Key success tips to study GPCR signaling using the HTRF advanced Phospho-ERK1/2 kit.

This technical note provides valuable tips for performing Advanced phospho-ERK1/2 assays on adherent cell lines expressing GPCRs and will ensure optimal results.

Abstract

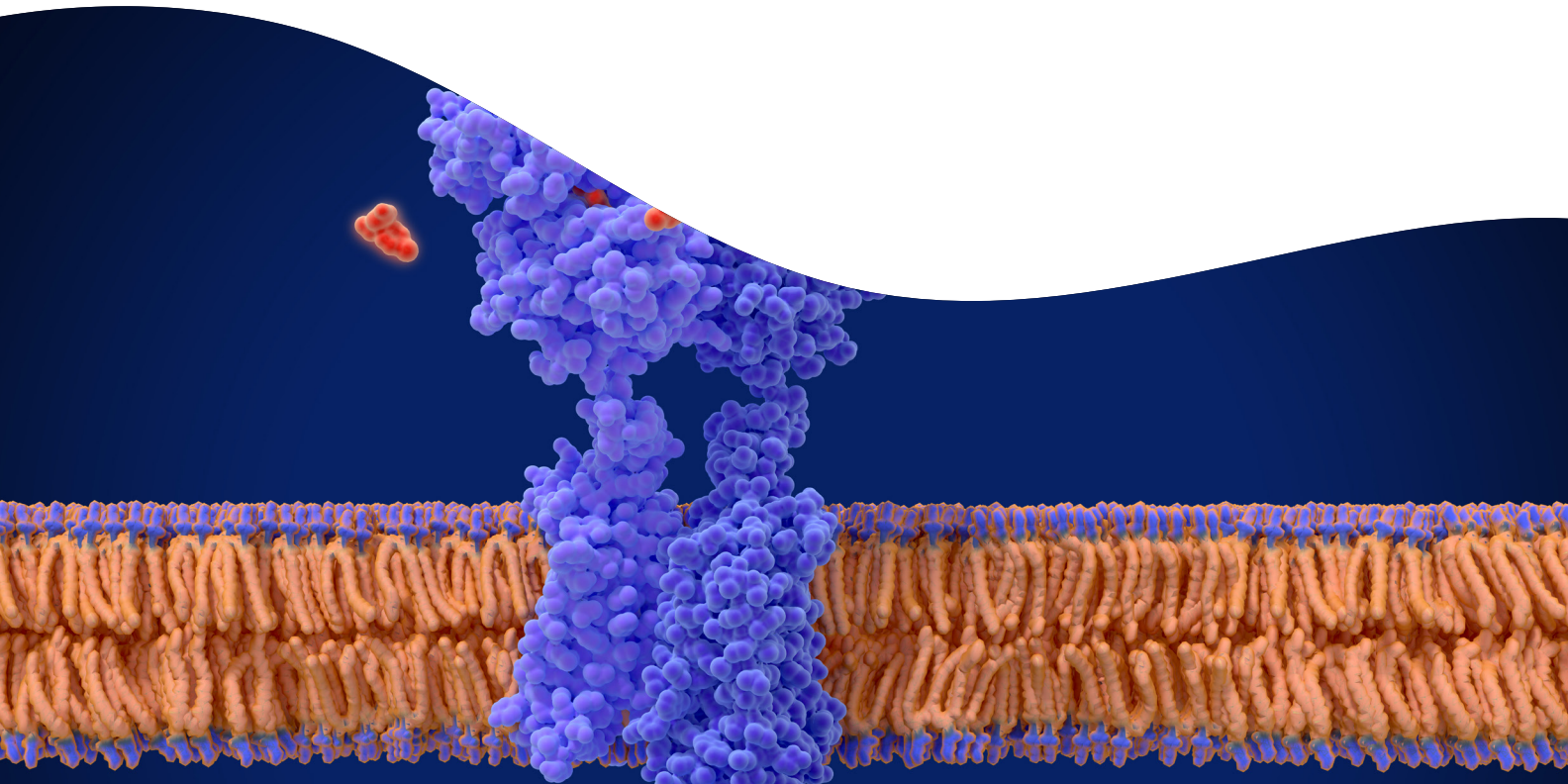
The HTRF® Advanced phospho-ERK1/2 kit is a highly sensitive and reliable tool for detecting ERK1/2 phosphorylation mediated by endogenous or overexpressed GPCRs in various cellular models. This technical note provides valuable tips for performing Advanced phospho-ERK1/2 assays on adherent cell lines expressing GPCRs and will ensure optimal results.

Introduction

Revvity's advanced phospho-ERK1/2 kit* is easy to use following the directions for assay and data analysis in the product insert. However, a deeper understanding of how to optimize various steps in the process and additional explanations of key technical information will help you obtain the best possible assay outcomes.

In the experiments that follow numerous conditions, from the preparation of working cells through cell lysis, were tested. The data demonstrate how optimizing conditions in these steps can ensure the best possible results.

*Advanced phospho-ERK1/2 (Thr202/Tyr204) kits : [64AERPEF (200 tests), 64AERPEG (500 tests), 64AERPEH (10K tests)].



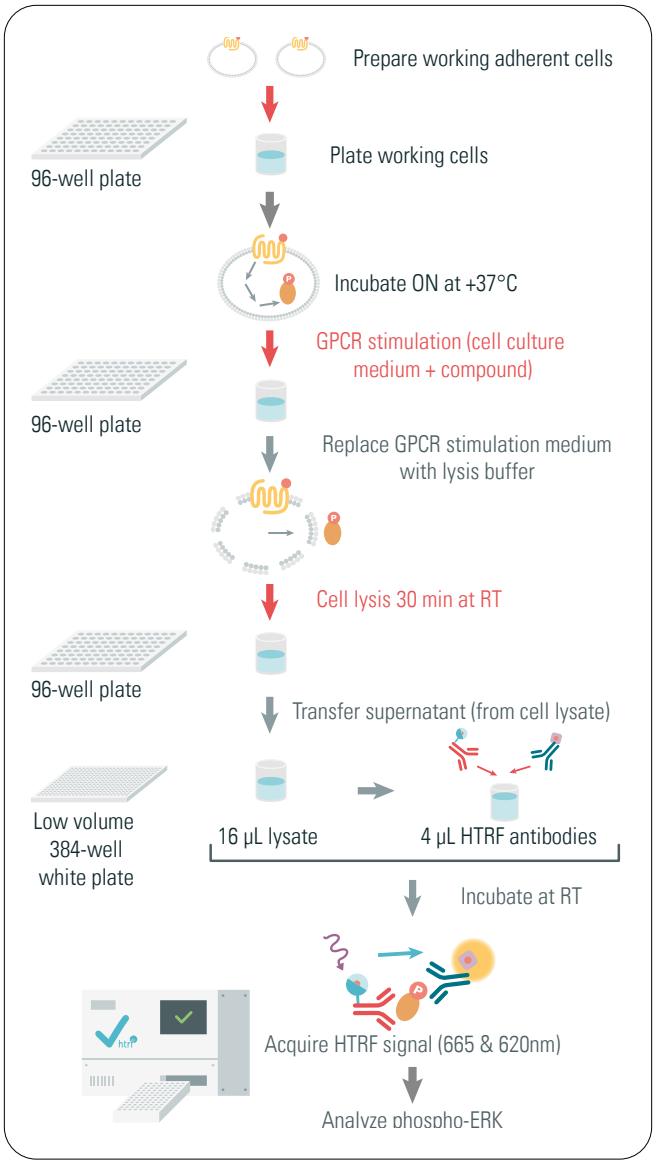


Figure 1: Process flow for detecting GPCR-mediated ERK1/2 phosphorylation.

Optimize preparation of working cells

TIP: Optimize cell density for the best signal amplitude

Evaluate cell densities from 1.5K to 100K cells/w to find the optimal condition to detect the best signal amplitude (Fig. 2).

A decrease in signal amplitude may be observed because of the hook effect. (See Fig. 2, blue bar.) This simply means that at least one of the detection reagents is depleted relative to the amount of phospho-ERK to be detected.

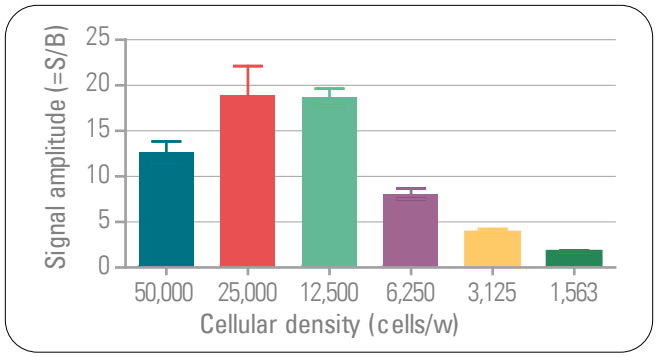


Figure 2: Detection of p-ERK with Advanced p-ERK1/2 kit using a range of cellular densities per well. A cell density of 50K cells/w produces the hook effect. 12.5K cells/w condition results in an optimal signal. A slightly lower cell density may be used if cells need to be saved.

TIP: Optimize the presence of serum in the cell culture medium

Evaluate several serum starvation conditions from no serum starvation to overnight serum starvation.

Most cells require the presence of serum in the cell culture medium for attachment and growth in microplates. The presence or overly-long absence of serum in the cell culture medium during the plating and stimulation step may considerably alter GPCR-mediated p-ERK detection in response to compounds. In the case shown in Fig. 3, both no serum starvation and overnight serum starvation conditions lead to a low signal amplitude due to a strong basal activity (see green and blue curves) resulting in incorrect pharmacological parameters (EC50). Most of the time starvation steps of about 1 to 2 hours produce the best results.

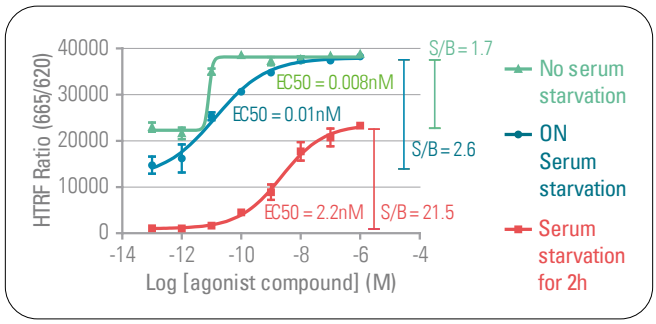


Figure 3: Detection of p-ERK with Advanced phospho-ERK1/2 kit using various cell plating conditions. In this experiment, a 2-hour serum starvation step (red curve) led to the best phospho-ERK detection (S/B and EC50).

TIP: Distribute compounds in the presence of cell culture medium

Do not remove cell culture medium from the working plates prior to distributing compounds on cells.

Medium removal during this step has a deleterious effect on phospho-ERK detection because of the stress caused to cells. In the absence of medium, a high basal activity essentially masks detection of any induced pharmacological dose response. When compound is added in the presence of medium, basal activity is low and a response can be detected. (See figure 4.)

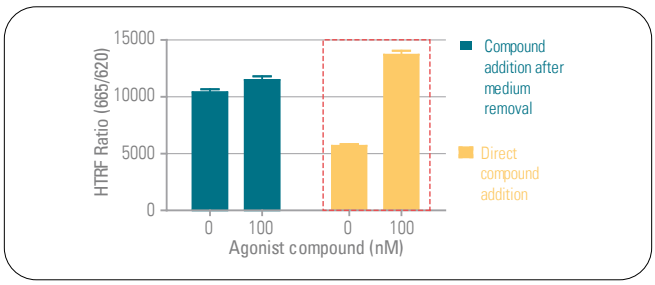


Figure 4: Detection of p-ERK with Advanced phospho-ERK1/2 kit. Comparison of pharmacological compound addition directly (orange bars) or after removal of medium (blue bars). Results show that direct compound addition in the presence of cell medium is mandatory to correctly detect dose response.

TIP: Optimize stimulation time

Evaluate stimulation times from 2 to 30 minutes to find the time that results in the best signal amplitude.

In general, it is a good practice to optimize stimulation time for other membrane receptor assays as well, such as tyrosine kinase receptors. You can also perform another optimization experiment by comparing incubation at room temperature and +37°C for various times during the stimulation step (data not shown).

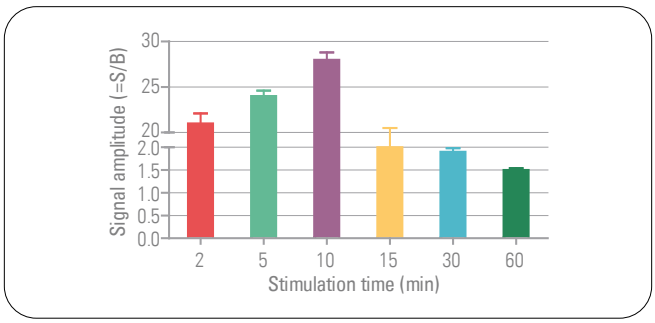


Figure 5: Detection of p-ERK with advanced Phospho ERK1/2 kit after stimulation for various times. Results shows that signal amplitude may be decreased by stimulating cells for too short (pink & green bars) or too long a time (yellow & brown bars).

Timing counts for the lysis step

TIP: Add lysis buffer shortly after removal of GPCR stimulation medium

A delay in the addition of lysis buffer after removing the GPCR stimulation medium can affect pharmacological compound dose response.

In this experiment, lysis buffer was added immediately (0=ref) or 5, 10 or 30 minutes after the removal of the GPCR stimulation buffer. When the addition of lysis buffer was delayed, cells' medium-free condition had a detrimental effect on the amount of ERK phosphorylation detected.

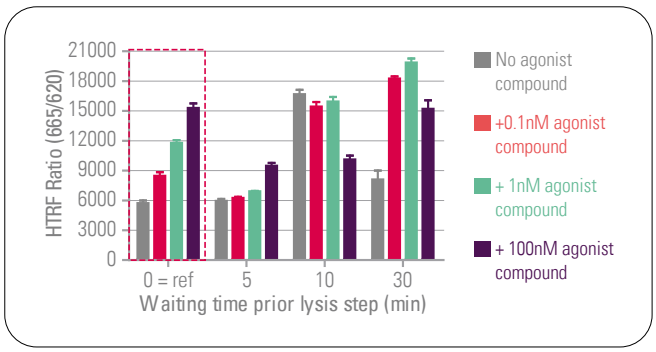


Figure 6: Effect of waiting time after stimulation buffer removal, prior to lysis step, with advanced phospho-ERK1/2 kit. Adding the lysis buffer immediately after medium removal is mandatory.

Conclusion

By carefully following the directions in the product insert and applying the tips in this technical note, you will obtain the best possible results with Revvity's HTRF Advanced phospho-ERK1/2 kit. While the focus here was on adherent cells expressing GPCRs, cells in suspension may be used by optimizing the protocol. Since optimal assay conditions are cell, GPCR and pharmacological compound dependent, it is important to perform these optimization steps for each new experiment.

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