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**4E-BP1 (Total) and 4E-BP1
(Phospho-Thr^{37/46}) TR-FRET Assay Kit**
Powered by Bioauxilium's THUNDER™ TR-FRET Technology

Item No. 500225

www.caymanchem.com

Customer Service 800.364.9897

Technical Support 888.526.5351

1180 E. Ellsworth Rd • Ann Arbor, MI • USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item Name	480 wells Quantity/Size
400271	Europium-Labeled 4E-BP1 (Phospho-Thr ^{37/46}) Antibody	1 vial/20 µl
400272	Acceptor-Labeled 4E-BP1 (Phospho-Thr ^{37/46}) Antibody	1 vial/80 µl
400298	Europium-Labeled 4E-BP1 (Total) Antibody	1 vial/5 µl
400299	Acceptor-Labeled 4E-BP1 (Total) Antibody	1 vial/20 µl
400211	Lysis Buffer 2 (5X)	4 vials/5 ml
400225	Detection Buffer (10X)	1 vial/250 µl
400297	4E-BP1 Positive Control Lysate	1 vial/500 µl
400257	Phosphatase Inhibitor Cocktail (100X)	1 vial/250 µl

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.



WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

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 kit booklet.

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 this kit is shown in the figures starting on page 33.

Before You Start

Please note the following:

ONLY white plates should be used for TR-FRET.

DO NOT modify the assay protocol or volumes.

DO optimize the cell density, serum starvation (optional), and stimulation or inhibition parameters.

ALWAYS use the included positive control lysate for every assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -80°C and used before the expiration date indicated on the outside of the box.

THUNDER™ General Information

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 homogeneous (no wash) TR-FRET technology. The kits are compatible with both adherent and suspended cells.

THUNDER™ TR-FRET Cell Signaling Assay Kits are based on Bioauxilium's enhanced proprietary time-resolved Förster resonance energy transfer (TR-FRET) technology. THUNDER™ assays can be read on most commercially available TR-FRET-compatible plate 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 blot and ELISA.

THUNDER™ TR-FRET Cell Signaling Assay Kits contain the essential reagents necessary to carry out the measurement of signaling proteins in cells, with the exception of the plate(s).

Materials Needed But Not Supplied

1. A plate reader equipped with a TR-FRET option
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of ultrapure water, with a resistivity of 18.2 M Ω -cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Culture plate: 96-well clear, flat-bottom polystyrene tissue culture-treated plate(s) for culturing cells when using the 2-plate (transfer) assay protocol. *NOTE: Do not use this type of plate for the 1-plate (all in one well) assay protocol.*
5. Detection plate: Half-area, 96-well white plate(s) for TR-FRET detection when using the 2-plate (transfer) assay protocol
6. Detection plate: Low-volume, 384-well white plate(s) for TR-FRET detection when using the 1- or 2-plate assay protocols
7. Adhesive sealing film for plates
8. Orbital microplate shaker

INTRODUCTION

Background

Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) is a member of the eIF4E-binding protein family and a repressor of translation.¹ Non-phosphorylated 4E-BP1 inhibits the initiation of mRNA translation by binding to eIF4E and preventing it from being incorporated into the 48S pre-initiation complex required for the recruitment of 40S ribosomal subunits to the 5' end of mRNA.^{1,2} It is inactivated by phosphorylation at positions Thr³⁷ and Thr⁴⁶ (phospho-Thr^{37/46}) by mammalian target of rapamycin complex 1 (mTORC1), or in an mTOR-independent manner by glycogen synthase kinase 3 β (GSK3 β) or leucine-rich repeat kinase 2 (LRRK2), and detaches from eIF4E, allowing translation to proceed.² 4E-BP1 has a tumor suppressing role in cancer cells by reducing the overall rate of translation and inhibiting the translation of certain transcripts, such as cell cycle progression and angiogenic factors.¹ However, its role can become pro-oncogenic under conditions of cellular stress in the tumor microenvironment by inducing a switch to cap-independent translation and increasing the translation of factors that are proangiogenic under hypoxic conditions. Cytoplasmic levels of phosphorylated (inactive) 4E-BP1 are negatively correlated with recurrence-free survival in patients with progesterone receptor-expressing breast cancer, while nuclear levels of non-phosphorylated (active) 4E-BP1 are positively correlated with recurrence-free survival.³ High levels of 4E-BP1 (phospho-Thr^{37/46}) are associated with poor prognosis in patients with early stage esophageal squamous cell carcinoma.⁴ 4E-BP1 (phospho-Thr^{37/46}) levels have also been used as a marker of protein synthesis.^{5,6}

About This Assay

This 4E-BP1 (Total) and 4E-BP1 (Phospho-Thr^{37/46}) TR-FRET Assay Kit uses a homogeneous TR-FRET assay method amenable to rapid measurement of total and phosphorylated protein levels in cells. This 4E-BP1 (Total) and 4E-BP1 (Phospho-Thr^{37/46}) TR-FRET Assay Kit is suitable for screening a large number of samples. The signal is stable at room temperature for at least 24 hours, affording flexibility in read times. The amount of reagents provided is sufficient for testing 96 total and 384 phosphorylated protein wells.

Principle Of This Assay

This assay is based on the traditional sandwich immunoassay principle (Figure 1, below). Following cell treatment, cells are 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 reactive to human samples.

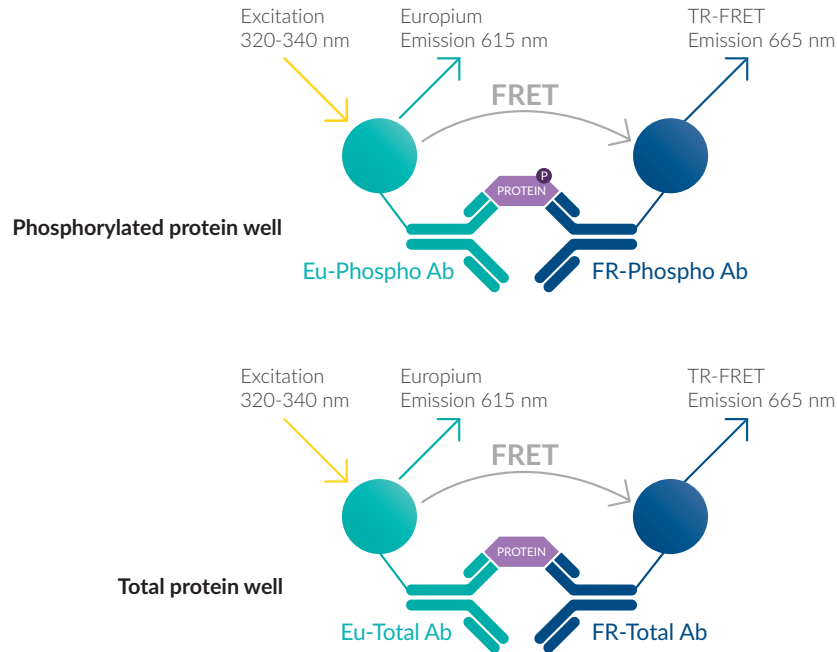


Figure 1. Schematic of the TR-FRET cell signaling assay principle

The first antibody in the pair used to detect the phosphorylated protein is labeled with a long-lifetime donor fluorophore (a europium chelate; Eu-Phospho Ab) and the second with a far-red acceptor fluorophore (FR-Phospho Ab). The same approach is used for the second antibody pair detecting the total protein (Eu-Total Ab and FR-Total Ab). The binding of the two matched and 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 Eu chelate molecules with a flash lamp (320 or 340 nm) or 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.

TR-FRET assays exhibit very low background fluorescence levels and high signal-to-background (S/B) ratios. The 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.

Assay Optimization

A critical step in performing any cell-based assay is the optimization of cell culture and treatment conditions. The following protocol 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 (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 suspended cells are generally acceptable for most cell lines. Of note, the optimal length of time for 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 Bioauxilium's website (www.Bioauxilium.com).

Reagent Preparation

The instructions described below are for testing the entire number of wells in each kit. Adjust volumes accordingly when testing fewer wells.

Bring all reagents to room temperature prior to use.

Centrifuge all tubes before use to improve recovery of content (2,000 x g, 10-15 seconds).

Mix the lysis and detection buffers and the positive control lysate by vortexing gently before use. Do NOT vortex the antibodies.

Use ultrapure water (18 MΩ-cm) to dilute the lysis and detection buffers.

NOTE: It is recommended to test all samples and controls at least in duplicate.

NOTE: ALWAYS include a positive control using the positive control lysate provided.

1. Supplemented Lysis Buffer

Supplemented Lysis Buffer 2 (1X) for the 2-Plate (Transfer) Assay Protocol with Adherent Cells: The supplemented Lysis Buffer 2 (1X) is designed for use in the 2-plate (transfer) assay protocol using adherent cells (see page 19). Each well requires 50 µl of supplemented Lysis Buffer 2 (1X). Dilute the Lysis Buffer 2 (5X) (Item No. 400211) with ultrapure water and add the Phosphatase Inhibitor Cocktail (100X) (Item No. 400257), which contains sodium fluoride (NaF), sodium orthovanadate (Na₃VO₄), and glycerophosphate at 100, 200, and 200 mM, respectively, to final NaF, Na₃VO₄, and glycerophosphate concentrations of 1, 2, and 2 mM, respectively. *NOTE: It is mandatory to supplement Lysis Buffer 2 with the Phosphatase Inhibitor Cocktail (100X).* Store unused Lysis Buffer 2 (1X) at 4°C; it will be stable for approximately two days.

OR

Supplemented Lysis Buffer 2 (5X) for the 2-Plate (Transfer) Assay Protocol with Suspension Cells or the 1-Plate Assay Protocol: The supplemented Lysis Buffer 2 (5X) is designed for use in the 2-plate (transfer) assay protocol using suspension cells (see page 22) or the 1-plate (all in one well) assay protocol for adherent or suspension cells (see page 27). Each well requires 10 μ l (transfer) or 3 μ l (all in one well) of supplemented Lysis Buffer 2 (5X). Directly add the Phosphatase Inhibitor Cocktail (100X) (Item No. 400257), which contains sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), and glycerophosphate at 100, 200, and 200 mM, respectively, to final NaF, Na_3VO_4 , and glycerophosphate concentrations of 5, 10, and 10 mM, respectively. *NOTE: It is mandatory to supplement Lysis Buffer 2 with the Phosphatase Inhibitor Cocktail (100X).* Store unused Lysis Buffer 2 (5X) at 4°C; it will be stable for approximately two days.

2. Detection Buffer (1X)

Dilute the 250 μ l of Detection Buffer (10X) (Item No. 400225) with 2.25 ml of ultrapure water. Store unused Detection Buffer (1X) at 4°C; it will be stable for approximately two days.

3. Antibody Detection Mix (4X)

Prepare and mix just before use. *NOTE: Due to the low reagent volumes, the antibodies are diluted with Detection Buffer (1X) directly in the vial for the Total-protein Antibody Mix (4X).*

NOTE: Each well requires 5 μ l of either Phospho-protein Antibody Mix (4X) or Total-protein Antibody Mix (4X).

Phospho-protein Antibody Mix (Eu-Phospho Ab + FR-Phospho Ab) (4X): Gently mix 20 μ l of Europium-Labeled 4E-BP1 (Phospho-Thr^{37/46}) Antibody (Eu-Phospho Ab; Item No. 400271) with 1,020 μ l of Detection Buffer (1X). Gently mix 80 μ l of Acceptor-Labeled 4E-BP1 (Phospho-Thr^{37/46}) Antibody (FR-Phospho Ab; Item No. 400272) with 960 μ l of Detection Buffer (1X). Gently mix the diluted Eu-Phospho Ab and FR-Phospho Ab solutions together.

OR

Total-protein Antibody Mix (Eu-Total Ab + FR-Total Ab) (4X): Add 255 μ l of Detection Buffer (1X) into the vial containing 5 μ l of Europium-Labeled 4E-BP1 (Total) Antibody (Eu-Total Ab; Item No. 400298). Add 240 μ l of Detection Buffer (1X) into the vial containing 20 μ l of Acceptor-Labeled 4E-BP1 (Total) Antibody (FR-Total Ab; Item No. 400299). Gently mix the diluted Eu-Total Ab and FR-Total Ab solutions together.

Store unused antibody detection mix working solutions at 4°C; they will be stable for approximately two days.

4. Positive Control Lysate

The 4E-BP1 Positive Control Lysate (Item No. 400297) is supplied ready to use. The thawed positive control lysate can be aliquoted, refrozen at -80°C, and thawed at least three more times.

TR-FRET Plate Reader Settings

We recommend reading the TR-FRET assays at two wavelengths, detecting both the emission from the Eu chelate donor fluorophore at 615 nm and the acceptor fluorophore at 665 nm. Table 1, below, provides instrument settings to be used as guidelines.

Parameter	TR-FRET-compatible Plate Reader	
	Flash lamp excitation	Laser excitation
Excitation filter	320 nm (or 340 nm)	N/A
Emission filter	615 nm (or 620 nm)	615 nm (or 620 nm)
Delay time	90 μ s	50 μ s
Flash energy level	100% or High	100%
Number of flashes	100	20
Window (integration time)	300 μ s	100 μ s

Table 1. Recommended TR-FRET plate reader settings

ASSAY PROTOCOL

Workflow

The THUNDER™ TR-FRET Cell Signaling Assay workflow consists of 3 simple steps.

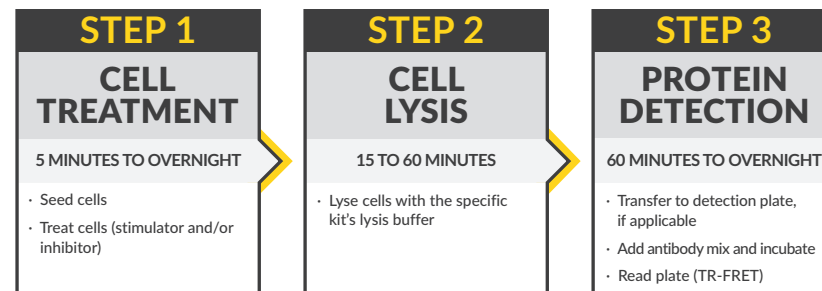
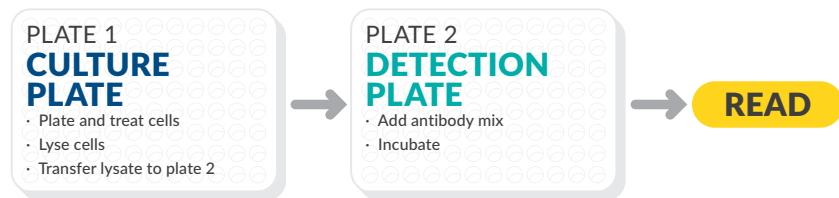


Figure 2. Assay workflow

Assay Summary

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

2-Plate (transfer) Protocol



1-Plate (all in one well) Protocol

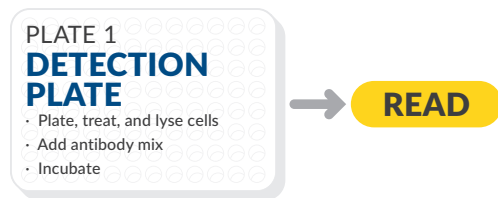


Figure 3. Assay summary Each assay uses the same total volume of 20 μ l for TR-FRET detection. The 2-plate protocol is conducted in either a half-area 96-well or low-volume 384-well plate(s), while the 1-plate protocol is conducted in a low-volume 384-well plate(s).

Performing the Assay: 2-plate (Transfer) Protocol

Adherent Cells

This is a transfer protocol that is conducted in two 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 volume of 20 μ l per well for TR-FRET detection. A summary of this protocol is provided in Table 2 (see page 24).

Cell Treatment

1. Dispense 50 μ l of cells at the pre-optimized density into a 96-well tissue culture-treated plate(s) in appropriate culture medium.
2. Incubate overnight at 37°C and 5% CO₂.
3. Cell Stimulation or Inhibition

Stimulation: Add 50 μ l of stimulator (2X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperature needs to be determined.*

No treatment: Add 50 μ l of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells.

OR

Inhibition: Add 25 μ l of inhibitor (4X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. Add 25 μ l of stimulator (4X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperatures need to be determined.*

No treatment: Add 25 μ l of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells. Add another 25 μ l of serum-free medium and incubate for the same amount of time, and at the same temperature, as treated cells.

Cell Lysis

1. Carefully remove the cell culture medium by aspirating the supernatant.
2. Immediately add 50 μ l of supplemented Lysis Buffer 2 (1X). The volume of supplemented Lysis Buffer 2 (1X) may be optimized from 25-100 μ l.
3. Incubate for 30 minutes at room temperature on an orbital plate shaker at 400 rpm. The incubation time in supplemented Lysis Buffer 2 (1X) may be optimized from 15-60 minutes.

NOTE: If samples cannot be measured immediately, store at -80°C.

TR-FRET Detection

1. Carefully pipette 15 μ l 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 plate.
2. It is recommended to add 15 μ l of EGFR Positive Control Lysate (undiluted) and 15 μ l of Lysis Buffer 2 (1X) (negative control) to separate assay wells.
3. Add 5 μ l of either Phospho-protein Antibody Mix (4X) or Total-protein Antibody Mix (4X) to each of the appropriate assay wells.
4. Cover the plate(s) with a plate sealer and incubate for 4 hours at room temperature.
5. Gently remove the adhesive plate sealer. Read on a TR-FRET-compatible plate reader. *NOTE: The same plate can be read several times without a negative effect on the assay performance, and the signal is stable for at least 24 hours at room temperature.*

NOTE: A summary of this pipetting protocol is provided in Table 4 (see page 26).

Suspension Cells

This is a transfer protocol that is conducted in two 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 volume of 20 μ l per well for TR-FRET detection. A summary of this protocol is provided in Table 3 (see page 25).

Cell Treatment

1. Dispense 20 μ l of cells at the pre-optimized density into a 96-well tissue culture-treated plate(s) in appropriate culture medium.
2. Directly proceed to cell stimulation or inhibition or incubate 2-4 hours at 37°C and 5% CO₂ prior to stimulation or inhibition. This step may be optimized.
3. Cell Stimulation or Inhibition

Stimulation: Add 20 μ l of stimulator (2X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperature needs to be determined.*

No treatment: Add 20 μ l of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells.

OR

Inhibition: Add 10 μ l of inhibitor (4X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. Add 10 μ l of stimulator (4X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperatures need to be determined.*

No treatment: Add 10 μ l of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells. Add another 10 μ l of serum-free medium and incubate for the same amount of time, and at the same temperature, as treated cells.

Cell Lysis

1. Add 10 μ l supplemented Lysis Buffer 2 (5X).
2. Incubate for 30 minutes at room temperature on an orbital plate shaker at 400 rpm. The incubation time in supplemented Lysis Buffer 2 (5X) may be optimized from 15-60 minutes.

NOTE: If samples cannot be measured immediately, store at -80°C.

TR-FRET Detection

Following cell lysis, proceed to the TR-FRET detection step as described for the standard 2-plate (transfer) protocol for adherent cells (see page 21).

2-Plate (Transfer) Assay Summary				
Step	Adherent Cells			
Cell Treatment	Stimulation	No Treatment	Inhibition	No Treatment
	50 µl cells	50 µl cells	50 µl cells	50 µl cells
	Incubate cells overnight			
	50 µl stimulator (2X)	50 µl serum-free medium	25 µl inhibitor (4X)	25 µl serum-free medium
	Incubate for pre-optimized time			
			25 µl stimulator (4X)	25 µl serum-free medium
			Incubate for pre-optimized time	
Cell Lysis	Remove media			
	50 µl supplemented Lysis Buffer 2 (1X)*			
	Incubate 30 minutes on an orbital shaker			
Protein Detection	15 µl lysate			
	5 µl Phospho-protein Antibody Mix (4X) OR Total-protein Antibody Mix (4X)			
	Cover and incubate 4 hours			
	Read TR-FRET signal			

Table 2. Assay summary for the 2-plate (transfer) protocol with adherent cells
 *The lysis buffer must be supplemented with the Phosphatase Inhibitor Cocktail (100X) as described in the Reagent Preparation section.

2-Plate (Transfer) Assay Summary				
Step	Suspension Cells			
Cell Treatment	Stimulation	No Treatment	Inhibition	No Treatment
	20 µl cells	20 µl cells	20 µl cells	20 µl cells
	20 µl stimulator (2X) OR incubate for 2-4 hours, then add 20 µl stimulator (2X)	20 µl serum-free medium OR incubate for 2-4 hours, then add 20 µl serum-free medium	10 µl inhibitor (4X) OR incubate for 2-4 hours, then add 10 µl inhibitor (4X)	10 µl serum-free medium OR incubate for 2-4 hours, then add 10 µl serum-free medium
	Incubate for pre-optimized time			
			10 µl inhibitor (4X)	10 µl serum-free medium
			Incubate for pre-optimized time	
			10 µl supplemented Lysis Buffer 2 (5X)*	
Cell Lysis	Incubate 30 minutes on an orbital shaker			
	15 µl lysate			
Protein Detection	5 µl Phospho-protein Antibody Mix (4X) OR Total-protein Antibody Mix (4X)			
	Cover and incubate 4 hours			
	Read TR-FRET signal			

Table 3. Assay summary for the 2-plate (transfer) protocol with suspension cells
 *The lysis buffer must be supplemented with the Phosphatase Inhibitor Cocktail (100X) as described in the Reagent Preparation section.

	Untreated Cells	Treated Cells	Positive Control	Negative Control
Cell lysate (untreated cells)	15 μ l			
Cell lysate (treated cells)		15 μ l		
Positive Control Lysate			15 μ l	
Lysis Buffer 2 (1X)				15 μ l
Phospho-protein Antibody Mix (4X) OR Total-protein Antibody Mix (4X)	5 μ l	5 μ l	5 μ l	5 μ l
Total assay volume	20 μl	20 μl	20 μl	20 μl

Table 4. Summary of pipetting protocol 2-Plate (transfer) assay protocol after lysis and prior to TR-FRET

Performing the Assay: 1-plate (All in One Well) Protocol

Adherent and Suspension Cells

This is an all-in-one-well protocol. No transfer step is needed. Conduct the assay in a white, low-volume 384-well assay plate(s) with a total assay volume of 20 μ l per well. A summary of this protocol is provided in Tables 5-7 (see pages 29-31).

Cell Treatment

1. Dispense 8 μ l of cells at the pre-optimized density in serum-free medium into a white, low-volume 384-well assay plate(s).
2. Cell Stimulation or Inhibition

Stimulation: Add 4 μ l of stimulator (3X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperature needs to be determined.*

No treatment: Add 4 μ l of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells.

OR

Inhibition: Add 2 μ l of inhibitor (6X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. Add 2 μ l of stimulator (6X) diluted in serum-free medium. Incubate for the pre-optimized time at either room temperature or 37°C. *NOTE: Optimal incubation temperatures need to be determined.*

No treatment: Add 2 μ l of serum-free medium. Incubate for the same amount of time, and at the same temperature, as treated cells. Add another 2 μ l of serum-free medium and incubate for the same amount of time, and at the same temperature, as treated cells.

Cell Lysis

1. Add 3 μ l of supplemented Lysis Buffer 2 (5X).
2. Incubate for 30 minutes at room temperature on an orbital plate shaker at 400 rpm. The incubation time in supplemented Lysis Buffer 2 (5X) may be optimized from 15-60 minutes.

NOTE: If samples cannot be measured immediately, store at -80°C.

TR-FRET Detection

1. Add 15 μ l of Positive Control Lysate (undiluted) and 15 μ l of Lysis Buffer 2 (1X) (negative control) to separate assay wells.
2. Add 5 μ l of either Phospho-protein Antibody Mix (4X) or Total-protein Antibody Mix (4X) prepared in Detection Buffer (1X) to each of the assay wells.
3. Cover the plate(s) with a plate sealer and incubate for 4 hours at room temperature.
4. Gently remove the adhesive plate sealer. Read on a TR-FRET-compatible plate reader. *NOTE: The same plate can be read several times without a negative effect on the assay performance, and the signal is stable for at least 24 hours at room temperature.*

1-Plate Assay Summary				
Step	Adherent or suspension cells			
White, low-volume 384-well plate(s)				
Cell Treatment	Stimulation	No treatment	Inhibition	No treatment
	8 μ l cells	8 μ l cells	8 μ l cells	8 μ l cells
	4 μ l stimulator (3X)	4 μ l serum-free medium	2 μ l inhibitor (6X)	2 μ l serum-free medium
	Incubate for pre-optimized time			
			2 μ l stimulator (6X)	2 μ l serum-free medium
			Incubate for pre-optimized time	
Cell Lysis	3 μ l supplemented Lysis Buffer 2 (5X)*			
	Incubate 30 minutes on an orbital shaker			
Protein Detection	15 μ l of 4E-BP1 Positive Control Lysate and 15 μ l of Lysis Buffer 2 (1X) to separate wells			
	5 μ l Phospho-protein Antibody Mix (4X) OR Total-protein Antibody Mix (4X)			
	Cover and incubate 4 hours			
	Read TR-FRET signal			

Table 5. Assay summary for the 1-plate (all in one well) protocol with adherent or suspension cells

*The lysis buffer must be supplemented with the Phosphatase Inhibitor Cocktail (100X) as described in the Reagent Preparation section.

	Untreated Cells	Treated Cells	Positive Control	Negative Control
Suspension cells	8 µl	8 µl		
Stimulator (3X)		4 µl		
Serum-free Medium	4 µl			
Positive Control Lysate			15 µl	
Lysis Buffer 2 (5X)	3 µl	3 µl		
Lysis Buffer 2 (1X)				15 µl
Phospho-protein Antibody Mix (4X) OR Total-protein Antibody Mix (4X)	5 µl	5 µl	5 µl	5 µl
Total assay volume	20 µl	20 µl	20 µl	20 µl

Table 6. Summary of pipetting protocol 1-Plate (all in one well) protocol using a stimulator

	Untreated Cells	Treated Cells	Positive Control	Negative Control
Suspension cells	8 µl	8 µl		
Inhibitor (6X)		2 µl		
Stimulator (6X)		2 µl		
Serum-free Medium	4 µl			
Positive Control Lysate			15 µl	
Lysis Buffer 2 (5X)	3 µl	3 µl		
Lysis Buffer 2 (1X)				15 µl
Phospho-protein Antibody Mix (4X) OR Total-protein Antibody Mix (4X)	5 µl	5 µl	5 µl	5 µl
Total assay volume	20 µl	20 µl	20 µl	20 µl

Table 7. Summary of pipetting protocol 1-Plate (all in one well) protocol using an inhibitor

Calculations

1. TR-FRET data are typically calculated and presented ratiometrically using the following formula:

$$[(665 \text{ nm}/615 \text{ nm}) \times 1,000]$$

2. Calculate the TR-FRET ratio for each well.
3. TR-FRET assays are homogeneous; do not subtract average negative control data (no lysate) from any other well readings.
4. For concentration-response curves, analyze data according to a nonlinear regression using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting.
5. Assay quality control: The undiluted 4E-BP1 Positive Control Lysate must generate an S/B ratio of at least 2 when compared to the negative control (Lysis Buffer 2 (1X) only). If this is not the case, your reader is not compatible with THUNDER™ TR-FRET Cell Signaling Assay Kits.

NOTE: The positive control lysate is provided as a control reagent, not for conducting a standard curve.

Performance Characteristics

Representative Data

Data shown here are examples of data typically generated with the 4E-BP1 (Total) and 4E-BP1 (Phospho-Thr^{37/46}) TR-FRET Assay Kit. 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.

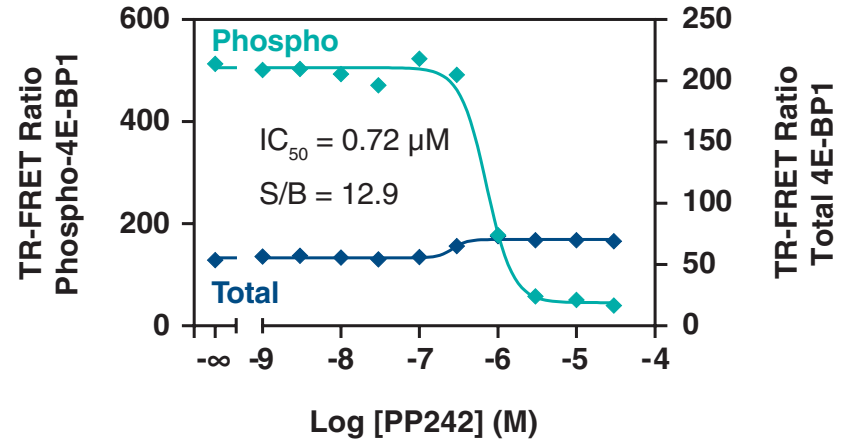


Figure 4. Inhibition of 4E-BP1 phosphorylation at Thr^{37/46} in A431 cells. A431 cells were seeded at 25,000 cells/well in triplicate and incubated with serial dilutions of the mTOR inhibitor PP242 for 3 hours at 37°C.

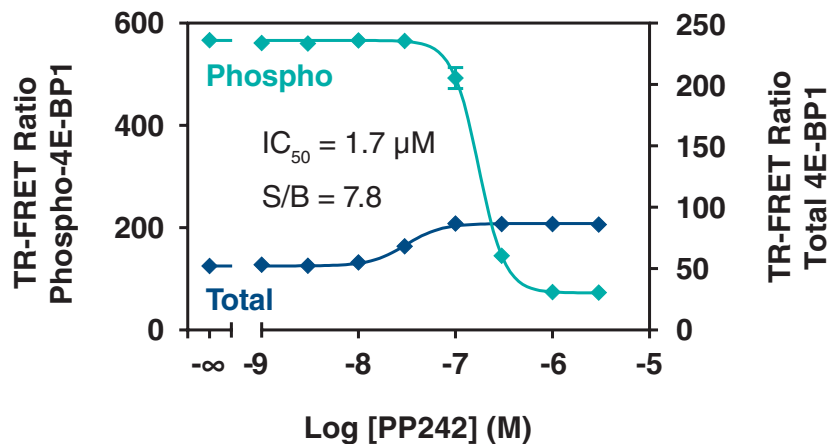


Figure 5. Inhibition of 4E-BP1 phosphorylation at Thr^{37/46} in HEK293 cells HEK293 cells were seeded at 60,000 cells/well in triplicate and incubated with serial dilutions of PP242 for 3 hours at 37°C.

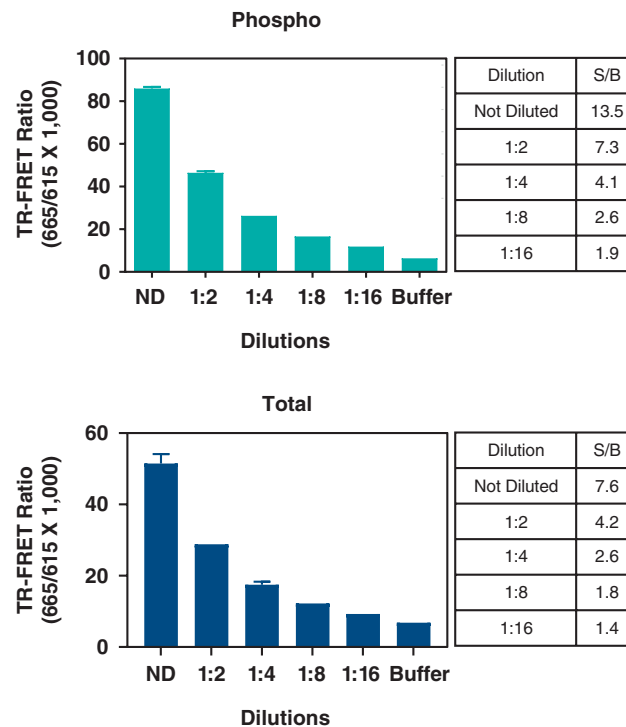


Figure 6. A431 cell lysate titration (QC Test) for phospho-Thr^{37/46} 4E-BP1 and total 4E-BP1 The 4E-BP1 (Total) and 4E-BP1 (Phospho-Thr^{37/46}) TR-FRET Assay Kit is routinely quality control tested using A431 cell lysates. A431 cells were cultured in a T175 flask to 70% confluence. Following cell lysis using 4 ml of Lysis Buffer 2 (1X) lysates were serially diluted with Lysis Buffer 2 (1X) and tested in triplicate in separate wells for total 4E-BP1 and phospho-Thr^{37/46}-4E-BP1.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Assay S/B ratio is <2 for the positive control lysate <i>versus</i> the negative control (<i>i.e.</i> Lysis Buffer (1X) alone)	<p>Plate reader and/or settings not suitable for TR-FRET assays</p> <p>Use of low-quality water for reagent preparation</p> <p>Use of black plates</p> <p>Plate read with the adhesive plate sealer</p>	<p>Use a filter-based instrument to read the plate(s).</p> <p>Ensure the correct excitation and emission filters and mirror module have been used.</p> <p>Use recommended instrument settings. Optimize the delay time, measurement window, and number of flashes.</p> <p>Only use ultrapure water for preparation of the Lysis and Detection Buffers.</p> <p>Only use white plates.</p> <p>The plate sealer MUST be removed before reading the plate(s).</p>

Problem	Possible Causes	Recommended Solutions
Low S/B ratio in the cellular experiment	<p>Suboptimal cell culture and/or treatment conditions</p> <p>Use of a different lysis buffer than the one included in the kit</p> <p>Lack of phosphatase inhibitors in the lysis buffer</p> <p>Use of low-quality water for reagent preparation</p> <p>Use of black plates</p>	<p>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.</p> <p>Optimize cell culture conditions. Too high OR low cell numbers can affect basal and maximal activation.</p> <p>Ensure the cell passage number is not too high OR low and that cells are behaving as expected (<i>i.e.</i> doubling time, viability).</p> <p>The lysis buffer MUST be supplemented with the Phosphatase Inhibitor Cocktail (100X) (final concentrations depend on the use of 1X or 5X lysis buffer). Additional phosphatase inhibitors and/or protease inhibitors are typically NOT required.</p> <p>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.</p> <p>Only use ultrapure water.</p> <p>Only use white, opaque plates.</p>

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NOTES

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