ASSAY PRINCIPLE

This assay is intended for the simple, rapid and direct detection of endogenous levels of ERK 1/2 in cells, only when phosphorylated at Thr202 and Tyr204. Upon activation, ERK 1/2 is phosphorylated and after lysis of the cell membrane, phospho-ERK1/2 (Thr202/Tyr204) can be detected using the kit reagents.

![FRET signal](image)

Figure 1: Principle of HTRF sandwich assay.

As shown here, phospho-ERK1/2 (Thr202/Tyr 204) is detected in a sandwich assay format using 2 different specific antibodies, one labelled with Eu**-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-ERK1/2 (Thr202/Tyr 204).

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-ERK1/2 (Thr202/Tyr 204) 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-ERK1/2 (Thr202/Tyr 204) 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/np-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
**TWO-PLATE ASSAY PROTOCOL FOR ADHERENT CELLS:**

1. Add 50 µL cells + 50 µL compounds to 96-well plate.
2. Remove supernatant.
3. Discard supernatant + 50 µL supplemented lysis buffer (1X) * to transfer 16 µL supernatant.
4. Incubate 2 h at RT.
5. Add 4 µL HTRF® pre-mixed antibodies.
6. Read & analyse.

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

**TWO-PLATE ASSAY PROTOCOL FOR SUSPENSION CELLS:**

1. Add 25 µL cells + 5 µL compounds + 10 µL supplemented lysis buffer (4X) * to transfer 16 µL supernatant.
2. Incubate 2 h at RT.
3. Add 4 µL HTRF® pre-mixed antibodies.
4. Read & analyse.

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

**ONE-PLATE ASSAY PROTOCOL:**

1. Add 8 µL cells + 4 µL compounds + 4 µL supplemented lysis buffer (4X) to 384-well white plate SV or HTRF 96-well SV plate.
2. Incubate 2 h at RT.
3. Add 4 µL HTRF® pre-mixed antibodies.
4. Read & analyse.

**FOR HTRF CERTIFIED READER**

For more information about HTRF® compatible readers and for set-up recommendations, please visit our website at:

www.cisbio.com/htrf-compatible-readers
### MATERIALS PROVIDED:

<table>
<thead>
<tr>
<th>KIT COMPONENTS</th>
<th>STORAGE</th>
<th>500 TESTS CAT# 64ERKPEG</th>
<th>10,000 TESTS CAT# 64ERKPEH</th>
<th>REF# (WHEN AVAILABLE SEPARATELY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control lysate (ready-to-use)</td>
<td>2-8°C until reconstituted</td>
<td>1 vial - Lyophilized</td>
<td>2 vials - Lyophilized</td>
<td>62ERKTDA</td>
</tr>
<tr>
<td>Phospho-ERK1/2 Cryptate antibody</td>
<td>2-8°C until reconstituted</td>
<td>1 vial - Lyophilized</td>
<td>1 vial - Lyophilized</td>
<td></td>
</tr>
<tr>
<td>Phospho-ERK1/2 d2 antibody</td>
<td>2-8°C until reconstituted</td>
<td>1 vial - Lyophilized</td>
<td>1 vial - Lyophilized</td>
<td></td>
</tr>
<tr>
<td>Blocking reagent* (stock solution 100X)</td>
<td>2-8°C</td>
<td>1 vial - 300 µL</td>
<td>1 vial - 6 mL</td>
<td>64KB1AAD (6mL)</td>
</tr>
<tr>
<td>Lysis buffer* # 1 (stock solution 4X)</td>
<td>2-8°C</td>
<td>1 vial - 8 mL</td>
<td>1 vial - 130 mL</td>
<td>64KL1FDF (130mL)</td>
</tr>
<tr>
<td>Detection buffer** (ready-to-use)</td>
<td>2-8°C</td>
<td>1 vial - 4 mL</td>
<td>1 vial - 50 mL</td>
<td></td>
</tr>
</tbody>
</table>

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

** The Detection Buffer is used to prepare working solutions of acceptor and donor reagents.

► PURCHASE SEPARATELY (small volume (SV) detection plate):
- HTRF® 96-well low volume plate Ref# 66PL96001
- HTRF® 384-well low volume plate Ref# 66PL384025

### STORAGE AND STABILITY

All reagents should be stored at 2-8°C until used. Once reconstituted, antibody and control solutions are stable 48 hours at 2-8°C, they can be frozen (at -60°C or below) and thawed one time. To avoid freeze/thaw cycles, aliquot working solutions into disposable plastic vials for storage at -60°C or below.
REAGENT PREPARATION

Allow all reagents to warm up to RT for at least 30 minutes before reconstitution.

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.

Reconstitute the control lysate with distilled water. See label indication for reconstitution. 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 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.

Reconstitute the Anti-phospho-ERK1/2 Cryptate and the Anti-phospho-ERK1/2 d2 with 1mL of detection buffer. Reconstituted antibody solutions are stable for 2 days at 4°C.

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

<table>
<thead>
<tr>
<th>500 TESTS KIT 64ERKPEG</th>
<th>10,000 TESTS KIT 64ERKPEH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phospho-ERK1/2 Cryptate antibody</strong></td>
<td></td>
</tr>
<tr>
<td>Once reconstituted the phospho ERK1/2 Cryptate antibody is ready to use.</td>
<td>1 volume Cryptate-antibody 19 volumes detection buffer</td>
</tr>
<tr>
<td>Dilute 20-fold the reconstituted antibody with detection buffer e.g add 19 ml of detection buffer to the 1 ml of reconstituted antibody solution</td>
<td></td>
</tr>
<tr>
<td><strong>Phospho-ERK1/2 d2 antibody</strong></td>
<td></td>
</tr>
<tr>
<td>Once reconstituted the phospho ERK1/2 d2 antibody is ready to use.</td>
<td>1 volume d2 antibody 19 volumes detection buffer</td>
</tr>
<tr>
<td>Dilute 20-fold the reconstituted antibody with detection buffer e.g add 19 ml of detection buffer to the 1 ml of reconstituted antibody solution</td>
<td></td>
</tr>
<tr>
<td><strong>Antibody mix</strong></td>
<td></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 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 of Cryptate-antibody solution.</td>
</tr>
</tbody>
</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 64ERKPEG | 10,000 TESTS KIT 64ERKPEH |
| Preparation of Supplemented Lysis buffer 4X |

1 volume
Blocking reagent

24 volumes
lysis buffer 4X

1 volume
Blocking reagent

24 volumes
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 64ERKPEG & 10,000 TESTS KIT 64ERKPEH |
| Preparation of lysis buffer 1X | Preparation of supplemented Lysis buffer 1X |

<table>
<thead>
<tr>
<th>500 tests</th>
<th>10,000 tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 volume</td>
<td>1 volume</td>
</tr>
</tbody>
</table>

lysis buffer 4X

3 volumes
distilled water

lysis buffer 4X

3 volumes
distilled water

1 volume
blocking reagent

99 volumes
lysis buffer 1X

1 volume
blocking reagent

99 volumes
lysis buffer 1X

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**

### FOR ADHERENT 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.

   - Cell seeding densities of 50 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 compound (2X) diluted in cell culture serum-free medium.

   - For most compound, incubation time is between 5 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.

4. Immediately add 50 µL of supplemented lysis buffer (1X) and incubate for at least 30 minutes at room temperature under shaking.

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.

### FOR SUSPENSION CELLS

1. Plate 25 µL of cells in 96 half-well plate in HBSS* or other appropriate medium.

2. Dispense 5 µL of compound (6X), diluted in HBSS or other appropriate medium.

3. Discard supernatant (for adherent cells).

### 96-well culture plate

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Non Treated Cell Lysate</th>
<th>Treated Cell Lysate</th>
<th>Control Lysate</th>
<th>Negative Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dispense 16 µL of non treated cell lysate</td>
<td>Dispense 16 µL of treated cell lysate</td>
<td>Dispense 16 µL of control lysate</td>
<td>Dispense 16 µL of supplemented lysis buffer(1X)</td>
</tr>
<tr>
<td>2</td>
<td>Add 2 µL of Phospho-ERK1/2 d2 antibody working solution to all wells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Add 2 µL of Phospho-ERK1/2 Cryptate antibody working solution to all wells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Cover the plate with a plate sealer. Incubate 2 h at room temperature. Maximum signal is reached after 2 h incubation time, and remains stable over a period of 24 hours. Therefore, readings can be made between 2 h and 24h of incubation time.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Remove the plate sealer and read on an HTRF compatible reader</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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.
# 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 15 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.

2. **Dispense 4 µL of compounds (3X) diluted in HBSS or other appropriate medium.** For most compound, incubation time is between 5 and 30 minutes at 37°C. We recommend a time course study to determine the optimal stimulation time.

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 2 h at room temperature. Maximum signal is reached after 2 h incubation time, and remains stable over a period of 24 hours. Therefore, readings can be made between 2 h and 24 h of incubation time. Set up your reader for Eu²⁺ Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on a compatible HTRF® reader.

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

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

<table>
<thead>
<tr>
<th>Non Treated Cell Lysate</th>
<th>Treated Cell Lysate</th>
<th>Negative Control</th>
<th>Control Lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dispense 8 µL of cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Add 4 µL HBSS or appropriate medium</td>
<td>Add 4 µL compound (3X)</td>
<td>Add 12 µL HBSS or appropriate medium</td>
<td>Dispense 16 µL control lysate</td>
</tr>
<tr>
<td>Add 4 µL supplemented lysis buffer (4X) - 30 min/RT.</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Add 2 µL of Phospho-ERK1/2 d2 antibody solution to all wells</td>
<td>Add 2 µL of Phospho-ERK1/2 Cryptate antibody solution to all wells</td>
<td>Cover the plate with a plate sealer. Incubate 2 h at room temperature. Maximum signal is reached after 2 h incubation time, and remains stable over a period of 24 hours. Therefore, readings can be made between 2 h and 24 h of incubation time.</td>
<td>Remove the plate sealer and read on an HTRF compatible reader</td>
</tr>
</tbody>
</table>

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/htrf-ratio-and-data-reduction

RESULTS

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 CHO-CCR5 cells (25,000 cells/well), using the two-plate assay protocol for adherent cells.

Cells were activated with Rantes for 30 minutes and then lysed with 50 µL of supplemented lysis buffer for 30 minutes at room temperature.

<table>
<thead>
<tr>
<th>log [compound] (M)</th>
<th>phospho-Erk 1/2 results</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (basal)</td>
<td>602  2.1</td>
</tr>
<tr>
<td>- 10.6</td>
<td>573  7.2</td>
</tr>
<tr>
<td>- 10.3</td>
<td>592  6.8</td>
</tr>
<tr>
<td>-10</td>
<td>588  1.6</td>
</tr>
<tr>
<td>- 9.6</td>
<td>645  1.5</td>
</tr>
<tr>
<td>- 9</td>
<td>955  3.5</td>
</tr>
<tr>
<td>- 8.3</td>
<td>1294 1.2</td>
</tr>
<tr>
<td>- 7.6</td>
<td>1471 2.2</td>
</tr>
<tr>
<td>- 7.3</td>
<td>1504 2.1</td>
</tr>
<tr>
<td>- 7</td>
<td>1512 1.8</td>
</tr>
<tr>
<td>Negative control</td>
<td>599  1.2</td>
</tr>
<tr>
<td>Control lysate</td>
<td>2390 0.7</td>
</tr>
</tbody>
</table>

EC50: 1.5nM
FREQUENTLY ASKED QUESTIONS / TROUBLESHOOTING PARAMETERS

<table>
<thead>
<tr>
<th>Using adherent cells, allow time for your cells to recover after plating</th>
<th>Allow cells to regain full signaling capacity by plating them at least 6 hours before starting the pharmacological treatment.</th>
</tr>
</thead>
</table>
| 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 resuspend 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 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 | Day1: Dispense 8 million cells in T175cm², add 25 mL of cell culture complete medium and incubate 2 days at 37°C, 5% CO2.
Day2: cell lysis
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 stimulation
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. |

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: i) 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.

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