TECHNICAL DATA SHEET

THUNDER™ Phospho-EGFR (Y1068) + Total EGFR
TR-FRET Cell Signaling Assay Kit

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CATALOG NUMBERS KIT-EGFRPT-500
400 points for phospho-EGFR and 100 points for total EGFR

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-EGFR (Y1068)** and **total EGFR** 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 **EGFR** phosphorylated at **Tyr1068** and another that recognizes **total** (both phosphorylated and unphosphorylated) **EGFR.**

SPECIES REACTIVITY

Human (Swiss-Prot Acc. P00533 and Entrez-Gene Id 1956).

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

TR-FRET ASSAY PRINCIPLE

The **Phospho-EGFR (Y1068) + Total EGFR** 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-EGFR (Y1068)** and **Total EGFR** 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-EGFR** or **total EGFR**) 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-EGFR (Y1068)** and **Total EGFR** in the cell lysate. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio.

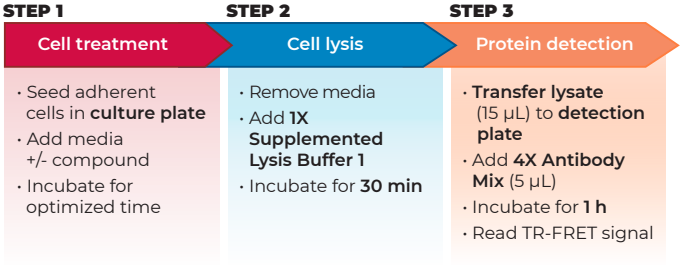

Figure 2 Assay workflow using the 2-plate (transfer) protocol.

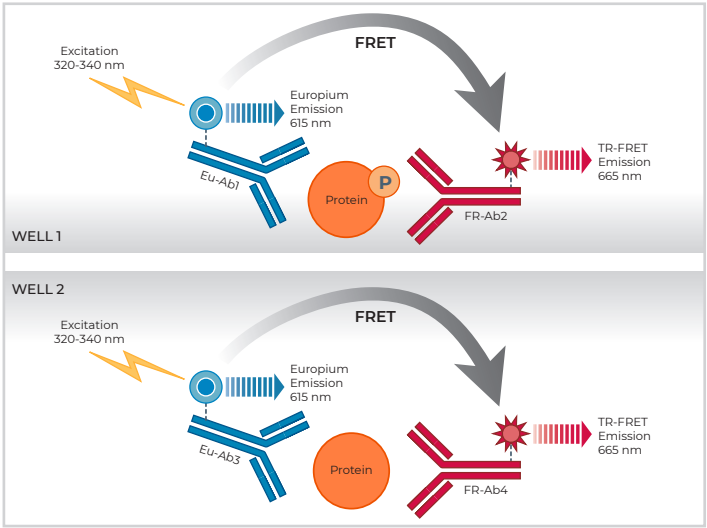

Fig. 1 Schematic representation of the TR-FRET cell signaling assay principle.

KIT COMPONENTS

KIT COMPONENTS	500 points*
Eu-labeled phospho-EGFR (Y1068) antibody (Eu-Ab1)	20 µL
Acceptor-labeled phospho-EGFR (Y1068) antibody (FR-Ab2)	80 µL
Eu-labeled total-EGFR antibody (Eu-Ab3)	5 µL
Acceptor-labeled total-EGFR antibody (FR-Ab4)	20 µL
Lysis Buffer 1 (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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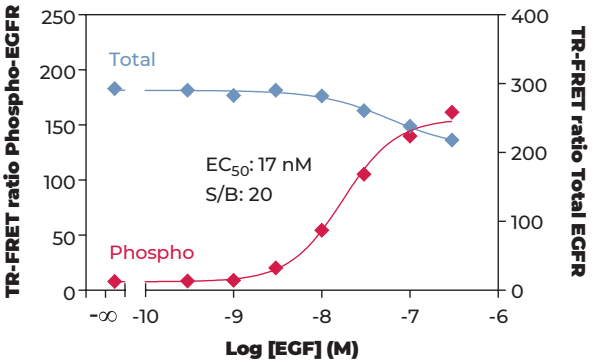
Phospho-EGFR (Y1068) + Total EGFR

VALIDATION DATA

This assay kit has been validated for the relative quantification of phospho-EGFR (Y1068) and total EGFR in A431 cell lysates using the 2-plate assay protocol.

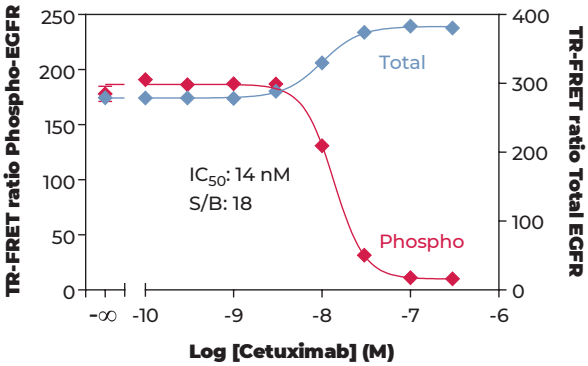
- Adherent cells were cultured overnight in a 96-well tissue culture plate (DMEM +10% FBS).
- Following cell treatment, the media was removed and cells were lysed with the 1X **Lysis Buffer 1** (50 µL) supplemented with the phosphatase inhibitors sodium fluoride (1 mM) and sodium orthovanadate (2 mM).
- 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-EGFR (Y1068) or Eu-Ab3 and FR-Ab4 (5 µL) for detection of total EGFR.
- The plate was incubated at RT for **1 hour** and the TR-FRET signal was recorded at 665 and 615 nm (EnVision®; lamp excitation).

STIMULATION OF PHOSPHO-EGFR (Y1068) IN A431 CELLS



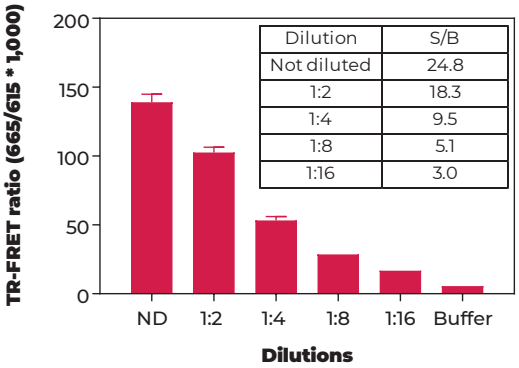
A431 cells (40,000 cells/well; in triplicate) were incubated with serial dilutions of EGF for 10 min at RT. Data show that treatment of A431 cells with EGF stimulates phosphorylation of EGFR at Y1068 but does not have a major effect on the levels of total EGFR.

INHIBITION OF PHOSPHO-EGFR (Y1068) IN A431 CELLS



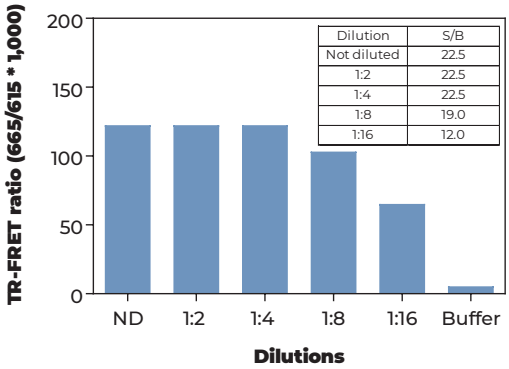
A431 cells (40,000 cells/well; in triplicate) incubated with serial dilutions of Cetuximab for 30 min at RT. Cells were then stimulated with 50 nM of EGF for 10 min at RT. Data show that treatment of A431 cells with Cetuximab inhibits phosphorylation of EGFR at Y1068 by EGF but does not have a major effect on the levels of total EGFR.

A431 CONTROL LYSATE TITRATION (QC TEST) PHOSPHO-EGFR (Y1068)



Dilution	S/B
Not diluted	24.8
1:2	18.3
1:4	9.5
1:8	5.1
1:16	3.0

A431 CONTROL LYSATE TITRATION (QC TEST) TOTAL EGFR



Dilution	S/B
Not diluted	22.5
1:2	22.5
1:4	22.5
1:8	19.0
1:16	12.0

Quality Control: the Phospho-EGFR (Y1068) + Total EGFR assay kit is routinely tested against EGF-treated A431 lysates. A431 cells were cultured in a T175 flask to 100% confluence and stimulated with 100 nM of EGF for 10 min at RT. Following cell lysis using 4 mL of 1X Lysis Buffer 1, lysates were serially diluted with 1X Lysis Buffer 1 and tested in triplicate and in separate wells for phospho-EGFR (Y1068) and total EGFR. Data show a linear relationship between lysate dilutions and TR-FRET ratio values. Note that due to the very high sensitivity of the Total EGFR kit, lysates from the T175 flask required at least a 1:4 pre-dilution in order to be within the dynamic assay range.

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

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7171 Frederick-Banting, Suite 2115, Montréal (Québec) H4S 1Z9 CANADA **bioauxilium.com** **1-866-610-6933 x 103**