

Cellul'erk - 500 tests Phosphorylated ERK1/2 detection using HTRF[®] technology HTRF[®] cell-based assay

Document reference : 64ERKPEG rev 00 (January 2010)

HTRF[®] package insert

Conjugate and buffer frozen set
Cellul'erk frozen set: 64ERKPEG

For in vitro research use only
Storage temperature: at $\leq -20^{\circ}\text{C}$

1. Introduction

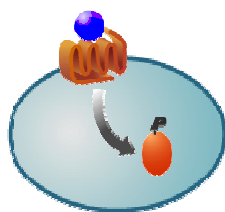
Cellul'erk enables simple, rapid and direct detection of phosphorylated extracellular signal regulated kinase (ERK1/2) in cells upon activation of a number of cell surface receptors.

A large variety of GPCRs may in particular activate the ERK signaling cascade. Given the importance of both upstream activators and downstream targets of ERK1/2, the control of ERK1/2 phosphorylation is crucial when studying the effect of an effector on a GPCR.

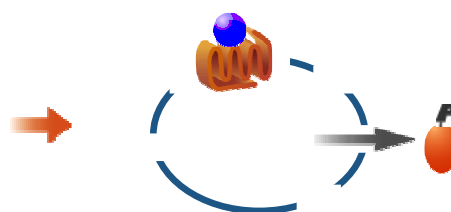
The assay using the truly homogeneous HTRF technology is antibodies based. This kit offers a new solution for high throughput screening of both agonists and antagonists of GPCRs expanding Cisbio Bioassays' offer for GPCRs investigation.

2. Assay description

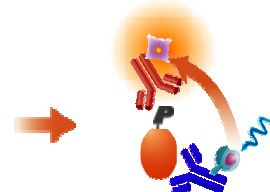
The HTRF assay is based on a sandwich immunoassay using an anti-phospho-ERK1/2 antibody labeled with d2 and an anti-ERK1/2 antibody labeled with Eu3+- Cryptate.



GPCR activation by ligand induces ERK1/2 phosphorylation



Lysis of the cell induces the release of phosphorylated ERK1/2



Detection of phosphorylated ERK1/2 with HTRF conjugates



3. Reagents description - storage and stability

Supplied reagents	500 tests
Low control (Erk unstimulated cell lysate)	1 X 150 µL frozen – ready to use
High control (Erk stimulated cell lysate)	1 X 150 µL frozen – ready to use
Erk lysis buffer (stock solution 4x)	4 X 2 mL frozen – ready to use (see §4)
Erk blocking reagent (lysis buffer supplement)	2 X 130 µL frozen – to dilute
Erk detection buffer	2 X 2 mL frozen – ready to use
Anti-Phospho ERK 1/2- d2 Stock solution	1 X 50 µL frozen – to dilute
Anti-ERK1/2 Eu ³⁺ - Cryptate Stock solution	1 X 50 µL frozen – to dilute

Storage: all components must be stored at –20°C or below.

To avoid freeze/thaw cycles, aliquote stock solutions into disposable plastic vials for storage at –20°C or below.

Reagents may be frozen and thawed once.

Thawed buffers can be stored at 2-8°C.

4. Preparation of the working solutions

The working solutions are prepared from stock solutions (§ 3) by following the instructions below:


Ligand	Dilute ligand stock solution using a serum free cell culture medium to prepare working solutions for activation step.
Low control	Ready to use.
High control	Ready to use.
Supplemented lysis buffer	<p>The preparation of the supplemented lysis buffer differs upon the protocol selected (two-plate or one-plate). Make sure to prepare the appropriate buffer for the assay protocol you have selected.</p> <p>Two-plate assay protocol (§ 5 & 5.1) Dilute 4 fold Erk lysis buffer stock solution with distilled water (e.g. take 1 mL of lysis buffer stock solution and add it to 3mL of distilled water. Mix gently). Then dilute 100 fold Erk blocking reagent in the above prepared lysis buffer (e.g. take 40 µL of Erk blocking stock solution and add it to 3960 µL of diluted lysis buffer. Mix gently).</p> <p>One-plate assay protocol (§ 7 & 7.1) Use the Erk lysis buffer stock solution without dilution. Dilute 25 fold Erk blocking reagent in Erk lysis buffer stock solution (e.g. take 100 µL of Erk blocking stock solution and add it to 2400 µL of lysis buffer. Mix gently)</p>
<i>It is recommended to prepare the required amount of supplemented lysis buffer just before running the assay</i>	
Anti-Phospho ERK1/2-d2	Dilute 20 fold with Erk detection buffer (e.g. take 10 µL of stock solution and add it to 190 µL of detection buffer. Mix gently)
Anti-ERK1/2 Eu ³⁺ -Cryptate	Dilute 20 fold with Erk detection buffer (e.g. take 10 µL of stock solution and add it to 190 µL of detection buffer. Mix gently)

Do not store working solutions- It is recommended to prepare the required amount of working solutions just before running the assay.

5. Two-plate Assay protocol

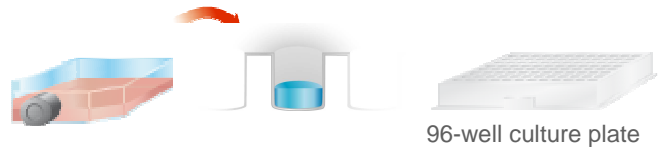
Day 1

Plate 50 μL of cells in 96-well tissue-culture treated plate and incubate overnight, at 37°C in CO_2 atmosphere.

 Dilute cells in growth medium using the appropriate cell density (cf. 6.1)


Depending on receptor a starving step with serum-free medium can be added.

1

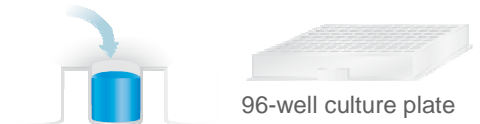


Day 2

Dispense 50 μL of ligand and incubate at room temperature.

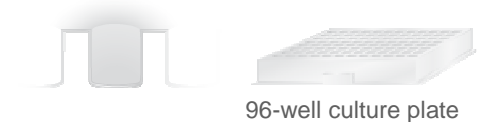
 Optimize the incubation time (cf. 6.2).

2




Remove carefully cell supernatant either by aspirating supernatant or flicking the plate.

3

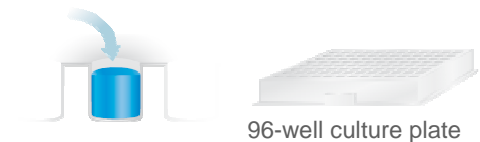


Add **immediately** 50 μL of supplemented lysis buffer.

 Use the appropriate supplemented lysis buffer and incubate at room temperature with shaking.

 Optimize the lysis incubation time.

4



Transfer 16 μL of cell lysate from the cell-culture plate to a 384-well small volume white plate.

5



Add 2 μL of each HTRF conjugates and incubate for 2 hours at room temperature (place a lid or a sealer on the plate during the incubation).

6



Read the plate on a compatible reader (readouts must be performed at 620 nm for the donor and 665 nm for the acceptor).

5.1. Standard protocol for two-plate assay protocol in 20 μ L final volume (after lysis step)

	Non stimulated cell lysate	Stimulated cell lysate	Low control	High control	Blank control	Negative control
Non stimulated cell lysate	16 μ L	-	-	-	-	-
Stimulated cell lysate	-	16 μ L	-	-	-	-
Low control	-	-	16 μ L	-	-	-
High control	-	-	-	16 μ L	-	-
Supplemented lysis buffer	-	-	-	-	16 μ L	16 μ L
Erk detection buffer	-	-	-	-	2 μ L	-
Anti-phospho-ERK1/2-d2	2 μ L	2 μ L	2 μ L	2 μ L	-	2 μ L
Anti-ERK1/2 Eu ³⁺ - Cryptate	2 μ L	2 μ L	2 μ L	2 μ L	2 μ L	2 μ L
Total volume	20 μ L	20 μ L	20 μ L	20 μ L	20 μ L	20 μ L

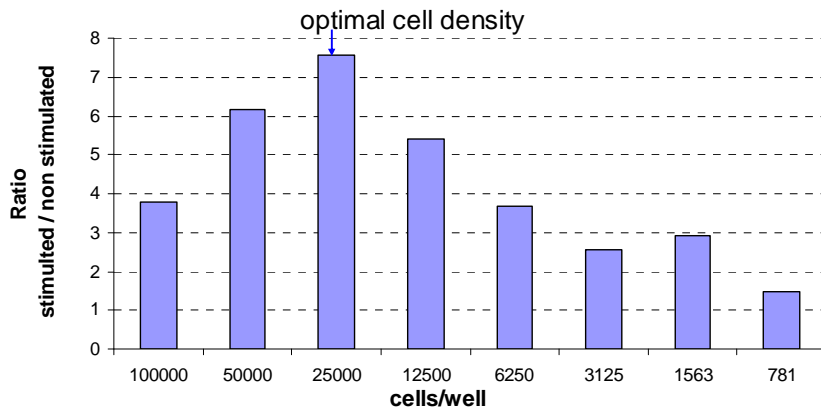
Blank control is used to check the Cryptate signal at 620 nm.

Negative control is used to check the Non specific signal.

6. Key parameters to optimize

Following graphs given as an example.

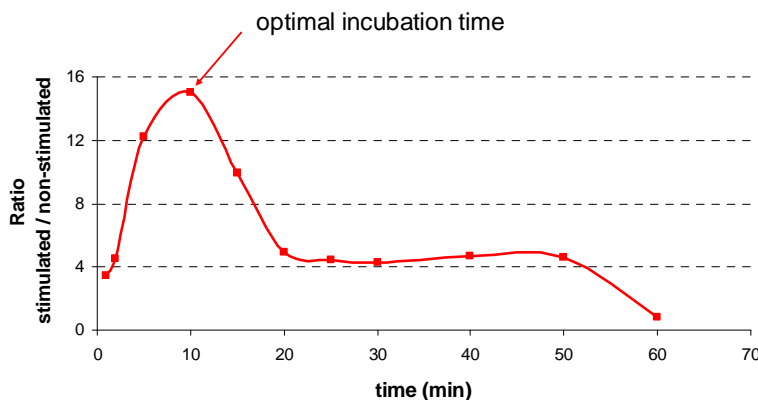
6.1. Cell density



Initial cell density has to be optimized in order to get the best assay window

*CHO-cell line
GPCR over expressed
Incubation time: 10min*

6.2. GPCR activation time



Ligand incubation kinetic must be carefully studied

*CHO-cell line
GPCR over expressed
Cell density: 25,000*

7. One-plate Assay protocol

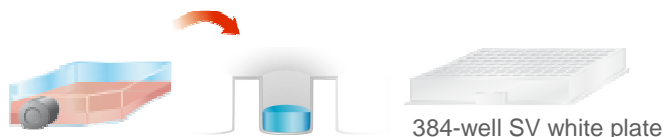
Day 1

Plate 8 μL of cells in a 384-well small volume white plate (tissue culture treated) and incubate overnight, at 37°C in CO₂ atmosphere.

⚠️ Dilute cells in growth medium using the appropriate cell density (cf. 7.1)

Depending on receptor a starving step with serum-free medium can be added.

1



Day 2

Dispense 4 μL of ligand and incubate at room temperature.

⚠️ Optimize the incubation time (cf. 7.2).

2



Add 4 μL of supplemented lysis buffer.

⚠️ Use the appropriate supplemented lysis buffer and incubate at room temperature with shaking.

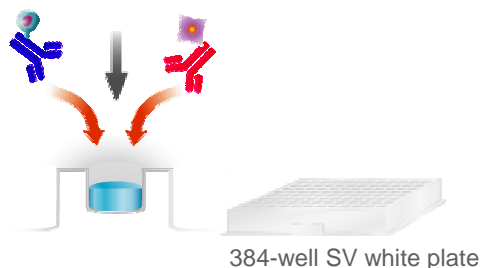
⚠️ Optimize the lysis incubation time.

3



Add 2 μL of each HTRF conjugates and incubate for 2 hours at room temperature (place a lid or a sealer on the plate during the incubation).

4



Read the plate on a compatible reader (readouts must be performed at 620 nm for the donor and 665 nm for the acceptor).

7.1. Standard protocol for a one-plate assay protocol in 20 μL final volume

	Non stimulated cells	Stimulated cells	Low control	High control	Blank control	Negative control
Cells	8 μL	8 μL	-	-	-	-
Serum free cell culture medium	4 μL	-	-	-	-	-
Ligand	-	4 μL	-	-	-	-
Low control	-	-	16 μL	-	-	-
High control	-	-	-	16 μL	-	-
Supplemented lysis buffer	4 μL	4 μL	-	-	16 μL	16 μL
Erk detection buffer	-	-	-	-	2 μL	-
Anti-phospho-ERK1/2-d2	2 μL	2 μL	2 μL	2 μL	-	2 μL
Anti-ERK1/2 Eu ³⁺ - Cryptate	2 μL	2 μL	2 μL	2 μL	2 μL	2 μL
Total volume	20 μL	20 μL	20 μL	20 μL	20 μL	20 μL

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

Negative control is used to check the Non specific signal.