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Product information:

Document reference : 64S6KPEG rev01 Aug-13

Phospho-p70S6K(Thr389)

500 tests

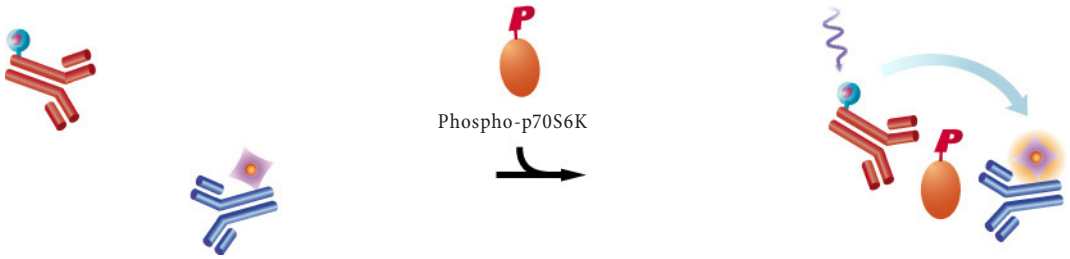
For research use only.

Not for use in therapeutic or diagnostic procedures.

Storage temperature: -60°C or below

1. ASSAY DESCRIPTION

This assay is intended for the simple, rapid and direct detection of endogenous levels of p70S6K in cells, only when phosphorylated at Thr389. Upon activation, p70S6K is phosphorylated and after lysis of the cell membrane, phospho-p70S6K(Thr389) can be detected using the kit reagents .

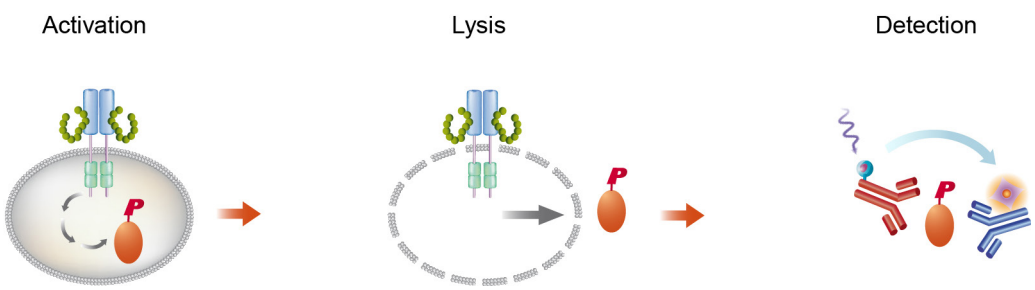


As shown here, phospho-p70S6K(Thr389) is detected in a sandwich assay format using 2 different specific antibodies, one labelled with Eu^{3+}-Cryptate (donor) and the second with d2 (acceptor).

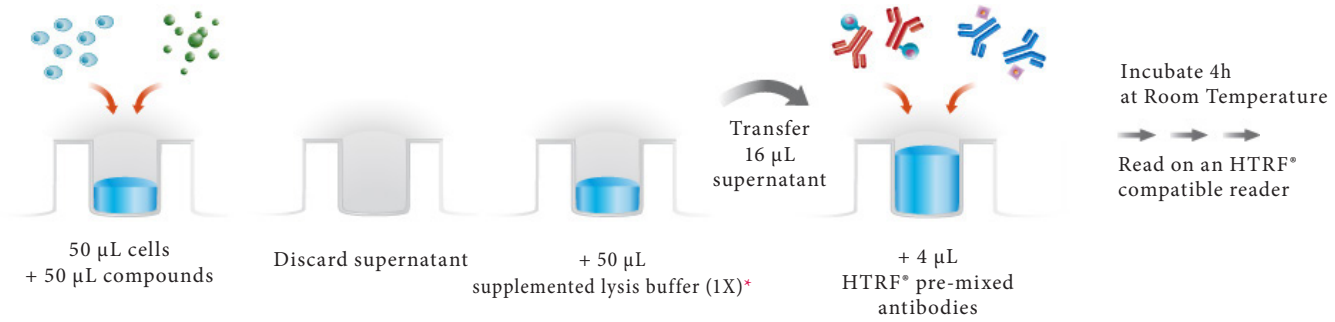
When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). The specific signal modulates positively in proportion to phospho-p70S6K(Thr389).

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-p70S6K(Thr389) 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-p70S6K(Thr389) 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.

2. PROTOCOL AT A GLANCE



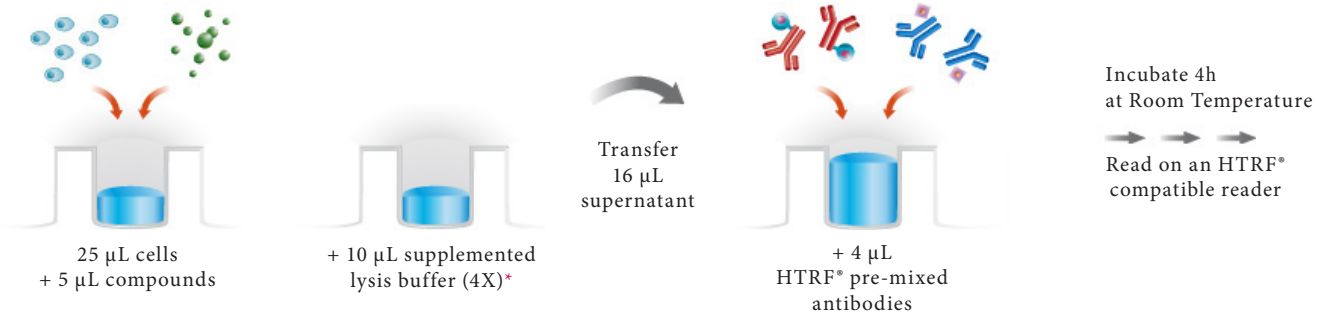
➥ Two-plate assay protocol (for adherent cells)



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

96-well plate → **Low volume detection plate**

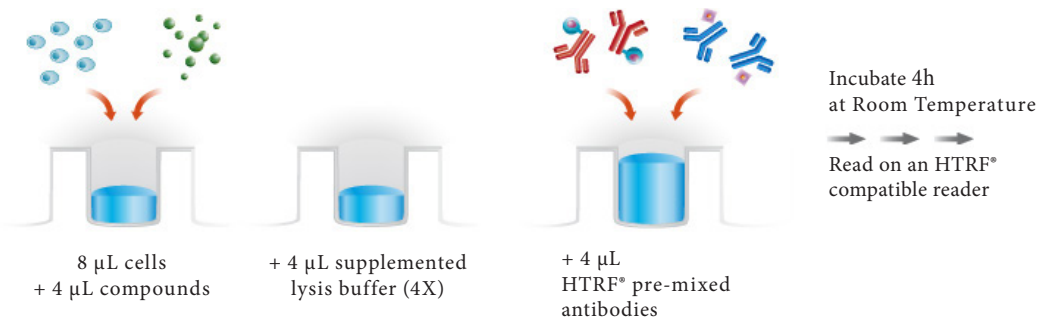
➥ Two-plate assay protocol (for suspension cells)



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

96-half well plate → **Low volume detection plate**

➥ One-plate assay protocol



384-well white plate SV

3. HTRF® REAGENTS

		Nb of vials	Volume per vial	Storage	Ref # (when available separately)
Phospho-p70S6K Cryptate antibody	 red cap	1	50 µL	-20°C or below	
Phospho-p70S6K d2 antibody	 blue cap	1	50 µL	-20°C or below	
Control lysate (ready-to-use)	 green cap	1	150 µL	-60°C or below	64S6KTDA
Blocking reagent* (stock solution 100X)	 purple cap	1	300 µL	-20°C or below	64KB1AAC (2 mL)
Lysis buffer* # 1 (stock solution 4X)	 transparent cap	4	2 mL	-20°C or below	64KL1FDF (130 mL)
Detection buffer (ready-to-use)	 orange cap	2	2 mL	-20°C or below	

* Amounts of reagents provided are sufficient for generating 50 µL of cell lysate per well. For higher volumes, extra vials can be ordered separately.

4. STORAGE STABILITY

Antibodies, control lysate and buffers should be stored frozen 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 -20°C or below) and thawed at least one more time.
Control lysate must be stored frozen at -60°C or below. Thawed control lysate can be refrozen (at -60°C or below) and thawed one more time.

5. 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.

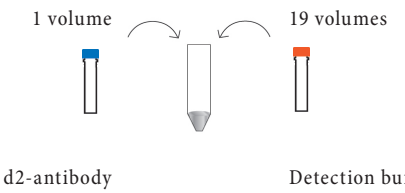
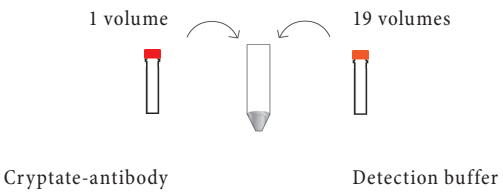
5.1. Control lysate solution: ready-to-use

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.

5.2. 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 500 and the 10,000 data point kit.

Antibody working solutions are stable for 2 days at 4°C. Dilute the antibodies with detection buffer. In practice:

Phospho-p70S6K d2 antibody	Phospho-p70S6K Cryptate antibody
	
Dilute 20-fold the frozen stock solution with detection buffer: e.g. add 0.95 mL of detection buffer to the 0.05 mL of d2-antibody stock solution.	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 Cryptate-antibody stock solution.

5.3. Preparation of supplemented lysis buffer

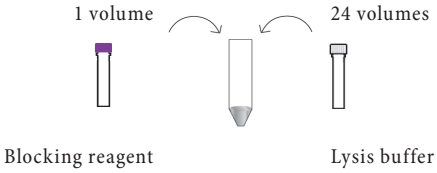
Make sure that the lysate has been generated by using the kit reagents.

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

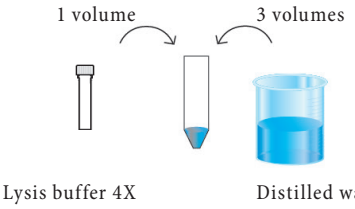
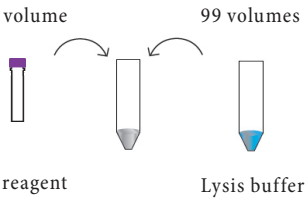
➥ SUPPLEMENTED LYSIS BUFFER 4X FOR TWO-PLATE ASSAY PROTOCOL ON SUSPENSION CELLS & ONE-PLATE ASSAY PROTOCOL

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

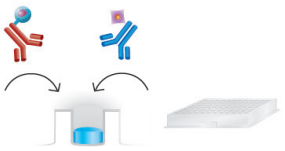
Two-plate protocol on suspension cells & One-plate assay protocol

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	
Preparation of lysis buffer 1x	Dilution of blocking reagent
	
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.

6. TWO-PLATE ASSAY PROTOCOL FOR ADHERENT CELLS & SUSPENSION CELLS

	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 HBSS* or other appropriate medium.	 96-well culture plate
	Cell seeding densities of 100 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.		
2	Dispense 50 µL of compounds (2X) diluted in cell culture serum-free medium	Dispense 5 µL of compounds (6X), diluted in HBSS* or other appropriate medium.	 96-well culture plate
	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.		
3	Remove carefully cell supernatant either by aspirating supernatant or by flicking the plate.	Do not remove HBSS* or other appropriate medium.	Discard supernatant (for adherent cells)  96-well culture plate
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. We recommend a time course study to determine the optimal lysis incubation time. 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 384-well small volume white plate.		 96-well culture plate 384-well SV plate
6	Add 4 µL of premixed antibody solutions (vol/vol) prepared in the detection buffer. Cover the plate with a plate sealer. Incubate 4h at room temperature. Maximum signal is reached after 4h incubation time, and remains stable over a period of 24 hours. Therefore, readings can be made between 4h and 24h of incubation time. Set up your reader for Eu3+ Cryptate and read the fluorescence emission at two different wavelenghts (665nm and 620nm) on a compatible HTRF® reader**.		 384-well SV plate

* If the suspension cells have to be treated for more than 30min, replace HBSS by cell culture medium without phenol red.

For more information about HTRF® compatible readers and for set-up recommendations, please visit our website at: www.cisbio.com/drug-discovery/htrf-compatible-readers

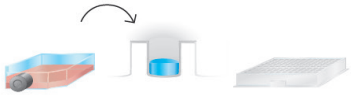
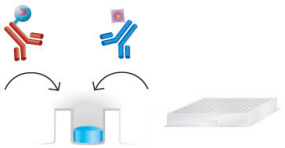
➥ STANDARD PROTOCOL FOR TWO-PLATE ASSAY PROTOCOL IN 20 µL FINAL VOLUME (AFTER LYSIS STEP)

	Non treated cell lysate	Treated cell lysate	Control lysate	Blank control	Negative control
Non treated cell lysate	16 µL	-	-	16 µL	-
Treated cell lysate	-	16 µL	-	-	-
Control lysate	-	-	16 µL	-	-
Supplemented lysis buffer (1X)			-	-	16 µL
Detection buffer	-	-	-	2 µL	-
Phospho-p70S6K d2 antibody	2 µL	2 µL	2 µL	-	2 µL
Phospho-p70S6K Cryptate antibody	2 µL	2 µL	2 µL	2 µL	2 µL
Total volume	20 µL	20 µL	20 µL	20 µL	20 µL

The blank control is used to check the Cryptate signal at 620 nm.

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.

7. ONE-PLATE ASSAY PROTOCOL

1	Plate 8 µL of cells in a 384-well small volume white plate in HBSS* or other appropriate medium. 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 can be included.	 384-well SV plate
2	Dispense 4 µL of compounds (3X) diluted in HBSS* or other appropriate 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.	 384-well SV plate
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	 384-well SV plate
4	Add 4 µL of premixed antibody solutions (vol/vol) prepared in the detection buffer. Cover the plate with a plate sealer. Incubate 4h at room temperature. Maximum signal is reached after 4h incubation time, and remains stable over a period of 24 hours. Therefore, readings can be made between 4h and 24h of incubation time. Set up your reader for Eu3+ Cryptate and read the fluorescence emission at two different wavelenghts (665nm and 620nm) on a compatible HTRF® reader**.	 384-well SV plate

* If the suspension cells have to be treated for more than 30min, replace HBSS by cell culture medium without phenol red.

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➥ STANDARD PROTOCOL FOR ONE-PLATE ASSAY PROTOCOL IN 20 µL FINAL VOLUME

	Non treated cell	Treated cell	Control lysate	Blank control	Negative control
Cells	8 µL	8 µL	-	8 µL	-
HBSS or other appropriate medium	4 µL	-	-	4 µL	12 µL
Compound	-	4 µL	-	-	-
Control lysate	-	-	16 µL	-	-
Supplemented lysis buffer (4X)	4 µL	4 µL	-	4 µL	4 µL
Detection buffer	-	-	-	2 µL	-
Phospho-p70S6K d2 antibody	2 µL	2 µL	2 µL	-	2 µL
Phospho-p70S6K Cryptate antibody	2 µL	2 µL	2 µL	2 µL	2 µL
Total volume	20 µL	20 µL	20 µL	20 µL	20 µL

The blank control is used to check the Cryptate signal at 620 nm.

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

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.

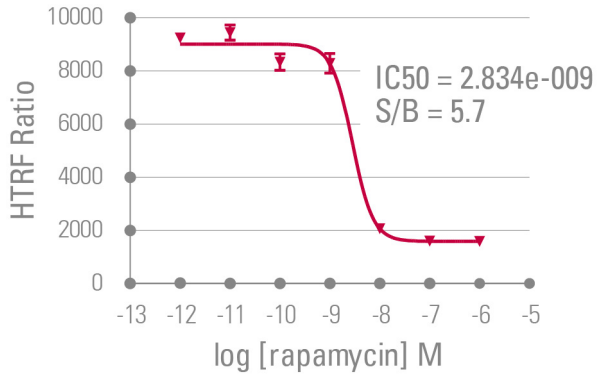
The curves are drawn up by plotting HTRF® Ratio versus the log [compound] concentrations.

Results on HEK293 cells (100 000 cells/well), using the two-plate assay protocol for adherent cells.

HEK293 cells were treated for 3H with increasing concentrations of Rapamycin. After stimulation with insulin 1µM for 30 minutes, HEK293 cells were then lysed with 50 uL of supplemented lysis buffer for 30 minutes at room temperature.

	phospho-p70S6K results	
log [compound] (M)	Ratio	CV %
-12	9238	1.7%
-11.0	9439	5.2%
-10.0	8328	6.4%
-9.0	8281	7.7%
-8.0	2060	1.7%
-7.0	1596	4.6%
-6.0	1589	4.3%

Negative control	952	5.0%
Control lysate	5416	1.0%



Ratio	$\frac{\text{Signal}_{665nm}}{\text{Signal}_{620nm}} \times 10^4$	Ratio must be calculated for each individual well.
CV%	$\frac{\text{Standard deviation}}{\text{Mean ratio}} \times 100$	The mean and standard deviation can then be worked out from ratio replicates.
For more information about data reduction, please visit our website at: www.cisbio.com/drug-discovery/htrf-ratio-and-data-reduction		

Technical support team can help you to set-up this protocol, please contact us at www.cisbio.com/drug-discovery/contact-us.

9. 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.	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: http://www.cisbio.com/drug-discovery/htrf-microplate-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	Day1: Dispense 8 million cells in T175cm^2, 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 with PBS (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 with PBS(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 -80°C. For long term conservation, aliquots should be stored in liquid nitrogen.

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