TECHNICAL DATA SHEET

THUNDER™ Phospho-4EBP1 (T37/T46) TR-FRET Cell Signaling Assay Kit

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CATALOG NUMBERS KIT-4EBP1P-100 (100 tests)
KIT-4EBP1P-500 (500 tests)

Store at -80°C
For research use only.
Not for use in diagnostic procedures.

PRODUCT DESCRIPTION

This assay kit measures intracellular levels of **phospho-4EBP1 (T37/T46)** protein in cell lysates using a simple, rapid and sensitive immunoassay based on the homogeneous (no-wash) THUNDER™ TR-FRET technology. The kit is compatible with both adherent and suspension cells.

SPECIFICITY

This assay kit contains two specific and selective antibodies, one that recognizes **4EBP1** phosphorylated at **Thr37** and **Thr46** and another that recognizes an invariant epitope of **4EBP1**.

SPECIES REACTIVITY

Human (Swiss-Prot Acc.: Q13541; Entrez-Gene Id: 1978).

Other species should be tested on a case-by-case basis.

TR-FRET ASSAY PRINCIPLE

The **Phospho-4EBP1 (T37/T46)** assay kit is a homogeneous time-resolved Förster resonance energy transfer (TR-FRET) sandwich immunoassay (Figure 1). The THUNDER™ Cell Signaling assay workflow consists of 3 steps (Figure 2). Following cell treatment, cells are first lysed with the specific Lysis Buffer provided in the kit. Then **Phospho-4EBP1 (T37/T46)** in the cell lysates is detected with a pair of fluorophore-labeled antibodies in a simple "add-incubate-measure" format (single-step reagent addition; no wash steps). One antibody is labeled with a donor fluorophore (Europium chelate; 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. Residual energy from the Eu chelate generates light at 615 nm. The signal at 665 nm is proportional to the concentration of **Phospho-4EBP1 (T37/T46)** in the cell lysate. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio.

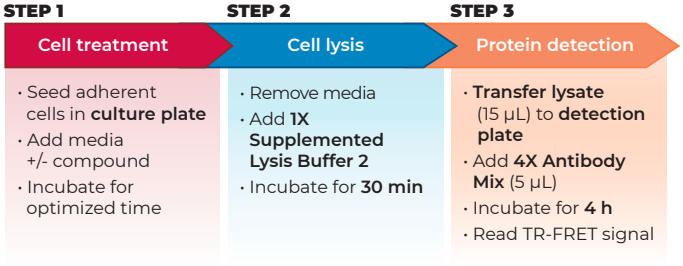


Figure 2 Assay workflow using the 2-plate (transfer) protocol.

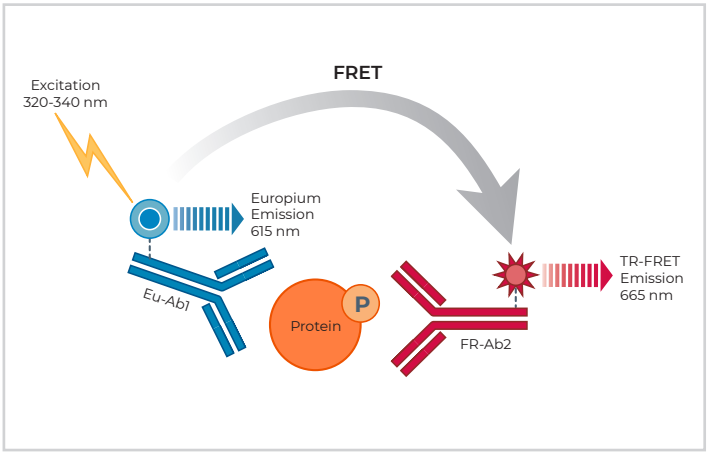


Figure 1 Schematic representation of the TR-FRET cell signaling assay principle.

KIT COMPONENTS

	100 points*	500 points*
Eu-labeled phospho-4EBP1 (T37/T46) antibody (Eu-Ab1)	5 µL	25 µL
Acceptor-labeled phospho-4EBP1 (T37/T46) antibody (FR-Ab2)	20 µL	100 µL
Lysis Buffer 2 (5X)	1 mL	5 mL
Detection Buffer (10X)	50 µL	250 µL
Positive control cell lysate	100 µL	500 µL

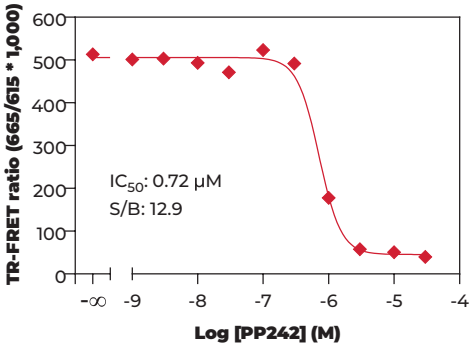
* The number of assay points is based on an assay volume of 20 µL in half-area 96-well or low-volume 384-well assay plates using the kit components at the recommended concentrations (refer to the User Manual).

VALIDATION DATA

This assay kit has been validated for the relative quantification of phospho-4EBP1 (T37/T46) in A431 and HEK293 cell lysates using the 2-plate assay protocol.

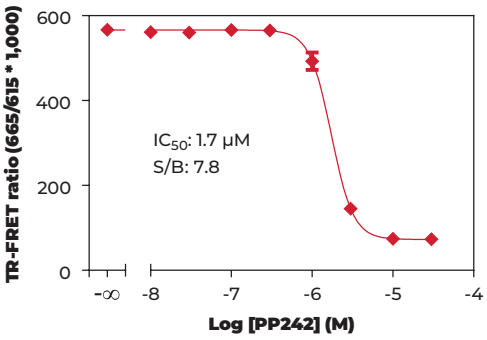
- Adherent cells were cultured overnight in a 96-well tissue culture plate (EMEM +10% FBS for HEK293; DMEM +10% FBS for A431). The plates used for HEK293 were coated with poly-L-lysine.
- Following cell treatment, the media was removed and cells were lysed with the **1X Lysis Buffer 2** (50 µL) supplemented with the phosphatase inhibitors sodium fluoride (1 mM) and sodium orthovanadate (2 mM).
- Following a **30-min** incubation at room temperature (RT) on an orbital shaker (400 rpm), lysates (15 µL) were then transferred to a 384-well assay plate followed by addition of the labeled antibodies Eu-Ab1 and FR-Ab2 (5 µL) for detection of phospho-4EBP1 (T37/T46).
- The plate was incubated at RT for **4 hours** and the TR-FRET signal was recorded at 665 and 615 nm (EnVision®; lamp excitation).

INHIBITION OF PHOSPHO-4EBP1 (T37/T46) IN A431 CELLS



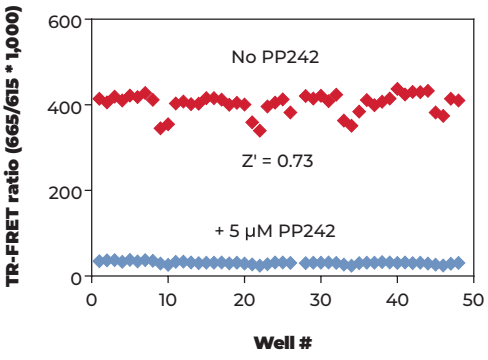
A431 cells (25,000 cells/well; in triplicate) were incubated with serial dilutions of PP242 for 3 hours at 37°C. Data show that treatment of A431 cells with PP242 inhibits phosphorylation of 4EBP1 at T37/T46.

INHIBITION OF PHOSPHO-4EBP1 (T37/T46) IN HEK293 CELLS



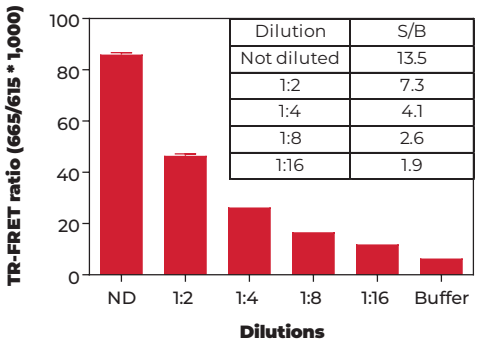
HEK293 cells (60,000 cells/well; in triplicate) were incubated with serial dilutions of PP242 for 3 hours at 37°C. Data show that treatment of HEK293 cells with PP242 inhibits phosphorylation of 4EBP1 at T37/T46.

Z'-FACTOR DETERMINATION IN A431 CELLS



A431 cells (25,000 cells/well) were incubated without or with 5 µM of PP242 for 3 hours at 37 °C. The Z' factor value was determined using a total of 48 wells for each treatment group. The Z'-factor value of 0.73 indicates that the assay is robust and suitable for HTS.

A431 CONTROL LYSATE TITRATION (QC TEST)



Dilution	S/B
Not diluted	13.5
1:2	7.3
1:4	4.1
1:8	2.6
1:16	1.9

Quality Control: the Phospho-4EBP1 (T37/T46) assay kit is routinely tested against A431 lysates. A431 cells were cultured in a T175 flask to 70% confluence. Following cell lysis using 4 mL of 1X Lysis Buffer 2, lysates were serially diluted with 1X Lysis Buffer 2 and tested in triplicate. Data show a linear relationship between lysate dilutions and TR-FRET ratio values.

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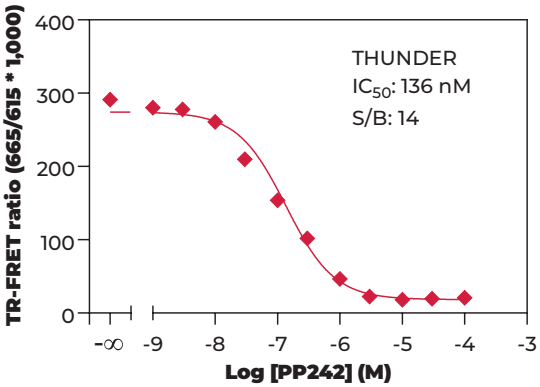
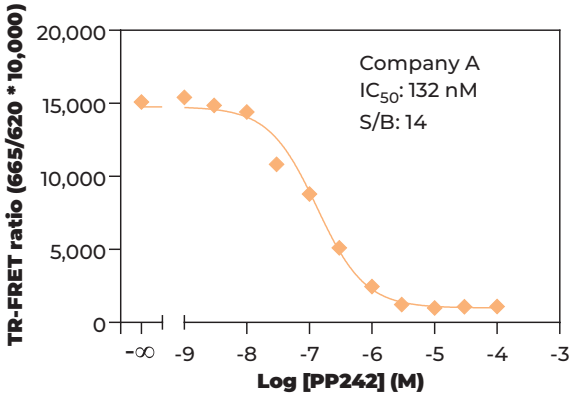
KIT BENCHMARKING – COMPARISON TO OTHER TR-FRET TECHNOLOGIES

As part of assay validation, the THUNDER™ Phospho-4EBP1 (T37/T46) Assay Kit was benchmarked against a competitive TR-FRET assay technology (Company A). Specifically, the 2 different assays were compared side-by-side for their capacity to detect basal phospho-4EBP1 (T37/T46) inhibition upon A431 cell treatment with PP242 using the 2 plate assay protocol. All reagents were prepared according to each manufacturer's recommendations. Following cell treatment, cells were lysed with the corresponding kit's 1X supplemented Lysis Buffer. Lysates were then tested on the same 384-well assay plate and according to the corresponding kit's standard protocol. The plate was read on an EnVision® (lamp excitation) following 4 hours of incubation. Data show that both TR-FRET assays exhibited comparable sensitivity (IC_{50} values) and S/B ratio.

THUNDER™ PHOSPHO-4EBP1 (T37/T46) ASSAY KIT



COMPANY A PHOSPHO-4EBP1 (T37/T46) ASSAY KIT



FOR MORE INFORMATION ON DEVELOPING AND OPTIMIZING TR-FRET CELL SIGNALING ASSAYS, CONSULT THE USER MANUAL.