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For in vitro research use only
Storage temperature : 2-8°C

HTRF® package insert

Document reference : 62P2APEB rev01 (July 2008)

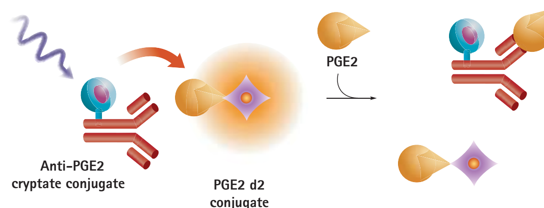
Packaging details :

	384-well low volume plate (20 µL)
62P2APEB	1,000 tests

1. Assay description and intended use

This kit is intended for the direct quantitative determination of prostaglandin E2 in buffered solution or cell culture supernatants.

Its principle is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). The method is a competitive immunoassay in which native PGE2 produced by cells, and d2-labeled PGE2 compete for binding to MAb anti-PGE2 labeled with Cryptate.



The HTRF® signal (i.e. energy transfer) is inversely proportional to the concentration of PGE2 in the calibrator or in the sample.

The PGE2 assay can be run under two different protocols:

1. The "supernatant" protocol (see §3), a standard PGE2 assessment in cell supernatant : i) cells are stimulated in a regular cell culture plate, and ii) cell supernatant is then transferred to the assay plate.
2. The "cell-based" protocol (see §4) is carried out in a single plate and allows the quantification of PGE2 directly on stimulated cells, without any transfer steps.

2. Background

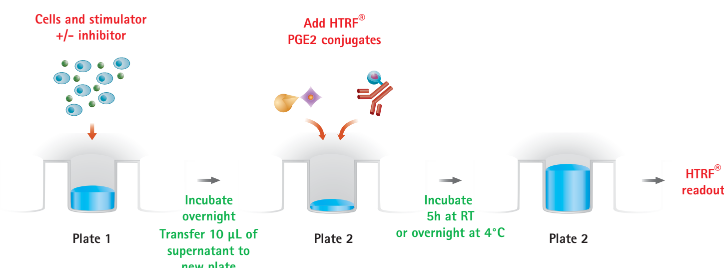
The production of prostaglandins begins with the liberation of arachidonic acid from membrane phospholipids by phospholipase A2 in response to inflammatory stimuli. The cyclooxygenases enzymes COX-1 and COX-2 then convert arachidonic acid to PGH2 (Prostaglandin H2). COX-1 is expressed constitutively and acts to maintain homeostatic function such as mucus secretion, whereas COX-2 is induced in response to an inflammatory stimuli. Further downstream, cell-specific prostaglandin synthases convert PGH2 into a series of prostaglandins including PGI2, PGF2, PGD2 and PGE2. PGE2 is produced by several cell types including macrophages, fibroblasts and some malignant cells and exerts its actions through 4 receptors EP1, EP2, EP3 and EP4. All these receptors are rhodopsin-type receptors with seven transmembrane-spanning domains. However each receptor is coupled to different G proteins and uses different second messenger signaling pathways.

Accounting on those receptors coupled to different G proteins, PGE2 has been shown to have both pro and anti-inflammatory actions. It induces vasodilatation by activating cAMP-coupled EP2 receptors on vascular smooth muscle and increases vascular permeability. But as inflammation progresses PGE2 promotes bronchodilatation.

PGE2 also inhibits T helper cells type 1 (Th1) cytokines productions such as IL-12, IFNγ, IL-2 and seems to favor Th2 type of immune response. Although it has also been described that PGE2 decreases IL-4 and IL-5 production as well as IgE production. PGE2 is involved in several pathologies such as periodontal disease and promotes tumor-cells survival.

3. Supernatant assay protocol

The supernatant assay protocol must be run in two distinct microplates : i) a culture plate for cell stimulation and ii) an assay plate in which PGE2 detection is carried out.



3.1. Supplied reagents and reconstitution

Allow the reagents to come to room temperature for at least 30 minutes before reconstitution.

Supplied reagents	Reagent reconstitution (stock solutions)		Working solutions
Anti- PGE2 - Cryptate**	1 vial Lyophilized*	⇨	Add 5 mL of reconstitution buffer to each vial. Mix gently.
PGE2 - d2	1 vial Lyophilized*	⇨	
PGE2 calibrator. Concentrated PGE2. See label indications for concentration after reconstitution	1 vial Lyophilized*	⇨	See label indications for reconstitution volume. Mix gently after reconstitution.
PGE2 control. Concentrated PGE2. See label indications for concentration after reconstitution.	1 vial Lyophilized*	⇨	
Reconstitution buffer 50 mM Phosphate buffer, pH 7.0, 0.8M KF	1 vial See volume on the label		
Diluent 50 mM Phosphate buffer, pH 7.0, 0.2 % BSA, 0.02 % NaN_3, preservatives	1 vial of 20 mL		

* All reagents were lyophilized in 50 mM phosphate buffer, pH 7, containing protease free BSA and stabilizers.

** The Cryptate conjugate concentration was optimized for a maximum assay sensitivity and to ensure an average counting of 40,000 cps at 620 nm (384-well low volume format), using the reference PHERAstar Plus reader (BMG LABTECH).

Precaution : HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the d2 and Cryptate-conjugates will impair the assay's quality.

3.2. Reagent storage and stability

All reagents should be stored at 2-8°C until reconstituted. Under proper storage conditions, they are stable until the expiry date indicated on the labels.

Reconstituted reagents are stable for one week at 4°C. They can be refrozen (at -80°C) and thawed twice.

3.3. Calibration curve preparation

Reconstitute the maximum calibrator according to the indications printed on the label and follow the dilution sequence shown in the following table to draw up the calibration curve. Dilution must be carried out with the diluent (or with freshly made PO_4 50 mM, BSA 0.2% pH7).

Calibrator	Calibrator concentration in pg/mL	Preparation
Cal 7	5,000	reconstituted calibrator
Cal 6	1,666.7	100 µL Cal 7 + 200 µL diluent
Cal 5	555.5	100 µL Cal 6 + 200 µL diluent
Cal 4	185.2	100 µL Cal 5 + 200 µL diluent
Cal 3	61.7	100 µL Cal 4 + 200 µL diluent
Cal 2	20.6	100 µL Cal 3 + 200 µL diluent
Cal 1	6.85	100 µL Cal 2 + 200 µL diluent
Cal 0	0	300 µL diluent

3.4. Sample preparation

Dilute all samples to be assayed with the diluent (or with freshly made PO4 50 mM, BSA 0.2% pH7). Consecutive dilutions should be made within the 0-5000 pg/mL range (working solution).

DMSO final concentration in the assay up to 2% can be used without affecting the assay quality.

3.5. Assay protocol for 384-well low volume plate (20 µL)

Dispense the reagents in the following order :

- 10 µL standard or sample
- 5 µL PGE2-d2
- 5 µL anti-PGE2 Cryptate

For negative control, replace the standard by 10 µL of diluent and PGE2-d2 by 5 µL of reconstitution buffer

For positive control, replace the standard by 10 µL of diluent

- Cover the plate with a plate sealer and incubate for 5 hours at room temperature*.
- Remove the plate sealer and read on a compatible HTRF® reader (more information about compatible reader at http://www.htrf.com/technology/htrfmeasurement/compatible_readers).

**** The incubation can be run overnight at 4°C in order to improve the assay sensitivity.***

3.6. Data reduction and example of a calibration curve

This data should not be substituted for that obtained in the laboratory

	A (665nm)	B (620nm)	Ratio (1)	Mean Ratio (2)	CV % (3)	Delta F % (4) (5h RT)
Negative control	1,533 1,475	37,354 36,011	410 410	410	0.1	
[calibrator] pg/mL						
0 positive control	53,216 53,089	33,277 33,776	15,992 15,718	15,855	1.2	3,767
6.8	52,850 54,528	33,966 35,379	15,560 15,413	15,486	0.7	3,677
20.6	52,079 50,308	35,949 33,518	14,487 15,009	14,748	2.5	3,497
61.7	45,653 45,522	35,781 35,307	12,759 12,893	12,826	0.7	3,028
185.2	33,178 34,230	35,619 35,773	9,315 9,569	9,442	1.9	2,203
555.5	19,095 18,991	36,474 36,650	5,235 5,182	5,208	0.7	1,170
1666.7	8,954 8,667	37,373 36,331	2,396 2,386	2,391	0.3	483
5000	4,401 4,251	38,426 37,613	1,145 1,130	1,138	0.9	178
PGE2 control	42,415 43,868	35,409 36,190	11,979 12,122	12,050	0.8	2,839

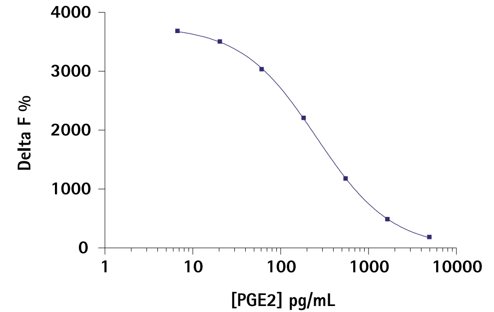
1. $\text{Ratio} = \frac{A_{665nm}}{B_{620nm}} \times 10^4$

2. $\text{Mean Ratio} = \frac{\Sigma \text{ratios}}{2}$

3. $\text{CV} = \frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$

4. $\text{Delta F} = \frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{neg}}{\text{Ratio}_{neg}} \times 100$

(Ratio_{neg} = negative control)



3.7. Assay characteristics

	Detection limit	EC_{50}
incubation 5 hours at RT	< 20 pg/mL (<57 pM)	250 pg/mL (0.7 nM)

3.8. Cross-reactivity

	Cross-reactivity in %
Prostaglandin E1	78.4
Prostaglandin E3	32.4
Sulprostone	11.4
6-keto prostaglandin F1 alpha	1.22
8-iso prostaglandin F2 alpha	0.46
PGF2 alpha	2

	Cross-reactivity in %
arachidonic acid	< 0.01
Prostaglandin A1	0.06
Prostaglandin B1	<0.01
Prostaglandin B2	< 0.01
Prostaglandin D2	< 0.01
Thromboxane B2	< 0.01

3.9. Assay flexibility and miniaturization

When used as suggested, the kit will provide sufficient reagents for 1,000 tests using a 384- well low volume plate in 20 µL final assay volume (HTRF® packaged basis).

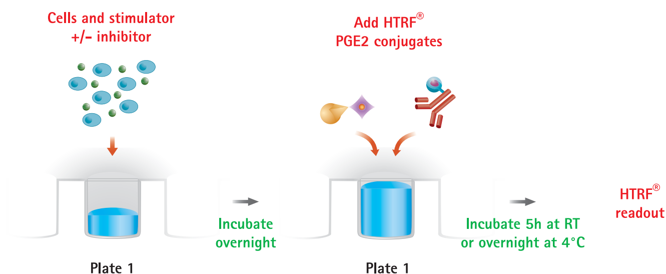
To move to other plate formats (96 half-well or 1536-well) and final volumes (100 µL to less than 10 µL), the volume of each assay component is simply proportionally adjusted in order to maintain the reagent concentrations as for the 20 µL final assay volume. For instance, in the case of the 1536-well format in 10 µL final volume, half as much material per well is used, thereby allowing 2,000 tests to be run. The performances of the HTRF® assay remain the same whatever the level of miniaturization.

Assay components	Volume proportion	Assay format: 1536-well (10 µL)	384-well low volume (20 µL)	96 half-well (100 µL)
Sample	2 volumes	5 µL	10 µL	50 µL
d2 conjugate	1 volume	2.5 µL	5 µL	25 µL
Cryptate conjugate	1 volume	2.5 µL	5 µL	25 µL
		2,000 tests	1,000 tests	200 tests

Plate references : 96 half-well plate (Costar # 3694 or equivalent), 384-well low volume plate (Greiner # 784076), 1536-well (Greiner # 782086).

4. Cell-based assay protocol

In this protocol, distribution has been modified in order to avoid the transfer step. Refer to § 3.



4.1. Reagent reconstitution

Refer to § 3.1.

4.2. Reagent storage and stability

Refer to § 3.2.

4.3. Calibration curve preparation

Reconstitute the maximum calibrator with the culture medium volume indicated on the label. Follow the same dilution sequence as shown in the table in § 3.3., **replacing the diluent by culture medium.**

4.4. Sample preparation

Cell density optimization is a key step in the PGE2 cell-based assay. Typically, the level of PGE2 produced by cells must fall within the 10-5000 pg/mL range.

Optimization consists of testing a broad range of cell concentrations (e.g. between 100 and 20,000 cells per well) in the presence or the absence of a direct activator of PGE2 production, e.g. LPS at 10 µg/mL.

In practice, resuspend the cells in the culture medium supplemented with the activator(s) or inhibitor(s) so that the desired number of cells will be dispensed under 10 µL.

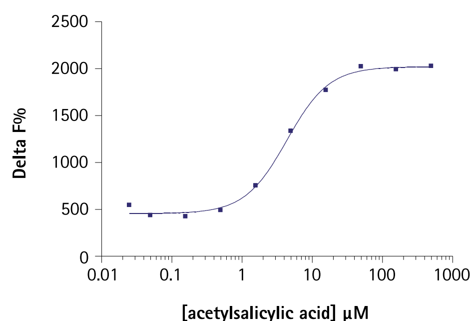
4.5. Cell-based assay protocol for 384-well low volume plates (20 µL)

Dispense the reagents in the following order:

- 10 µL standard or cell suspension
- Incubate the plate for 18 to 24 hours at 37°C in a CO_2 incubator with a fully humidified atmosphere.
- 5 µL PGE2-d2
- 5 µL anti-PGE2-Cryptate
- Incubate the plate for 5 hours at room temperature or overnight at 4°C
- Read on a compatible HTRF® reader (more information about compatible reader at htrf-assays.com/readers).

4.6. Case study : IC50 determination of acetylsalicylic acid in U937 promonocytic cells

U937 promonocytic cells were differentiated into macrophage-like adherent cells by a 24 hour culture in the presence of 10 nM phorbol 12-myristate 13-acetate (PMA). Cells were then dissociated and washed with fresh medium and distributed at the density of 20,000 cells per well. Cells were stimulated with lipopolysaccharides (LPS) at 10 µg/mL/well. Acetylsalicylic acid was included in the culture at different concentrations (between 0.1 µM and 500 µM). The day after, 5 µL of each HTRF® conjugate were added. The plates were then incubated 5 hours at room temperature before reading on a PHERAstar Plus® (BMG Labtech).



	IC 50 µM
Acetylsalicylic acid	4.1

This assay could be used to determine inhibitors of PGE2 production in cellular conditions. In all instances, cell density should be carefully optimized, as the secretion of PGE2 may vary between cell types.

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