MANUAL

PHOSPHO-BAD (SER112) KITS

Part # 64BADPET

Test Size#: 1 x 96 tests (64BADPET)

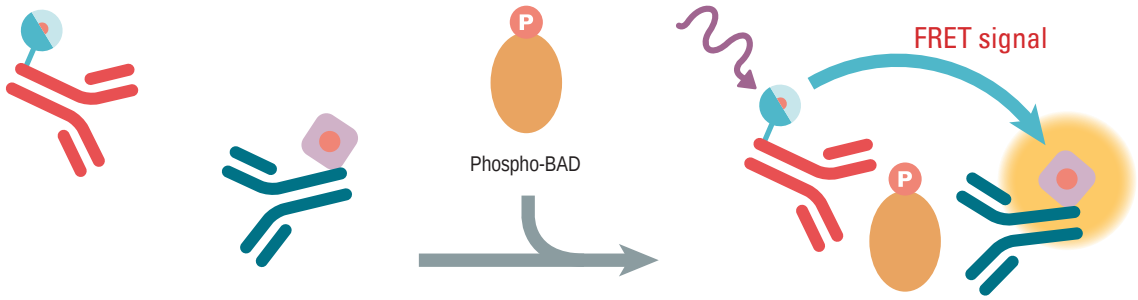
Revision: #06 of September 2023

Store at: ≤-60°C

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

ASSAY DESCRIPTION

This assay is intended for the simple, rapid and direct detection of endogenous levels of BAD in cells, only when phosphorylated at Ser112. Upon activation, BAD is phosphorylated and after lysis of the cell membrane, phospho-BAD (Ser112) can be detected using the kit reagents.



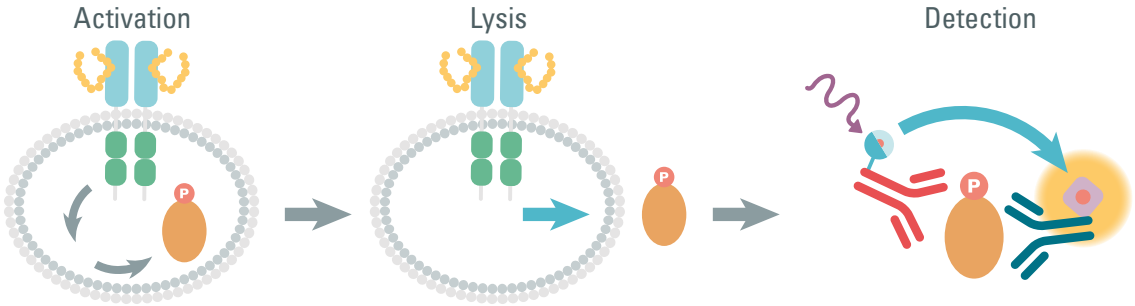
As shown here, phospho-BAD (Ser112) is detected in a sandwich assay format using 2 different specific antibodies, one labelled with Eu^{3+}-Cryptate (donor) and the second with d2 (acceptor).

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 phospho-BAD (Ser112).

If you are using adherent cells please refer to the "transfer" assay manual where cells are plated, stimulated and lyzed in the cell culture plate then lysates are transferred to a HTRF® 96 well-detection plate for the detection of phospho-BAD (Ser112) by HTRF reagents.

If you are using suspension cells please refer to the "no transfer" assay manual, where detection of phospho-BAD (Ser112) with HTRF reagents is performed in a single HTRF 96-well detection plate used for plating, stimulation and detection (no washing steps required).

ASSAY AT A GLANCE



HTRF REAGENTS

		Nb	Volume per vial	Storage
Phospho-BAD Eu Cryptate antibody	red cap	1	10 µL	≤-16°C
Phospho-BAD d2 antibody	blue cap	1	10 µL	≤-16°C
Control lysate (ready-to-use)	green cap	1	150 µL	≤-60°C
Blocking reagent* (stock solution 100X)	purple cap	1	300 µL	≤-16°C
Lysis buffer* # 3 (stock solution 4X)	transparent cap	1	2 mL	≤-16°C
Detection buffer (ready-to-use)**	orange cap	1	0.5 mL	≤-16°C
HTRF 96 well detection plate		1		RT

* Amounts of reagents provided are sufficient for generating 8 mL of cell lysate.

** The Detection Buffer is used to prepare working solutions of acceptor and donor reagents.

STORAGE STABILITY

Antibodies, control lysate and buffers should be stored frozen at ≤-60°C 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

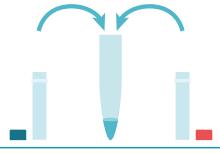
Allow all reagents to thaw before use.
We recommend centrifuging the vials gently after thawing, before pipeting the stock solutions.

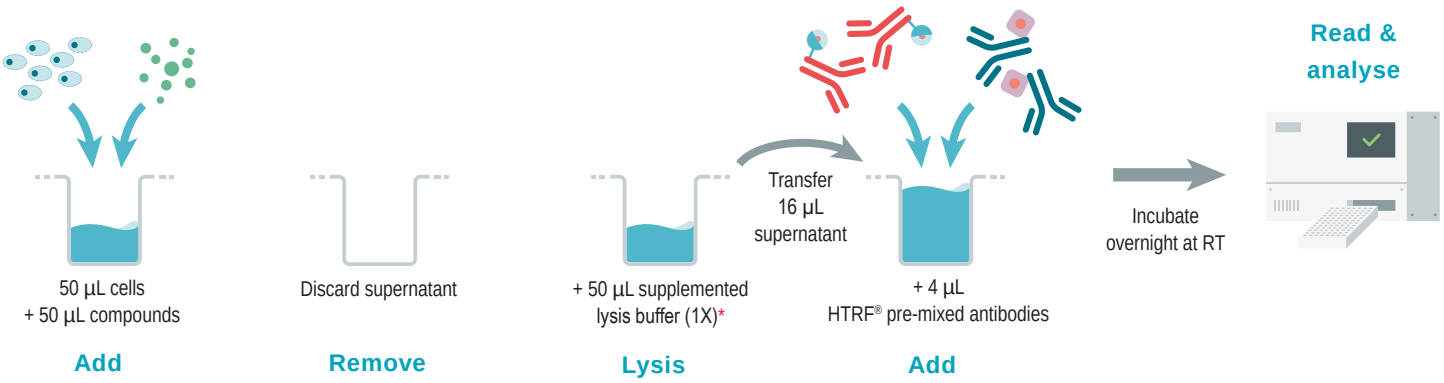
CONTROL LYSATE SOLUTION: READY-TO-USE

The control cell 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.

PREPARATION OF ANTIBODY WORKING 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. Be careful, as working solution preparation for antibodies may differ between the different kit sizes.
Antibody working solutions are stable for 2 days at 2-8°C.
Dilute the antibodies with detection buffer, and just prior to dispensing, pre-mix (1vol/1vol) the Phospho-BAD Eu Cryptate antibody & the Phospho-BAD d2 antibody. In practice:

Phospho-BAD d2 antibody working solutions	Phospho-BAD Eu Cryptate antibody working solutions	Pre-mixed Phospho-BAD Eu Cryptate antibody & Phospho-BAD d2 antibody working solutions
Acceptor-antibody (10 µL) 200 µL detection buffer 	Cryptate-antibody (10 µL) 200 µL detection buffer 	1 volume Acceptor-antibody working solution 1 volume Cryptate-antibody working solution 
Add 200 µL of detection buffer to the 10 µL of Acceptor-antibody stock solution.	Add 200 µL of detection buffer to the 10 µL of Cryptate-antibody stock solution.	Mix gently the Phospho-BAD Eu Cryptate antibody and Phospho-BAD d2 antibody working solutions: add 210 µL of Phospho-BAD d2 antibody to 210 µL of Phospho-BAD Eu Cryptate antibody



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

For other culture formats, please refer to the recommended volumes in the table below

Cell culture microplate/flask	Cell seeding	Volume of lysis buffer after medium removal
96-well	25 - 100 x 10^3 cells / well	50 µL
24-well	140 - 550 x 10^3 cells / well	250 µL
12-well	290 - 1,150 x 10^3 cells / well	500 µL
6-well	700 - 2,800 x 10^3 cells / well	1,5 mL

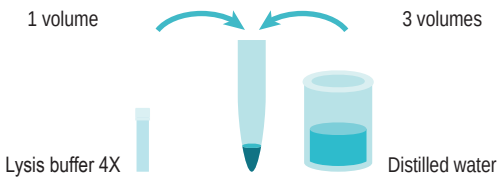
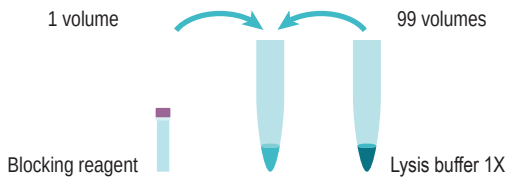
PREPARATION OF SUPPLEMENTED LYSIS BUFFER FOR «TRANSFER» ASSAY MANUAL ON ADHERENT CELLS

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 supplemented lysis buffer before running the assay, working solutions are stable for 2 days at 2-8°C.

Determine the amount of supplemented lysis buffer needed for the experiment. For example, in a 96-well culture plate, use 50 µL of lysis buffer. Prepare 1X lysis buffer solution and then dilute the blocking reagent stock solution 100-fold with this lysis buffer 1X. In practice:

Transfer assay manual on adherent cells	
Preparation of lysis buffer 1x	**Dilution of the blocking reagent**
	
Dilute the "lysis buffer 4X" 4-fold with distilled water to prepare lysis buffer 1X: Take 2 mL of lysis buffer 4X and add it to 6 mL of distilled water. Mix gently.	Dilute the "blocking reagent" 100-fold with "Lysis buffer 1X": Take 80 µL of "Blocking reagent stock solution" and add it to 7.92 mL of lysis buffer 1X. Mix gently.

TRANSFER ASSAY MANUAL FOR ADHERENT CELLS

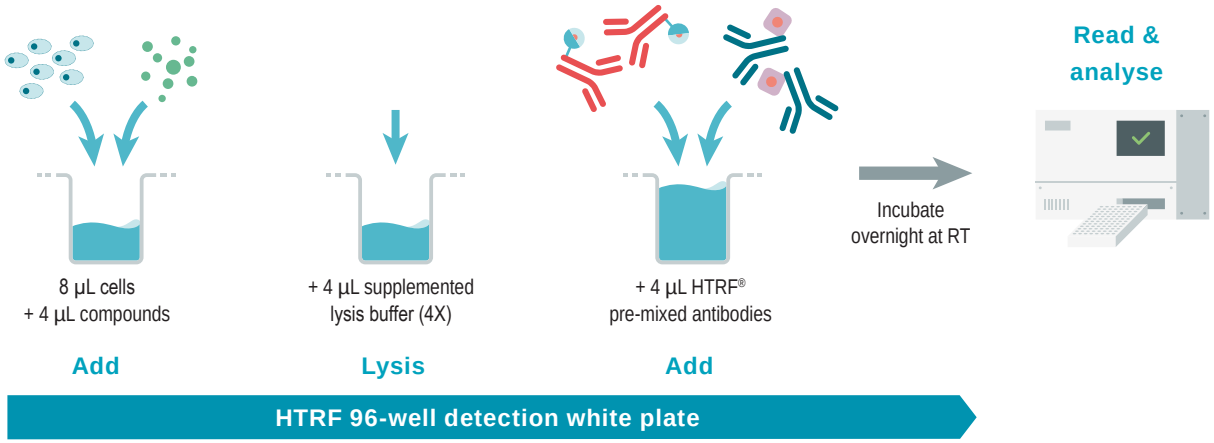
	GENERAL LAB WORK PRIOR USING REVVITY KIT: CELLS PREPARATION	
1	Plate 50 µL of cells in 96-well tissue-culture treated plate in appropriate growth medium and incubate overnight, at 37°C in CO2 atmosphere. Cell seeding densities of 25 K cells/well are generally sufficient for most cell lines, but optimization of cell seeding densities is recommended. Depending on receptor a starving step with serum-free medium could be essential.	 96-well culture plate
2	Dispense 50 µL of compounds (2X) diluted in cell culture serum-free medium* 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.	 96-well culture plate
3	Remove carefully cell supernatant either by aspirating supernatant or by flicking the plate.	Discard supernatant (for adherent cells)  96-well culture plate
	PHOSPHO-BAD DETECTION USING REVVITY KIT	
4	Immediately add 50 µL of supplemented lysis buffer (1X) and incubate for at least 30 minutes at room temperature under shaking. 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.	 96-well culture plate
5	After homogenization by pipeting up and down, transfer 16 µL of cell lysate from the 96-well cell-culture plate to the HTRF 96-well detection plate.	 96-well culture plate 96-well 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 overnight at room temperature. Set up your reader for Eu^{3+} Cryptate and read the fluorescence emission at two different wavelenghts (665nm and 620nm) on a compatible HTRF reader**.	 96-well 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 TRANSFER 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	Dispense 4 µL of Premix antibody solutions: Phospho-BAD d2 antibody & Phospho-BAD Eu Cryptate antibody			
Step 3	Cover the plate with a plate sealer. Incubate overnight 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 control lysate signal / non-specific signal should be greater than 2.



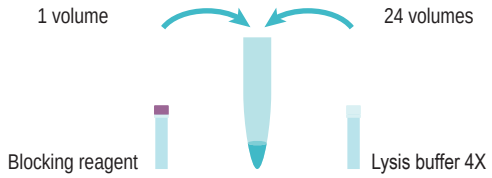
PREPARATION OF SUPPLEMENTED LYSIS BUFFER «NO TRANSFER» ASSAY MANUAL ON SUSPENSION CELLS

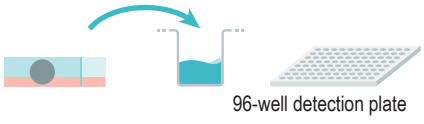
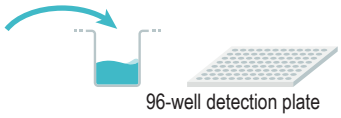
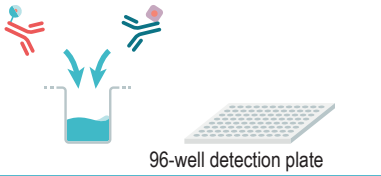
Make sure that the lysate has been generated by using the kit reagents.

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 supplemented lysis buffer before running the assay, working solutions are stable for 2 days at 2-8°C.

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

No transfer assay manual on suspension cells

Dilute the "blocking reagent stock solution" 25-fold with "Lysis buffer 4X". e.g. take 80 µL of "Blocking reagent stock solution" and add it to 1.92 mL of lysis buffer 4X. Mix gently.

	GENERAL LAB WORK PRIOR USING REVVITY KIT: CELLS PREPARATION	
1	Dispense 8 µL of cells in the HTRF 96-well detection plate in your appropriate medium. Cell seeding densities of 8 K 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.	
2	Dispense 4 µL of compounds (3X) diluted in your appropriate medium. For most compounds, incubation time is between 10 and 30 minutes at 37°C. We recommend a time course study to determine the optimal stimulation time.	
	PHOSPHO-BAD DETECTION USING REVVITY KIT	
3	Add 4 µL of supplemented lysis buffer (4X). Use the appropriate supplemented lysis buffer and incubate for at least 30 minutes at room temperature under shaking. We recommend a time course study to determine the optimal lysis incubation time	
4	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 wavelenghts (665nm and 620nm) on a compatible HTRF reader*.	

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

STANDARD MANUAL FOR NO TRANSFER ASSAY MANUAL IN 20 µL FINAL VOLUME

		Non treated cell lysate	Treated cell lysate	Negative control	Control lysate
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
PHOSPHO-BAD DETECTION STEPS	Step 3	Add 4 µL of supplemented lysis buffer (4X) and incubate for at least 30 minutes / RT			-
	Step 4	Dispense 4 µL of Premix antibody solutions: Phospho-BAD d2 antibody & Phospho-BAD Eu Cryptate antibody			
	Step 5	Cover the plate with a plate sealer. Incubate overnight 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 (readings on PHERAstarFS with flash lamp). Results may vary from one HTRF® compatible reader to another.

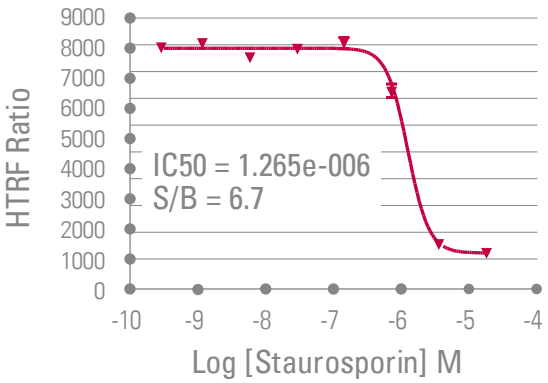
The curves are drawn up by plotting HTRF® Ratio versus the log [compound] concentrations.

Results on MCF-7 cells (25 000 cells/well), using the two-plate assay manual for adherent cells.

MCF-7 cells were treated for 3H with increasing concentrations of staurosporin. After stimulation with PMA 0.8µM for 30 minutes, MCF-7 cells were then lysed with 50 uL of supplemented lysis buffer for 30 minutes at room temperature.

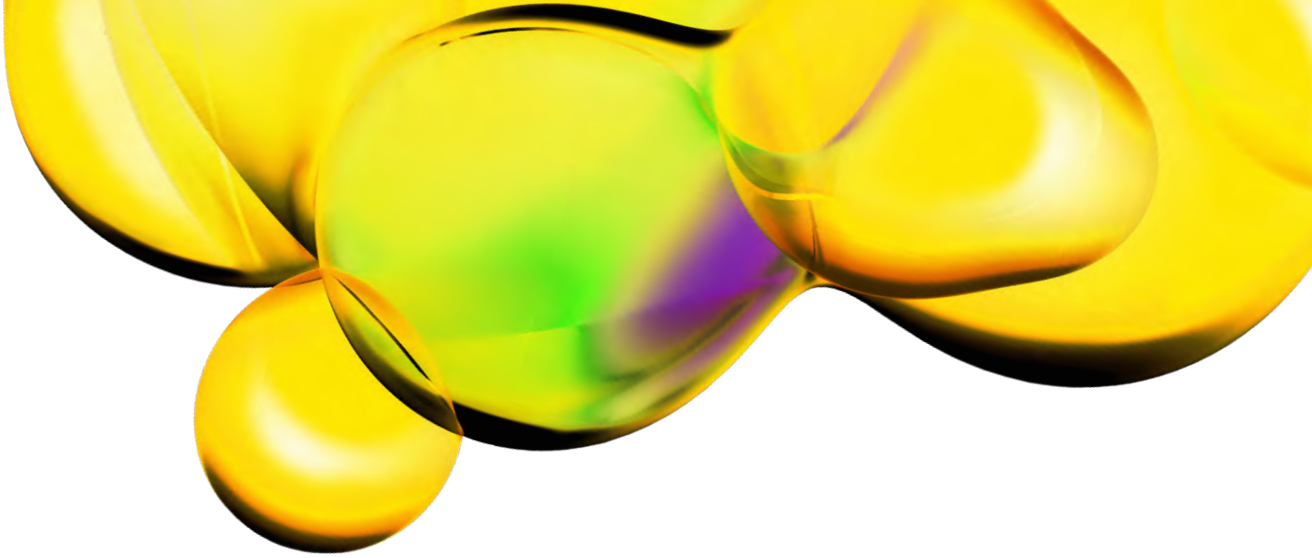
	phospho-BAD results	
log [compound] (M)	Ratio	CV %
-9.5	8013	1.0%
-8.9	8153	0.0%
-8.2	7680	0.0%
-7.5	7972	0.0%
-6.8	8174	1.0%
-6.1	6544	2.0%
-5.4	1478	4.0%
-4.7	1189	1.0%

Negative control	974	10.0%
Control lysate	4783	2.0%



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 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 50 µL. 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 interesrcan 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-BAD 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. For long term conservation, aliquots should be stored in liquid nitrogen.



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