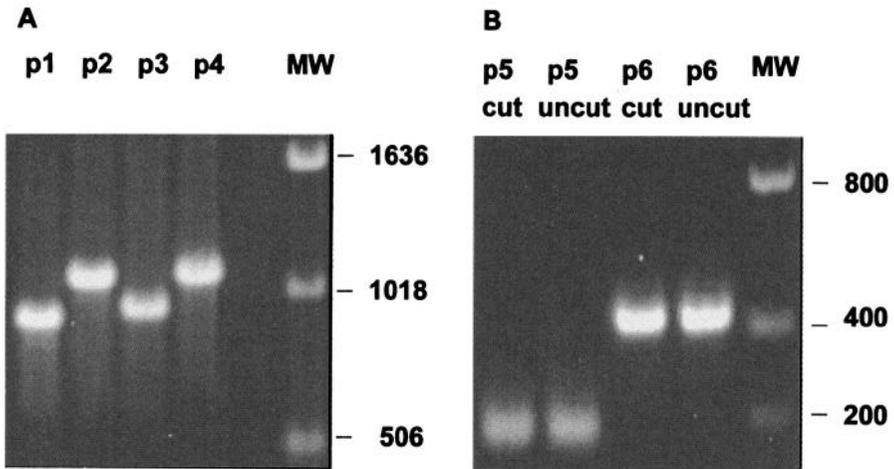


Fig. 1. Ethidium bromide-stained symmetric PCR products amplified using cycling profiles for templates (A) 750–2000 bp and (B) ≤ 500 bp. Note in B that templates were amplified both uncut, and cut by restriction digest.

profile (see profile 1, **Subheading 3.1.2.**) for 750–2000 bp templates, demonstrating robust cycling conditions. Cycling profile 3 for templates ≤ 500 bp was used to amplify 143- and 432-bp templates from plasmids p5 and p6 using custom primer pairs (**Fig. 1B**) (see **Note 4**). Separate amplifications with insert intact and excised from plasmid were performed. Ethidium bromide staining does not show improved size specificity using restricted insert as template under these optimized conditions, although overextension is more likely for smaller templates if conditions are not optimized.

1.1.5. Asymmetric Probe Generation

Having validated cycling conditions, predominantly antisense [α - ^{32}P] dCTP labeled cDNA probes are amplified using a 1:100 forward/reverse primer asymmetric molar ratio. Exponential amplification occurs until limited forward primers are consumed, after which antisense cDNAs are linearly amplified to a relative abundance over dsDNA in proportion to the remaining number of thermal cycles. To increase specific activity, [dCTP] is reduced to 0.025X the 200 μM concentration each of other nucleotides. **Figure 2** demonstrates typical radiolabel incorporation and mass generation for approx 1000 base probes through a range of [dCTP], which is optimal at 2–5 μM . A >20X yield of probe mass is typically generated from 10 ng dsDNA template at 5 μM dCTP (**3**).

As shown in **Fig. 3**, although asymmetric PCR yields less product than symmetric amplification because of limited forward primer and reduced [dCTP],

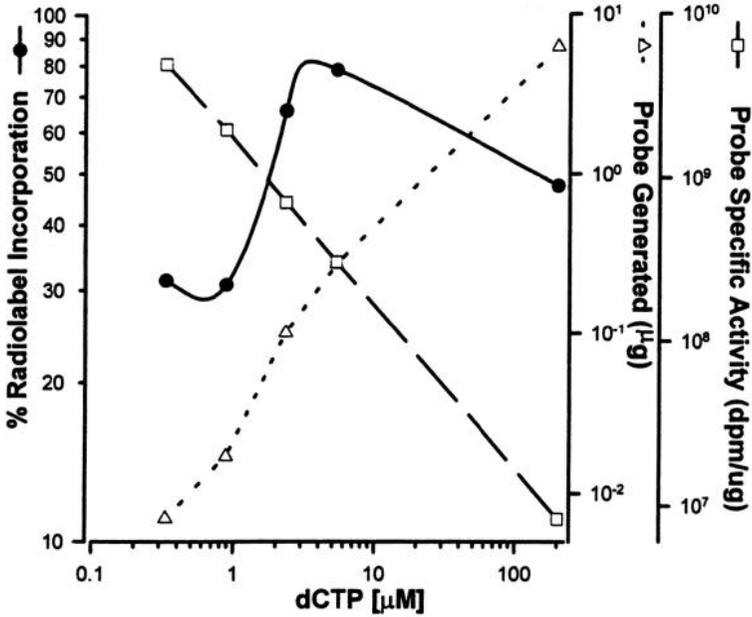


Fig. 2. Effect of [dCTP] on radiolabel incorporation, probe mass, and specific activity. Results were obtained for amplification of a 936 bp target using protocol 1.

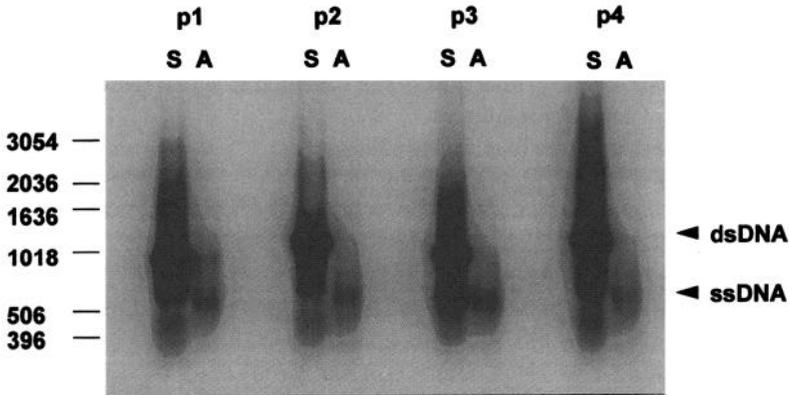


Fig. 3. Determination of single-stranded vs double-stranded product formation under symmetric vs asymmetric amplification conditions. Templates p1-p4 were amplified under symmetric (S) and asymmetric (A) PCR conditions in the absence of radiolabeled dCTP. 10 μL of each reaction product was then resolved in a 1% TAE gel and transferred to nylon membrane before probing with respective symmetrically amplified radiolabeled probes in order to detect both forward and reverse strand products. Note that ssDNA apparent MW is approx 2/3 that of dsDNA.

signal from ssDNA increases to >90% of total signal for asymmetric products, up dramatically from <20% for symmetric products.

1.1.6. Measurement of Radiolabel Incorporation.

Small aliquots of amplified product are dried on ion exchange filter disks and washed with sodium phosphate buffer to remove unincorporated radiolabel. Percent incorporation is determined by scintillation counting against washed and unwashed disks in order to ascertain whether sufficient incorporation, and thus sufficient probe mass generation, has occurred. Probes are ready for use without further cleanup, which is a distinct advantage over other conventional probe generation methods.

2. Materials

- 1 Autoclave
2. NaOH, FW 40.0, Sigma (St. Louis, MO, cat no S0899).
3. HCl, 12.0 M, Sigma (cat. no. H7020)
4. 0.5 mL thin-walled reaction tubes, Perkin Elmer (Foster City, CA) (8010180).
5. Sterile water, molecular-biology grade
6. *Taq* DNA polymerase, 5 U/ μ L, Gibco-BRL (Gaithersburg, MD, cat no 18038042)
7. 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), provided with *Taq*
8. 50 mM MgCl₂, provided with *Taq*
9. 10 mM dATP in Tris-HCl pH 7.5, Gibco-BRL (cat no 18252015)
10. 10 mM dCTP in Tris-HCl pH 7.5, Gibco-BRL (cat no 18253013)
11. 10 mM dGTP in Tris-HCl pH 7.5, Gibco-BRL (cat. no. 18254011).
12. 10 mM dTTP in Tris-HCl pH 7.5, Gibco-BRL (cat. no. 18255018).
13. Custom oligonucleotide primers, Midland Certified Reagent (Midland, TX).
14. Recombinant plasmid templates.
15. Mineral oil, Perkin Elmer (cat. no 1862302).
16. Trizma base, FW 121.1, Sigma (cat. no. T8524).
17. Acetic acid, Sigma (cat. no. A6283)
18. Disodium EDTA, FW 372.2, Sigma (cat. no. E5134).
19. Glycerol, FW 92.1, Sigma (cat. no. G5516)
20. Lauryl sulfate, sodium salt (SDS), FW 288.4, Sigma (cat. no. L4390).
21. Bromophenol blue, FW 691.9, Sigma (cat. no. B5525).
22. Ethidium bromide, FW 394.3, Sigma (cat. no. E7637).
23. Agarose, DNA analytical grade, Bio-Rad (Melville, NY) (cat no 1620125)
24. kb DNA MW ladder, 0.2–12 kb, Gibco-BRL (cat no. 15615016)
25. [α -³²P] dCTP 3000 Ci/mmol, 10 μ Ci/ μ L, Amersham (Arlington Heights, IL, cat. no. PB10205)
26. Aluminum foil
27. Anion-exchange paper circles, Whatman (Clifton, NJ, cat. no. DE81).
28. Na₂HPO₄, FW 142.0, Sigma (cat. no. S9763).
29. Scintillation vials.

- 30 Scintillation fluid, Fisher (Pittsburgh, PA, cat. no. SX184).
31. PCR cycler, Perkin Elmer (model TC1 or 480).
- 32 Submarine gel apparatus with gel tray and comb, Bio-Rad.
33. Power supply, Bio-Rad 300 or 200/2.0.
34. Transparent lucite radiation shield.
- 35 Scintillation counter

2.1. Stock Solutions and Instrumentation Requirements

1. 0.5 M EDTA, pH 8.0: Weigh 16.8 g disodium EDTA into 80 mL sterile water, and pH to 8.0 with concentrated or pelleted (approx 2 g) NaOH. Adjust volume to 100 mL and autoclave to sterilize
2. 10% SDS Dissolve 50 g lauryl sulfate, sodium salt in 500 mL sterile water and pH to 7.2 with HCl. This solution does not require further sterilization.

2.1.1. For Template and Oligonucleotide Working Stocks

Recombinant plasmid working stocks should be diluted in sterile water to 10 ng/ μ L and stored in 200- μ L aliquots at -20°C . Primers should be at least 17 bases, with either a G or C residue in 2 of the last 3 bases of the 3' terminus, with annealing temperatures of $55\text{--}60^{\circ}\text{C}$, with approx 50% GC content (*see Note 5*), having no secondary structure and minimal primer dimer formation. Given the mass of primers in optical density units (*see Note 6*), solubilize in sterile water to 1 $\mu\text{g}/\mu\text{L}$ using an A_{260} of 20 $\mu\text{g}/\text{OD unit} \cdot \text{cm}$. Using an estimate of 330 g/mol per base, prepare 20 μM working stocks by further dilution as necessary. Prepare an additional 200 nM stock of forward primer for asymmetric amplification by diluting 1 μL of 20 μM stock to 100 μL with sterile water. Store stocks at -20°C .

2.1.2. For PCR Cycling

The step cycle, which holds temperature plateaus for the time interval programmed, should be used for both symmetric and asymmetric amplifications.

2.1.3. For Symmetric Amplification

1. 2.5 mM dA/C/G/TTP Combine 20 μL each of 10 mM dATP, dCTP, dGTP, and dTTP. Store at -20°C in 20- μL aliquots to prevent freeze/thaw degradation of repeatedly used bulk stocks (*see Note 7*).
2. 50 mM MgCl_2 and 10X PCR buffer Dispense these reagents, provided with *Taq* DNA polymerase, in 50- μL aliquots and store at -20°C

2.1.4. For Agarose/TAE Electrophoresis of Products

1. 5X TAE buffer: Bring 24.2 g Trizma base, 5.71 mL acetic acid, and 10 mL 0.5 M EDTA pH 8.0 to 1 L using sterile water (*see Note 8*). Store at room temperature.
2. 10 mg/mL ethidium bromide: Dilute ethidium bromide to 10 mg/mL with sterile water and store protected from light at room temperature.

- 3 10X Sample buffer: To a 1.5-mL Eppendorf tube add 500 μL glycerol, 200 μL 0.5 M EDTA pH 8.0, 100 μL 10% SDS, 10 mg bromophenol blue, and 200 μL sterile water (*see Note 9*). Store at room temperature.
- 4 Sterile 0.5-mL thin-walled tubes: Sterilize tubes with caps open by autoclaving

2.1.5. For Asymmetric Amplification

1. 2.5 mM dA/G/TTP Combine 20- μL volumes each of 10 mM dATP, dGTP, dTTP, and water. Store at -20°C in 20- μL aliquots.
2. 0.25 mM dCTP. Dilute 20 μL 10 mM dCTP to 80 μL with sterile water. Then dilute 10 μL of the 2.5 mM dCTP solution to 100 μL with sterile water. Store at -20°C in 10- μL aliquots

2.1.6. For Measuring Radiolabel Incorporation.

1. 0.5 M Na_2HPO_4 pH 7.5: Bring 71.0 g Na_2HPO_4 to 1 L with sterile water and pH to 7.5. Store at room temperature.

3. Methods

3.1. General Methods for Amplification

The following methods apply generally for probes regardless of size, with the exception of thermal cycling profiles and possible restriction digest excision of templates less than 500 bases. Three different cycling profiles are recommended, one for 750–2000 base probes, another for 500–1000 base probes, and a third for ≤ 500 base probes. Size criterion is based upon *Taq* incorporation of approx 1000 bases/min, and profile overlap allows simultaneous robust amplification of approx 1000 base templates with either longer or shorter templates, in separate tubes, using the appropriate thermal profile.

3.1.1. Determine Amount of Plasmid to Amplify

Using a 10-ng/ μL working stock, the microliter volume used will equal the size in kb of recombinant plasmid.

3.1.2. PCR Cycling Programs

Program the appropriate step cycle thermal profiles into a PCR cycler:

3.1.2.1. PROFILE 1 750–2000 BASES

1. Symmetric amplification: 94°C 1 min, 45°C 1 min, 72°C 2 min, 30 cycles, linked to 4°C soak.
2. Asymmetric amplification. 94°C 1 min, 45°C 1 min, 72°C 2 min, 40 cycles, linked to 4°C soak.

3.1.2.2. PROFILE 2: 500–1000 BASES

1. Symmetric amplification: 94°C 1 min, 45°C 1 min, 72°C 1 min, 30 cycles, linked to 4°C soak

2. Asymmetric amplification. 94°C 1 min, 45°C 1 min, 72°C 1 min, 40 cycles, linked to 4°C soak

3.1.2.3. PROFILE 3: ≤500 BASES

1. Symmetric amplification: 94°C 30 s, 45°C 30 s, 72°C 30 s, 30 cycles, linked to 4°C soak
2. Asymmetric amplification 94°C 30 s, 45°C 30 s, 72°C 30 s, 50 cycles, linked to 4°C soak.

3.1.3. Generation of Symmetric Products

1. Thaw PCR reagents, except *Taq* (see **Note 10**) on ice. Mix each reagent prior to use. Aspirate template stock, rather than vortex, to prevent shearing of plasmid. Do not mix *Taq*. Add volumes in order shown in **Table 1** to a sterile 0.5-mL tube.
2. Cap and transfer tube to cyclor. Amplify using appropriate step cycle thermal profile

3.1.4. Agarose/ TAE Separation of Symmetric Products

Cast an agarose/TAE gel (see **Note 11**) of sufficient thickness to create well spaces for up to 20 μL loading volume, allowing approx 2 mm between casting tray and bottom of comb:

1. Clean gel-casting tray and seal ends with tape. Position comb such that bottom of teeth are approx 2 mm above casting tray
2. Transfer agarose to microwave-safe glass container and bring to appropriate volume with 1X TAE. Add 10 μL ethidium bromide solution (see **Note 12**). Microwave for 2–3 min with frequent mixing until solution is clear and all agarose is melted. Pour solution into casting tray and allow to cool and harden for 45 min
3. After 45 min, overlay agarose gel with 1X TAE. Allow 5 min for liquid to soak into wells. Carefully remove comb by lifting straight up. Remove tape and place gel in tank under 1X TAE buffer, oriented such that samples migrate through the length of the gel toward the positive (red) electrode.
4. Thaw Gibco kb DNA ladder on ice and mix. Transfer 4 μL ladder and 5 μL sterile water to fresh 0.5-mL tube and add 1 μL 10X sample buffer.
5. When symmetric amplification is complete, transfer each 48 μL aqueous volume to a fresh 0.5-mL tube avoiding mineral oil layer above. Then transfer 10–20 μL of this to a fresh tube containing 1–2 μL 10X sample buffer. Load ladder and samples into wells.
6. To rapidly move DNA into gel, initially run at high voltage (50 V for 30 min on Bio-Rad rigs; see **Note 13**). Continue at this voltage until bromophenol dye front migrates 1/2–2/3 down gel. Alternatively decrease voltage to as low as 0.5 V/cm for overnight run if desired
7. Wearing eye protection, view ethidium bromide-stained DNA bands using a short-wave UV wand (see **Note 14**). Products should be prominent and discretely resolved into tight bands, with minimal smearing. Estimate size and mass of symmetric product band(s) by comparison to ladder (see **Note 15**).

Table 1
Symmetric PCR Reaction Mix

Add in the following order	μL	Final conditions
10 X Buffer	5.0	10 mM Tris, 50 mM KCl, pH 8.4
50 mM MgCl_2	1.5	1.5 mM MgCl_2
2.5 mM dATP/dCTP/dGTP/dTTP	4.0	10 nmol each
Primer oligo F (20 pmol/ μL)	1.5	30 pmol
Primer oligo R (20 pmol/ μL)	1.5	30 pmol
Sterile H_2O	To 50 total	
Plasmid	1 $\mu\text{L}/\text{kb}$	10 ng/kb template
<i>Taq</i> polymerase	1.0	5 U
Mineral oil	25	

3.1.5. Asymmetric Probe Amplification

1. Thaw radiolabel behind transparent lucite protective shield. Aspirate to mix. Do not vortex.
2. Thaw PCR reagents, except *Taq* (see Note 10), on ice and mix prior to use. Aspirate template stock, rather than vortex, to prevent shearing of plasmid. Do not mix *Taq*. Add volumes in order shown in Table 2 to a sterile 0.5-mL tube (see Note 16). Work behind lucite shield during and after addition of radiolabel.
3. Cap and transfer tube to cyclor. Amplify using appropriate step cycle thermal profile.

3.1.6. Radiolabel Incorporation Measurement

Work behind lucite shield. On completion of the reaction, remove 48 μL of the products from below the oil layer into a fresh tube. Dilute to 200 μL with sterile water and mix by aspiration. Check probe incorporation of label as follows:

1. Spot 1 μL of products onto each of three DE81 disks (placed on tin foil) and allow to dry for 5 min.
2. Transfer one disk to a scintillation vial for estimation of total radiolabel.
3. Transfer the two other disks to a 50-mL conical tube. Wash the two disks four times with 0.5 M Na_2HPO_4 pH 7.5 (10–15 mL), standing for 5 min each time.
4. Finally rinse the discs in water, methanol (twice), and air-dry for 3–5 min. Transfer disks to separate scintillation vials, add 10 mL scintillation fluid, and count both “washed” and “total” disks in a scintillation counter for 1 min.
5. Calculate incorporation using the mean of washed counts against total counts. Typical incorporation is 65–70% for all probes. Probe mass should be sufficient to provide 5.0×10^5 to 1.0×10^6 dpm/mL media used to hybridize probe with membrane-bound RNA.
6. Store in lucite container at -20°C .
7. At the time of probe use, heat at 95°C for 5 min, then snap chill on ice 3 min before addition to hybridization buffer.

Table 2
Asymmetric PCR Reaction Mix

Add in the following order	μL	Final conditions
10X Buffer	5.0	10 mM Tris, 50 mM KCl, pH 8.4
50 mM MgCl_2	1.5	1.5 mM MgCl_2
2.5 mM dATP/dGTP/dTTP	4.0	10 nmol each
0.25 mM dCTP	1.0	0.25 nmol
Primer Oligo F (0.2 pmol/ μL)	1.5	0.3 pmol
Primer Oligo R (20 pmol/ μL)	1.5	30 pmol
Sterile H_2O	To 50 total	
Plasmid	1 $\mu\text{L}/\text{kb}$	10 ng/kb template
$[^{32}\text{P}]\text{dCTP}$ 3000 Ci/mmol, 10 $\mu\text{Ci}/\mu\text{L}$	5.0	50 μCi , 0.01666 nmol
<i>Taq</i> polymerase	1.0	5 U
Mineral oil	25	

4. Notes

1. Glycerol stocks of transformed cells (50% glycerol, 50% liquid culture media) can be stored indefinitely at -70°C for future inoculation of culture media. A variety of commercially available plasmid isolation kits, such as those offered by Qiagen, offer plasmid extracts of purity satisfactory for use as template in PCR.
2. Universal primers, which anneal to sites on plasmid sequences bordering inserts, are effective for probe generation for use in Northern analysis but, by definition, preclude amplification of a subregion in the cloned insert. Probe use in applications such as cDNA library, genomic, or recombinant plasmid screening, is also compromised because of the presence of plasmid sequence in the probe. Additionally, cDNAs amplified from template region of inserts will have discrete 5' ends but overextended 3' ends may incorporate neighboring sequence or plasmid-linker arm sequence, particularly for small probes. Such additional sequence incorporation in this small fraction of the cDNA pool can be eliminated, as demonstrated in Fig. 4, by amplifying template restricted from vector.
3. Primers designed for use in both asymmetric probe generation and RT-PCR amplification having melting temperatures $>60^\circ\text{C}$ will still anneal specifically if template is limited to recombinant plasmid, rather than cDNA library or genomic DNA. The higher melting temperature (T_m) promotes a greater proportion of binding to available template at 45°C (approx 15°C below the T_m) and so enhances yield.
4. Plasmids p5 and p6, containing partial ovine clearance natriuretic clearance factor receptor and ovine guanylate cyclase type B natriuretic factor receptor cDNAs (8), respectively, were provided by G. Peter Aldred, University of Melbourne, Australia.
5. G and C residues contribute greater hydrogen bonding and thus increase melting temperature. If GC content is low, extending primer length will increase melting temperature.

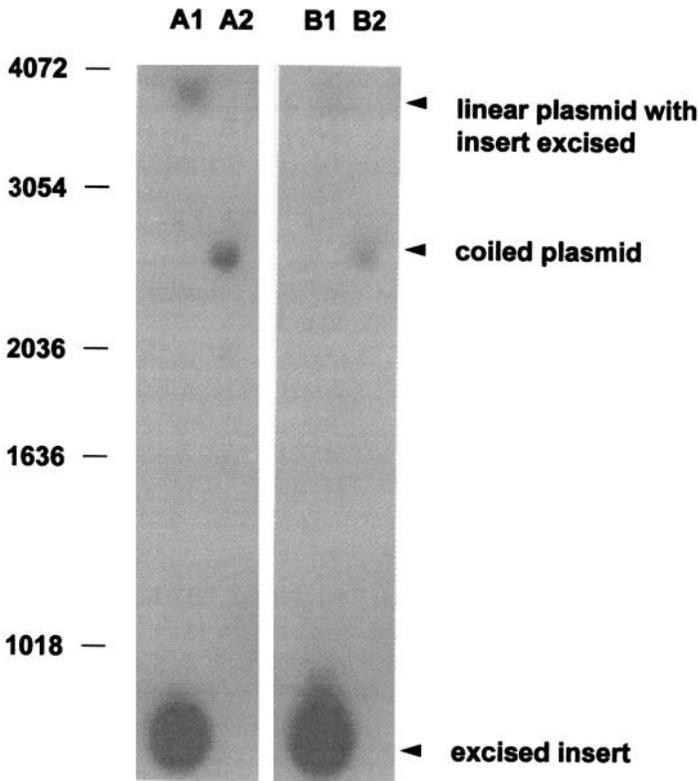


Fig. 4. Plasmid-vector detection by probes generated from intact plasmid template and from plasmid with insert excised. 3.9 kb plasmid with 718-bp insert removed by restriction enzyme digestion was resolved alongside intact, coiled recombinant plasmid (Lanes A1 and A2, respectively), transferred to membrane, and hybridized with asymmetric PCR probe amplified with primers annealing to the termini of the insert of intact plasmid template. Lanes B1 and B2 contain the same analytes as A1 and A2 but were hybridized with probe amplified with the same primers using restriction enzyme digested plasmid, i.e., free insert. Signal from linearized host plasmid in A1 is significantly higher than that in B1. Thus even when using insert specific primers, cutting insert from plasmid template prior to probe generation will reduce detection of false positives when performing transformed colony screening.

6. Synthesized primers, commercially available through various vendors such as Midland Certified Reagent Company (Midland, TX), are usually sent at ambient temperature in a lyophilized state.
7. Degradation of nucleotides usually occurs after 3–6 mo storage at -20°C . Reactions incorporating $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ in the presence of diluted dCTP are more sensitive to nucleotide instability and fail before conventional PCR.

- 8 Resultant 1X TAE solution is 40 mM Tris acetate, 1 mM EDTA.
- 9 Resultant 1X concentration is 5% glycerol, 10 mM 0.5 M EDTA, 0.1% SDS, 0.1 mg/mL bromophenol blue.
10. Remove *Taq* from -20°C immediately prior to adding it to reaction mix and return directly to -20°C storage.
11. Resolve products > 1000 bases in 1% agarose/TAE and <1000 bases in 2% agarose/TAE.
12. Alternatively, 10 μL ethidium bromide/500 mL can be added to submarine tank buffer, which will prevent formation of a gradient formed by migrating ethidium bromide cast into gel. When adding ethidium bromide into buffer rather than casting in gel, allow time for ethidium bromide to penetrate gel and intercalate with DNA samples before viewing with UV light (about 30 min).
13. Voltage conditions will depend on the gel rig, namely the distance between electrodes. The values given (50 V for 30 min = 25 Vhr) are for a Bio-Rad DNA SubCell with 30 cm between electrodes. Thus the total Vhr will need to be adjusted if your electrodes are at a different distance. Higher voltages should not be used, particularly on small rigs because of excess heating.
- 14 Negatively charged dsDNA, intercalated with ethidium bromide, migrates downward toward anode as excess ethidium bromide migrates upward toward cathode.
15. kb DNA ladder (Gibco) contains 100 ng 1636 bp dsDNA/ μL . For low mass products, use low-mass DNA ladder (Gibco), which contains 40 ng 400 bp dsDNA/ μL .
- 16 Equal dpm/ μg is incorporated for all probes, but shorter probes incorporate less radiolabel per strand. Specific activity may be further increased by increasing radiolabel added. Further dilution of dCTP to 2 μM will also increase specific activity, however, a small number of reactions may fail to generate product (3)

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Optimization of a Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Mass Assay for Low-Abundance mRNA

Jacqueline M. Cale, Cynthia E. Shaw, and Ian M. Bird

1. Introduction

Whereas Northern analysis is a standard procedure by which abundant levels of mRNA can be quantified and characterized by size, there is a limit to the sensitivity of this technique, even with the best probes and the use of poly(A+) mRNA enrichment (*see* Chapters 28 and 30). At these times, it is necessary to move to more sensitive techniques. RNase protection assays or equivalent are much more sensitive but are technically difficult to perform, and require expensive dedicated equipment as well as production of riboprobes. More recently, there has been a predominance of the use of equally sensitive reverse transcription-polymerase chain reaction (RT-PCR) methodologies, which rely on the now more universally available PCR cycler and otherwise inexpensive and easily performed Southern Blot analytical methodology.

Whereas RT-PCR has many advantages, it is important also to know the limitations. There are actually two levels of RT-PCR assay, regarded as quantitative and semiquantitative. Quantitative assays are accurate and precise. They usually compare amplification of the unknown message with amplification of a synthetic deletion mutant RNA added at known amounts. This competitive type of assay allows for variations in reverse transcription efficiency and *Taq* efficiency between assays and therefore yields information on the true level of mRNA transcripts. Standard curves are highly reproducible, so full curves can be run less often with only a few standards run in every assay (1). However, they rely on the formation of deletion constructs and reverse transcription of the cDNA to provide the RNA competitor. Semiquantitative assays (2) do not

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usually include such a competitor and so are easier to set up, and can be accurate (i.e., detect fold increases in mRNA) but not precise or as highly reproducible.

A particular concern of semiquantitative assays has been possible variation in reverse transcription products between tubes. In the past, this has been a particular problem since reverse transcription and PCR reactions were performed separately and additional volumetric errors would arise from transfer of RT products to the PCR tube. In the case of a competitive/quantitative assay, this would be compensated for since both unknown and competitor products would be equally affected. Attempts are often made to allow for this by coamplification of an internal control such as actin, GAPDH, and so on. However, there is a problem with this since the primers for the unknown and the internal control are different and so may bind with different efficiencies. Furthermore, the use of additional internal control primer sets in subsequent PCR will also potentially disturb the kinetics of amplification of the unknown sequence both through mispriming or, more likely, the more rapid amplification of the GAPDH or actin target because of the greater abundance of those internal control mRNA species. Thus, the target of interest may fail to amplify efficiently before the amplified internal control sequence has consumed all the reagents.

Whereas competitive assays may be the preferred method, the recent development of a single-tube RT-PCR method based on combined AMV-RT with *Taq* polymerase in a PCR buffer (2) has overcome many, but not all, of the limitations of semiquantitative PCR assays. Since AMV-RT can operate at temperatures up to 60°C, and do so in PCR buffer, then transfer errors are eliminated, as well as problems with secondary structure that can occur during reverse transcription at lower temperatures. We have been using such assays recently to “quantify” low levels of mRNA in as little as 0.1 µg total RNA (3,4). These assays are very easy to perform once established, and give highly reproducible results within an assay. By monitoring the results from a pooled RNA standard within each and every assay we have been able to easily identify the very few assays that perform poorly (because of reagent/enzyme failure). Using a single-tube assay and operating at higher reverse transcription temperature, we have not found it necessary to provide an internal control per tube for reverse transcription. This is, in part, also because of our use of an optimized RNA extraction procedure that consistently yields high-quality RNA (Chapter 28) together with always dispensing reagents as a homogenous master mix, so further eliminating tube-to-tube pipeting errors. The only normalization necessary is for errors in the initial RNA spectrophotometric quantification, i.e., as applies to Northern blots. This can be achieved separately by slot-blot analysis of RNA from the same sample for a housekeeping gene or, as described here, 28S rRNA.

With the precautions and changes described, semiquantitative RT-PCR assays can be reliable, accurate, and reasonably reproducible. We describe below the procedure to set up such an assay for a new target, detecting from 10^3 – 10^8 cDNA products of reverse transcription. Restated, the assumption of the assay is that RNA quality is consistent, and reverse transcription efficiency is constant between samples. The assay will reveal changes in RNA relative to control, i.e., is accurate, but not the true quantity of RNA, i.e., is not precise. Should further precision be required, the assay can be rapidly established as described and then standards can be replaced with synthetic cRNA followed by addition of a fixed quantity of deletion-cRNA competitor into each tube.

2. Materials

Purchase all reagents as molecular-biology grade, i.e., RNase- and DNase-free.

2.1. RT-PCR Assay and Southern Analysis

1. DNA thermal cycler, Perkin Elmer (Foster City, CA).
2. AMV reverse transcriptase (2.5 U/ μ L), Gibco-BRL (Gaithersburg, MD) (cat. no. 18020024).
3. *Taq* DNA polymerase (5 U/ μ L), Gibco-BRL (cat. no. 18038042).
4. 10X PCR Buffer, Supplied with *Taq* DNA polymerase.
5. $MgCl_2$ (50 mM), Supplied with *Taq* DNA polymerase.
6. 10 mM dATP in 1 mM Tris-HCl, pH 7.5, Gibco-BRL (cat. no. 18252015).
7. 10 mM dCTP in 1 mM Tris-HCl, pH 7.5, Gibco-BRL (cat. no. 18253013).
8. 10 mM dGTP in 1 mM Tris-HCl, pH 7.5, Gibco-BRL (cat. no. 18254011).
9. 10 mM dTTP in 1 mM Tris-HCl, pH 7.5, Gibco-BRL (cat. no. 18255018).
10. Forward and reverse primers, Midland Certified Reagent Co (Midland, TX).
11. Molecular-biology grade water, 5 Prime \rightarrow 3 Prime, (Boulder, CO).
12. Sterile 0.5-mL PCR reaction tubes, Perkin Elmer
13. Sterile 1.5-mL microcentrifuge tubes.
14. Sterile pipet tips.
15. Insert-containing plasmid at known concentration (see Note 1).
16. Total RNA samples and controls at or above 1 μ g/5 μ L concentration

2.2. Gel Electrophoresis

See Chapter 30, Subheading 2. (materials 16–24, 32, 33).

2.3. Transfer to MagnaGraph Membrane

See Chapter 29, Subheading 2. (materials 2, 3, 5, 7, 26, 28, 32, 35).

1. Concentrated hydrochloric acid.

2.4. Southern Hybridization

See Chapter 29, Subheading 2. (materials 6, 10–13, 19, 21, 22, 27).

2.5. Quantification

- 1 X-ray film cassettes and Amersham (Arlington Heights, IL) Hyperfilm MP or phosphorimager equipment

2.6. 28S rRNA Slot-Blot Analysis

- 1 Biodot SF cell—slot blot apparatus, Bio-Rad (Melville, NY) (cat. no. 170 6542).
2. Quick Spin™ columns, Boehringer Mannheim (cat. no. 1273922)
- 3 T4 polynucleotide kinase, Gibco-BRL (cat no 18004010).
- 4 5X kinase buffer, supplied with T4 polynucleotide kinase.
- 5 ³²P-γATP, >6000Ci/mmol, Amersham (cat. no PB10218)
- 6 28S oligonucleotide: 5'-AAA ACG ATC AGA GTA GTG GTA TTT CAC CG -3,' (Clonetech, Palo Alto, CA, cat. no. 90341)
7. Disodium EDTA.
8. Formaldehyde.
- 9 Tris base.

2.7. Solutions and Reagents for RT-PCR Assay

Wear gloves when working with RNA, preparing solutions, or using chemicals classified as irritants. Keep hands clean, and prepare all solutions and reagents using autoclaved or sterile glassware, microcentrifuge tubes, reaction tubes, and pipet tips. Also keep instruments and work area clean. In general, molecular-biology grade water (RNase and DNase free) should be used for all RT-PCR assays. Solutions for Southern blotting of PCR products can be prepared with Nanopure (18 Ω) sterile water, and solutions for RNA dilution and loading to slot blots should be prepared with molecular-biology grade or DEPC water (*see* Chapter 28, Subheading 2.1.).

2.7.1. RT-PCR Assay

All RT-PCR reagents should be aliquoted to smaller volumes to minimize damage from repeated freeze/thaw. *Taq* polymerase should be stored long term at -20°C and reverse transcriptase at -70°C. Working stocks of both enzymes can be stored at -20°C.

- 1 2.5 mM dA, C, G, TTPs: Allow each reagent to completely thaw on ice and vortex each thoroughly before mixing together. Place equal volumes of 10 mM dATP, dCTP, dGTP, and dTTP in a sterile 1.5-mL microfuge tube. Vortex and aliquot approx 50 μL into sterile 0.5-mL tubes clearly labeled with contents and date. Store at -20°C
- 2 *Forward and reverse primers.* Primers should be approx 17 bases or greater, with either a G or C residue in 2 of the last 3 bases of the 3' terminus, with annealing temperatures of 55–65°C, but within 2°C of each other (*see Note 2*). In addition, primers should have approx 50% GC content with no stable secondary structure and minimal primer-dimer formation. Given the quantity of primers in

Table 1
Example of Plasmid Stock Solution Calculations

No. of bases:					
plasmid	+	insert	=	total bases	
2960		301		3261	
Molecular weight:					
total bases	×	no of strands	×	mol wt per base	= total mol wt
3261		2		330	= 2 152 260
Mol/μL in working stock:					
ng/μL	×	convert to g/μL	÷	total mol wt	= mol/μL
10		10^{-9}		2 152 260	= 4.65×10^{-15}
Copies/μL in working stock:					
mol/μL	×	avogadros	×	number	= copies/μL
4.65×10^{-15}		6.022		10^{23}	= 2.80×10^9
μL for 100×10^{10} copies:					
copies	÷	copies/μL	=	μL stock needed	
100×10^{10}		2.80×10^9		357.4	
Prepare top standard A:					
μL stock	+	μL H ₂ O	=	final volume (μL)	
357.4		142.6		500	

optical density units, solubilize in molecular-biology grade water to 1 μg/μL (using an A_{260} of 20 μg/OD unit) and keep on ice. Using an estimate of 330 g/mol per base, dilute an aliquot of forward and reverse primers at 1 μg/μL to a final concentration of 20 pmol/mL. Aliquot approx 30 μL into sterile 0.5-mL tubes clearly labeled with contents and date. Store at -20°C.

- 10 X PCR buffer*: Aliquot approx 75 μL into sterile 0.5-mL tubes clearly labeled with contents and date. Store at -20°C.
- MgCl₂, 50 mM*: Aliquot approx 50 μL into sterile 0.5-mL tubes clearly labeled with contents and date. Store at -20°C.
- Plasmid dilution series*: Determine the concentration in μg/μL for the most concentrated stock plasmid and insert of interest. Working on ice, dilute the most concentrated stock to either 10 or 50 ng/μL using molecular-biology water. The working stock solution should be 10 ng/μL for plasmid plus insert of <4.0 kb (for >4.0 kb, see Note 1). Perform calculations in Table 1 specific to the combined plasmid and insert of interest. Dilute the plasmid with molecular-biology grade water to get the desired number of copies. The calculations in Table 1 are an example from our ovine ecNOS RT-PCR assay (4). Prepare the remaining dilution series standards as outlined in Table 2 and distribute as 100-μL aliquots. Store at -20 or -70°C.

Table 2
Preparation of Plasmid Dilution Series (Standards)

Standard	Amount	+	Molecular biology H ₂ O	Copies/ 5 μ L
Standard A	Calculated from Table 1		Calculated from Table 1	10 ¹⁰
Standard B	50 μ L A		450 μ L	10 ⁹
Standard C	50 μ L B		450 μ L	10 ⁸
Standard D	50 μ L C		450 μ L	10 ⁷
Standard E	50 μ L D		450 μ L	10 ⁶
Standard F	50 μ L E		450 μ L	10 ⁵
Standard G	50 μ L F		450 μ L	10 ⁴
Standard H	50 μ L G		450 μ L	10 ³
Control	—		500 μ L	0

2.7.2. Gel Electrophoresis

See Chapter 30, Subheading 2.1.4., for detailed solutions information.

2.7.3. Vacuum Blotting to Membrane

1. *Depurinating solution:* 0.25 M HCl. Using a 10-mL pipet or a graduated cylinder, add 24 mL of concentrated HCl to 1 L of nanopure or ultrapure water
2. *Denaturing solution:* 0.15 M NaCl, 0.05 M NaOH. Weigh 8.8 g NaCl and 2.02 g NaOH into 1 L of nanopure or ultrapure water. Autoclave on liquid cycle.
3. *Neutralizing solution:* 0.15 M NaCl, 0.1 M Tris, pH 7.5. Weigh 8.8 g NaCl and 12.1 g Tris base into 800 mL nanopure or ultrapure water, and pH to 7.5 with concentrated HCl. Make up to 1 L. Autoclave on liquid cycle.
4. *20X SSC:* 3.0 M NaCl, 0.3 M trisodium citrate, pH 7.0. Weigh 175.3 g NaCl and 88.2 g Na₃ Citrate and dissolve in 800 mL nanopure or ultrapure water. Adjust pH to 7.0 and adjust volume to 1 L. Autoclave on liquid cycle.

2.7.4. Southern Hybridization

Use the same solutions as for Northern hybridization. See Chapter 29, Subheading 2.1.4., for detailed solutions information.

2.8. Solutions and Reagents for Slot Blot Analysis

1. *0.5 M EDTA pH 8.0:* Weigh 16.8 g disodium EDTA into 80 mL DEPC water, and adjust pH to 8.0 with concentrated or pelleted (approx 2 g) NaOH. Adjust volume to 100 mL and autoclave to sterilize.
2. *10 mM Tris (pH 7.5)/1 mM EDTA (TE buffer pH 7.5):* Dissolve 0.121 g Tris base and 0.037 g disodium EDTA in 80 mL sterile water. pH to 7.5 and make up to 100 mL. Filter sterilize at 0.2 μ m

3. Methods

3.1. RT-PCR Mass Assay

Optimization of an RT-PCR mass assay includes four essential steps that we recommend be completed in the following order: optimization of annealing temperature, optimization of Mg^{2+} concentration, optimization of cycle number, and generation of a standard curve. While setting up RT-PCR reactions, keep reagents on ice until they are returned to storage at $-20^{\circ}C$ (*see Note 3*). Use fresh pipet tips between all reagents, standards, and samples. Precision and accuracy are key in this procedure; use care when pipetting since these are small volumes.

3.1.1. Optimizing Annealing Temperature

This amplification is run in the absence of reverse transcriptase since reverse transcriptase does not affect annealing, but inhibits *Taq* polymerase activity. An excess concentration of template is tested alongside an intermediate level since the temperature sensitivity should differ between the two template concentrations. The lower amount of template will be more sensitive to changes in annealing temperature, enabling one to detect the optimal annealing temperature. For each annealing temperature tested, a separate amplification will have to be run (a total of four amplifications). Choose a basal annealing temperature that is $10^{\circ}C$ below the lowest oligonucleotide T_m . Initially run amplifications that anneal at this basal temperature, 2, 4, and $6^{\circ}C$ above this temperature (*see Note 4*).

- 1 Clearly label a 0.5-mL PCR reaction tube for each amplification to be run
- 2 Pipet 5 μL each of the 10^{10} copies/5 μL or 10^6 copies/5 μL standards into their respective reaction tubes
- 3 Make a master mix to ensure that each tube has an equal concentration of reagents. Make the master mix for more tubes than actually needed **Table 3** can be used as a guide. The number of tubes (n) is two at each temperature in this case, so calculate a master mix for three total tubes. Place the specified amount of each reagent in a sterile 1.5-mL microcentrifuge tube
- 4 Vortex master mix to ensure that reagents are equally concentrated throughout.
- 5 Distribute 45 μL of master mix to the two reaction tubes Briefly vortex each reaction tube to equally mix the template and reagents. Centrifuge each of the reaction tubes briefly to eliminate any bubbles and to clear liquid from the side walls of the tubes.
- 6 Overlay each reaction with 30 μL of mineral oil.
- 7 *Step cycle* using the program in **Table 4**.
- 8 Pipet 45 μL of the PCR products from under the mineral oil and place into sterile, clearly labeled 0.5-mL tubes If products are not to be immediately separated on a gel, store at $-20^{\circ}C$.

Table 3
Master Mix Guide for Annealing Temperature Optimization

Reagent	For one tube	Calculated for three tubes	Final concentration
10X PCR buffer	5 μL	15 μL	1X PCR buffer
MgCl ₂ 50 mM	1.5 μL	4.5 μL	1.5 mM
dNTPs 2.5 mM each	4 μL	12 μL	0.20 mM each (A, C, G, T)
Forward primer, 20 pmol/ μL	1.5 μL	4.5 μL	30 pmol
Reverse primer, 20 pmol/ μL	1.5 μL	4.5 μL	30 pmol
<i>Taq</i> polymerase	1 μL	3 μL	5.0 U
H ₂ O	30.5 μL	91.5 μL	to 50 μL
Total master mix	45 μL	—	—

Table 4
Step Cycle Program

Thirty-five cycles of amplification

Denature at 94°C (time varies with length of insert [*see Note 5*])

Anneal
 Exp 1: basal temp. ($T_m - 10^\circ\text{C}$) for 30 s
 Exp 2: basal temp. + 2°C for 30 s
 Exp 3: basal temp. + 4°C for 30 s
 Exp 4: basal temp. + 6°C for 30 s

Extend at 72°C (time varies with length of insert [*see Note 5*])

Link to 4°C soak

Soak at 4°C (no longer than overnight)

- 9 Separate 10 μL of each product (with 1 μL of DNA 10X sample buffer) on a 1.5 or 2% TAE gel (see **Note 6**) until the loading dye is about half-way down the gel (see Chapter 30, Subheading 3.1.4., for details on gel electrophoresis methodology).
- 10 Turn off power supply and remove gel and gel tray to a glass dish. Photograph the gel in the gel tray on a UV light box. Use the photograph to determine the proper annealing temperature as illustrated in **Fig. 1**.

3.1.2. Optimizing [Mg²⁺]

This reaction is run in the presence of an intermediate amount of either plasmid template (10⁶ copies) or total RNA (1.0 μg). Both the plasmid template and RNA sample concentrations should be sensitive to changes in [Mg²⁺]. Results will differ since DNA template does not require reverse transcription, and only *Taq* sensitivity is revealed. We recommend trying four (final) con-

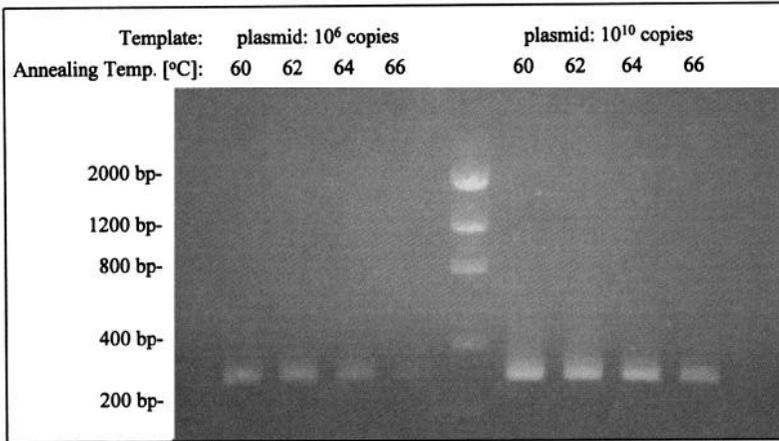


Fig. 1. Effect of annealing temperature on PCR quantification: These representative data clearly show the greater sensitivity of product loss with increasing temperature when initial template is more limiting. In this case we chose an annealing temperature of 62°C for subsequent assays. At this temperature and below, temperature changes did not affect product yield, whereas further increases in temperature above 62°C resulted in increasing loss of product.

centrations of MgCl₂: 1.5, 2.0, 2.5, and 3.0 mM. Run a total of eight amplifications (2 templates × 4 concentrations). Choose optimum conditions based primarily on results from RNA template.

1. Clearly label a 0.5-mL PCR reaction tube for each amplification to be run.
2. Pipet 5 μL of the 10⁶ copies/5 μL and 1.0 μg/5 μL into their respective reaction tubes (*see Table 5*).
3. Pipet the specified amount of 50 mM MgCl₂ (from 1.5–3 mL) into the reaction tubes containing template. Add molecular-biology grade water so that all reaction tubes have the same volume (outlined in **Table 5**).
4. Make a master mix to ensure that each tube has an equal concentration of reagents. Make the master mix for more tubes than actually needed. **Table 6** can be used as a guide. The number of tubes (*n*) is eight in this case, so calculate a master mix for nine total tubes. Place the specified amount of each reagent in a sterile 1.5-mL microcentrifuge tube.
5. Vortex master mix to ensure that reagents are equally concentrated throughout.
6. Distribute 42 μL of master mix to each reaction tube. Briefly vortex each reaction tube to equally mix the template and reagents. Centrifuge each of the reaction tubes briefly to eliminate any bubbles and to clear liquid from the side walls of the tubes.
7. Overlay each reaction with 30 μL of mineral oil.
8. Step cycle the reaction. Reverse transcribe according to **Table 7** and link to the program determined in the first assay optimization step (**Subheading 3.1.1**).

Table 5
Template and MgCl₂ Distribution for [Mg²⁺] Optimization

Template (5 µL).	DNA 10 ⁶ cps	RNA 1.0 µg						
MgCl ₂ (µL)	1.5	1.5	2.0	2.0	2.5	2.5	3.0	3.0
H ₂ O (µL)	1.5	1.5	1.0	1.0	0.5	0.5	0.0	0.0

Table 6
Master Mix Guide for [Mg²⁺] Optimization

Reagent	For one tube	Calculated for nine tubes	Final concentration
10X PCR buffer	5 µL	45 µL	1X PCR buffer
dNTPs 2.5 mM each	4 µL	36 µL	0.20 mM each (A, C, G, T)
Forward primer, 20 pmol/µL	1.5 µL	13.5 µL	30 pmol
Reverse primer, 20 pmol/µL	1.5 µL	13.5 µL	30 pmol
Taq polymerase	1 µL	9 µL	5.0 U
Reverse transcriptase	1 µL	—	2.5 U
H ₂ O	28 µL	252 µL	to 50 µL
Total master mix	42 µL	—	—

Table 7
Reverse Transcription and Step Cycle Program

Reverse transcription—1 cycle
Anneal at 62°C for 5 min
Reverse transcribe at 50°C for 10 min
Denature at 94°C for 2 min
Link to PCR amplification program optimized in Subheading 3.1.1. —
35 cycles
Link to 4°C soak

- 9 Pipet 45 µL of each PCR product from under the mineral oil and place into a sterile, clearly labeled 0.5-mL tube. If products are not to be immediately separated on a gel, store at -20°C.
10. Separate 10 µL of each product (with 1 µL of DNA 10X sample buffer) on a 1.5 or 2% TAE gel (*see Note 6*) until the loading dye is about half-way down the gel (*see Chapter 30, Subheading 3.1.4.*, for details on gel electrophoresis methodology)

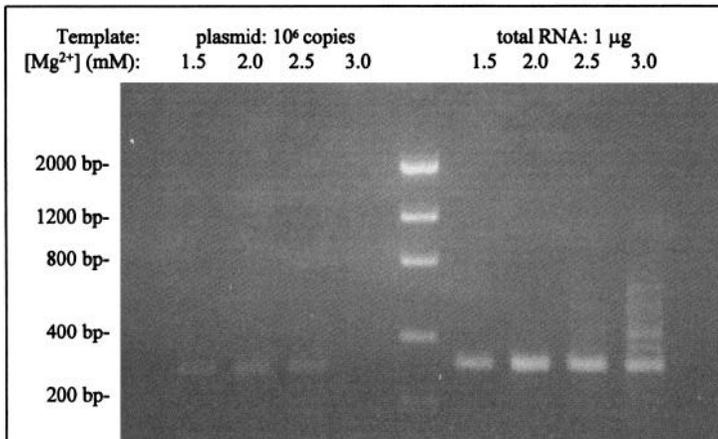


Fig. 2. Effect of Mg²⁺ concentration on PCR quantification: For both DNA and RNA starting materials, both 2.0 and 2.5 mM Mg²⁺ gave the best yields of product. However, looking more closely at the products from RNA amplification, concentrations of 2.5 and 3.0 mM Mg²⁺ also gave rise to additional higher molecular-weight byproducts. Thus, the optimum Mg²⁺ concentration for maximum yield with maximum specificity of product was 2.0 mM Mg²⁺, which was used in all subsequent assays.

- Turn off power supply and remove gel and gel tray to glass dish. Photograph the gel in the gel tray on a UV light box. Use this photograph to determine the proper [Mg²⁺] as illustrated in Fig. 2.

3.1.3. Optimizing Amplification Cycle Number

A high standard is run alongside an intermediate standard and the cycle number is varied from 25 to 35 cycles with two cycle intervals. Reverse transcriptase is included even in the absence of RNA template since it inhibits *Taq* activity, presumably by competitively binding to the oligo/template pair but remaining inactive. The lower amount of template will be more sensitive to changes in cycle number because of its position in the middle of the standard curve, enabling one to detect an optimal cycle number. The higher standard allows one to detect a difference between the high and intermediate standards that will produce an optimum range for quantification of data. A water control is also run in this experiment. This amplification requires one to remain close to the cycler so that reactions can be removed promptly after the specified number of cycles has passed. There are 13 reactions (2 templates \times 6 intervals + 1 water control).

- Clearly label a 0.5-mL PCR reaction tube for each amplification to be run.
- Pipet 5 μL each of the 10^6 copies/5 μL or 10^8 copies/5 μL into their respective reaction tubes (see Table 9).

Table 8
Master Mix Guide for Cycle Number Optimization

Reagent	Calculated for		Final concentration
	For one tube	15 tubes	
10X PCR buffer	5 μL	75 μL	1X PCR buffer
MgCl ₂ 50 mM	(2 μL)	(30 μL)	(2 mM-adjust)
dNTPs 2.5 mM each	4 μL	60 μL	0.20 mM each (A, C, G, T)
Forward primer, 20 pmol/ μL	1.5 μL	22.5 μL	30 pmol
Reverse primer, 20 pmol/ μL	1.5 μL	22.5 μL	30 pmol
Taq polymerase	1 μL	15 μL	5.0 U
Reverse transcriptase	1 μL	15 μL	2.5 U
H ₂ O	(29 μL)	(435 μL)	(to 50 μL -adjust)
Total master mix	45 μL	—	—

Table 9
Template Distribution and Cycle Number Guide

Copy no	10 ⁶	10 ⁸	H ₂ O										
Cycle no.	25	25	27	27	29	29	31	31	33	33	35	35	35
Counter	9	9	7	7	5	5	3	3	1	1	4°C	4°C	4°C

3. Make a master mix to ensure that each reaction tube has an equal concentration of reagents (*see Table 8*). Make the master mix for more tubes than actually needed. In general, for an assay exceeding 10 tubes, where n is the number of tubes, make enough master mix for $n + 2$. For illustrative purposes, 2 mM final MgCl₂ is shown in the remaining assay examples *but this should be adjusted according to results from Subheading 3.1.2*. Place the specified amount of each reagent in a sterile 1.5-mL microcentrifuge tube.
4. Vortex master mix to ensure that reagents are equally concentrated throughout.
5. Dispense 45 μL of master mix to each reaction tube. Briefly vortex each reaction tube to equally mix the template and reagents. Centrifuge each of the reaction tubes briefly to eliminate any bubbles and to clear liquid from the side walls of the tubes.
6. Overlay each reaction with 30 μL of mineral oil.
7. Cycle the reaction as in the second optimization assay (reverse transcribe, step cycle amplify, 4°C soak). Start all reactions at the same time and remove when the counter displays the remaining cycles indicated in **Table 9** (*see Note 7*).
8. Pipet 45 μL of the PCR products from under the mineral oil and place into sterile, clearly labeled 0.5-mL tubes. If products are not to be immediately separated on a gel, store at -20°C .

9. Separate 10 μL of each product (with 1 μL of DNA 10X sample buffer) on a 1.5 or 2% TAE gel (*see Note 6*) until the loading dye is about half-way down the gel (*see Chapter 30, Subheading 3.1.4., for details on gel electrophoresis methodology*).
10. Turn off power supply and remove gel and gel tray to glass dish. Photograph the gel in the gel tray on a UV light box. Continue as in **Subheading 3.1.3.1.**

3.1 3.1. SOUTHERN BLOTTING

1. Prepare the gel for transfer: Carefully push gel out of mold and into the glass dish by pressing on the top end with even pressure along length of gel.
2. Add equal volumes of nanopure water and 20X SSC in a sterile glass bottle to make 10X SSC.
3. Incubate the gel in the following solutions for the specified amount of time. The gel must be completely submerged for each incubation period. Remove each solution with a suction device taking care not to damage the gel.
 - a. Depurinate: 0.25 *N* HCl for 10 min.
 - b. Denature: 0.15 *M* NaCl, 0.05 *M* NaOH for 30 min.
 - c. Neutralize: 0.15 *M* NaCl, 0.1 *M* Tris, pH 7.5 for 30 min.
 - d. Equilibrate: 10X SSC for 15 min.
4. Vacuum blot the gel to MagnaGraph hybridization membrane (Molecular Separations, Westboro, MA), for 90 min at 5–7 psi. (*See Chapter 29, Subheading 3.1.3., for methodological details on vacuum blotting.*)
5. Turn off the vacuum and remove the gel from the vacuum apparatus.
6. Use a UV lamp to illuminate the membrane (short wave). **Important:** *Wear protective eye goggles*. With a pencil, mark corners of sealing mask, lane openings, and mass ladder.
7. Remove the sealing mask and then crosslink the damp Southern membrane (125 mJ). Date and label the membrane with a pencil. Store at -20°C in a heat-sealed bag.
8. Prehybridize the membrane overnight, as detailed in Chapter 29, Subheading 3.1.4.
9. Probe membrane with asymmetric PCR-radiolabeled probe (*see Chapter 30, Subheadings 3.1.5. and 3.1.6., for details on generating asymmetric PCR probes*).
10. Follow the hybridization protocol outlined in Chapter 29, Subheading 3.1.4. When working with radioactivity, wear gloves, and keep materials behind a protective shield.
11. Hybridize overnight or at least 6 h.
12. Discard used probe in radioactive liquid waste. Wash once with 20 mL 2X SSC/0.1% SDS, 15 min, 42°C , and discard as radioactive liquid waste.
13. Wash blot with 20 mL 0.1X SSC/0.1% SDS, 30 min, 42°C , in hybridization oven. Discard first wash as radioactive liquid waste.
14. Wash blot with 0.1X SSC/0.1% SDS, 30 min, 42°C , in hybridization oven.
15. Dry the blot on a clean paper towel.
16. Wrap the blot in plastic wrap, making sure that it is not wet. Cover with screen protective film (supplied with scanning equipment) and scan on a direct radioimaging scanner for the required amount of time (*see Note 8*).

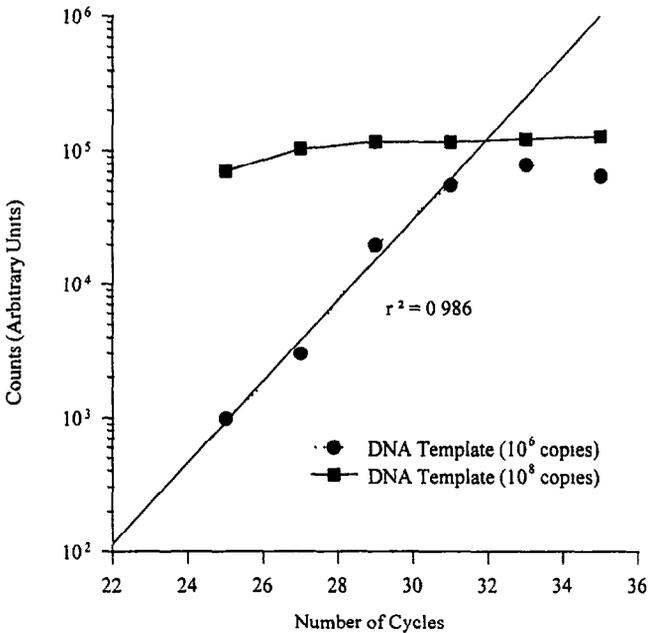


Fig. 3. Effect of cycle number on PCR quantification. These data show the sensitivity of lower template to change in cycle number. Between 25 and 32 cycles there is a steady increase of amplified product. At 32 cycles and beyond the formation of product tails off for the lower concentration template. Choose a cycle number at least two cycles less than the end of the linear range for product formation as a cycle number for all subsequent reactions (in this case, 28 cycles of PCR amplification).

17. Tape the blot (still covered in plastic wrap) down on a film cassette and expose to film for the required amount of time determined by the radioimaging scan (see Note 8).
18. Quantify signal from method of choice by scanning densitometry, correcting for background.
19. Generate a plot of cycle number vs densitometry counts from the Southern blot as in Fig. 3. Select a cycle number as described in the legend.

3.1.4. Generation of a Standard Curve

In this assay, the complete plasmid dilution series standard curve (see Sub-heading 2.7.1.) is run alongside a total RNA dilution series. In addition to the water control, a control RNA sample is run in the absence of reverse transcriptase (RT-). All other samples are run in the presence of reverse transcriptase (RT+). This amplification has 15 reactions.

1. Make the additional RNA dilution series in clearly labeled, sterile 0.5-mL tubes. Starting with a concentration of 1 µg/5 µL, make dilutions of 0.5 µg/5 µL,

Table 10
Master Mix Guide for Generation of Standard Curve

Reagent	Calculated for		
	For one tube	17 tubes	Final concentration
10X PCR buffer	5 μL	85 μL	1X PCR buffer
MgCl ₂ , 50 mM	(2 μL)	(34 μL)	(2.0 mM-adjust)
dNTPs 2.5 mM each	4 μL	68 μL	0.20 mM each (A, C, G, T)
Forward primer, 20 pmol/ μL	1.5 μL	25.5 μL	30 pmol
Reverse primer, 20 pmol/ μL	1.5 μL	25.5 μL	30 pmol
<i>Taq</i> polymerase	1 μL	17 μL	5.0 U
H ₂ O	(30 μL)	(510 μL)	(to 50 μL -adjust)
Total master mix	45 μL	—	—

0.1 $\mu\text{g}/5 \mu\text{L}$ (used as RT+ and RT-), 0.05 $\mu\text{g}/5 \mu\text{L}$, and 0.01 $\mu\text{g}/5 \mu\text{L}$. Use the following list as a guide

1 $\mu\text{g}/5 \mu\text{L}$	Dilute from concentrated RNA stock to 50 μL
0.5 $\mu\text{g}/5 \mu\text{L}$	15 μL (1 $\mu\text{g}/5 \mu\text{L}$) + 15 μL molecular biology grade H ₂ O
0.1 $\mu\text{g}/5 \mu\text{L}$	6 μL (0.5 $\mu\text{g}/5 \mu\text{L}$) + 24 μL molecular biology grade H ₂ O
0.05 $\mu\text{g}/5 \mu\text{L}$	15 μL (0.1 $\mu\text{g}/5 \mu\text{L}$) + 15 μL molecular biology grade H ₂ O
0.01 $\mu\text{g}/5 \mu\text{L}$	6 μL (0.05 $\mu\text{g}/5 \mu\text{L}$) + 24 μL molecular biology grade H ₂ O

- Clearly label a 0.5-mL PCR reaction tube for each amplification to be run.
- Pipet 5 μL of each template into their respective reaction tubes. Also pipet 1 μL of molecular-biology grade H₂O into the RT-reaction tube
- Make a master mix to ensure that each reaction tube has an equal concentration of reagents (see **Table 10**). Do not yet add reverse transcriptase to the mix. Make the master mix for more tubes than actually needed. In general, for an assay exceeding 10 tubes, where n is the number of tubes, make enough for $n + 2$. Dispense 45 μL mix into the single RT-RNA control tube
- Add $n+1$ μL (16 μL , in this case) of AMV-RT to the remaining master mix and vortex.
- Dispense 46 μL into the rest of the reaction tubes. Briefly vortex each reaction tube to equally mix the template and reagents. Centrifuge each of the reaction tubes briefly to eliminate any bubbles and to clear liquid from the side walls of the tubes.
- Overlay each reaction with 30 μL of mineral oil.
- Cycle (reverse transcription, PCR amplification, 4°C soak) the reaction using the [Mg²⁺] and cycle number chosen in the previous experiments from **Sub-headings 3.1.2.** and **3.1.3.**, respectively.

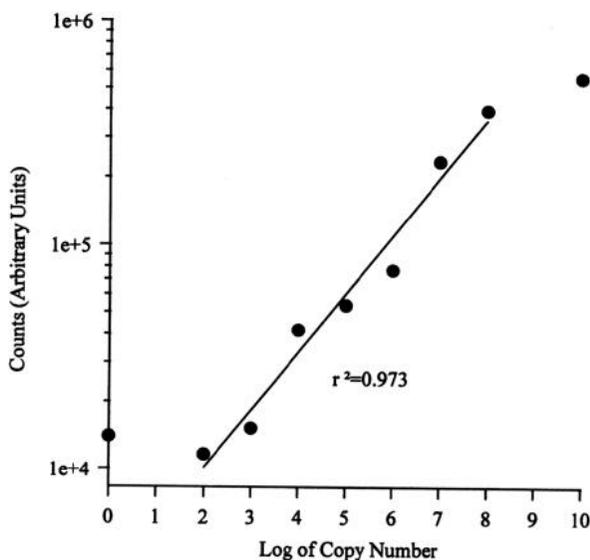
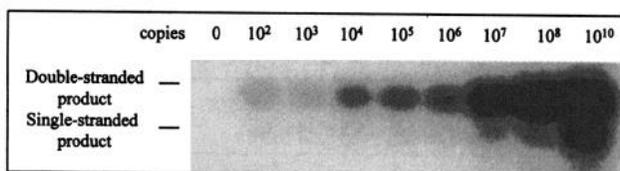


Fig. 4. Quantification of plasmid dilution standard curve: initial copy number is plotted against counts from the phosphorimager scan (log-log scale). The high correlation of the regression line indicates that range of quantification is accurate between 10^3 and 10^8 copies. The 10^{10} standard appears to tail off and is not in the linear range of accurate quantification. This will also happen at lower standards (10^8 , 10^7) if the amplification cycle number is too high. If this occurs, reduce the cycle number by two and rerun the experiment.

9. Pipet 45 μL of the PCR products from under the mineral oil and place into sterile, clearly labeled 0.5-mL tubes. If products are not to be immediately separated on gel, store at -20°C .
10. Separate 10 μL of each product (with 1 μL of DNA 10X sample buffer) on a 1.5 or 2% TAE gel (*see Note 6*) until the loading dye is about half-way down the gel (*see Chapter 30, Subheading 3.1.4.*, for details on gel electrophoresis methodology).
11. Turn off power supply and remove gel and gel tray to glass dish. Photograph the gel in the gel tray on a UV light box.
12. Prepare the gel for transfer: Continue as outlined in **Subheading 3.1.3.1**.

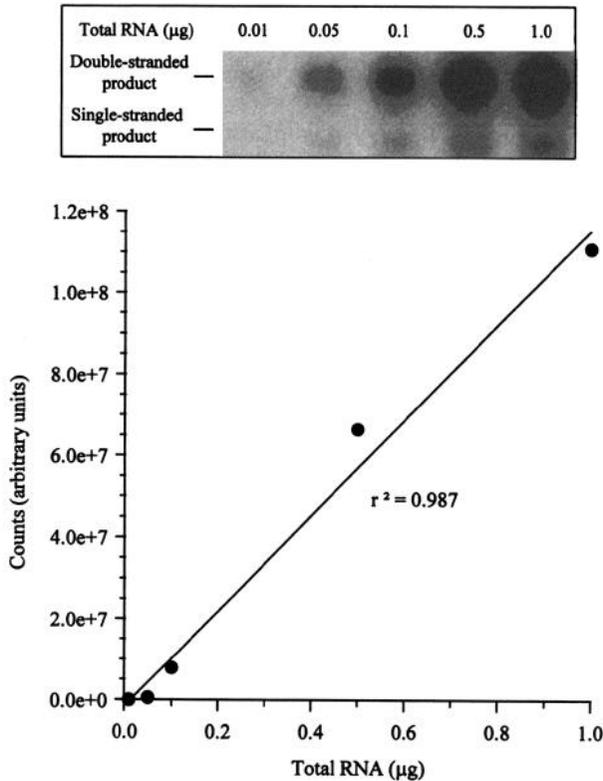


Fig. 5. Quantification of RNA dilution curve: These data show a representative RNA dilution and the resulting signal as detected by Southern blotting and hybridization to *OecNOS* asymmetric PCR probe. Total RNA is plotted against counts from the phosphorimaging scan (linear scales). The high correlation of the regression line indicates that 0.05–1 µg of total RNA may be accurately quantified. We chose 0.1 µg of RNA since the counts for this standard fell well below that of the top standard. It is not best to choose a concentration that is too small. Alternatively, if the signal is so high that less than 0.01 µg of RNA is needed, Northern analysis may be more appropriate.

13. Generate a plot of log of plasmid copy number vs densitometric counts from the Southern blot as in Fig. 4. Generate a plot of total RNA concentration vs densitometry counts from the Southern blot as in Fig. 5.

3.2. Slot-Blot Analysis

Slot blotting of as little as 1 µg RNA per slot is sufficient for normalization of data using end-labeled 28S rRNA oligonucleotide probe. This method takes some practice if one is not familiar with it. Prepare the apparatus first. Calculate volumes for 1 µg of total RNA before taking aliquots for analysis and keep on ice.

3.2.1. Slot-Blot Setup

1. Soak the lid, gasket, and support base in DEPC water before use (overnight preferably). Rinse in ethanol and dry before use
2. Cut MagnaGraph membrane to size using template and soak with three precut filter pads in 20X SSC. Place in apparatus so the filter paper fits in the recess and the membrane is sandwiched between the lid and the gasket. Tighten the thumb screws to ensure vacuum seal.
3. From the vacuum tap to the slot blotter fit in this order: vacuum regulator, 250 mL conical flask trap, three-way tap with bleed (needle) valve, and slot blotter.
4. Place 125 μL 20X SSC in each well. Wait until samples are in the water bath before continuing.

3.2.2. Preparation of Samples

1. Aliquot 1 μg of sample and make up to 10 μL with molecular-biology grade H_2O . Add 40 μL TE pH 7.5 (alternatively, take samples up to 45 μL molecular biology grade H_2O , but add 5 μL 10X TE pH 7.5).
2. Denature samples by adding 35 μL 20X SSC and 20 μL formaldehyde. Heat all samples for 15 min in a 60°C water bath.
3. Remove all samples to ice and wait 3 min.

3.2.3. Loading Samples to Membrane

1. Open the bleed valve and the vacuum regulator valve. Turn the three way tap so the slot blotter is not subject to vacuum. Set the vacuum to read 1 psi only, with the vacuum regulator valve closed. Open the bleed valve three more turns.
2. Turn the three way tap to allow vacuum to the membrane. Retighten/check the screws. The liquid (**Subheading 3.2.1., step 4**) should take at least 7.5 min to drain. If not, readjust bleed valve.
3. Turn three way tap to allow air flow through both the bleed valve but isolate the slot blotter.
4. Load samples to wells (105 μL each), and reset the tap to apply vacuum to samples. Allow to draw through (at least 7.5 min). Do not allow the pipet tips to touch the membrane, causing a false signal.
5. Apply 20X SSC to each well (150 μL) to wash samples on. Continue vacuum until all are drained. If any drain early keep the membrane wet by spotting with 20X SSC.
6. Disassemble apparatus and crosslink the membrane 120 mJ. Store in a heat-sealed bag at -20°C until hybridization.

3.2.4. Generation of End-Labeled 28S Oligonucleotide Probe

The PCR thermal cycler can be used for all incubations in this process. One may also use water baths. Since two incubations are needed (65°C, then 37°C), the cycler is a more efficient tool. Program the incubations as soak programs or time delay programs linked to 4°C soak.

1. Dilute oligonucleotide to 100 ng/ μ L in molecular biology grade water.
2. Add to a 0.5-mL reaction tube: 12 μ L molecular biology grade water and 2 μ L oligonucleotide
3. Incubate 65°C, 2 min, then immediately place on ice
4. Add 5 μ L 5X Kinase buffer, 5 μ L [³²P- γ]dATP, and 1 μ L T4 Kinase (10 U/ μ L)
5. Mix reaction mixture by repeated pipeting action. Incubate 37°C for 30 min.
6. Stop labeling reaction by addition of 1 μ L of 0.5 M EDTA (final concentration of 15 mM). Place on ice (*see Note 9*). Add 24 μ L of TE pH 7.5 (final volume 50 μ L).
7. Purify the labeled oligonucleotide using the Quick Spin columns. Follow the manufacturers instructions.
8. Determine activity of labeled oligonucleotide. Place 1 μ L of the purified oligonucleotide in 10 mL of scintillation fluid. Using a scintillation counter, determine dpm for ³²P. Use enough labelled oligonucleotide for 10⁶ dpm per mL of hybridization buffer.
9. Store at -20°C. Labeled probe can be stored at -20°C for approx 1 wk.

3.2.5. Hybridization with Labeled Probe

1. Prehybridize the membrane overnight, as outlined in Chapter 29, Subheading 3.1.4
2. Immediately before using the probe, pellet any particulate matter present by centrifugation for 5 min. Transfer volume of probe to be used to fresh PCR tube and incubate 95°C for 2 min. Chill on ice for 3 min.
3. Add ³²P-oligonucleotide probe to prehybridization solution
4. Hybridize overnight or at least 6 h.
5. Discard used probe in radioactive liquid waste. Wash once with 20 mL 2X SSC/0.1% SDS, 15 min, 42°C, and discard as radioactive liquid waste
6. Wash blot with 20 mL 0.1X SSC/0.1% SDS, 30 min, 42°C, in hybridization oven. Discard first wash as radioactive liquid waste.
7. Wash blot with 20 mL 0.1X SSC/0.1% SDS, 30 min, 42°C, in hybridization oven
8. Dry the blot on a clean paper towel. Wrap the blot in plastic wrap
9. Expose to phosphorimager or film overnight and then expose to film for several days as appropriate. Quantify using scanning densitometry, correcting for background.

4. Notes

1. The plasmid plus insert stock solution should be made up, if possible, to between 0.5 and 1.0 μ g/ μ L. The working stock should be a dilution of the concentrated stock to 10 ng/ μ L. If the plasmid plus insert sequence is longer than 4.0 kb, a more concentrated working stock (approx 50 ng/ μ L) will be needed.
2. The T_m used is that calculated from the primers design. A calculated T_m may vary for the same oligonucleotide from one manufacturer to the next according to the allowance for neighboring groups. Therefore, we recommend using the T_m given by the software package "Primer Designer" (Durham, NC), version 1.01 (1990) or later, Scientific and Educational Software (*see also Note 4*).
3. All PCR reagents and templates should be kept on ice throughout the procedure. RNA should be kept thawed for minimum time. Make one master mix of the

reagents so that an equal concentration is present in each of the reaction tubes. To ensure maximum performance from enzymes, do not take them out of the freezer until you need to add them to the master mix, and put them back right after use. It is also helpful to vortex all reagents and templates before use, since they have been frozen at low temperatures of -20 or -70°C . Gently vortex RNA samples (setting 4–5); shearing or deterioration of the strands will occur at higher speeds.

- 4 T_m may be different according to method of calculation. Therefore, if no apparent change in products is observed, then repeat experiment over a wider temperature range until the top annealing temperature tested shows a loss of product relative to products from lower annealing temperatures.
5. Generally, the following list can be used to determine denaturing and extending periods for PCR amplification:

Length of insert	< 500 bases	500–750 bases
Denature	30 s	1 min
Extend	30 s	1 min

Whereas we have successfully run assays for products from 200–750 bases, we recommend generating products of 300–500 bases using the shorter program

6. For products <500 bases, use a 2% gel. For products between 500 and 1000 bases, a 1–1.5% gel can be used. The mass ladder will indicate the product size if no standard is available
- 7 On a Perkin Elmer TCI or 480 cycler, the counter displays the number of cycles remaining not including the cycle that is presently running. After the 25th cycle has been completed, 10 cycles remain, however the counter reads 9 since a cycle is already running
8. Exposure must be sufficient to allow detection of the lower standards as well as the upper standards. Our experience is that 5- to 15-min exposures to phosphorimager are sufficient when there is moderate signal and the probe/radioactivity is fresh. The time needed to expose to film is approx four times longer than used for phosphorimaging.
9. At this point, one may skip the binding column if abundant message is expected or if background is not usually a problem (depending on membrane). In that case bring the labeled product up to 200 μL with molecular-biology grade H_2O (after stopping the reaction with 0.5 M EDTA). Use half to all of the final volume in the hybridization procedure.

Acknowledgments

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