Evaluation of the CisBio Cellul'erk HTRF® kit as a tool to investigate receptor pharmacology

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Introduction

- Conventional GPCR activity assays such as calcium flux, cAMP and GTPγs accumulation are routinely used in medium to high throughput formats to investigate receptor pharmacology. Lower throughput methods such as western blotting, immunoprecipitation and immunocytochemistry have traditionally been used to investigate pathways further downstream such as ERK phosphorylation.
- ERK phosphorylation can be activated by G-protein dependant and independent pathways as shown in the literature[1,2]. Hence, it is important to have an ERK phosphorylation assay and not solely rely on assays measuring upstream signalling pathways.
- We have evaluated the use of the CisBio Cellul'erk HTRF® kit as a tool to detect lysophosphatidic acid (LPA) mediated ERK1/2 phosphorylation via the LPA-1 receptor in primary human lung fibroblasts (HLFs) using a 384-well assay format.
- We also used phospho-ERK1/2 (pERK1/2) specific antibodies in an imaging assay format to further correlate results with the HTRF® kit as well as the calcium assay.

Methods and Materials

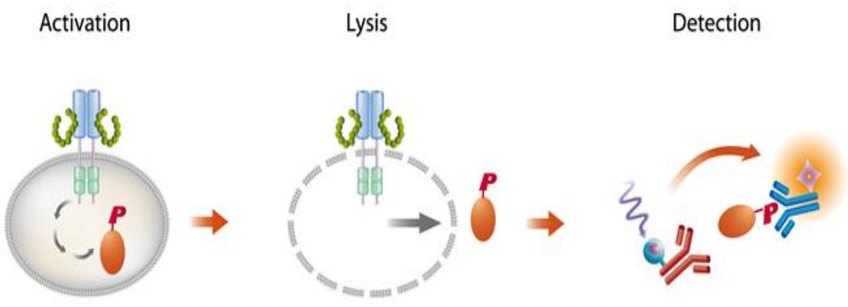
Calcium Fluo-4 Assay

Methods as described by manufacture's instructions. Briefly, HLFs (8,000 cells well^{-1}) were plated in 384-well black walled, clear bottom plates. Incubated for 24h at 37ºC/5% CO_2. After 24h, cells were washed and incubated with serum free media for 4-6h. Fluo-4 calcium dye was added, incubated for 45min and antagonist added. Plate further incubated for 30min. Agonist then added using the FDSS7000 (Hamamatsu) and plate read for 2min with 1s increments.

pERK Imaging Assay

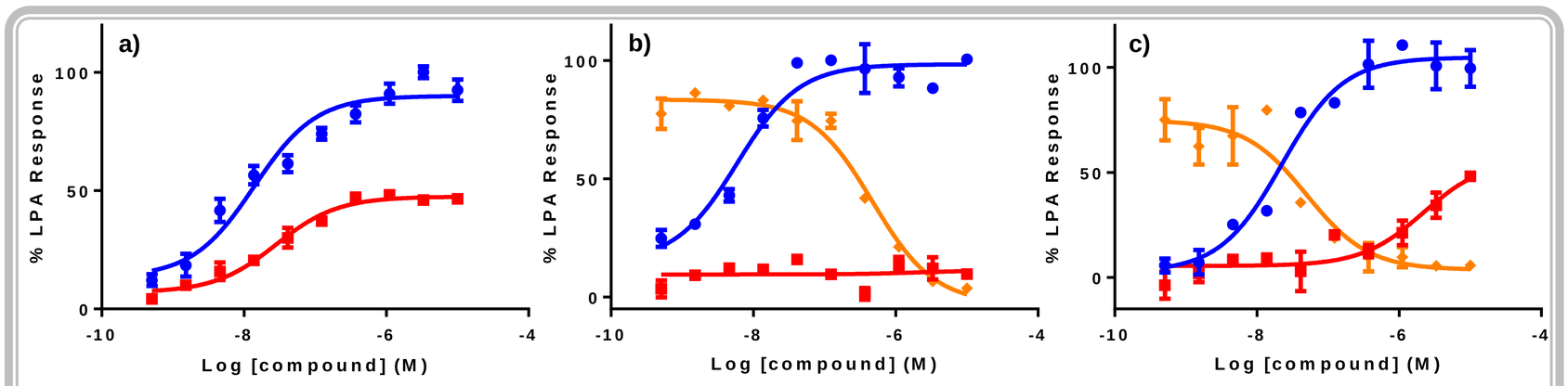
HLFs were plated at 3000 cells well^{-1} in 384-well black walled, clear bottom plates. Incubated for 24h at 37ºC/5% CO_2. After which, cells were washed and serum starved for 24h. Antagonist was added to the cells and incubated for 30min at 37ºC. Agonist was then added for 5min at 37ºC. Cells were then fixed with 4% formaldehyde for 15min. Cells washed and incubated with permeabilising block (20% FBS + 0.2% Tween-20 + PBS) for 2h. pERK1/2 primary antibody (Cell Signalling technology, #4370) was added and plate incubated at 4ºC overnight. Primary antibody washed off and Alexa Fluor® 488 secondary (Invitrogen# A11008) was added along with nuclear dye (Hoescht) for 1h at 37ºC. Secondary antibody washed and plate imaged using the InCell 2000 (GE Healthcare) using DAPI filter (x0.1ms) and FITC filter (x1.3ms) for pERK1/2 staining. Images analysed for cell intensity using the IN Cell Analyser software.

Cellul'erk HTRF® Assay



Methods as described by manufacturer's instructions. HLFs were plated at 8,000 cells well^{-1} in a 384-well culture plate and incubated overnight at 37ºC/5% CO_2. Cells were then washed with serum free media and starved for 24h. Antagonist was added to the cells and incubated for 30min at 37ºC. Agonist was then added for 5min at 37ºC. Cell supernatant was carefully removed and supplemented lysis buffer was added. Plate was further incubated for 25min at RT with shaking. Cell lysate was transferred to a small volume 384-well plate. HTRF® conjugates were added and plate incubated for 2h at RT with shaking. Plate was then read on the EnVision (Perkin Elmer).

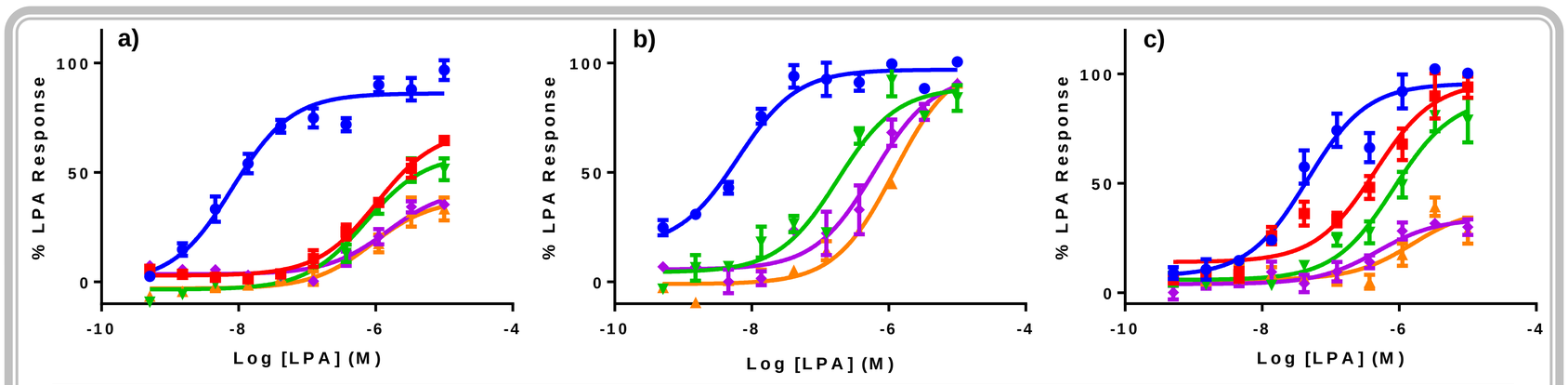
Results



	● LPA (-PTX)	■ LPA (+PTX)	◆ U0126
(a) Calcium Assay	14.61nM	29.48nM	N/A
(b) Cellul'erk HTRF® Assay	6.48nM	>10µM	472.07nM
(c) pERK Imaging Assay	19.46nM	232.30nM	51.69nM

Figure 1 and Table 1. Concentration response to LPA +/- 100ng/ml Pertussis Toxin (PTX), Inhibition profile of U0126 (ERK1/2 specific inhibitor) in the pERK assay formats. PTX treatment was carried out during the serum starvation step. An EC_{80} concentration of LPA was used against U0126 in the pERK assays. EC_{50} & IC_{50} values calculated appropriately.

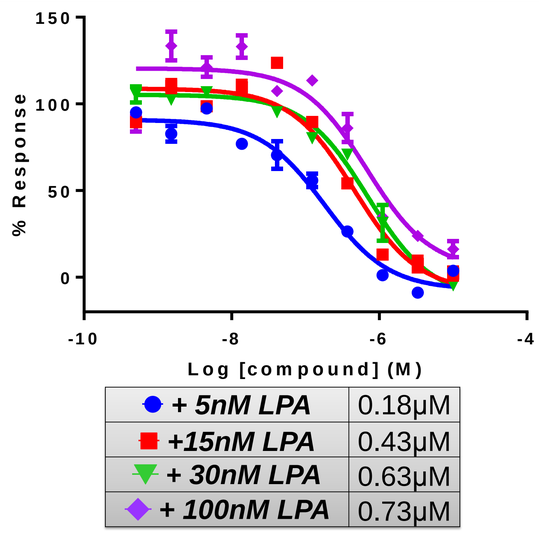
- Effect of PTX treatment shows that the LPA response is $G_{\alpha i}$ dependant to varying degrees in the different assays.
- U0126 displays a full inhibition profile in both pERK1/2 assay formats.



	● Control	■ + 30nM QBV	▼ + 100nM QBV	◆ + 500nM QBV	▲ + 1µM QBV
(a) Calcium Assay	8.14nM	0.71µM	0.88µM	1.04µM	1.48µM
(b) Cellul'erk HTRF® Assay	6.26nM	Not Determined	0.18µM	0.60µM	1.20µM
(c) pERK Imaging Assay	39.09nM	0.37µM	0.48µM	0.83µM	1.57µM

Figure 2 and Table 2. Concentration response curves of LPA in the presence of varying concentrations of the LPA-1 antagonist NVP-QBV971-NX-3 [(R)-2-(4'-(4-(((1-(2-chlorophenyl)ethoxy)carbonyl)amino)-3-methylisoxazol-5-yl)-[1,1'-biphenyl]-4-yl)acetic acid]. EC_{50} values are calculated and presented in Table 2.

- The calcium response mediated by LPA-1 becomes less potent and efficacious with increasing concentrations of the LPA-1 antagonist.
- In the Cellul'erk HTRF® Assay, a rightward shift in LPA potency is observed with increasing concentrations of the antagonist. A Schild Plot of this data (not shown) gives a pA_2 value of 726pM.
- The pERK1/2 imaging assay data appears to mimic the Cellul'erk ® HTRF assay data at low concentrations of the antagonist. At higher concentrations of the antagonist a decrease in the maximal response is observed similar to what is seen in the calcium assay.



● *+ 5nM LPA*	0.18µM
■ *+15nM LPA*	0.43µM
▼ *+ 30nM LPA*	0.63µM
◆ *+ 100nM LPA*	0.73µM

Figure 3 and Table 3. Concentration response curves of the LPA-1 antagonist NVP-QBV971-NX-3 in the presence of varying LPA concentrations in the Cellul'erk HTRF® Assay. IC_{50} values are calculated and presented in Table 3.

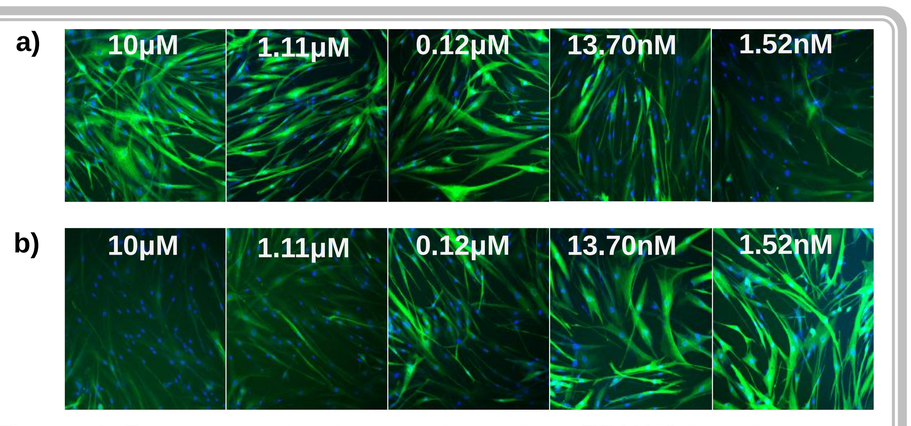


Figure 4. Representative images from the pERK1/2 imaging assay. An Alexa Fluor® 488 secondary antibody (Green) is used against the primary pERK1/2 antibody. Nuclei are stained with Hoescht (Blue). pERK1/2 activation can be seen as function of cell intensity. a) A concentration dependant pERK1/2 activation response can be seen when cells are stimulated with varying LPA concentrations. b) A concentration dependant inhibition of the pERK1/2 response can be seen when cells are treated with varying concentrations of the LPA-1 receptor antagonist NVP-QBV971-NX-3 (challenged with 30nM of LPA).

Discussion

- We have validated the Cellul'erk HTRF® assay in an LPA-1 receptor mediated pathway using primary human lung fibroblasts.
- We have compared the data from the Cellul'erk HTRF® assay with the commonly used calcium assay. Data between the two functional assays correlated well and variances may be attributed to the different signalling pathways. The results also highlight that ERK1/2 phosphorylation could be due to a different pathway, independent of $G_{\alpha i}$ signalling.
- While the pERK1/2 imaging assay has the advantage of being able to visualise the activity, the Cellul'erk HTRF® assay proved to be more sensitive and robust at picking up ERK1/2 phosphorylation.
- We conclude that the Cellul'erk HTRF® assay is an attractive option for a highly sensitive, fast and medium throughput format to detect ERK1/2 phosphorylation and could potentially be used as a front line assay for drug discovery.

References

1. ROSETHORNE, E. M. & CHARLTON, S. J. (2011) Agonist-biased signaling at the histamine H4 receptor: JNJ7777120 recruits beta-arrestin without activating G proteins. Mol.Pharmacol., 79, (4) 749-757.
2. LEFKOWITZ, R. J. & SHENOY, S. K. (2005) Transduction of receptor signals by beta-arrestins. Science, 308, (5721) 512-517.