HTRF Alpha-Tubulin Housekeeping kit: An indispensable complement to cell signaling assays to properly interpret compound effect.

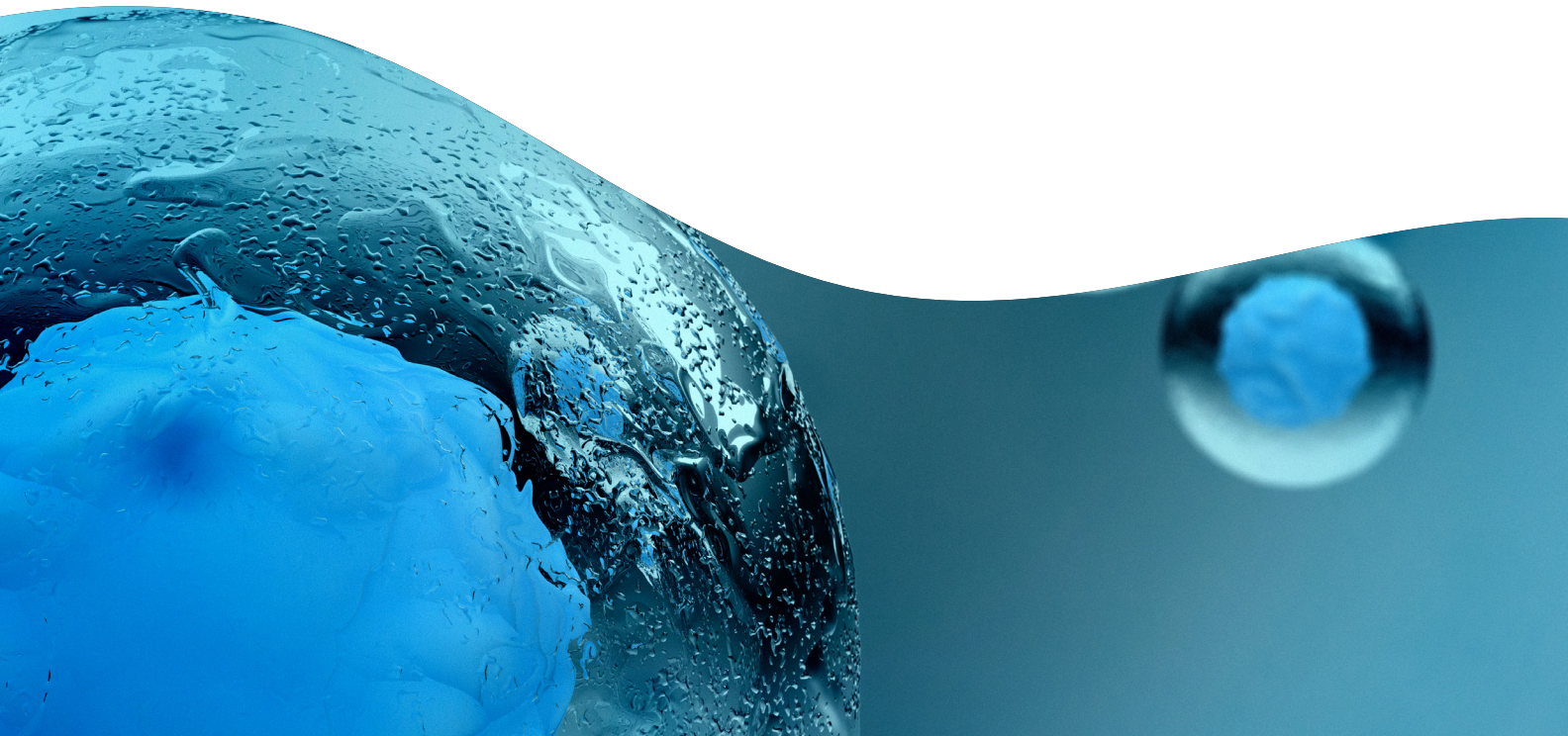
This application note demonstrates that a complete picture of the compound's mechanism of action requires information from all 3 assays: The level of phosphorylated protein, total protein and alpha-tubulin.

Abstract

All Revvity cell signaling assays are built on the same format, and the alpha-tubulin assay is no exception to the rule. The HTRF® Alpha-Tubulin Housekeeping kit (#64ATUBPEG/H) is based on a homogeneous TR-FRET immunoassay format and measures the endogenous level of the protein in cell and tissue lysates.

Alpha-tubulin is one of the most commonly used housekeeping proteins. Measuring levels of alpha-tubulin is essential to understand the compound's mechanism of action and effect on the phosphorylation event. Its concentration is proportional to cell number and total protein content, and is therefore used as an internal control to correct for signal changes caused either by experimental variability or by changes in global protein amount induced by cell treatment.

Through different case studies, this application note explains how to analyze a set of data generated on the same lysate using HTRF Alpha-Tubulin Housekeeping, phospho- and total protein assays. It also demonstrates the need to combine all three assays to correctly interpret the effect of compound(s).



Workflow: Multi-Parametric detection on the same lysate

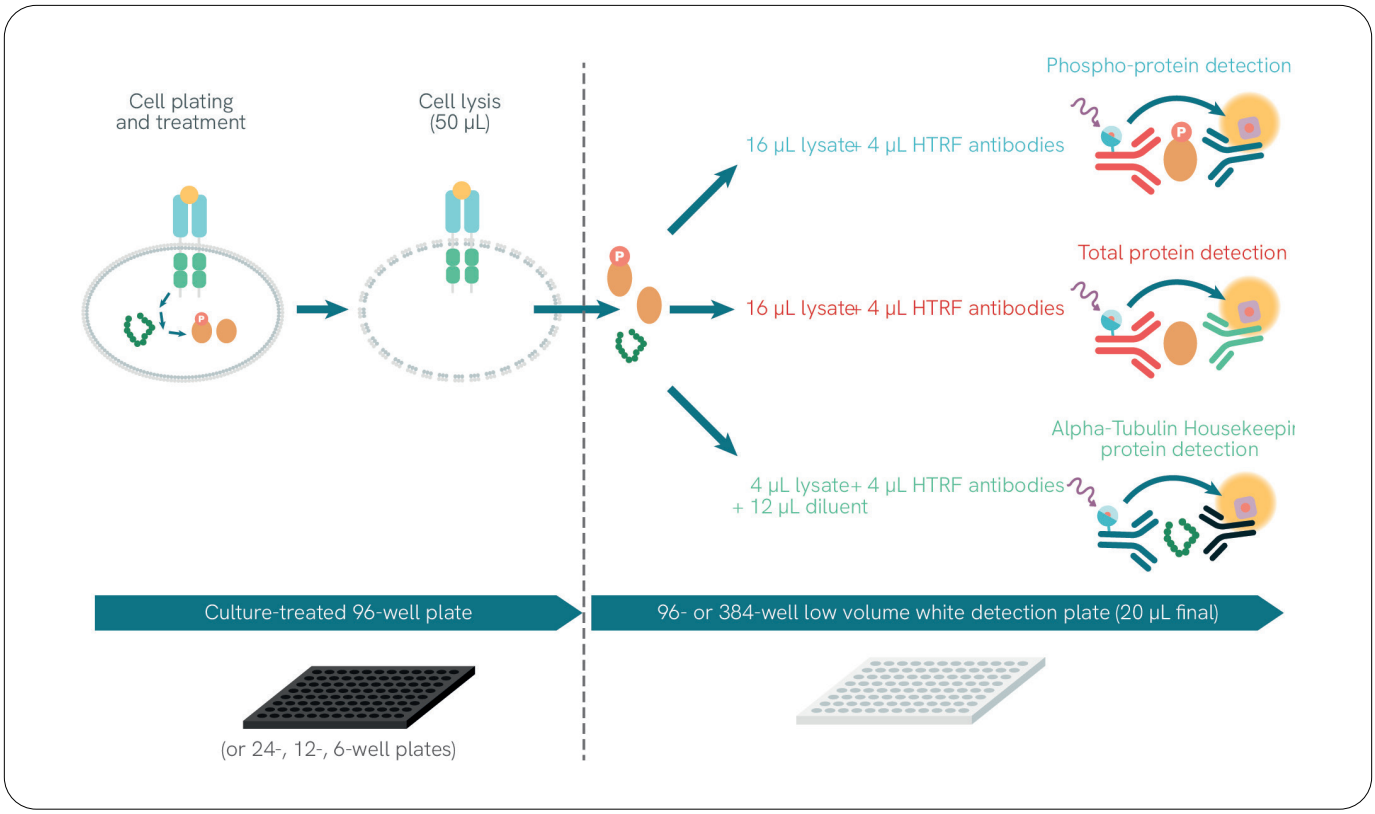


Figure 1: Assay workflow

After treatment with the compound(s) of interest for the desired time period, cells are lysed in the 96-well culture plate using 50 µL of the appropriate lysis buffer (please refer to www.revvity.com/htrf-lysis-buffer-compatibility). Depending on your needs, cell treatment and lysis can also be carried out in other culture formats (please refer to the technical note www.revvity.com/guidelines-cell-culture-and-lysis). The low sample volume of 4 µL and the compatibility with all Cell Signaling lysis buffers enables a multi-parametric analysis with the detection of the housekeeping protein α-tubulin parallel with the phospho- and/or total protein(s) of interest on the same lysate.

Assay optimization

The alpha-tubulin, phospho-protein and total protein content will be measured from the same sample. It must be remembered that a careful optimization of the cell density is required, to ensure that all 3 analytes will be tested at a concentration compatible with their respective assay's linear range. Too high a cell density can result in one analyte being outside the linear assay range, and its subsequent underestimation.

Alternatively, the volume of lysis buffer can be increased/decreased, or the lysate can be diluted with the lysis buffer just before detection.

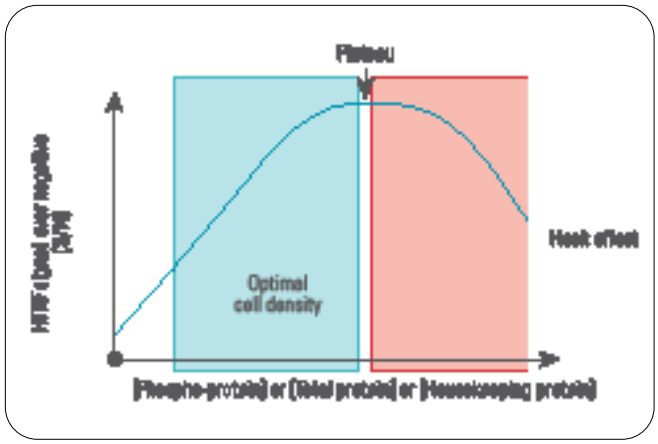


Figure 2: Assay optimization

Data handling

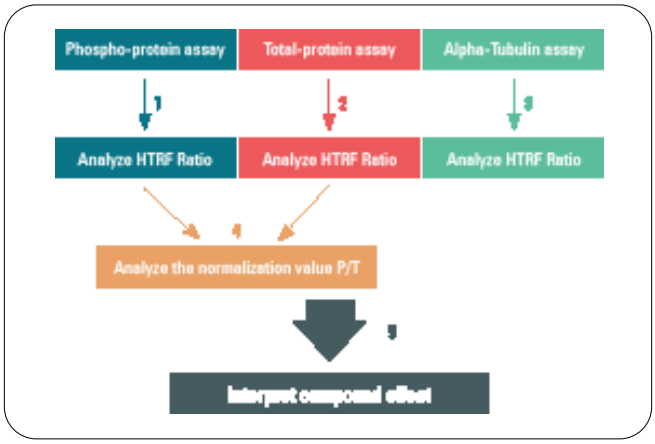


Figure 3: Data handling

Data analysis begins with the calculation of the HTRF ratios, for each well independently and for each assay:
HTRF Ratio = (signal $_{665nm}$ / signal $_{620\ nm}$) x 104.

The mean and error values (CV% and SEM) are then determined for each replicate, which is generally done for each dose of compound.

At this stage, it is essential to examine independently each of the three sets of data (the phospho-protein, the total protein and the housekeeping protein α-tubulin) in order to detect any signal modulation which could be caused by cell treatment.

An alpha-tubulin signal modulation can stem from several causes:

1. The compound tested induced a modulation of the total protein content, for example by inhibiting the global protein synthesis.
2. The compound turns out to be toxic, causing a cell detachment and thus a decrease in the overall number of cells prior to lysis.
3. The signal modulation may simply be due to experimental variability, such as slight but significant differences in the number of cells plated or volume of lysis buffer dispensed.

In the case of signal modulations on both the phospho- and the total protein, it is necessary to calculate the normalization value (P/T) representing the true proportion of phosphorylated protein compared to the total protein present within the sample: Normalization value (P/T) = (HTRF Ratio $_{Phospho}$ / HTRF Ratio $_{Total}$) x 100.

IT is finally the combination of all these data that will allow you to properly understand and interpret the effect of compound(s) on the protein of interest. Different case studies are detailed just after to illustrate this approach.

Case studies

Experimental methods

All assays were performed using the standard two-plate assay protocol for adherent cells (for more information, please refer to the phospho-/total protein assay kit package inserts). Briefly, cells were plated in 96-well plates for 24 hours, treated with increasing concentrations of compounds, and the cell supernatant was removed before lysis with supplemented lysis buffer #4 for 30 minutes. The appropriate volume of lysate was sequentially dispensed into low volume detection white plate and the corresponding kit reagents were dispensed for the combined analysis of the phospho-protein, the total protein, and α-tubulin (64ATUBPEG/H).

Case study #1: Phospho-ERK modulation by the MEK inhibitor PD98059

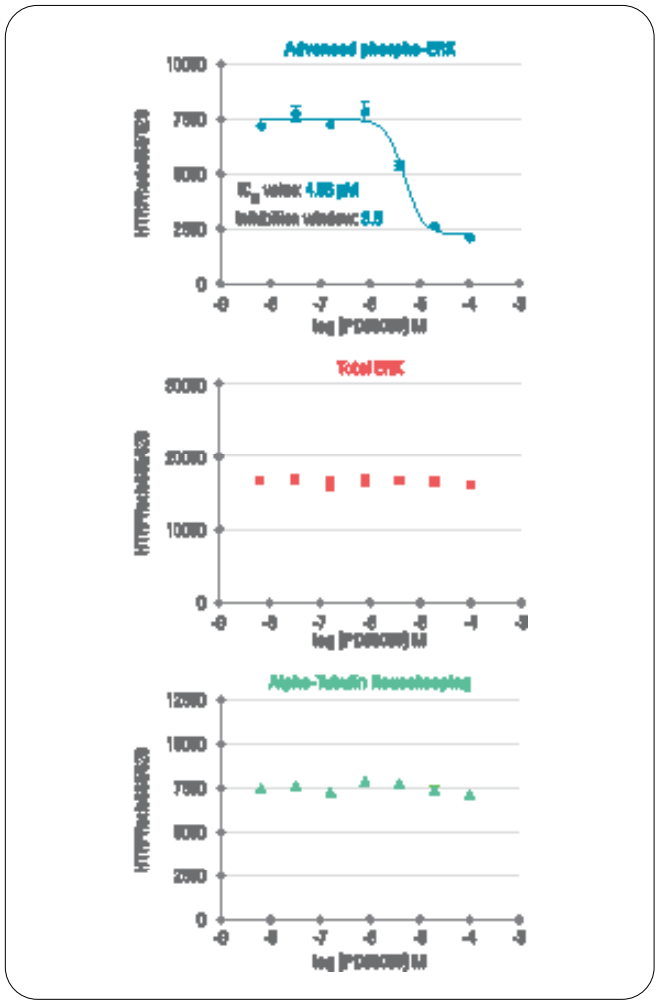


Figure 4: Phospho-ERK modulation by the MEK inhibitor

HeLa cells (50K/well) were treated with PD98059 for 30 minutes. Phospho- and total ERK were detected using the HTRF Advanced ERK phospho-T202 /Y204 and ERK total kits (64AERPET/G/H and 64NRKPET/G/H).

Increasing doses of PD98059 lead to increased inhibition of ERK phosphorylation. Since inhibition of ERK phosphorylation occurs without a decrease in total ERK or alpha-tubulin levels, toxicity can be excluded from the list of possible mechanisms of action. Thus, it can be ascertained that PD98059 acts to decrease ERK phosphorylation primarily by targeting MEK, the kinase upstream of ERK.

In this case, there is no need to calculate the normalization values phospho-/total ERK to interpret the results. Therefore, pharmacological parameters such as the IC_{50} value and the inhibition window can be directly calculated from the sigmoidal dose-response curve of the phospho-ERK assay.

Case Study #2: Phospho-AKT modulation by IGF-1

HEK293 cells (100K/well) were treated with human IGF-1 for 10 minutes. Phospho- and total AKT were detected using the HTRF AKT phospho-S473 and AKT total kits (64AKSPET/G/H and 64NKTPET/G/H).

Cell stimulation with IGF-1 induces an increase in AKT phosphorylation on Ser473 and a down-regulation of AKT expression level, both in a dose-dependent manner with similar EC_{50} and IC_{50} values. On the other hand, the level of α-tubulin remained unchanged, suggesting that the decrease in total AKT was not caused by an inhibition of global protein synthesis or by experimental issues.

In this case, the normalization value phospho-/total AKT was calculated to account for the compound's effect on the overall total AKT levels. It was therefore possible to conclude that IGF-1 leads to a 6-fold increase in the proportion of phosphorylated AKT proteins, with an EC_{50} value of 1.01 nM.

This example clearly illustrates the need to analyze the phospho- and total protein of interest, as well as α-tubulin, in order to correctly understand the effect of compounds on the protein. This case scenario provides compelling evidence that both the total assay and the alpha-tubulin assay should be used to gain a full understanding of the compound's mechanism of action.

Case Study #3: Phospho-EIF2α modulation by Palmitate

HepG2 cells (50K/well) were treated overnight with palmitate. Phospho- and total EIF2α were detected using the HTRF EIF2α phospho-S52 and EIF2α total kits (64EF2PEG/H and 64NEFPEG/H).

Palmitate is a saturated fatty acid well known to induce ER stress in hepatocytes, leading to the activation of the kinase PERK which in turn phosphorylates EIF2α.

Overnight cell treatment with palmitate does not seem to induce a significant and dose-dependent increase in phospho-EIF2α levels.

Conversely, high concentrations of the molecule clearly trigger a fall in total EIF2α and α-tubulin levels in a similar way, with a 3 to 3.5-fold decrease using the dose of 800 µM.

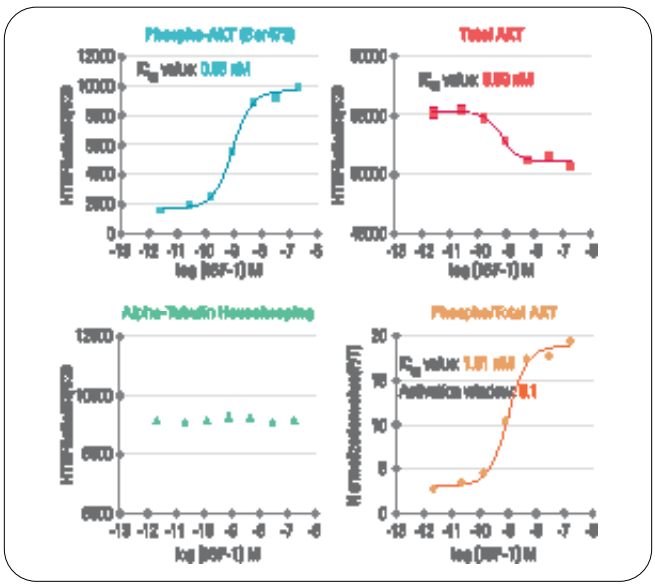


Figure 5: Phospho-AKT modulation by IGF-1

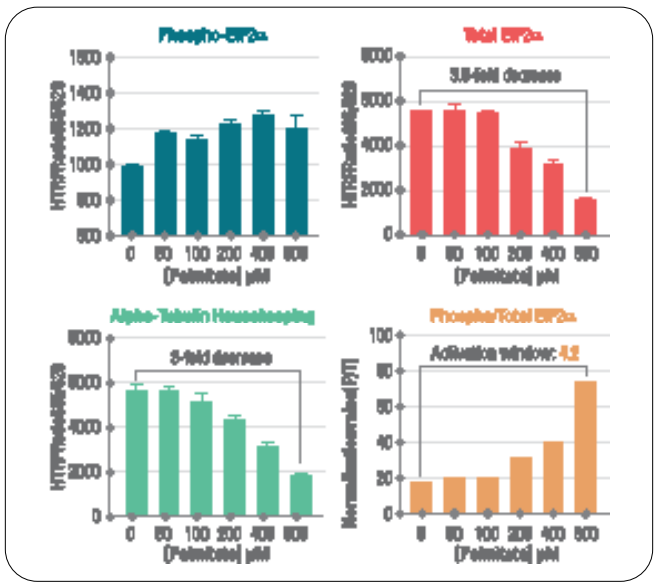


Figure 6: Phospho-EIF2α modulation by palmitate

Together these data enabled the conclusion that the loss of signal obtained on total EIF2α is not caused by a specific down-regulation of its expression level but is due to a toxic effect of palmitate using concentrations above 100 µM. Thus, high doses induced a cell detachment that was clearly visible under the microscope, and the suspended cells were removed before lysis.

The normalization value phospho-/total EIF2α was therefore calculated to understand the effect of palmitate on EIF2α phosphorylation. Notwithstanding the toxicity of fatty acid, the normalization data (P/T) clearly establishes that palmitate induces a significant and dose-dependent increase in the proportion of phosphorylated EIF2α proteins, with a greater than 4-fold change using the highest concentration of the molecule.

Once again, this example demonstrates the importance of performing all three assays in conjunction to be able to properly interpret the effect of the molecule on the phosphorylation status of the protein of interest. Especially in this case, the Alpha-Tubulin Housekeeping assay was instrumental in understanding why the total protein was modulated.

Conclusion

This application note demonstrates that a complete picture of the compound's mechanism of action requires information from all 3 assays: the level of phosphorylated protein, total protein and alpha-tubulin.

The case studies presented clearly highlight that each of the three assays gives a different level of information, and all are complementary, meaning that the total protein assay cannot be substituted for the α-tubulin assay and vice versa.

Finally, the lysis buffer compatibility and the low sample volume needed for detection offer the advantage of enabling the three assays to be performed on the same lysate derived from a single well.

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