MANUAL

Total AP2 KITS

Part #: 64AP2TPEB & 64AP2TPEC

Test Size#: 1,000 TESTS (64AP2TPEB), 20,000 TESTS (64AP2TPEC)

Revision: #02 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 AP2 in cells. AP2 is produced by cells and, after lysis of the cell membrane, total-AP2 can be detected using the kit reagents. This total protein assay is used for monitoring the steady state protein level in various cells.

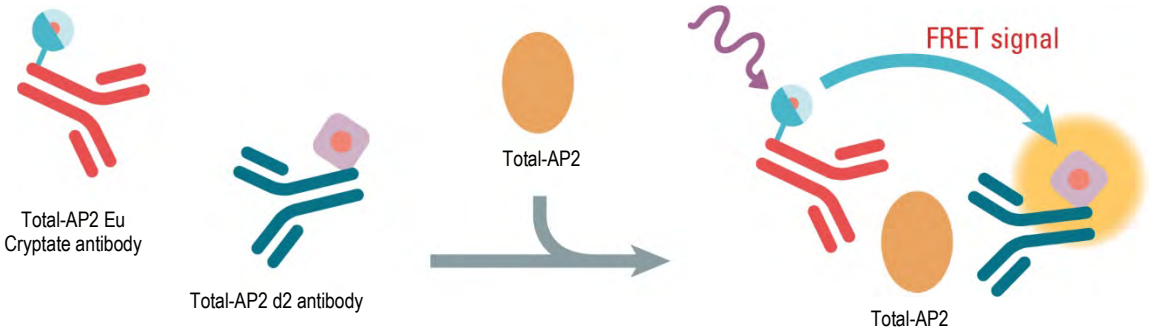


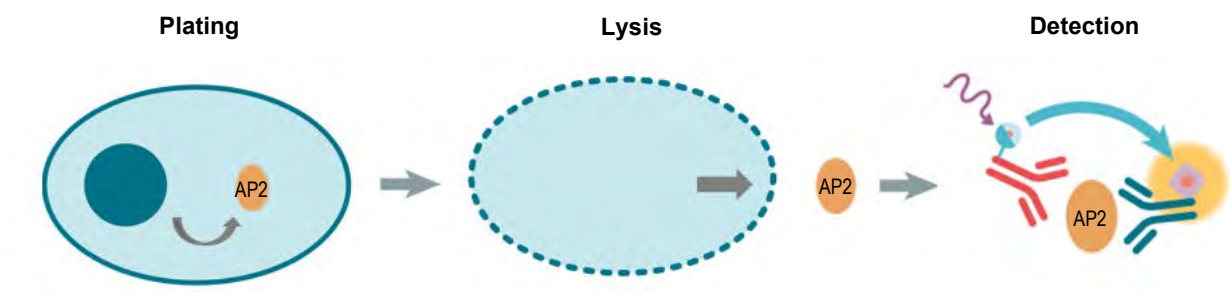
Figure 1: Principle of the HTRF sandwich assay

As shown here, total-AP2 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 antibodies 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 increases in proportion to total-AP2 expression.
The assay can be run under a two-plate assay manual, where cells are plated and lysed in the same culture plate. Lysates are then transferred to the assay plate for the detection of total-AP2 by HTRF® reagents. This manual enables the cells' viability and confluence to be monitored. It can also be further streamlined to a one-plate assay manual. Detection of total-AP2 with HTRF® reagents is performed in a single plate used for plating, stimulation, and detection. No washing steps are required. This manual, HTS designed, allows miniaturization while maintaining HTRF® quality.

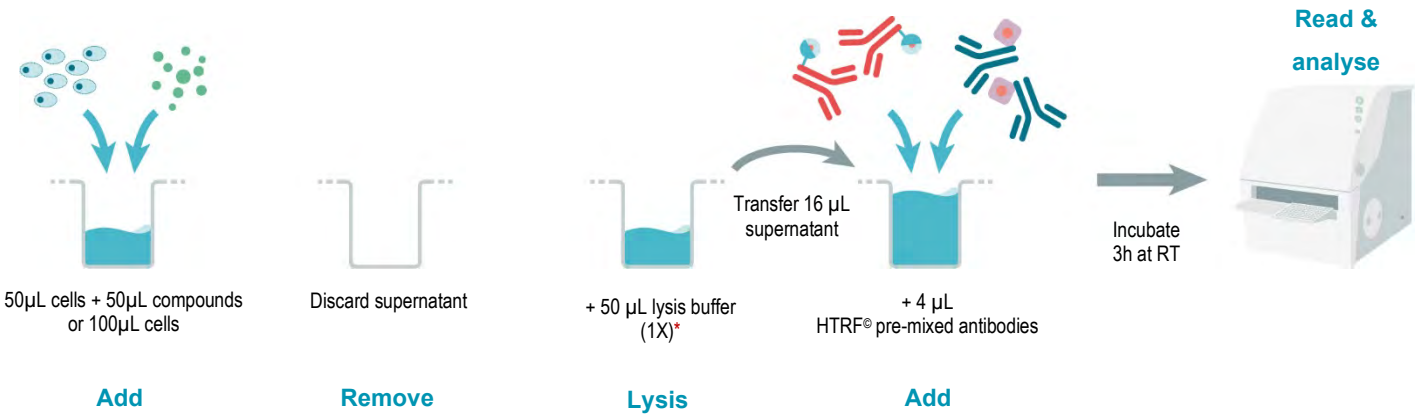
For tissue derived samples, please refer to the technical note: "Optimize your HTRF® cell signaling assays on tissues" on www.revvity.com

Our technical support team can help you to set up this manual, or another. Please contact us at www.revvity.com

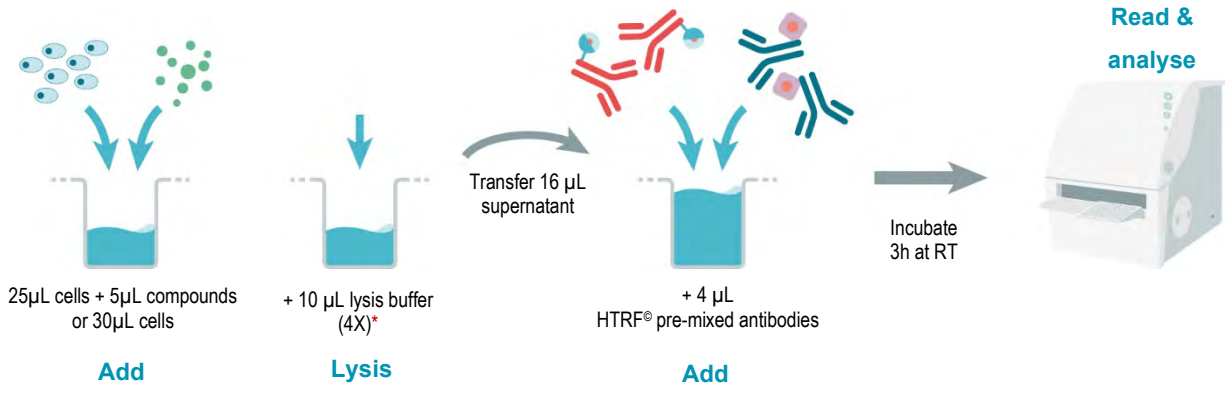
MANUAL AT A GLANCE



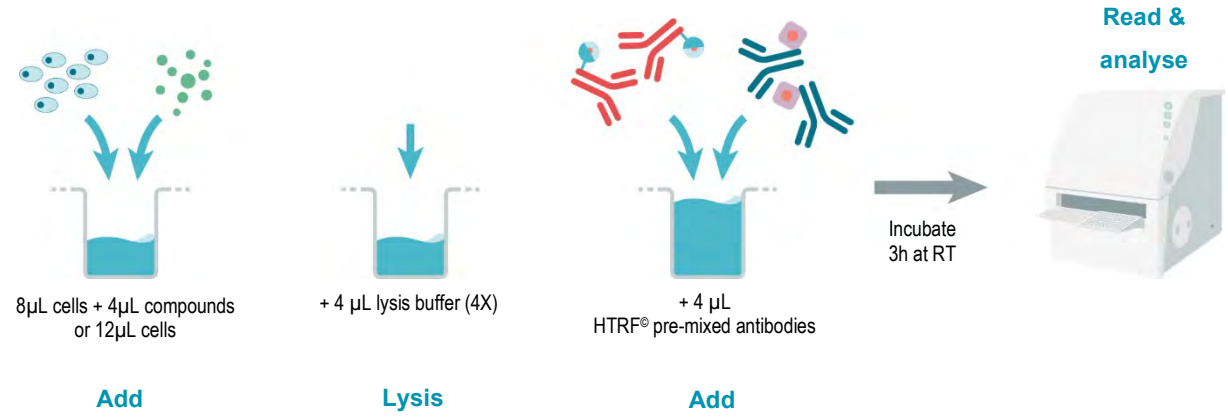
► TWO-PLATE ASSAY MANUAL FOR ADHERENT CELLS:



► TWO-PLATE ASSAY MANUAL FOR SUSPENSION CELLS:



► ONE-PLATE ASSAY MANUAL:



*Depending on the cell lines used, the lysis volume 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.

► 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	1,000 TESTS CAT# 64AP2TPEB		20,000 TESTS CAT# 64AP2TPEC	
Control lysate (ready-to-use)	≤-60°C	Green cap	1 vial - 150µL	Green cap	3 vials - 150µl
AP2 total kit Eu cryptate antibody (20X)	≤-16°C	Red cap	1 vial - 100µL	Red cap	2 vials - 1mL
AP2 total kit d2 antibody (20X)	≤-16°C	Blue cap	1 vial - 100µL	Blue cap	2 vials - 1mL
Lysis buffer* #4 (4X)	2-8°C	Clear cap	1 vial - 16mL	White cap	2 vials -130mL
Phospho-total protein detection buffer** (ready-to-use)	1,000 tests ≤-16°C 20,000 tests 2-8°C	Orange cap	4 vials - 2mL	Red cap	2 vials - 50mL

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

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 (2mL) should be stored frozen until needed. Thawed lysis buffer and detection buffer can be stored at 2-8°C on your premises.

Thawed antibodies are stable for 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 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.

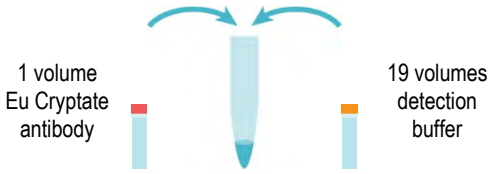
Thaw the control lysate. Mix gently, and then the control lysate is ready to use.

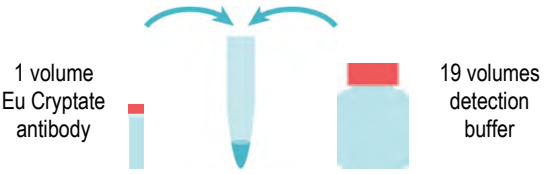
TO PREPARE WORKING ANTIBODY SOLUTIONS:

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

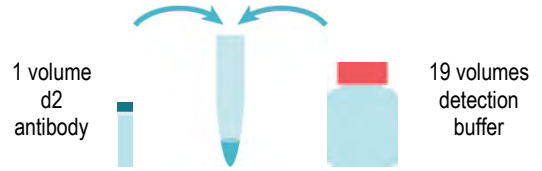
Europium Cryptate-antibodies will impair the assay's quality. Be careful, as the working solution preparation for the antibodies may differ between the 1,000 and 20,000 test data point kit.

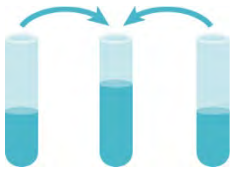
Antibody working solutions are stable for 2 days at 2-8°C. Dilute the antibodies with detection buffer. In practice:

<table>
<tr><th>1,000 TESTS KIT CAT# 64AP2TPEB</th><th>20,000 TESTS KIT CAT# 64AP2TPEC</th></tr>
<tr><td colspan="2">AP2 total kit Eu cryptate antibody</td></tr>
<tr><td> 

Dilute the frozen stock solution with detection buffer 20-fold, e.g. add 0.95 mL of detection buffer to 0.05 mL of Eu Cryptate-antibody stock solution.</td><td> 

Dilute the frozen stock solution with detection buffer 20-fold, e.g. add 19mL of detection buffer to the 1mL of Eu Cryptate-antibody stock solution.</td></tr>
<tr><td colspan="2">AP2 total kit d2 antibody</td></tr>
<tr><td> 

Dilute the frozen stock solution with detection buffer 20-fold, e.g. add 0.95 mL of detection buffer to 0.05 mL of d2-antibody stock solution.</td><td> 

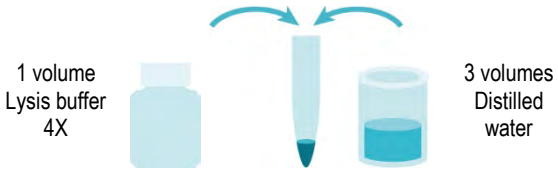
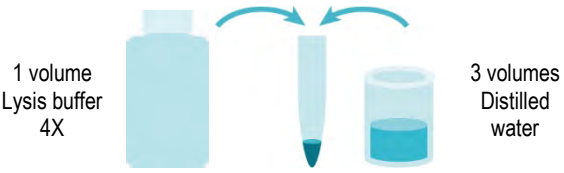
Dilute the frozen stock solution with detection buffer 20-fold, e.g. add 19mL of detection buffer to 1mL of d2-antibody stock solution.</td></tr>
<tr><td colspan="2">Antibody mix</td></tr>
<tr><td>It is recommended to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2-antibody solution to 1 volume of Eu Cryptate-antibody solution.</td><td>  </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 supplemented 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.

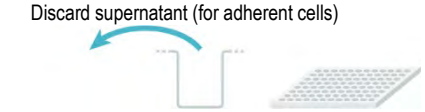
► *Lysis buffer 1X for two-plate assay manual on adherent cells*

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:

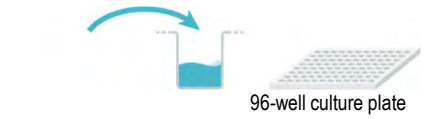
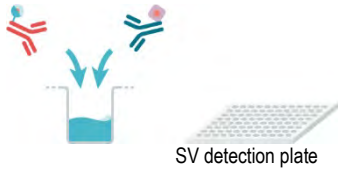
<table>
<tr><th colspan="2">TWO- PLATE ASSAY MANUAL ON ADHERENT CELLS</th></tr>
<tr><th colspan="2">1,000 TESTS KIT CAT# 64AP2TPEB & 20,000 TESTS KIT CAT# 64AP2TPEC</th></tr>
<tr><td colspan="2">Preparation of lysis buffer 1X</td></tr>
<tr><td>1,000 TESTS</td><td>20,000 TESTS</td></tr>
<tr><td> 
</td><td> 
</td></tr>
<tr><td colspan="2">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>
</table>

TWO PLATE ASSAY MANUAL

GENERAL LAB WORK PRIOR USING REVVITY KIT: CELLS PREPARATION

	FOR ADHERENT CELLS	FOR SUSPENSION CELLS	
1	Plate 50 µL of cells in 96-well tissue-culture treated plate in appropriate growth medium and incubate overnight, at 37°C in CO2 atmosphere. If no stimulation is required, plate 100 µL of cells instead.	Plate 25 µL of cells in 96 half-well plate in your appropriate medium. If no stimulation is required, plate 30 µL of cells instead.	 96-well culture plate
	Cell seeding densities of 100K cells/well are generally sufficient for most cell lines, but the optimization of cell seeding densities is recommended. Depending on the receptor, a starving step with serum-free medium may be essential.		
2	If stimulation is required, dispense 50 µL of compound (2X) diluted in cell culture serum-free medium.	If stimulation is required, dispense 5 µL of compound (6X), diluted in your appropriate medium.	 96-well culture plate
	For most compounds, incubation time is 4h at 37°C. We recommend a time course study to determine the optimal stimulation time.		
3	Carefully remove cell supernatant either by aspirating the supernatant or by flicking the plate.	Do not remove your appropriate medium.	Discard supernatant (for adherent cells)  96-well culture plate

TOTAL-AP2 DETECTION USING REVVITY KIT

	FOR ADHERENT CELLS	FOR SUSPENSION CELLS	
4	Immediately add 50 µL of lysis buffer (1X) and incubate for at least 30 minutes at room temperature under shaking.	Immediately add 10 µL of lysis buffer (4X) and incubate for at least 30 minutes at room temperature under shaking.	 96-well culture plate
	Use the appropriate lysis buffer and incubate at room temperature under shaking. Lysis incubation time may be optimized. Lysis volume can be decreased down to 25 µL.		
5	After homogenization by pipetting up and down, transfer 16µL of cell lysate from the 96-well cell-culture plate to a small volume (SV) white detection plate. Depending on the cell lines used, it may be necessary to dilute the cell lysate to ensure samples are within the assay linear range.		 96-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 for 3 hours at room temperature. Set up your reader for Eu^{3+} Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on an HTRF® compatible reader.		 SV detection plate

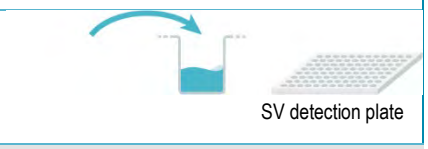
► ***Standard manual for two-plate assay manual in 20 µL final volume (after lysis step)***

	NON TREATED CELL LYSATE	TREATED CELL LYSATE	CONTROL LYSATE	NEGATIVE CONTROL
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 lysis buffer(1X)
Step 2 	Add 4 µL of premixed AP2 total kit d2 antibody + AP2 total kit Eu cryptate antibody solution to all wells			
Step 3 	Cover the plate with a plate sealer. Incubate for 3h at room temperature.			
Step 4 	Remove the plate sealer and read on an HTRF compatible reader			

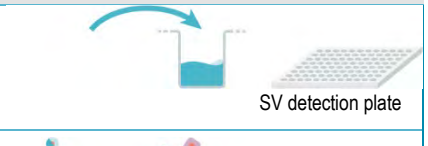
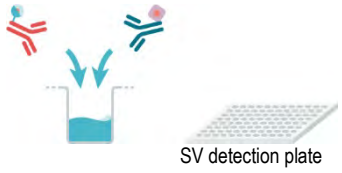
The Negative control is used to check the non-specific signal. The ratio between the control lysate signal / non-specific signal should be greater than 2.

ONE PLATE ASSAY MANUAL

GENERAL LAB WORK PRIOR USING REVVITY KIT: CELLS PREPARATION

1	Plate 8 µL of cells in a small volume (SV) white detection plate in your appropriate medium. If no stimulation is required, plate 12 µL of cells instead. Cell seeding densities of 100K cells/well are generally sufficient for most cell lines, but optimization of cell seeding densities is recommended. A starving step with serum-free medium can be included.	
2	If stimulation is required, dispense 4 µL of compounds (3X) diluted in your appropriate medium. For most compounds, the incubation time is 4h at 37°C. We recommend a time course study to determine the optimal stimulation time.	

TOTAL-AP2 DETECTION USING REVVITY KIT

3	Add 4 µL of lysis buffer (4X). Use the appropriate lysis buffer and incubate for at least 30 minutes at room temperature under shaking. Lysis incubation time may be optimized.	
4	Add 4 µL of premixed antibody solutions (vol/vol) prepared in the detection buffer. Cover the plate with a plate sealer. Incubate for 3 hours at room temperature. Set up your reader for Eu^{3+} Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on an HTRF® compatible reader.	

► ***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
GENERAL LAB WORK	Step 1	Dispense 8 µL of cells		-	
	Step 2	Add 4 µL of your appropriate medium	Add 4 µL of compound (3X)	Add 12 µL of your appropriate medium	Dispense 16 µL of control lysate
TOTAL-β-ARRESTIN 1 DETECTION	Step 3	Add 4 µL of lysis buffer (4X) - 30 min/RT.			-
	Step 4	Add 4 µL of premixed AP2 total kit d2 antibody + AP2 total kit Eu cryptate antibody solution to all wells			
	Step 5	Cover the plate with a plate sealer. Incubate for 3h at room temperature.			
	Step 6	Remove the plate sealer and read on an HTRF compatible reader			

The Negative control is used to check the non-specific signal. The ratio between the 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

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

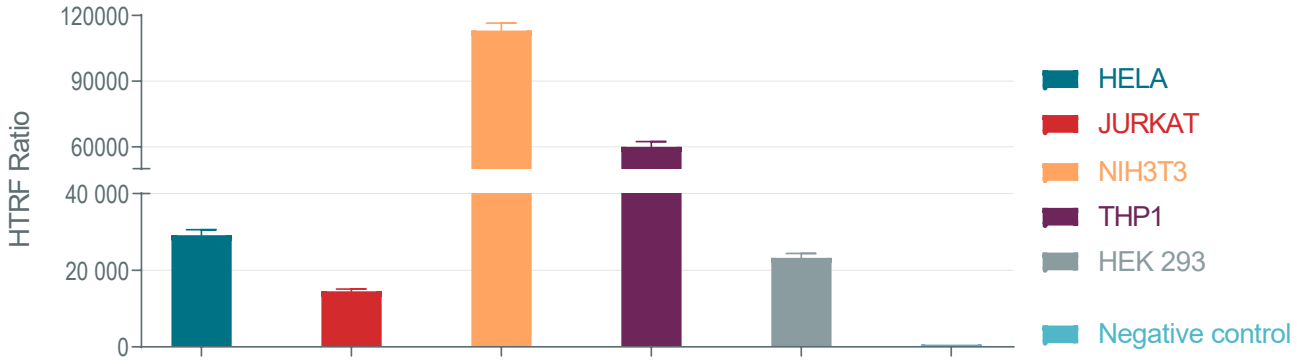
The results are drawn by plotting HTRF® ratio versus cell lysates from the various cell lines in which total AP2 is expressed.

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 you work in the optimal conditions.

The results shown here with various cell lysates were previously normalized with the HTRF Alpha-tubulin Housekeeping cellular kit.

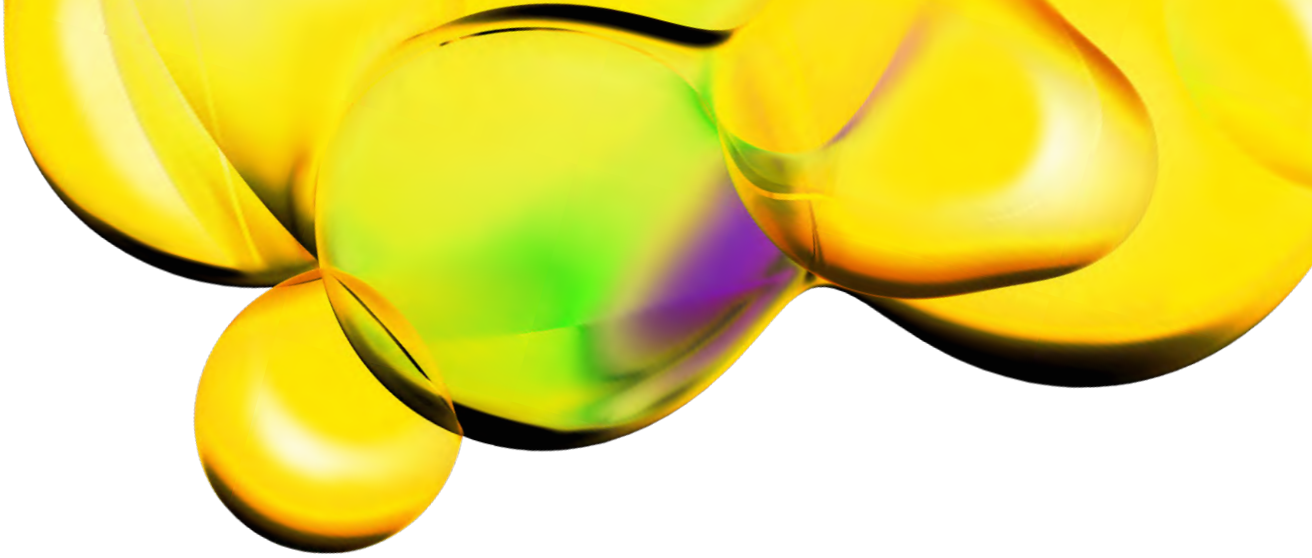
Cells were grown in T175cm^2 culture flasks, then lysed with lysis buffer #4 (1X) for 30 minutes at room temperature under shaking. For each condition, a triplet was done by transferring 16µL of each cell lysate into a 384-well plate for the detection. Lysate dilutions were done to ensure that work took place within the linear detection range of the HTRF AP2 total immunoassay kit.

Samples	Mean HTRF Ratio	CV%
HELA cells	29157	5%
JURKAT cells	14542	4%
NIH3T3 cells	113097	3%
THP1 cells	59999	4%
HEK293 cells	23262	5%
Negative control	600	2%



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
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 to using the AP2 total Revvity 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 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 the 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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