MANUAL

Technology: HTRF™ Pathway Readout

HTRF Human phospho-IRAK4 (Thr345) Detection Kit

Part number	64IRK4T45PEG	64IRK4T45PEH
Test size	500 tests	10,000 tests

Storage: ≤-60°C

Version: 01 **Date:** January 2025

ASSAY PRINCIPLE

This assay is intended for the simple, rapid and direct detection of endogenous levels of IRAK4 in cells, only when phosphorylated at Thr345. Upon activation, IRAK4 is phosphorylated and after lysis of the cell membrane, phospho-IRAK4 (Thr345) can be detected using the kit reagents.

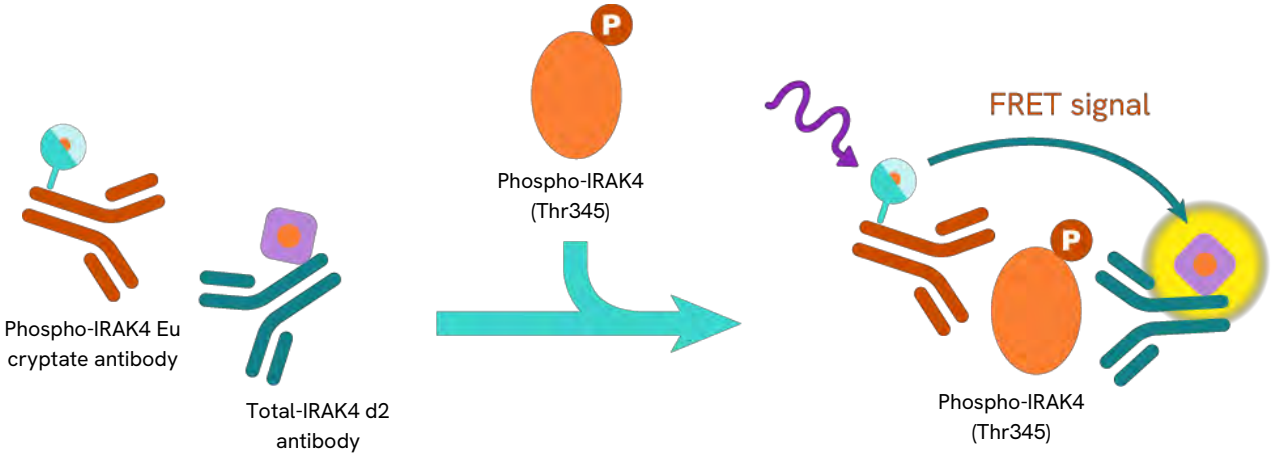


Figure 1: Principle of HTRF sandwich assay.

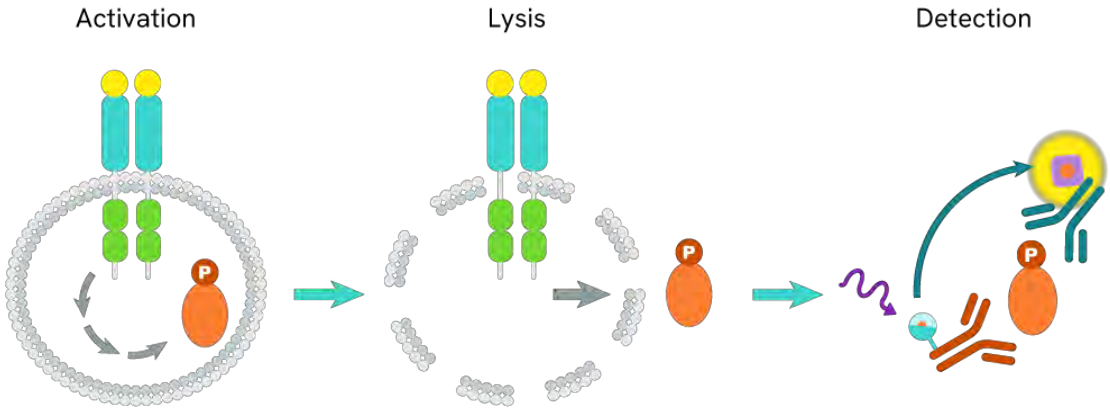
As shown here, phospho-IRAK4 (Thr345) is detected in a sandwich assay format using 2 different specific antibodies, one labelled with Eu^{3+}-Cryptate (donor) and the second with d2 (acceptor). **One antibody is selected for its specific binding to the phosphorylated motif on the protein, the second for its ability to recognize the total protein independently of its phosphorylation state.**

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-IRAK4 (Thr345).

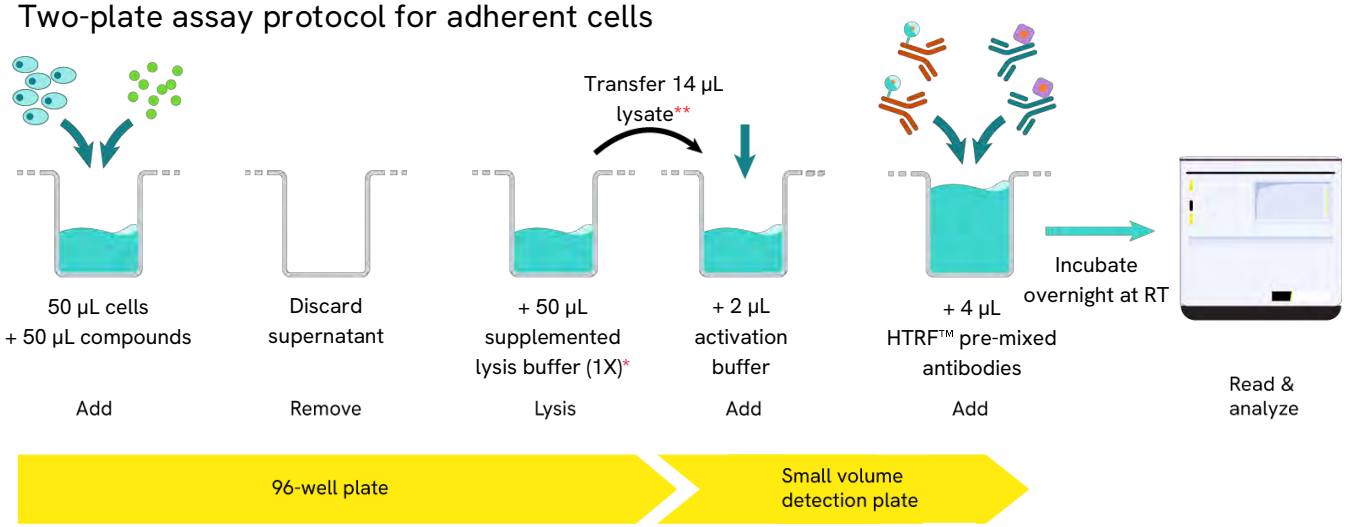
The assay can be run under a two-plate assay protocol, where cells are plated, stimulated and lysed in the same culture plate. Lysates are then transferred to the assay plate for the detection of phospho-IRAK4 (Thr345) by HTRF™ reagents. This protocol gives the cells viability and confluence to be monitored.

For tissue derived samples, please refer to the technical note: "Optimize your HTRF™ cell signaling assays on tissues".
Technical support team can help you to set-up this protocol or another one. Please contact us.

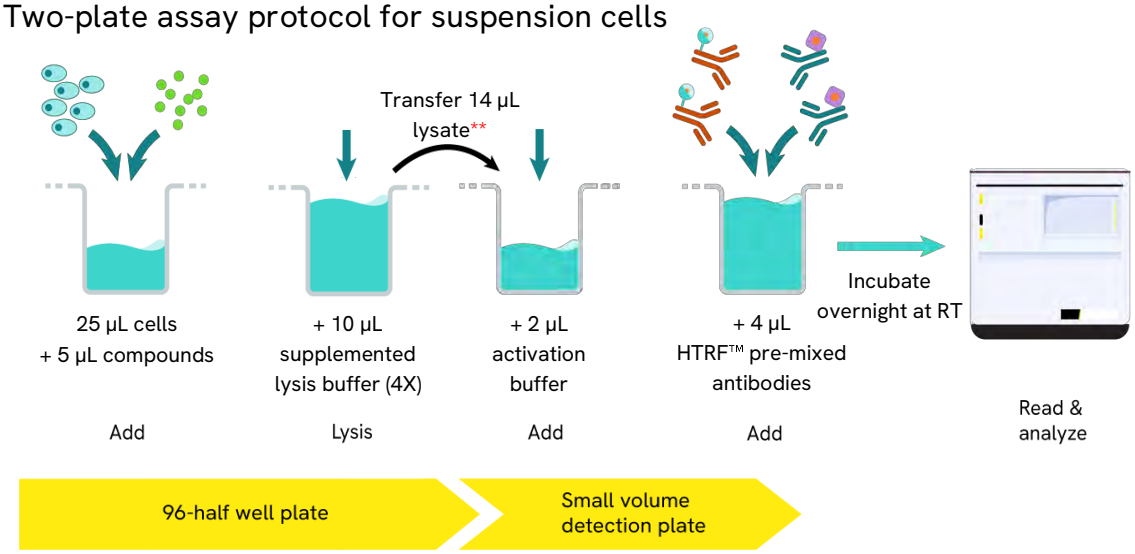
PROTOCOL AT A GLANCE



Two-plate assay protocol for adherent cells



Two-plate assay protocol for suspension cells



* Depending on cell lines used, volume of lysis should be optimized.
** Depending on cell lines used, it can be necessary to dilute the cell lysate to ensure samples are within the assay linear range.

For HTRF certified reader

For more information about HTRF™ compatible readers and for set-up recommendations, please visit our website.

MATERIAL PROVIDED

KIT COMPONENTS	STORAGE	500 TESTS			10,000 TESTS		
Control lysate (ready-to-use)	≤-60°C		green cap	1 vial-150 µL		green cap	2 vials-150 µL
Phospho-IRAK4 Eu cryptate antibody	≤-16°C		red cap	1 vial-50 µL		red cap	1 vial-1 mL
Phospho-IRAK4 d2 antibody	≤-16°C		blue cap	1 vial-50 µL		blue cap	1 vial-1 mL
Blocking reagent* (stock solution 100X)	≤-16°C		purple cap	1 vial-300 µL		purple cap	3 vials-2 mL
Lysis buffer * #1 (stock solution 4X)	≤-16°C		transparent cap	4 vials-2 mL		white cap	1 vial-130 mL
Detection buffer** (ready-to-use)	≤-16°C		orange cap	2 vials-2 mL		red cap	1 vial-50 mL
Activation buffer (ready-to-use)	≤-16°C		Yellow cap	1 vial-1mL		white cap	1 vial-20mL

* 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.

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 protocol 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 / Activation buffer	2-8°C until the expiration date indicated on the package
Antibodies*	2-8°C for 48h or freeze at -16°C or below until the expiration date indicated on the package for long term storage
Protein/standard/Control Lysate*	freeze at -60°C or below until the expiration date indicated on the package for long term storage

*For Antibodies, Protein, Standard & control lysate, Stock solutions may be thawed and frozen only once. Freeze in aliquots to avoid multiple freeze/thaw cycles (once aliquoted, single use of the reagent).

Volume of antibodies aliquots should not be under 10µL. Volume of Protein, Standard & control lysate aliquots should not be under 20µL.

REAGENT PREPARATION

Allow all reagents to thaw before use. We recommend centrifuging the vials gently after thawing, before pipetting the stock solutions. Prepare the working solutions from stock solutions by following the instructions below.

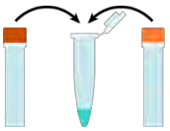
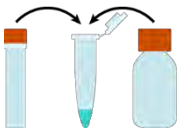
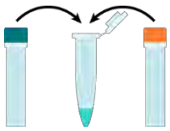
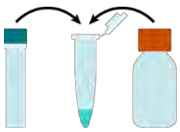
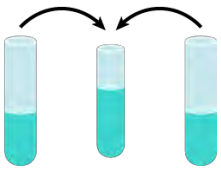
To prepare working control lysate solution

The control lysate is only provided as an internal assay control to check the quality of the results obtained. The window between 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

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

<table>
<tr><th>500 TESTS KIT</th><th>10,000 TESTS KIT</th></tr>
<tr><td colspan="2">Phospho-IRAK4 Eu cryptate antibody</td></tr>
<tr><td>1 volume Eu Cryptate-antibody
 
19 volumes detection buffer</td><td>1 volume Eu Cryptate-antibody
 
19 volumes detection buffer</td></tr>
<tr><td>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 Eu Cryptate-antibody stock solution.</td><td>Dilute 20-fold the frozen stock solution with detection buffer e.g add 19 ml of detection buffer to the 1 ml of Eu Cryptate-antibody stock solution.</td></tr>
<tr><td colspan="2">Phospho-IRAK4 d2 antibody</td></tr>
<tr><td>1 volume d2 antibody
 
19 volumes detection buffer</td><td>1 volume d2 antibody
 
19 volumes detection buffer</td></tr>
<tr><td>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 d2-antibody stock solution.</td><td>Dilute 20-fold the frozen stock solution with detection buffer e.g add 19 mlof detection buffer to the 1 ml of d2-antibody stock solution.</td></tr>
<tr><td colspan="2">Antibody mix</td></tr>
<tr><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 ofEu Cryptate-antibody solution.
  </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 ofEu Cryptate-antibody solution.</td></tr>
</table>

To prepare supplemented lysis buffer

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

Supplemented lysis buffer differs between the protocols. Make sure to use the appropriate supplemented lysis buffer depending on the chosen protocol'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 two-plate assay protocol on suspension cells

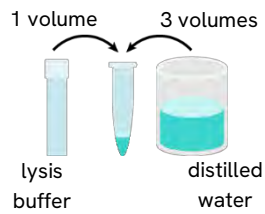
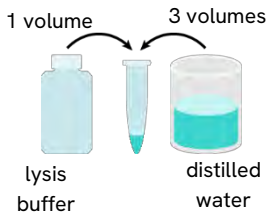
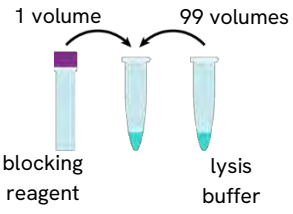
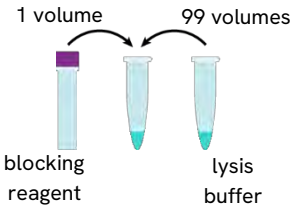
Determine the amount of supplemented lysis buffer needed for the experiment. Each well requires 4 µL of supplemented lysis buffer for one-plate assay protocol and 10 µL for two-plate assay protocol on suspension cells. Dilute the blocking reagent stock solution 25-fold with lysis buffer 4X. In practice:

TWO-PLATE PROTOCOL ON SUSPENSION CELL	
500 TESTS KIT	**10,000 TESTS KIT**
Preparation of Supplemented Lysis buffer 4X	
	

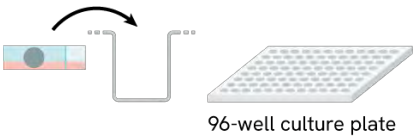
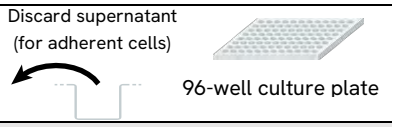
Dilute the "blocking reagent stock solution" 25-fold with "lysis buffer 4X" e.g take 0.1 ml of "Blocking reagent stock solution" and add it to 2.4 ml of lysis buffer 4X. Mix gently.

Supplemented Lysis buffer 1X for two-plate assay protocol on adherent cells

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:

TWO-PLATE ASSAY PROTOCOL ON ADHERENT CELLS			
500 TESTS KIT & 10,000 TESTS KIT			
Preparation of lysis buffer 1X		**Preparation of supplemented lysis buffer 1X**	
500 tests	10,000 tests	500 tests	10,000 tests
			
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.		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.	

TWO-PLATE ASSAY PROTOCOL

	GENERAL LAB WORK PRIOR USING HTRF 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.	Plate 25 µL of cells in 96 half-well plate in yourappropriate medium.	 96-well culture plate
	Cell seeding densities of 50-400K 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	Dispense 50 µL of compound (2X) diluted in cellculture serum-free medium.	Dispense 5 µL of compound (6X), diluted in yourappropriate medium.	 96-well culture plate
	For most compound, incubation time is between 30min and 20 hours at 37°C. We recommend a time course study to determine the optimal stimulation time.		
3	Remove carefully cell supernatant either by aspirating supernatant or by flicking the plate.	Do not remove your appropriate medium.	 Discard supernatant (for adherent cells) 96-well culture plate
	PHOSPHO-IRAK4 (Thr345) DETECTION USING HTRF KIT		
	FOR ADHERENT CELLS	**FOR SUSPENSION CELLS**	
4	Immediately add 50 µL of supplemented lysis buffer(1X) and incubate for at least 30 minutes at room temperature under shaking.	Immediately add 10 µL of supplemented lysis buffer(4X) and incubate for at least 30 minutes at room temperature under shaking.	 96-well culture plate
	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 14 µL of cell lysate from the 96-well cell-culture plate to a small volume (SV) white detection plate. Depending on cell lines used, it can be necessary to dilute the cell lysate to ensure samples are within theassay linear range		 96-well culture plate SV detection plate
6	Add 2 µL of activation buffer and 4 µL of premixed antibody solutions (vol/vol) prepared in the detection buffer.Cover the plate with a plate sealer. Incubate overnight at room temperature. Set up your reader for Eu^{3+} Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on a compatible HTRF™ reader.		 SV detection plate

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

		NON TREATED CELL LYSATE	TREATED CELL LYSATE	CONTROL LYSATE	NEGATIVE CONTROL
Step 1		Dispense 14 µL of non treated cell lysate	Dispense 14 µL of treated cell lysate	Dispense 14 µL of control lysate	Dispense 14 µL of supplemented lysis buffer(1X)
Step 2		Add 2 µL of activation buffer to all wells			
Step 3		Add 2 µL of Phospho-IRAK4 d2 antibody working solution to all wells			
Step 4		Add 2 µL of Phospho-IRAK4 Eu cryptate antibody working solution to all wells			
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.

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

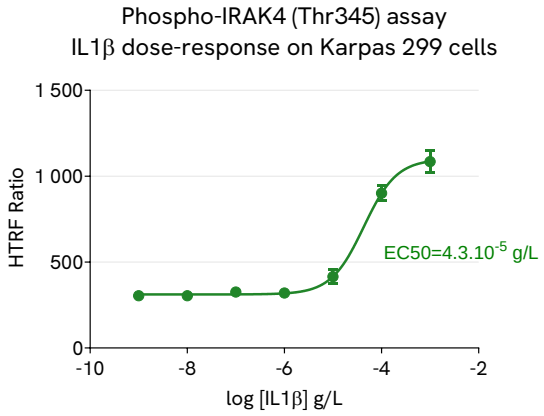
For more information about data reduction, please visit our website.

RESULTS

These data should be considered only as an example. Results may vary from one HTRF™ compatible reader to another. The curves are drawn up by plotting HTRF™ Ratio versus the log [compound] concentrations.

The curves are drawn up by plotting HTRF™ Ratio versus the log [compound] concentrations.
Results on Karpas 299 cells plated at 400,000 cells per well, using the two-plate assay protocol for suspension cells.
Cells were treated with increasing concentrations of IL1β for 45 min. Cells were then lysed with supplemented lysis buffer #1 (1X) for 30 minutes at room temperature.
14 µL of lysates were transferred in a plate to detect phospho-IRAK4.

		PHOSPHO-IRAK4 (Thr345)	
[IL1β] (ng/mL)	**Log([IL1β]) (g/L)**	**Mean HTRF Ratio**	**CV%**
0,001	-9	304	3%
0,01	-8	304	1%
0,1	-7	326	8%
1	-6	320	3%
10	-5	415	10%
100	-4	901	5%
1000	-3	1085	6%
Negative		279	3%
Control lysate		3051	2%



GENERAL LAB WORK PRIOR USING HTRF 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 beforestarting the pharmacological treatment.
Depending on the pathway, a serum starving step could be essential toreduce the basal level activity. This step should be optimized case-by- case.	Advice on cell culture conditions prior using HTRF™ kit: For adherent cells Before treating the cells with compounds, remove culture media from the plate andreplace 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 hoursup 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 andtyrosine phosphatases The lysis buffer is effective for creating cell extract under non denaturing conditionsfrom both plated cells and cells pelleted from suspension cultures.
Using the two-plate assay protocol, a low signal can often be improvedby adjusting lysis volumes.	In most cases, a typical adherent cell line grown in 96-well plates is readily detected ina lysis volume of 50µL. However, the lysis volume can be adjusted from 25 µL to 200µL.
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 assayperformances.
Parameters such as cell density, stimulation time and lysis incubationtime 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 phosphorylationof the readout of interscan vary from one cell line to another. Depending on the type of treatment, and the temperature, the stimulation time can vary widely. Becauseof 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 ofthis, 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
Assaying for multiple targets from a single lysate.	The two-plate assay protocol 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 Phospho-IRAK4 (Thr345) HTRF™ 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 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

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

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