APPLICATION NOTE

A smart HTRF phospho-protein platform to maximize anticancer drug discovery: From 2D, 3D cell cultures to xenografts.

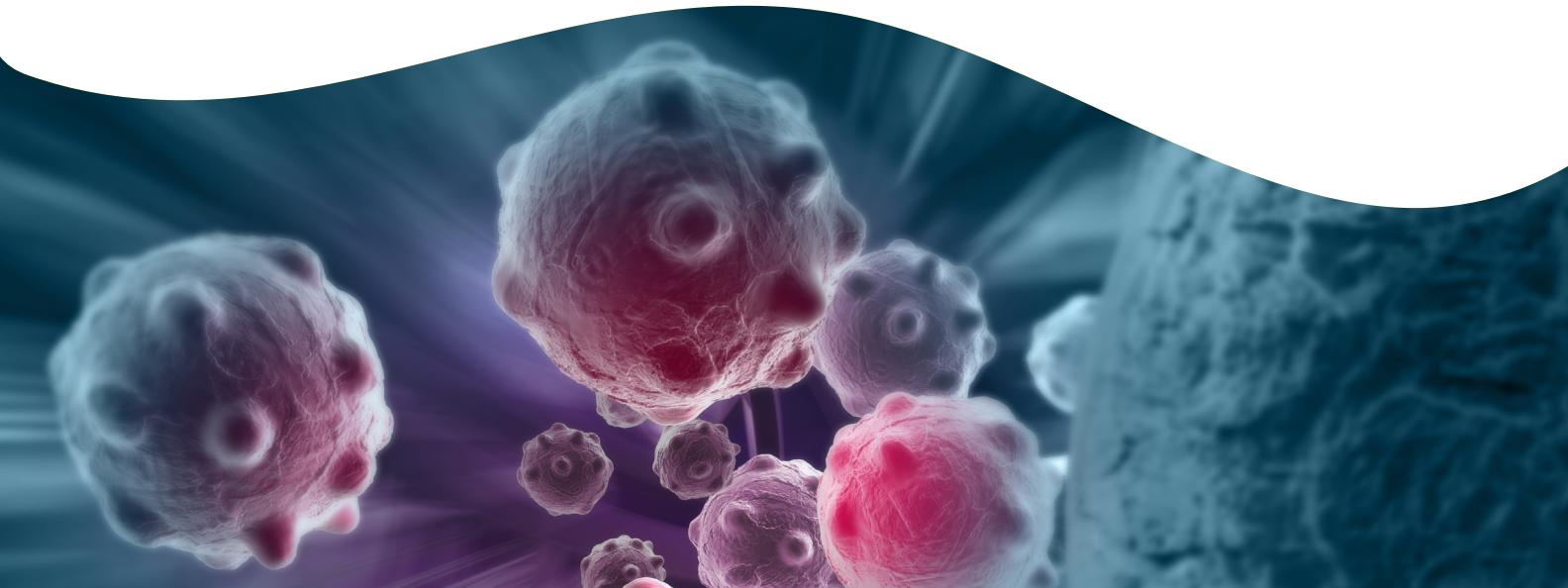
This application note illustrates the use of HTRF phospho-/total protein assays to analyze cell signaling pathways on *in vitro* and *in vivo* sample types routinely used in the anticancer drug discovery process.

Abstract

Revvity offers a comprehensive line of HTRF® cell signaling assays for studying protein post-translational modifications. This application note illustrates the use of HTRF phospho-/total protein assays to analyze cell signaling pathways on *in vitro* and *in vivo* sample types routinely used in the anticancer drug discovery process. 2D tumor cell cultures, 3D tumor cell cultures and xenograft models established with the human BxPC-3 pancreatic tumor cell line were treated with the anti-EGFR tyrosine kinase inhibitor (TKI) erlotinib (Fig. 1). Analysis of the phospho- and total proteins ERK1/2 and AKT was then performed using HTRF assays. A protocol and guidelines to correctly perform HTRF experiments on each model and to analyze data in order to obtain reliable results are also provided.

Introduction

Two-dimensional (2D) cell culture on flat plastic surfaces using immortalized human cancer cell lines is the traditional method for studying cancer *in vitro*. 2D cell-based assays are fast and easy to set up, easily automated and inexpensive, and quickly provide some valuable information. Therefore, high-throughput screening (HTS) on 2D-cell cultures is frequently the starting point for identifying novel antitumor drugs.



However, cell monolayers possess an abnormal flattened structure that is not physiologically relevant, and therefore do not mimic the complexity of a tumor and its microenvironment *in vivo*. This explains the difficulties that may be encountered downstream with translation *in vivo*: many of the potentially active drugs discovered on 2D cell cultures do not perform as well in preclinical animal model systems and subsequently display a disappointing success rate in the clinic.

To bridge the gap between simplistic *in vitro* 2D cell culture models and time-consuming and expensive *in vivo* tumor xenograft models, various *in vitro* biomimetic three-dimensional (3D) cell culture systems have been developed over the past few years. Relevant extracellular matrix components now allow creating realistic 3D tumor models that are used more and more in secondary screening programs to evaluate the antitumor effect of compounds.

To easily and rapidly translate anticancer drug discovery research from *in vitro* to *in vivo* models, researchers need robust biochemical assays compatible with models with different levels of complexity, ranging from 2D cell cultures to animal models. The aim of this application note was to demonstrate that HTRF phospho-/total protein assays are suitable for the accurate analysis of cell signaling pathways in 2D, 3D tumor cell cultures and xenograft models, allowing rapid access to precise pharmacological parameters and reproducible statistical data.

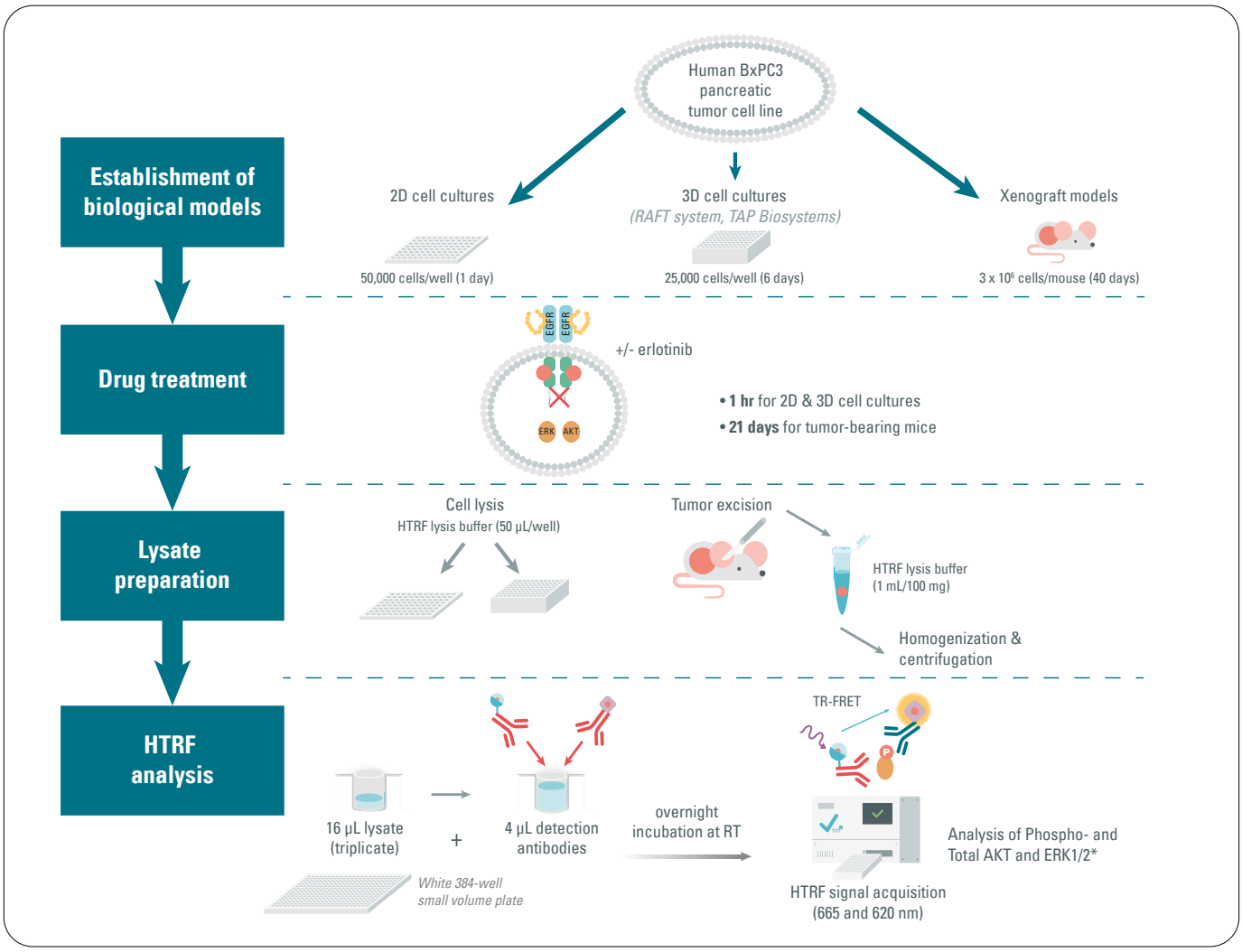


Figure 1: Experimental flow chart

**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*

Materials & methods

Establishment of biological models

2D cell cultures were generated by plating 50,000 BxPC-3 cells in 96-well culture plates in complete culture medium for 24 hr at 37°C, 5% CO2.

3D cell cultures were produced using the RAFT system (kindly provided by TAP Biosystems) by following manufacturer's instructions. Briefly, 25,000 BxPC-3 cells were incorporated in a collagen hydrogel in RAFT 96-well plates and cultured in complete culture medium for 6 days at 37°C, 5% CO2.

Tumor xenograft models were established as described in the application note "HTRF phospho-assays reveal subtle drug-induced effects in tumor xenografts. A method of choice beyond Western blot".

Immunostaining of 2D and 3D tumor cell cultures

To control the correct formation of 3D tumor models, cell cultures were stained for 20 min in the dark with Krebs buffer containing 50 nM of a d2-labeled anti-extracellular EGFR antibody and 2 µg/mL of Hoechst. After 4 washing steps, cells were visualized using an epifluorescence microscope (200X magnification).

Treatments

2D and 3D tumor cell cultures were treated for 1 hr with increasing concentrations of erlotinib (in triplicate). Five minutes before the end of the treatment, cells were incubated with 2 nM of Epidermal Growth Factor (EGF).

Five tumor-bearing mice received a daily oral dose of erlotinib (100 mg/kg) for 21 days (treated group), while five others weren't treated during this period (control group).

Preparation of lysates

For cell cultures, the medium was removed and cells were lysed with 50 µL of HTRF supplemented phospho-/total protein lysis buffer, as described in the kit package inserts.

Tumor xenograft lysates were prepared as described in the technical note "Best practices for analyzing tumor xenografts with HTRF phospho-assays".

HTRF Phospho-/Total protein assays

The cell culture lysate collected from one well was used to detect the four targets of interest. The lysate was used neat for the detection of phospho-AKT, diluted 1:2 in lysis buffer for the detection of total AKT and total ERK, and diluted 1:4 in lysis buffer for the detection of phospho-ERK.

To prepare the tumor lysates for assay, the protein concentration of each sample was determined and then normalized for each HTRF assay: 0.25 mg/mL for Total-ERK and Total AKT; 0.5 mg/mL for Advanced phospho-ERK and 1 mg/mL for Phospho-AKT.

HTRF assays were performed as described in the kit package inserts. Briefly, 16 µL of neat or diluted lysate were dispensed in triplicate in a white 384-well small volume plate and 4 µL of pre-mixed HTRF antibodies were added. A negative control was included for each assay by replacing the lysate with lysis buffer. After overnight incubation at room temperature, the HTRF signal (665 and 620 nm) was recorded on a PHERAstar® FS reader (BMG Labtech) using flash lamp excitation.

HTRF data handling & analysis

The HTRF Ratio was calculated for each well using the

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

following formula:

Means, standard errors of the mean (SEM) and coefficients of variation (CV) were determined for each triplicate. Phospho-protein assay results were then normalized to those obtained with the corresponding total protein assay, as described in the application note "A guideline for HTRF® cell-based phospho-protein data normalization".

For *in vitro* cell culture experiments, IC50 values were obtained from sigmoidal inhibition curves by plotting HTRF signal vs. log [erlotinib] (GraphPad Prism). Pharmacological assay windows were also calculated by dividing the signal at the top of the curve by the one at the bottom of the curve.

Results of *in vivo* experiments 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

Each HTRF assay carried out on the three different tumor models performed very well, as demonstrated by the clearly distinct HTRF signals obtained on samples compared to negative controls, with pharmacological assay windows ranging from 4 to 11 (Fig. 2 and 3).

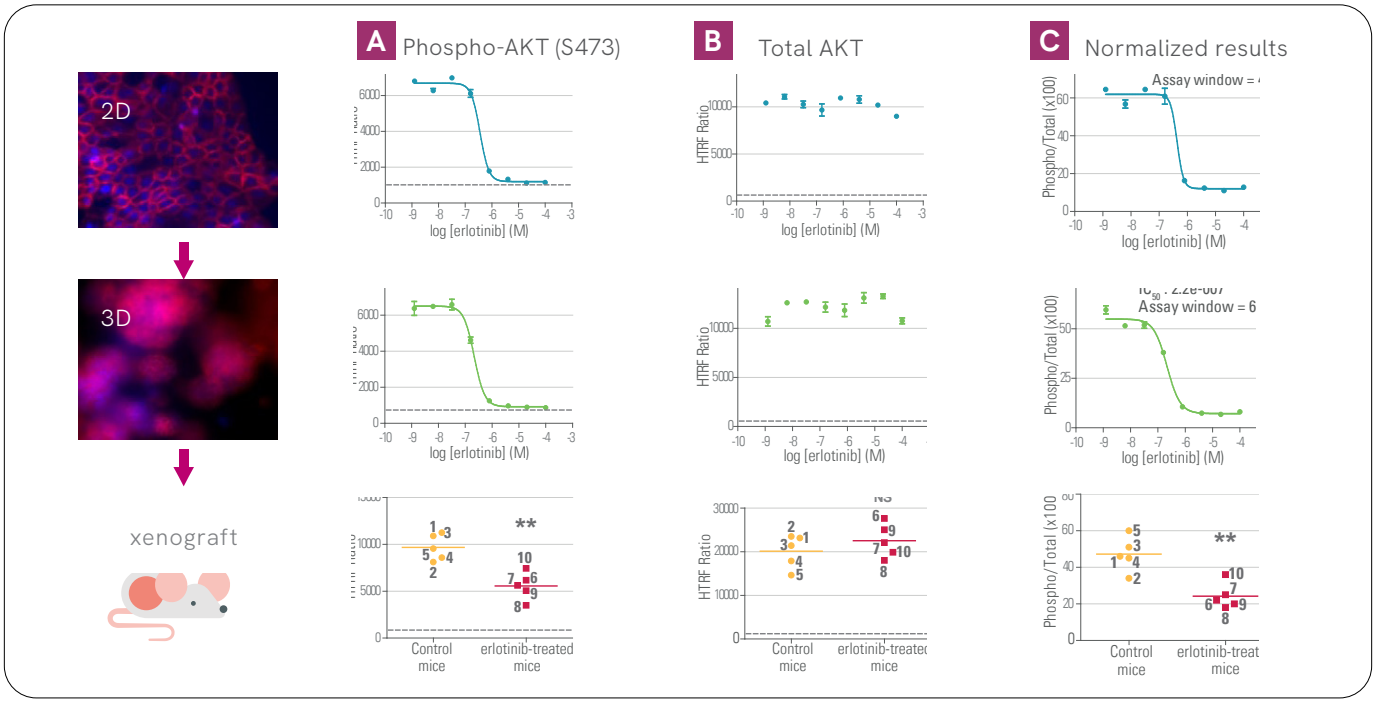


Figure 2: Analysis of AKT phosphorylation in lysates of 2D, 3D tumor cell cultures and xenograft models treated with erlotinib.
a. HTRF Phospho-AKT (Ser473) assay;
b. HTRF Total AKT assay;
c. Normalized results (phospho/total)
Grey dotted lines correspond to the signal of the negative controls. Error bars represent SEM. Data presented on xenografts correspond to the mean of four independent analyses.

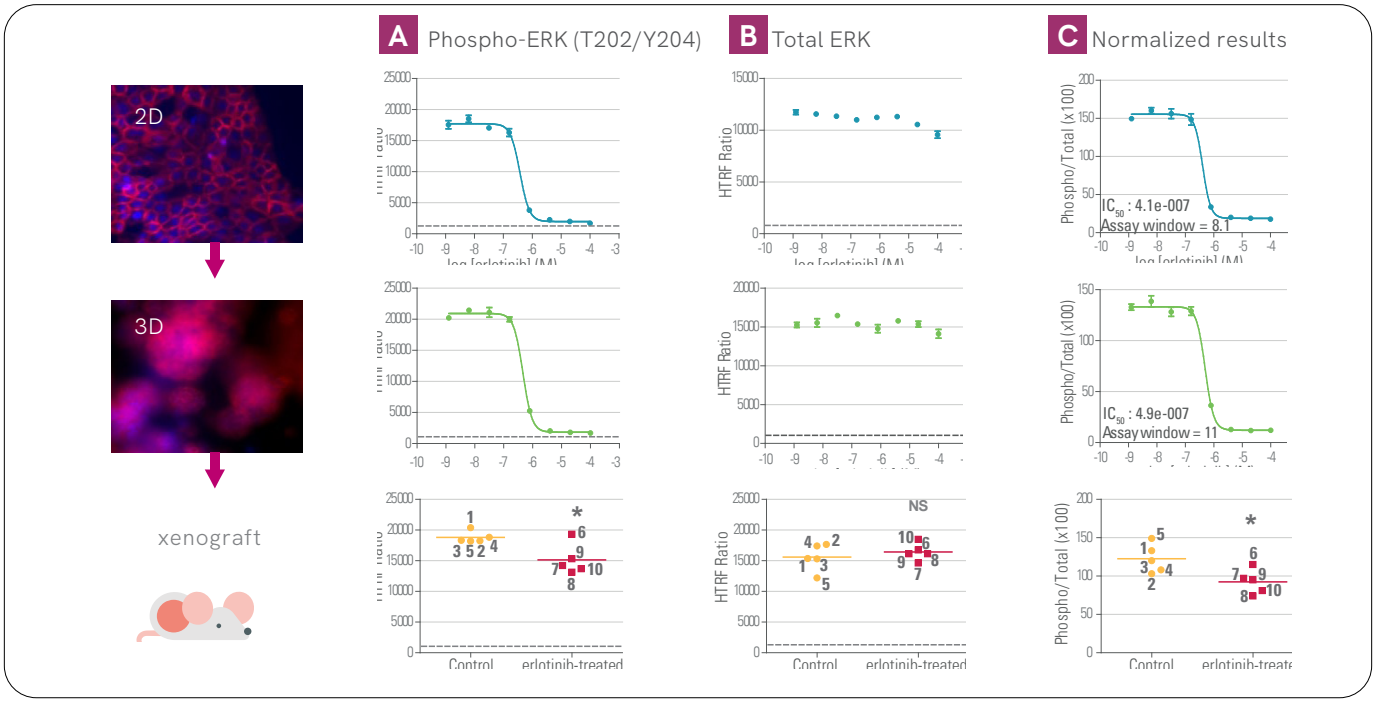


Figure 3: Analysis of ERK phosphorylation in lysates of 2D, 3D tumor cell cultures and xenograft models treated with erlotinib.
a. HTRF Advanced phospho-ERK (Thr202/Tyr204) assay;
b. HTRF Total ERK assay;
c. Normalized results (phospho/total).
Grey dotted lines correspond to the signal of the negative controls. Error bars represent SEM. Data presented on xenografts correspond to the mean of four independent analyses.

After a 1-hr treatment, erlotinib induced a complete inhibition of AKT and ERK phosphorylation in 2D and 3D tumor cell cultures (Fig. 2a and 3a), while the expression level of both proteins remained unchanged (Fig. 2b and 3b). These results are in agreement with the literature (F. Lange et al., 2012). The potency of erlotinib to inhibit ERK and AKT signaling was similar in 2D and 3D models, with IC50 values comprised between 2.2 x 10-7 and 4.9 x 10-7 M (Fig. 2c and 3c). Unlike *in vitro* experiments, the TKI induced a moderate but significative inhibition of AKT and ERK signaling *in vivo* after 21 days of treatment (Fig. 2 and 3). These results correlate well with the literature (E. Buck et al., 2006).

Moreover, HTRF phospho-/total protein assays showed excellent reproducibility. The mean intra-assay CV calculated for the four assays on 2D- and 3D-cell culture models was 4.2%. The mean inter-assay CV determined for the same four assays, on four independent analyses of tumor xenograft lysates, was 14.2%.

Discussion

HTRF is a time-saving, homogeneous technique. The "one add-and-read" assay protocols are very easy and fast compared to traditional heterogeneous methods like Western blot and ELISA. Moreover, HTRF is a sample-saving method. The volume of 2D or 3D cell lysate generated in one well (50 µL) was sufficient to perform the four assays in parallel from the same treated sample. For the xenograft samples, only 32 µg of proteins were necessary to detect the same four targets.

As shown in the data, the HTRF cell signaling assays carried out on the three different tumor models provided clearly distinguishable signals versus negative controls and excellent intra- and inter-assay reproducibility. Thus, the accurate and reliable results don't need to be reproduced many times, further conserving precious samples.

Conclusion

This application note demonstrates that HTRF phospho-/total protein assays are universal and highly versatile. They are compatible with various biological tumor models, ranging from conventional *in vitro* 2D cell cultures, to realistic 3D cell tissues, and finally to *in vivo* tumor xenograft models. Therefore, they can be easily implemented in labs at every step of the drug discovery process (from HTS to secondary screenings and preclinical studies). In conclusion, the HTRF phospho-/total protein assay platform maximize the transition of anticancer drug discovery research programs from *in vitro* to *in vivo* tumor models.

References

Lange F, Rateitschak K, Kossow C, et al. Insights into erlotinib action in pancreatic cancer cells using a combined experimental and mathematical approach. World J Gastroenterol. 2012; 18(43): 6226-6234.

Buck E, Eyzaguirre A, Brown E, et al. Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-cell lung, pancreatic, colon, and breast tumors. Mol Cancer Ther. 2006; 5: 2676-2684.

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