APPLICATION NOTE

Best practices for analyzing tumor xenografts with HTRF phospho assays.

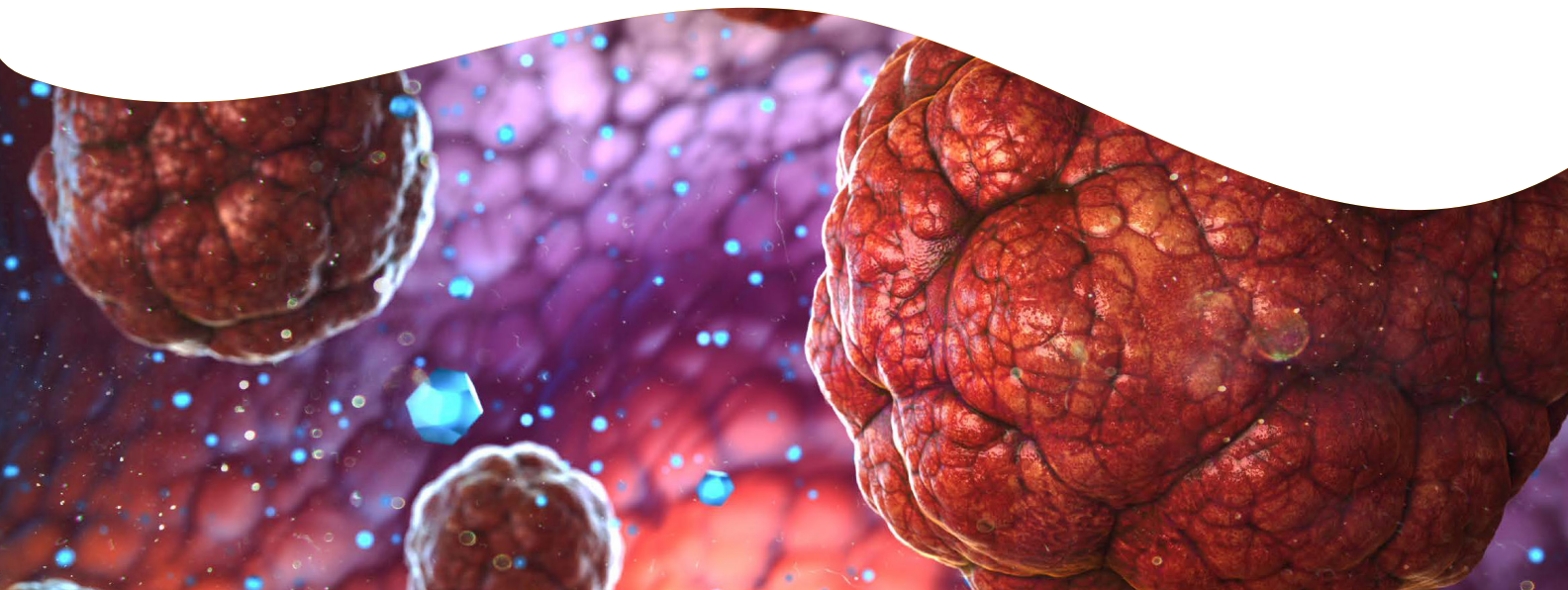
This technical note provides procedures and guidelines for performing HTRF phospho-protein assays on tumor xenografts, with a particular focus on the preparation of tumor lysates and working samples.

Abstract

HTRF® is a highly reproducible, reliable technique for evaluating the *in vivo* efficacy of novel anti-tumor therapeutics using tumor xenografts. This technical note provides procedures and guidelines for performing HTRF phospho-protein assays on tumor xenografts, with a particular focus on the preparation of tumor lysates and working samples.

Introduction

Revvity HTRF phospho-/total protein kits are designed to be robust, reliable, and easy to use. However, proper preparation and handling of tumor xenografts, lysates, and working samples is essential. By following the directions in this technical note precisely, you will save time and be assured of optimal, reliable results.



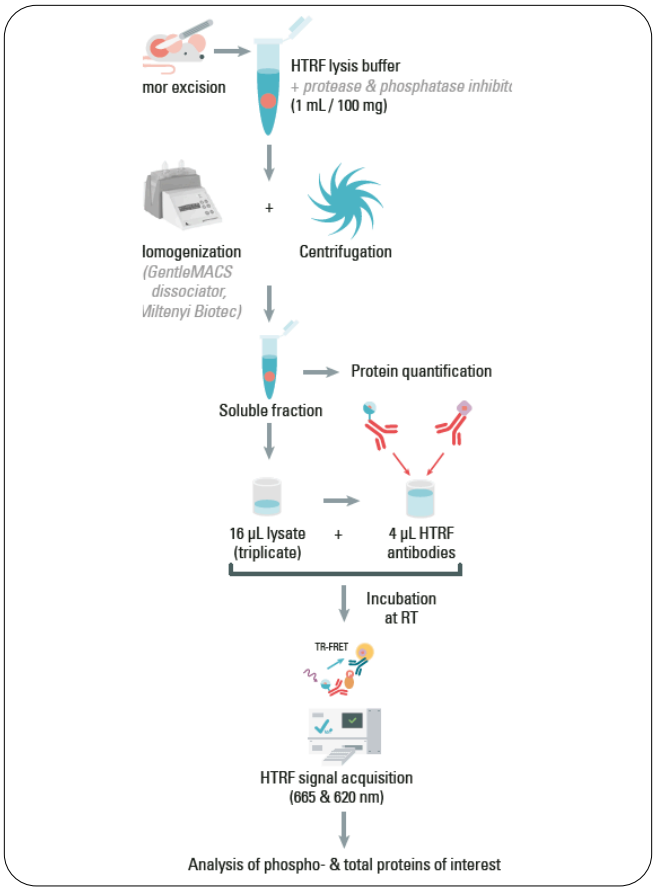


Figure 1: HTRF process flow for tumor xenograft analysis

Preparation of tumor xenograft lysates

Be sure to keep samples on ice until transfer into the HTRF detection plate and addition of HTRF antibodies. Use of incorrect lysis buffer, dilution of lysis buffer, or improper temperature control of tumor xenografts and lysates may affect the accuracy of your results.

Reagents & devices

- GentleMACS™ Dissociator in association with GentleMACS M tubes (Miltenyi Biotec)
- Phospho-/total protein lysis buffer (Revvity Bioassays) provided in every HTRF kit (dilute lysis buffer stock solution 4-fold in distilled water)
- Complete Protease Inhibitor Cocktail Tablets, EDTA-free and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche, product Nos. 04693159001 and 04906845001) (dilute 1 tablet of each in 10 mL of 1X lysis buffer)

Procedure

1. Excise the tumor xenografts and store at -80°C.
2. Before homogenization, place tumors on ice and weigh in order to adjust the volume of lysis buffer to the tumor weight (1 mL/100 mg).
   a. The minimum volume of lysis buffer required in the GentleMACS M tube is 300 µL and the maximum is 10 mL. If necessary, divide the tumor into two pieces or decrease the volume of lysis buffer (1 mL/200 mg).
   b. Prepare 1X lysis buffer on ice and supplement it with protease and phosphatase inhibitor cocktail tablets.
3. Homogenize the tumor using the GentleMACS Dissociator. Transfer the tumor into the M Tube and add the volume of lysis buffer required. Run "Protein" program (~1 min.) If one run is not sufficient to entirely homogenize the tumor (e.g. with big tumors), run the program another time.
4. After lysis, keep the tubes on ice and then centrifuge for 10 min at 16,000 g, 4°C. Collect soluble fractions, aliquot (to avoid freeze/thaw cycles) and store at -80°C.

Determine optimal working protein concentrations

In this step, you first determine the initial protein concentration of each sample and normalize samples to be able to compare the results between control (untreated) and treated mice. Then prepare serial dilutions of sample lysate to determine the linear range of protein concentrations for the assay. The goal is to work with a protein concentration that gives a sufficient signal (S/B) but which is still in the assay's linear range.

Reagents & devices

- QuantiPro BCA Assay kit (SIGMA)
- Phospho-/total protein lysis buffer (Revvity Bioassays) provided in every HTRF kit (dilute lysis buffer stock solution 4-fold in distilled water)
- Phospho-/total protein blocking reagent (Revvity Bioassays) provided in every HTRF kit (dilute blocking reagent stock solution 100-fold in 1X lysis buffer)

Procedure

1. Use one aliquot of tumor lysate to determine the protein concentration of each sample (with the QuantiPro BCA Assay kit from SIGMA).
2. Before HTRF analysis, thaw samples on ice and then dilute to the same initial concentration of proteins (e.g. 1 mg/mL) in 1X lysis buffer prepared on ice and supplemented with the blocking reagent.
3. Prepare several 1:2 serial dilutions of each normalized sample in the same lysis buffer and test to define a range of optimal protein concentrations that ensures operation in the assay's linear range (as illustrated in Fig. 1).
4. Based on linearity results, prepare working samples for assay.

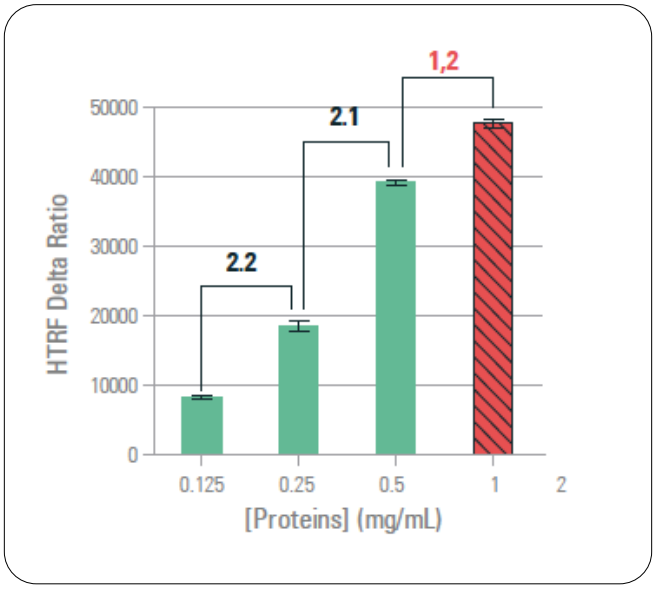


Figure 2: Example of sample dilutions performed on a human pancreatic BxPC3 tumor xenograft lysate using HTRF total AKT assay. By calculating the multiplying factor between the htrf delta ratio of two serial dilutions, it is possible to conclude that, in this case, the signal is linear up to 0.5 Mg/ml (green bars).

Perform and analyze HTRF assays

For HTRF detection, please follow kit instructions. As mentioned in the package insert, perform incubation with HTRF antibodies in the detection plate at room temperature.

1. Calculate HTRF Ratio = (665 signal / 620 signal) x 10,000 for each well.
2. Calculate Delta Ratio = HTRF Ratio $_{sample}$ - Mean HTRF Ratio $_{negative.}$
3. Calculate Mean Delta Ratio, Standard deviation (SD) and CV% (= SD/Mean Delta Ratio) for each replicate.

*negative = tumor lysate dilution replaced by 1X lysis buffer supplemented with the blocking reagent

Conclusions

This technical note provides detailed procedures and guidelines for HTRF assays on tumor xenografts. Focusing on careful preparation of lysates and working samples using the right reagents and conditions, as described, will ensure that you obtain accurate results that you can interpret correctly.

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