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# THUNDER™

## TR-FRET CELL SIGNALING ASSAY KITS

**GENERIC USER MANUAL - VERSION 2**

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**This is a generic User Manual for all THUNDER™ Cell Signaling Assay kits. For kit-specific information, please refer to the Technical Data Sheet of the kit, available at https://bioauxilium.com/tr-fret-cell-signaling-assay-kits/**

**An electronic version of the manual is available at https://bioauxilium.com/resources/**

## 1 INTENDED USE

The THUNDER™ TR-FRET Cell Signaling Assay Kits are designed for the semi-quantitative measurement of phosphorylated and/or total (both phosphorylated and unphosphorylated) proteins in cell lysates using the homogeneous (no-wash) TR-FRET technology. The kits are compatible with both adherent and suspension cells.

## 2 STORAGE AND STABILITY

All kits are shipped on blue ice. Immediately upon receiving the kit, store it at -80°C. Kits have a shelf-life of one year upon receipt when stored and handled as described. The kit expiration date is indicated on the box label.

## 3 PRECAUTIONS

**Please read these instructions carefully prior to beginning the assay.**

- Although these instructions were carefully written and checked, Bioauxilium Research, Inc., cannot accept responsibility for problems encountered when using this User Manual. Suggestions for improvement will be gratefully accepted.
- This User Manual must be read in its entirety before using these products.
- These kits are sold based on the number of points. A "point" simply refers to a single assay well.
- Do not mix or substitute reagents or materials from other kit lots or kits. Kits are quality control tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
- We cannot guarantee the performance of the product outside the conditions detailed in this User Manual.
- The kits are designed for the detection of endogenous cellular proteins across a wide variety of cell lines. However, until each cell line in particular is tested, the possibility of the presence of undetectable levels of the target protein cannot be excluded.
- Users should ensure that their cell line has measurable levels of the target protein. Expression levels of signaling proteins in different cell types vary widely. The cell line used for the assay validation of each kit is shown in the corresponding Technical Data Sheet (available at https://bioauxilium.com/resources/).

## 4 THUNDER™ GENERAL INFORMATION

The THUNDER™ Cell Signaling Assay Kits are based on the Time-Resolved Förster Resonance Energy Transfer (TR-FRET) technology. THUNDER™ assays can be read on most commercially available TR-FRET compatible microplate readers (a list of suitable TR-FRET readers can be found at www.Bioauxilium.com). TR-FRET based assays are homogeneous because they do not require any washing or separation steps. In addition, the THUNDER™ assays use a standardized, simple and rapid "add-incubate-measure" protocol with a single-step reagent addition. This streamlined assay protocol dramatically decreases hands-on time and provides a powerful alternative to cumbersome, error prone and time-consuming techniques such as Western blotting and ELISA.

There are 3 types of kits available:

- The **Phospho-Protein Kits** detect the relative amounts of a specific phosphorylated target protein.
- The **Total Protein Kits** detect the relative amounts of a specific target protein regardless of its phosphorylation status. These kits can be used to normalize data obtained with the matched Phospho-protein kits or to monitor protein expression levels.
- The **Phospho + Total Protein Kits** provide a novel opportunity to simultaneously detect with a single kit matched phosphorylated and total proteins in separate wells within the same microplate.

The THUNDER™ assay kits contain the essential reagents necessary to carry out the measurement of signaling proteins in cells.

## 5 ASSAY PRINCIPLE

All THUNDER™ assays are based on the traditional sandwich immunoassay principle (Figure 1, below). Following cell treatment, cells are first lysed with the specific Lysis Buffer provided in the kit. Then, the target protein in the cell lysates is detected with a pair of fluorophore-labeled antibodies.

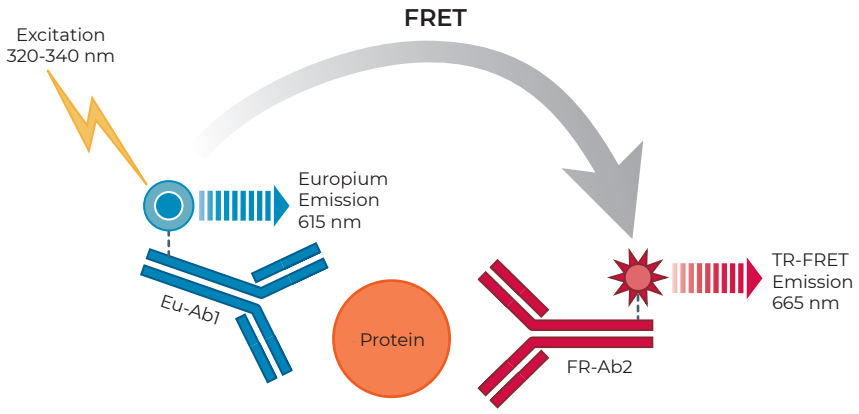



**FIGURE 1** Schematic representation of the TR-FRET cell signaling assay principle.

The first antibody is labelled with a long-lifetime Europium chelate donor (Eu-Ab1) and the second with a far-red acceptor fluorophore (FR-Ab2). The binding of the two labeled antibodies to distinct epitopes on the target protein takes place in solution and brings the two dyes into close proximity. Excitation of the donor Europium chelate molecules with a flash lamp (320 or 340 nm) or a laser (337 nm) triggers a FRET from the donor to the acceptor molecules, which in turn emit a TR-FRET signal at 665 nm. The signal at 665 nm is proportional to the concentration of target protein in the cell lysate. Residual energy from the Eu chelate generates light at 615 nm, which can be used as an internal standard to normalize light emitted at 665 nm.

Because these long-lived fluorescence signals are detected in a time-resolved manner, TR-FRET assays exhibit very low background fluorescence levels and high signal-to-background (S/B) ratios. Another key advantage of TR-FRET assays is that data can be expressed and analyzed as either the signal at 665 nm or the 665 nm/615 nm ratio. The ratiometric measurement further increases assay reproducibility and robustness.

## 6 KIT CONTENTS

### PHOSPHO OR TOTAL PROTEIN ASSAY KITS

| Component | Amount (100 points) | Amount (500 points) |
|---|---|---|
| Europium-labeled antibody (Eu-Ab1) | 5 µL (1 clear tube, red cap) | 25 µL (1 clear tube, red cap) |
| Acceptor-labeled antibody (FR-Ab2) | 20 µL (1 brown tube, blue cap) | 100 µL (1 brown tube, blue cap) |
| Lysis Buffer (5X) | 1 mL (1 tube, black cap) | 5 mL (4 tubes; black cap) |
| Detection Buffer (10X) | 50 µL (1 tube, yellow cap) | 250 µL (1 tube, yellow cap) |
| Positive control lysate | 100 µL (1 tube; clear cap) | 200 µL (1 tube; clear cap) |
| Phosphatase Inhibitor Cocktail (100X) | 50 µL (1 tube, green cap) | 250 µL (1 tube, green cap) |

### PHOSPHO + TOTAL PROTEIN ASSAY KITS

| Kit components | 500 points (400 phospho + 100 total) |
|---|---|
| Europium-labeled phospho-protein antibody (Eu-Ab1) | 20 µL (1 clear tube, red cap) |
| Acceptor-labeled phospho-protein antibody (FR-Ab2) | 80 µL (1 brown tube, blue cap) |
| Europium-labeled total-protein antibody (Eu-Ab3) | 5 µL (1 clear tube, red cap) |
| Acceptor-labeled total-protein antibody (FR-Ab4) | 20 µL (1 brown tube, blue cap) |
| Lysis Buffer (5X) | 5 mL (4 tubes; black cap) |
| Detection Buffer (10X) | 250 µL (1 tube; yellow cap) |
| Positive control lysate | 200 µL (1 tube; clear cap) |
| Phosphatase Inhibitor Cocktail (100X) | 250 µL (1 tube; green cap) |

* The number of assay points (wells) is based on an assay volume of 20 µL in half-area 96-well or low-volume 384-well microplates using the kit components at the recommended concentrations

## 7 MATERIALS REQUIRED BUT NOT SUPPLIED

| Item | Recommended source | Catalog No. |
|---|---|---|
| Ultrapure laboratory grade water<br>Glass-distilled or deionized water is not acceptable | Many options available | NA |
| **Culture plate**<br>96-well clear flat bottom polystyrene TC-treated microplate, for culturing cells when using the 2-plate assay protocol.<br>Do not use this plate for the one-plate assay protocol. | Corning | 3595 |
| **Detection plate (96-well microplate option)**<br>Half-area 96-well microplate, white, for TR-FRET detection when using the 2-plate assay protocol. | Greiner | 675075 |
| | Corning | 3693 |
| **Detection plate (384-well microplate option)**<br>Low-volume 384-well microplate, white, for TR-FRET detection when using the 2- or 1-plate assay protocols. | PerkinElmer | 6007290 |
| | Greiner | 784075 |
| | Corning | 4513 |
| Multi- and single-channel pipettes | Many options available | NA |
| Adhesive sealing film for plates | Many options available | NA |
| Orbital plate shaker | Many options available | NA |
| A plate reader equipped with the TR-FRET option | Many options available | NA |

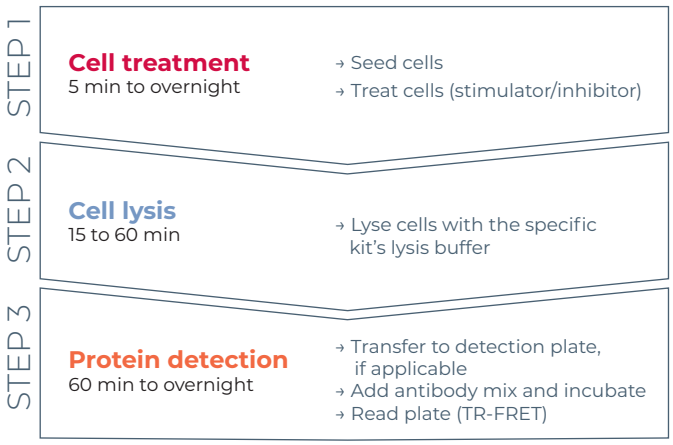
## 8 ASSAY OPTIMIZATION GUIDELINES

A critical step in performing any cell-based assay is the optimization of cell culture and treatment conditions. The following Manual assumes that both the cell number and treatment conditions have been previously optimized, as these key parameters often vary for each cell line. It is therefore strongly recommended to optimize these parameters in order to maximize the assay signal and ensure optimum performance with a high S/B ratio.

Cell number, serum-starvation step (optional) and stimulation or inhibition time (at either room temperature or 37°C) should be optimized for each cell line and target protein. Cell numbers that are too high or too low can negatively influence the activation of intracellular signaling pathways. Cell seeding densities of 40,000-80,000 cells/well for adherent cells or 100,000-200,000 cells/well for suspension cells are generally acceptable for most cell lines. Of note, the optimal time of stimulation can vary widely among cell lines, from a few minutes to more than one hour. As such, a time course study is strongly recommended to determine the optimal stimulation time, ideally at both room temperature and 37°C, since incubation temperature has an effect on the kinetics of target protein stimulation. Additional assay development guidelines are available on Bio**auxilium**'s website (www.bioauxilium.com).

## 9 ASSAY WORKFLOW

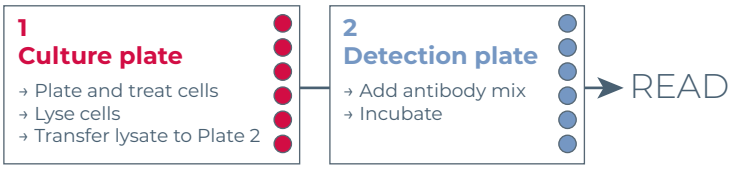
The THUNDER™ Cell Signaling Assay workflow consists of 3 simple steps:



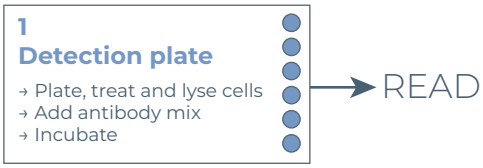

## 10 SUMMARY OF ASSAY PROTOCOLS

The THUNDER™ Cell Signaling Assays can be run using using one of two possible protocols.

**2-Plate (transfer) protocol**




**1-Plate (all-in-one-well) protocol**




Regardless of the assay protocol used, assays are optimized to be run in either half-area 96-well or low-volume 384-well plates using the same total volume (20 µL).

## >> 2-PLATE ASSAY SUMMARY

| Step | Adherent cells | | Suspension cells | |
|---|---|---|---|---|
| | 96-WELL CULTURE PLATE | | | |
| **CELL TREATMENT** | **STIMULATION** | **INHIBITION** | **STIMULATION** | **INHIBITION** |
| | 50 µL cells | 50 µL cells | 20 µL cells | 20 µL cells |
| | Incubate cells overnight | Incubate cells overnight | 20 µL 2X stimulator* | 10 µL 4X inhibitor* |
| | 50 µL 2X stimulator* | 25 µL 4X inhibitor* | Incubate for optimized time | Incubate for optimized time |
| | Incubate for optimized time | Incubate for optimized time | | 10 µL 4X stimulator* |
| | | 25 µL 4X stimulator* | | Incubate for optimized time |
| | | Incubate for optimized time | | |
| **CELL LYSIS** | Remove media | | 10 µL 5X Supplemented Lysis Buffer** | |
| | 50 µL 1X Supplemented Lysis Buffer** | | Incubate 30 min | |
| | Incubate 30 min | | | |
| | WHITE, HALF-AREA 96-WELL PLATE OR LOW-VOLUME 384-WELL PLATE | | | |
| **PROTEIN DETECTION** | 15 µL lysate | | | |
| | 5 µL 4X Antibody Detection Mix | | | |
| | Incubate 1 hour to overnight | | | |
| | Read TR-FRET signal | | | |

** The untreated cells receive the same volume of serum-free medium and are incubated for the same amount of time, and at the same temperature, as the treated cells.*

*** The 1X Lysis Buffer must be supplemented with the Phosphatase Inhibitor Cocktail diluted at 1X, whereas the 5X Lysis Buffer must be supplemented with the Phosphatase Inhibitor Cocktail diluted at 5X.*

### >> 1-PLATE ASSAY SUMMARY

| Step | Adherent or suspension cells | |
|---|---|---|
| | WHITE, LOW-VOLUME 384-WELL PLATE | |
| **CELL TREATMENT** | **STIMULATION** | **INHIBITION** |
| | 8 µL cells | 8 µL cells |
| | 4 µL 3X stimulator* | 2 µL 6X inhibitor* |
| | Incubate for optimized time | Incubate for optimized time |
| | | 2 µL 6X stimulator* |
| | | Incubate for optimized time |
| **CELL LYSIS** | 3 µL 5X Supplemented Lysis Buffer** | |
| | Incubate 30 min | |
| **PROTEIN DETECTION** | 5 µL 4X Antibody Detection Mix | |
| | Incubate 1 hour to overnight | |
| | Read TR-FRET signal | |

** The untreated cells receive the same volume of serum-free medium and are incubated for the same amount of time, and at the same temperature, as the treated cells.*

*** The 5X Lysis Buffer must be supplemented with the Phosphatase Inhibitor Cocktail diluted at 5X.*

## 11 REAGENT PREPARATION

- The instructions described below are for testing the entire number of assay points in each kit. Adjust volumes accordingly for testing of fewer assay points (applicable to the 500-point kits only).
- Bring all reagents to room temperature prior to use.
- Centrifuge all tubes before use to improve recovery of content (2000x g, 10-15 sec).
- ONLY use ultrapure water (Milli-Q® grade water; 18 MΩ·cm) to dilute Lysis and Detection Buffers.
- NOTE: It is recommended to test all samples and controls at least in duplicate.
- NOTE: It is MANDATORY to supplement the Lysis Buffer with the Phosphatase Inhibitor Cocktail at the specified concentration.
- NOTE: Always include in your detection plate a positive control using the positive control lysate included in the kit.

| Solution | Instructions and storage conditions | | |
|---|---|---|---|
| **1a**<br>1X Supplemented Lysis Buffer<br>(for the 2-plate assay protocol with adherent cells) | Each well requires 50 µL of 1X Supplemented Lysis Buffer, which contains 1 mM NaF, 2 mM Na3VO4 and 2 mM beta-glycerophosphate.<br>NOTE: it is mandatory to supplement the 1X Lysis Buffer with the 100X Phosphatase Inhibitor Cocktail diluted at 1X.<br>Dilute the 5X Lysis Buffer and 100X Phosphatase Inhibitor Cocktail to 1X with ultrapure water.<br>For example: add 1 mL of 5X Lysis Buffer to 3.95 mL of ultrapure water and 50 µL of 100X Phosphatase Inhibitor Cocktail. | | |
| | The unused 1X Lysis Buffer may be stored at +4°C for 2 days. | | |
| **1b**<br>5X Supplemented Lysis Buffer<br>(for the 2-plate assay protocol with suspension cells or the 1-plate assay protocol) | Each well requires 10 µL (2-plate assay protocol) or 3 µL (1-plate assay protocol) of 5X Supplemented Lysis Buffer, which contains 5 mM NaF, 10 mM Na3VO4 and 10 mM beta-glycerophosphate.<br>NOTE: it is mandatory to supplement the 5X Lysis Buffer with the 100X Phosphatase Inhibitor Cocktail diluted at 5X.<br>Directly dilute the 100X Phosphatase Inhibitor Cocktail to 5X with the 5X Lysis Buffer.<br>For example: add 50 µL of 100X Phosphatase Inhibitor Cocktail to 0.95 mL of 5X Lysis Buffer. | | |
| | The unused 5X Lysis Buffer may be stored at +4°C for 2 days. | | |
| **2**<br>1X Detection Buffer<br>(for dilution of labeled antibodies) | Dilute the 10X Detection Buffer to 1X with ultrapure water.<br>For example: add 50 µL of 10X Detection Buffer to 450 µL of ultrapure water. | | |
| | The unused 1X Detection Buffer may be stored at +4°C for 2 days. | | |
| **3**<br>4X Antibody Detection Mix in 1X Detection Buffer | Prepare and mix just before use.<br>Each well requires 5 µL of 4X Antibody Detection Mix. | | |
| | **For the 100-point kit**<br>(Phospho or Total protein kit)<br>NOTE: due to the low reagent volumes in the 100-point kit, the antibodies are diluted with 1X Detection Buffer directly in the vial.<br>**a**. Add 255 µL of 1X Detection Buffer into the vial containing 5 µL of Eu-Ab1 stock solution.<br>**b.** Add 240 µL of 1X Detection Buffer into the vial containing 20 µL of FR-Ab2 stock solution.<br>**c.** Mix gently 260 µL of pre-diluted Eu-Ab1 with 260 µL of pre-diluted FR-Ab2. | **For the 500-point kit**<br>(Phospho or Total protein kit)<br>**a.** Mix gently 1,275 µL of 1X Detection Buffer with the 25 µL of Eu-Ab1 stock solution.<br>**b.** Mix gently 1,200 µL of 1X Detection Buffer with the 100 µL of FR-Ab2.<br>**c.** Mix gently 1,300 µL of pre-diluted Eu-Ab1 with 1,300 µL of pre-diluted FR-Ab2. | **For the 500-point kit**<br>(Phospho + Total protein kit)<br>Phospho-protein (Eu-Ab1 + FR-Ab2 Mix):<br>**a.** Mix gently 1,020 µL of 1X Detection Buffer with the 20 µL of Eu-Ab1 stock solution.<br>**b.** Mix gently 960 µL of 1X Detection Buffer with the 80 µL of FR-Ab2.<br>**c.** Mix gently 1,040 µL of pre-diluted Eu-Ab1 with 1,040 µL of pre-diluted FR-Ab2.<br>Total protein (Eu-Ab3 + FR-Ab4 Mix):<br>Proceed as for the 100-point kit. |
| | The unused 4X antibody working solutions may be stored at +4°C for 2 days. | | |
| **4**<br>Positive Control Lysate | The control lysate is supplied ready to use. | | |
| | The thawed control lysate can be aliquoted, refrozen at -80°C and thawed at least three more times. | | |

## 12 TR-FRET PLATE READER SETTINGS

We recommend reading the TR-FRET assays at two wavelengths, detecting both the emission from the Europium chelate donor fluorophore at 615 nm, and the acceptor fluorophore at 665 nm. The following instrument settings are provided as guidelines.

| | TR-FRET Compatible Plate Reader | |
|---|---|---|
| **Parameter** | **Flash lamp excitation** | **Laser excitation** |
| Excitation filter | 320 nm (or 340 nm) | Not applicable |
| Emission filter | 615 nm (or 620 nm) | 615 nm (or 620 nm) |
| Delay time | 90 µs | 50 µs |
| Flash energy level | 100% or High | 100% |
| Number of flashes | 100 | 20 |
| Window (integration time) | 300 µs | 100 µs |

# 13 STANDARD 2-PLATE ASSAY PROTOCOL FOR ADHERENT CELLS

**This is a transfer protocol that is conducted in 2 different types of plates: cell culture and lysis are conducted in a 96-well culture plate(s), whereas detection is conducted in either a white, half-area 96-well assay plate(s) or a white, low-volume 384-well assay plate(s), with a total assay volume of 20 µL per well for TR-FRET detection. A summary of this protocol is provided in page 8.**

## CELL TREATMENT STEP

1. Dispense 50 µL of cells (at the pre-optimized density) into a 96-well tissue culture-treated plate in appropriate culture medium.
2. Incubate overnight at 37°C, 5% $CO_2$.
3. **For cell STIMULATION:**
   a. Add 50 µL of stimulator (2X) diluted in serum-free medium.
   b. Incubate for pre-optimized time at either room temperature (RT) or 37°C. Optimal incubation temperature needs to be determined.
4. **For cell INHIBITION:**
   a. Add 25 µL of inhibitor (4X) diluted in serum-free medium.
   b. Incubate for pre-optimized time at either RT or 37°C.
   c. Add 25 µL of stimulator (4X) diluted in serum-free medium.
   d. Incubate for pre-optimized time at either RT or 37°C.

## CELL LYSIS STEP

1. REMOVE carefully the cell culture medium by aspirating the supernatant.
2. CRITICAL STEP: immediately add 50 µL of 1X Supplemented Lysis Buffer. Lysis Buffer volume (25-50 µL) may be optimized.
3. Incubate for 30 min at RT under shaking (orbital plate shaker set at 400 rpm; moderate agitation). Lysis incubation time (15-60 min) may be optimized.

PAUSE POINT: lysates can be frozen at -80°C or used immediately for target protein detection.

## TR-FRET DETECTION STEP

1. TRANSFER STEP: carefully pipette 15 µL of cell lysate from the 96-well culture plate to a well of either a white, half-area 96-well or a white, low-volume 384-well microplate.
2. RECOMMENDED: add 15 µL of Positive Control Lysate (undiluted) and 15 µL of 1X Lysis Buffer (negative control) to separate assay wells.
3. Add 5 µL of 4X Antibody Detection Mix (Eu-Ab1 + FR-Ab2) to each of the assay wells.

NOTE: If you are using a Phospho + Total protein Detection Kit, add to separate wells containing 15 µL of lysate either 5 µL of 4X Eu-Ab1 + FR-Ab2 for detection of the phospho-protein or 5 µL of 4X Eu-Ab3 + FR-Ab4 for detection of the total protein.

4. Cover the plate with a plate sealer and incubate between 1 and 18 hours at RT, depending on the assay kit (see the corresponding Technical Data Sheet).
5. CRITICAL STEP: gently remove the adhesive plate sealer. Read plate on a TR-FRET compatible microplate reader.

NOTE: The same plate can be read several times without a negative effect on the assay performance.

# 14 STANDARD 2-PLATE ASSAY PROTOCOL FOR SUSPENSION CELLS

**This is a transfer protocol that is conducted in 2 different types of plates: cell culture and lysis are conducted in a 96-well culture plate(s), whereas detection is conducted in either a white, half-area 96-well assay plate(s) or a white, low-volume 384-well assay plate(s), with a total assay volume of 20 µL per well for TR-FRET detection. A summary of this protocol is provided in page 8.**

## CELL TREATMENT STEP

1. Dispense 20 µL of cells (at the pre-optimized density) into a 96-well tissue culture-treated plate in appropriate culture medium.
2. Directly proceed to cell treatment or incubate 2-4 hours at 37°C in a 5% $CO_2$ atmosphere. This step may be optimized.
3. **For cell STIMULATION:**
   a. Add 20 µL of stimulator (2X) diluted in serum-free medium.
   b. Incubate for pre-optimized time at either room temperature (RT) or 37°C. Optimal incubation temperature needs to be determined.
4. **For cell INHIBITION:**
   a. Add 10 µL of inhibitor (4X) diluted in serum-free medium.
   b. Incubate for pre-optimized time at either RT or 37°C.
   c. Add 10 µL of stimulator (4X) diluted in serum-free medium.
   d. Incubate for pre-optimized time at either RT or 37°C.

## CELL LYSIS STEP

1. Add 10 µL of 5X Supplemented Lysis Buffer.
2. Incubate for 30 min at RT under shaking (orbital plate shaker at 400 rpm). Lysis incubation time (15-60 min) may be optimized.

PAUSE POINT: lysates can be frozen at -80°C or used immediately for target protein detection.

## TR-FRET DETECTION STEP

Following cell lysis, proceed to the TR-FRET detection step as described for the standard 2-plate assay protocol for adherent cells.

### SUMMARY OF PIPETTING PROTOCOL FOR THE 2-PLATE ASSAY PROTOCOL AFTER THE LYSIS STEP

| | Untreated cells | Treated cells | Positive control | Negative control |
|---|---|---|---|---|
| **Cell lysate (untreated cells)** | 15 µL | | | |
| **Cell lysate (treated cells)** | | 15 µL | | |
| **Positive Control Lysate** | | | 15 µL | |
| **1X Supplemented Lysis Buffer** | | | | 15 µL |
| **4X Antibody Detection Mix** | 5 µL | 5 µL | 5 µL | 5 µL |
| **Total assay volume** | 20 µL | 20 µL | 20 µL | 20 µL |

# 15 STANDARD 1-PLATE ASSAY PROTOCOL FOR ADHERENT OR SUSPENSION CELLS

**This is an all-in-one-well protocol (no transfer step is needed) that is conducted in a 20-µL total assay volume and in a single white, low-volume 384-well assay plate(s) with a total assay volume of 20 µL per well. A summary of this protocol is provided in page 9**

## CELL TREATMENT STEP

1. Dispense 8 µL of cells (at the pre-optimized density), in appropriate serum-free culture medium, into a white, low-volume 384-well assay plate(s).
2. **For cell STIMULATION:**
   a. Add 4 µL of stimulator (3X) diluted in culture serum-free medium.
   b. Incubate for pre-optimized time at either room temperature (RT) or 37°C. Optimal incubation temperature needs to be determined.
3. **For cell INHIBITION:**
   a. Add 2 µL of inhibitor (6X) diluted in serum-free medium.
   b. Incubate for pre-optimized time at either RT or 37°C.
   c. Add 2 µL of stimulator (6X) diluted in serum-free medium.
   d. Incubate for pre-optimized time at either RT or 37°C.

## CELL LYSIS STEP

1. Add 3 µL of 5X Supplemented Lysis Buffer.
2. Incubate for 30 min at RT under shaking (orbital plate shaker at 400 rpm). Lysis incubation time (15-60 min) may be optimized.

PAUSE POINT: lysates can be frozen at -80°C or used immediately for target protein detection.

## TR-FRET DETECTION STEP

1. RECOMMENDED: add 15 µL of Positive Control Lysate (undiluted) and 15 µL of 1X Lysis Buffer (negative control) to separate assay wells.
2. Add 5 µL of 4X Antibody Detection Mix (Eu-Ab1 + FR-Ab2) prepared in 1X Detection Buffer to each of the assay wells.
3. Cover the plate with a plate sealer and incubate between 1 and 18 hours at RT, depending on the assay kit (see the corresponding Technical Data Sheet).
4. CRITICAL STEP: gently remove the adhesive plate sealer. Read plate on a TR-FRET compatible microplate reader.

## PIPETTING PROTOCOL FOR TESTING A **STIMULATOR** WITH THE 1 PLATE ASSAY PROTOCOL

| | Untreated cells | Treated cells | Positive control | Negative control |
|---|---|---|---|---|
| Suspension cells | 8 µL | 8 µL | - | - |
| Culture media | 4 µL | - | - | - |
| 3X Stimulator | - | 4 µL | - | - |
| Positive Control Lysate | - | - | 15 µL | - |
| 5X Supplemented Lysis Buffer | 3 µL | 3 µL | - | - |
| 1X Supplemented Lysis Buffer | - | - | - | 15 µL |
| 4X Antibody Detection Mix | 5 µL | 5 µL | 5 µL | 5 µL |
| Total assay volume | 20 µL | 20 µL | 20 µL | 20 µL |

**PIPETTING PROTOCOL FOR TESTING AN INHIBITOR WITH THE 1 PLATE ASSAY PROTOCOL**

| | Untreated cells | Treated cells | Positive control | Negative control |
|---|---|---|---|---|
| Suspension cells | 8 µL | 8 µL | - | - |
| Culture media | 4 µL | | - | - |
| 6X Inhibitor | - | 2 µL | - | - |
| 6X Stimulator | - | 2 µL | - | - |
| Positive Control Lysate | - | - | 15 µL | - |
| 5X Supplemented Lysis Buffer | 3 µL | 3 µL | - | - |
| 1X Supplemented Lysis Buffer | - | - | - | 15 µL |
| 4X Antibody Detection Mix | 5 µL | 5 µL | 5 µL | 5 µL |
| Total assay volume | 20 µL | 20 µL | 20 µL | 20 µL |

## 16 DATA ANALYSIS

1. TR-FRET data are typically calculated and presented ratiometrically using the following formula:
   **[(665 nm/615 nm) x 1,000]**
2. Alternatively, the signals at 665 nm can be used directly to process your data.
3. Calculate the TR-FRET ratio for each well.
4. Since TR-FRET signal is read in a time-resolved mode, background substraction is usually not necessary.
5. For concentration-response curves, analyze data according to a nonlinear regression using the 4 -parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting.
6. Assay quality control: the undiluted Positive Control Lysate must generate an S/B ratio of at least 2 when compared to the negative control (1X Lysis Buffer only). If this is not the case, your reader is not compatible with the THUNDER™ kits.

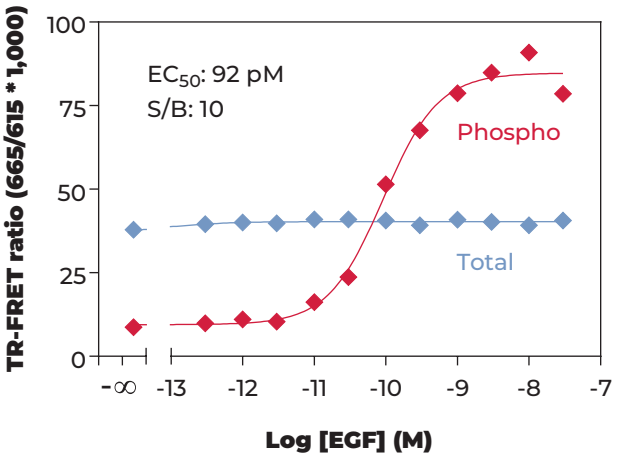
NOTE The Positive Control Lysate is provided as a control reagent, not for conducting a standard curve.

## 17 REPRESENTATIVE DATA

Data shown here are examples of data typically generated with the THUNDER™ Cell Signaling Assay Kits. The TR-FRET signal was recorded at 665 and 615 nm (EnVision®; lamp excitation) using the recommended plate reader settings. Note that both the TR-FRET ratios and S/B ratios will vary from one TR-FRET compatible reader to another. In addition, note that excitation with a laser (337 nm) generates higher counts and, usually, higher S/B ratios.

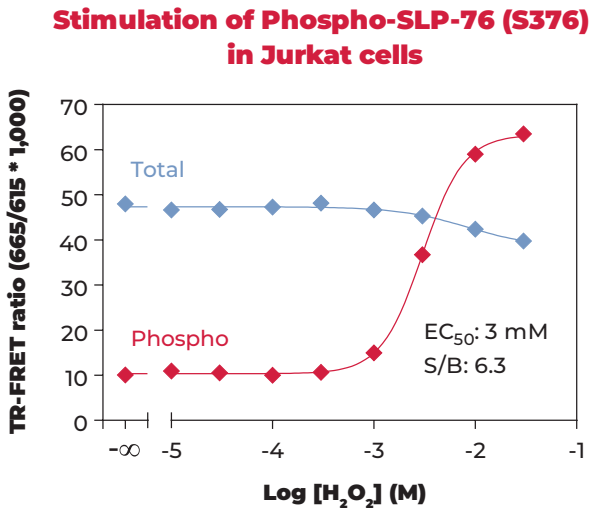
### DATA OBTAINED WITH THE 2-PLATE (TRANSFER) PROTOCOL USING ADHERENT CELLS

**Stimulation of Phospho-ERK1/2 (T202/Y204) in HEK293 cells**



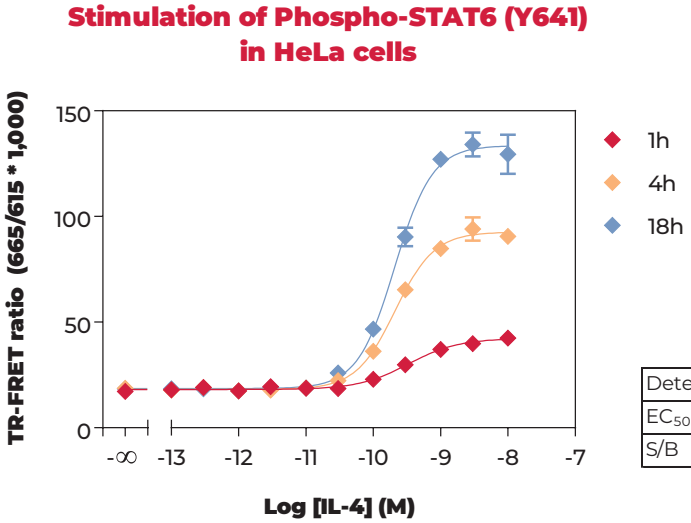

HEK293 cells (50 µL) were seeded at 50,000 cells/well in a 96-well culture plate and cultured overnight at 37°C, 5% CO2, in DMEM with 10% FBS. Cells were then treated with EGF (50 µL), diluted in DMEM without serum, for 10 min at RT. Following removal of the media from the wells, cells were lysed with 50 µL/well of 1X Supplemented Lysis Buffer 1, on an orbital shaker (400 rpm) for 30 min at RT. The lysates (15 µL) were then transferred to a white low-volume 384-well assay plate and analyzed for phospho-ERK1/2 (T202/Y204) and total ERK1/2 using the corresponding assay kits. The plate was read after a 4-hour incubation period at RT.

## DATA OBTAINED WITH THE 2-PLATE (TRANSFER) PROTOCOL USING SUSPENSION CELLS




Jurkat cells (20 µL), resuspended in RPMI without serum, were seeded at 400,000 cells/well in a 96-well culture plate and treated with $H_2O_2$ (20 µL) for 15 min at RT. Cells were then lysed with 10 µL/well of 5X Supplemented Lysis Buffer 2, on an orbital shaker (400 rpm) for 30 min at RT. The lysates (15 µL) were then transferred to a white low-volume 384-well assay plate and analyzed for phospho-SLP-76 (S376) and total SLP-76 using the corresponding assay kits. The plate was read after an 18-hour incubation period at RT.

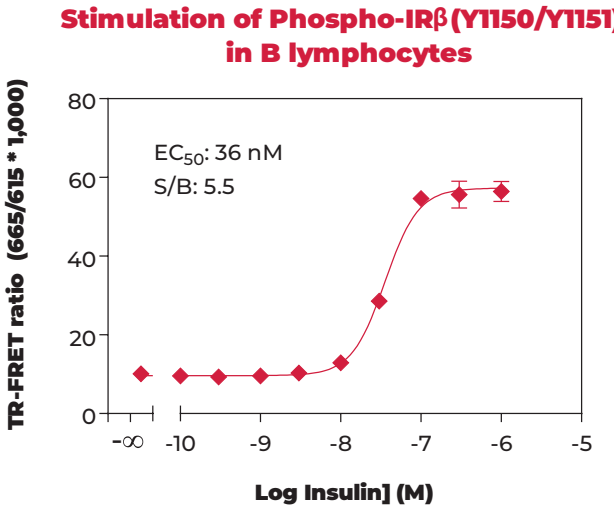
## DATA OBTAINED WITH THE 1-PLATE (ALL-IN-ONE-WELL) PROTOCOL USING ADHERENT CELLS




| Detection time (h) | 1 | 4 | 18 |
|---|---|---|---|
| $EC_{50}$ (nM) | 0,33 | 0,21 | 0,21 |
| S/B | 2,3 | 5 | 7,2 |

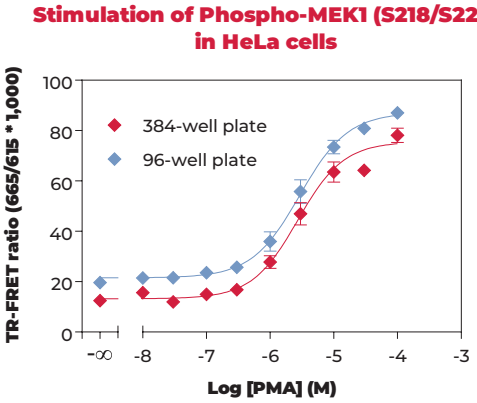
HeLa cells (8 µL), resuspended in DMEM without serum, were seeded at 160,000 cells/well in a white low-volume 384-well assay plate and immediately treated with IL 4 (4 µL) for 20 min at RT. Cells were then lysed with 3 µL/well of 5X Supplemented Lysis Buffer 2, on an orbital shaker (400 rpm) for 30 min at RT. The lysates (15 µL) were then directly analyzed for phospho-STAT6 (Y641) using the corresponding assay kit. The plate was read after 1, 4 and 18 hours of incubation at RT.

## DATA OBTAINED WITH THE 1-PLATE (ALL-IN-ONE-WELL) PROTOCOL USING SUSPENSION CELLS

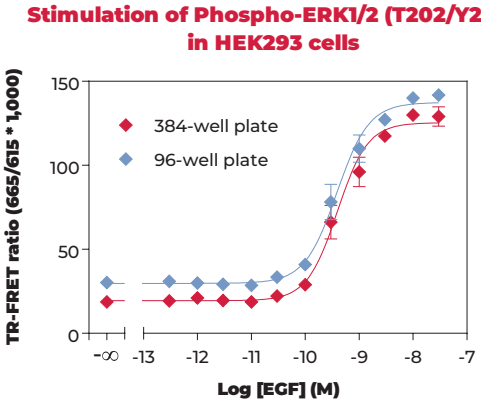



B lymphocytes (8 µL), resuspended in RPMI without serum, were seeded at 40,000 cells/well in a white low-volume 384-well assay plate and immediately treated with Insulin (4 µL) for 20 min at 37°C. Cells were then lysed with 3 µL/well of 5X Supplemented Lysis Buffer 4, on an orbital shaker (400 rpm) for 30 min at RT. The lysates (15 µL) were then directly analyzed for phospho-IRβ (Y1150/Y1151) using the corresponding assay kit. The plate was read after a 4-hour incubation period at RT.

## COMPARISON OF DATA OBTAINED WITH THE HALF-AREA 96-WELL PLATE VERSUS THE LOW-VOLUME 384-WELL PLATE




| Plate | 384-well | 96-well |
|---|---|---|
| $EC_{50}$ (µM) | 2.72 | 2.93 |
| S/B | 6.3 | 4.4 |




| Plate | 384-well | 96-well |
|---|---|---|
| $EC_{50}$ (nM) | 0.39 | 0.38 |
| S/B | 6.9 | 4.7 |

Following cell lysis, lysates (15 µL) were transferred to either a half-area 96-well plate or a low-volume 384-well plate. Phospho-protein was then detected using the corresponding 4X Antibody Detection Mix (5 µL).

## 18 TROUBLESHOOTING

| Problem | Possible causes | Recommended solution |
|---|---|---|
| **Assay S/B ratio <2 for the positive control lysate versus the negative control (i.e., the 1X Lysis Buffer alone).** | • Microplate reader and/or settings not suitable for TR-FRET assays.<br>• Use of low-quality water for reagent preparation.<br>• Use of black microplates.<br>• Plate read with the adhesive plate sealer. | • Use a filter-based instrument to read the plate.<br>• Ensure that you have the correct excitation and emission filters and mirror module.<br>• Use initially the recommended instrument settings. Optimize the delay time, measurement window and number of flashes.<br>• ONLY use ultrapure water for preparation of the Lysis and Detection Buffers.<br>• ONLY use white microplates.<br>• The plate sealer MUST be removed before reading the plate. |
| **Low S/B ratio in the cellular experiment.** | • Sub-optimal cell culture and/or treatment conditions.<br>• Use of a different Lysis Buffer than the one included in the kit.<br>• Lack of phosphatase inhibitors in the Lysis Buffer.<br>• Use of low-quality water for reagent preparation.<br>• Use of black microplates. | • Use the positive control lysate to determine whether the poor signal comes from the kit reagents or from the cellular experimental conditions used in the assay.<br>• Optimize cell culture conditions. Too high or low cell numbers can affect basal and maximal activation.<br>• Ensure the cell passage number is not too high or too low and that cells are behaving as expected (i.e., doubling time, viability).<br>• ONLY use the specific Lysis Buffer included in the kit.<br>• The Lysis Buffer MUST be supplemented with the Phosphatase Inhibitor Cocktail included in the kit. DO NOT use different phosphatase inhibitor cocktails. Additional phosphatase inhibitors and/or protease inhibitors are typically not required unless otherwise stated in the corresponding Technical Data Sheet.<br>• The assay S/B ratio might be increased by decreasing the volume of Lysis Buffer used to lyse the cells to 25 µL to increase the target protein concentration in the lysate.<br>• ONLY use ultrapure water.<br>• ONLY use white, opaque microplates. |

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**THUNDER™** TR-FRET CELL SIGNALING ASSAY KITS

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