



HTRF HUMAN MOUSE TOTAL SMAD4 DETECTION KITS

PROTOCOL

Part # 64SMAD4TPEG & 64SMAD4TPEH

Test Size#: 500 tests (64SMAD4TPEG), 10,000 tests (64SMAD4TPEH)

Revision: 01 of March/2022 **Store at:** $\leq -60^{\circ}\text{C}$

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 SMAD4 protein in cells, SMAD4 is produced by cells and after lysis of the cell membrane, Total SMAD4 can be detected using the kit reagents. This total protein assay is used for monitoring the steady state protein level in the cell - ideal for normalization when analyzing the phosphorylation level of the corresponding protein.

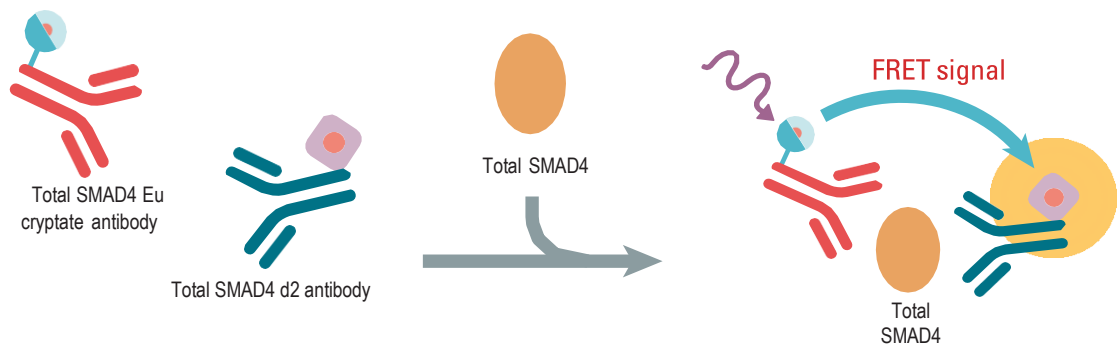


Figure 1: Principle of HTRF sandwich assay.

As shown here, Total SMAD4 protein 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 Total SMAD4 protein.

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 Total SMAD4 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 Total SMAD4 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.

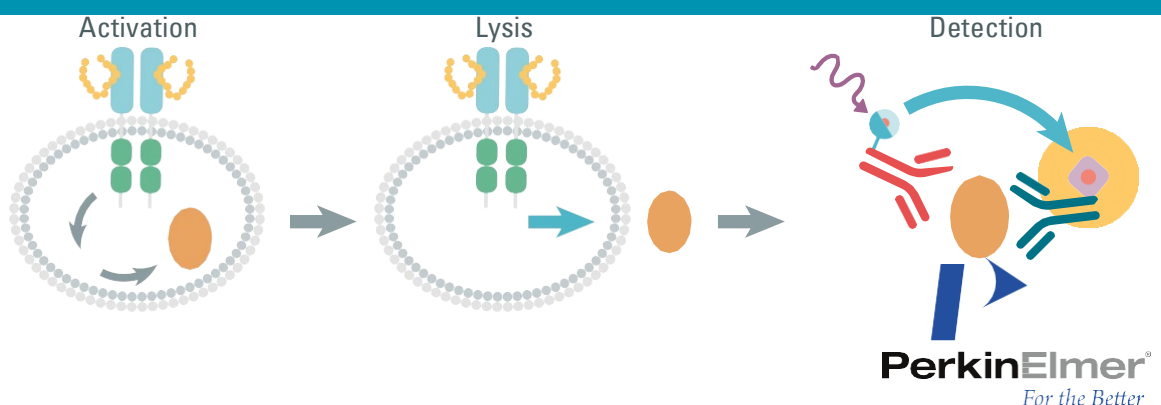
For tissue derived samples, please refer to the technical note: "Optimize your HTRF[®] cell signaling assays on tissues" on

<http://learn.cisbio.com/lp-optimize-your-htrf-cell-signaling-assays-on-tissues>

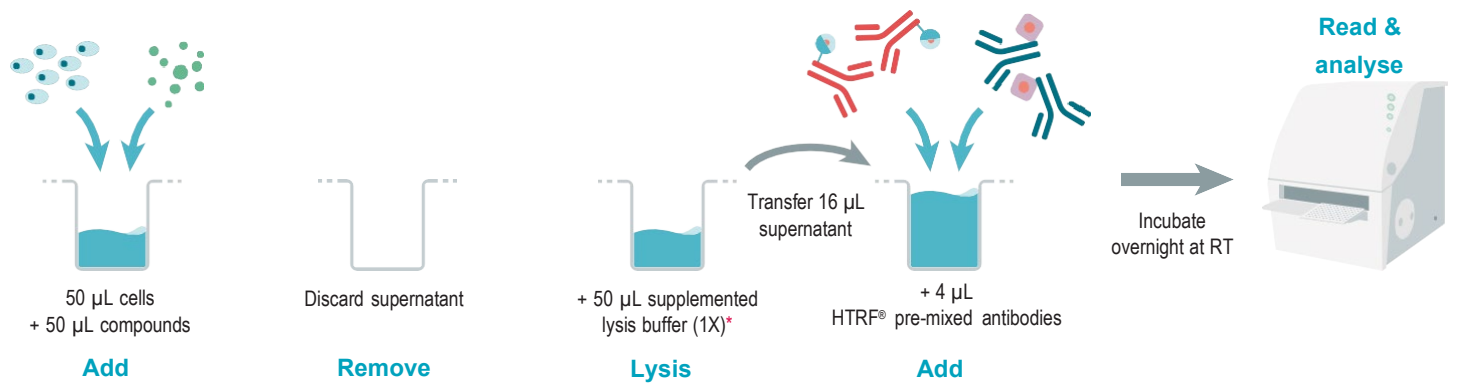
Technical support team can help you to set-up this protocol or another one.

Please contact us at www.cisbio.com/contact-us

PROTOCOL AT A GLANCE



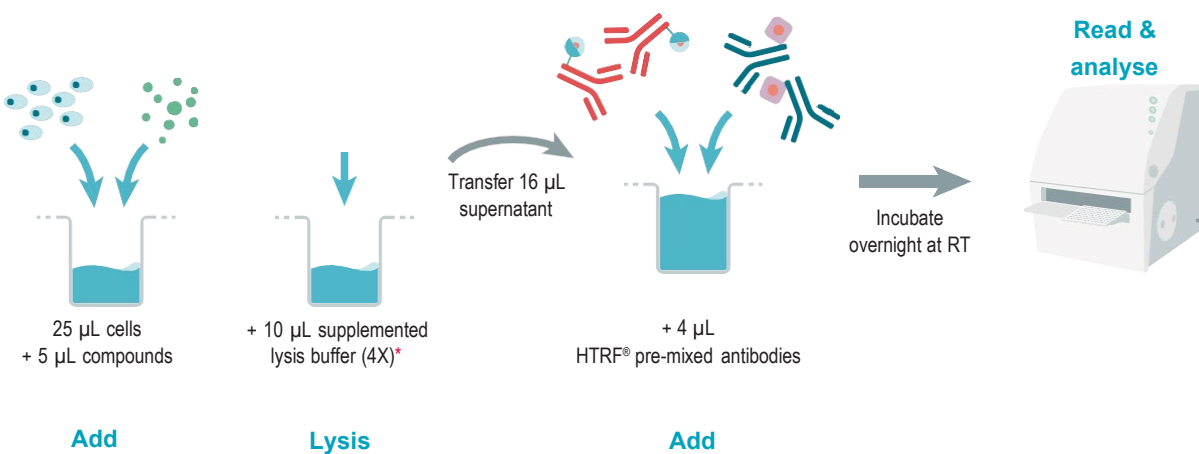
▶ TWO-PLATE ASSAY PROTOCOL FOR ADHERENT CELLS:



96-well plate

Small volume detection plate

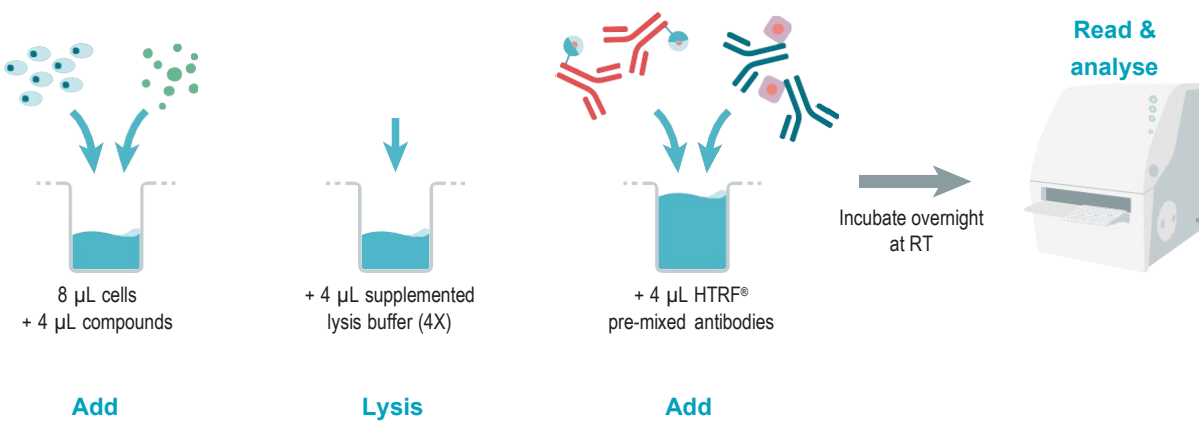
▶ TWO-PLATE ASSAY PROTOCOL FOR SUSPENSION CELLS:



96-half well plate

Small volume detection plate

▶ ONE-PLATE ASSAY PROTOCOL:



Small volume detection plate













* 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# 64SMAD4TPEG		10,000 TESTS CAT# 64SMAD4TPEH	
Control lysate (ready-to-use)	≤-60°C	 green cap	1 vial-150 µL	 green cap	2 vials-150 µL
Total SMAD4 Eu cryptate antibody	≤-16°C	 red cap	1 vial-50 µL	 red cap	1 vial-1mL
Total SMAD4 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.

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 :

500 TESTS KIT 64SMAD4TPEG		10,000 TESTS KIT 64SMAD4TPEH	
Total SMAD4 Eu cryptate antibody			
<p>1 volume Eu Cryptate- antibody</p> <p>19 volumes detection buffer</p> <p>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.</p>	<p>1 volume Eu Cryptate- antibody</p> <p>19 volumes detection buffer</p> <p>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.</p>		
Total SMAD4 d2 antibody			
<p>1 volume d2 antibody</p> <p>19 volumes detection buffer</p> <p>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.</p>	<p>1 volume d2 antibody</p> <p>19 volumes detection buffer</p> <p>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.</p>		
Antibody mix			
<p>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.</p>	<p>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.</p>		

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*

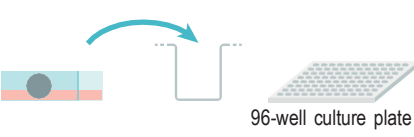
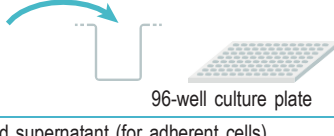
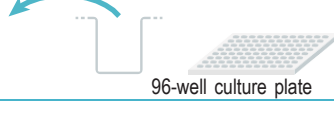
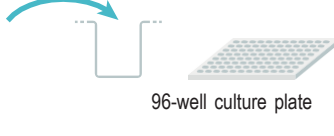

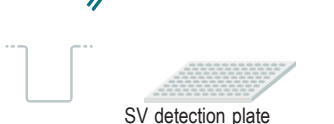
Determine the amount of supplemented lysis buffer needed for the experiment. Each well requires 4 µL of supplemented lysis buffer for one-plate assay protocol and 10 µL for two-plate assay protocol on suspension cells. Dilute the blocking reagent stock solution 25-fold with lysis buffer 4X. In practice:

TWO-PLATE PROTOCOL ON SUSPENSION CELL & ONE-PLATE ASSAY PROTOCOL	
500 TESTS KIT 64SMAD4TPEG	10,000 TESTS KIT 64SMAD4TPEH
Preparation of Supplemented Lysis buffer 4X	
<p>1 volume Blocking reagent</p> <p>24 volumes lysis buffer 4X</p>	<p>1 volume Blocking reagent</p> <p>24 volumes lysis buffer 4X</p>
<p>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.</p>	

► *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 64SMAD4TPEG & 10,000 TESTS KIT 64SMAD4TPEH			
Preparation of lysis buffer 1X		Preparation of supplemented Lysis buffer 1X	
<p>500 tests</p> <p>1 volume lysis buffer 4X</p> <p>3 volumes distilled water</p>	<p>10,000 tests</p> <p>1 volume lysis buffer 4X</p> <p>3 volumes distilled water</p>	<p>500 tests</p> <p>1 volume blocking reagent</p> <p>99 volumes lysis buffer 1X</p>	<p>10,000 tests</p> <p>1 volume blocking reagent</p> <p>99 volumes lysis buffer 1X</p>
<p>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.</p>		<p>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.</p>	

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 CO ₂ atmosphere.	Plate 25 μL of cells in 96 half-well plate in your appropriate medium.	 96-well culture plate
	Cell seeding densities of 100K cells/well for one day incubation or 50K cells/well for 2 days incubation 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 min to 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
TOTAL SMAD4 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 pipetting up and down, transfer 16 μL of cell lysate from the 96-well cell-culture plate to a small volume (SV) white detection plate.		 96-well culture plate SV 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		
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 wavelengths (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 Total SMAD4 d2 antibody working solution to all wells			
Step 3	Add 2 μL of Total SMAD4 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	<p>Plate 8 μL of cells in a small volume (SV) white detection plate in your appropriate medium.</p> <p>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 can be included.</p>
2	<p>Dispense 4 μL of compounds (3X) diluted in your appropriate medium.</p> <p>For most compound, incubation time is between 30 min to 1 hour at 37°C.</p> <p>We recommend a time course study to determine the optimal stimulation time.</p>
TOTAL SMAD4 DETECTION USING CISBIO KIT	
3	<p>Add 4 μL of supplemented lysis buffer (4X).</p> <p>Use the appropriate supplemented lysis buffer and incubate for at least 30 minutes at room temperature under shaking.</p> <p>Lysis incubation time may be optimized.</p>
4	<p>Add 4 μL of premixed antibody solutions (vol/vol) prepared in the detection buffer.</p> <p>Cover the plate with a plate sealer.</p> <p>Incubate overnight at room temperature.</p> <p>Set up your reader for Eu3+ Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on a compatible HTRF® reader.</p>

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

		NON TREATED CELL LYSATE	TRE ATED 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
TOTAL SMAD4 DETECTION STEPS	Step 3	Add 4 μL of supplemented lysis buffer (4X) - 30 min/RT.			
	Step 4	Add 2 μL of Total SMAD4 d2 antibody solution to all wells			
	Step 5	Add 2 μL of Total SMAD4 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.

8 DATA REDUCTION & INTERPRETATION

1. Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal } 665 \text{ nm}}{\text{Signal } 620 \text{ nm}} \times 10^4$$

2. Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

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

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.

The signal linearity is dependent both on the cell line and on the total protein detected. A cell density experiment is highly recommended to ensure working in optimal conditions.

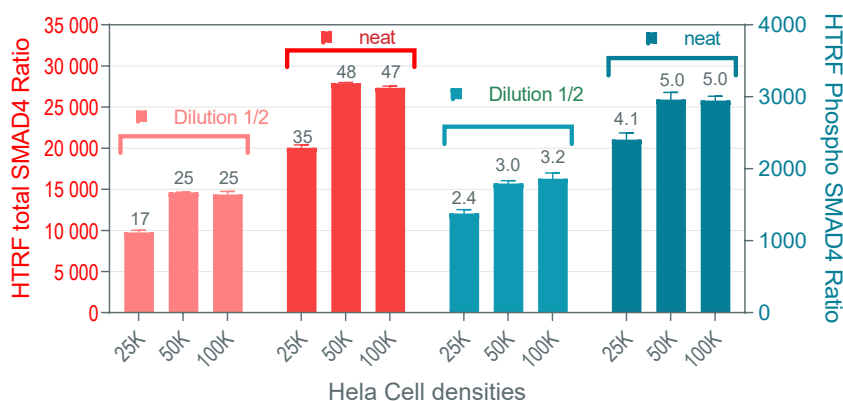
Results on Hela cell line at 3 different densities (25K, 50K and 100 Kcells/well) using the two-plate assay protocol for adherent cells:

Cells were seeded in a 96w plate and incubated for 2 days at 37°. Each condition was then lysed with supplemented lysis buffer #4 (1X) for 30 minutes at room temperature. The cell lysates were tested pure and ½ diluted as shown on the figure below.

Note that 16 µL of lysates can be transferred in a first plate to detect total-SMAD4, and 16 µL of lysates in a second plate to detect phospho-SMAD4 using the HTRF phospho-SMAD4 detection kit - Cat # 64SMAD4T7PEG, 64SMAD4T7PEH

Cell density (cells/well)	Dilution	Total-SMAD4		Phospho-SMAD4 Thr277	
		Mean HTRF Ratio	CV%	Mean HTRF Ratio	CV%
25,000	neat	20048	1.8	2406	3.8
	1/2	9745	2.7	1379	3.7
50,000	neat	27886	0.4	2960	3.4
	1/2	14621	0.3	1795	2.2
100,000	neat	27331	0.8	2948	2
	1/2	14365	2.7	1861	4.4
Negative		576	0.5	591	3.0
Control lysate		51085	0.7	9440	3.8

HTRF Total SMAD4 signal
Hela densities in 96w plate



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 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.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 hela cell lysates example of T175 flask	General lab work - prior using Total SMAD4 Cisbio kit: Day1: Dispense 8 million of Hela 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

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.

Copyright 2020 Cisbio. All rights reserved.

FOR MORE INFORMATION

Europe and other countries +33(0)466-796-705 U.S. and Canada 1-888-963-4567 China +86-21-5018-9880
Japan +81-(0)43-306-8712 Visit www.cisbio.com to find a list of our regional distributors