HTRF® PHOSPHO-ASSAYS REVEAL SUBTLE DRUG-INDUCED EFFECTS IN TUMOR XENOGRAFTS. A METHOD OF CHOICE BEYOND WESTERN BLOT

APPLICATION NOTE

ABSTRACT Cisbio offers a comprehensive line of HTRF phospho- and total protein assays to study post-translational cellular modifications. These versatile, homogeneous assays are suitable for a variety of sample types, including primary cell lines and tumor xenografts. Combining an HTRF phospho-assay with its associated total protein 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 compares the use of HTRF phospho- and total protein assays with traditional Western blot for analyzing cell signaling pathways in tumor xenografts. The data demonstrates that HTRF is a faster, simpler, and more accurate technique for assessing protein phosphorylation and for revealing subtle drug-induced modulations than Western blotting.

INTRODUCTION

Cell signaling pathways involving phospho-proteins are often over-activated in cancer, leading to aberrant cell proliferation and survival. Because of this relationship, protein phosphorylation is frequently assessed while developing novel anti-tumor therapeutics, such as tyrosine kinase inhibitors (TKI) or biologics such as therapeutic monoclonal antibodies.

Initial in vitro 2D-cell culture studies provide useful information about the pharmacological effect of molecules. Optionally, because they are more relevant physiologically, in vitro 3D-cell culture models can be used to increase the number of valuable drug candidates, before preclinical studies on animals. Subsequently, human tumor xenograft-bearing mice are used routinely as predictive models to evaluate the in vivo efficacy of such therapeutic drugs prior to human clinical trials.

Western blotting is the standard technique for analyzing proteins but it is labor intensive, involving significant hands-on time and as long as 24 hours to generate results. HTRF phospho-assays allow researchers to accurately assess the level of the phosphorylated protein of interest in as little as 4 hours, while conserving precious samples.

HTRF phospho-assays were first developed and successfully adapted for cell-based applications. The aim of this study was to demonstrate that this product line is also perfectly suited for analyzing tumor xenografts.

MATERIALS & METHODS

Human pancreatic tumor xenografts were established in immunodeficient mice, with or without treatment, with the anti-EGFR TKI erlotinib (Tarceva). HTRF phospho-/total protein assays* and Western blot analyses were then performed on tumor lysates to analyze phospho- and total ERK1/2 and AKT proteins (as shown in Fig. 1).

**Phospho-AKT (Ser473) [64AKSPEG], Total-AKT [64NKTPEG], Advanced phospho-ERK T202/Y204) [64AERPEG], and Total-ERK [64NRKPEG] Cellular Assay Kits were used in these studies.*

ESTABLISHMENT OF TUMOR XENOGRAFTS AND MICE TREATMENT

Human BxPC3 pancreatic tumor cells (3 x 10^6) were injected in the right flank of ten six-week-old female nude mice. After 40 days, tumor-bearing mice were randomized in two groups: a control group (five non-treated mice) and a treated group (five mice with daily oral dose of erlotinib, 100 mg/kg). After 21 days of treatment, mice were sacrificed and tumors were excised and stored at -80°C.

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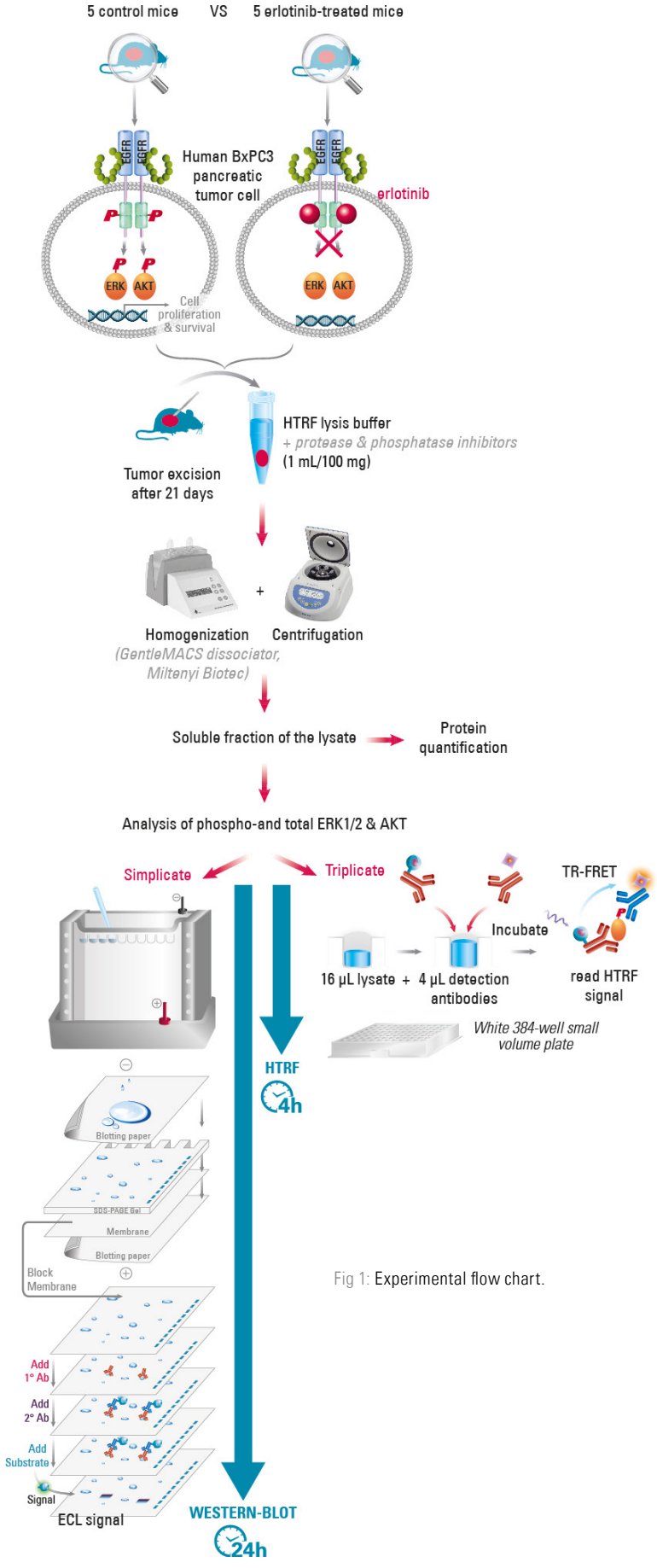

Fig 1: Experimental flow chart.

PREPARATION OF LYSATES AND WORKING SAMPLES

Tumor xenograft lysates were prepared according to the procedure described in the technical note, "Best practices for analyzing tumor xenografts with HTRF phospho-protein assays". The initial protein concentration of each lysate was determined and normalized for both the HTRF and Western blot assays. Serial dilutions of sample lysate were then tested to determine the linear range of protein concentration and prepared for use in the HTRF assays.

HTRF PHOSPHO-/TOTAL PROTEIN ASSAYS

To perform the HTRF assays, 16 µL of diluted samples were dispensed in triplicate into a 384-well white, small volume microplate, and 4 µL of pre-mixed d2 and cryptate antibodies were added. A negative control was included in each assay by replacing the lysate with supplemented lysis buffer. After overnight incubation at room temperature, the HTRF signal was recorded on a PheraSTAR® FS reader (BMG Labtech) with flash lamp excitation.

WESTERN BLOT ASSAYS

Each sample was mixed with Laemmli buffer and boiled at 100°C for 5 min. Proteins (24 µg/line) were then separated by SDS-PAGE using pre-cast gels and transferred to PVDF membranes using the iBlot® dry blotting system (Life Technologies). Membranes were cut into two sections to analyze ERK1/2 and AKT separately. Each section was blocked in Tris-buffered saline - 0.1% Tween-20 (TBS-T) with 5% nonfat dry milk for 1 hr. After the TBS-T washing step, membranes were incubated with the same anti-phospho-antibody used in the HTRF kit and diluted to 1 µg/mL in TBS-T with 5% BSA.

After overnight incubation at 4°C, membranes were washed and incubated with the appropriate HRP-conjugated secondary antibody diluted in the blocking solution according to manufacturer recommendations. After a 2-hr incubation, membranes were washed and incubated with a chemiluminescent substrate for 5 min. Bands were visualized with an imaging system.

After detection of the phosphorylated protein, membranes were stripped for 15 min, washed in TBS, and the corresponding total protein was detected following the same protocol by restarting from the blocking step and using the same anti-total antibody used in the HTRF assay.

DATA HANDLING

HTRF assays:

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

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

- The Signal to Background (S/B) was then determined as follows:

$S/B = HTRF\ Ratio_{sample} / HTRF\ Ratio_{negative\ control}$

To ensure good assay performance, the S/B needs to be higher than 2.

- The HTRF Delta Ratio (specific signal) was calculated for each well using the following formula:

$HTRF\ Delta\ Ratio = HTRF\ Ratio_{sample} - HTRF\ Ratio_{negative\ control}$

- Means, Standard Deviations (SD), and Coefficients of Variation (CV) were then determined for each triplicate.

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

Western blot assays:

The signal was carefully assessed to be certain that it was not saturated (to ensure linearity). Bands were quantified using GeneTools analysis software (Syngene). Relative quantification was performed by assigning 100% intensity to one of the bands of the control group.

DATA ANALYSIS & INTERPRETATION

Results obtained using HTRF or Western blot were analyzed using an unpaired t test (GraphPad Prism) in order to evaluate the statistical significance of the treatment. (NS = not significant; * = significant; ** = very significant)

RESULTS

HTRF ASSAY PERFORMANCE

Four independent analyses of AKT phosphorylation were performed on different days using different aliquots of lysates and different lots of HTRF Phospho-AKT (Ser473) assay kits (Fig. 2).

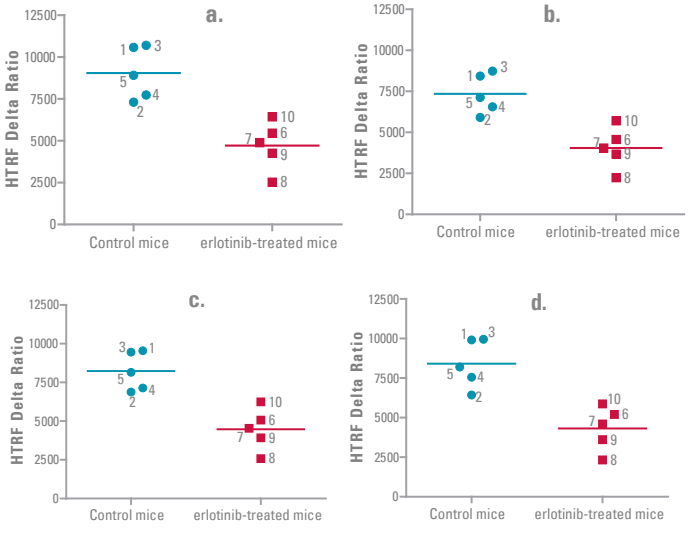

Fig 2: HTRF Phospho-AKT (Ser473) data obtained from four distinct assays on control & erlotinib-treated tumor lysates, analyzed by unpaired t-tests.

Remarkably, similar fluorescent signals were obtained in each independent assay. The control and treated mice respectively received the same ranking in t-tests in each analysis. Additionally, the data obtained with HTRF Phospho-AKT assays demonstrates excellent intra- and inter-assay reproducibility (Table 1).

Intra-assay Reproducibity	Inter-assay Reproducibility
CV < 7%	CV < 9%

Table 1: Intra- and inter-assay reproducibility of HTRF Phospho-AKT (Ser473) assay over four independent analyses of tumor lysates from control and treated mice.

The overall four independent analyses comparing control and treated mice led to the same conclusion: erlotinib treatment induces a very significant decrease of AKT phosphorylation in vivo (Table 2) without modifying its expression level (data on Total AKT is not shown here). These results are in agreement with literature on this topic (E. Buck et al., 2006; Diep CH et al, 2011).

Analysis	p value	Significance
a.	0.0019	**
b.	0.0029	**
c.	0.0019	**
d.	0.0022	**

Table 2: Significance of the in vivo effect of erlotinib on the decrease of AKT phosphorylation, determined by HTRF.

COMPARISON OF WESTERN BLOT AND HTRF ASSAYS

Data for both the Western blot and HTRF phospho-ERK assays were used to evaluate the effect of erlotinib on ERK1/2 (Fig. 3).

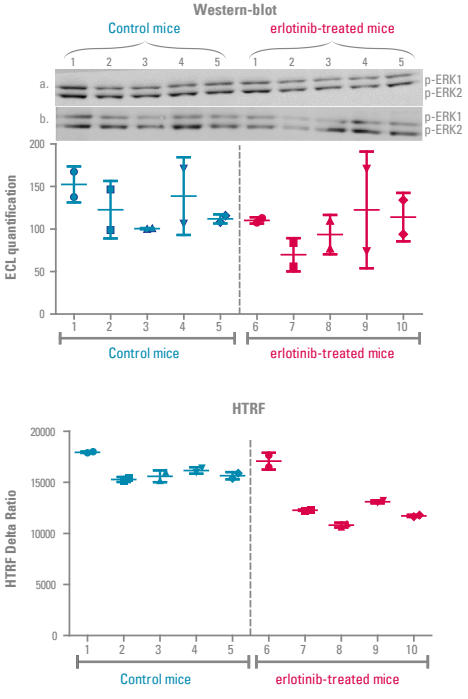

Fig 3: Side-by-side analysis of phospho-ERK1/2 (Thr202/Tyr204) in tumor lysates using Western blot and HTRF (two independent analyses a. & b.). Error bars represent standard deviations.

Using two independent side-by-side analyses, a marked difference was observed in the inter-assay reproducibility of the two techniques, and thus the significance of the results, as shown in Table 3.

Technology	Analysis	Inter-assay	p value	Significance
Western blot	a.	0% < CV < 56% Mean CV = 22%	0.0588	NS
	b.		0.3224	NS
HTRF	a.	0% < CV < 5% Mean CV = 2%	0.0396	*
	b.		0.0227	*

Table 3: Inter-assay reproducibility of Western-blot and HTRF techniques over two independent side-by-side analyses (a. and b.) of phospho-ERK1/2 in tumor lysates. Significance of the in vivo effect of erlotinib on the decrease of ERK1/2 phosphorylation as determined by both techniques.

Based on the Western blot results, a researcher could conclude that the in vivo effect of erlotinib on the reduction of ERK1/2 phosphorylation is non-significant. The lack of reproducibility of the Western blot technique would thus ultimately lead to an incorrect interpretation of the effect of the molecule.

In contrast, the HTRF data is highly reproducible and clearly highlights a significant inhibition of phospho-ERK1/2 by erlotinib without any modification of its expression level (data on Total ERK1/2 not shown here). The interpretation of HTRF results is in agreement with literature on this topic (E. Buck et al., 2006; Diep CH et al, 2011).

DISCUSSION

Western blot is a time-consuming, heterogeneous technique that is inherently variable. It requires numerous incubation and wash steps, and inconsistencies in protein migration, separation and transfer within and between assays are common. Additionally, samples are generally analyzed in singleton, further reducing reliability. There is no defined method for quantification of bands, and problems with technique (such as signal saturation) can make them even more difficult to quantify correctly.

These and other factors reduce the reproducibility and overall reliability of the technique, as demonstrated in this application note. In our experiments, Western blot results were difficult to interpret with confidence with only two independent analyses. In fact, the data would have led to a false negative interpretation of the effects of the molecule. Numerous additional analyses would be required to generate statistically significant results, consuming precious samples and time.

In contrast, Cisbio's HTRF phospho-/total protein kits are easy "one mix-and-read" assays that provide a reliable and rapid alternative to Western blot. Data is measureable directly and provides a direct relative quantification. This homogeneous, miniaturized technique reduces assay steps and leads rapidly to accurate and reliable results that do not need to be reproduced many times.

Robust design and extensive quality control contribute to the excellent reproducibility observed, within and between assays, and between kit lots. In the study presented here, this led to a correct interpretation of the effect of the molecule with only two independent analyses (in accordance with literature), conserving samples and saving time.

CONCLUSIONS

This application note demonstrates that HTRF phospho- and total protein assays are suitable for analyzing tumor xenograft lysates. HTRF provides a more convenient, more robust and sample-saving analysis of protein phosphorylation in tumor xenografts than Western blotting. Most importantly, highly reproducible HTRF assay results lead to correct data interpretation, essential in evaluating the in vivo efficacy of candidate anti-tumor therapeutics.

REFERENCES

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