TECHNICAL NOTE

Best practices for analyzing brain samples with HTRF phospho assays for neuroscience.

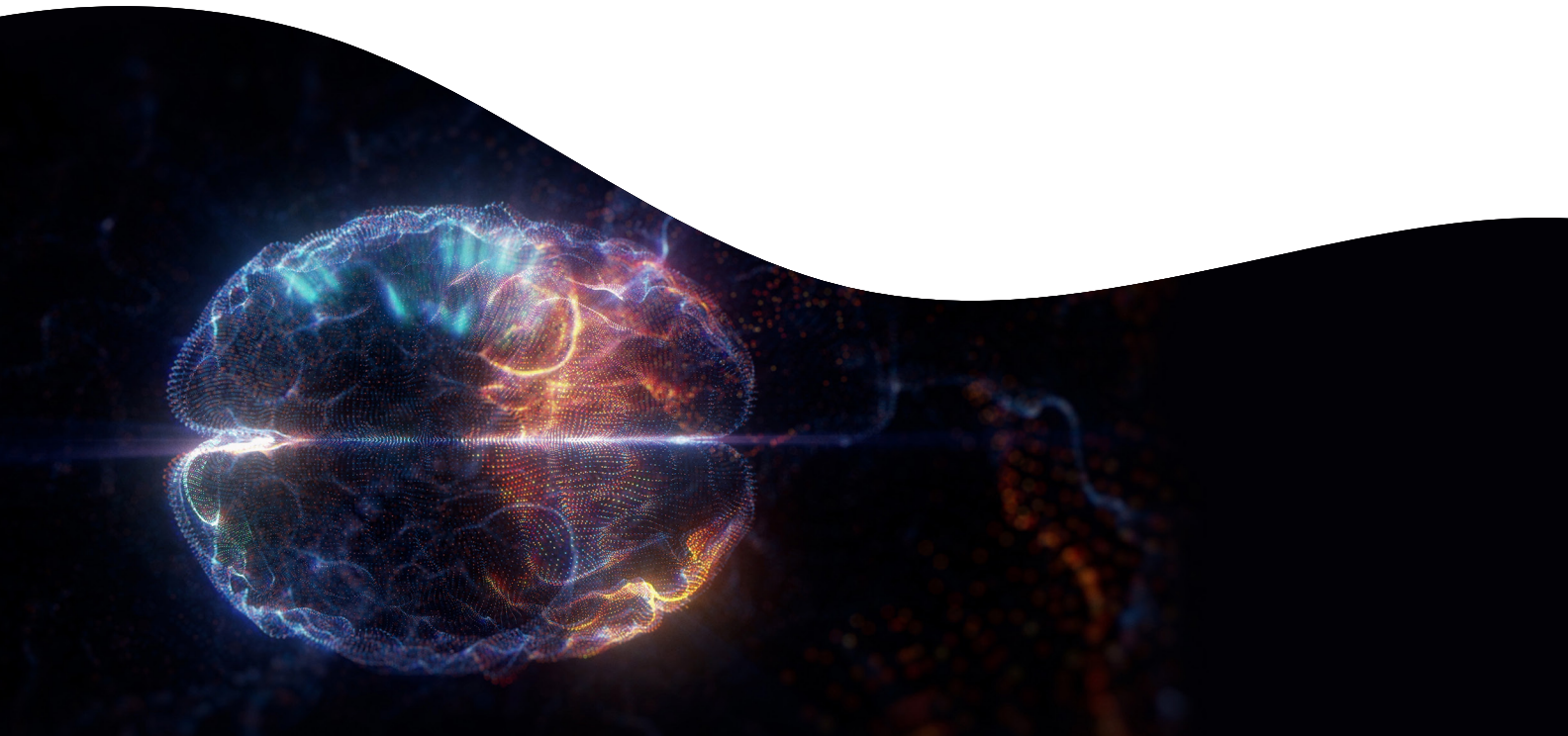
Focusing on the careful preparation of lysates and working samples using the right reagents and conditions, this technical note will ensure that you obtain accurate results that you can interpret correctly.

Abstract

Revvity's HTRF® assays for Neurosciences are based on a highly reproducible, reliable technique for the accurate measurement of Neuromarkers of interest using brain samples. This technical note provides procedures and guidelines for performing HTRF phospho-protein assays on brain samples, with a special focus on the preparation of brain lysates and working samples.

Introduction

Revvity's HTRF phospho-/total protein kits are designed to be robust, reliable, and easy to use. However, the proper preparation and handling of brain extracts, 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.



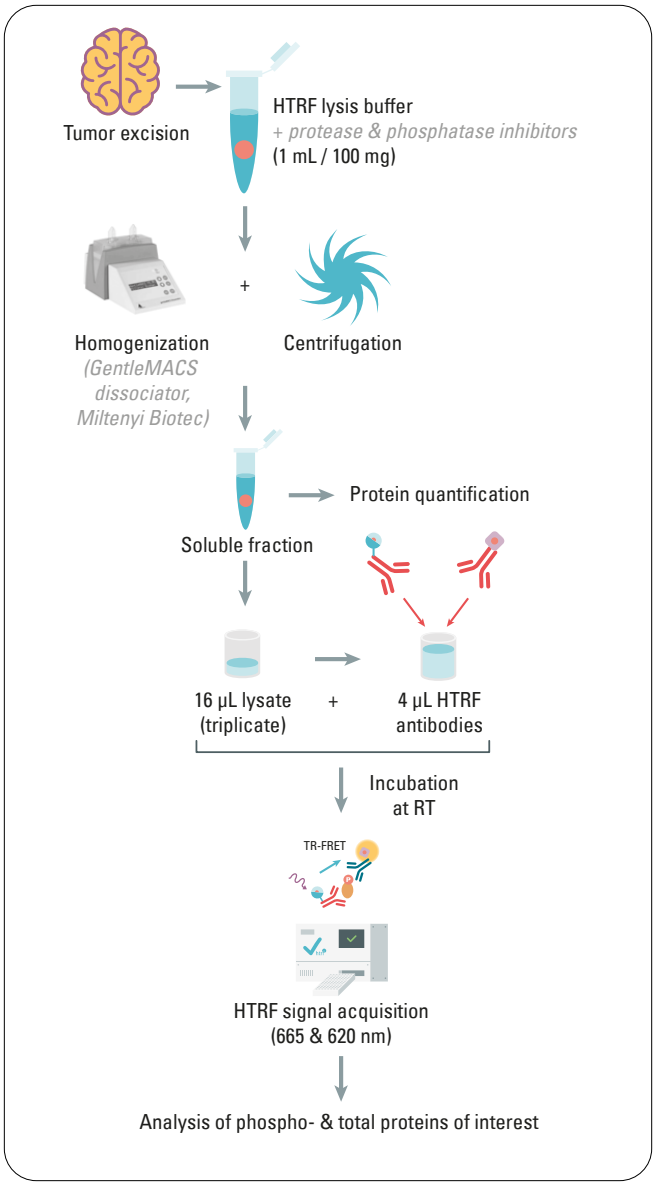


Figure 1: HTRF process flow for tissue analysis

Preparation of brain lysates for cns applications

Be sure to keep samples on ice until their transfer into the HTRF detection plate (96, 384 or 1536 wells) and the addition of HTRF reagents. Use of an incorrect lysis buffer, dilution of lysis buffer, or improper temperature control of structures and lysates will affect the accuracy of your results.

Reagents and devices

- GentleMACS™ Dissociator in association with GentleMACS™ M tubes (Miltenyi Biotec) or sonicator (e.g. Sonifier®)
- Phospho-/total protein lysis buffer (Revvity Bioassays): dilute lysis buffer stock solution 4-fold in distilled water
- Cellular kinase blocking reagent 100X (Revvity Bioassays): dilute blocking reagent stock solution 100-fold in 1X lysis buffer

Procedure (Fig.1)

1. Excise the tissue and store at -80°C
2. Before homogenization, place the brain sample on ice and weigh it in order to adjust the volume of lysis buffer to the tissue weight (1 mL/100 mg)
   a. The minimum volume of lysis buffer required in the GentleMACS™ M tube is 1 mL and the maximum is 10 mL
   b. Prepare 1X lysis buffer on ice and supplement it with blocking reagent at 1X
3. Homogenize the tissue using the GentleMACS™ Dissociator. Transfer the tissue into the M Tube and add the required volume of lysis buffer. Run "Protein" program (~1 min.) If one run is not sufficient to entirely homogenize the tissue, run the program a second time. Sonication can be used as an alternative method.
4. After lysis, keep the tubes on ice and then centrifuge for 10 min at 1800 g, 4°C. Collect soluble fractions, aliquot (to avoid freeze/thaw cycles) and store at -80°C.

Determine optimal working protein concentrations

This step is designed to ensure that you work with a protein concentration that gives sufficient signal (S/B) while remaining in the linear range of the assay. The first step is to determine the initial protein concentration in each sample and normalize samples to be able to compare the results between the control (untreated) and treated mice. Then prepare serial dilutions of sample lysates to determine the linear range of protein concentrations for the assay.

Reagents and devices

- Bradford assay or BCA assays
- Phospho-/total protein lysis buffer (Revvity Bioassays) provided in the HTRF kit (dilute lysis buffer stock solution 4-fold in distilled water)
- Cellular kinase blocking reagent 100X (Revvity Bioassays) (dilute blocking reagent stock solution 100-fold in 1X lysis buffer)

Procedure

1. Use one aliquot of tissue lysate to determine the protein concentration in each sample (with the Bradford assay or BCA assays).
2. Before HTRF analysis, thaw samples on ice and then dilute to the same initial concentration of proteins (e.g. 2 mg/mL to 125µg /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 within the assay's linear range.
4. Based on the linearity results, prepare working samples for assay.
5. Fig. 2 shows an example with Tau aggregation assay. As you can see, for high protein concentrations (above 1.86 mg/ml), the signal decreases, meaning the Hook effect is beginning, and we are no longer in the linear range of detection. The actual assay range is between 0.033 and 1.86 mg/ml.

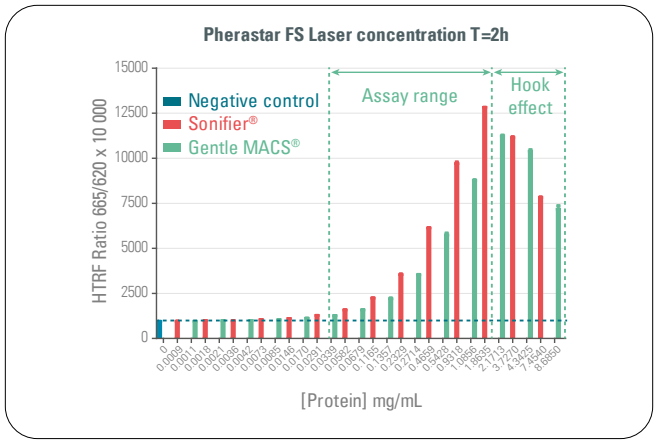


Figure 2: Example with TAU aggregation assay.

Perform and analyze HTRF assays

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

1. Calculate HTRF ratio = (665 signal/620 signal) x 10,000 for each well.
2. Compare the HTRF ratio from positive sample versus negative side by side to determine signal to noise.

Conclusions

This technical note provides detailed procedures and guidelines for HTRF assays on brain samples. Focusing on the 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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