TECHNICAL DATA SHEET

THUNDER™ Phospho-CREB (S133) + Total CREB
TR-FRET Cell Signaling Assay Kit

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CATALOG NUMBERS KIT-CREBPT-500
400 points for phospho-CREB and 100 points for total CREB

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

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PRODUCT DESCRIPTION

This assay kit measures intracellular levels of **phospho-CREB (S133)** and **total CREB** 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 **CREB** phosphorylated at **Ser133** and another that recognizes **total** (both phosphorylated and unphosphorylated) **CREB**.

SPECIES REACTIVITY

Human, Mouse (Swiss-Prot Acc.: P16220; Entrez-Gene Id: 1385).

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

TR-FRET ASSAY PRINCIPLE

The **Phospho-CREB (S133) + Total CREB** 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-CREB (S133)** and **Total CREB** 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-CREB** or **total CREB**) 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-CREB (S133)** and **Total CREB** in the cell lysate. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio.

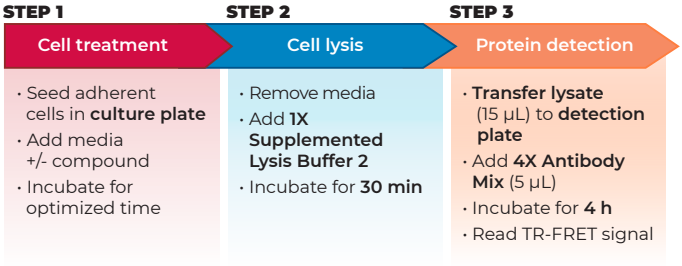

Figure 2 Assay workflow using the 2-plate (transfer) protocol.

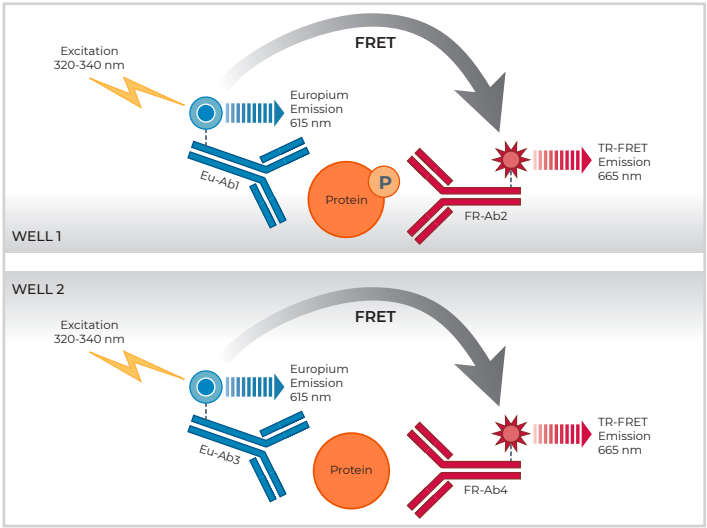

Fig. 1 Schematic representation of the TR-FRET cell signaling assay principle.

KIT COMPONENTS

	500 points*
Eu-labeled phospho-CREB (S133) antibody (Eu-Ab1)	20 µL
Acceptor-labeled phospho-CREB (S133) antibody (FR-Ab2)	80 µL
Eu-labeled total-CREB antibody (Eu-Ab3)	5 µL
Acceptor-labeled total-CREB antibody (FR-Ab4)	20 µL
Lysis Buffer 2 (5X)	5 mL
Detection Buffer (10X)	250 µL
Positive control cell lysate	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).

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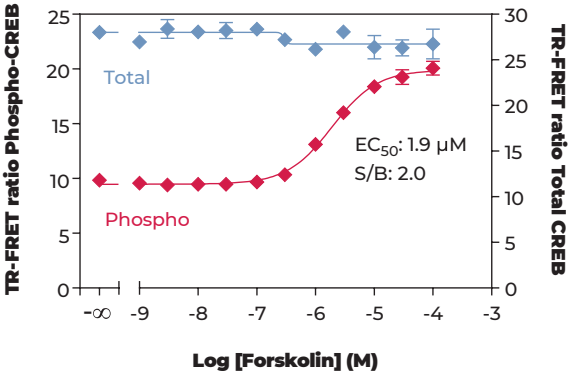
Phospho-CREB (S133) + Total CREB

VALIDATION DATA

This assay kit has been validated for the relative quantification of phospho-CREB (S133) and total CREB in A431, NIH3T3 and B lymphocyte cell lysates using the 2-plate assay protocol.

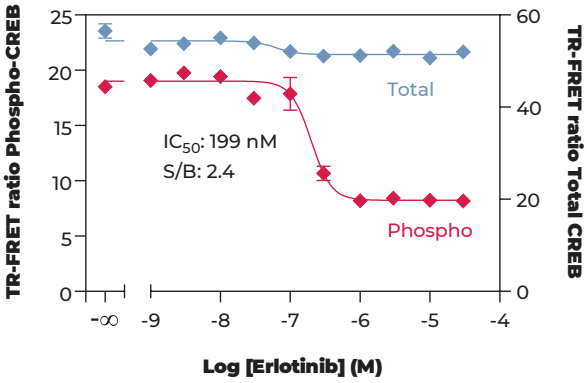
- Adherent cells were cultured overnight in a 96-well tissue culture plate before treatment, whereas B lymphocytes were centrifuged and resuspended at the desired density in RPMI without serum before treatment. Culture media used were: DMEM +10% FBS for A431; DMEM + 10% CBS for NIH3T3; RPMI+15% FBS for B lymphocytes.
- For adherent cells, 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).
- For B lymphocytes, cells were lysed by adding 5X **Lysis Buffer 2** (with phosphatase inhibitors), to a final concentration of 1X.
- 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-CREB (S133) or Eu-Ab3 and FR-Ab4 (5 µL) for detection of total CREB.
- The plate was incubated at RT for **4 hours** and the TR-FRET signal was recorded at 665 and 615 nm (EnVision®; lamp excitation).

STIMULATION OF PHOSPHO-CREB (S133) IN NIH3T3 CELLS



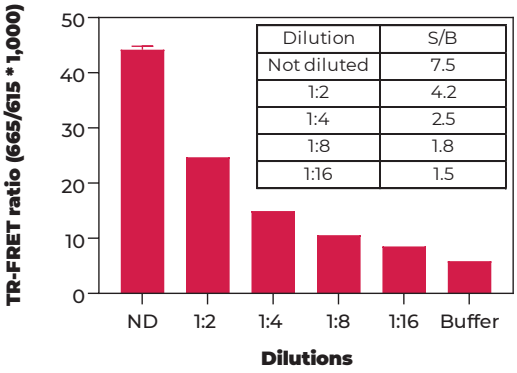
NIH3T3 cells (50,000 cells/well; in triplicate) were incubated with serial dilutions of Forskolin for 30 min at 37°C. Data show that treatment of NIH3T3 cells with Forskolin stimulates phosphorylation of CREB at S133 but does not affect the levels of total CREB.

INHIBITION OF PHOSPHO-CREB (S133) IN A431 CELLS



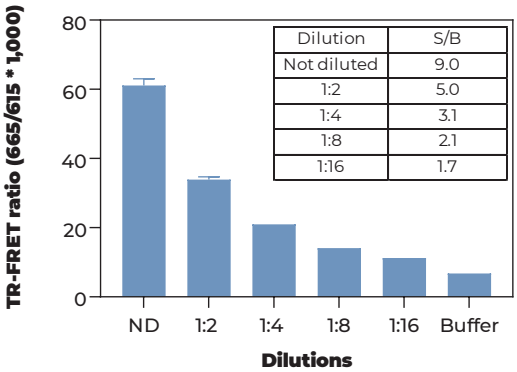
A431 cells (50,000 cells/well; in triplicate) were incubated with serial dilutions of the inhibitor Erlotinib for 30 min at 37°C. Cells were then stimulated with 1 nM of EGF for 10 min at 37°C. Data show that treatment of A431 cells with Erlotinib inhibits phosphorylation of CREB at S133 by EGF, but does not affect the levels of total CREB.

B LYMPHOCYTE CONTROL LYSATE TITRATION (QC TEST) PHOSPHO-CREB (S133)



Dilution	S/B
Not diluted	7.5
1:2	4.2
1:4	2.5
1:8	1.8
1:16	1.5

B LYMPHOCYTE CONTROL LYSATE TITRATION (QC TEST) TOTAL CREB



Dilution	S/B
Not diluted	9.0
1:2	5.0
1:4	3.1
1:8	2.1
1:16	1.7

Quality Control: the Phospho-CREB (S133) + Total CREB assay kit is routinely tested against NECA-treated B lymphocyte lysates. B lymphocyte cells (80 millions in 8 mL of media) were incubated in a T-flask with 300 nM of NECA (8 mL at 2X) for 30 min at RT. Following cell lysis using 4 mL of 5X Lysis Buffer 2 (1X final), lysates were serially diluted with 1X Lysis Buffer 2 and tested in triplicate and in separate wells for phospho CREB (S133) and total CREB. Data show a linear relationship between lysate dilutions and TR-FRET ratio values.

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

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