MANUAL

HTRF PHOSPHO- LRRK2 (Ser935) DETECTION KIT

Part # 6FLRKPEG & 6FLRKPEH

Test Size#: 500 tests (6FLRKPEG), 10,000 tests (6FLRKPEH)

Revision: #07 of September 2023 **Store at:** ≤-60°C

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

ASSAY PRINCIPLE

This assay is intended for the simple, rapid and direct detection of endogenous levels of LRRK2 in cells, only when phosphorylated at Ser935. After lysis of the cell membrane, phospho-LRRK2 (Ser935) can be detected using the kit reagents.

As shown here, phospho-LRRK2 (Ser935) is detected in a sandwich assay format using 2 different specific antibodies, one labelled with Eu^{3+}-Cryptate (donor) and the second with d2 (acceptor).

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 specific signal modulates positively in proportion to phosphoLRRK2 (Ser935).

The assay can be run under a two-plate assay manual, where cells are plated, stimulated and lysed in the same culture plate. Lysates are then transferred to the assay plate for the detection of phospho-LRRK2 (Ser935) by HTRF® reagents. This manual gives the cells viability and confluence to be monitored.

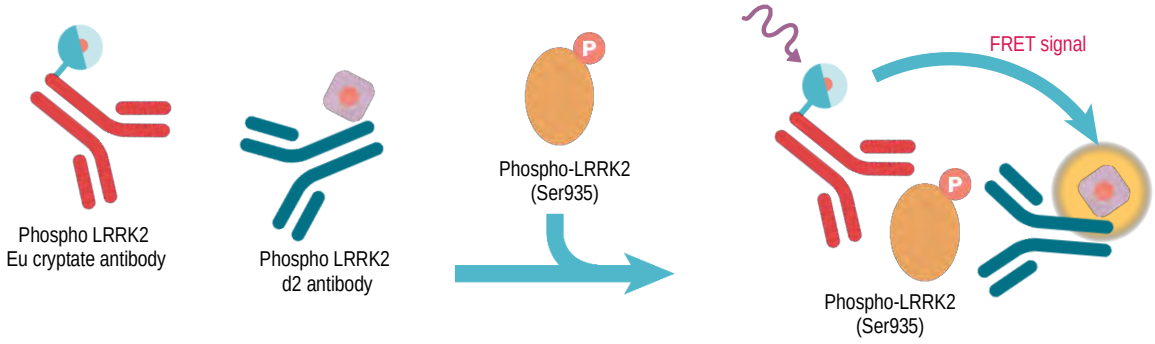
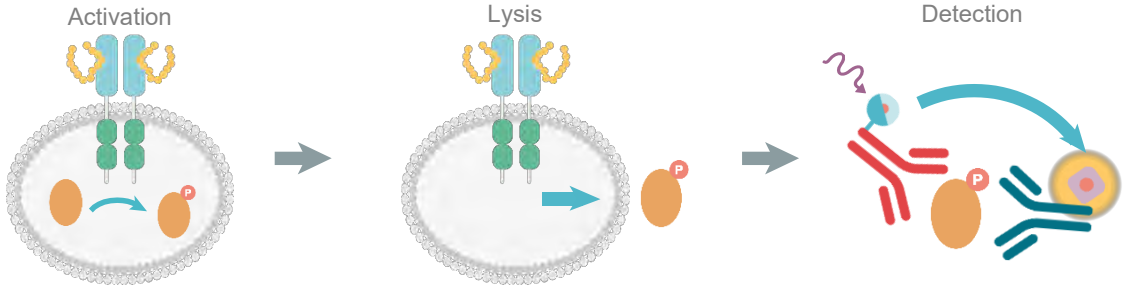


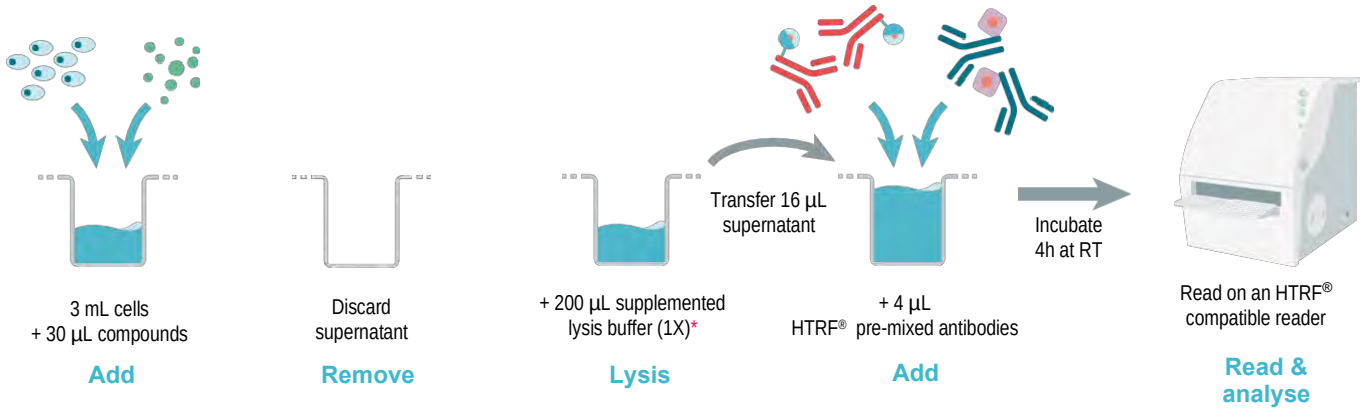
Figure 1. Principle of HTRF sandwich assay.

For tissue derived samples, please refer to the technical note: “Optimize your HTRF® cell signaling assays on tissues” on www.revvity.com
Technical support team can help you to set-up this manual or another one.
Please contact us at www.revvity.com

MANUAL AT A GLANCE



► TWO-PLATE ASSAY MANUAL FOR ADHERENT CELLS:



* Depending on cell lines used, volume of lysis should be optimized. It can also be necessary to dilute the cell lysate to ensure samples are within the assay linear range.
Technical support team can help you to set-up this manual, please contact us at www.revvity.com

► FOR HTRF CERTIFIED READER

For more information about HTRF® compatible readers and for set-up recommendations, please visit our website at: www.revvity.com

MATERIALS PROVIDED

KIT COMPONENTS	STORAGE	500 TESTS			10,000 TESTS		
Phospho LRRK2 Cryptate antibody	≤-16°C		red cap	1 vial-50 µL		red cap	1 vial-1 mL
Phospho LRRK2 d2 antibody	≤-16°C		blue cap	1 vial-50 µL		blue cap	1 vial-1 mL
Control lysate (ready-to-use)	≤-60°C		green cap	1 vial-150 µL		green cap	2 vials-150 µL
Blocking reagent* (stock solution 100X)	≤-16°C		purple cap	1 vial-300 µL		purple cap	3 vials-2 mL
Lysis buffer * #4 (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

* Amounts of reagents provided are sufficient for generating 50 µL of cell lysate per well.
** The Detection Buffer is used to prepare working solutions of acceptor and donor reagents.

► PURCHASE SEPARATELY

96-well or 384-well small volume (SV) detection microplates - For more information about microplate recommendations, please visit our website at: www.revvity.com

STORAGE AND STABILITY

Antibodies, control lysate and buffers should be stored frozen until use.
Thawed lysis buffer, detection buffer and blocking reagent can be stored at 2-8°C in your premises.
Thawed antibodies are stable 48 hours at 2-8°C; they can be refrozen (at ≤-16°C) and thawed at least one more time.
Control lysate must be stored frozen at ≤-60°C. Thawed control lysate can be refrozen (at ≤-60°C) and thawed one more time.

REAGENT PREPARATION

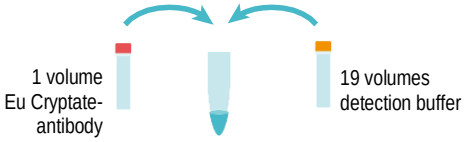
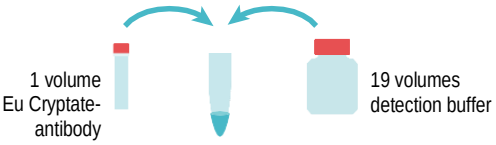
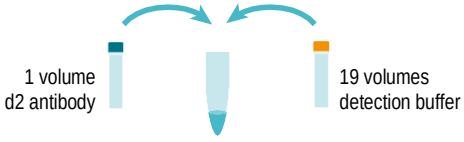
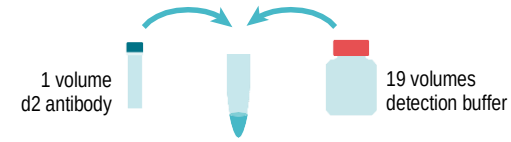
Allow all reagents to thaw before use.
We recommend centrifuging the vials gently after thawing, before pipeting the stock solutions.
Prepare the working solutions from stock solutions by following the instructions below.

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 window between control lysate and negative control should be greater than 2.

TO PREPARE WORKING ANTIBODY SOLUTIONS

HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the d2 and Cryptate-antibodies will impair the assay's quality.
Antibody working solutions are stable for 2 days at 4°C. Dilute the antibodies with detection buffer. In practice:

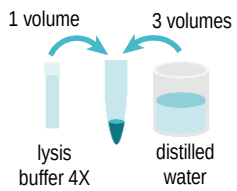
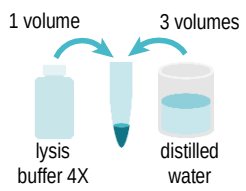
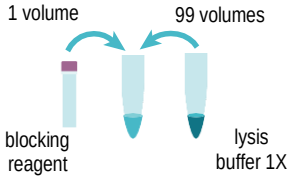
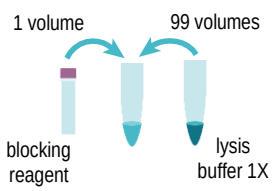
500 TESTS KIT	10,000 TESTS KIT
Phospho LRRK2 Eu Cryptate antibody	
	
Dilute 20-fold the frozen stock solution with detection buffer: e.g. add 0.95 mL of detection buffer to the 0.05 mL of Cryptateantibody stock solution.	Dilute 20-fold the frozen stock solution with detection buffer: e.g. add 19 mL of detection buffer to the 1 mL of Cryptate antibody stock solution
Phospho LRRK2 d2 antibody	
	
Dilute 20-fold the frozen stock solution with detection buffer: e.g. add 0.95 mL of detection buffer to the 0.05 mL of d2- antibody stock solution	Dilute 20-fold the frozen stock solution with detection buffer: e.g. add 19 mL of detection buffer to the 1 mL of d2-antibody stock solution.

TO PREPARE SUPPLEMENTED LYSIS BUFFER

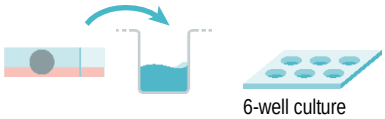
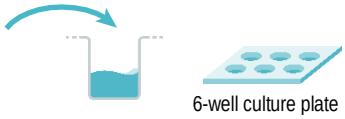
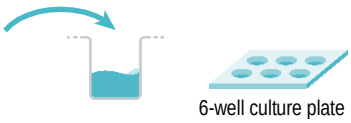
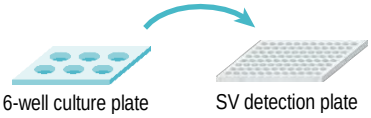
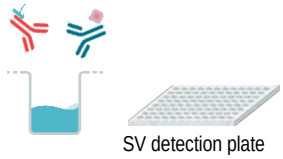
Make sure that the lysate has been generated by using the kit reagents.
Prepare the required amount of supplement lysis buffer before running the assay, working solutions are stable for 2 days at 2-8°C.

► **Supplemented Lysis buffer 1X for two-plate assay manual 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:

TWO-PLATE ASSAY MANUAL ON ADHERENT CELLS			
500 TESTS KIT & 10,000 TESTS KIT			
Preparation of lysis buffer 1X		Dilution of blocking reagent	
500 tests	10,000 tests	500 tests	10,000 tests
			
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.		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.	

TWO PLATE ASSAY MANUAL – FOR ADHERENT CELLS

	GENERAL LAB WORK USING HTRF KIT	
1	Plate 3 mL of cells in 6-well tissue-culture treated plate in appropriate growth medium and incubate overnight, at 37°C in CO2 atmosphere.	 6-well culture plate
	Cell seeding densities of 2,000,000 cells/well are generally sufficient for most cell lines, but optimization of cell seeding densities is recommended. Depending on receptor a starving step with serum-free medium could be essential.	
2	Dispense 30 µL of compounds (100X) diluted in cell culture serum-free medium	 6-well culture plate
	For most compound, incubation time is between 10 and 30 minutes at 37°C. We recommend a time course study to determine the optimal stimulation time.	
3	Remove carefully cell supernatant either by aspirating supernatant.	Discard supernatant (for adherent cells)  6-well culture plate
	PHOSPHO-LRRK2 (Ser935) DETECTION USING HTRF KIT	
4	Immediately add 200 µL of supplemented lysis buffer (1X) and incubate for at least 30 minutes at room temperature under shaking.	 6-well culture plate
	Use the appropriate supplemented lysis buffer and incubate at room temperature with shaking. We recommend a time course study to determine the optimal lysis incubation time.	
5	After homogenization by pipeting up and down, transfer 16 µL of cell lysate from the 96-well cell-culture plate to a 384-well small volume white plate. Depending on cell lines used, it can be necessary to dilute the cell lysate to ensure samples are within theassay linear range	 6-well culture plate SV detection plate
6	Add 4 µL of premixed antibody solutions (vol/vol) prepared in the detection buffer. Cover the plate with a plate sealer. Incubate 4h at room temperature. Maximum signal is reached after 4h incubation time, and remains stable over a period of 24 hours. Therefore, readings can be made between 4h and 24h of incubation time. Set up your reader for Eu^{3+} Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on a compatible HTRF® reader**.	 SV detection plate

**For more information about HTRF® compatible readers and for set-up recommendations, please visit our website at: www.revvity.com

► Standard manual for two-plate assay manual in 20 µL final volume (after lysis step)

	NON-TREATED CELL LYSATE	TREATED CELL LYSATE	CONTROL LYSATE	NEGATIVE CONTROL	BLANK CONTROL
Step 1	Dispense 16 µL of non-treated cell lysate	Dispense 16 µL of treated cell lysate	Dispense 16 µL of control lysate	Dispense 16 µL of supplemented lysis buffer(1X)	Dispense 16 µL of non-treated cell lysate
Step 2	Add 2 µL of Phospho LRRK2 d2 antibody working solution to all wells				Add 2 µL of Detection buffer
Step 3	Add 2 µL of Phospho LRRK2 Eu cryptate antibody working solution to all wells				
Step 4	Cover the plate with a plate sealer. Incubate 4h at room temperature.				
Step 5	Remove the plate sealer and read on an HTRF compatible reader				

The blank control is used to check the Cryptate signal at 620 nm.
The Negative control is used to check the non-specific signal. The ratio between control lysate signal / non-specific signal should be greater than 2.

DATA REDUCTION & INTERPRETATION

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.

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

For more information about data reduction, please visit www.revvity.com

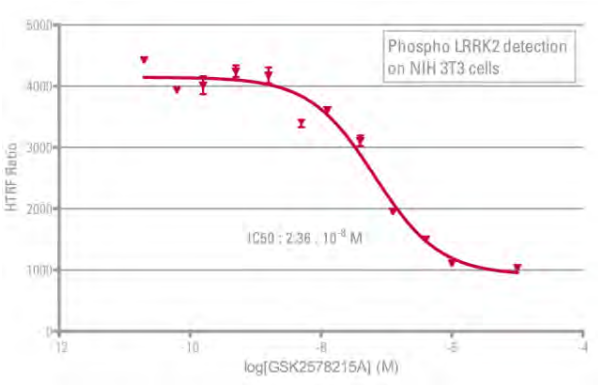
RESULTS

These data should be considered only as an example. Results may vary from one HTRF® compatible reader to another.

The results are drawn up by plotting HTRF® Ratio.

Results obtained on NIH 3T3 cells (500,000 cells/well), using the two-plate assay manual for adherent cells NIH 3T3 cells were treated for 2 hours at 37°C with increasing concentrations of GSK2578215A, an LRRK2 inhibitor.

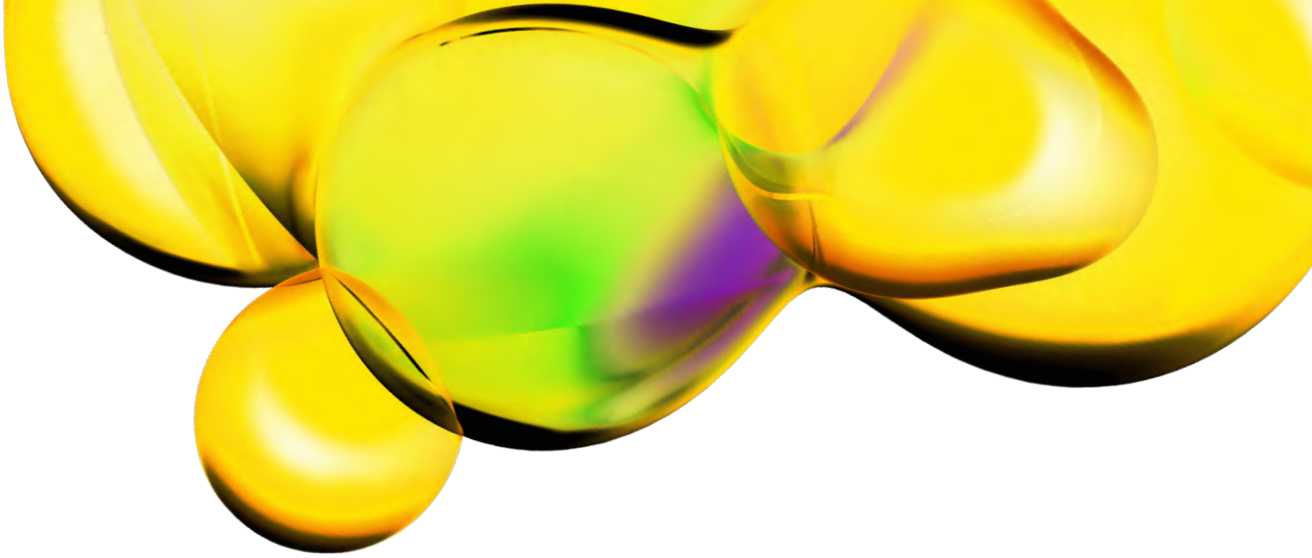
	Phospho-LRRK2	
Log[GSK2578215A] (M)	Mean HTRF Ratio	CV%
-10.7	4 423	0%
-10.2	3 938	1%
-9.8	4 013	5%
-9.3	4 242	3%
-8.8	4 175	4%
-8.3	3 388	3%
-7.9	3 609	1%
-7.4	3 106	4%
-6.9	1 952	2%
-6.4	1 502	1%
-6	1 107	1%
-5	1 036	1%
Negative control	361	2%
Control lysate	4435	2%



Technical support team can help you to set-up this manual, please contact us at www.revvity.com

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

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 beforestarting the pharmacological treatment.
Depending on the pathway, a serum starving step could be essential toreduce the basal level activity. This step should be optimized case-by- case.	Advice on cell culture conditions prior using HTRF® kit: For adherent cells Before treating the cells with compounds, remove culture media from the plate andreplace 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 hoursup 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 andtyrosine phosphatases The lysis buffer is effective for creating cell extract under non denaturing conditionsfrom both plated cells and cells pelleted from suspension cultures.
Using the two-plate assay manual, a low signal can often be improvedby adjusting lysis volumes.	In most cases, a typical adherent cell line grown in 96-well plates is readily detected ina lysis volume of 50µL. However, the lysis volume can be adjusted from 25 µL to 200µL.
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 assayperformances.
Parameters such as cell density, stimulation time and lysis incubationtime 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 phosphorylationof the readout of interesrcan vary from one cell line to another. Depending on the type of treatment, and the temperature, the stimulation time can vary widely. Becauseof 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 ofthis, 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.revvity.com
Assaying for multiple targets from a single lysate.	The two-plate assay manual indicates the use of 16µL of lysate per well, whereas the 96-well cell culture microplate would generate 50µL (or more) of lysate. Therefore, a typical cell lysate can be assayed for many targets, given that temporal and expression level constraints can vary from one target to another.
Batch production of cell lysates example of T175 flask	General lab work - prior using Phospho-LRRK2 HTRF® kit: Day1: Dispense 8 million cells in T175cm2, add 25 mL of cell culture complete medium and incubate 2 days at 37°C, 5% CO2. Day3: cell stimulation Remove cell culture medium by aspiration, wash once (do not detach the cells), add 5 mL of agonist (1x) diluted in FCS free medium and incubate at 37 ° C, 5% CO2, for the optimized time. Day3: 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, centrifuge 10 min, 2400 rcf at RT, recover cell lysate supernatant and store aliquots at -60°C or below. For long term conservation, aliquots should be stored in liquid nitrogen.



Manufactured by Cisbio Bioassays - Parc Marcel Boiteux - 30200 Codolet – FRANCE

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