HTRF HUMAN PHOSPHO JAK1 Y1034/1035 DETECTION KITS

PROTOCOL

Part # 64JAK1Y10PEG & 64JAK1Y10PEH

Test Size#: 500 tests (64JAK1Y10PEG), 10,000 tests (64JAK1Y10PEH)

Revision: 01 of November/2021 **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 JAK1 in cells, only when phosphorylated at Tyr1034/1035. Upon activation, JAK1 is phosphorylated and after lysis of the cell membrane, phospho JAK1 Y1034/1035 can be detected using the kit reagents.

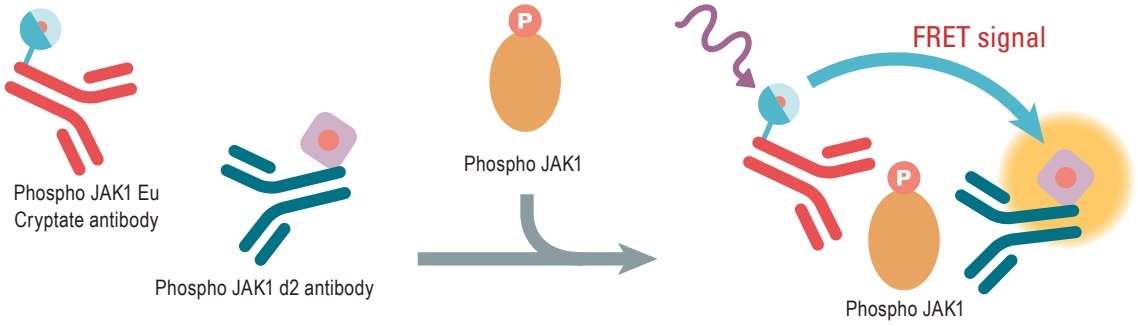


Figure 1: Principle of HTRF sandwich assay.

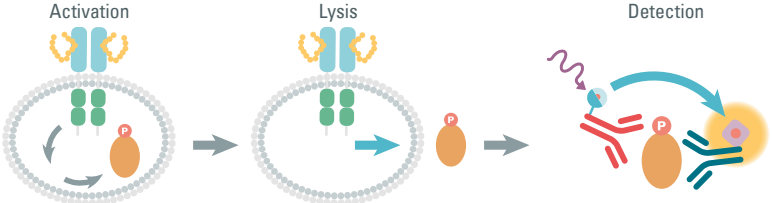
As shown here, phospho JAK1 Y1034/1035 is detected in a sandwich assay format using 2 different specific antibodies, one labelled with Eu^{3+}-Cryptate (donor) and the second with d2 (acceptor). One antibody is selected for its specific binding to the phosphorylated motif on the protein, the second for its ability to recognize the 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 JAK1 Y1034/1035.

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 JAK1 Y1034/1035 by HTRF® reagents. This protocol gives the cells viability and confluence to be monitored. It can also be further streamlined to a one-plate assay protocol. Detection of phospho JAK1 Y1034/1035 with HTRF® reagents is performed in a single plate used for plating, stimulation and detection. No washing steps are required. This protocol, 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 http://learn.cisbio.com/lp-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 at www.cisbio.com/contact-us

PROTOCOL AT A GLANCE



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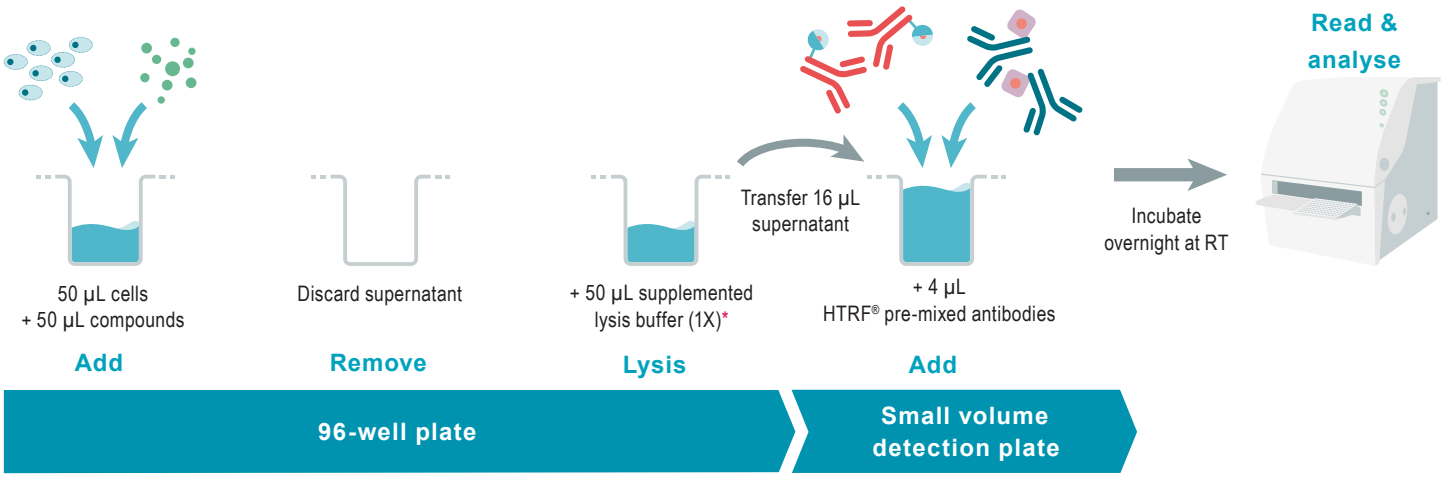
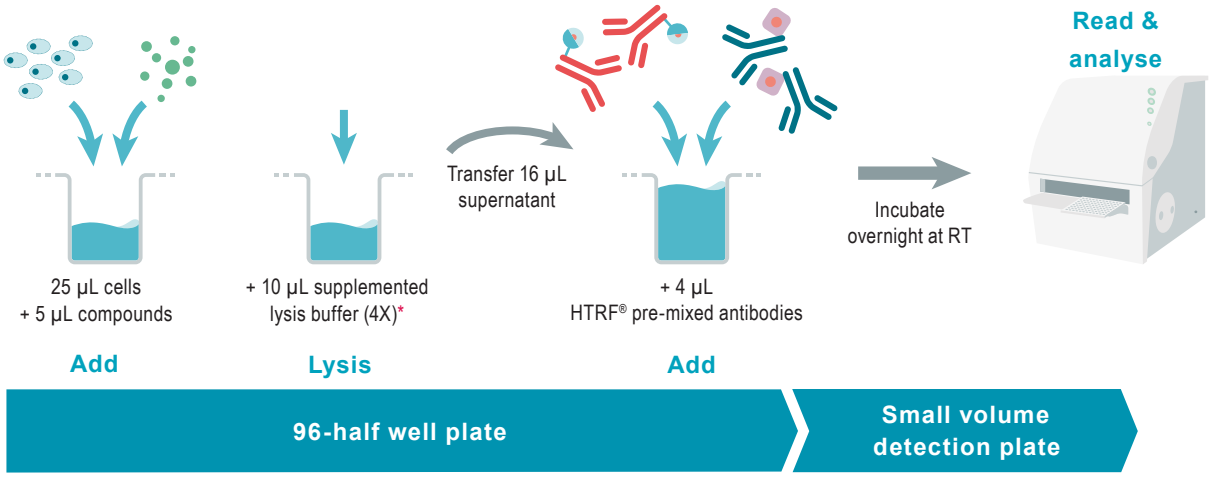
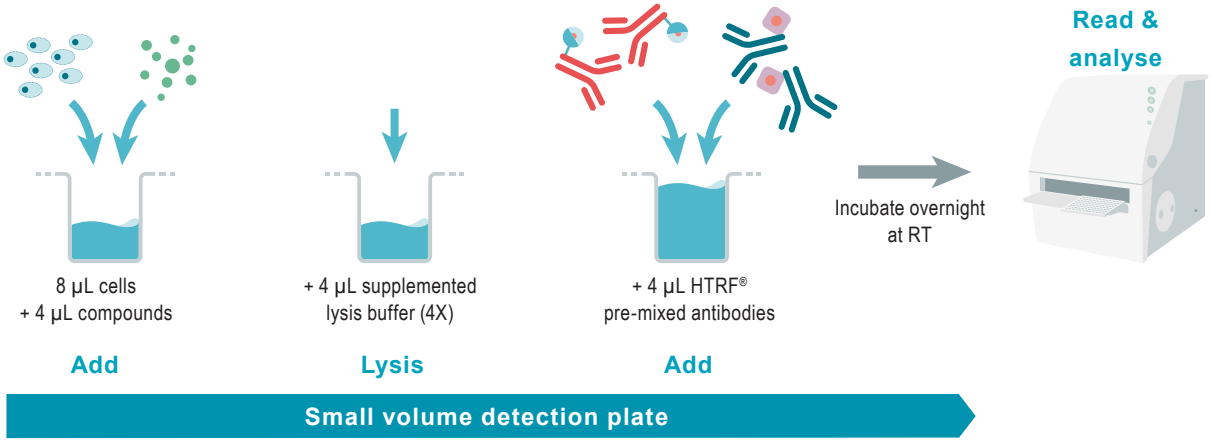
► TWO-PLATE ASSAY PROTOCOL FOR ADHERENT CELLS:



► TWO-PLATE ASSAY PROTOCOL FOR SUSPENSION CELLS:



► ONE-PLATE ASSAY PROTOCOL:



* 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 at:www.cisbio.com/readers

MATERIALS PROVIDED:

KIT COMPONENTS	STORAGE	500 TESTS CAT# 64JAK1Y10PEG		10,000 TESTS CAT# 64JAK1Y10PEH	
Control lysate (ready-to-use)	≤-60°C	green cap	1 vial - 150 µL	green cap	2 vials - 150 µL
Phospho JAK1 Eu Cryptate antibody	≤-16°C	red cap	1 vial - 50 µL	red cap	1 vial - 1 mL
Phospho JAK1 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* # 4 (stock solution 4X)	≤-16°C	transparent cap	4 vials - 2 mL	white cap	1 vial - 130 mL
Detection buffer** (ready-to-use)	≤-16°C	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.cisbio.com/content/microplates-recommendations

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	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.

4 REAGENT PREPARATION

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

Prepare the working solutions from stock solutions by following the instructions below.

TO PREPARE WORKING CONTROL LYSATE SOLUTION

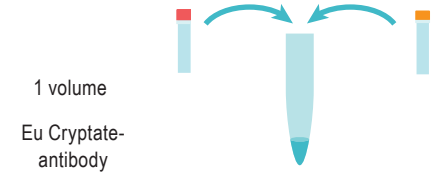
The control lysate is only provided as an internal assay control to check the quality of the results obtained. The window between control lysate and negative control should be greater than 2.

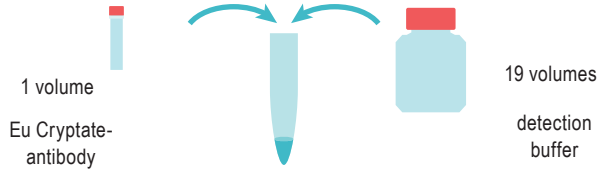
Thaw the control lysate. Mix gently, the control lysate is ready to use.

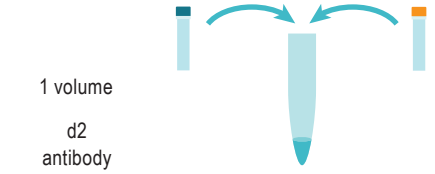
TO PREPARE WORKING ANTIBODY SOLUTIONS:

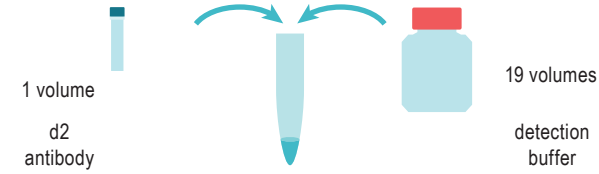
HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the d2 and Eu Cryptate-antibodies will impair the assay's quality. Be careful, as working solution preparation for antibodies may differ between the 500 and 10,000 tests data point kit.

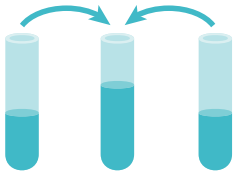
Antibody working solutions are stable for 2 days at 2-8°C. Dilute the antibodies with detection buffer. In practice:

<table>
<tr><th>500 TESTS KIT 64JAK1Y10PEG</th><th>10,000 TESTS KIT 64JAK1Y10PEH</th></tr>
<tr><td colspan="2">Phospho JAK1 Eu Cryptate antibody</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 JAK1 d2 antibody</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 ml of 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 of Eu Cryptate-antibody solution.</td><td>  </td><td>It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2-antibody solution to 1 volume of Eu Cryptate-antibody solution.</td></tr>
</table>

TO PREPARE SUPPLEMENTED LYSIS BUFFER:

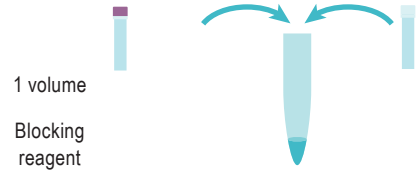
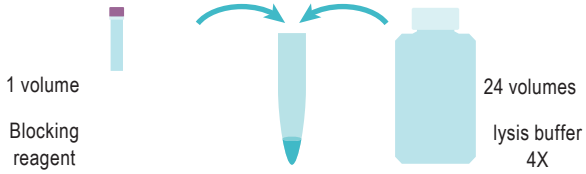
Make sure that the lysate has been generated by using the kit reagents.
Supplemented lysis buffer differs between the protocols. Make sure to use the appropriate supplemented lysis buffer depending on the chosen protocol's specification.

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 & one-plate assay protocol*

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 & ONE-PLATE ASSAY PROTOCOL

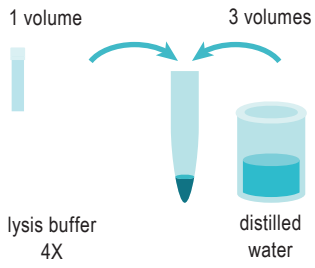
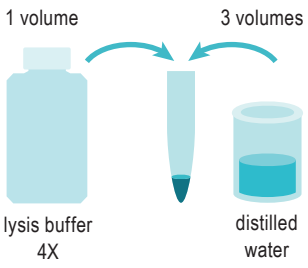
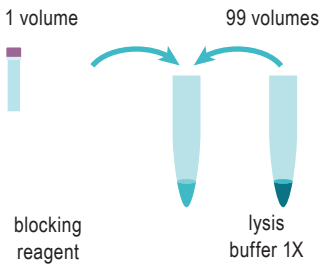
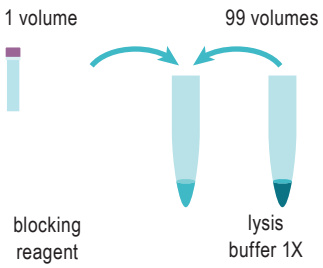
500 TESTS KIT 64JAK1Y10PEG	10,000 TESTS KIT 64JAK1Y10PEH
Preparation of Supplemented Lysis buffer 4X	
	
Dilute the "blocking reagent stock solution" 25-fold with "lysis buffer 4X" e.g take 0.1 ml of "Blocking reagent stock solution" and add it to 2.4 ml of lysis buffer 4X. Mix gently.	

► *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 64JAK1Y10PEG & 10,000 TESTS KIT 64JAK1Y10PEH

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 CISBIO 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 your appropriate medium.	 96-well culture plate
	Cell seeding densities of 300K 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 cell culture serum-free medium	Dispense 5 µL of compound (6X), diluted in your appropriate medium.	 96-well culture plate
	For most compound, incubation time is between 30 minutes and 1 hour 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

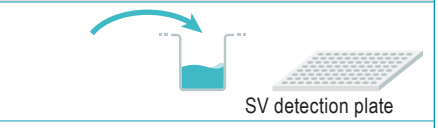
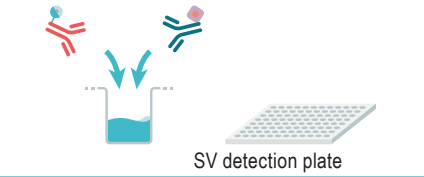
PHOSPHO JAK1 DETECTION USING CISBIO KIT

	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 16 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 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 overnight at room temperature. Set up your reader for Eu3+ Cryptate and read the fluorescence emission at two different wavelenghts (665nm and 620nm) on a compatible HTRF® reader.		 SV detection plate

► *Standard protocol for two-plate assay protocol 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 JAK1 d2 antibody working solution to all wells			
Step 3	Add 2 µL of Phospho JAK1 Eu Cryptate antibody working solution to all wells			
Step 4	Cover the plate with a plate sealer. Incubate overnight 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 CISBIO KIT: CELLS PREPARATION	
1	Plate 8 µL of cells in a small volume (SV) white detection plate in your appropriate medium. Cell seeding densities of 150K 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 compound, incubation time is between 30 minutes and 1 hour at 37°C. We recommend a time course study to determine the optimal stimulation time.	
	PHOSPHO JAK1 DETECTION USING CISBIO 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. 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 overnight at room temperature. Set up your reader for Eu3+ Cryptate and read the fluorescence emission at two different wavelenghts (665nm and 620nm) on a compatible HTRF® reader.	

► ***Standard protocol for one-plate assay protocol 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 JAK1 DETECTION STEPS	Step 3	Add 4 µL of supplemented lysis buffer (4X) - 30 min/RT.			-
	Step 4	Add 2 µL of Phospho JAK1 d2 antibody solution to all wells			
	Step 5	Add 2 µL of Phospho JAK1 Eu Cryptate antibody solution to all wells			
	Step 6	Cover the plate with a plate sealer. Incubate overnight at room temperature.			
	Step 7	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.cisbio.com/data-reduction

RESULTS

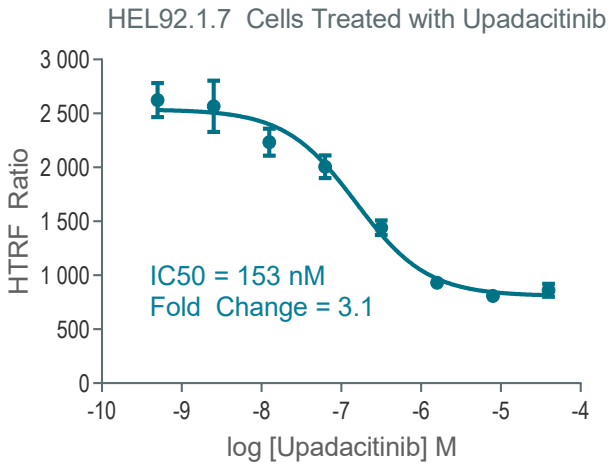
These data should be considered only as an example. Results may vary from one HTRF® compatible reader to another.

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

Results on HEL92.1.7 cells (300,000 cells/well), using the two-plate assay protocol for suspension cells.

Cells were treated with increasing concentrations of Upadacitinib for 1H, followed by a stimulation step with Pervanadate 100 µM,IL4 100 ng/ml and IFNg 100 ng/mL for 30 minutes and then lysed with supplemented lysis buffer #4 for 30 minutes at room temperature.

		Phospho-JAK1 Y1034/1035	
[Upadacitinib] (nM)	Log [Upadacitinib] (M)	Mean HTRF Ratio	CV%
0,00	-9,3	2624	6,0%
1,28	-8,6	2567	10,0%
6,4	-7,9	2233	6,0%
32	-7,2	2006	5,0%
160	-6,5	1441	5,0%
800	-5,8	932	2,0%
4000	-5,1	808	1,0%
20000	-4,6	818	1,0%
Negative		644	5,0%
Control lysate		3589	2,0%



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 Cisbio 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 protocol, 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 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.cisbio.com/htrf-compatible-readers
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 JAK1 Cisbio 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.

PERVANADATE
PHOSPHO-TYR INHIBITOR APPENDIX
Preparation of 30 mM pervanadate solution

APPENDIX

COMPONENT

REAGENT	VOLUME (µL)
Hydrogen peroxide (30%)	6
Sodium orthovanadate 200 mM	15
PBS	79
Total volume of Pervanadate 30 mM	100

PREPARATION:

1. Mix 15 µL of uncolored sodium orthovanadate 200 mM in 79 µL of PBS
2. Add 6 µL hydrogen peroxide 30% to previous mixture
3. Incubate the mixture at least 10 min at room temperature in the dark before adding to the cells

STORAGE AND STABILITY:

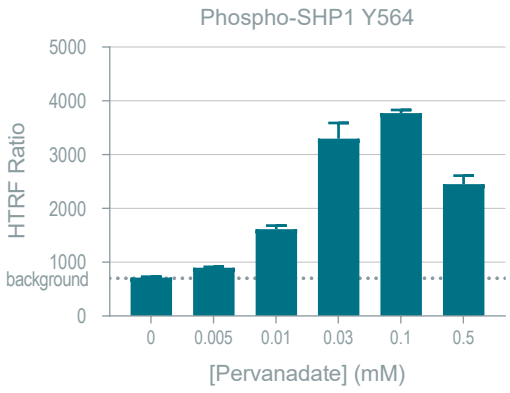
This working Pervanadate solution is not stable over time. Therefore, prepare extemporaneously fresh pervanadate solution away from light and use it immediately.

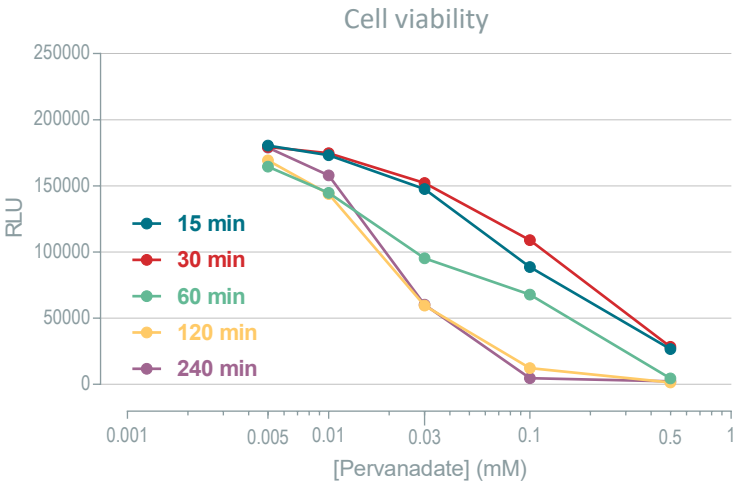
Discard the remaining unused solution.

OPTIMIZATION OF PERVANADATE CONCENTRATION AND INCUBATION TIME:

Jurkat cells were first incubated with increasing concentrations of pervanadate for 30 min. After the lysis step, SHP1 phosphorylation was assessed using the HTRF phospho SHP1 Y564 kit. As indicated on the histogram, only a weak HTRF signal was detectable without pervanadate. The optimal phosphorylation signal was obtained with concentration of pervanadate ranging from 0.03mM up to 0.1 mM. In addition to phospho-SHP1 detection, cell viability was also examined using Cell-TiterGlo assay (Promega).

As illustrated on the curves, exceeding 0.03 mM of pervanadate and 30 minutes incubation time, strongly impacted cell viability. Altogether, the recommended optimal condition of pervanadate usage is 30 µM during 30 min at 37°C-5%CO2.





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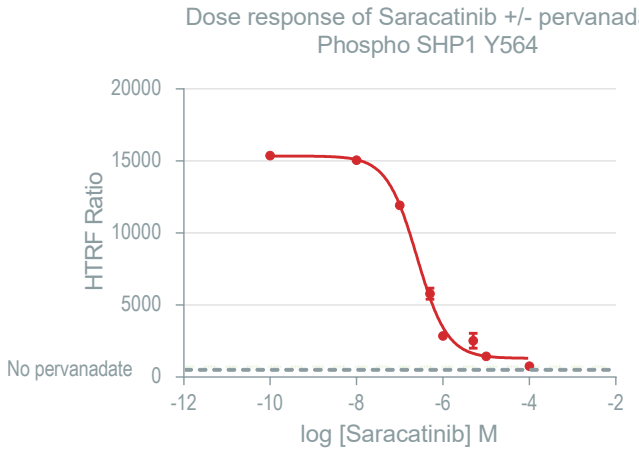
PHARMACOLOGICAL ILLUSTRATION:

Jurkat suspension cells were seeded at 100,000 cells/well in a 96 well half area plate, and incubated for 24h at 37°C, 5% CO2 with increasing concentrations of Saracatinib.

30 min before lysis, Jurkat cells were incubated with Pervanadate (30µM).

The dose response inhibition of saracatinib on SHP1 phosphorylation could only be appreciated in the presence of pervanadate. Without pervanadate, no response could be observed. Note that same results were obtained with Phospho-SHP2 (Tyr542) kit, when used in Jurkat cells.



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