MANUAL

Technology: HTRF® GPCRs

IP-one ELISA

Part number	72IP1PEA	72IP1PED
Test size	96 wells	480 wells

Storage: 2-8°C

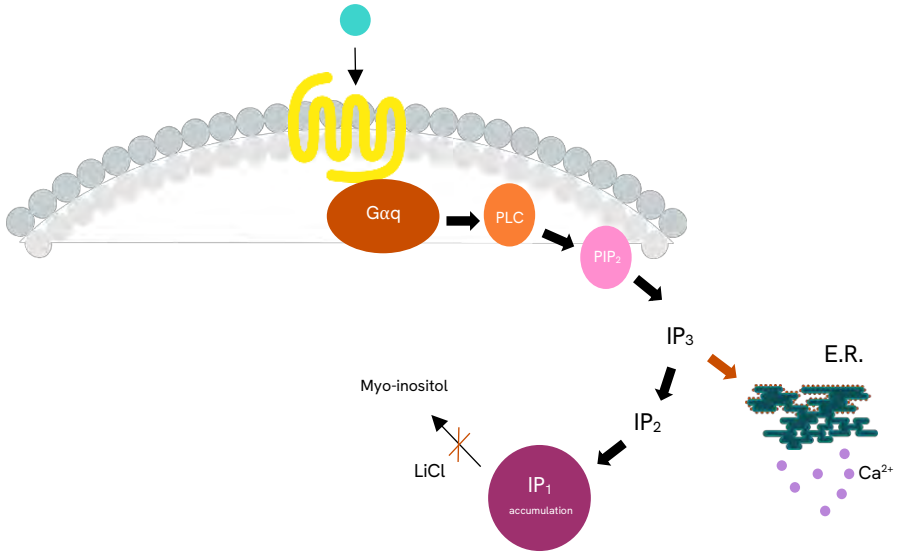
Version: 06 **Date:** February 2024

ABOUT IP1

The IP-One ELISA assay has been designed to monitor the activation of Phospholipase C (PLC) coupled receptors, which carry information within the cell.

Among these, the Gq coupled GPCRs represent the most important family of receptors which can activate the b subtype of the PLC family (1). Other receptor types, like protein tyrosine kinase receptors (2), antigen or immunoglobulin receptors (3) or collagen receptors (4), are known to activate another PLC subtype, PLC-g.

Whatever the receptor family, the activation triggers the release of D-myo-inositol 1,4,5 trisphosphate (IP3) results in in a transient increase of intracellular Ca^{2+}. The IP3 lifetime within the cell is very short (less than 30 sec) before it is transformed into IP2 and IP1. When LiCl is added to the culture medium, the degradation of IP1 into myo-inositol is inhibited, and IP1 can therefore accumulate in the cell. Then, after receptor activation, IP1 can be precisely quantified using the IP-One assay.

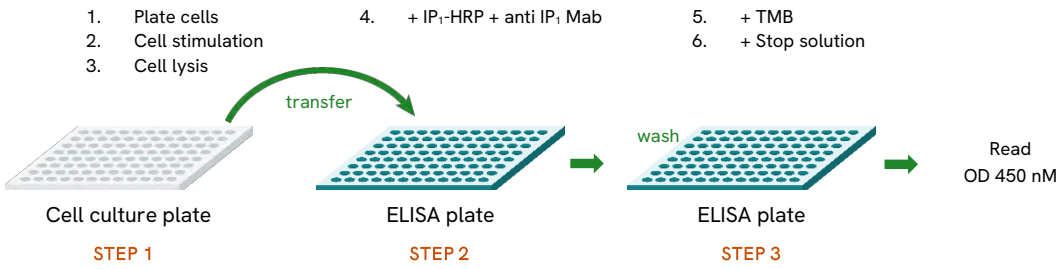


1. Thomsen, W., Frazer, J., and Unett, D. (2005) CurrOpin Biotechnol 16, 655-665
2. Berridge, M. J. (1993) Nature 361, 315-325
3. Rhee, S. G. (2001) Annu Rev Biochem 70, 281-312
4. Gibbins, J. M. (2004) J Cell Sci 117, 3415-3425

ABOUT THE ASSAY

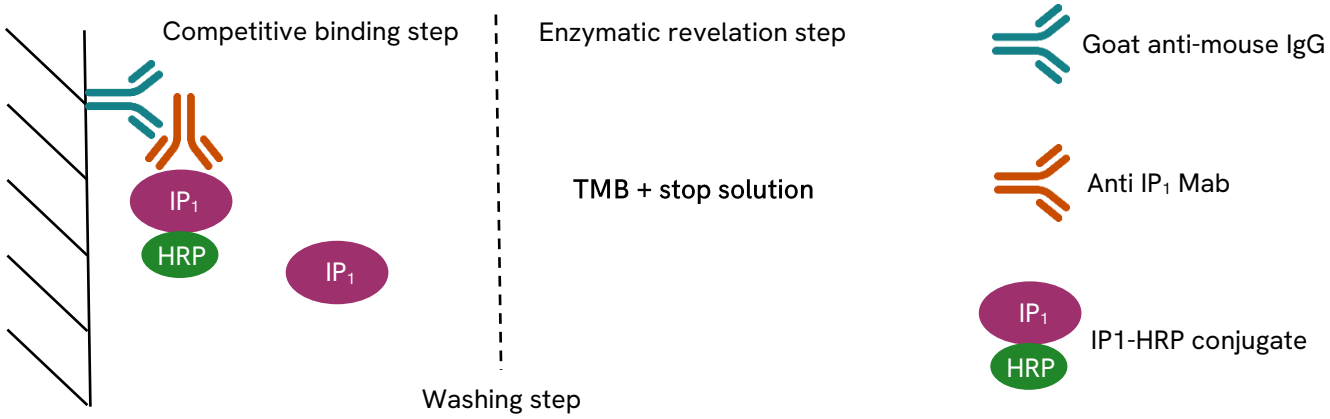
The IP-One ELISA Kit contains all the reagents necessary to measure the IP1 produced in the cell after GPCR activation.

a. Cell based assay



Step 1	
1	Cells are plated in appropriate cell culture plate (overnight incubation).
2	Cells are stimulated by a ligand or the drug of interest (1 hour of incubation).
3	Cells are lysed (30 min of incubation).
Step 2	
4	After transfer of the supernatant into the ELISA plate supplied with the kit, add IP1-HRP conjugate and anti-IP1 Mab (3 hours of incubation).
Step 3	
5	After a washing step, add TMB (from 20 to 30 min of incubation).
6	Add stop solution, and read at 450 nm with optical correction.

b. Elisa assay



The IP-One ELISA kit is a competitive immunoassay for the quantitative determination of D-myo-inositol 1 phosphate (IP1). This assay is based on the competition between free IP1 and IP1-HRP (Horse-Radish Peroxidase) conjugate for a limited number of binding sites on an anti-IP1 monoclonal antibody.

After a washing step, the revelation is carried out by the addition of HRP substrate TMB (3, 3′, 5, 5′-Tetramethylbenzidine). The reaction is stopped and the optical density (OD) is read at 450 nm with an optional correction between 610 nm et 650 nm (correction for optical imperfections in the plate).

KIT CONTENTS

a. Kit components

The kit contains all the components necessary to perform 96 tests.

Microplate (12 strips of 8 wells)	pre-coated with goat Anti-mouse IgG
Plate cover	2 adhesive strips
Zip bag	for storage of unused strips
IP1-HRP Conjugate	1 vial (2.8 mL)
Anti-IP1 Monoclonal antibody (Mab)	1 vial (lyophilised)
IP1 calibrator	1 vial (lyophilised)
Stimulation buffer 5X	1 vial (8 mL)
Diluent	1 vial (15 mL)
Concentrated wash solution (Tween 20)	1 vial (10 mL)
TMB	1 vial (15 mL)
Lysis reagent 20%	1 vial (2 mL)
Stop solution	1 vial (22 mL)

b. Storage and utilization

The kit must not be used beyond the expiry date indicated on the kit label. Do not mix or substitute reagents with those from other batches.

The kit has to be stored at 2-8°C. Bring all the reagents to room temperature for at least 30 minutes before opening. The reagents can be used immediately after reconstitution. Mix gently all the reagents before use.

c. Recommendation prior using Revvity kit

The stimulation buffer supplied with the kit has been validated on several GPCR models (see the composition below).

If you wish to use your own stimulation buffer:

- do not forget to add LiCl in order to prevent IP1 degradation (50 mM recommended)
- do not use phosphate salts which interfere in the IP1 assay

Stimulation buffer composition 1X: 10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM Glucose, 50 mM LiCl pH=7.4.

ADDITIONAL ITEMS REQUIRED

- Cell culture plate (96 or 24 wells) appropriate for adherent cells
- A plate reader capable of measuring absorbance at 450 nm with wavelength correction set between 610 nm and 650 nm
- Deionized or distilled water for all the dilutions
- Pipettes, pipette tips and multi-channel pipette
- A microplate shaker capable of maintaining a speed of 200+/- 50 rpm
- Plate washer
- Disposable bottles or tubes for diluting calibrator

SAFETY PREPARATION

Some components of this kit contain corrosive products (see symbol on the reagent label). Use with caution and wear suitable protection.

REAGENT PREPARATION

a. Working solutions

The working solutions are prepared following the instructions on next page:

Reagent	Preparation
IP1-HRP Conjugate Diluent TMB Stop solution	Ready-to-use solution
Monoclonal antibody (anti-IP1 MAb)	Add X mL diluent (see information on the vial)
IP1 calibrator (50 µM after reconstitution)	
Stimulation buffer 5X	Dilution 1/5 in water - e.g. 4 mL stimulation buffer + 16 mL water
Concentrated wash solution	Dilution 1/666 in water (e.g. 1 mL concentrated solution + 665 mL water) (Solution 1X : 0.05% Tween 20)
Lysis reagent 20%	Dilution with diluent (see cell assay) to: 1% (e.g. 0.2 mL lysis reagent + 3.8 mL diluent) 2.5% (e.g. 0.4 mL lysis reagent + 2.8 mL diluent)

b. Preparation of standard solutions

Reconstitute the calibrator with diluent (see indications on the label for the reconstitution volume). After reconstitution the calibrator is 50 µM IP1. Prepare the standard solutions C1 to C5 by successive dilutions of calibrator with diluent. Unused concentrated calibrator (50 µM) can be aliquoted and stored at –20°C (see next section).

Standard solution IP1	Preparation	Standard concentration	
		Working solution (nM)	Final in assay (nM)
C5	100 µL reconstituted calibrator + 400 µL diluent	10 000	5 000
C4	100 µL C5 + 500 µL diluent	1 666	833
C3	100 µL C4 + 300 µL diluent	416	208
C2	100 µL C3 + 300 µL diluent	104	52
C1	100 µL C2 + 200 µL diluent	34	17
C0	Diluent	0	0

c. Preparation of standard solutions

Microplate: Return unused strips to the foil pouch containing the dessicant pack and close it hermetically. Then, store this pouch in the zip bag supplied with the kit.

Reagents		Storage	Stability
Microplate (in zip bag) Diluent TMB		+ 4°C	1 month
Stop solution		+ 4°C	Until expiry date (see the label)
IP1 calibrator (50 µM) Anti-IP1 Mab	Reconstituted	-20°C	3 months
Stimulation buffer	Diluted (1X)	+ 4°C	1 day
	Concentrated (5X)		Until expiry date (see the label)
Lysis reagent	Diluted solution 1% or 2.5%	+ 4°C	Until expiry date (see the label)
	Concentrated 20%		
Concentrated wash solution (Tween 20)	Concentrated	+ 4°C	Until expiry date (see the label)
	Diluted (1X)		1 week

PROCEDURES

a. Cell based assay (example for adherent cells)

Day 1: dispense cells into cell culture plate

96 wells

Cell concentration recommended:
80,000 cells/well (200 µL)

24 wells

Cell concentration recommended:
400,000 cells/well (500 µL)

Overnight
37°C + 5% CO2

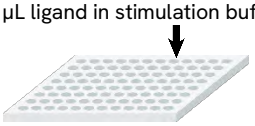
Day 2: remove cell supernatant

Invert the plate or aspirate the supernatant.

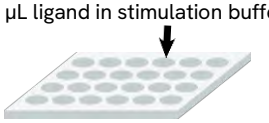
15 µL stimulation buffer or compound
15 µL ligand in stimulation buffer

Stimulation step

100 µL stimulation buffer or compound
100 µL ligand in stimulation buffer



At this stage do not leave the cells too long without buffer (see below for more information).

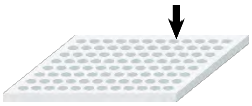


1 hour
(37°C + 5% CO_2)

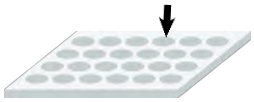
1 hour
(37°C + 5% CO_2)

Lysis step

30 µL lysis reagent 1%



50 µL lysis reagent 2.5%



30 min
(37°C + 5% CO_2)

30 min
(37°C + 5% CO_2)

Transfer into ELISA coated plate (50 µL/well)

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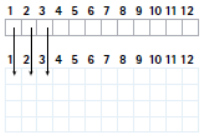
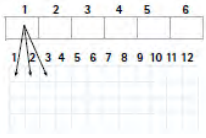




ELISA assay

ELISA assay

Some advices to ensure good results:

- To have a good cell response, it is very important to reduce the time between cell supernatant removal step and the stimulation step. To do this, you should add 15 µL (96 well plate) or 100 µL (24 well plate) of stimulation buffer very quickly before adding the ligand.
- It is necessary to check the basal level in your cell assay.

b. ELISA assay procedure

It is recommended that all samples and standards be assayed in duplicate except the blank and TA wells, which can be run in singlet.

It is important to add the different reagents in the order indicated on this table.

Bring all the reagents to room temperature for at least 30 minutes before opening (see page 4, "Reagentsstability after utilization").

NSB: Non Specific Binding

Blank: Substrate blank

TA: Total Activity

	A	B
1	C0	C0
2	C1	C1
3	C2	C2
4	C3	C3
5	C4	C4
6	C5	C5
7	NSB	NSB
8	Blank	TA

		Standard	Sample	NSB	Blank	TA
Solid phase and IP1 measurement	Standard solutions C0 to C5	50 µL	...	...	...	...
	Cell supernatant	...	50 µL	...	...	...
	IP1-HRP Conjugate	25 µL	25 µL	25 µL	...	...
	Anti-IP1 MAb	25 µL	25 µL	...	...	...
	Diluent	...	...	75 µL	...	...

Cover the plate and Incubate 3 hours RT under shaking (200 +/- 50 rpm)

Reagent excess elimination	Wash 6 times with wash solution 1x(250 µL / well)					
Enzymatic activity revelation	TMB	100 µL	100 µL	100 µL	100 µL	100 µL
	IP1-HRP Conjugate	...	...	...	...	5 µL

Incubate from 20 to 30 min RT in dark environment

Enzymatic signal stabilization	Stop solution	100 µL	100 µL	100 µL	100 µL	100 µL

Reading at 450 nm (with correction at 620 nm for example)

Reading must be done within 2 hours after stop reaction (dark environment).

RESULTS

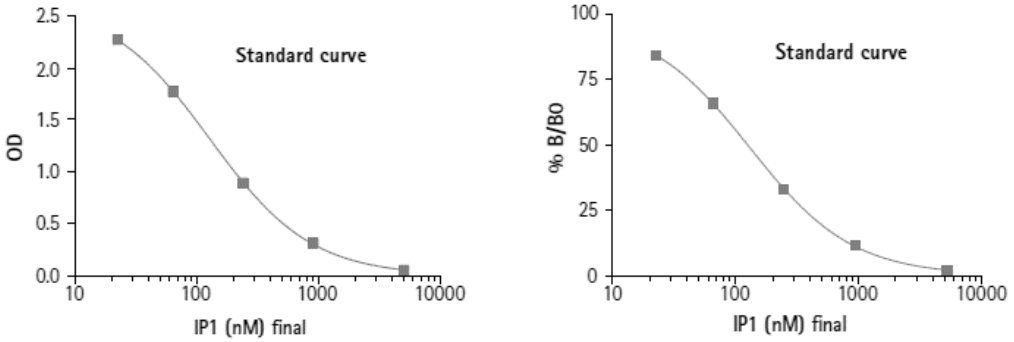
a. Calculation of results

- For each well subtract the OD at 620 nm from the OD at 450 nm
- Average the duplicate readings for each standard, sample and NSB
- Calculate the average net optical density for each standard and sample.

  Average net OD = Average bound OD – Average NSB OD
- Calculate % B/BO = divide the net OD for each standard and sample by standard CO net OD and multiply by 100

b. Data reduction (example)

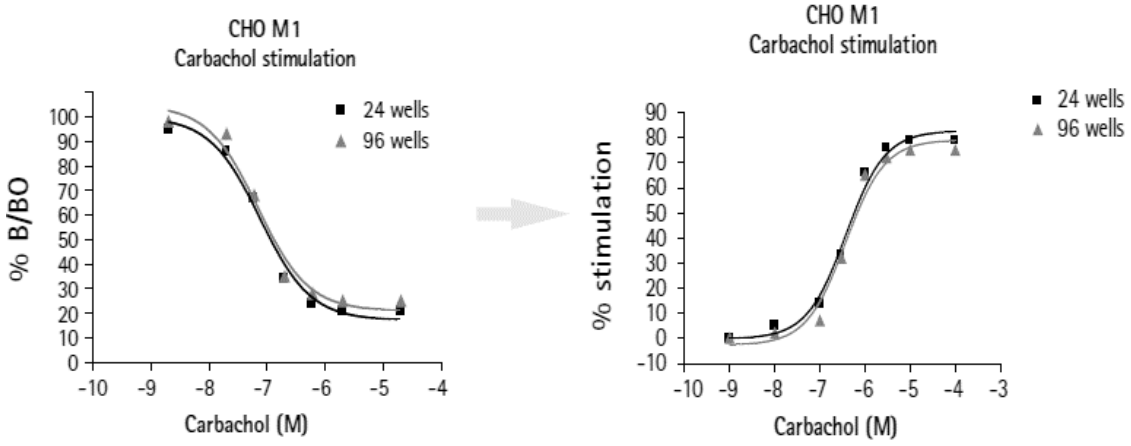
Standards Final concentration	450 nm	620 nm	450-620 nm	Average	Average net	CV	B/BO
CO 0 nM	2.690 2.718	0.041 0.041	2.649 2.677	2.663	2.654	0.7	100%
C1 17 nM	2.378 2.297	0.039 0.041	2.339 2.256	2.298	2.289	2.6	86%
C2 52 nM	1.850 1.814	0.039 0.041	1.811 1.773	1.792	1.783	1.5	67%
C3 208 nM	0.944 0.941	0.039 0.039	0.905 0.902	0.904	0.895	0.2	34%
C4 833 nM	0.349 0.369	0.041 0.040	0.308 0.329	0.319	0.310	4.7	12%
C5 5,000 nM	0.094 0.095	0.039 0.040	0.055 0.055	0.055	0.046	0.0	2%
NSB	0.049 0.049	0.039 0.041	0.010 0.008	0.009		15.7	
Blank	0.051	0.041	0.010				
TA	4.456	0.042	4.414				



c. Cell dose response

The figures below show the dose response obtained on a CHO-M1 stable line stimulated with Carbachol (comparison between cell culture in 96 wells and 24 wells) expressed either in % B/BO or % stimulation.

$$\% \text{ stimulation} = \left(1 - \frac{\text{OD (stimulated cells)}}{\text{OD (non stimulated cells)}}\right) \times 100$$



	24 wells	96 wells
EC_{50}	3.764e-007	3.711e-007

d. Assay characteristics

EC_{50} (final)	Sensitivity (final)
110 nM	10 nM

nM IP1 (final)	CV Intra-assay (n=19)	CV Inter-assay (n=18)
75	2.6%	6.6%
500	2.9%	8.6%
2 500	4.2%	7.0%

The assay does not show any cross-reactivity with myo-inositol, IP2, IP3, IP4, PIP2 and PIP3 for concentrations up to 50 µM.
*IP-One is covered by an international patent application

REACH European regulations and compliance
This product and/or some of its components include a Triton concentration of 0.1% or more and as such, it is concerned by the RE ACH European regulations. We recommend researchers using
this product to act in compliance with REACH and in particular: to only use the product for in vitro research in appropriate and controlled premises by qualified researchers, ii) to ensure the collection and the treatment of subsequent waste,
and iii) to make sure that the total amount of Triton handled does not exceed 1 ton per year.

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