

www.htrf-assays.com

HTRF® package insert

Document reference : 62IP1PEB rev05 (January 2006)

Packaging details :

	384-well low volume plate (20 µL)
62IP1PEB	1,000 tests

1. Assay description and intended use

This kit is intended for the direct quantitative determination of myo-Inositol 1 phosphate (IP1), and has been optimized in order to measure IP1 directly on cultured cells.

This assay is based on a monoclonal antibody specific for IP1 labeled with Eu Cryptate, competing with both native IP1 produced by cells and IP1 coupled to the dye d2. The specific signal (i.e. energy transfer) is inversely proportional to the concentration of IP1 in the calibrator or in the cell lysate. As for all other HTRF® assays, data reduction using the fluorescence ratio (665 nm/620 nm) eliminates possible photophysical interference and means the assay is unaffected by the usual medium conditions and colored compounds.

2. Background

GPCRs carry information within the cell via two major signaling pathways : the activation of Gs or Gi protein coupled receptors results in a variation of the cyclic AMP (cAMP) level, whereas the activation of Gq protein coupled receptors results in a transient increase of intracellular Ca²⁺, triggered by inositol (1,4,5) tri-phosphate (IP3).

After the GPCR Gq activation, the lifetime of IP3 is very short (less than 30 sec) before being transformed into IP2 and then IP1. IP1 is accumulated in the cell when LiCl is added to the culture medium. After activation of the GPCR, IP1 can be precisely quantified using the IP-One assay.

The HTRF® IP-One assay has been evaluated on various GPCR models, using either stable or transient cell lines (e.g. Muscarinic M1, Purinergic P2Y1), as well as chimeric systems (Gα16, Gq19). Results obtained with reference agonists or antagonists were similar to those obtained with the reference radioactive assay (cf poster on "www.htrf-assays.com/htrf/news.php4").

The IP-One assay represents a new solution for Gq investigation under HTS conditions.

3. Assay procedure in brief

The IP1 quantification must be carried out on cells plated and cultivated the day before the experiment.

Day 1 :

- cell preparation: cells are plated in tissue culture treated white microplates (see HTRF® recommended references). Plated cells must then be incubated overnight at 37°C in a cell culture incubator.

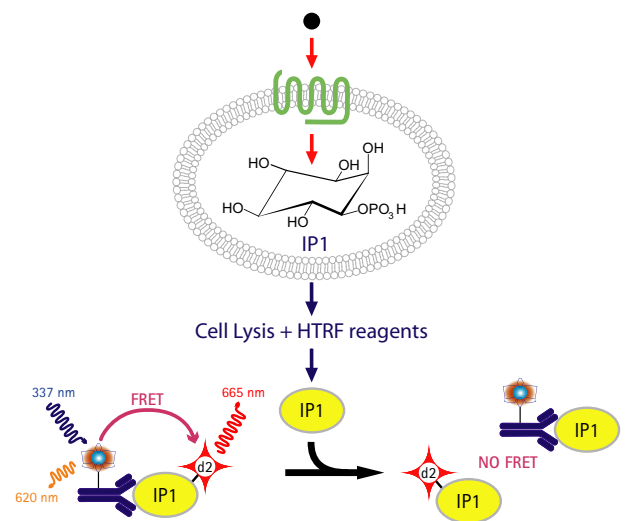
Day 2 :

- cell stimulation: after removal of the cell supernatant, cells are stimulated for 30 minutes by the addition of the compound diluted in the stimulation buffer provided in the kit.
- IP-One assay: both HTRF® conjugates are dispensed successively into the wells. Plates can be read on an HTRF® compatible reader after 1 hour of incubation.

Recommended plates

The IP-One assay is designed to be carried out in tissue culture treated white microplates (from 96 to 384 wells). The use of the following plates is highly recommended :

Manufacturer	Plate type	IP-One assay volume	Reference
Greiner*	96-well plate, white, TC	100 µL	655 083
Greiner	384-well plate, white, TC	40 µL	781 080
Greiner	384-well small volume plate, white, TC	20 µL	784 080



4. Reagent preparation

Allow the reagents to warm up at room temperature for at least 30 mins before reconstitution.

Supplied reagents	Stock solution preparation	Working solutions
anti-IP1 Cryptate 1 vial, lyophilized	Reconstitute each vial with 1 mL of distilled water . Mix gently.	⇒ For each conjugate, dilute 1 volume of stock solution in 4 volumes of Conjugate & lysis buffer.
IP1 -d2 1 vial, lyophilized		
IP1 calibrator 1 vial, lyophilized	See indications on label for reconstitution volume (distilled water). Mix gently.	⇒ See calibration curve preparation for further dilution ⇒ To be used directly after reconstitution
IP1 control 1 vial, lyophilized		
Conjugate & lysis buffer 1 vial of 13mL		Ready to use
IP1 stimulation buffer 5x* 1 vial of 8 mL		Make a 1/5 dilution in distilled water to prepare the 1x Stimulation buffer (e.g. for 10 mL: take 2mL of stimulation buffer 5x and add 8mL of distilled water - Mix gently)

The IP1 stimulation buffer provided with the kit has been validated on several GPCR models by Cisbio. Once diluted 1/5 in distilled water, its composition is the following one: Hepes 10 mM, CaCl₂ 1 mM, MgCl₂ 0.5 mM, KCl 4.2 mM, NaCl 146 mM, glucose 5.5 mM, LiCl 50 mM pH 7.4. It is used to dilute the IP1 standards, the agonist, antagonist or test compounds.

- *If you wish to use your own stimulation buffer :
- 50 mM of LiCl is recommended in order to prevent IP1 degradation in the cells
 - Phosphate buffer is not recommended in this stimulation buffer because it interferes in the IP-One assay.

Never mix the conjugate working solutions.

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 quality.

5. Reagent storage and stability

	Storage conditions	Stability
Lyophilized reagents	4°C until reconstitution	Until expiry date indicated on the labels
Stock solutions and working solutions*	4°C	4 days
	frozen (-20°C)	3 months. May be frozen and thawed once

*It is recommended to dispense the remaining solutions of calibrator and conjugates into disposable plastic vials for storage conditions after first use.

6. Calibration curve preparation

Follow the dilution sequence shown in the following table to prepare the calibrators. Dilutions must be carried out with the 1X stimulation buffer. The calibration curve covers an average range of 44 to 44,000 nM.

Calibrator	Preparation	[IP1] working solution nM	[IP1] final in assay nM
Cal 6	50 µL reconstituted calibrator + 200 µL 1x stimulation buffer	44,000	22,000
Cal 5	50 µL Cal 6 + 150 µL 1x stimulation buffer	11,000	5,500
Cal 4	50 µL Cal 5 + 150 µL 1x stimulation buffer	2,750	1,375
Cal 3	50 µL Cal 4 + 150 µL 1x stimulation buffer	688	344
Cal 2	50 µL Cal 3 + 150 µL 1x stimulation buffer	172	86
Cal 1	50 µL Cal 2 + 150 µL 1x stimulation buffer	43	21.5
Cal 0	150 µL 1x stimulation buffer	0	0

7. Assay procedure

7.1. Generation of the calibration curve and controls (384-well low volume plate, 20 µL final volume)

These controls and calibrators do not require cells, but must be run in a similar plate to the rest of the assay.

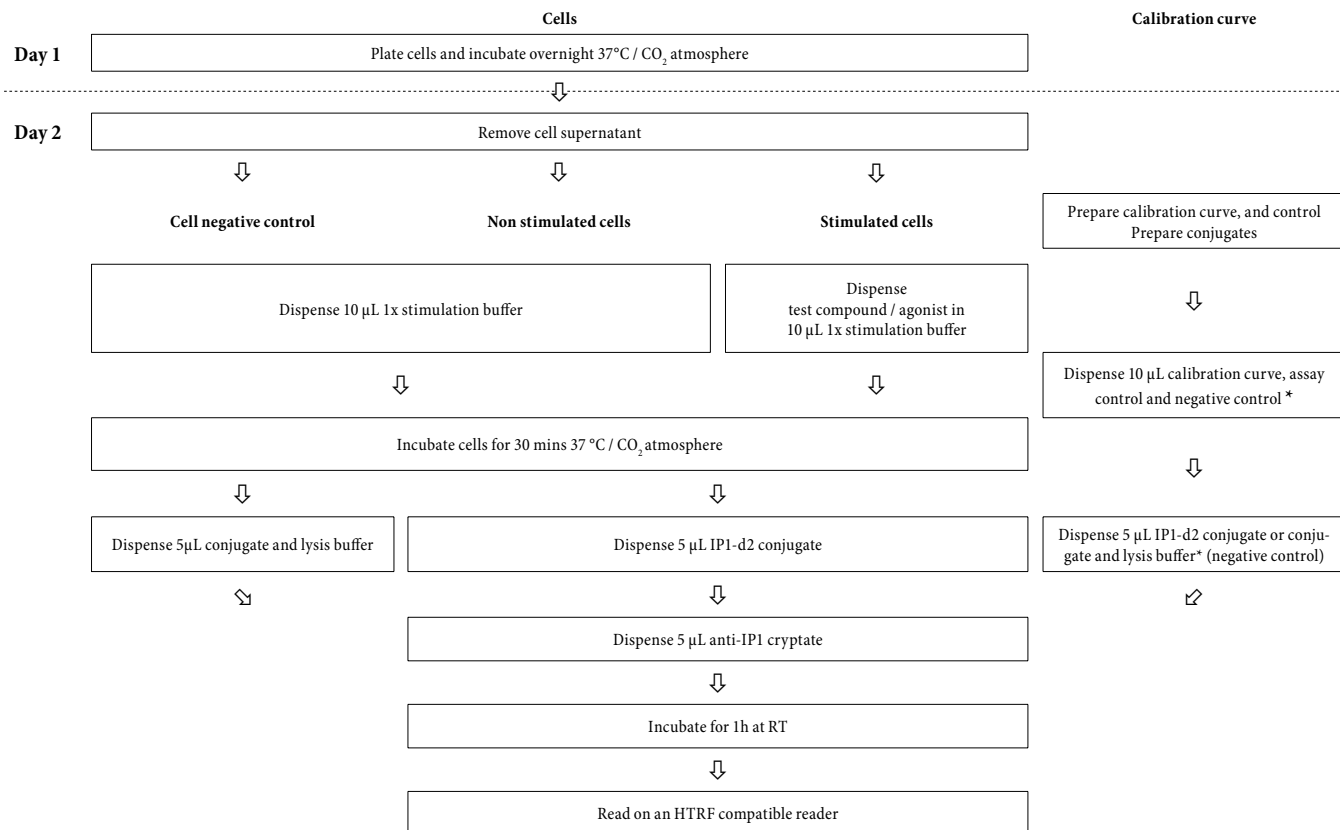
Negative control	Calibration curve	Assay control	The assay control validates the accuracy of the calibration curve. The concentration deduced from the delta F obtained should fall into the concentration range given on the vial label.
10 µL 1x stimulation buffer	10 µL IP1 calibrator	10 µL IP1 control	
5 µL conjugate & lysis buffer	5 µL IP1 d2	5 µL IP1 d2	
5 µL anti-IP1 Cryptate	5 µL anti-IP1 Cryptate	5 µL anti-IP1 Cryptate	

Incubate for 1h at RT.

Read on an HTRF® compatible reader.

HTRF signal is stable over a 24 hour period at RT.

7.2. Protocol (384-well low volume plate, 20 µL final volume)



Place lid on plate during each incubation. Remove the lid for readout

* See distribution sequence § 7.1.

8. Data reduction

Results are calculated from the 665nm / 620nm ratio and expressed in Delta F. An example of data reduction is given in the table below (readout on RUBYstar). Draw up the assay calibration curve by plotting delta F% versus IP1 concentration, as shown in the graph below.

	A (665nm)	B (620nm)	Ratio (1)	Mean ratio (2)	CV % (3)	Delta F % (4)
Negative control	7,819 7,494	162,857 152,444	480 492	486	1.7	
[calibrator] nM	[IP1] final in assay nM					
0	0	86,247 85,167	146,657 145,599	5,881 5,849	5,865	0.4
Positive control						1,107
43	21.5	78,544 79,910	141,045 143,411	5,569 5,572	5,570	0.0
						1,047
172	86	71,319 68,895	143,131 141,722	4,983 4,861	4,922	1.7
						913
687	344	55,228 49,412	146,684 145,606	3,765 3,394	3,579	7.3
						637
2,750	1,375	31,523 28,981	154,746 146,973	2,037 1,972	2,004	2.3
						313
11,000	5,500	15,085 15,573	146,038 154,836	1,033 1,006	1,019	1.9
						110
44,000 (Maximum calibrator)	22,000	10,085 10,418	146,194 146,692	690 710	700	2.1
						44
IP1 control		41,040 39,178	149,239 146,652	2,750 2,671	2,711	2.0
						458

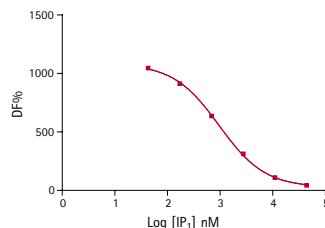
$$1. \text{ Ratio} = \frac{A_{665\text{nm}}}{B_{620\text{nm}}} \times 10^4$$

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

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

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

(Ratio_{neg} = negative control)



9. Assay characteristics

Specificity of the assay : the assay does not show any cross reaction with Myo-inositol, PIP2, IP2, IP3, IP4 or PIP3 up to a concentration of 50µM.

Analytical characteristics of the assay :

EC 50	S/B
500 nM (final concentration)	10

(Readout on RUBYstar reader, BMG LABTECH)

Plate readout may be carried out several times over a 24 hour period without altering the performances of the assay.

10. Assay miniaturization

When used as recommended, the kit provides sufficient reagents for 1,000 tests using a 384-well low volume plate in 20 µL final assay volume.

If other plate formats are used (96 half-well or 384-well), the cell density has to be optimized according to the surface of the well. The volume of each assay component must be proportionally adjusted in order to maintain the reagent concentrations as for the 20 µL final assay volume.

Assay components	Volume proportion	Format				
		96-well (100 µL)	384-well (40 µL)	384-well (20 µL)	384-well low volume (20 µL)	384-well low volume (10 µL)
Cells / well		80,000	30,000	15,000 to 30,000	8,000 to 15,000	8,000
Compound in IP1 stimulation buffer 1X	2 volumes	50 µL	20 µL	10 µL	10 µL	5 µL
conjugate & lysis buffer	1 volume	25 µL	10 µL	5 µL	5 µL	2.5 µL
anti-IP1 Cryptate	1 volume	25 µL	10 µL	5 µL	5 µL	2.5 µL
		200 tests	500 tests	1,000 tests	1,000 tests	2,000 tests

11. Cell density optimization – Agonist and antagonist dose responses

The choice of the plates and the cell density optimization are key steps when using the in IP-One kit. A wide range of cell densities must be assessed (see above table), in the presence or absence of agonist. The optimum cell density is the number of cells per well which leads to the highest signal amplitude between the IP1 basal state (non stimulated cells) and the level reached when cells are stimulated.

Figure 1 represents an example of a cell density optimization in a 96-well white TC plate (Greiner 655 083) on a CHO-M1 stable cell line stimulated with acetylcholine for 30 mins at 37°C. The optimum cell density was 80,000 cells per well and the EC50 of the acetylcholine was found to be 71 nM.

Figure 2 shows the dose response of an antagonist (atropine) obtained on a CHO-M1 stable line stimulated with acetylcholine (at EC80 dose). In this experiment, the IC50 of atropine was found to be 12.5 nM.

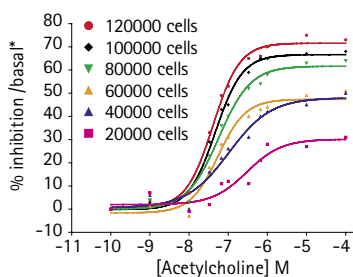


Figure 1

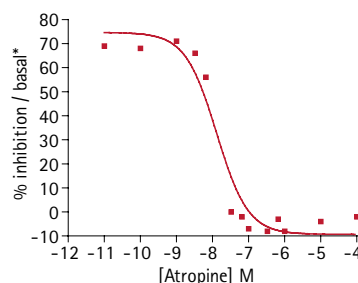


Figure 2

$$* \% \text{ inhibition / basal} = \left[1 - \frac{\text{DF (stimulated cells)}}{\text{DF (non stimulated cells)}} \right] \times 100$$