I. SCOPE OF THE TEST

The PerkinElmer Life Sciences Inositol-1,4,5-Trisphosphate (IP₃) [³H] Radioreceptor Assay Kit is designed to measure IP₃ extracted from tissues and cell suspensions. Suggested sample preparation methods are included. It is the researcher’s responsibility to select, optimize, and validate the sample preparation method and the sample source(s). The kit includes a blanking solution, [³H]-labeled IP₃ tracer, membrane preparation containing IP₃ receptor, purified IP₃ standard, and the assay tubes and centrifuge racks used in the assay.

II. BACKGROUND INFORMATION

Inositol-1,4,5-trisphosphate (IP₃) is an intracellular second messenger which mobilizes intracellular calcium stores in response to cell stimulation by neurotransmitters, hormones, and other agonists (reviewed in refs. 1 and 2). Receptors for IP₃ have been found in endoplasmic reticulum (ER)³⁴ and other cell organelles⁵⁶. The binding of IP₃ to receptors on the ER causes the opening of calcium channels allowing calcium ions to leak from the ER into the cytosol⁵⁷. Increasing IP₃ levels produces a large variety of cell responses, depending on the cell type under study. Some of the effects include muscle contraction, plasma membrane depolarization, and aggregation and serotonin release from platelets (reviewed in ref. 8). IP₃ may also play an important role in memory⁹¹⁰.

The immediate precursor of IP₃ is the phospholipid phosphatidylinositol-4,5-bisphosphate (PIP₂), which resides in the cell's plasma membrane. PIP₂ is produced by the phosphorylation of phosphatidylinositol at the 4-position and the 5-position of the inositol headgroup (see Figure 1). PIP₂ is cleaved to IP₃ by a phosphodiesterase, specifically referred to as phosphoinositidase¹¹. In addition to forming IP₃, purified phosphoinositidase has been shown to cleave PIP₂ to a cyclic form of inositol trisphosphate in which one phosphate group is attached at both the 1- and 2-position¹².
A. This cyclic inositol-1,2,4,5-trisphosphate has been found in cells but it is much less effective at mobilizing calcium and exhibits lower binding than IP₃ to receptors. Cyclic inositol-1,2,4,5-trisphosphate is stable at neutral pH. However, extraction methods such as the one given in this manual, which use strong acid, convert the cyclic compound to IP₃.

IP₃ is inactivated by a specific phosphatase which removes the phosphate at the 5-position to give inositol-1,4-bisphosphate. Hydrolysis by other specific phosphatases ultimately leads to the formation of D-myo-inositol, which is recycled to produce more phosphatidylinositol. IP₃ can also be phosphorylated at the 3-position, producing inositol-1,3,4,5-tetrakisphosphate (IP₄). This compound may be involved in control of calcium entry across the plasma membrane. IP₄ is degraded in much the same manner as IP₃, or it can be further phosphorylated in some cells to produce IP₅ and IP₆.

III. ASSAY METHODS

Most methods for the determination of IP₃ require purification of cell extracts by ion exchange chromatography followed by chemical or enzymatic phosphate hydrolysis. Quantitation of inositol by gas chromatography, mass spectrometry, or enzymatic assay; or the spectrophotometric analysis of inorganic phosphate is then performed. Using the enzyme inositol-1,4,5-trisphosphate 3-kinase and [γ³²P]ATP with partially purified cell extracts, the formation of [³²P]IP₄ has also been used as a measure of IP₃ levels. In other methods, precursors of IP₃ are labeled with [³²P] phosphate or [³H]inositol, and inositol polyphosphates are separated by HPLC using radioactivity to locate peaks. The mass of IP₃ recovered is calculated from the specific activity of the phosphoinositide precursor. All of these methods are slow, expensive or tedious to perform.

Based on radioreceptor assay protocols using membrane preparations from adrenal cortex and cerebellum, PerkinElmer Life Sciences has developed a sensitive, specific, and easy-to-use radioreceptor assay kit for the rapid quantitative determination of D-myo-inositol-1,4,5-trisphosphate.
IV. PRINCIPLE OF THE METHOD

The basic principle of radioreceptor assay (RRA) is competitive ligand binding, where a radioactive ligand competes with a non-radioactive ligand for a fixed number of receptor binding sites. Unlabeled ligand from standards or samples and a fixed amount of tracer (labeled ligand) are allowed to react with a constant and limiting amount of receptor. Decreasing amounts of tracer are bound to the receptor as the amount of unlabeled ligand is increased.

The PerkinElmer radioreceptor assay kit uses a membrane preparation derived from calf cerebellum which contains the IP$_3$ receptor. This receptor binds natural inositol trisphosphate (IP$_3$) and radiolabeled, tritiated inositol trisphosphate ([$^3$H]IP$_3$), also included in the kit. The amount of [$^3$H]IP$_3$ bound to the receptor is measured by centrifuging the membranes into a pellet at the bottom of a centrifuge tube and counting the amount of radioactivity in the pellet. The concentration of both receptors and [$^3$H]IP$_3$ have been selected for optimum reproducibility and sensitivity. The addition of unlabeled IP$_3$, added to the incubation mixture as either a standard or unknown sample, competes with [$^3$H]IP$_3$ for binding to the receptor and lowers the amount of radioactivity in the membrane pellet. This is illustrated in Figure 2.

![Figure 2](image_url)

A determination of non-specific binding is required for the quantitation of ligand added in samples or standard solutions. Non-specific binding is caused by tracer which is not bound to the receptor but which remains attached to other parts of the membrane or the tube. This is measured by saturating the receptor with cold ligand and measuring the amount of tracer which remains. In this kit, non-specific binding is determined by saturating the receptor with a large amount of inositol hexaphosphate (IP$_6$) rather than IP$_3$. This reagent is supplied as the Blanking Buffer. As Figure 3 shows, Blanking Buffer produces the same non-specific binding as high concentrations of IP$_3$.

Using IP$_3$ standards of known concentration, a standard (dose-response) curve is produced. This curve is used to determine the concentration of IP$_3$ in unknown samples by interpolation.
V. REAGENT DESCRIPTION

THIS KIT IS INTENDED FOR LABORATORY USE ONLY AND NOT FOR DIAGNOSTIC PURPOSES. All necessary reagents are supplied for 192 assay tubes plus two total count determinations if the suggested assay protocol is followed.

<table>
<thead>
<tr>
<th>192 Tubes</th>
<th>Kit Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 vials</td>
<td>Receptor Preparation, each vial containing 37 KBq (1 µCi) [3H]IP&lt;sub&gt;3&lt;/sub&gt; Tracer, lyophilized</td>
</tr>
<tr>
<td>2 vials</td>
<td>IP&lt;sub&gt;3&lt;/sub&gt; Standard, lyophilized</td>
</tr>
<tr>
<td>1 vial</td>
<td>Blanking Solution, 1.5 mL</td>
</tr>
<tr>
<td>1 bottle</td>
<td>Assay Buffer, 100 mL</td>
</tr>
<tr>
<td>2 racks</td>
<td>Containing 96 Minitubes each</td>
</tr>
<tr>
<td>4</td>
<td>Foam Centrifuge Racks</td>
</tr>
</tbody>
</table>

The IP<sub>3</sub> IRA kit is shipped at 2°-8°C and, upon receipt, components should be stored as directed. Component stability and handling precautions are described below. The storage of components under conditions other than those recommended may result in reduced shelf life.

**NOTE:** Sodium azide has been added as an antibacterial agent where appropriate. The National Institute for Occupational Safety and Health has issued a bulletin citing the potential explosion hazard due to the reaction of sodium azide with copper, lead, brass, or solder in plumbing systems. Although sodium azide has been added at a minimal concentration, it is still recommended that drains be flushed with large amounts of water if the plumbing system is used to dispose of those reagents. Copper-free and lead-free discharge lines should be used whenever possible. Decontamination procedures should be followed prior to maintenance work on drain lines which have been used for disposal of azide-containing reagents.

A. Receptor Preparation/[3H]IP<sub>3</sub> Tracer

The Receptor Preparation/Tracer is supplied lyophilized. It contains 37 KBq (1 µCi) of [3H]IP<sub>3</sub> complexed with the receptor. Stored protected from light at 2°-8°C in the unopened vial, the complex is stable for at least one month from the date of receipt. After reconstitution with the addition of 2.5 mL of distilled water, the Receptor Preparation/Tracer...
is stable for at least 5 days at 2°-8°C. For long term storage, it is recommended that the reconstituted material be stored frozen. At -20°C the reconstituted Receptor Preparation/Tracer is stable for at least one month. Freezing and thawing three times produces no loss of activity.

INSTRUCTIONS RELATING TO THE HANDLING, USE, STORAGE, AND DISPOSAL OF THIS RADIOACTIVE MATERIAL

This radioactive material may be received, acquired, possessed, and used only by research laboratories for *in vitro* laboratory tests not involving internal or external administration of the material, or the radiation therefrom, to human beings or animals. Its receipt, acquisition, possession, use, and transfer are subject to the regulations and a general license of the U.S. Nuclear Regulatory Commission or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

1. All radioactive materials must be labeled and secured in specifically designated posted areas. Records of receipt and survey must be maintained.

2. All work with these materials must be carried out only in authorized areas.

3. Prohibit mouth pipetting of radioactive materials.

4. There must be no smoking or eating within the work area.

5. Hands must be washed after handling radioactive materials.

6. Any spilled material must be wiped up quickly and thoroughly and the contaminated substances transferred to a suitable receptacle. The surfaces involved must be washed thoroughly with an appropriate decontaminant. Monitor to ensure the area has been effectively decontaminated.

7. When use of the tracer reagent has been completed, empty and decontaminate the vial. This radioactive material can be discarded into the sanitary sewerage system using copious amounts of water to ensure a minimal discharge concentration.

8. Prior to disposal of the empty, uncontaminated kit and tracer containers in unrestricted areas, remove or deface the radioactive material labels or otherwise clearly indicate that the containers no longer contain radioactive material.

9. Inform all personnel who will work with this tracer that it contains radioactive material.

10. Radioactive material must be labeled to show that it is $^3$H, the quantity, “Caution Radioactive Material” and the radiation symbol.

11. Under a general license, it is only permissible to possess up to 10 exempt quantities of radioactive material, and exempt quantities may not be combined to create a container with more than an exempt quantity.

B. IP$_3$ Standard

The Standard is supplied lyophilized. Store in the dark at -20°C. For use in the assay, reconstitute the material with
2.0 mL of distilled water. The solution contains 120 pmol/mL (120 nM) D-myoinositol-1,4,5-trisphosphate in 1 mM EDTA, 1 mM EGTA, pH 7.5. Mix well before use in assay. Once reconstituted, stored at -20°C in the dark, the solution is stable for at least one month.

C. Blanking Solution

The Blanking Solution is 2% (W/V) inositol hexaphosphate (IP₆) in 20 mM EDTA, 20 mM EGTA, 0.05% sodium azide, pH 7.5. Stored at 2°-8°C, the solution is stable for at least two months.

D. Assay Buffer

The Assay Buffer contains 0.05% sodium azide, 5 mM EDTA, 5 mM EGTA, and 50 mM sodium TAPS buffer, pH 8.6. The assay buffer is stable for at least two months at 2°-8°C.

VI. MATERIALS REQUIRED BUT NOT SUPPLIED

In addition to the reagents supplied with the kit, the following materials are required:

1. Pipettors and/or pipets that accurately and precisely deliver the required volumes.
2. 12 x 75 mm polystyrene or polypropylene tubes.
3. Test tube rack.
4. Vortex mixer.
5. Centrifuge with swinging bucket rotor.
7. Distilled water.
8. 7 mL glass counting vials and caps (Kimble part number 74503-7)
9. 0.15 M sodium hydroxide solution.
10. Liquid Scintillation Cocktail. Atomlight® or Formula-989® are strongly recommended for use in this assay. Studies have shown that some other cocktails are unsuitable.

This list does not include materials needed for extraction of IP₃, which is listed in sample handling Section VII.
**VII. PREPARATION OF SAMPLES**

A. **Collection and Storage**

It is recommended that all samples be processed immediately after collection and assayed as soon as possible. IP$_3$ appears to be stable for at least 24 hours at 2°-8°C in 1 M trichloroacetic acid (TCA) and after solvent extraction of the TCA. However, stability may depend on the tissue extracted and the levels of residual solvent. It is up to the individual investigator to determine optimal extraction and storage conditions. We recommend polypropylene containers for extraction and storage.

B. **Equipment and Reagents Required**

- Trichloroacetic acid, 100% (w/v) in water
- Trioctylamine ((C$_8$H$_{17}$)$_3$N)
- 1,1,2-Trichloro-1,2,2-trifluoroethane (Cl$_2$FCCF$_2$Cl, referred to as TCTFE throughout the rest of the manual, is available from Aldrich, J. T. Baker, and other chemical supply companies.)
- Distilled water
- Polypropylene centrifuge tubes or microfuge tubes
- Polypropylene or polystyrene graduated pipets
- Polyethylene transfer pipets
- Homogenizer
- Low speed centrifuge (capable of generating at least 1,000 x g)
- Vortex mixer

C. **Extraction of IP$_3$**

In each extraction experiment, it is good practice to assay a *method blank* consisting of distilled water which has been extracted along with samples. This practice will assure the user that non-specific interfering substances have not been introduced from solvents, etc. If an alternate sample preparation procedure is employed, it is the user’s responsibility to validate the system and to ensure that proper recovery and matrix corrections are made. The extraction procedure described below is based on a procedure employed by Challiss, et al. 24 for the extraction of IP$_3$ from rat cerebral cortex slices.

The waste solvent solution produced in the extraction procedure should be stored in a tightly capped glass bottle and disposed of as hazardous material according to local ordinances.

1. **Solid Tissues, Blood, and Cell Suspensions in Plasma or Serum**

   This procedure is designed for the extraction of materials with high protein concentration.

   a. Dilute 1 volume of 100% trichloroacetic cold distilled water.
      
      (1 mL + 5 mL water). This will produce a TCA solution with a concentration of approximately 1 M. Keep on ice until ready to use.

   b. Add 5 mL of ice cold 1 M TCA solution for each 1 gram (wet weight) of tissue or each 1 mL of blood, plasma or serum. Homogenize solid tissues thoroughly at 0°-4°C. Vortex thoroughly after addition of TCA to blood, plasma, or serum; and incubate for 15 minutes on ice.
c. Centrifuge for 10 minutes at 0°-4°C at 1,000 x g, or centrifuge for 1 minute in a microfuge (10,000-15,000 x g).

d. Remove supernatant and discard pellet. Incubate supernatant solution for 15 minutes at room temperature.

2. Cell Suspensions in Media Containing No More Than 10% Serum.

This procedure is designed for the extraction of materials with low protein concentration and where levels of IP₃ are expected to be near the limits of detectability with this assay. Due to the higher levels of salts which may be present in these extracts, corrections for the matrix will probably be required. This is discussed further in the appendix.

a. Place the cell suspension on ice. Add 0.2 volumes (0.2 mL for each 1 mL of cell suspension) of ice cold 100% TCA solution and vortex thoroughly. Incubate in ice for 15 minutes.

b. Centrifuge for 10 minutes at 0°-4°C at 1,000 x g, or centrifuge for 1 minute in a microfuge (10,000-15,000 x g).

c. Remove supernatant and discard pellet. Incubate supernatant solution for 15 minutes at room temperature.

D. Removal of Trichloroacetic Acid from Extracts

1. On the day of use prepare a solution of TCTFE-trioctylamine, 3:1 (3 mL TCTFE + 1 mL trioctylamine). Store this solution at room temperature in a tightly capped glass container until needed. Because trioctylamine sticks to glass, only polypropylene or polystyrene pipets should be used to transfer solutions containing this compound.

2. In a polypropylene tube, add 2 mL of TCTFE-trioctylamine for each 1 mL of TCA extract. Cap the tube and shake vigorously for about 15 seconds, or mix by vigorous trituration - drawing up and expelling the entire contents of the tube using a polyethylene transfer pipet - for 15 seconds. To prevent leaking, use screw-cap tubes when shaking.

3. Allow the mixture to sit for three minutes at room temperature. Two layers should be visible; a clear aqueous top layer and a slightly cloudy bottom layer. If the solutions do not completely separate or you wish to speed up the separation, low speed centrifugation can be used (1,000 x g for 5 minutes).

4. Using a transfer pipet, or pipet tip, carefully remove the top layer, which contains the IP₃. DO NOT DRAW UP ANY OF THE LOWER, SOLVENT LAYER.

The most effective way to remove the top layer is:

a. Hold the tube so that it is tipped at a 45° or greater angle.

b. Place the pipet or pipet tip so that it touches the top of the tube and just touches
the top of the aqueous layer.

c. Aspirate off the top layer keeping the pipet tip close to the top of the aqueous layer as it is removed.

Carefully examine the contents of the pipet or pipet tip for immiscible solvent as you are aspirating the aqueous top layer. Discharge any solvent which may have been removed along with this layer.

The solvent extraction step will produce a reduction in the volume of the aqueous layer. Start with at least 20% greater volume than required for assay.

5. After extraction, the samples should be stored on ice until assayed. If samples are determined to have levels of IP₃ which are too high to assay, they should be diluted with distilled water. Control extracts of buffer or tissue culture medium should be diluted in the same manner. Recovery from the entire extraction procedure is usually greater than 80%. The extracted samples should have a pH around 4.5.

DO NOT PERFORM A SECOND TCTFE-TRIOCTYLAMINE EXTRACTION ON THE ORIGINALLY EXTRACTED SAMPLE. THIS WILL LEAD TO A SIGNIFICANT LOSS OF IP₃. If an additional solvent extraction is required (to remove remaining trioctylamine, for example) pure TCTFE can be used without IP₃ loss.

VIII. PROTOCOL

1. WARNING: THIS PRODUCT CONTAINS A CHEMICAL KNOWN TO THE STATE OF CALIFORNIA TO CAUSE CANCER. (NOTE: [³H] TRACER)

2. An aliquot of an appropriate control should be assayed to account for any method blank that may arise as a result of the extraction or chromatographic steps. It is the user’s responsibility to check and correct for non-specific matrix and solvent effects. If the solution tested for IP₃ interferes with the assay due to high salt or other components, it may be necessary to generate an entire standard curve using this solution rather than water. In this case, the IP₃ standard should also be reconstituted with 2 mL of this solution instead of water. Refer to the appendix for an example of data analysis of IP₃ standard curve in tissue culture medium.

3. Inadequate centrifugation time or speed may result in incomplete precipitation of bound counts. Inadequate centrifugation speed or prolonged inversion after decanting may cause the pellets to become dislodged from the bottom of the tubes, resulting in incomplete retention of bound counts. Although good results can be obtained by centrifuging at 1,000 x g, maximum sedimentation and, therefore, greatest sensitivity is achieved at forces greater than 1,500 x g. A longer centrifugation time (e.g., 15-20 minutes) can compensate for lower centrifugation speeds.

4. For maximum sensitivity, all solutions should be kept on ice until used. The entire minitube rack should also be placed on ice to keep the tubes cold during set-up.

5. To control day-to-day variability, it is recommended that a standard curve be run each day samples are being assayed.

6. Only use pencil (4B or softer) or india ink pens to mark the writing patch at the top of the minitubes. Other
inks will dissolve in the scintillation cocktail and may lower counting efficiency. Use gloves when handling the tubes to prevent erasure of pencil markings.

7. The foam centrifuge racks are designed to fit a Sorvall H1000B swinging bucket rotor. For other centrifuge rotors, these racks may have to be cut with a razor blade to fit properly. The racks should fit snugly, but be easily removed after centrifugation.

8. After centrifugation, when decanting supernates from the reaction tubes, it is very important to remove all liquid from the tubes. Residual liquid remaining as unbound counts in the assay will cause a loss of sensitivity at the low concentration end of the standard curve.

9. To easily remove a minitube from a foam centrifuge rack, first loosen the tube by pushing it up from the bottom of the rack with a thumb.

10. A high-quality scintillation cocktail is necessary for best results. The addition of basic aqueous solutions to some cocktails may produce quenching and chemiluminescence. Atomlight or Formula-989 are strongly recommended for use in this assay.

B. Reagent Preparation

1. Concentrated Receptor Preparation/Tracer

Reconstitute one vial of lyophilized Receptor Preparation/Tracer with 2.5 mL of cold distilled water. Swirl the vial thoroughly to insure that the membranes are uniformly suspended. Allow the Receptor Preparation/Tracer to sit on ice for 15 minutes before using.

2. Working Receptor/Tracer

A Working Receptor/Tracer solution is prepared by diluting the Concentrated Receptor Preparation/Tracer 1:15 (v/v) with Assay Buffer. Always mix the Concentrated Receptor Preparation/Tracer before withdrawing the required volume. Dilute only enough Concentrated Receptor Preparation/Tracer for use in each assay. For example, in performing a 20 tube assay, dilute 0.5 mL Concentrated Receptor Preparation/Tracer with 7.5 mL of Assay Buffer. Do not store and reuse Working Receptor/Tracer solution. Store unused portion of Concentrated Receptor Preparation/Tracer for several days at 2°-8°C or for longer periods at -20°C.

3. Standards

Add 2 mL of distilled water to one vial of lyophilized IP3 Standard, cap, and mix well by inverting. This will produce an IP3 solution with 120 pmol/mL (12 pmol/0.1 mL). Prepare a series of standards using distilled water as diluent. Polystyrene or polypropylene tubes (12 x 75 mm) are convenient for the dilutions. The set of standards described below range from 0.12 pmol/0.1 mL to 12 pmol/0.1 mL. Use standards A through H for the assay. Return unused portion of reconstituted standard concentrate to -20°C after use.
The Working Receptor/Tracer suspension must be thoroughly mixed by inversion of the container several times prior to addition. Repeat mixing by inversion for every 16 assay tubes. Add 400 µL of this solution to all tubes and to the two counting vials (total counts). Vortex each tube thoroughly for 3-4 seconds.

### Suggested Dilution Scheme For IP₃

<table>
<thead>
<tr>
<th>Tube</th>
<th>Concentration (pmol/0.1 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Undiluted standard</td>
</tr>
<tr>
<td>B</td>
<td>0.5 mL of standard a + 0.75 mL distilled water</td>
</tr>
<tr>
<td>C</td>
<td>0.5 mL of standard b + 0.50 mL distilled water</td>
</tr>
<tr>
<td>D</td>
<td>0.5 mL of standard c + 0.50 mL distilled water</td>
</tr>
<tr>
<td>E</td>
<td>0.5 mL of standard d + 0.50 mL distilled water</td>
</tr>
<tr>
<td>F</td>
<td>0.5 mL of standard e + 0.50 mL distilled water</td>
</tr>
<tr>
<td>G</td>
<td>0.5 mL of standard f + 0.75 mL distilled water</td>
</tr>
<tr>
<td>H</td>
<td>Distilled water only</td>
</tr>
</tbody>
</table>

### Assay

1. Label duplicate minitubes for blank, standards, and samples with a pencil. Set up two 7 mL counting vials for total counts.

2. Add 100 µL Blanking Solution to the two tubes labeled blank.

3. Add 100 µL of distilled water to the two tubes labeled zero standard.

4. Add 100 µL of each sample and diluted standard to appropriate tubes.

5. Tap the minitube rack to allow all liquid in the tubes to migrate to the bottom.

6. Place tubes in pre-chilled foam centrifuge racks. Press tubes to the bottom of the rack. Wrap rack and tubes with plastic wrapping to reduce evaporation. Incubate for one hour at 2°-8°C.

7. Centrifuge tubes (NOT TOTAL COUNTS) at 4°-6°C for 15-20 minutes at 1,500 - 2,500 x g.

8. NOTE: Decant the supernatants of all tubes (NOT TOTAL COUNTS) into a waste basin (for radioactive waste) by inverting the foam centrifuge racks and shaking sharply downward. Allow the tubes to remain upside down for a total of 3 minutes. Tap vigorously twice during this time period to remove residual supernatant on absorbent paper. If the blotting paper appears more than damp, tap the tubes again on fresh absorbent paper. Make sure that all tubes have good contact with the absorbent paper.

9. Add 50 µL of 0.15 M sodium hydroxide to each tube. Vortex each tube for 2-3 seconds.

10. Incubate the tubes at room temperature for 10 minutes. Vortex each tube again for 5
seconds. The membrane pellet should be totally dissolved at this time.

12. Drop each minitube into a counting vial. Add 5 mL of Atomlight or Formula-989 scintillation cocktail to all vials (including TOTAL COUNTS). Cap the vials tightly and mix the contents of each vial thoroughly. After mixing, the cocktail should appear homogeneous. The following suggestions will make mixing easier:

a. Add the cocktail carefully to fill the minitube, with additional cocktail overflowing into the counting vial.

b. The most efficient mixing occurs when air bubbles are used to mix the aqueous solution with the cocktail. This is accomplished by inverting the vials and allowing trapped air bubbles to rise to the bottom of the minitubes. For best results use repeated slow inversions combined with rapid shaking of vials held horizontally.

c. Many vials can be mixed at once using a proper rack and a flat plate held over the top, which holds the vials in place.

d. For uniform counting efficiency between vials, the level of cocktail should be the same inside and outside the minitube. This can be achieved by inverting the vials slowly once or twice followed by mild shaking of each individual vial held at a 45° angle.

13. Place vials in scintillation counter. Wait approximately 15 minutes before counting to insure that all of the vials have reached uniform temperature. Count for at least two minutes. Shorter counting times may produce counting errors caused by static electricity.

Table I - IP₃ Assay Protocol Schematic
(All volumes are in microliters)

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Blanking Solution</th>
<th>Distilled Water</th>
<th>Standard Samples</th>
<th>Receptor/Tracer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Counts</td>
<td>1-2</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Non-specific Binding</td>
<td>3-4</td>
<td>100</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>“0” Standard Standards</td>
<td>5-6</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>7-20</td>
<td>---</td>
<td>100</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>21, 22, etc.</td>
<td>---</td>
<td>---</td>
<td>100</td>
</tr>
</tbody>
</table>

After adding diluted Receptor/Tracer to tubes, vortex and incubate one hour at 2°-8°C. Add 0.4 mL diluted Receptor/Tracer directly to two counting vials for Total Counts. Centrifuge at 4°C for 15 to 20 minutes at 1,500 - 2,500 x g. Decant all tubes EXCEPT TOTAL COUNTS, and blot off excess liquid. Solubilize pellets in 50 µL of 0.15 M NaOH. Place tubes in counting vials. Add 5 mL scintillation cocktail. Mix. Count in liquid scintillation counter for two minutes.
IX. CALCULATIONS

After counting has been completed, the concentration of IP$_3$ in the samples is determined from a standard curve. The following method is suggested. (See Table II for sample calculations.)

A. If all tubes have been counted for the same period of time, use the total accumulated counts; otherwise, correct all raw counts to counts per minute (CPM).

B. Average the counts for each set of duplicates.

C. Calculate the average NET counts for all standards and samples by subtracting from each the average non-specific binding counts (tubes 3-4).

D. Determine the normalized percent bound ($\% \text{ B/B}_o$) for each standard and sample as follows:

$$\% \text{ B/B}_o = \frac{\text{Net CPM of Standard or Sample}}{\text{Net CPM of “0” Standard}} \times 100\%$$

E. Using semi-logarithmic graph paper, plot $\% \text{ B/B}_o$ for each standard versus the corresponding concentration of IP$_3$ added (in picomoles per 0.1 mL). See Figure 4 for typical standard curve.

F. Determine the concentration in each sample by interpolation from the standard curve. The concentration obtained must then be corrected for dilution, recovery, etc. to determine the original concentration in the sample.

NOTE: Any samples with a concentration of IP$_3$ determined to be above the range of the standard curve may be diluted with distilled water and re-assayed. The values obtained are then multiplied by the appropriate dilution factor.
### Table II - Sample Calculations

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>CPM</th>
<th>Average</th>
<th>Net CPM</th>
<th>% B/B</th>
<th>Sample Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Counts</td>
<td>1 2</td>
<td>9476 9364</td>
<td>9420</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blank</td>
<td>3 4</td>
<td>279 370</td>
<td>324</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>&quot;0&quot; Standard</td>
<td>5 6</td>
<td>3275 3194</td>
<td>3234</td>
<td>2910</td>
<td>100</td>
</tr>
<tr>
<td>0.12 pmol</td>
<td>7 8</td>
<td>2862 3032</td>
<td>2947</td>
<td>2623</td>
<td>90.1</td>
</tr>
<tr>
<td>0.3 pmol</td>
<td>9 10</td>
<td>2796 2627</td>
<td>2712</td>
<td>2388</td>
<td>82.0</td>
</tr>
<tr>
<td>0.6 pmol</td>
<td>11 12</td>
<td>2384 2400</td>
<td>2392</td>
<td>2068</td>
<td>71.0</td>
</tr>
<tr>
<td>1.2 pmol</td>
<td>13 14</td>
<td>1996 2122</td>
<td>2059</td>
<td>1735</td>
<td>59.6</td>
</tr>
<tr>
<td>2.4 pmol</td>
<td>15 16</td>
<td>1558 1540</td>
<td>1549</td>
<td>1225</td>
<td>42.1</td>
</tr>
<tr>
<td>4.8 pmol</td>
<td>17 18</td>
<td>1124 1052</td>
<td>1088</td>
<td>764</td>
<td>26.2</td>
</tr>
<tr>
<td>12.0 pmol</td>
<td>19 20</td>
<td>683 730</td>
<td>706</td>
<td>382</td>
<td>13.1</td>
</tr>
<tr>
<td>Sample</td>
<td>21 22</td>
<td>1336 1334</td>
<td>1335</td>
<td>1011</td>
<td>34.7</td>
</tr>
</tbody>
</table>

3.3 pmol per 0.1 mL
X. LIMITATIONS

The following compounds have been checked for receptor competition.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Competition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inositol-1,4,5-trisphosphate</td>
<td>100</td>
</tr>
<tr>
<td>Inositol-1-phosphate</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cyclic-inositol-1:2 monophosphate</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Inositol-1,4-bisphosphate</td>
<td>0.02</td>
</tr>
<tr>
<td>Inositol-1,3,4-trisphosphate</td>
<td>0.03</td>
</tr>
<tr>
<td>Inositol-2,4,5-trisphosphate</td>
<td>6.8</td>
</tr>
<tr>
<td>Cyclic-1:2,4,5-trisphosphate</td>
<td>&lt; 13.0</td>
</tr>
<tr>
<td>Inositol-1,3,4,5-tetrak isophosphate</td>
<td>2.3</td>
</tr>
<tr>
<td>Inositol pentaphosphate</td>
<td>5.5</td>
</tr>
<tr>
<td>Inositol hexaphosphate (phytic acid)</td>
<td>0.3</td>
</tr>
<tr>
<td>Inositol-1,4,5-tristhiophosphate</td>
<td>11.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IP₃ Added (pmol)</th>
<th>Average IP₃ Measured (pmol)</th>
<th>Standard Deviation</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
XI. PERFORMANCE

A. Recovery and Accuracy

Tissue culture medium RPMI 1640 containing 10% fetal calf serum, 1% glutamine, 1% sodium pyruvate and $10^6$ U937 cells/mL was spiked with several concentrations of IP$_3$. Five aliquots of each concentration (1 mL per aliquot) were extracted as described in VII, Preparation of Samples. Measurements by radioreceptor assay produced the results summarized in Table III.

**Table III**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra pmol (X ± S.D.)</th>
<th>Inter pmol (X ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.11 ± 0.05</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td>B</td>
<td>0.68 ± 0.14</td>
<td>0.56 ± 0.10</td>
</tr>
<tr>
<td>C</td>
<td>1.68 ± 0.07</td>
<td>1.71 ± 0.15</td>
</tr>
<tr>
<td>D</td>
<td>6.65 ± 0.40</td>
<td>7.71 ± 0.87</td>
</tr>
</tbody>
</table>

B. Reproducibility of the Assay

Intra-assay reproducibility was determined by the experiment described in XI, A, in which multiple replicates ($n = 5$) of various spiked cell suspension samples were measured in a single assay. Inter-assay reproducibility was determined by assaying these samples in four separate assays over a four-day period with a number of receptor/tracer and standard preparations. Samples for inter-assay reproducibility were aliquoted, fast frozen on dry ice, and stored at -20°C until assayed. The following results were obtained:
C. Linearity

Tissue culture medium described in XI, A, containing $10^6$ U937 cells/mL was spiked with 7.15 pmol/0.1 mL IP$_3$. After extraction, the concentrations of IP$_3$ in these solutions along with 2-, 4-, and 8-fold dilutions were measured with the PerkinElmer IP$_3$ RRA. As a control, the medium without cells was also extracted and diluted. The results are shown in Figure 5.

D. Sensitivity

Defined as the mass corresponding to twice the standard deviation of the zero binding, the sensitivity of the system was found to be approximately 0.1 pmol.
XII. REFERENCES

APPENDIX

In order to determine the effect of tissue culture medium on the assay, the following experiment was performed. One mL of ice cold 100% (w/v) trichloroacetic acid was mixed with 5 mL of cold medium RPMI 1640. After 15 minutes on ice, the solution was incubated at room temperature for an additional 15 minutes. This solution was then extracted with 12 mL of TCTFE:trioctylamine, 3:1. The two phases were allowed to completely separate and the top aqueous layer was removed. IP₃ from a concentrated stock solution was diluted into distilled water and extracted RPMI. Two-fold serial dilutions in water and extracted RPMI were made, and 0.1 mL of each dilution was assayed as described in Protocol, Section VIII. The results are summarized in Table V.

<table>
<thead>
<tr>
<th>pmol IP₃ Added</th>
<th>Net CPM Distilled Water</th>
<th>% B/B₀</th>
<th>Net CPM Extracted RPMI</th>
<th>% B/B₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2118</td>
<td>100</td>
<td>1567</td>
<td>100</td>
</tr>
<tr>
<td>0.35</td>
<td>1700</td>
<td>80.3</td>
<td>1250</td>
<td>79.8</td>
</tr>
<tr>
<td>0.7</td>
<td>1454</td>
<td>68.7</td>
<td>1058</td>
<td>67.6</td>
</tr>
<tr>
<td>1.4</td>
<td>1138</td>
<td>53.8</td>
<td>804</td>
<td>51.3</td>
</tr>
<tr>
<td>2.8</td>
<td>780</td>
<td>36.9</td>
<td>572</td>
<td>36.5</td>
</tr>
<tr>
<td>5.6</td>
<td>436</td>
<td>20.6</td>
<td>382</td>
<td>24.4</td>
</tr>
<tr>
<td>11.2</td>
<td>276</td>
<td>13.0</td>
<td>221</td>
<td>14.1</td>
</tr>
<tr>
<td>22.4</td>
<td>199</td>
<td>9.4</td>
<td>142</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Figure 6
The results in Table V show that the undiluted extracted medium inhibits binding of tracer to the receptor. Therefore, the amount of IP$_3$ extracted from cells in medium cannot be directly determined using a standard curve made up in water. However, the sensitivity of the assay is not affected by the presence of extracted medium. This can be seen by comparing the competitive binding curves, as shown in Figure 6.

Instead of producing a complete standard curve in extracted tissue culture medium, an alternate method may be suitable for IP$_3$ determination. In this procedure, the standard curve uses IP$_3$ in water and a control measurement is performed using extracted medium. A ratio is calculated by dividing CPM bound in water (B$_0$) by CPM bound in extracted medium.

\[ R = \frac{\text{CPM-water}}{\text{CPM-medium}} = \frac{2118}{1567} = 1.35 \]

All values obtained from samples extracted in tissue culture medium are multiplied by this ratio to produce an adjusted CPM. The adjusted CPM values are used to determine IP$_3$ levels, as described in Calculations, Section IX. The calculations and results are shown in Table VI.
Table VI shows that, at least for RPMI 1640, an accurate estimation of IP$_3$ can be made using a water based standard curve and a CPM correction factor. However, it is up to the individual researcher to confirm that this method of correction is suitable.

This correction factor may not be appropriate for solutions containing serum, due to the presence of IP$_3$ in the serum. A standard curve prepared in extracted RPMI 1640 plus 10% fetal calf serum gave acceptable measurements of IP$_3$ when the level obtained in “0 added” was subtracted from the other values. The data is shown in Table VII.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.91</td>
<td>0</td>
</tr>
<tr>
<td>0.12</td>
<td>1.01</td>
<td>0.10</td>
</tr>
<tr>
<td>0.3</td>
<td>1.15</td>
<td>0.24</td>
</tr>
<tr>
<td>0.6</td>
<td>1.46</td>
<td>0.55</td>
</tr>
<tr>
<td>1.2</td>
<td>2.12</td>
<td>1.21</td>
</tr>
<tr>
<td>2.4</td>
<td>3.34</td>
<td>2.43</td>
</tr>
<tr>
<td>4.8</td>
<td>4.93</td>
<td>4.02</td>
</tr>
<tr>
<td>12.0</td>
<td>10.34</td>
<td>9.43</td>
</tr>
</tbody>
</table>