APPLICATION NOTE

HTRF phospho-total lysis buffer: a universal alternative to RIPA lysis buffers.

This application note compares the use of the HTRF phospho-total lysis buffer with commonly used RIPA lysis buffers for the analysis of cell signaling pathways in different types of samples, presenting data that supports the efficacy and superiority of the HTRF phospho-total lysis buffer.

Abstract

Revvity offers a comprehensive line of HTRF® phospho- and total protein assays to investigate cell signaling pathways. These versatile, homogeneous assays are suitable for a variety of sample types, including primary cell lines and tumor xenografts. Each HTRF assay includes a common and essential step that relies on the ability of the lysis buffer to release the protein of interest. This protein, either phosphorylated or not, is then detected by an HTRF sandwich immunoassay.

This application note compares the use of Revvity's HTRF phospho-total lysis buffer with commonly-used RIPA lysis buffers for the analysis of cell signaling pathways in different types of samples. The data demonstrate that HTRF phospho-total lysis buffer is as efficient as RIPA lysis buffers to perform protein extraction and is compatible with both simple and more complex biological samples. Moreover, the HTRF phospho-total lysis buffer delivers higher quality HTRF results than RIPA lysis buffers. Finally the HTRF phospho-total Revvity lysis buffer is not only suitable for HTRF, but also for other analytical methods such as Western blot and ELISA.

Introduction

Any biochemical investigation of biological events that control cell fate, in more or less advanced relevant models, requires cell membrane solubilization first, then the release of cellular protein content. Of course the accessibility of proteins from the different cellular compartments, whether cytoplasmic or nuclear proteins, is essential. For some time, many research laboratories have utilized RIPA lysis buffer (radioimmunoprecipitation assay buffer) as a stringent enough lysis buffer, capable of liberating proteins from the cytoplasm and from the nucleus. However, there are many closely related formulations of RIPA buffer in use. This may result in variability in scientific data from lab to lab, leading to confusion or misunderstanding.

In the course of the development of HTRF phospho-proteins assays, Revvity has carefully optimized a phospho-total lysis buffer that recapitulates lysis efficacy and maximizes HTRF assay.

The aim of this study was to demonstrate that the Revvity's HTRF phospho-total lysis buffer is perfectly suited to release cytoplasmic and nuclear proteins. The protein extraction efficacy of the lysis buffer is comparable to different commercially available RIPA buffers when applied to 2D or 3D cell cultures, tumor xenografts or tissues. As expected, the performance of HTRF assays is better with the optimized HTRF phospho-total lysis buffer than with the RIPA buffers. In addition to its suitability for various biological samples, HTRF phospho-total lysis buffer delivers valuable results in other applications such as Western blot and ELISA. Considered together, HTRF phospho-total lysis buffer is an excellent alternative to RIPA lysis buffer for biochemical investigations.

Materials & methods

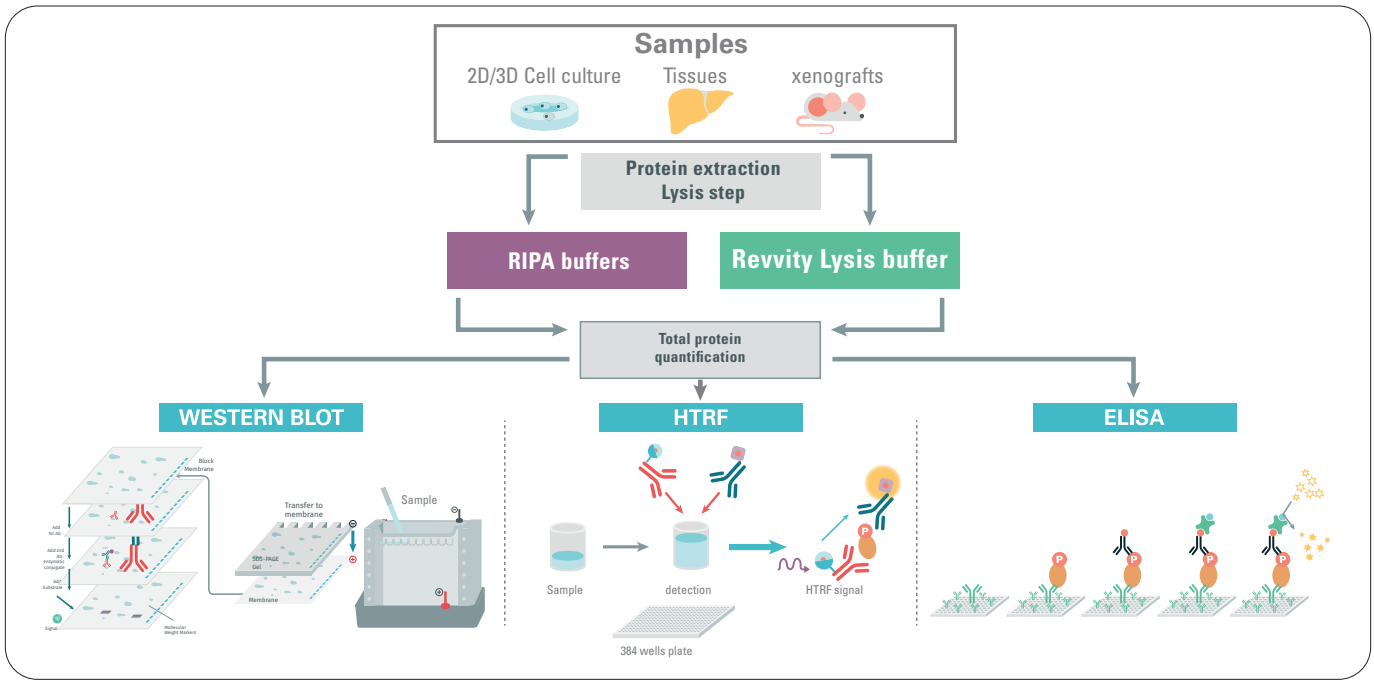
Biological models & reagents

Cell lines: Hela, BXPC3, HEK293 cells were purchased from ATCC.

Tumor xenografts and tissues from mice were kindly provided by Dr. Thierry Chardès, IRCM Montpellier.

Innovative Biomimesys® scaffolds for **3D cell culture** were kindly provided by the company CELENYS. For more information, visit www.celenys.com

HTRF Phospho-assays: Revvity Bioassays, Phospho-AKT (Ser473) [64AKSPEG], Advanced phospho-ERK (T202/Y204) [64AERPEG]; phospho-STAT3 [62AT3PEG]; phospho-CREB [64CREPEG]; phospho-EGFR [64EG1PEG]



HTRF phospho-total lysis buffer: Revvity, #64KL4FDF supplemented with blocking reagent #64KB1AAC

RIPA buffers were purchased from: Cell Signaling Technologies (CST) # 9806 (R1), Millipore # 20-188 (R2), Thermo Fisher Scientific # 89900 (R3) and Sigma Aldrich # R 0278 (R4). See Table 1 for buffer composition.

Table 1: Composition of different commercially available RIPA lysis buffers.

	R1	R2	R3	R4
Tris-HCl, pH 7.4	20 mM	50 mM	25 mM	50 mM
NaCl	150 mM	150 mM	150 mM	150 mM
Na_2EDTA	1 mM	1 mM	-	-
EGTA	1 mM	-	-	-
NP-40	1%	1%	1%	1%
Na-DOC	1%	-	1%	0.5%
SDS	-	-	0.1%	0.1%

ELISA kits were purchased from CST: Pathscan Phospho-p38 MAPK Sandwich ELISA Kit #7946S, PathScan® Phospho-mTOR (Ser2448) Sandwich ELISA #7976S.

Cell culture conditions

Table 2: Experimental conditions.

HTRF assays	Cell line	Number of cells/well	Agonist	Treatment time
P-ERK	HEK293	100,000	EGF	5 min
P-STAT3	Hela	50,000	IFNα	30 min
P-AKT	HEK293	100,000	IGF-1	10 min
P-CREB	HEK293	100,000	Forskolin	30 min
P-EGFR	BXPC3	50,000	EGF	5 min

Preparation of lysates

For 2D and 3D cell lysates, please refer to the procedure described in the application note [A smart HTRF phospho-protein platform to maximize anticancer drug discovery: from 2d, 3d cell cultures to xenografts]. For tumor xenograft and tissue lysates, please refer to the procedure described in the technical note [Best practices for analyzing tumor xenografts with HTRF® phospho assays].

Total protein quantification

Protein concentration was determined using the QuantiPro BCA Assay kit from SIGMA.

HTRF Phospho-/Total protein assays and Western blot assays

Please refer to the application note entitled "HTRF phopsho-assays reveal subtle drug-induced effects in tumor xenografts, a method of choice beyong western blot".

ELISA assays

Hela cells (2 million) were incubated in a 10 cm Petri dish for 48 hr, then either left untreated for phospho-mTor analysis or anisomycin-treated for phospho-p38. 500 µL of each lysis buffer was added for 5 min. Then the cells were scraped off the plates, transferred to tubes, and centrifuged at 10,000 rpm for 10 min. Then serial lysate dilutions were prepared prior to the subsequent steps, performed following CST's instructions.

Data handling

HTRF assays

Learn more about HTRF data reduction on our website.

Western blot assays

Chemiluminescent signal was acquired on a G: Box imaging system equipped with GeneTools analysis software (Syngene).

ELISA assays

The Optical Density at 450 nm (OD_{450}) was measured using the iEMS MF microplate reader (Labsystems, Helsinki, Finland).

Results

HTRF Phospho-total lysis buffer is as efficient as RIPA lysis buffers to perform cell lysis & protein extraction

We first compared the capacity of HTRF phospho-total lysis buffer versus RIPA lysis buffers to release proteins from various cell type models. As shown in Figure 1, the protein concentration was comparable among the different lysis buffers, indicating that HTRF phospho-total lysis buffer is as efficient as the RIPA lysis buffers to liberate proteins from cells.

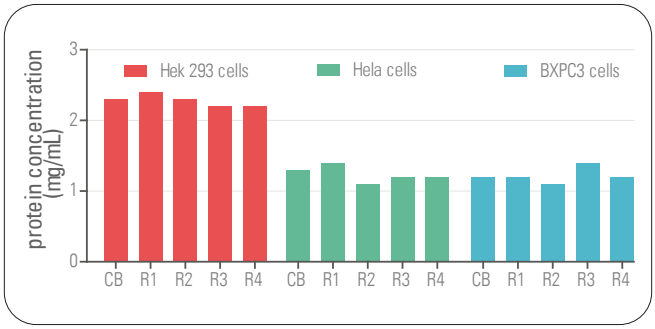


Figure 1: Comparison of protein concentration obtained after 2D cell culture lysis using HTRF phospho-total lysis buffer (CB) and four different RIPA lysis buffers (R1-R4).

HTRF Phospho-total lysis buffer efficiently releases protein content from more complex biological samples

We also evaluated whether the HTRF phospho-total lysis buffer performed effectively enough to induce lysis of complex samples. We monitored the protein concentration obtained on samples derived from a mouse liver, from a tumor xenograft and from 3D cell culture, subjected either to HTRF phospho-total lysis buffer and RIPA buffer 4. As illustrated in Figure 2, the HTRF phospho-total lysis buffer (CB) delivers amounts of proteins comparable to RIPA buffer 4, e.g. around 20 mg/mL from mouse liver, almost 10 mg/mL for tumor xenografts and around 2mg/mL for 3D cell culture. Thus the HTRF phospho-total lysis buffer is suited for lysing complex biological samples.

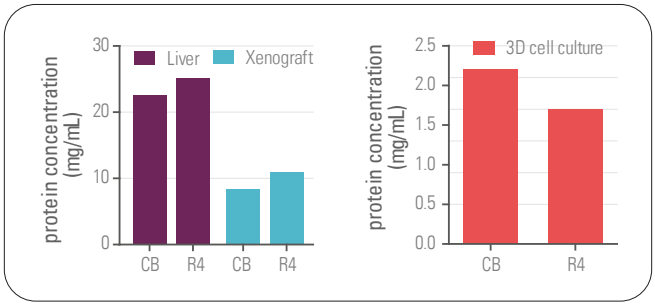


Figure 2: Comparison of protein concentration obtained after lysis of liver tissue, tumor xenograft and 3D cell culture, using HTRF phospho-total lysis buffer (CB) and different RIPA lysis buffers (for ripa lysis buffers details, see table 1).

HTRF Phospho-total HTRF assays perform better using HTRF Phospho-total lysis buffer than ripa lysis buffers

Next we addressed whether HTRF phospho-assays are compatible with RIPA lysis buffers. To do so, we studied the performance of HTRF advanced Phospho-ERK, Phospho-AKT, Phospho-CREB, Phospho-EGFR and Phospho-STAT3 assays using the four RIPA lysis buffers (R1-R4) and the HTRF phospho-total lysis buffer (CB).

Even though HTRF Phospho-CREB performed identically whichever RIPA buffer was used, some discrepancies were noticed with the HTRF Phospho-AKT (Figure 3 and 4). In contrast, the HTRF phospho-total lysis buffer performed consistently better in both assays, supporting its good capacity to release nuclear proteins such as CREB, or other transcription factors like STAT3 or even ERK (Figure 3). Finally, a particular focus on the basal level of phospho-AKT indicates that the assay performs at a higher level of sensitivity when using HTRF Phospho-Total lysis buffer (Figure 4B). Table 3 summarizes the signal to background ratio (S/B) of these HTRF-phospho assays using the different lysis buffers. The data further demonstrate that HTRF phospho-total lysis buffer is better suited for use in these HTRF phospho-assays than any of the RIPA lysis buffers.

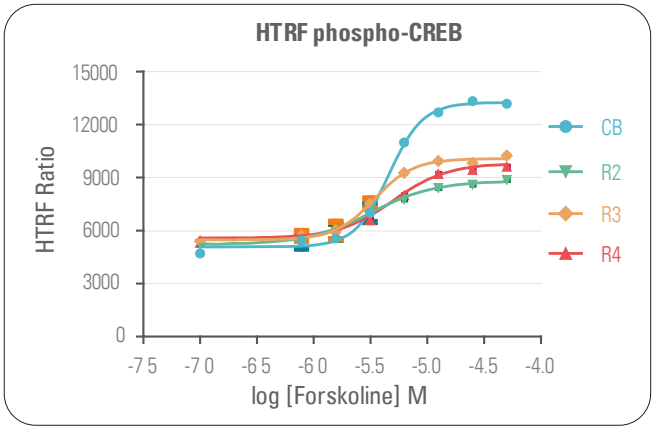


Figure 3: Comparison of HTRF phospho CREB assay performance, using HTRF phospho-total lysis buffer (#64KL1FDF) and four different RIPA lysis buffers.

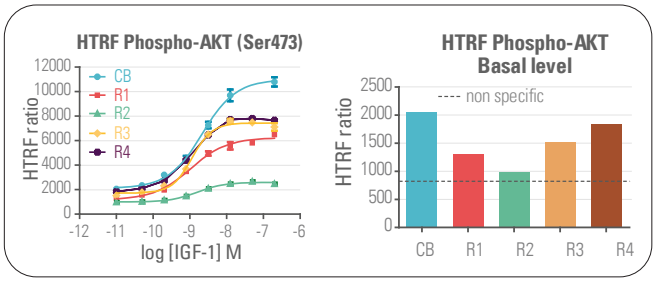


Figure 4: HTRF phospho AKT assay performance induced by IGF-1 dose response, using HTRF phospho-total lysis buffer and four different RIPA lysis buffers (A). Comparison of HTRF sensitivity between the different lysis buffers (B).

Table 3: Performance of HTRF assays. Pharmacological signal to background (S/B) were calculated by dividing the maximum HTRF signal (given by the highest stimulated condition) by the minimum HTRF signal (given by the unstimulated-basal condition).

S/B	CB	R1	R2	R3	R4
Advanced P-ERK	3.8	2.3	2.3	3.3	2.4
P-STAT3	7.7	4.7	5.5	4.2	5.2
P-AKT	5.3	5	2.5	4.7	4.2
P-CREB	2.6	nd	1.7	1.82	1.8
P-EGFR	4.7	3.5	3.3	3.4	3.2

HTRF Phospho-total lysis buffer is compatible with other analytical methods, such as elisa or western blot

Finally we addressed if the HTRF phospho-total lysis buffer could be utilized for other biochemical investigation methods. Since Western blot and ELISA are the most conventional approaches to study signaling pathways and protein phosphorylation, these two methods were selected.

First we performed serial lysate dilutions obtained either with the HTRF phospho-total lysis buffer or with the ELISA lysis buffer included in each kit. The results shown on Figures 5A and 5B demonstrate that phospho-mTOR and phospho-p38 ELISAS performed equivalently whether the HTRF phospho-total lysis buffer or the ELISA lysis buffer was used.

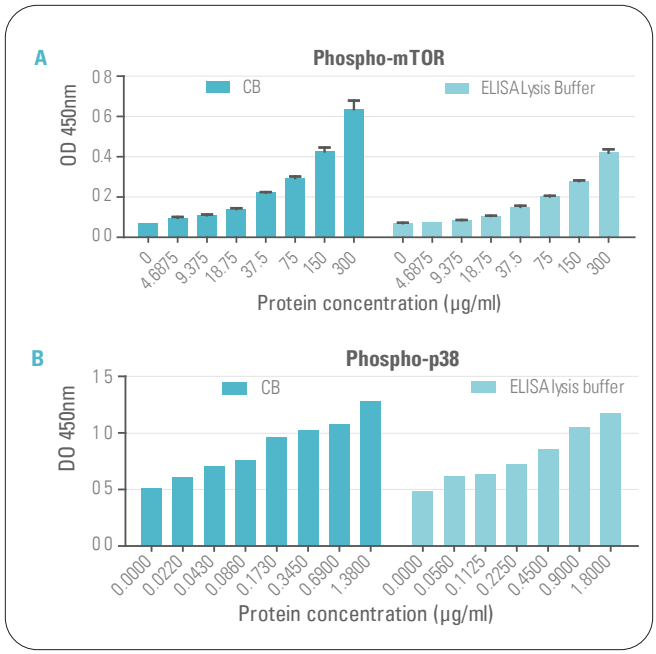


Figure 5: Detection of phospho-mtor (A) and phospho-p38 (B) by ELISA using HTRF phospho-total lysis buffer and ELISA lysis buffer.

The results obtained by Western blot on Phospho-STAT3 and Phospho-AKT (Figures 6A and 6B) demonstrate that HTRF phospho-total lysis buffer is not only as efficient as RIPA buffers to release proteins from various biological samples but is also compatible with other methods.

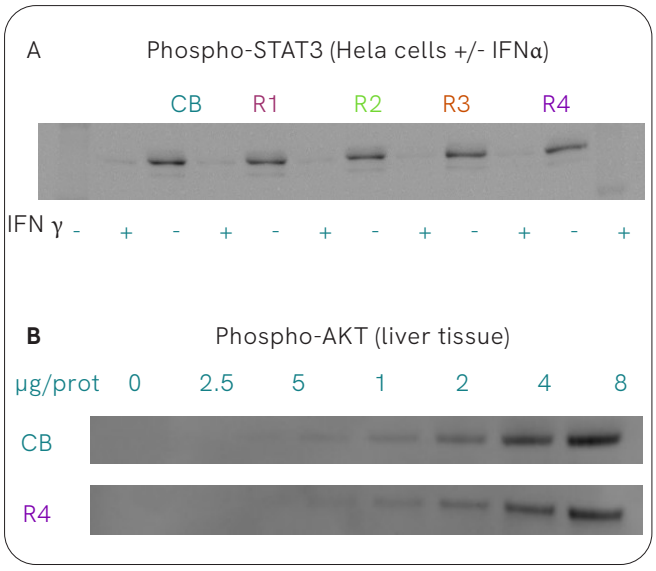


Figure 6: Detection of phospho-STAT3 (A) and phospho-AKT (B) by western blot using the HTRF phospho-total lysis buffer and RIPA R4 lysis buffer.

Conclusions

HTRF phospho-/total protein assay kits are easy “one mix-and-read” assays, based on the use of an optimized gentle lysis buffer. Here we provide evidence that the HTRF Phospho-total lysis buffer delivers many advantages over commonly-used RIPA lysis buffers. First, HTRF phospho-total lysis buffer efficiently releases cytoplasmic and nuclear proteins. Additionally, HTRF phospho-total lysis buffer is compatible with biological samples of varying complexity from standard cell lines to tumor xenografts and tissues. Finally, HTRF phospho-total lysis buffer outperforms the RIPA buffers in HTRF assays, and is also perfectly suited for other methods of analysis. In summary, Revvity’s HTRF phospho-total lysis buffer is a universal lysis buffer that can be broadly applied to biochemical studies.

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