MANUAL

HTRF HUMAN IRF-5 DETECTION KITS

Part # 64HIRF5PEG & 64HIRF5PEH

Test Size#: 500 TESTS (64HIRF5PEG), 10,000 TESTS (64HIRF5PEH)

Revision: #04 of January 2024 **Store at:** ≤-60°C

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

ASSAY PRINCIPLE

This kit is intended for the simple and rapid quantification of endogenous levels of IRF-5 in cells. IRF-5 protein is produced by cells and after lysis of the cell membrane, the level of IRF-5 produced by cells can be detected using the kit reagents.

Following lysis of cells, the IRF-5 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 IRF-5 present in the sample, thereby generating FRET. Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the IRF-5 protein concentration (Fig. 1)

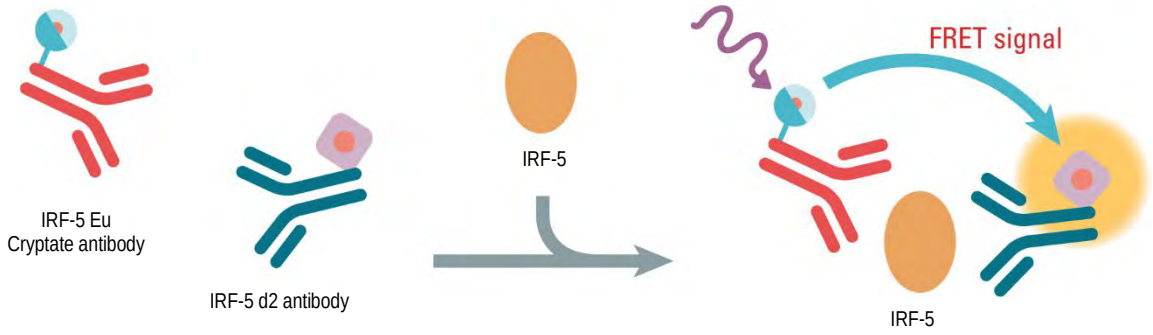
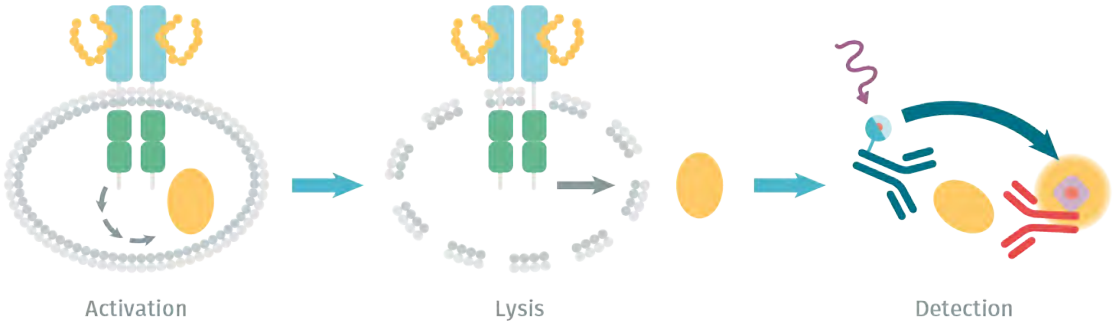
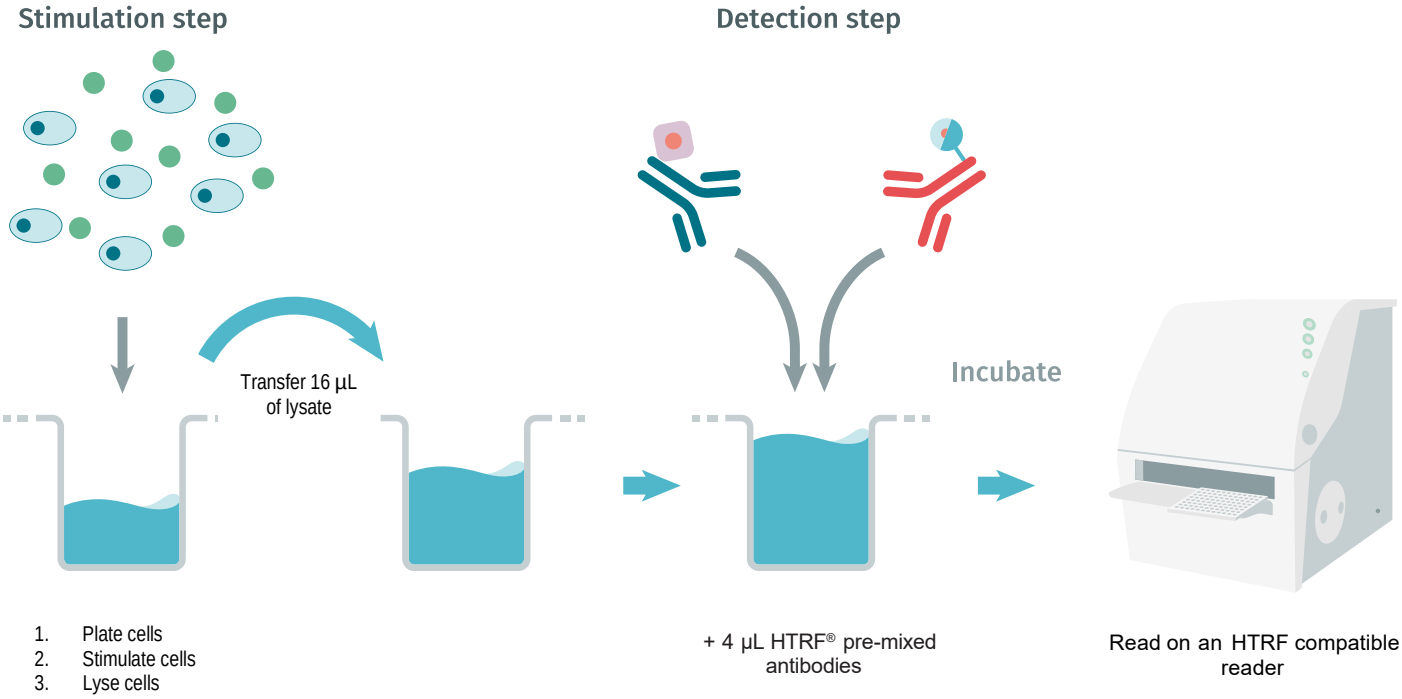


Figure 1: Principle of HTRF IRF-5 sandwich assay

MANUAL 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# 64HIRF5PEG		10,000 TESTS CAT# 64HIRF5PEH	
IRF-5 Eu Cryptate Antibody (stock solution 50X)	≤-16°C	Orange cap	1 vial - 20 µL	Red cap	1 vial – 0.4 mL
IRF-5 d2 Antibody (stock solution 50X)	≤-16°C	Blue cap	1 vial - 20 µL	Purple cap	1 vial – 0.4 mL
IRF-5 Control lysate (ready-to-use)	≤-60°C	Green cap	1 vial – 150 µL	Green cap	2 vials – 150 µL
Lysis buffer* # 2 (stock solution 4X)	≤-16°C	Transparent cap	4 vials – 2 mL	White cap	1 vial – 130 mL
Detection buffer ** (ready-to-use)	≤-16°C	Red cap	2 vials – 2 mL	Red cap	1 vial – 50 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.revvity.com

*Small volume (SV) detection microplates.
For more information about microplate recommendations, please visit our website at: www.revvity.com

STORAGE AND STABILITY

Storage upon reception:
Store the kit at -60°C or below until the expiration date indicated on the package.

Storage and stability of thawed material:
When you are ready to use the kit, take the reagents out and prepare them following the manual provided in this document. Unused thawed reagents can be stored and conserved for future use. Refer to the table below for storage options and corresponding shelf life.

	Storage after Thawing/reconstitution
Lysis Buffer / Blocking Reagent / Detection buffer	2-8°C until the expiration date indicated on the package
Antibodies*	2-8°C for 48h or freeze at -16°C or below until the expiration date indicated on the package for long term storage
Protein/standard /Control Lysate*	freeze at -60°C or below until the expiration date indicated on the package for long term storage

*For Antibodies, Protein, Standard & control lysate, Stock solutions may be thawed and frozen only once. Freeze in aliquots to avoid multiple freeze/thaw cycles (once aliquoted, single use of the reagent). Volume of antibodies aliquots should not be under 10µL. Volume of Protein, Standard & control lysate aliquots should not be under 20µL.

REAGENT PREPARATION

Allow all reagents to thaw before use. We recommend centrifuging the vials gently after thawing, before pipetting 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 the control lysate and the negative control should be greater than 2.

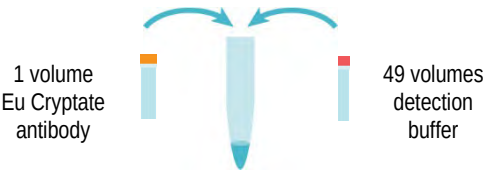
Thaw the control lysate. Mix gently, and then the control lysate is ready to use.

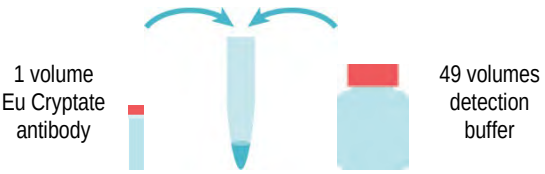
TO PREPARE WORKING ANTIBODY SOLUTIONS:

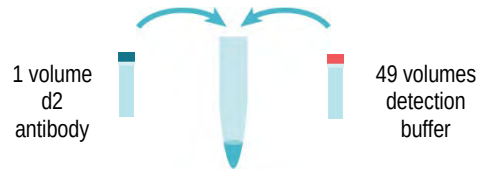
HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the d2 or

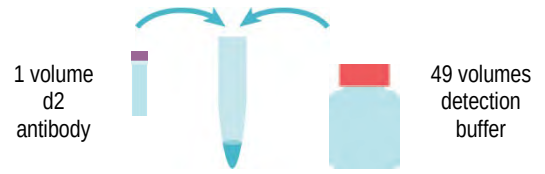
Europium Cryptate-antibodies will impair the assay's quality. Be careful, as the working solution preparation for the antibodies may differ between the 500 and 10,000 tests data point kit.

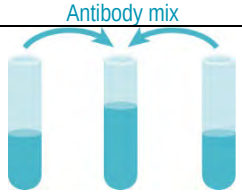
Antibody working solutions are stable for 2 days at 2-8°C. Dilute the antibodies with detection buffer. In practice:

<table>
<tr><th>500 TESTS KIT CAT# 64HIRF5PEG</th><th>10,000 TESTS KIT CAT# 64HIRF5PEH</th></tr>
<tr><td colspan="2">IRF-5 Eu cryptate antibody</td></tr>
<tr><td> 

Dilute the frozen stock solution with detection buffer 50-fold, e.g. add 0.98 mL of detection buffer to 0.02 mL of Eu-antibody stock solution.</td><td> 

Dilute the frozen stock solution with detection buffer 50-fold, e.g. add 19.6mL of detection buffer to the 0.4mL of Eu-antibody stock solution.</td></tr>
<tr><td colspan="2">IRF-5 kit d2 antibody</td></tr>
<tr><td> 

Dilute the frozen stock solution with detection buffer 50-fold, e.g. add 0.98 mL of detection buffer to 0.02 mL of d2-antibody stock solution.</td><td> 

Dilute the frozen stock solution with detection buffer 50-fold, e.g. add 19.6mL of detection buffer to 0.4mL of d2-antibody stock solution.</td></tr>
<tr><td colspan="2">Antibody mix</td></tr>
<tr><td>It is recommended 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 Eu Cryptate-antibody solution.</td><td> 
It is recommended 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 Eu Cryptate-antibody solution.</td></tr>
</table>

TO PREPARE LYSIS BUFFER:

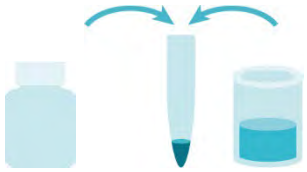
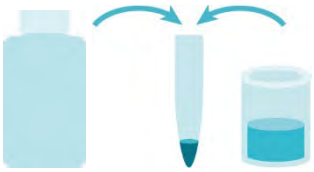
Make sure that the lysate has been generated by using the kit reagents.

The lysis buffer differs between the manuals. Make sure you use the appropriate lysis buffer depending on the chosen manual's specifications.

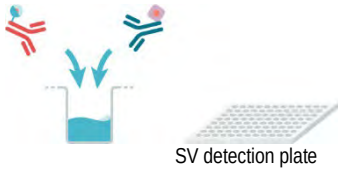
Prepare the required amount of lysis buffer before running the assay. Working solutions are stable for 2 days at 2-8°C.

- ***Lysis buffer 1X for two-plate assay manual on adherent cells***

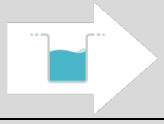
Determine the amount of lysis buffer needed for the experiment. Each well generally requires 50µL of lysis buffer 1X. Prepare a lysis buffer solution 1X and then distribute on adherent cells. In practice:

TWO- PLATE ASSAY MANUAL ON ADHERENT CELLS	
500 TESTS KIT CAT# 64HIRF5PEG & 10,000 TESTS KIT CAT# 64HIRF5PEH	
Preparation of lysis buffer 1X	
500 TESTS	10,000 TESTS
1 volume Lysis buffer 4X  3 volumes Distilled water .	1 volume Lysis buffer 4X  3 volumes Distilled water
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.	

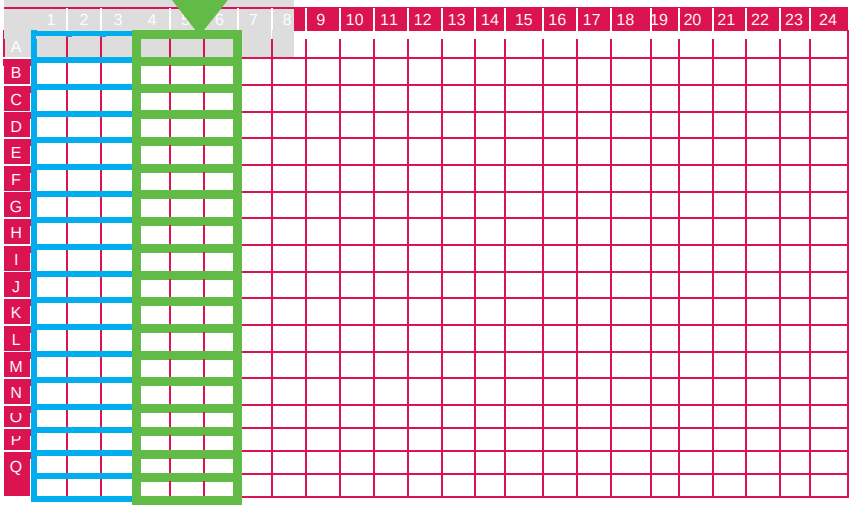
IRF-5 ASSAY MANUAL

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.	 96-well culture plate
Transfer 16 µL of cell lysate treated from the 96-well plate to a 384-small volume (SV) white detection plate	 96-well culture plate 384-SV 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 Eu^{3+} Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on a compatible HTRF® reader.	 SV detection plate

STANDARD MANUAL FOR SAMPLES OR CONTROL DETECTION

	SAMPLES OR CONTROL LYSATES	
Step 1 	Dispense 16 µL of each sample into each sample well	Dispense 16 µL of “control lysate” into each well
Step 2 	Add 2 µL of IRF-5 d2 antibody working solution to all wells	
Step 3 	Add 2 µL of IRF-5 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 Sample ***Sample 1*** 4 µL pre-mixed Anti-IRF-5 antibodies	Repeat Well A1	Repeat Well A1	16 µL Control lysates ***Control lysate*** 4 µL pre-mixed Anti-IRF-5 antibodies	Repeat Well A4	Repeat Well A4
B	16 µL Sample 1 ***Sample 2*** 4 µL pre-mixed Anti-IRF-5 antibodies	Repeat Well B1	Repeat Well B1			
C	16 µL Sample 1 ***Sample 3*** 4 µL pre-mixed Anti-IRF-5 antibodies	Repeat Well C1	Repeat Well C1			
D	16 µL Sample ... ***Sample ...*** 4 µL pre-mixed Anti-IRF-5 antibodies	Repeat Well D1	Repeat Well D1			
E	16 µL Sample ... ***Sample ...*** 4 µL pre-mixed Anti-IRF-5 antibodies	Repeat Well E1	Repeat Well E1			
F	16 µL Sample ... ***Sample ...*** 4 µL pre-mixed Anti-IRF-5 antibodies	Repeat Well F1	Repeat Well F1			
G	16 µL Sample ... ***Sample ...*** 4 µL pre-mixed Anti-IRF-5 antibodies	Repeat Well G1	Repeat Well G1			
H	16 µL Sample ... ***Sample ...*** 4 µL pre-mixed Anti-IRF-5 antibodies	Repeat Well H1	Repeat Well H1			



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.

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

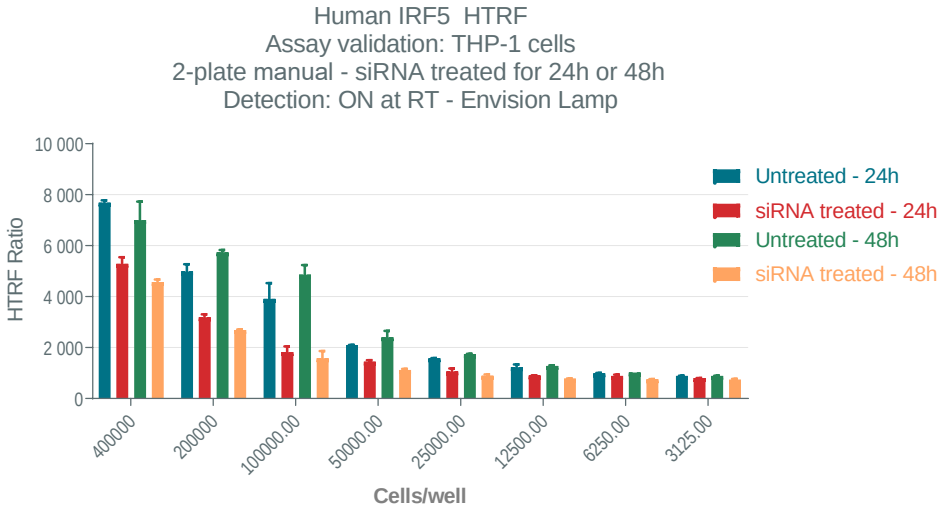
For more information about data reduction, please visit www.revvity.com

RESULTS

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

THP-1 cells were plated at different cell densities under 50 µl in 96-well plates in complete culture medium. For untreated cell, 10 µl of completed culture medium was added. For siRNA treated, 10 µl containing a mix of Lipofectamine® RNAiMax/siRNA for IRF5 was added. Cells were then incubated for 24h and 48h at 37°C, 5% CO2.

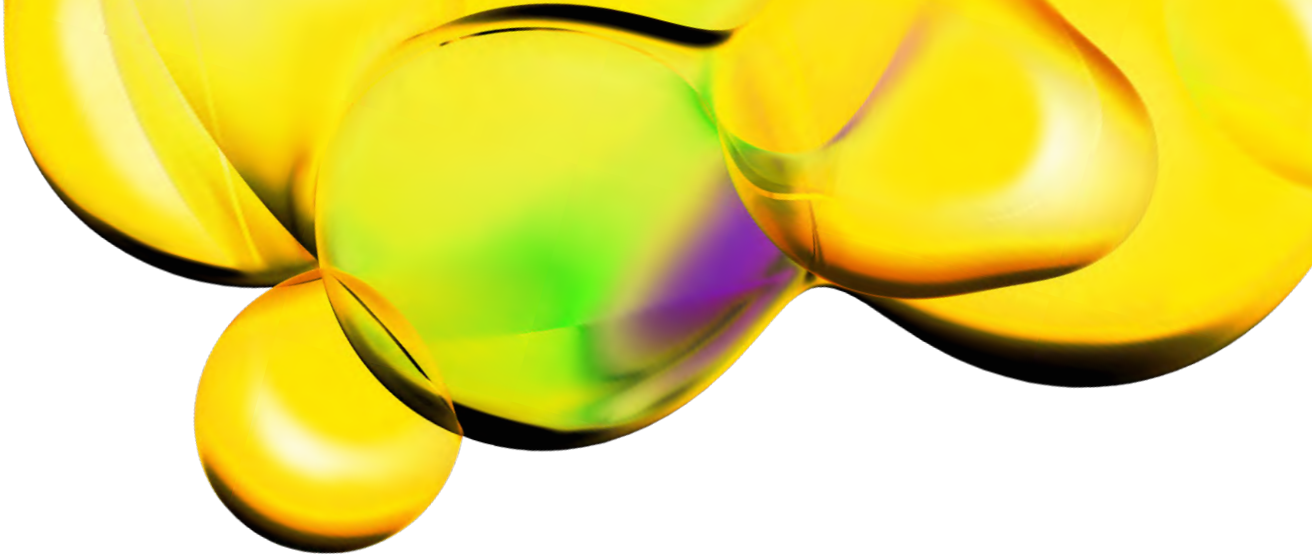
After incubation, cells were lysed with 20 µL of supplemented lysis buffer #2 at 4X for 30 minutes at RT under gentle shaking, and 16 µL of lysate were transferred into a low volume white microplate before the addition of 2 µL of the HTRF d2 detection reagent and 2 µL HTRF Eu-K detection reagent. The HTRF signal was recorded after ON incubation.



Cells/well	Assay window
400,000	13.7
200,000	8.9
100,000	7.0
50,000	3.7
25,000	2.8
12,500	2.2
6,250	1.8
3,125	1.6

GENERAL LAB WORK PRIOR USING REVVITY 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 may be essential to reduce the basal level activity. This step should be optimized case-by- case.	Advice on cell culture conditions prior to using the Revvity 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 The 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 provided in the kit. Lysates generated with HTRF® buffers can be used in other technologies, like Western Blot. 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 manual, 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 setup may seriously impair the results. For information about HTRF® compatible readers and for setup recommendations, please visit our website at: www.revvity.com
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.



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

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