TDP-43 AGGREGATION KITS

PROTOCOL

Part # 62TDP43PEG & 62TDP43PEH

Test size#: 500 tests (62TDP43PEG) and 10,000 tests (62TDP43PEH) - assay volume: 20 µL

Revision: 02-July2020

Store at: -60°C or below (62TDP43PEG); -60°C or below (62TDP43PEH)

For research use only. Not for use in diagnostic procedures.

ASSAY PRINCIPLE

This kit is intended for the simple and rapid quantification of all forms of endogenous levels of TDP-43 protein aggregation in cells. Aggregated TDP-43 protein is produced by cells and after lysis of the cell membrane. The level of TDP-43 aggregation produced by cells can be detected using the kit reagents.

Following a disaggregation procedure for samples to extract TDP-43 from the aggregation, the antigen is detected in a sandwich assay format using 2 different specific antibodies, one labeled with Europium Cryptate (donor) and the second with d2 (acceptor).

The detection principle is based on HTRF® technology. When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). The two antibodies bind to the TDP-43 present in the sample, thereby generating FRET. Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the TDP-43 protein concentration (Fig. 1)

Signal intensity of sample containing TDP-43 protein aggregates is compared to signal intensity of the same sample following the disaggregation procedure. The ratio obtained between TDP-43 disaggregated/ TDP-43 aggregated on the same sample is proportional to level of aggregation of TDP-43.

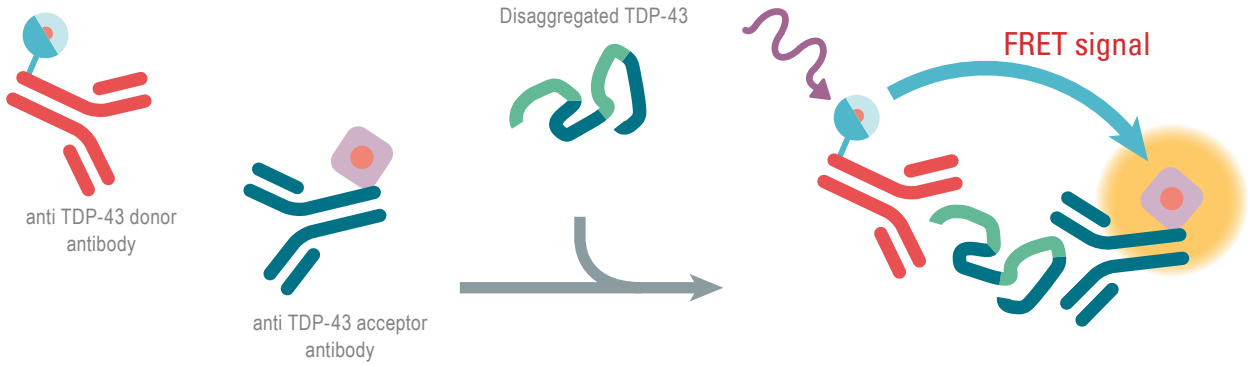
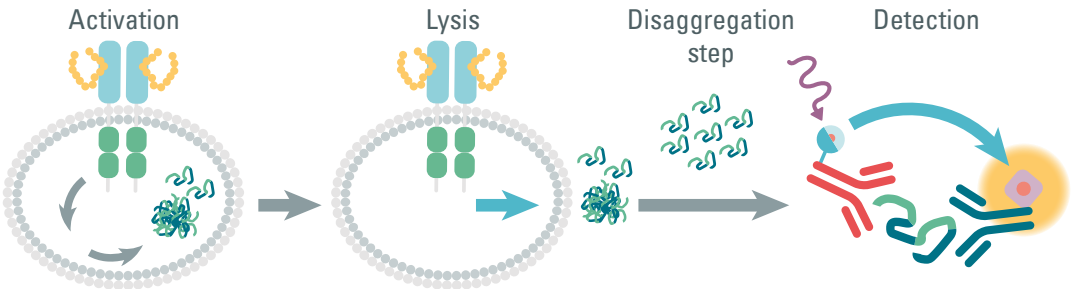
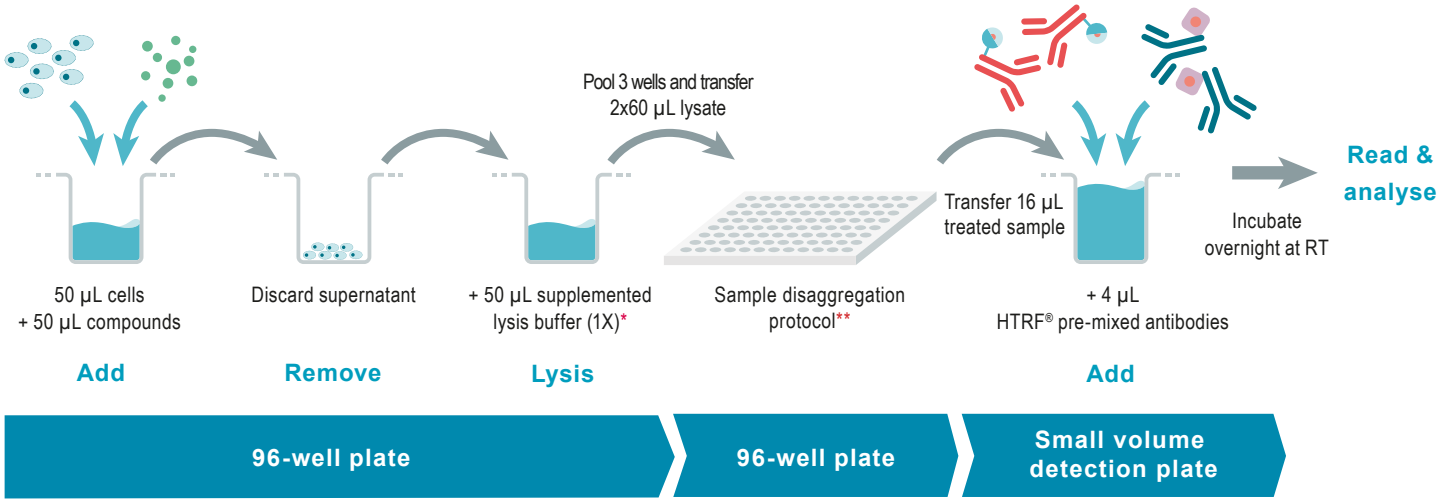


Figure 1: Principle of HTRF TDP-43 sandwich assay.

PROTOCOL AT A GLANCE





* Depending on cell lines used, volume of lysis should be optimized. Depending on cell lines used, it can be necessary to dilute the cell lysate to ensure samples are within the assay linear range

** see page 5

MATERIALS PROVIDED:

KIT COMPONENTS	STORAGE	500 TESTS * CAT # 62TDP43PEG		10,000 TESTS * CAT # 62TDP43PEH	
TDP-43 Eu Cryptate Antibody (stock solution 50X)	≤-16°C	orange cap	1 vial - 20 µL	red cap	1 vial - 0.4 mL
TDP-43 d2 Antibody (stock solution 50X)	≤-16°C	blue cap	1 vial - 20 µL	purple cap	1 vial - 0.4 mL
TDP-43 Control lysate (ready-to-use)	≤-60°C	green cap	1 vial - 250 µL	green cap	2 vials - 250 µL
Blocking reagent* (stock solution 100X)	≤-16°C	purple cap	1 vial - 300 µL	purple cap	3 vials - 2 mL
Lysis buffer* # 1 (stock solution 4X)	≤-16°C	transparent cap	4 vials - 2 mL	white cap	1 vial - 130 mL
Detection buffer ** (ready-to-use)	≤-16°C	orange cap	2 vials - 2 mL	red cap	1 vial - 50 mL
Disaggregation buffer A (ready-to-use)	2°-8°C	green cap	2 vials - 1.8 mL	green cap	1 vial - 120 mL
Disaggregation buffer B (ready-to-use)	2°-8°C	red cap	2 vials - 1.8 mL	red cap	1 vial - 120 mL
Control detection buffer C (ready-to-use)	2°-8°C	yellow cap	4 vials - 1.8 mL	yellow cap	1 vial - 120 mL

* When used as advised, the two available kit sizes will provide sufficient reagents for 500 and 10,000 tests respectively in 20 µL final. Assay volumes can be adjusted proportionally to run the assay in 96 or 1536 well microplates.
** The Detection buffer is used to prepare working solutions of acceptor and donor reagents.

PURCHASE SEPARATELY:

- HTRF®-Certified Reader. Make sure the setup for Eu Cryptate is used.

For a list of HTRF-compatible readers and set-up recommendations, please visit www.cisbio.com/compatible-readers

- Small volume (SV) detection microplates - .

For more information about microplate recommendations, please visit our website at: cisbio.com/microplates-recommendations

STORAGE AND STABILITY

Store the kit at -60°C or below.
Under proper storage conditions, reagents are stable until the expiry date indicated on the label.
Diluent and detection buffer are shipped frozen, but can be stored at 2-8°C in your premises.

Reagents

To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below .

Thawed diluent and detection buffer can be stored at 2-8°C in your premises.

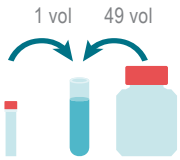
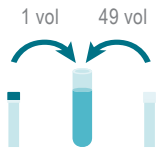
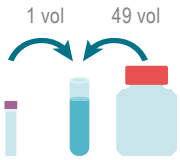
REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare reagents in the specified buffers. The use of an incorrect diluent may affect reagent stability and assay results.
- Thaw the frozen reagents at room temperature, allow them to warm up to room temperature for at least 30 mins before use
- We recommend centrifuging the vials gently after thawing, before pipeting the stock solutions.
- Take care to prepare stock and working solutions according to the directions for the kit size you have purchased.

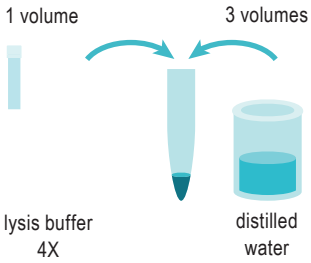
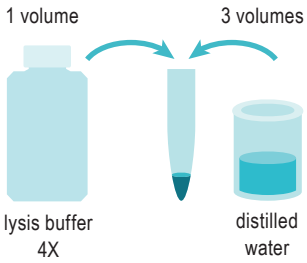
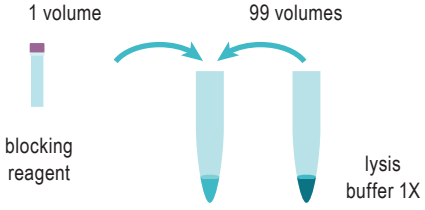
TO PREPARE ANTIBODY WORKING SOLUTIONS:

Each well requires 2 µL of TDP-43-Eu Cryptate Antibody and 2 µL of TDP-43-d2 Antibody.
Prepare the two antibody solutions in separate vials.

<table>
<tr><th colspan="2">500 TESTS KIT - 62TDP43PEG</th><th colspan="2">10,000 TESTS KIT - 62TDP43PEH</th></tr>
<tr><td colspan="4">TDP-43 Eu Cryptate antibody</td></tr>
<tr><td>Dilute 50-fold the 50X stock solution (thawed reagent) of human TDP-43 Eu Cryptate antibody with Detection buffer: add 1 volume of Eu Cryptate antibody stock solution in 49 volumes of detection buffer (e.g. 20 µL of Eu Cryptate antibody stock solution + 980 µL of detection buffer).</td><td> 
</td><td> 
</td><td>Dilute 50-fold the 50X stock solution (thawed reagent) of human TDP-43 Eu Cryptate antibody with Detection buffer: add 1 volume of Eu Cryptate antibody stock solution in 49 volumes of detection buffer (e.g. 0.4 mL of Eu Cryptate antibody stock solution + 19.6 mL of detection buffer).</td></tr>
<tr><td colspan="4">TDP-43 d2 antibody</td></tr>
<tr><td>Dilute 50-fold the 50X stock solution (thawed reagent) of human TDP-43 d2 antibody with Detection buffer: add 1 volume of d2 antibody stock solution in 49 volumes of detection buffer (e.g. 20 µL of d2-antibody stock solution + 980 µL of detection buffer).</td><td> 
</td><td> 
</td><td>Dilute 50-fold the 50X stock solution (thawed reagent) of human TDP-43 d2 antibody with Detection buffer: add 1 volume of d2 antibody stock solution in 49 volumes of detection buffer (e.g. 0.4 mL of d2 antibody stock solution + 19.6 mL of detection buffer).</td></tr>
<tr><td colspan="4">Antibody mix</td></tr>
<tr><td>It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2 antibody solution to 1 volume of Cryptate antibody solution (e.g. 1 mL of d2 antibody + 1 mL of Cryptate antibody).</td><td colspan="2">  </td><td>It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2 antibody solution to 1 volume of Cryptate antibody solution (e.g. 20 mL of d2 antibody + 20 mL of Cryptate antibody).</td></tr>
</table>

TO PREPARE SUPPLEMENTED LYSIS BUFFER FOR ASSAY PROTOCOL ON ADHERENT CELLS:

Determine the amount of supplemented lysis buffer needed for the experiment. Each well requires generally 50 µL of supplemented lysis buffer. Prepare a lysis buffer solution 1X and then dilute the blocking reagent stock solution 100-fold with this lysis buffer 1X.
In practice:

<table>
<tr><th colspan="2">500 TESTS KIT - 62TDP43PEG</th><th colspan="2">10,000 TESTS KIT - 62TDP43PEH</th></tr>
<tr><td colspan="4">Lysis buffer 1X</td></tr>
<tr><td>Dilute the “lysis buffer 4X” 4-fold with distilled water to prepare lysis buffer 1X.
E..g. take 1.25 mL of lysis buffer 4X and add it to 3.75 mL of distilled water. Mix gently..</td><td> 
</td><td> 
</td><td>Dilute the “lysis buffer 4X” 4-fold with distilled water to prepare lysis buffer 1X.
E..g. take 1.25 mL of lysis buffer 4X and add it to 3.75 mL of distilled water. Mix gently.</td></tr>
<tr><td colspan="4">Supplemented Lysis buffer 1X</td></tr>
<tr><td>Dilute the “blocking reagent” 100-fold with “Lysis buffer 1X”.
E.g. take 0.05 mL of “Blocking reagent stock solution” and add it to 4.95 mL of lysis buffer 1X. Mix gently.</td><td colspan="2"> 
</td><td>Dilute the “blocking reagent” 100-fold with “Lysis buffer 1X”.
E.g. take 0.05 mL of “Blocking reagent stock solution” and add it to 4.95 mL of lysis buffer 1X. Mix gently.</td></tr>
</table>

TO PREPARE WORKING CONTROL LYSATE SOLUTION

The control lysate is only provided as an internal assay control to check the quality of the results obtained.

The Aggregated ratio of this control should be around 2 corresponding to a median detection of TDP-43 aggregates.

Thaw the control lysate. Mix gently, the control lysate is ready to use.

TO PREPARE SAMPLES:

IMPORTANT RECOMENDATIONS:

- The samples should be diluted (we recommed to test several dilutions) in lysis buffer (1X) to ensure samples are within the assay linear range.
- The efficacy of disaggregation step can be verify comparing the aggregated ratio of the control lysate (around 2) with the tested sample.
- It is mandatory to collect and store samples into polypropylene microtubes and to treat samples into polypropylene plate or microtubes.
- To determine an aggregated ratio in samples TDP-43 have to be extracted from the aggregates using the disaggregation procedure described below.

SAMPLE ACTIVATION PROCEDURE

Each detection well requires 16 µL of activated samples.

⇨Add 1 volume (10 µL recommended) of the Disaggregation buffer A or Control detection buffer C.

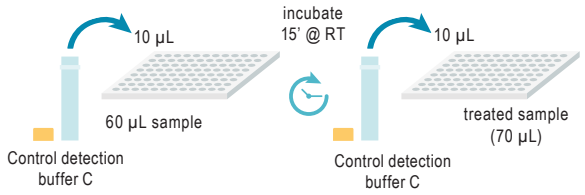
⇨Mix pipetting up and down 2 or 3 times (or vortex in tubes).

⇨Incubate for 15 minutes at room temperature.

⇨Add 1 volume (10 µL recommended) of the Disaggregation buffer B or Control detection buffer C.

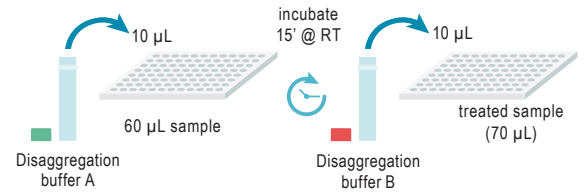
⇨Mix gently.

CONTROL PROTOCOL



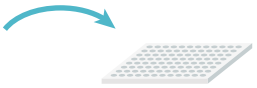
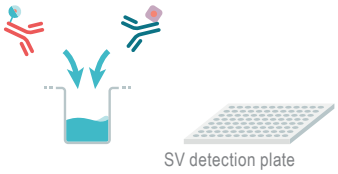
First step: add Control detection buffer C to the sample
(e.g. add 10 µL of Control detection buffer C to 60 µL of sample.)
Incubation: 15' at room temperature.
Second step: Add Control detection buffer C to the treated sample
(e.g. add 10 µL of Control detection buffer C to 70 µL of treated sample.)

DISAGGREGATION PROTOCOL



First step: add Disaggregation buffer A to the sample
(e.g. add 10 µL of Disaggregation buffer A to 60 µL of sample.)
Incubation: 15' at room temperature.
Second step: Add Disaggregation buffer B to the treated sample
(e.g. add 10 µL of Disaggregation buffer B to 70 µL of treated sample.)

TDP-43 AGGREGATION ASSAY PROTOCOL

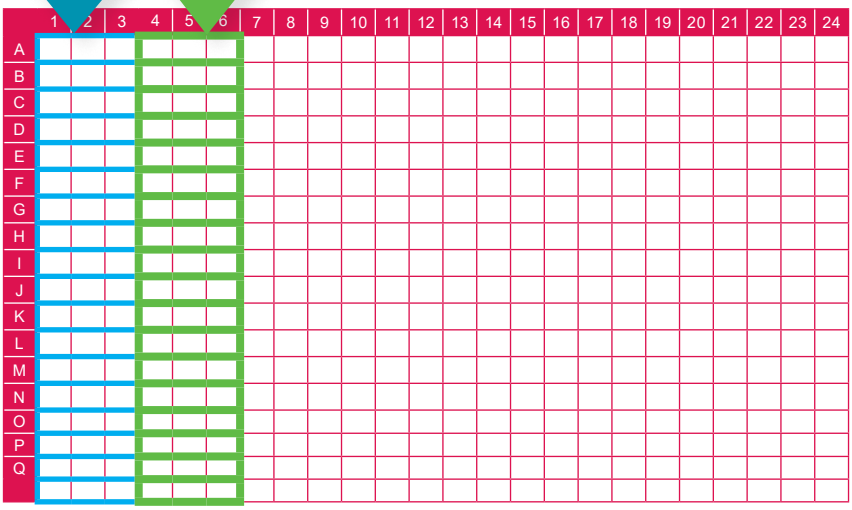
Add 50µL of supplemented lysis buffer (1X) to the plated cells and incubate for at least 30 minutes at room temperature under shaking. Use the appropriate supplemented lysis buffer. Lysis incubation time may be optimized. Lysis volume can be decreased down to 25 µL.	
After homogenization by pipeting up and down, pool 3 wells Transfer 2 x 60 µL from the 96-well cell-culture plate into 2 wells of a 96-well plate	
Add 10 µL of the Disaggregation buffer A or Control detection buffer C and homogeneize by pipetting up and down. Incubate 15 min at room temperature Add 10 µl of the Disaggregation buffer B or Control detection buffer C.	
Transfer 16 µL of cell lysate treated from the 96-well plate to a 384-small volume (SV) white detection plate	
Add 4 µL of premixed antibody solutions (vol/vol) prepared in the detection buffer. Cover the plate with a plate sealer. Incubate overnight at room temperature. Set up your reader for Eu3+ Cryptate and read the fluorescence emission at two different wavelenghts (665nm and 620nm) on a compatible HTRF® reader.	

STANDARD PROTOCOL FOR DISAGGREGATION OR CONTROL DETECTION

		Sample	Disaggregation buffer A	Disaggregation buffer B	Control detection buffer C	Control detection buffer C
Sample & kit Control lysate	Disaggregation	60 µL	10 µL	10 µL		
	Control	60 µL	-		10 µL	10 µL

	Samples	
Step 1	Dispense 16 µL of each sample “control” into each sample control well	Dispense 16 µL of each sample “disaggregeted” into each dissagregated sample well
Step 2	Add 2 µL of TDP-43 d2 antibody working solution to all wells	
Step 3	Add 2 µL of TDP-43 Eu Cryptate antibody working solution to all wells	
Step 4	Seal the plate and incubate ON @ RT Following incubation, the signal remains stable over a period of 48 hours.	
Step 5	Remove the plate sealer and read on an HTRF® compatible reader	

	1	2	3	4	5	6
A	16 µL Control ***Control lysate*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well A1	Repeat Well A1	16 µL Disaggregated ***Control lysate*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well A4	Repeat Well A4
B	16 µL Sample 1 ***Sample 1*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well B1	Repeat Well B1	16 µL Disaggregated ***Sample 1*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well B4	Repeat Well B4
C	16 µL Sample 1 ***Sample 2*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well C1	Repeat Well C1	16 µL Disaggregated ***Sample 2*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well C4	Repeat Well C4
D	16 µL Sample 1 ***Sample 3*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well D1	Repeat Well D1	16 µL Disaggregated ***Sample 3*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well D4	Repeat Well D4
E	16 µL Sample 1 ***Sample ..*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well E1	Repeat Well E1	16 µL Disaggregated ***Sample ..*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well E4	Repeat Well E4
F	16 µL Sample 1 ***Sample ..*** 4 µL pre-mixed Anti-TDP-43 antibodie	Repeat Well F1	Repeat Well F1	16 µL Disaggregated ***Sample ..*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well F4	Repeat Well F4
G	16 µL Sample 1 ***Sample ..*** 4 µL pre-mixed Anti-TDP-43 antibodie	Repeat Well G1	Repeat Well G1	16 µL Disaggregated ***Sample ..*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well G4	Repeat Well G4
H	16 µL Sample 1 ***Sample ..*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well H1	Repeat Well H1	16 µL Disaggregated ***Sample ..*** 4 µL pre-mixed Anti-TDP-43 antibodies	Repeat Well H4	Repeat Well H4



DATA REDUCTION

1. Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2. Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

3. Calculate the Aggregated Ratio for each sample.

$$\text{Aggregated Ratio} = \frac{\text{Ratio Dissagregated Sample}}{\text{Ratio Control Sample}}$$

For more information about data reduction, please visit http://www.cisbio.com/htrf-ratio-and-data-reductio

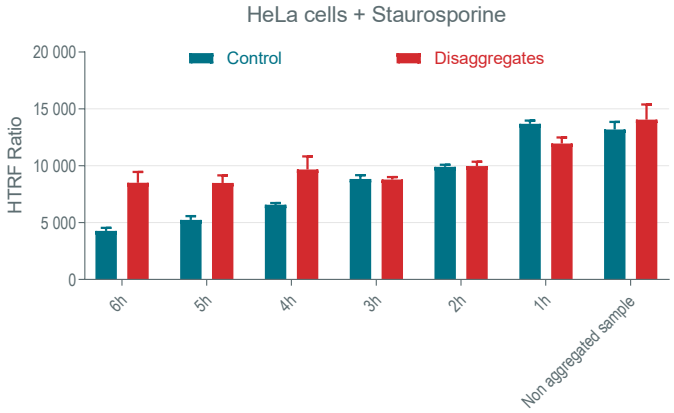
RESULTS

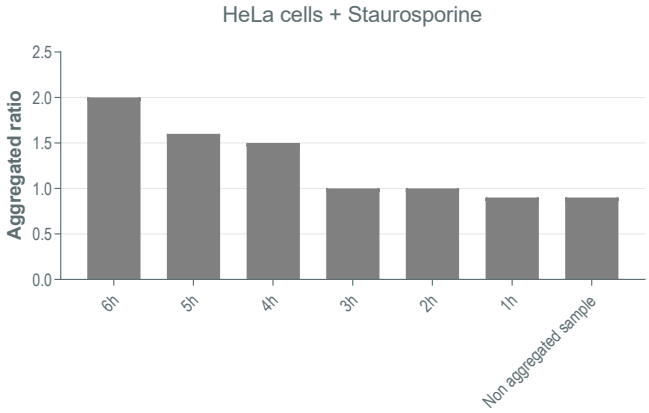
These data should be considered only as an example (readings on PHERAstarFS with flash lamp). Results may vary from one HTRF® compatible reader to another.

The data are drawn up by plotting HTRF® Ratio versus sample treated in control and disaggregation condition, then Aggregated ratio per sample.

The signal linearity is dependent both on the cell line and on the total protein detected. A cell density experiment is highly recommended to ensure working in optimal conditions.

Results on HeLa cells (50,000 cells/well), using the assay protocol for adherent cells. Cells were treated according to kinetic of staurosporine treatment from 1 to 6 hours at 1 µM and then lysed with supplemented lysis buffer #1 (1X) for 30 minutes at room temperature. 16 µL of control or disaggregated lysates were transferred in a small volume detection plate to detect TDP-43 using the HTRF TDP-43 aggregation assay - Cat # 62TDP43PEG, 62TDP43PEH. In these tested conditions, the aggregated ratio increases gradually from 4 to 6h under staurosporine treatment.





	Control		Disaggregates		
Samples	Mean HTRF Ratio	CV %	Mean HTRF Ratio	CV %	Aggregated Ratio
6h	4270	6.4	8503	11.3	2
5h	5243	6.4	8488	7.9	1.6
4h	6581	2.2	9678	11.9	1.5
3h	8843	3.7	8799	2.4	1
2h	9909	1.9	9981	3.8	1
1h	13699	2	11966	4.4	0.9
Non aggregated sample	13192	5	14059	9.5	0.9

GENERAL LAB WORK PRIOR USING CISBIO KIT: CELLS AND LYSATE PREPARATION: FREQUENTLY ASKED QUESTIONS / TROUBLESHOOTING PARAMETERS

Using adherent cells, allow time for your cells to recover after plating	Allow cells to regain full signaling capacity by plating them at least 6 hours before starting the pharmacological treatment.
Depending on the pathway, a serum starving step could be essential to reduce the basal level activity. This step should be optimized case-by-case.	Advice on cell culture conditions prior using Cisbio kit: - For adherent cells Before treating the cells with compounds, remove culture media from the plate and replace it with serum-free media before incubating from 2 hours up to overnight at 37°C. - For suspension cells Starvation step should be carried out in the flask. Harvest cells by centrifugation and re-suspend cells at a suitable cell density in serum-free media, incubate from 2 hours up to overnight at 37°C.
Generation of lysates	Ensure that the lysates used for the assay have been generated by using the HTRF® lysis buffer supplemented with the HTRF® blocking reagent, provided in the kit. Lysates generated with HTRF® buffers can be used in other technologies, like Western-blot. The blocking reagent contains only phosphatase inhibitors that prevent dephosphorylation of phosphorylated proteins from active serine/threonine and tyrosine phosphatases The lysis buffer is effective for creating cell extract under non denaturing conditions from both plated cells and cells pelleted from suspension cultures.
Using the two-plate assay protocol, a low signal can often be improved by adjusting lysis volumes.	In most cases, a typical adherent cell line grown in 96-well plates is readily detected in a lysis volume of 50µL. However, the lysis volume can be adjusted from 25 µL to 200 µL.
Using an improper cell density can induce poor sensitivity and low signal	Check that the cell density is correct. Too high or low cell numbers can affect assay performances
Parameters such as cell density, stimulation time and lysis incubation time should be optimized for each cell line used.	The assay can be used for many adherent and non-adherent cell types, including transfected cell lines and primary cells. However, the expression and phosphorylation of the readout of interest can vary from one cell line to another. Depending on the type of treatment, and the temperature, the stimulation time can vary widely. Because of this, we recommend a time course study to determine the optimal compound incubation time. Depending of the nature of your cells, lysis time may vary from 30' to 1h. Because of this, we also recommend determination of the optimal time.
Fluorescence reading	Using an inappropriate set-up may seriously impair the results. For information about HTRF® compatible readers and for set-up recommendations, please visit our website at: www.cisbio.com/htrf-compatible-readers
Batch production of cell lysates example of T175 flask	General lab work - prior using HTRF® kit: Day1: Dispense 3 million cells in T175cm2, add 25 mL of cell culture complete medium and incubate 3 days at 37°C, 5% CO2. Day4: cell stimulation Remove cell culture medium by aspiration, wash once (do not detach the cells), add 5 mL of compound (1x) diluted in medium and incubate at 37°C, 5% CO2, for the optimized time . Day4: cell lysis Remove stimulation medium, wash once (do not detach the cells), add 3 ml of 1X HTRF® lysis buffer supplemented with the HTRF® blocking reagent for 30 min at Room Temperature under orbital shaking. Transfer the cell lysate to a 15 mL vial and store aliquots at -60°C or below. For long term conservation, aliquots should be stored in liquid nitrogen.

FOR MORE INFORMATION
Europe and other countries +33(0)466-796-705 U.S. and Canada 1-888-963-4567 China +86-21-5018-9880
Japan +81-(0)43-306-8712 Visit www.cisbio.com to find a list of our regional distributors

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