**TECHNICAL DATA SHEET**

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

**CATALOG NUMBERS**

KIT-4EBPIPT-500

- 400 points for phospho-4EBP1 and 100 points for total 4EBP1

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) and total 4EBP1 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 antibody pairs, one that recognizes 4EBP1 phosphorylated at Thr37 and Thr46 and another that recognizes total (both phosphorylated and unphosphorylated) 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) + Total 4EBP1 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) and Total 4EBP1 in the cell lysates are detected in separate wells with two pairs of fluorophore-labeled antibodies in a simple 'add-incubate-measure' format (single-step reagent addition; no wash steps). For detection of the phosphorylated protein, one antibody is labeled with a donor fluorophore (Europium chelate; Eu-Ab1) and the second with a far-red acceptor fluorophore (FR-Ab2). The same approach is used for the second antibody pair detecting the total protein (Eu-Ab3 and FR-Ab4). The binding of the two matched labeled antibodies to distinct epitopes on the target protein (either phospho-4EBP1 or total 4EBP1) 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) and Total 4EBP1 in the cell lysate. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio.

**STEP 1**  
Seed adherent cells in culture plate  
Add media  
+/- compound  
Incubate for optimized time

**STEP 2**  
Remove media  
Add 1X Supplemented Lysis Buffer 2  
Incubate for 30 min

**STEP 3**  
Transfer lysate [15 μL] to detection plate  
Add 4X Antibody Mix [5 μL]  
Incubate for 4 h  
Read TR-FRET signal

**KIT COMPONENTS**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eu-labeled phospho-4EBP1 (T37/T46) antibody (Eu-Ab1)</td>
<td>20 μL</td>
</tr>
<tr>
<td>Acceptor-labeled phospho-4EBP1 (T37/T46) antibody (FR-Ab2)</td>
<td>80 μL</td>
</tr>
<tr>
<td>Eu-labeled total-4EBP1 antibody (Eu-Ab3)</td>
<td>5 μL</td>
</tr>
<tr>
<td>Acceptor-labeled total-4EBP1 antibody (FR-Ab4)</td>
<td>20 μL</td>
</tr>
<tr>
<td>Lysis Buffer 2 (5X)</td>
<td>5 mL</td>
</tr>
<tr>
<td>Detection Buffer (10X)</td>
<td>250 μL</td>
</tr>
<tr>
<td>Positive control cell lysate</td>
<td>500 μL</td>
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</tbody>
</table>

*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) and total 4EBP1 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 to separate wells of either the labeled antibodies Eu-Ab1 and FR-Ab2 (5 µL) for detection of phospho-4EBP1 (T37/T46) or Eu-Ab3 and FR-Ab4 (5 µL) for detection of total 4EBP1.
• 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
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, but does not have a major effect on the levels of total 4EBP1.

INHIBITION OF PHOSPHO-4EBP1
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, but does not have a major effect on the levels of total 4EBP1.

A431 CONTROL LYSATE TITRATION (QC TEST)
PHOSPHO-4EBP1 (T37/T46)

Quality Control: the Phospho-4EBP1 (T37/T46) + Total 4EBP1 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 and in separate wells for phospho-4EBP1 (T37/T46) and total 4EBP1. Data show a linear relationship between lysate dilutions and TR-FRET ratio values.

A431 CONTROL LYSATE TITRATION (QC TEST)
TOTAL 4EBP1

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