APPLICATION NOTE

A guideline for HTRF® cell-based phospho-protein data normalization

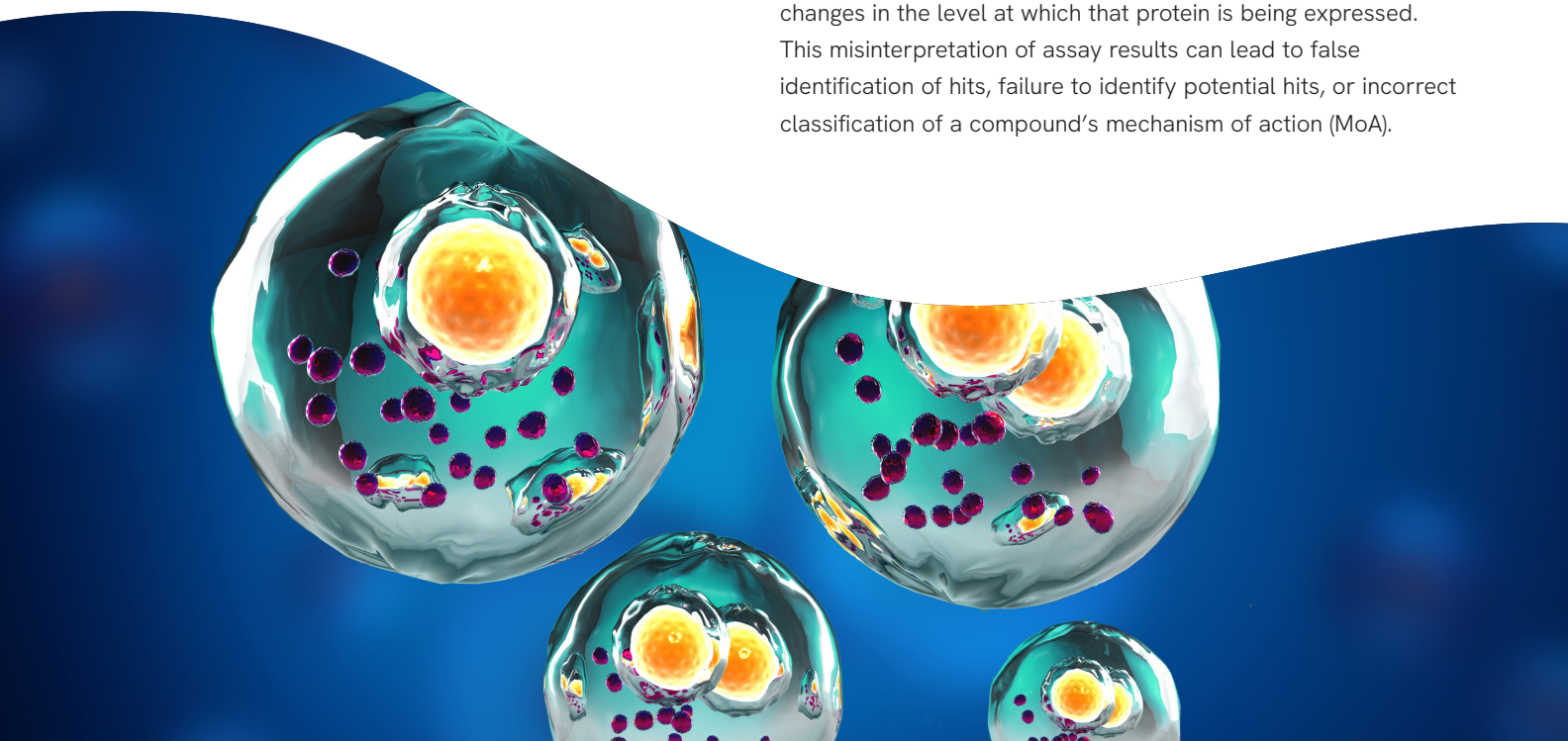
This application note illustrates how to calculate a normalization value and interpret the results correctly when using htrf phosphospecific and total protein assays

Abstract

Revvity offers a comprehensive line of cellular assays to study a variety of post-translational modifications, such as phosphorylation. These products include both phospho-specific and total protein assays. Combining a phospho-specific assay with its associated total assay enables researchers to accurately identify candidate molecules that regulate phosphorylation, as well as to characterize their mechanism of action by performing the appropriate data analysis. This application note illustrates how to calculate a normalization value and interpret the results correctly when using HTRF phospho-specific and total protein assays. While data is presented for AKT and MEK, the concept of handling and interpreting data is similar for all phospho- and methyl-specific assays.

Introduction

Protein phosphorylation is frequently assessed while developing potent therapeutic drugs, as it has been identified as an underlying mechanism for a variety of diseases. Researchers use protein-specific phospho-assays, such as HTRF-based cellular assays, to quantify the level of the phosphorylated protein-of-interest in a given pathway-of-interest to determine signal transduction. Without also assessing the total protein level - phosphorylated and non-phosphorylated -, researchers may conclude incorrectly that a compound acts to up- or down-regulate phosphorylation, when in fact action may be due to changes in the level at which that protein is being expressed. This misinterpretation of assay results can lead to false identification of hits, failure to identify potential hits, or incorrect classification of a compound's mechanism of action (MoA).



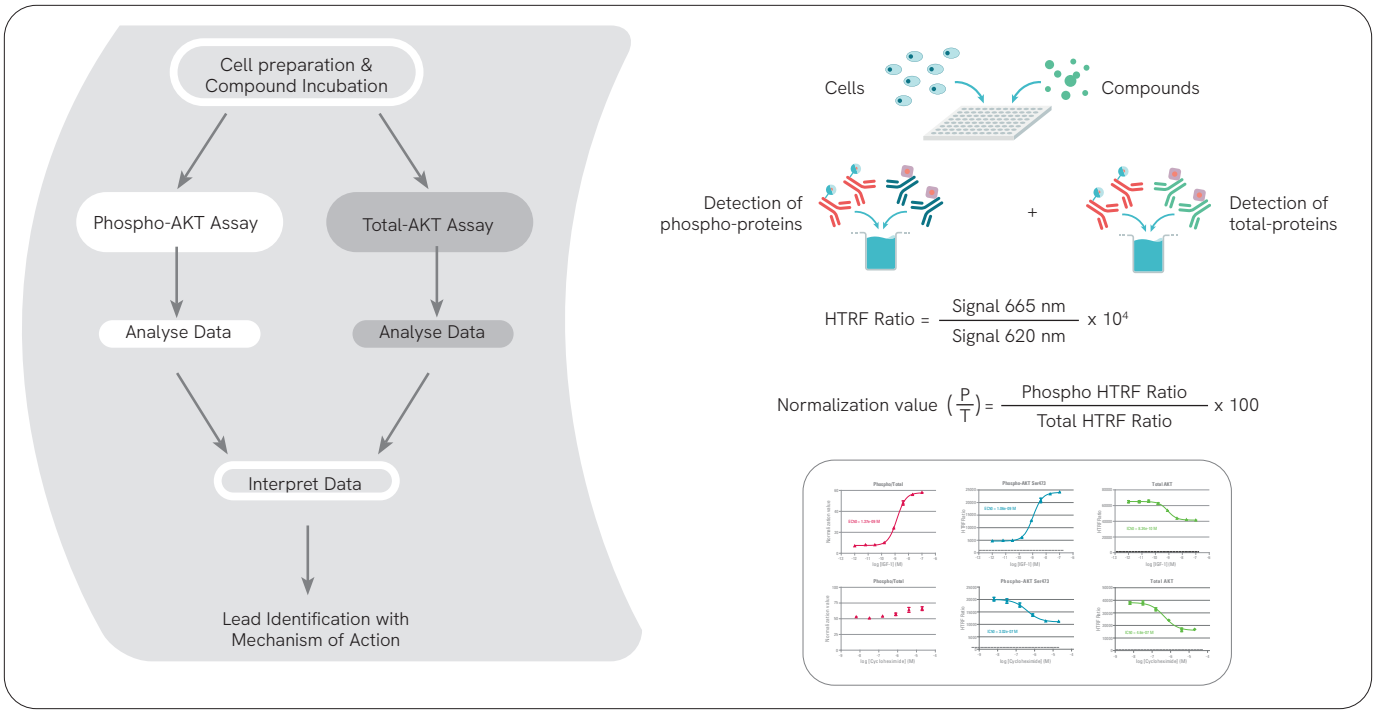


Figure 1: Streamlined assessment of total and phospho-specific protein level.

The combination of HTRF phospho-specific assays and total protein assays provides researchers a streamlined process to analyse protein phosphorylation from low to high-throughput.

Cells are incubated with the compound(s) of interest in a single plate, cells are lysed, and then cellular lysates are dispensed to an assay detection plate with two detection wells for each sample, one to measure total protein of interest, the other to measure protein phosphorylation.

The goal of this application note is to provide valuable guidelines for analyzing and interpreting results efficiently, obtained by combining HTRF phospho- and total protein assays. AKT and MEK phosphorylation are used as examples. By following these guidelines, researchers can expect to obtain the most reliable results possible while deriving as much information as possible from their samples, including deciphering a compound's basic MoA.

HTRF assays total and phospho-specific

Several assays were performed for this study: two Revvity AKT-related HTRF assays, total-AKT and phospho-AKT Ser473 and two Revvity MEK HTRF assays, total MEK and phospho-MEK1Ser218/222.

The total assays detect the total level of the relevant protein, both phosphorylated and unphosphorylated, and can be used in conjunction with phospho-specific assays to monitor changes in protein expression versus changes in protein phosphorylation.

Products used: AKT phospho-S473 kit (64AKSPEG), AKT total kit (64NKTPEG), MEK1 phospho-S218/222 kit (64ME1PEG), and MEK1 total kit (64NE1PEG).

Experimental methods: All assays were performed using the standard two-plate HTRF assay protocol for adherent cells. To summarize: cells were incubated with compounds in a single 96-well plate, the cells lysed, and transferred to two different assay plates, one to measure phospho-AKT, the other to measure total-AKT, and the appropriate HTRF reagents added for detection. For more detailed protocols please visit the web pages for the referenced products.

HEK293 cells were used for all AKT assays at 100,000 cells/well. Cells were incubated with various concentrations of either: insulin-like growth factor 1 (IGF-1), a known AKT activator, for 10 minutes; or cycloheximide, a known protein synthesis inhibitor, for 16 hours. Hela cells were used for all MEK1 assays at 100,000 cells/well. Cells were stimulated with various concentrations of epidermal growth factor (EGF), a known activator of MEK1, for 5 minutes.

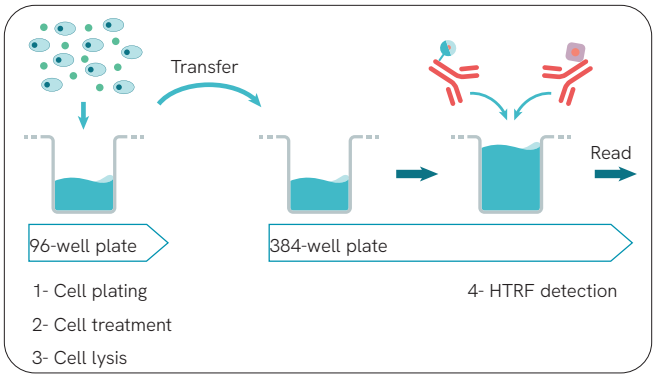


Figure 2: Standard two-plate assay protcol.

Assay optimization

Cell lysate derived from a single well is used to perform both phospho-specific and total assays. Thus both assays should be optimized in conjunction to define optimal conditions, such as the number of cells per well, to ensure that both assays are operating in the linear range of detection and have a sufficient S/B >2.

Data handling

Phospho- and total protein data evaluation begins with the calculation of HTRF ratios. The HTRF ratio should be calculated for each well independently, and these values should be used for any subsequent statistical analysis, such as determining mean or standard error of the mean.

Find out more about calculating HTRF ratios at http://www.cisbio.com/drug-discovery/htrf-ratio-and-data-reduction

- The HTRF Ratio is calculated for each well using the following formula:

$$\text{HTRF Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

The normalization value is calculated using the formula below following the calculation of HTRF ratios for both the phospho- and total assays. This is a relative value that represents the proportion of phosphorylated protein compared to the total protein present within the sample.

- The normalization value is then calculated for each well as follows:

$$\text{Normalization value } \left(\frac{P}{T}\right) = \frac{\text{Phospho HTRF Ratio}}{\text{Total HTRF Ratio}} \times 100$$

Interpreting phospho-AKT/Total-AKT data using the normalization value

Cells were treated with either a known activator of AKT phosphorylation (IGF-1) or an inhibitor of protein synthesis (cycloheximide). Phospho-AKT and total-AKT assays were performed. Data was expressed both as HTRF ratios for each individual assay and as normalization values (Fig. 3A & 3B).

As expected, treatment of cells with IGF-1 (Fig. 3A) increases AKT phosphorylation levels on serine 473 significantly in a dose-dependent manner, with an EC50 value of 1.06 nM. IGF-1 also down-regulates AKT expression, with an IC50 value of 0.8 nM. The normalization values, P-AKT/T-AKT calculated using data from both assays shows that the proportion of phosphorylated versus non-phosphorylated AKT increases significantly upon IGF-1 stimulation.

Cycloheximide treatment (Fig. 3B) results in decreased AKT phosphorylation as well as decreased AKT expression with similar IC50 values. If the total-AKT assays had not been performed, and only the phospho-AKT assay results were evaluated, then cycloheximide could have been identified incorrectly as an inhibitor of AKT phosphorylation.

Data interpretation: By performing both assays and expressing the data as normalization values, it is clear that the level of AKT phosphorylation is not modulated by cycloheximide treatment (Fig.3B). Thus, cycloheximide can be identified correctly as an inhibitor of AKT expression with an IC50 value of 460 nM, but that it does not inhibit phosphorylation.

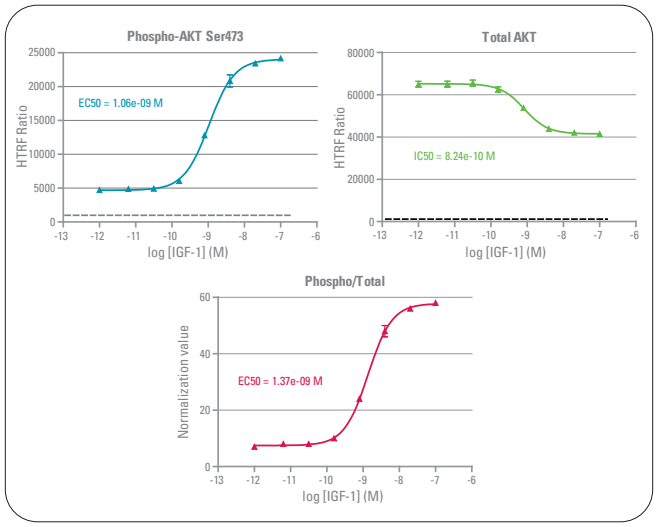


Figure 3A: Compound modulation of AKT phosphorylation and AKT expression.

Phospho-AKT Ser473 & Total-AKT results following growth factor stimulation. HEK293 cells were stimulated with IGF-1 for 10 minutes. Cells were subsequently lysed, cellular lysates transferred to two assay plates, and phospho-AKT and total-AKT assays performed using standard protocols. Results are reported as HTRF ratios for the phospho and total-AKT assays, as well as expressed as normalization values (phospho/total). Black dotted lines correspond to the signal of negative controls (no cells).

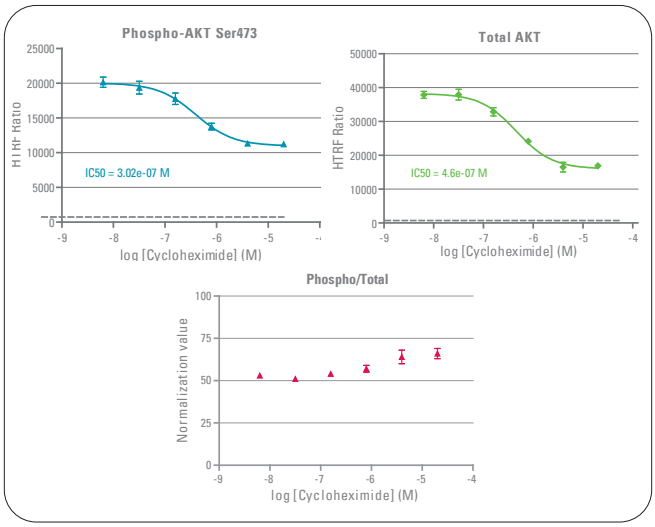


Figure 3B: Compound modulating only AKT expression.

Phospho-AKT Ser473 & Total-AKT results following inhibition of protein synthesis. HEK293 cells were treated with Cycloheximide for 16 hours. Cells were subsequently lysed, cellular lysates transferred to two assay plates, and phospho-AKT and total-AKT assays performed using standard protocols. Results are reported as HTRF ratios for the phospho and total-AKT assays, as well as expressed as normalization values (phospho/total). Black dotted lines correspond to the signal of negative controls (no cells).

Is the normalization value sufficient to properly identify a compound's MoA?

In a word: No. The normalization value is a relative value that combines the results of two independent biological cellular events that can be modulated differently. It is therefore essential to examine all three sets of results, total-AKT HTRF ratios, phospho-AKT HTRF ratios, and normalization values, to identify a compound's MoA correctly. To illustrate this premise, data from the IGF-1 stimulation of AKT phosphorylation shown in Fig. 4A is compared with MEK1 assay results upon stimulating cells with EGF (Fig. 4B).

As described earlier, IGF-1 cell stimulation modulates AKT normalization values positively (Fig. 4A). Upon EGF cell stimulation, MEK1 normalization values are also modulated positively (Fig. 4B). From this, can we conclude that the mechanism of action is similar because the normalization profiles are similar? In fact, such a conclusion would be incorrect.

Data interpretation: Examining all three data sets shows clearly that, although EGF and IGF-1 have similar normalization profiles, they have very distinct MoAs. While IGF-1 stimulation modulates AKT phosphorylation positively and modulates AKT expression negatively, EGF stimulation induces only positive modulation in MEK1 phosphorylation. It has no effect on MEK1 expression. This clearly demonstrates the need to assess all three data sets to gain complete understanding of a compound's effects and thus to be able to draw the most accurate conclusions.

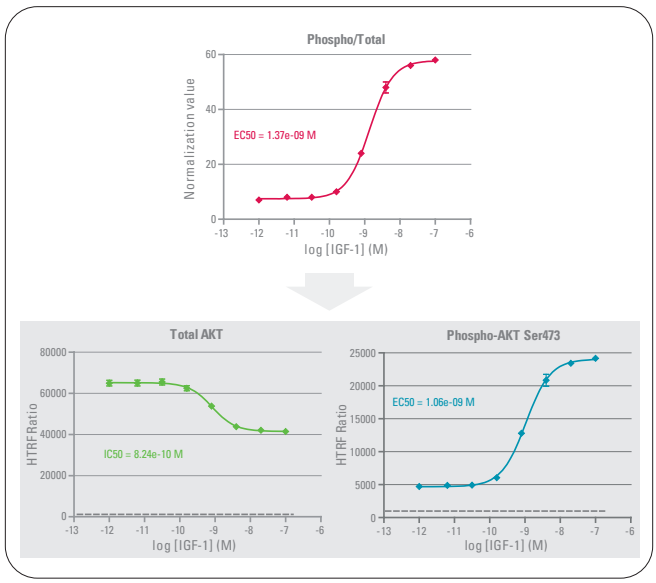


Figure 4A: Effects of growth factor stimulation on AKT phosphorylation.

HEK293 cells were stimulated with IGF-1 for 10 minutes. Cells were subsequently lysed, cellular lysates transferred to two assay plates, and phospho-AKT and total-AKT assays performed using standard protocols. Results are reported as both HTRF ratios for AKT phospho- and total assays, as well as expressed as normalization values. Black dotted lines correspond to the signal of the negative control (no cells).

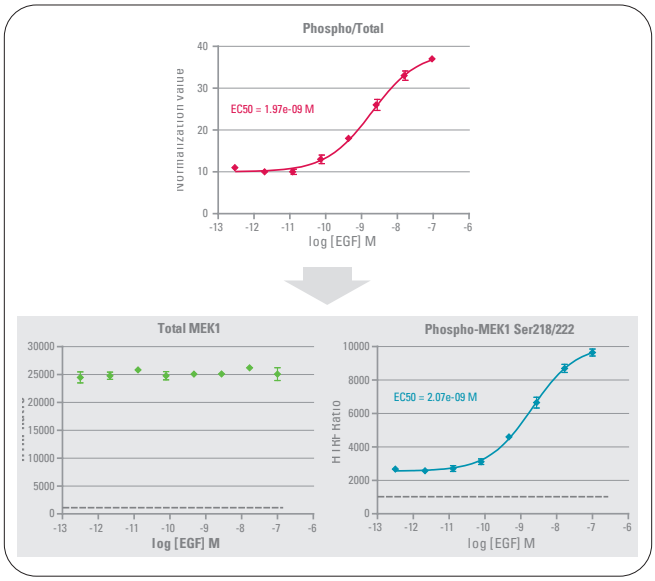


Figure 4B: Effects of growth factor stimulation on MEK1 phosphorylation.

Hela cells were stimulated with EGF for 5 minutes. Cells were subsequently lysed, cellular lysates transferred to two assay plates, and phospho-MEK1 and total-MEK1 assays performed using standard protocols. Results are reported as both HTRF ratios for MEK1 phospho- and total assays, as well as expressed as normalization values. Black dotted lines correspond to the signal of the negative control (no cells).

Conclusions

Performing phospho-specific and total protein assays in conjunction, and properly analyzing and interpreting the data, enables researchers to accurately identify compounds that modulate the phosphorylation of specific proteins as well as to elucidate the mechanism by which it acts. These results illustrate the importance of conducting a side-by-side evaluation of phospho-specific and total protein assay data, as well as compiling the data from both assays to express normalization values. This progressive analysis ensures the accuracy of hit identification and provides insight into each compound's MoA.

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