Description

The present assay system provides the quantitative determination of 13,14-dihydro-15-keto-prostaglandin-F2α (PGFM). PGFM can be assayed in the range 0.6-150 pg/tube. Each kit contains materials sufficient for 100 assay tubes, permitting the construction of one standard curve and assay of 24 unknowns in triplicate.

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Introduction

Prostaglandin F2α (PGF2α) plays an important role in various biological functions and in pathological processes. Because of this, there are efforts in research to obtain some correlation between PGF2α concentration and the normal or pathological functions investigated. Like other primary prostaglandins, however, PGF2α is a substrate for the dehydrogenase enzyme that converts it into 13,14-dihydro-15-keto-PGF2α (PGFM), the main metabolite found in the circulation. It is accepted generally that the only reliable way for monitoring the endogeneous production of prostanoids is to measure metabolites, rather than parent compounds.

The current PGFM RIA kit combines the advantages of radioiodine labelled tracer 13,14-dihydro-15-keto-PGF2α-[125I]-tyrosine-methyl ester (PGFM-[125I]TME) with a highly sensitive and specific antibody to provide a rapid, simple and sensitive method for the determination of PGFM concentration with about ten times as high a sensitivity as that obtained with the most sensitive assays using tritium-labelled tracer.

Principle of the method

This assay is based on the competition between unlabelled PGFM and a fixed quantity of 125I-labelled PGFM for a limited number of binding sites on PGFM specific antibody. Allowing to react a fixed amount of tracer and antibody with different amounts of unlabelled ligand the amount of tracer bound by the antibody will be inversely proportional to the concentration of unlabelled ligand.

In this kit, separation of bound from free tracer is achieved by precipitating the bound antigen with polyethylene glycol (PEG...
After centrifugation the supernatant is removed and the bound radioligand is counted in a gamma-counter equipped with a well-type NaI (Tl) scintillation crystal. Results obtained from the standards are used to construct a standard dose-response curve that enables the amount of unlabelled ligand in the sample to be calculated.

Contents of the kit

1 vial  TRACER
0.3 ml per vial, containing about 75kBq 
PGFM-[\textsuperscript{125}I]TME in ethanolic solution

1 vial  STANDARD, lyophilised, 
containing 10.0 ng PGFM 
in buffer with 0.01% thimerosal

1 vial  ANTISERUM, lyophilised, 
containing polyclonal PGFM antiserum (rabbit) 
with 0.01 % thimerosal.

1 vial  TRACER BUFFER, Ready to use. 
10.5 ml per vial, containing 0.01% thimerosal

1 bottle  ASSAY BUFFER CONCENTRATE 
15 ml per bottle, containing 0.01% thimerosal

1 bottle  POLYETHYLENE-GLYCOL 6000, SOLUTION 
(PEG 6000) 
Ready to use. 
105 ml per bottle, containing 0.01 % thimerosal

Materials and equipment required

Round bottom polystyrene or polypropylene assay tubes, about 12 x 75 mm
Test tube racks
Plastic film to cover tubes
Precision pipettes (100, 500 and 1000 µl)
Vortex mixer
Refrigerator
Refrigerated centrifuge
Gamma counter

Recommended tools and equipment
repeating pipettes

Preparation of reagents

\textsuperscript{125}I-TRACER
One vial of the tracer concentrate contains 75 kBq of PGFM-[\textsuperscript{125}I]TME in ethanolic solution. Pour the whole quantity of the tracer buffer into the tracer vial. The resulting solution contains 75 kBq of the tracer in 50 mM phosphate buffered saline, pH 7.3, with 0.3% \( \gamma \)-globulin, 0.02% Triton X-100 and 0.01% thimerosal. The diluted solution is stable until expiry date, if stored at -20 °C.

ANTISERUM
The antiserum was raised in rabbit against a bovine serum albumin conjugate of PGFM. Stored at 2-8 °C, the lyophilised antiserum is stable until expiry date. 
Reconstitute the antiserum by adding 10 ml of distilled water with gentle mixing to avoid foaming. Make sure that the lyophilised material is in solution. After reconstitution, the solution contains PGFM antiserum of appropriate binding ability in 50 mM phosphate buffer, pH 7.3, with 0.1% gelatin and 0.01% thimerosal. This solution should be stored at 2-8 °C. Under these conditions
the solution is stable until at least the date of expiry. Do not store reconstituted antiserum deep-frozen.

**STANDARD**
Reconstitute the lyophilised standard by adding exactly 1.0 ml distilled water. Make ensure that the lyophilised material is in solution. The resulting solution contains 10 ng of PGFM per ml in 50 mM phosphate buffer, pH 7.3 with 0.1% gelatin and 0.01% thimerosal. Stored at 2-8 °C this stock solution is stable until the date of expiry. The appropriate aliquot of the standard concentrate is diluted to obtain a series of standard dilutions according to the suggested dilution scheme shown later. Diluted standard solutions must not be stored.

**ASSAY BUFFER CONCENTRATE**
To prepare assay buffer for use in the assay system add 60 ml distilled water to the bottle after warming it to room temperature and mix thoroughly. The diluted assay buffer contains 50 mM phosphate buffer, pH 7.3 with 0.1% gelatin and 0.01% thimerosal. Stored at 2-8 °C diluted buffer is stable until the date of expiry.

**TRACER BUFFER**
This solution contains 0.3% human gamma-globulin, 0.02% Triton X-100 and 0.01% thimerosal in 50 mM phosphate buffered saline, pH 7.3 and is stable at 2-8 °C until the date of expiry.

**POLYETHYLENE-GLYCOL 6000 SOLUTION**
This reagent contains 20% polyethylene-glycol (PEG 6000) in 50 mM phosphate buffer, pH 7.3, with 0.01% thimerosal as preservative. Stored at 2-8°C, the solution is stable until the date of expiry.

---

**Preparation of samples prior to assay**
Contrary to primary prostaglandins whose concentrations increase rapidly when tissues are injured, „de novo” prostanoid synthesis does not usually alter the physiological levels of prostaglandin metabolites therefore the sampling procedure does not need any special precaution. However the general problem of radioimmunoassay, i.e. that the inhibition of binding to the antibody can be affected by factors other than the analyte concentration itself holds for PGFM RIA, too. Because of this, preparation of samples prior to assay is a prerequisite to the radioimmunological determination of prostaglandins. Out of the various useful sample preparation methods, the most reliable solid-phase extraction technique using Sep-Pak C₁₈ Cartridges (Waters Ass.) has been applied successfully in our laboratory with a slight modification of the Powell’s method for preparation of human plasma samples prior to radioimmunoassay. This procedure is provided for guidance only, and it remains the investigator’s responsibility to validate his experimental method.

**Extraction on Sep-Pak C₁₈ Cartridges**
1. Pretreat the cartridge according to manufacturer’s instructions.
2. Dilute 1 ml plasma with 4 ml water, then acidify to pH 3.0 with the addition of about 100 µl 2 M citric acid (Ceck pH individually, if needed).
3. Apply sample to the cartridge with a syringe and pass through with gentle pressure (about 1 ml/min).
4. Wash with 10 ml water.
5. Wash with 10 ml 10% ethanol.
6. Wash with 10 ml petroleum ether or n-hexane.
7. Elute PG-s with 10 ml ethyl-acetate.
8. Add 1 ml water to ethyl-acetate, shake, and separate the water phase. Repeat this procedure once again.

9. Dry the ethyl-acetate extract under vacuum at room temperature.

10. Dissolve the residue in assay buffer and measure PG-level immediately. If RIA measure cannot be performed immediately, store samples in ethylacetate below -20°C and proceed with steps 9-10 immediately before the assay.

Using this method the extraction efficiency is over 90%, thus the measurement of this value by adding labelled marker may generally be omitted.

Precaution: Plasma or serum samples cannot by any means assayed directly.

Warning! Never use the cartridges more than once with plasma samples.

Assay procedure

Preparation of PGFM working standards

To prepare standard dilution assay buffer provided with the kit must be used. The suggested dilution scheme to cover a standard curve range of 0.6 to 150 pg is shown in Table 1.

<table>
<thead>
<tr>
<th>Tube</th>
<th>volume of the standard dilution</th>
<th>volume of assay buffer</th>
<th>Amount of standard (pg/tube)</th>
</tr>
</thead>
<tbody>
<tr>
<td>s</td>
<td>1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>150 of sol. s</td>
<td>850</td>
<td>150</td>
</tr>
<tr>
<td>B</td>
<td>200 of sol. A</td>
<td>400</td>
<td>50</td>
</tr>
<tr>
<td>C</td>
<td>200 of sol. B</td>
<td>400</td>
<td>16.7</td>
</tr>
<tr>
<td>D</td>
<td>200 of sol. C</td>
<td>400</td>
<td>5.6</td>
</tr>
<tr>
<td>E</td>
<td>200 of sol. D</td>
<td>400</td>
<td>1.9</td>
</tr>
<tr>
<td>F</td>
<td>200 of sol. E</td>
<td>400</td>
<td>0.6</td>
</tr>
</tbody>
</table>

vial "s" is prepared by reconstituting the lyophilised standard with 1.0 ml distilled water.

Note: All volumes are in microliter.

Radioimmunoassay protocol

For a quick guide refer to Table 2.

Day 1

1) Prepare all reagents as described previously prior to performing the assay.

2) Equilibrate all reagents except PEG 6000 solution to room temperature and mix before use. (Refer to Table 2. for steps 3-16.)

3) Label triplicate tubes according to Table 2. (Determinations can equally be performed using duplicates.)

4) Pipette 300 µl of assay buffer into tubes 4-6 (non-specific binding tubes)

5) Pipette 200 µl of assay buffer into tubes 7-9 (total bound or zero standard tubes)

6) Pipette 100 µl of assay buffer into all remaining tubes except 1-3 (total count tubes)

7) Pipette 100 µl of each diluted standard in triplicate (A through F into tubes 10-27).
8) Pipette 100 µl of each sample in triplicate into tubes 28-100.
9) Pipette 100 µl of tracer solution into each tube.
10) Pipette 100 µl of antiserum into all tubes except 1-6 and vortex thoroughly for 2-5 seconds. (Note: If drops remained on the wall of tubes after vortexing, centrifuge the tubes at 1000 x g for a few seconds.)
11) Incubate the tubes at 4°C overnight (16-20 hours).

Day 2
12) Shake the cold PEG 6000 solution vigorously and pipette 1 ml into tubes 4-100. Vortex the tubes for a few seconds.
13) Equilibrate the tubes at 4°C for 10 minutes.
14) Centrifuge the tubes at 4°C and 2000 xg for 20 minutes. See Note 1.
15) Remove the supernatant (See Note 2.)
16) Count the radioactivity for at least 60 seconds in a gamma-counter.
17) Calculate the concentrations as described in Calculation of results.

Note 1.
\[ g = 1.118 \times 10^5 \times (\text{rpm})^2 \times (\text{arm-length of the centrifuge in cm}) \]
The tubes should be centrifuged for a longer time or at a higher rpm, if the supernatant is not clear enough.

Note 2.
The supernatant can be decanted provided that drops are removed from the wall of tube. However, aspiration with the aid of a simple plastic tip is more convenient. Care should be taken not to stir up the precipitate.

Table 2. Assay Protocol, Pipetting Guide (all volumes in microliters)

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Reagents</th>
<th>Total count</th>
<th>Non specific binding</th>
<th>Zero standard</th>
<th>Standard A-F</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-3</td>
<td>4-6</td>
<td>7-9</td>
<td>10-27</td>
<td>28-100</td>
</tr>
<tr>
<td>Buffer</td>
<td></td>
<td>300</td>
<td>200</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Tracer</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Antiserum</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Vortex mix
Incubate overnight (16-20 hours) at 4°C

PEG 6000 | 1000 | 1000 | 1000 | 1000

Vortex mix
Incubate for 10 minutes at 4°C

Centrifuge for 20 minutes at 4°C and 2000 xg

Remove the supernatant.

Count all tubes

**Calculation of results**
The calculation is illustrated using representative data. The assay data collected should be similar to those shown in Table 3. Calculate the average counts per minute (CPM) for each triplicate of assay tubes.
Calculate the percent \( B_0/T \) for zero standard \( (S_0) \) by using the following equation:

\[
% B_0/T = \frac{S_0 \text{ cpm} - \text{NSB cpm}}{T \text{ cpm} - \text{NSB cpm}} \times 100
\]

Calculate the normalized percent binding for each standard and sample by using the following equation:

\[
% B/B_0 = \frac{S_{A-F} \text{ / sample cpm} - \text{NSB cpm}}{S_0 \text{ cpm} - \text{NSB cpm}} \times 100
\]

Using semi-logarithmic graph paper plot \( B/B_0 \% \) for each standard versus the corresponding concentration of PGFM. Figure 1 shows a typical standard curve.

Determine the PGFM concentration of the unknown samples by interpolation from the standard curve. Do not extrapolate values beyond the standard curve range.

Table 3. Typical Assay Data

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Tube No</th>
<th>Count cpm</th>
<th>Average cpm</th>
<th>Average net cpm</th>
<th>B/B0 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1 2 3</td>
<td>25065 24016 24444</td>
<td>24508</td>
<td>23528</td>
<td></td>
</tr>
<tr>
<td>Non-specific binding</td>
<td>4 5 6</td>
<td>939 971 1030</td>
<td></td>
<td>980</td>
<td></td>
</tr>
<tr>
<td>( S_0 ) 0 pg/tube</td>
<td>7 8 9</td>
<td>13873 14320 13625</td>
<td>13939</td>
<td>12959</td>
<td>100</td>
</tr>
<tr>
<td>( S_A ) 0.6 pg/tube</td>
<td>10 11 12</td>
<td>13093 13472 13057</td>
<td>13207</td>
<td>12227</td>
<td>94.4</td>
</tr>
<tr>
<td>( S_B ) 1.9 pg/tube</td>
<td>13 14 15</td>
<td>11840 12147 12688</td>
<td>12225</td>
<td>11245</td>
<td>86.8</td>
</tr>
<tr>
<td>( S_C ) 5.6 pg/tube</td>
<td>16 17 18</td>
<td>9558 9675 9804</td>
<td>9679</td>
<td>8699</td>
<td>67.1</td>
</tr>
<tr>
<td>( S_D ) 16.7 pg/tube</td>
<td>19 20 21</td>
<td>6854 7144 7084</td>
<td>7027</td>
<td>6047</td>
<td>46.7</td>
</tr>
<tr>
<td>( S_E ) 50 pg/tube</td>
<td>22 23 24</td>
<td>4851 4928 4768</td>
<td>24849</td>
<td>3869</td>
<td>29.9</td>
</tr>
<tr>
<td>( S_F ) 150 pg/tube</td>
<td>25 26 27</td>
<td>3266 3376 3381</td>
<td>3341</td>
<td>2361</td>
<td>18.2</td>
</tr>
</tbody>
</table>
**Characterization of the assay**

**Assay parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSB/T</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>B_0/T</td>
<td>50 ± 10%</td>
</tr>
<tr>
<td>ED-50</td>
<td>14.9 ± 2.9 pg/tube</td>
</tr>
</tbody>
</table>

**Specificity**

Cross reactivity was defined in per cent by weight at the 50% displacement level.

- 13,14-dihydro-6,15-diketo-PGE_2: 2.6%
- 13,14-dihydro-15-keto-PGE_2: 0.1%
- PGA_2: 0.001%
- PGD_2: < 0.001%
- PGE_1: < 0.001%
- PGE_2: < 0.001%
- PGF_1α: < 0.001%
- PGF_2α: < 0.001%
- 6-keto-PGF_1α: < 0.001%
- 6-keto-PGE_1: < 0.001%
- Thromboxane B_2: < 0.001%

**Additional information**

**Storage**

Store this kit between 2 and 8 °C.

**Availability**

From stock.

**Shelf life**

The shelf life of kit reagents is 8 weeks from the date of manufacturing. The actual expiry date is given on package label and in the quality certificate. Components from various lots or from kits of different manufacturers should not be mixed or interchanged.
Precautions and warnings

This kit should only be used for research purposes.

Radioactivity

This kit contains radioactive material. Receipt, acquisition, possession, or use of radioactive materials are subject to regulations, and a licence of (inter)national authorizing bodies. It is the responsibility of the user to ensure that local regulations or codes of practice are satisfied.

Related products

Radioimmunoassay systems

<table>
<thead>
<tr>
<th>Product Description</th>
<th>Catalogue Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandin $F_{2\alpha}$ [$^{125}$I] RIA kit</td>
<td>RK-15</td>
</tr>
<tr>
<td>6-keto-PGF$<em>{1\alpha}$/2,3-dinor-6-keto-PGF$</em>{1\alpha}$ [$^{125}$I] RIA kit</td>
<td>RK-16M</td>
</tr>
<tr>
<td>Thromboxane $B_2$/2,3-dinor-TXB$_2$ [$^{125}$I] RIA kit</td>
<td>RK-17M</td>
</tr>
<tr>
<td>Prostaglandin $E_2$ [$^{125}$I] RIA kit</td>
<td>RK-25M</td>
</tr>
<tr>
<td>Bicyclo-prostaglandin $E_2$ [$^{125}$I] RIA kit</td>
<td>RK-28</td>
</tr>
<tr>
<td>11-Dehydrothromboxane $B_2$ [$^{125}$I] RIA kit</td>
<td>RK-67M</td>
</tr>
<tr>
<td>Prostaglandin $D_2$ [$^3$H] RIA kit</td>
<td>HTK-2</td>
</tr>
<tr>
<td>Prostaglandin $F_{2\alpha}$ [$^3$H] RIA kit</td>
<td>HTK-3</td>
</tr>
</tbody>
</table>

INSTITUTE OF ISOTOPES Co. Ltd.
H-1535 Budapest, P.O.Box. 851.
Tel.: (36-1) 392-2577
Fax: (36-1) 395-9247

WEB SITE: http://www.izotop.hu
Technical e-mail: immuno@izotop.hu
Commercial e-mail: commerce@izotop.hu