MANUAL

HTRF TOM20 DETECTION KITS

Part # 64TOM20PEG & 64TOM20PEH

Test Size#: 500 TESTS (64TOM20PEG), 10,000 TESTS (64TOM20PEH)

Revision: #02 of September 2023 **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 TOM20 protein in cell lysates and is compatible to the HTRF Ubiquitin phospho-S65 kit. For generation of lysates, please refer to the phosphoprotein kit guidelines.

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 TOM20 present in the sample, thereby generating FRET. Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the TOM20 protein concentration (Fig. 1)

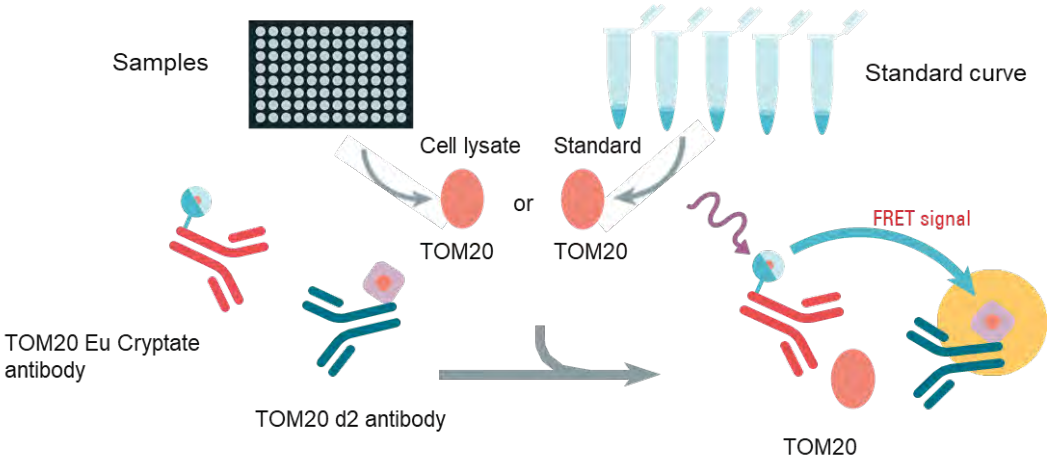
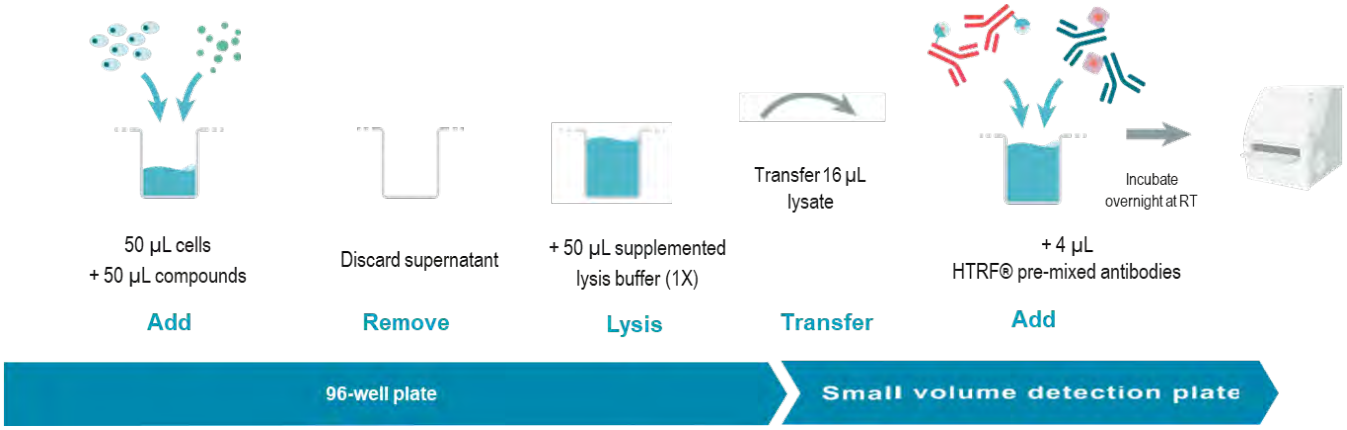


Figure 1: Principle of HTRF TOM20 sandwich assay

MANUAL AT A GLANCE



Make sure to use the set-up for Eu 3+ Cryptate. For more information about set-up and compatible HTRF® readers, please visit our website at: www. .com

MATERIALS PROVIDED :

KIT COMPONENTS	STORAGE	500 TESTS CAT# 64TOM20PEG		10,000 TESTS CAT# 64TOM20PEH	
TOM20 Eu Cryptate Antibody (stock solution 50X)	≤-16°C	Orange cap	1 vial – 20 µL	Red cap	1 vial – 0.4 mL
TOM20 d2 Antibody (stock solution 50X)	≤-16°C	Blue cap	1 vial – 20 µL	Purple cap	1 vial – 0.4 mL
TOM20 Standard (ready-to-use as STD7)	≤-60°C	Green cap	1 vial – 600 µL	Green cap	2 vials – 600 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	Transparent 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

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

STORAGE AND STABILITY

Store the kit at ≤-60°C. Under appropriate storage conditions, reagents are stable until the expiry date indicated on the label.

TOM antibodies and standard can be frozen and thawed only twice. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at ≤-60°C. Thawed detection buffer #3, Lysis buffer #4 and Blocking reagent can be stored at 2-8°C on your premises.

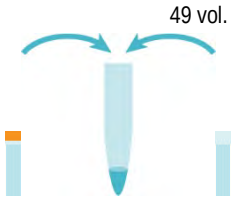
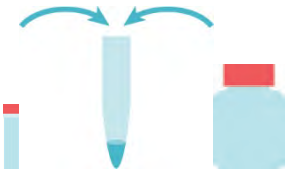
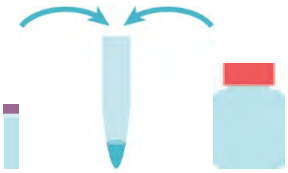
REAGENT PREPARATION

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 WORKING ANTIBODY SOLUTIONS:

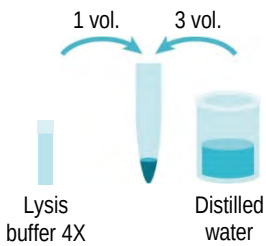
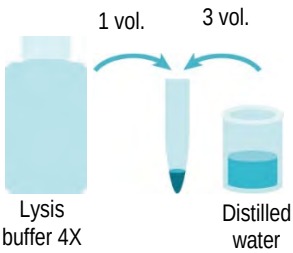
Each well requires 2 µL of TOM20-Eu Cryptate Antibody and 2 µL of TOM20-d2 Antibody. Prepare the two antibody solutions in separate vials.

<table>
<tr><th colspan="2">500 TESTS</th><th colspan="2">10,000 TESTS</th></tr>
<tr><td colspan="4">TOM20 Eu Cryptate antibody</td></tr>
<tr><td>Dilute 50-fold the 50X stock solution (thawed reagent) of human TOM20 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 TOM20 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">TOM20 d2 antibody</td></tr>
<tr><td>Dilute 50-fold the 50X stock solution (thawed reagent) of human TOM20 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 TOM20 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 colspan="2">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>  </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 USED FOR CELL LYSIS AND STANDARD DILUENT:

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</th><th colspan="2">10,000 TESTS</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> 
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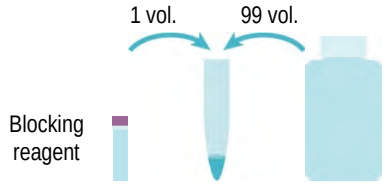
<tr><td colspan="4">Supplemented Lysis buffer 1X</td></tr>
<tr><td>Dilute the “blocking reagent” 100-fold with “Lysis buffer 1X”.

E.g. take 0.05 mL of “Blocking reagent stock solution” and add it to 4.95 m of lysis buffer 1X. Mix gently.</td><td> 
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E.g. take 0.05 mL of “Blocking reagent stock solution” and add it to 4.95 m of lysis buffer 1X. Mix gently.</td></tr>
</table>

TO PREPARE STANDARD WORKING SOLUTIONS

• Each well requires 16 µL of standard

• Dilute the standard stock solution serially with supplemented lysis buffer #4 (1X).

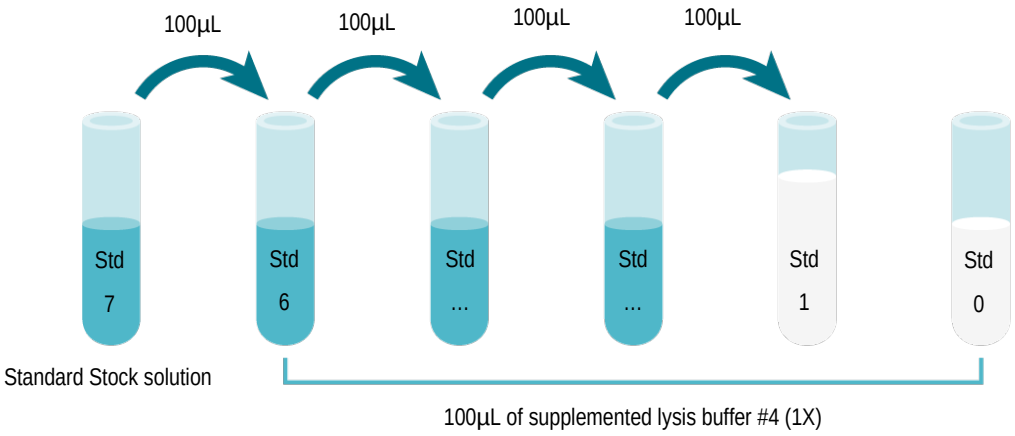
• In order to counteract any standard sticking, we recommend changing tips between each dilution.

A recommended standard dilution procedure is listed and illustrated below:

The standard stock solution is already the high standard (Std 7) for the top of the curve. Use the high standard (Std 7) to prepare the standard curve using 1/2 serial dilutions as follows:

• Dispense 100 µL of supplemented lysis buffer #4 (1X) in each vial from Std 6 to Std 0.

• Add 100 µL of standard (Std 7) to 100 µL of supplemented lysis buffer #4 (1X), mix gently and repeat the 1/2 serial dilution to make standard solutions: std6, std5, std4, std3, std2, std1.



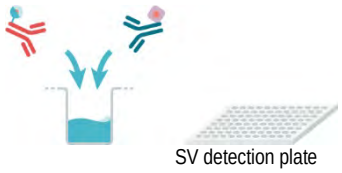
STANDARD	SERIAL DILUTIONS	TOM20 WORKING SOLUTIONS (pg/mL)
Standard 7	Standard Stock solution	5 000
Standard 6	100 µL standard 7 + 100 µL supplemented lysis buffer #4 (1X)	2 500
Standard 5	100 µL standard 6 + 100 µL supplemented lysis buffer #4 (1X)	1 250
Standard 4	100 µL standard 5 + 100 µL supplemented lysis buffer #4 (1X)	625
Standard 3	100 µL standard 4 + 100 µL supplemented lysis buffer #4 (1X)	312
Standard 2	100 µL standard 3 + 100 µL supplemented lysis buffer #4 (1X)	156
Standard 1	100 µL standard 2 + 100 µL supplemented lysis buffer #4 (1X)	78
Standard 0	100 µL supplemented lysis buffer #4 (1X)	0

TO PREPARE SAMPLES:

► *IMPORTANT RECOMENDATIONS:*

- Working with 50,000 cells per well and 50µL lysis buffer per well should give a cell lysate falling within the assay linear range. Using higher cell densities, the samples should be diluted (we recommend testing several dilutions) in lysis buffer (1X) to ensure samples are within the assay linear range.
- It is mandatory to collect and store samples into polypropylene microtubes.

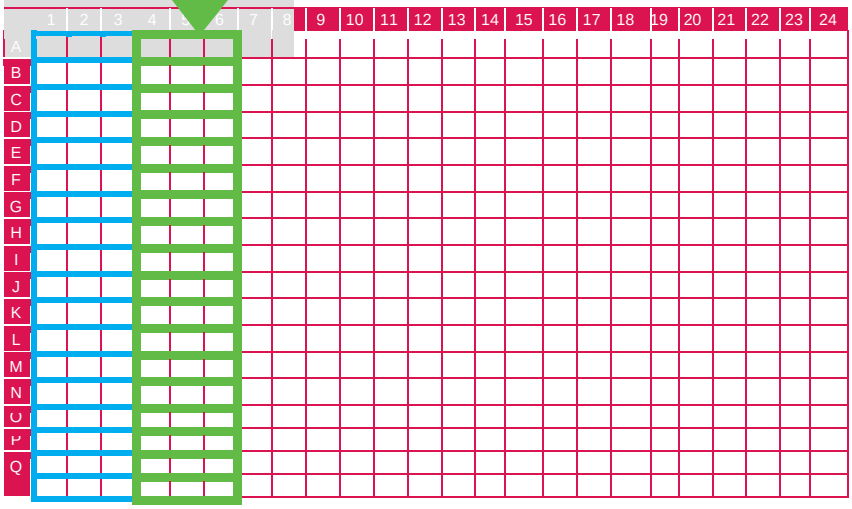
TOM20 ASSAY MANUAL

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 volume and incubation time may be optimized.	 96-well culture plate
After homogenization by pipeting up and down 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 Eu3+ Cryptate and read the fluorescence emission at two different wavelenghts (665nm and 620nm) on a compatible HTRF® reader.	 SV detection plate

STANDARD MANUAL FOR DETECTION

	SAMPLES	
Step 1	Dispense 16 µL of each dilution of the standard (triplicates)	Dispense 16 µL of each lysate sample (triplicates)
Step 2	Add 2 µL of TOM20 d2 antibody working solution to all wells	
Step 3	Add 2 µL of TOM20 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 Negative ***Supplemented Lysis Buffer*** 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well A1	Repeat Well A1	16 µL ***Sample 1*** 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well A4	Repeat Well A4
B	16 µL STD1 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well B1	Repeat Well B1	16 µL ***Sample 2*** 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well B4	Repeat Well B4
C	16 µL STD2 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well C1	Repeat Well C1	16 µL ***Sample 3*** 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well C4	Repeat Well C4
D	16 µL STD3 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well D1	Repeat Well D1	16 µL ***Sample 4*** 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well D4	Repeat Well D4
E	16 µL STD41 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well E1	Repeat Well E1	16 µL ***Sample ..*** 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well E4	Repeat Well E4
F	16 µL STD5 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well F1	Repeat Well F1	16 µL ***Sample ..*** 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well F4	Repeat Well F4
G	16 µL STD6 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well G1	Repeat Well G1	16 µL ***Sample ..*** 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well G4	Repeat Well G4
H	16 µL STD7 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well H1	Repeat Well H1	16 µL Disaggregated ***Sample ..*** 4 µL pre-mixed Anti-TOM20 antibodies	Repeat Well H4	Repeat Well H4



DATA REDUCTION & INTERPRETATION

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

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

3. Calculate the delta ratio of the acceptor and donor emission signals for each individual well. The Standard 0 (Negative control) plays the role of an internal assay control.

Delta Ratio = Ratio Standard or sample - Ratio Standard 0

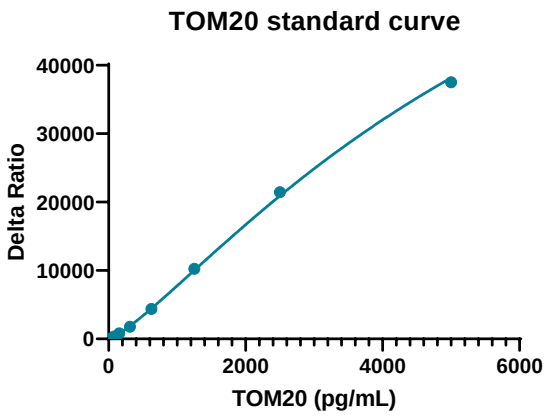
For more information about data reduction, please visit www.revvity.com

RESULTS

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

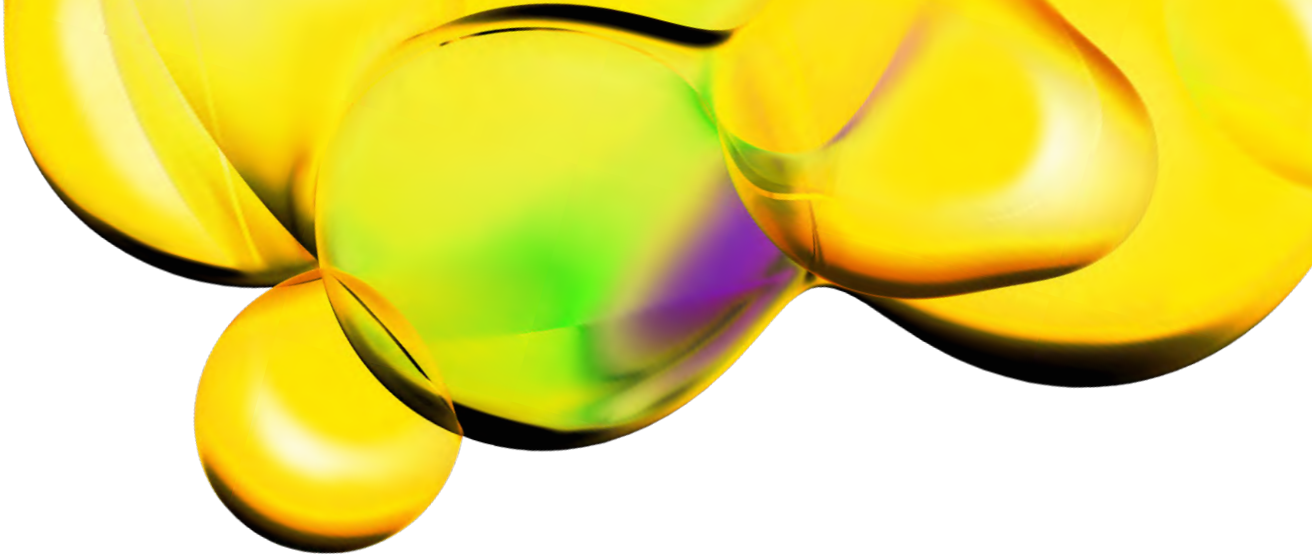
Standard curve fitting with the 4 Parameter Logistic (4PL) model (with $1/Y^2$ weighting):

	Ratio (1)	CV (2)	**Delta Ratio**
Standard 0 - Negative control	715	4%	0
Standard 1 - 78 pg/mL	1052	4%	338
Standard 2 - 156 pg/mL	1538	3%	823
Standard 3 - 312 pg/mL	2510	4%	1795
Standard 4 - 625 pg/mL	5102	4%	4388
Standard 5 - 1 250 pg/mL	10971	5%	10256
Standard 6 - 2 500 pg/mL	22166	1%	21451
Standard 7 - 5 000 pg/mL	38224	3%	37510



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 evvity 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 m , 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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