APPLICATION NOTE

Sensitive detection of GPCR-Mediated ERK1/2 phosphorylation in various cellular models with the HTRF advanced Phospho-ERK1/2 kit.

This application note demonstrates how the Advanced Phospho-ERK1/2 (Thr202/ Tyr204) kit is suitable for ERK signaling pathway investigations mediated by GPCR stimulation with pharmacological compounds.

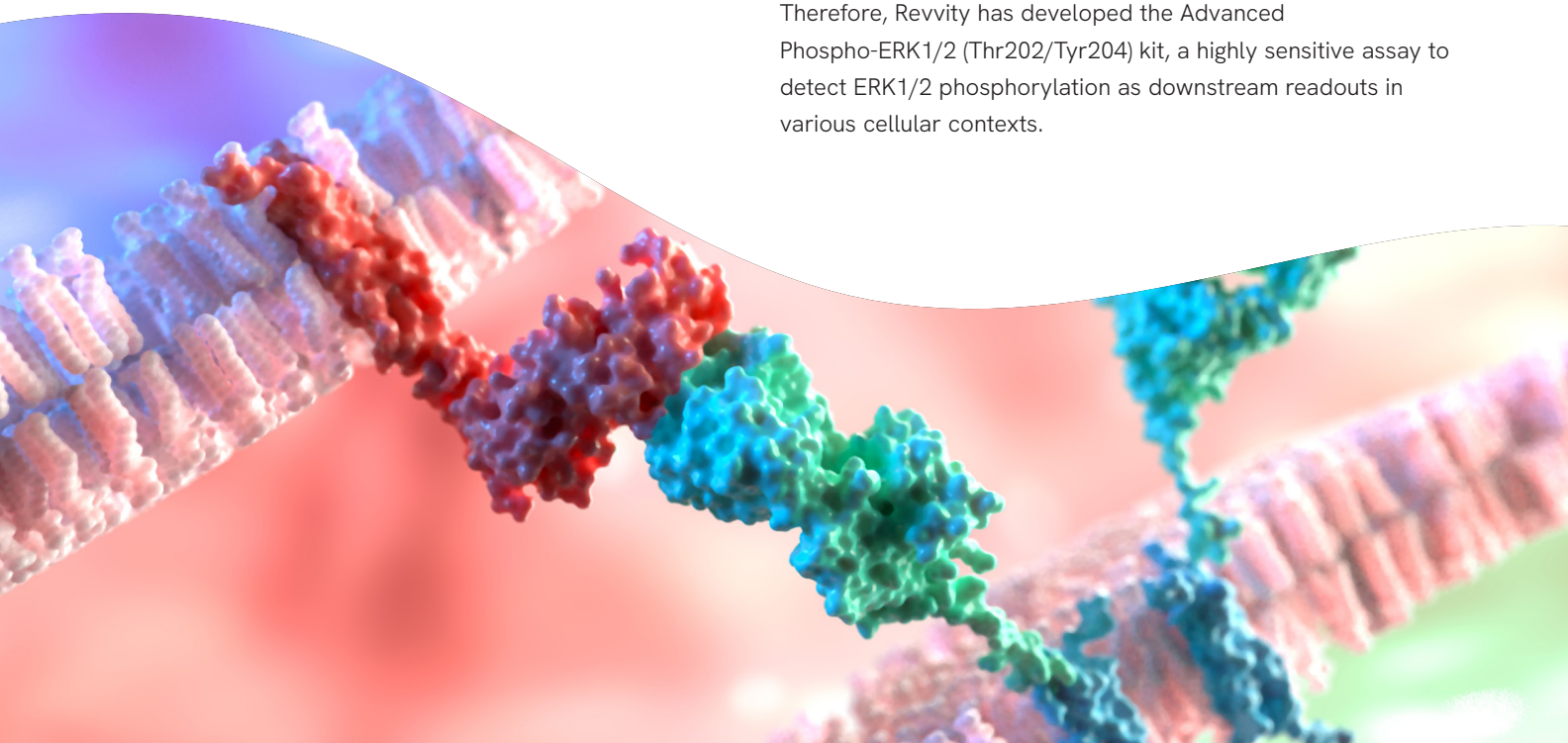
Abstract

Revvity offers a comprehensive line of HTRF® assays to study GPCR signaling pathways. These include GPCR second messenger assays for cAMP and IP1 investigations as well as kits for phospho-protein assays for downstream readouts. This application note shows the ability of the Advanced Phospho-ERK1/2 (Thr202/Tyr204) kit to detect ERK1/2 phosphorylation in different cellular models. The data demonstrate that the Advanced Phospho-ERK1/2 assay provides very sensitive detection of ERK1/2 phosphorylation mediated by G-protein coupled receptor (GPCR) activation in both generic and physiological cellular models with overexpressed and endogenous GPCRs.

Introduction

G protein-coupled receptors (GPCRs) are seven-transmembrane domain receptors. GPCRs represent a high percentage of investigational drug targets because of their involvement in many diseases. GPCRs have two principal signal transduction pathways: the cyclic AMP (cAMP) and the phosphatidylinositol (IP).

Revvity offers cAMP and IP-One HTRF kits as assays to measure second messenger accumulations as upstream readouts of reference in biochemical and cell-based assays. However, it is well-known that effectors other than cAMP and IP are involved in the GPCR signaling pathways. Also, several receptors are not optimally coupled through the cAMP or IP pathways. Therefore, Revvity has developed the Advanced Phospho-ERK1/2 (Thr202/Tyr204) kit, a highly sensitive assay to detect ERK1/2 phosphorylation as downstream readouts in various cellular contexts.



The HTRF Advanced Phospho-ERK1/2 kit is a versatile tool to detect cellular ERK1/2 phosphorylation mediated by GPCR activation. The miniaturized assay format and the robust signal amplitude are tailored for both research and high-throughput screening. The assay provides a powerful link between upstream (cAMP, IP) and downstream (P-ERK) GPCR signaling pathway readouts, allowing the use of HTRF technology as a universal sample-saving technology to characterize the effects of pharmacological compounds on GPCRs.

The aim of this study was to demonstrate that Advanced Phospho-ERK1/2 (Thr202/Tyr204) kit is suitable to detect ERK phosphorylation (P-ERK) mediated by overexpressed or endogenous GPCRs in various cellular contexts (from stable cell lines to tumor patient-derived cells).

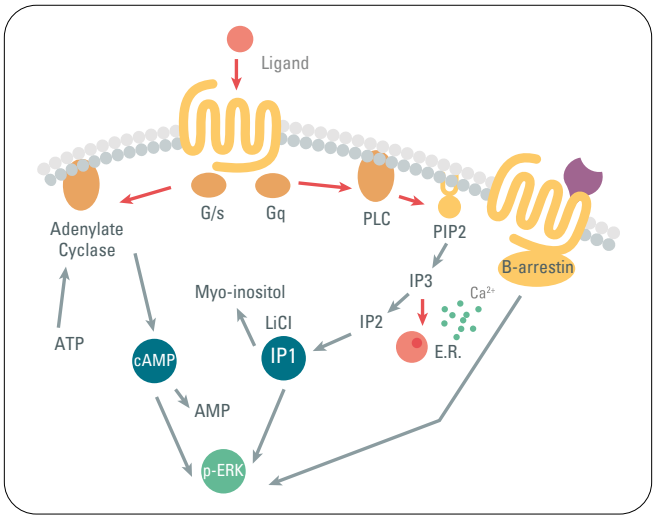


Figure 1: Main readouts for GPCR signaling pathway investigations including P-ERK.including P-ERK.

Materials and methods

Assays using Revvity's Advanced Phospho-ERK1/2 kit were performed using selected cell lines in order to evaluate the assay's ability to detect GPCR-mediated ERK1/2 phosphorylation.

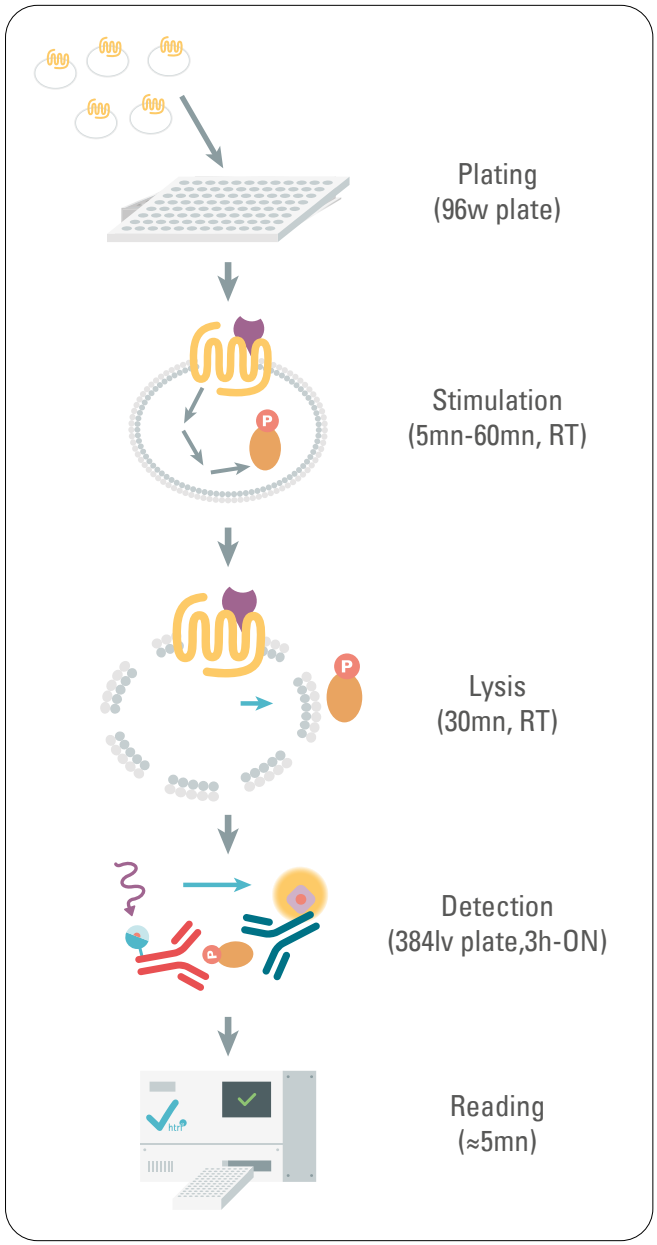


Figure 2: Advanced Phospho-ERK1/2 (Thr202/Tyr204) kit assay principle.

As described below, we compared the detection of ERK1/2 protein phosphorylation in conditions where GPCRs are overexpressed or at endogenous levels. To accomplish this, Advanced Phospho-ERK1/2 assays were performed using stable cell lines, immortalized cell lines, cancer cells and a tumor patient-derived cell type stimulated with appropriate agonists to evaluate receptor-mediated downstream ERK1/2 phosphorylation. Details of cell lines used and receptor types are included in the Results section of this application note.

* Revvity's Advanced ERK phospho-T202/Y204 kits [64AERPEF (200 tests), 64AERPEG (500 tests), 64AERPEH (10,000 tests)] were used in these studies.

Growth and optimization of cells

All cell lines used in this series of experiments were adherent cells. Cells were plated in culture medium the day before experiments in white 96-well clear bottom plates then incubated at 37°C for 24 hours.

Several experiments were done to optimize cell density and serum starvation conditions (not shown here). Cells were stimulated with commercial pharmacological compounds of reference. Time and temperature for stimulations were also optimized. The Advanced Phospho-ERK1/2 assays were performed under these optimized conditions.

Preparation of lysates

The lysis step was done with HTRF lysis buffer supplemented with HTRF blocking reagent, which are provided in the Advanced Phospho-ERK1/2 kit.

Advanced Phospho-ERK1/2 assays

16 µL of lysates were dispensed in triplicates into a small volume white 384-well plate. Then 2 µL of d2 (acceptor) and 2 µL of Eu3+-Cryptate (donor) antibodies provided in the Advanced Phospho-ERK1/2 (Thr202/Tyr204) kit were added. A negative control was included in each assay by replacing the lysate with supplemented HTRF lysis buffer.

After overnight incubation at room temperature, the HTRF signal was recorded on a PheraSTAR FS reader (BMG Labtech) with flash lamp excitation.

Data handling and analysis

Learn more about HTRF data reduction at www.cisbio.com/htrf-ratio-and-data-reduction.

Results

The detection of phospho-ERK with the Advanced Phospho-ERK1/2 kit mediated by GPCR stimulation is shown using:

- Engineered stable cell lines overexpressing SNAP-tagged GPCRs (HEK293, HEK293A and CHO-K1 cells)
- Immortalized cells (HEK293A) expressing endogenous GPCRs
- Cancer cells, isolated from a primary tumor of human colorectal adenocarcinoma expressing endogenous GPCRs
- Tumor patient-derived cells, isolated from hepatic metastases of human colorectal cancer expressing endogenous GPCRs

Various GPCR families were tested as models: chemokine receptors (ex CCR1), proteinase activated receptors (ex PAR1), VIP receptors (ex VPAC1), opioid receptors (ex µopioid), neurotensin receptors (ex NTS2), lysophospholipid receptors (ex S1P1) and ghrelin receptor (GHSR1A).

As shown in Fig. 3, EKR1/2 protein phosphorylation is readily detected using the Advanced P-ERK1/2 assay for all conditions tested. The EC50* was in good correlation with affinity values from the literature. S/B calculated were robust enough to screen compounds.

*Note: Slight variations of EC50 values have been detected depending on cell background and expression of GPCRs (overexpressed vs endogenous).

Discussion

Revvity's Advanced Phospho-ERK1/2 (Thr202/Tyr204) kit is an easy "one mix-and-read" assay that provides a very sensitive technique to detect ERK1/2 phosphorylation mediated by overexpressed or endogenous GPCR activation in various cell types This study illustrates that P-ERK is a reliable downstream readout of GPCR signaling pathways.

As shown in this application note, the assay readily detects EKR1/2 protein phosphorylation mediated by GPCR stimulation with agonist compounds not only in stable cell lines over-expressing GPCRs but also in more physiological cellular models. It is a reliable method delivering accurate and reproducible results, allowing the determination of pharmacological parameters such as EC50 values.

Conclusions

This application note demonstrates that Revvity's Advanced Phospho-ERK1/2 (Thr202/Tyr204) kit is suitable for ERK signaling pathway investigations mediated by GPCR stimulation with pharmacological compounds. The kit's high sensitivity enables detection of ERK phosphorylation in cell lines of varying complexity. As the data shows, the kit is suitable for investigations using cellular models with both overexpressed and endogenous GPCRs. Additionally, the miniaturized assay format combined with a robust S/B make the kit an optimal choice for a both research and high throughput screening.

In summary, the Advanced Phospho-ERK1/2 (Thr202/Tyr204) kit is an excellent and versatile tool for studying the downstream effect of pharmacological compounds on GPCRs.

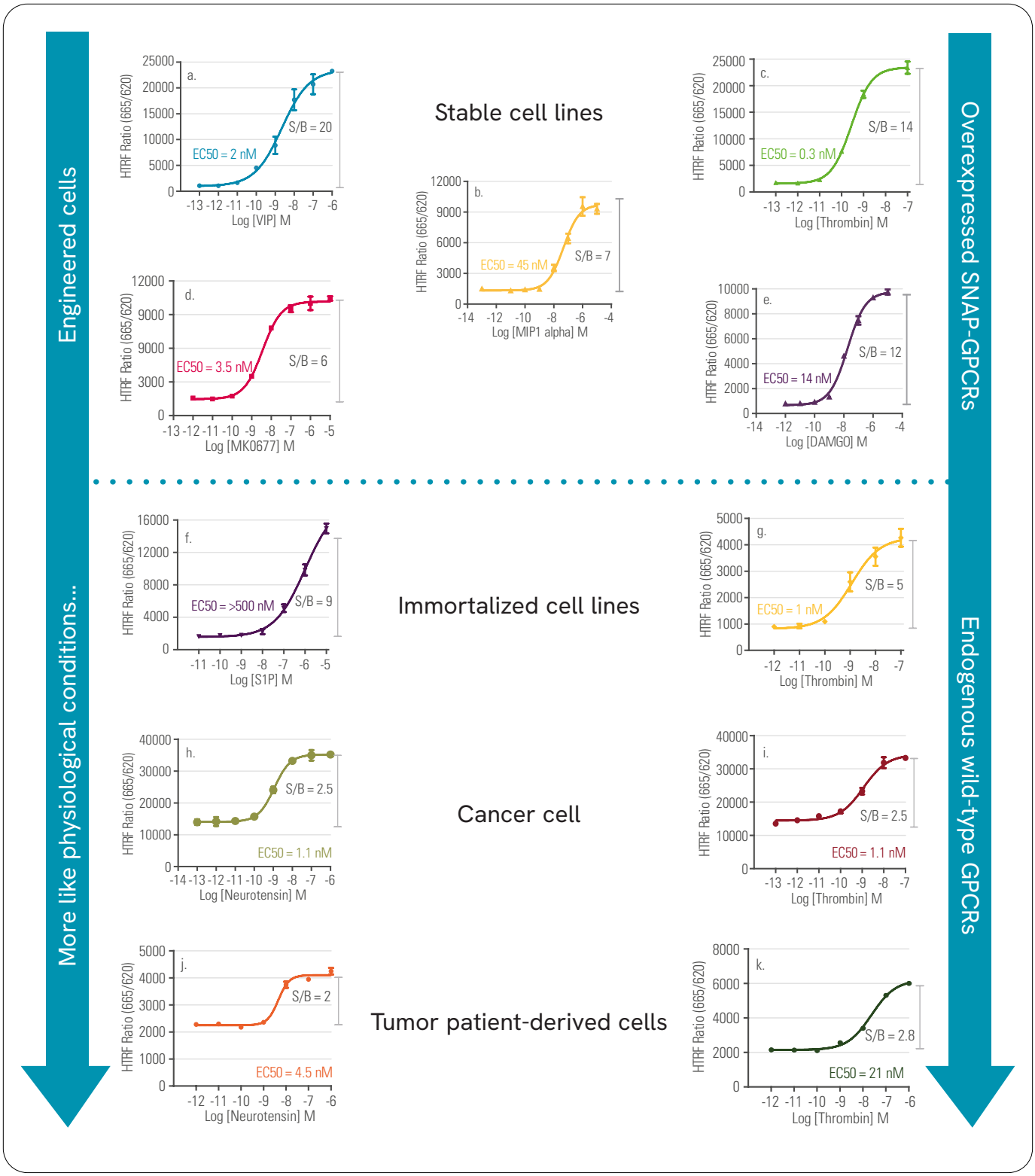


Figure 3: Detection of ERK1/2 protein phosphorylation mediated by agonist-stimulated overexpressed and endogenous GPCRs with Advanced Phospho-ERK1/2 (Thr202/Tyr204) kit. **a.** Stimulation of SNAP-VPAC1 in CHO-K1 cells with VIP. **b.** Stimulation of SNAP-CCR1 in HEK293 cells with MIP-1 alpha. **c.** Stimulation of SNAP-PAR1 in HEK293A cells with thrombin. **d.** Stimulation of SNAP-GHSR1A in HEK293 cells with MK0677. **e.** Stimulation of SNAP-μ Opioïd in CHO-K1 cells with DAMGO. **f.** Stimulation of lysophospholipid S1P receptors with S1P in HEK293A cells. **g.** Stimulation of proteinase activated receptors with thrombin in HEK293A cells. **h.** Stimulation of neurotensin receptors with neurotensin in HT29 cells. **i.** Stimulation of proteinase activated receptors with thrombin in HT29 cells. **j.** Stimulation of neurotensin receptors with neurotensin in tumor patient-derived cells. **k.** Stimulation of proteinase activated receptors with thrombin in tumor patient-derived cells.

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