MANUAL

HTRF HUMAN PAN PHOSPHO-EGFR DETECTION KITS

Part # 64HR1PEG & 64HR1PEH

Test Size#: 500 tests (64HR1PEG), 10,000 tests (64HR1PEH)

Revision: #09 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 EGFR in cells, only when phosphorylated on Tyr residues. Upon activation, EGFR is phosphorylated and after lysis of the cell membrane, phospho-EGFR can be detected using the kit reagents.

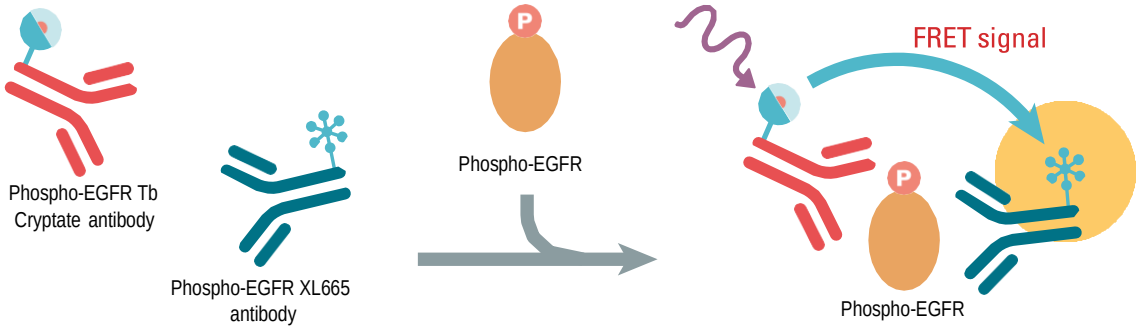


Figure 1: Principle of HTRF sandwich assay.

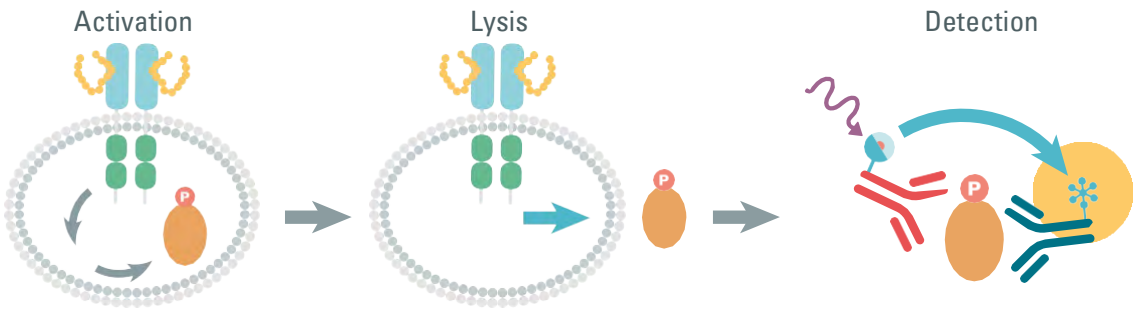
As shown here, phospho-EGFR is detected in a sandwich assay format using 2 different specific antibodies, one labelled with Tb^{3+}-Cryptate (donor) and the second with XL665 (acceptor).

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 phospho-EGFR.

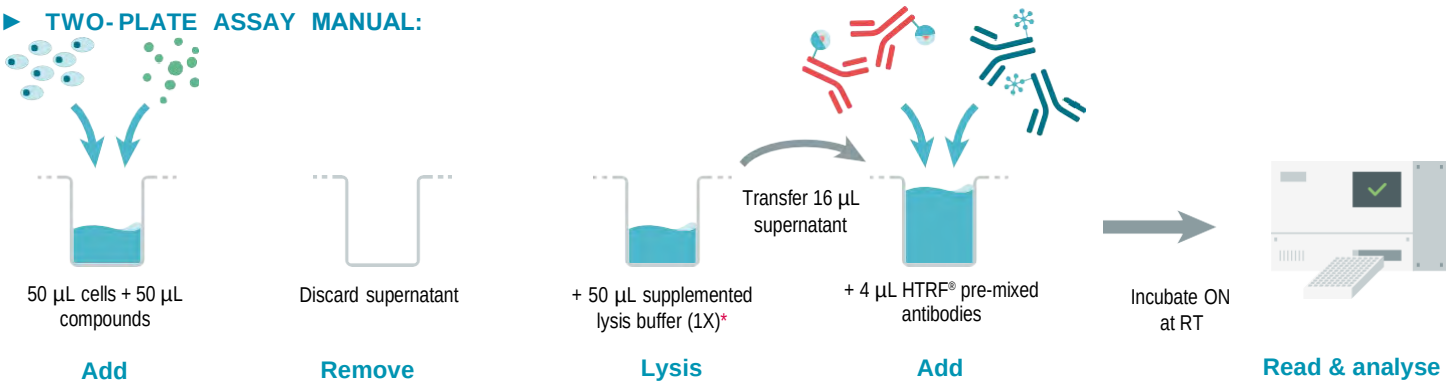
The assay can be run under a two-plate assay manual, where cells are plated, stimulated, and lysed in the same culture plate. Lysates are then transferred to the assay plate for the detection of phospho-EGFR by HTRF® reagents. This manual gives the cells viability and confluence to be monitored. It can also be further streamlined to a one-plate assay manual. Detection of phospho-EGFR 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
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

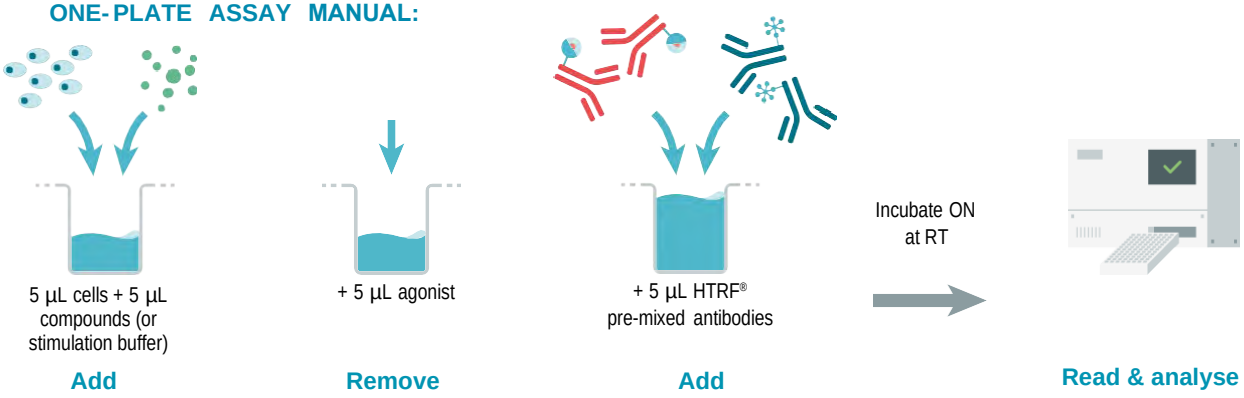


► TWO-PLATE ASSAY MANUAL:



96-well plate → Small volume detection plate

ONE-PLATE ASSAY MANUAL:



Small volume detection plate

* Depending on cell lines used, volume of lysis should be optimized.

► 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 CAT# 64HR1PEG		10,000 TESTS CAT# 64HR1PEH	
Control lysate (ready-to-use)	≤-60°C	green cap	1 vial - 150 µL	green cap	2 vials - 150 µL
Phospho-EGFR Tb Cryptate antibody	≤-60°C	red cap	1 vial - 50 µL	red cap	1 vial - 1 mL
Phospho-EGFR XL665 antibody	≤-60°C	blue cap	1 vial - 50 µL	blue cap	1 vial - 1 mL
Stimulation buffer (stock solution 5X) **For one plate manual only**	≤-16°C	yellow cap	2 vials - 1.5 mL	green cap	1 vials - 40 mL
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) **For two plate manual only**	≤-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

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 -60°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

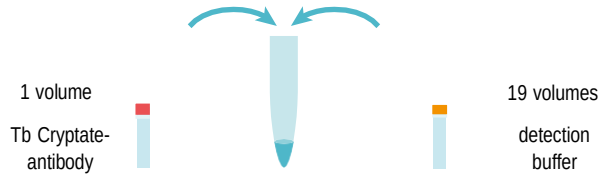
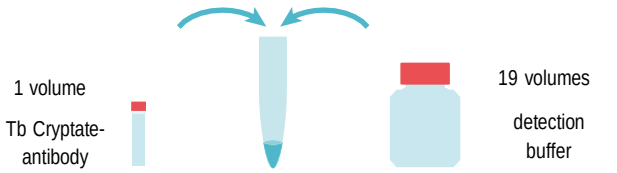
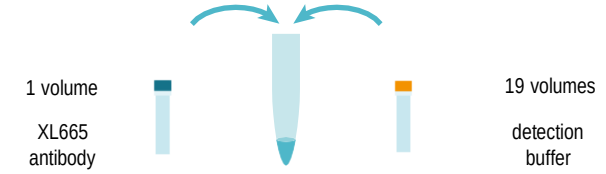
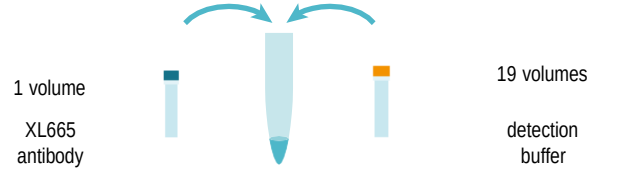
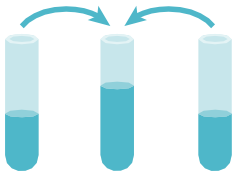
The control lysate is only provided as an internal assay control to check the quality of the results obtained. The window between control lysate and negative control should be greater than 2.

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

TO PREPARE WORKING ANTIBODY SOLUTIONS (Two-plate manual):

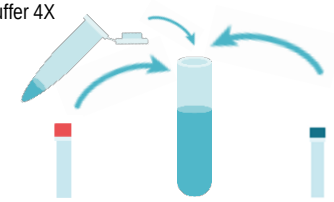
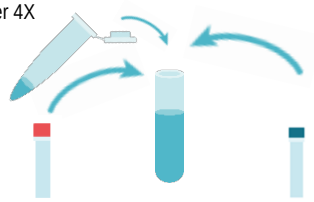
HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the XL665 and Tb Cryptate-antibodies will impair the assay's quality. Be careful, as working solution preparation for antibodies may differ between the 500 and 10,000 tests data point kit.

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

500 TESTS KIT 64HR1PEG	10,000 TESTS KIT 64HR1PEH
Phospho-EGFR Tb 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 Tb Cryptate-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 Tb Cryptate-antibody stock solution.
Phospho-EGFR XL665 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 XL665-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 XL665-antibody stock solution.
Antibody mix	
It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of XL665-antibody solution to 1 volume of Tb Cryptate-antibody solution.	 It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of XL665-antibody solution to 1 volume of Tb Cryptate-antibody solution.

TO PREPARE WORKING ANTIBODY SOLUTIONS (ONE-PLATE ASSAY MANUAL):

Dilute the antibodies with supplemented lysis buffer 4X. In practice:

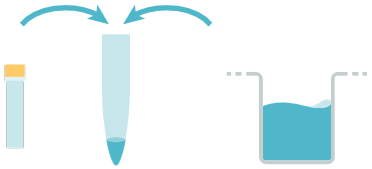
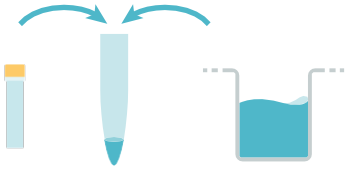
500 TESTS KIT 64HR1PEG	10,000 TESTS KIT 64HR1PEH
Phospho-EGFR Tb Cryptate antibody and Phospho-EGFR XL665 antibody	
Supplemented Lysis Buffer 4X 48 volumes 1 volume Tb Cryptate-antibody 1 volume XL665 antibody  Dilute the frozen cryptate and XL665 stock solutions 50-fold with supplemented lysis buffer 4X (e.g. add 0.05 mL of the cryptate conjugate and 0.05 mL of the XL665- conjugate to 2.4mL of supplemented lysis buffer 4X).	Supplemented Lysis Buffer 4X 48 volumes 1 volume Tb Cryptate-antibody 1 volume XL665 antibody  Dilute the frozen cryptate and XL665 stock solutions 50-fold with supplemented lysis buffer 4X (e.g. add 1 mL of the cryptate conjugate and 1 mL of the XL665- conjugate to 48 mL of supplemented lysis buffer 4X).

TO PREPARE STIMULATION BUFFER (ONE-PLATE ASSAY MANUAL ONLY):

This buffer is used to prepare cell suspension and compound dilutions.

Prepare the required amount of stimulation buffer before running the assay.

Determine the amount of stimulation buffer needed for the experiment. Each well requires 15 µL of stimulation buffer. Dilute the stimulation buffer stock solution 5-fold with distilled water. In practice:

500 TESTS KIT 64HR1PEG	10,000 TESTS KIT 64HR1PEH
Preparation of Stimulation buffer	
1 volume stimulation buffer 4 volumes distilled water  Dilute the “stimulation buffer 5X” 5-fold with distilled water. e.g. take 1.5 mL of stimulation buffer 5X and add 6 ml of distilled water.	1 volume stimulation buffer 4 volumes distilled water 

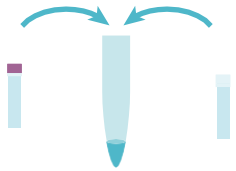
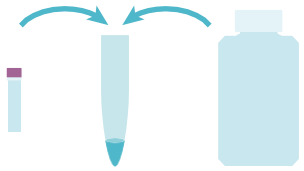
TO PREPARE SUPPLEMENTED LYSIS BUFFER:

Make sure that the lysate has been generated by using the kit reagents.
Supplemented lysis buffer differs between the manuals. Make sure to use the appropriate supplemented lysis buffer depending on the chosen manual’s specification.

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

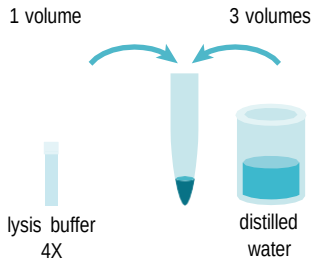
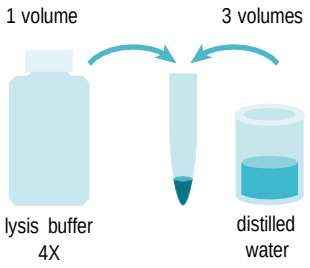
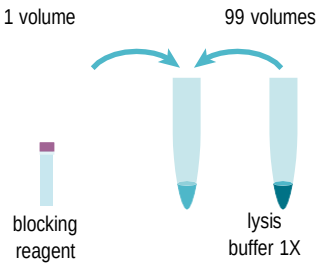
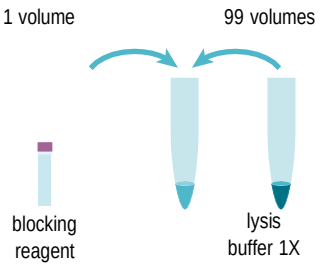
► *Supplemented Lysis buffer 4X for one-plate assay manual*

Determine the amount of supplemented lysis buffer needed for the experiment. Each well requires 5 µL of supplemented lysis buffer for one-plate assay manual. Dilute the blocking reagent stock solution 25-fold with lysis buffer 4X. In practice:

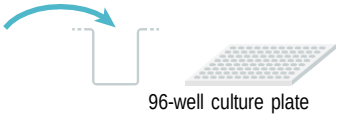
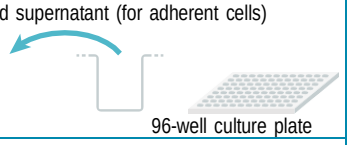
ONE-PLATE ASSAY MANUAL	
500 TESTS KIT 64HR1PEG	10,000 TESTS KIT 64HR1PEH
Preparation of Supplemented Lysis buffer 4X	
1 volume Blocking reagent 24 volumes lysis buffer 4X  Dilute the “blocking reagent stock solution” 25-fold with “lysis buffer 4X” e.g take 0.1 ml of “Blocking reagent stock solution” and add it to 2.4 ml of lysis buffer 4X. Mix gently.	1 volume Blocking reagent 24 volumes lysis buffer 4X 

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

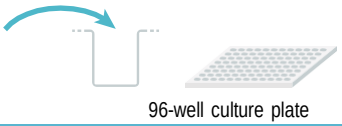
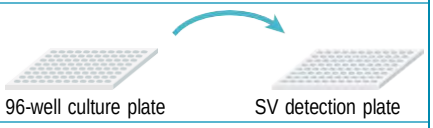
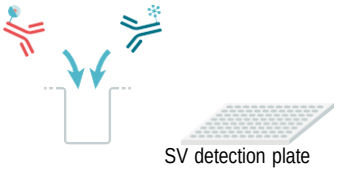
Determine the amount of supplemented lysis buffer needed for the experiment. Each well requires generally 50 µL of supplemented lysis buffer. Prepare a lysis buffer solution 1X and then dilute the blocking reagent stock solution 100-fold with this lysis buffer 1X. In practice:

<table>
<tr><th colspan="4">TWO-PLATE ASSAY MANUAL</th></tr>
<tr><th colspan="4">500 TESTS KIT 64HR1PEG & 10,000 TESTS KIT 64HR1PEH</th></tr>
<tr><td colspan="2">Preparation of lysis buffer 1X</td><td colspan="2">Preparation of supplemented Lysis buffer 1X</td></tr>
<tr>
<td>500 tests
 
</td>
<td>10,000 tests
 
</td>
<td>500 tests
 
</td>
<td>10,000 tests
 
</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>
<td colspan="2">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>

GENERAL LAB WORK PRIOR USING REVVITY KIT: CELLS PREPARATION

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. Cell seeding densities of 50K 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	Activation dose response: Dispense 50 µL of agonist (2X) diluted in cell culture serum-free medium. For most compound, incubation time is between 10 and 30 minutes at 37°C. Inhibition dose response: Pre-treat cells with 25 µL of compounds (3X) diluted in cell culture serum-free medium. Incubation time is between 30min and 2h at 37°C. Then stimulate cells with 25 µL agonist concentration (4X) diluted in cell culture serum-free medium, Determine the concentration of the agonist to use. We recommend a time course study to determine the optimal stimulation time.	
3	Carefully remove cell supernatant either by aspirating supernatant or by flicking the plate.	

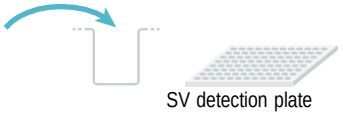
PHOSPHO-EGFR DETECTION USING REVVITY KIT

4	Immediately add 50 µL of supplemented lysis buffer (1X) and incubate for at least 30 minutes at room temperature. Use the appropriate supplemented lysis buffer and incubate at room temperature with shaking. Lysis incubation time may be optimized. Lysis volume can be decreased down to 25 µL.	
5	After homogenization by pipeting up and down, transfer 16 µL of cell lysate from the 96-well cell-culture plate to a small volume (SV) white 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 ON at room temperature. Set up your reader for Tb3+ Cryptate and read the fluorescence emission at two different wavelenghts (665nm and 620nm) on a compatible HTRF® 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
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)
Step 2	Add 2 µL of Phospho-EGFR XL665 antibody working solution to all wells			
Step 3	Add 2 µL of Phospho-EGFR Tb Cryptate antibody working solution to all wells			
Step 4	Cover the plate with a plate sealer. Incubate ON at room temperature.			
Step 5	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 control lysate signal / non-specific signal should be greater than 2.

	GENERAL LAB WORK PRIOR USING REVVITY KIT: CELLS PREPARATION	
1	Plate 5 µL of cells in a small volume (SV) white detection plate in stimulation buffer. Cell seeding densities of 10K 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 can be included.	 SV detection plate
2	Activation dose response: Dispense 5µl of the stimulation buffer then add 5 µL of agonist (3X) diluted in stimulation buffer. For most compound, incubation time is between 10 and 30 minutes at 37°C. Inhibition dose response: Pre-treat cells with 5 µL of compounds (2X) diluted in the stimulation buffer, for 30min to 2h at 37°C. Then stimulate cells with 5 µL agonist (3X) diluted in the stimulation buffer, during the appropriate time, usually at the concentration equivalent to the agonist EC80. We recommend a time course study to determine the optimal stimulation time.	 SV detection plate
	PHOSPHO-EGFR DETECTION USING REVVITY KIT	
3	Add 5 µL of premixed antibody solutions (vol/vol) prepared in supplemented lysis buffer 4X. Cover the plate with a plate sealer. Incubate ON at room temperature. Set up your reader for Tb3+ Cryptate and read the fluorescence emission at two different wavelenghts (665nm and 620nm) on a compatible HTRF® reader.	 SV detection plate

► ***Standard manual for one-plate assay manual in 20 µL final volume***

		NON TREATED CELL LYSATE	TRE ATED CELL LYSATE	NEGATIVE CONTROL	CONTROL LYSATE
GENERAL LAB WORK	Step 1	Dispense 5 µL of cells		-	
	Step 2	Add 5 µL of stimulation buffer	Add 5 µL of compound (2X)	Add 15 µL of stimulation buffer	Dispense 15 µL of control lysate
PHOSPHO-EGFR DETECTION STEPS	Step 3	Add 5 µL of agonist (3X)		-	
	Step 4	Add 5 µL of premixed conjugates to all wells			
	Step 5	Cover the plate with a plate sealer. Incubate ON 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 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

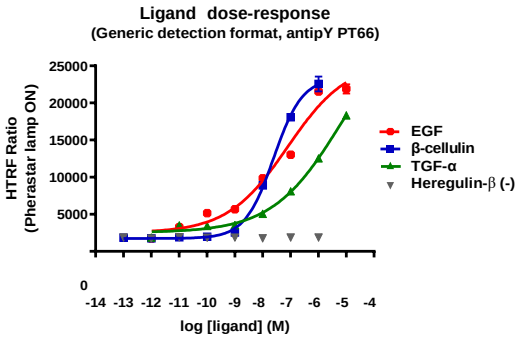
These data should be considered only as an example. Results may vary from one HTRF® compatible reader to another.

DOSE RESPONSE EXPERIMENT

Results obtained on A431 cells (25,000 cells) activated with various agonists for 10 minutes, using the two-plate assay manual are presented below. Cells were lysed with 50 µL of supplemented lysis buffer for 30 minutes at room temperature. Results may vary from one HTRF® compatible reader to another. Heregulin-β was used as a negative control.

The activation curve is drawn up by plotting ratio versus the log [compound] concentration:

[Agonist] Log(M)	EGF		β-cellulin		TGF-α		Heregulin-β	
	Ratio (1)	CV % (2)	Ratio (1)	CV % (2)	Ratio (1)	CV % (2)	Ratio (1)	CV % (2)
10-13			1835	0.4			1864	1.2
10-12	1733	3.3	1766	7.5	1863	0.4	1715	0.9
10-11	3261	3.9	1909	2.2	3486	0.3	1911	2.9
10-10	5152	0.0	1971	1.4	3429	1.4	1814	3.3
10-9	5675	3.2	2547	0.0	3523	2.0	1838	2.0
10-8	9834	2.5	8894	0.7	5043	6.2	1751	0.7
10-7	13017	0.0	18066	0.6	8082	1.2	1869	1.1
10-6	21511	0.3	22549	4.4	12512	1.0	1872	2.1
10-5	21879	2.9			18315	1.2		



	EGF	β-cellulin	TGF-α
EC50	7.799e-008	2.469e-008	6.335e-006
S/B	12.6	12.5	9.8

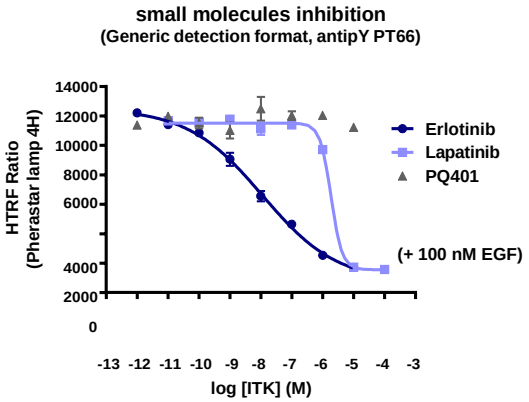
INHIBITION EXPERIMENT

Results were obtained on A431 cells (25 000 cells/well) treated with compounds for 30min, then stimulated for 10 minutes by EGF used at 100nM. Cells were lysed with 50 µL of supplemented lysis buffer for 30 minutes at room temperature.

PQ401 was used as a negative control.

The inhibition curve is drawn up by plotting ratio versus the log [compound] concentration.

[Compounds]	erlotinib		lapatinib	
M / Log(M)	Ratio (1)	CV % (2)	Ratio (1)	CV % (2)
-12	12214	1		
-11	11408	2	11657	4
-10	10844	2	11581	3
-9	9063	9	11776	4
-8	6552	9	11128	6
-7	4643	8	11387	0
-6	2530	4	9717	5
-5	1707	8	1719	5
-4			1564	2

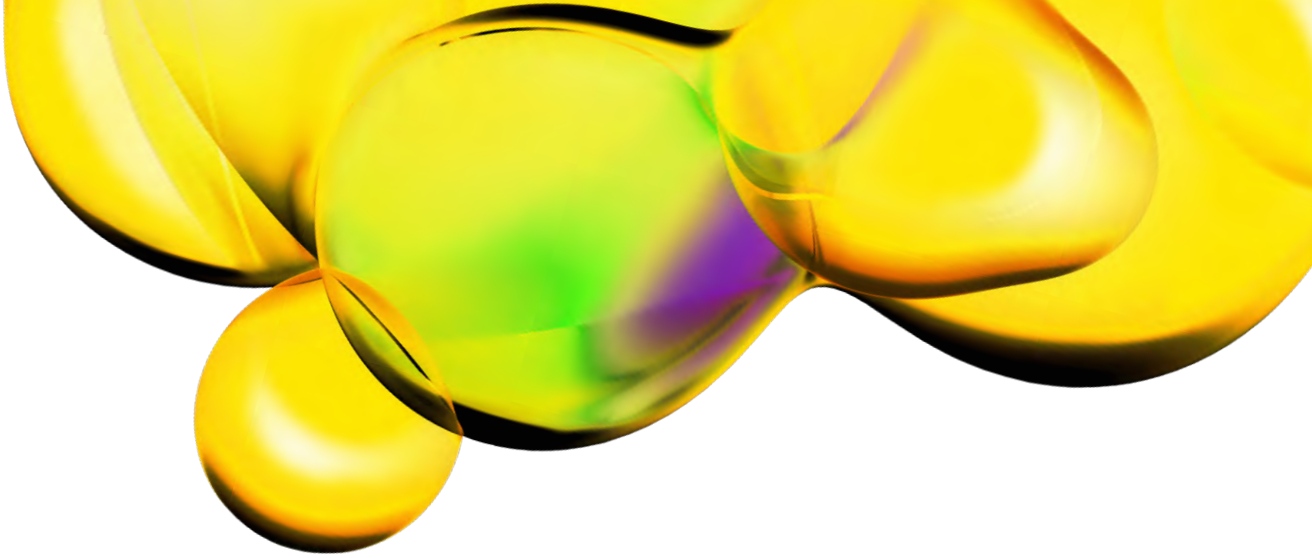


	Erlotinib	Lapatinib
IC50	1.132e-008	1.868e-006
Ki	5e-09	8.2e-07
S/B	7.1	7.4

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 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 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 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 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 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. Phospho-EGFR detection using Revvity kit: 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.

REACH European regulations and compliance
This product and/or some of its components include a Triton concentration of 0.1% or more and as such, it is concerned by the REACH European regulations. We recommend researchers using this product to act in compliance with REACH and in particular: to only use the product for in vitro research in appropriate and controlled premises by qualified researchers, ii) to ensure the collection and the treatment of subsequent waste, and iii) to make sure that the total amount of Triton handled does not exceed 1 ton per year.

This product contains material of biologic origin. Use for research purposes only. Do not use in humans or for diagnostic purposes. The purchaser assumes all risk and responsibility concerning reception, handling and storage.
The use of the cell line will be done with appropriate safety and handling precautions to minimize health and environmental impact.



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

www.revvity.com

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com