Protein Disaggregation kit enables accurate HTRF detection of phosphorylated aggregation-prone proteins.

Key features

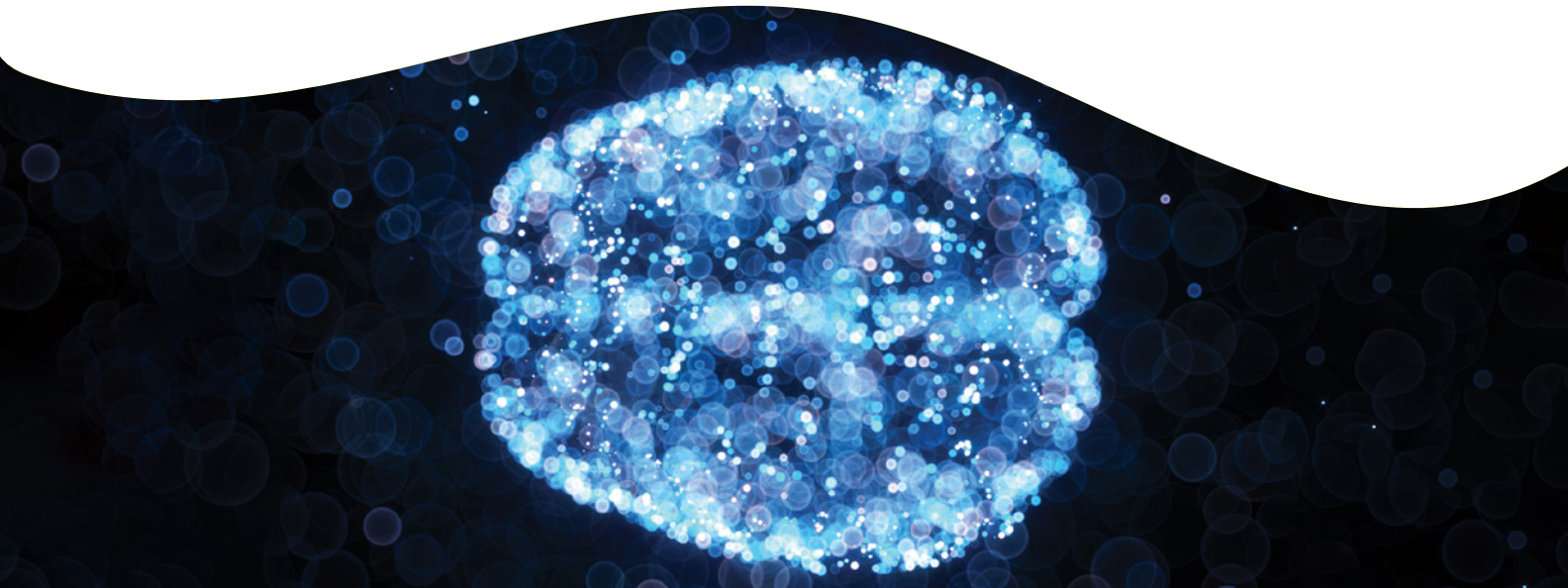
This technical note explains how to easily combine the Protein Disaggregation kit with the HTRF phosphorylation kits to ensure an accurate measurement of protein phosphorylation events in aggregated samples.

Introduction

HTRF® phosphorylation immunoassays allow for the detection of different levels of phosphorylated protein in cell lysates and may reflect signaling pathway activation or dysregulation leading to pathological states.

In the field of neuroscience, protein aggregation is a common marker of several neurodegenerative diseases that can be associated with an abnormal or exacerbated phosphorylation status (e.g., TAU, α-synuclein). In the context of protein aggregation, the assessment of phosphorylation events may be altered as a consequence of a lack of accessible phospho epitopes, leading to an underestimation. This challenge is common to all antibody-based detection techniques and requires insoluble/soluble protein fractionation steps.

As detailed in this technical note, combining the Protein Disaggregation kit and HTRF phospho immunoassays provides a new, easy-to-use, and reliable approach to detect all phosphorylation events of a protein of interest as demonstrated with TDP-43 protein, which is prone to aggregate in a pathological context.



Preparation of disaggregated samples

Prerequisite

Make sure the instructions provided in the package insert are followed. Use the appropriate supplemented lysis buffer and adjust the protocol to the adherent or suspension cell conditions. Depending on the cell lines used, the volume of lysis should be optimized and it might be necessary to dilute the cell lysate to ensure samples are within the assay's linear range. Make sure you have enough volume of samples for each condition tested, including the disaggregation procedure and the phospho HTRF assay of interest. After lysis, it is mandatory to collect and store samples in polypropylene microtubes or plates for treatment.

Principle

Once the samples are lysed, a rapid disaggregation step is performed on the samples (15 min) without any centrifugation step. After transfer of the treated samples, the standard protocol of the phospho HTRF immuno-assay is run by following the instructions provided in the kit's manual (Fig.1).

Reagents and device

- Protein disaggregation kit (Revvity #64DAGGRPET & 64DAGGRPEG)
- Phospho-Total protein Lysis buffer 1 to 5 (4X) respectively (Revvity #64KL1FDF, 64KL2FDF, 64KL3FDF, 64KL4FDF, 64KL5FDF): dilute lysis buffer stock solution 4-fold in distilled water for adherent cells protocol.
- Cellular kinase blocking reagent (100X) (Revvity #64KB1AAC): dilute blocking reagent stock solution 100-fold in 1X lysis buffer for adherent cells protocol or 25-fold in 4X lysis buffer for suspension cells protocol.
- ProxiPlate-384 Plus, White 384-shallow well (Revvity #6008280/9)

Procedure (Fig.2)

- Prepare supplemented lysis buffer with blocking reagent for suspension or adherent cells protocol as described in the kit manual.
- In a 96-well culture plate, add 50 µL of supplemented lysis buffer (1X) for adherent cells or 10 µL (4X) for suspension cells and incubate for at least 30 minutes at room temperature under shaking.
- After lysis, homogenize by pipetting up and down and pool at least three wells of the same condition for the detection of one targeted protein (multiply the number of wells proportionally to the kits being tested).
- Transfer 2 x 60 µL of sample into two vials or two plate wells.
- Add 10 µL of the Disaggregation buffer A or Control detection buffer C and homogenize by vortexing preferentially or pipetting up and down (at least three times).
- Incubate for 15 min at room temperature.

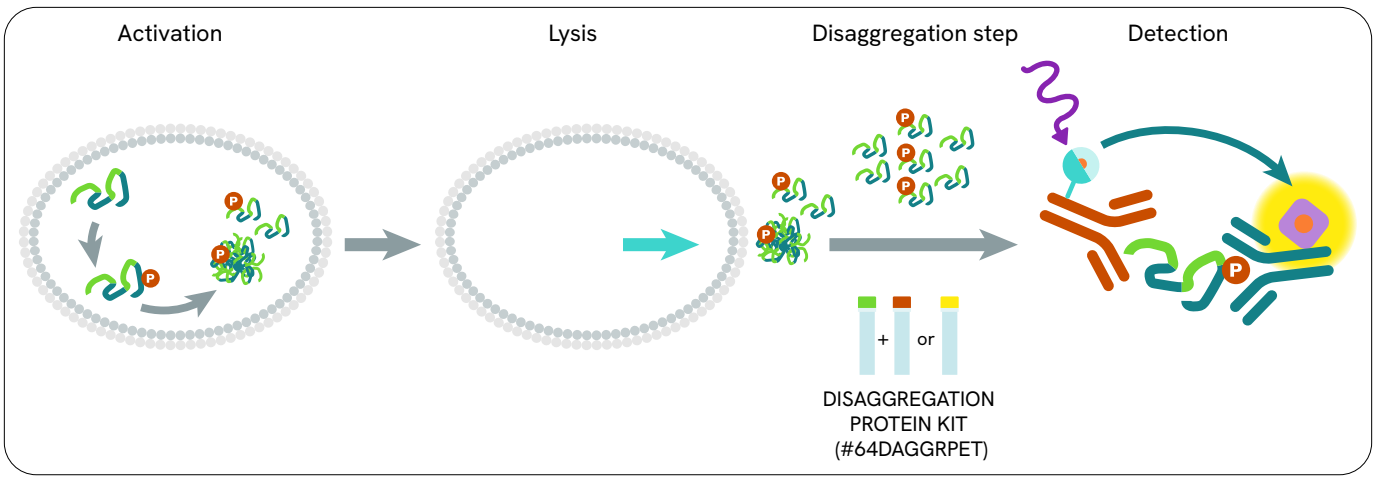


Figure 1: Protocol overview.

- Add 10 µl of the Disaggregation buffer A or Control detection buffer C and mix by vortexing preferentially or pipetting up and down (at least three times).
- After the disaggregation steps, samples are ready for phospho-total HTRF detection following the kit's instructions.
- For HTRF detection, please accurately follow the kit instructions. As mentioned in the package insert, transfer the appropriate volume of treated sample with protein disaggregation reagents in the assay plate to perform the HTRF measurement. Add HTRF reagents (Eu Cryptate and d2-labeled antibodies) and incubate the plate as recommended before reading on an HTRF-compatible reader.

Analyze HTRF phospho assay after disaggregation protocol

After reading the fluorescence emission at 665 nm and 620 nm on a compatible HTRF reader:

1. Calculate HTRF ratio = (665 signal/620 signal) x 10 000 for each well
2. Compare the HTRF ratio from the sample treated with Control detection buffer C (Control) versus the sample treated with Disaggregation buffer A+B (Disaggregated) to reveal any additional level of phosphorylated protein that was masked in the aggregated sample.
3. The aggregation level of total protein can be checked using the same protocol but with the Total HTRF assay of the targeted protein enabling calculation of an Aggregated Ratio
4. Aggregated Ratio = Ratio Disaggregated Sample / Ratio Control Sample

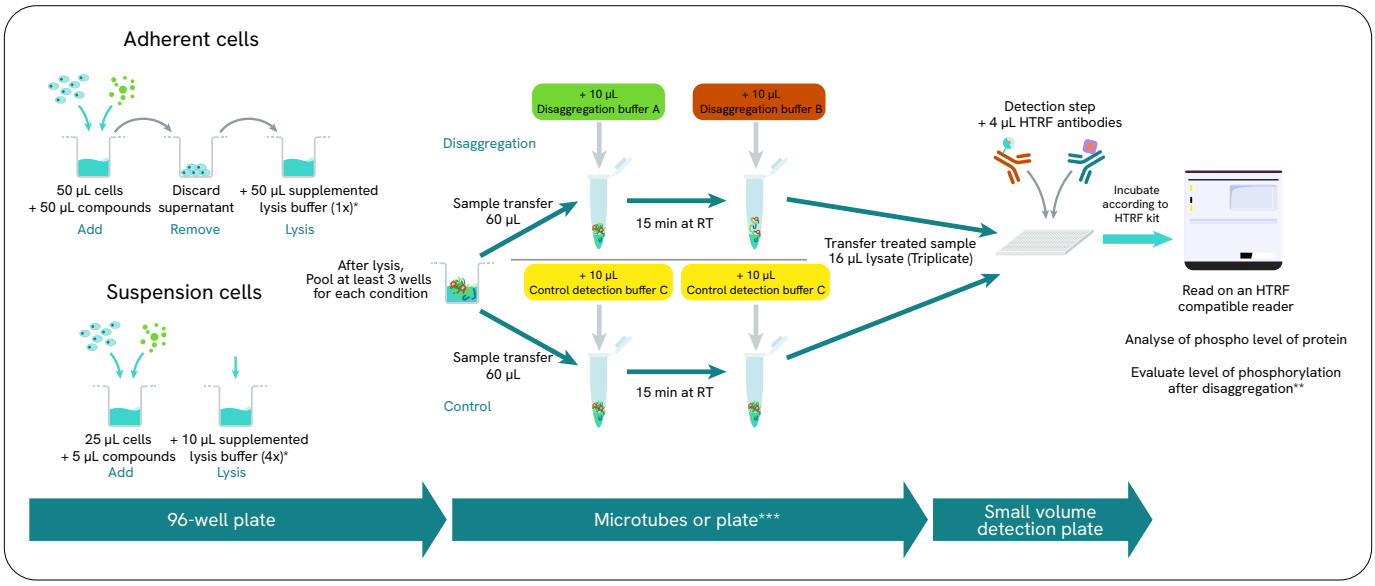


Figure 2: Workflow for protein disaggregation and HTRF® detection.
*Depending on the cell lines used, the volume of lysis should be optimized. Depending on the cell lines used, it may be necessary to dilute the cell lysate to ensure samples are within the assay linear range.
**Perform the same protocol to analyze the total level of protein and calculate the aggregated ratio.
***The sample disaggregation protocol was optimized for microtubes but it can be performed in microplates providing you are pipetting up and down at least three times.

Example with HTRF TDP-43 phospho Ser409/410

Staurosporine causes TDP-43 aggregation, as described previously (TDP-43 aggregation kits #62TDP43PEG) and confirmed below in Figure 3b, but also phosphorylation of TDP-43. The combination of the Protein Disaggregation kit and HTRF TDP-43 phospho S409/410 enables the detection of TDP-43 phosphorylation levels (4.5 fold change) induced by staurosporine (1 µM, 6h) in Hela cells (Fig. 3a). In the absence of disaggregation treatment, detection of TDP-43 phosphorylation events are not measurable (refer to Control condition) because of the lack of accessible phospho epitopes. The use of this disaggregation step provides an easy to implement and reliable solution to accurately measure protein phosphorylation levels in the context of protein aggregation.

On the other hand, Calyculin A treatment (100 nM, 30 min) induces phosphorylation of TDP-43 in Hela cells (Fig. 3c) that is detectable in Control C condition and after the disaggregation steps (A+B). Evaluation of the level of aggregation in these conditions with the TDP-43 aggregation kit (#62TDP43PEG) shows that there is no aggregation (Fig. 3d). In this case, detection of the level of phosphorylation is reliable even in the absence of disaggregation treatment.

Conclusion

This technical note provides detailed procedures to ensure the correct detection of protein phosphorylation in the context of aggregation. Because the phosphorylation of TDP-43 in aggregates is a pathological hallmark of TDP-43 proteinopathies, the use of the Protein Disaggregation kit prior to HTRF phospho assay detection is required for a reliable interpretation of the modulation of phosphorylation levels and could enable the identification of novel therapeutic drugs.

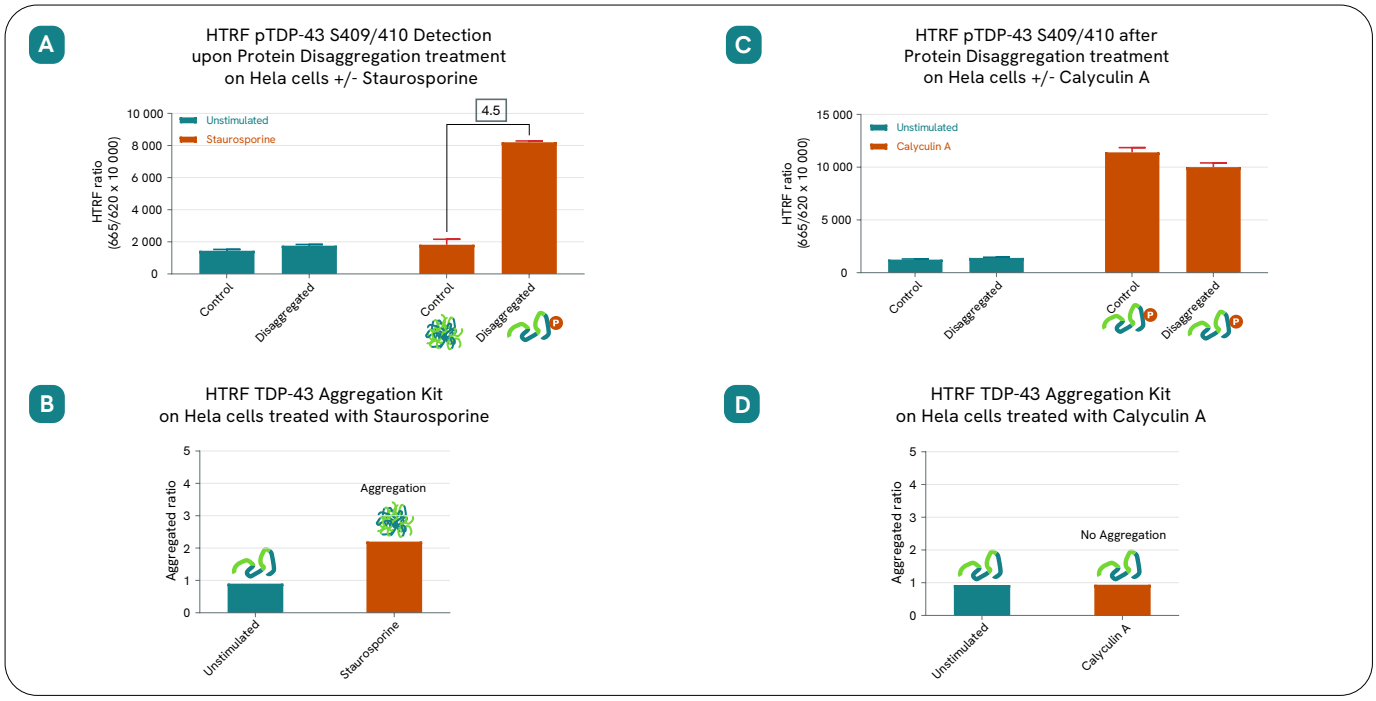


Figure 3: Example with TDP-43 pS409/410 assay.

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com