CAT. & Size	A1060011S (1,000 tests)	VKEYBIO-01-2024
	A1060011L (10,000 tests)	For Research Use Only
Storage at	-60°C or below	Not For Diagnostic Or Therapeutic Use

KeyTec® TR-FRET

Phospho-ERK1/2 (T202/Y204) Kit

Instruction Manual

1. Introduction

The KeyTec® TR-FRET Phospho-ERK1/2 (T202/Y204) Kit is designed for detect the level of phosphorylated ERK1/2 (T202/Y204) protein in cell lysates. It is based on sandwich immunoassay model and utilizes TR-FRET technology, known for its ease of use, homogeneity (no wash), low background, high sensitivity, robustness.

The detection principle is based on TR-FRET technology. A pair of anti Phospho-ERK1/2 (T202/Y204) protein antibodies, each labeled with KeyTec® TR-FRET Solar Eu[1] and KeyTec® TR-FRET LA[2], bind to the same antigen Phospho-ERK1/2 (T202/Y204) protein. This binding brings the donor molecule into proximity with the acceptor molecule. Excitation of the donor will result in the generation of the TR-FRET signal at 665 nm, proportional to the concentration of Phospho- ERK1/2 (T202/Y204) protein. (Figure 1)

*[1] KeyTec® TR-FRET Solar Eu: TR-FRET Donor Molecule

*[2] KeyTec® TR-FRET LA: TR-FRET Acceptor Molecule

Figure 1. KeyTec® TR-FRET Phospho-protein Assay Principle

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- Sample: Lysates of suspension or adherent cells.
- Specificity: Recognizes specifically the Phospho-AKT (S473) protein.
- Species: Validated for Human and mouse (UniProtKB：P27361，P28482; Entrez-Gene ID：5595, 5594); Validation for other species is pending.

2. Components

Components	Storage	A1060011S (1,000 tests*3)	A1060011L (10,000 tests*3)
mAb anti-phospho-ERK1/2 (T202/Y204) - Solar Eu	⩽ -60 °C	1 vial 25 µL/vial	1 vial 250 µL/vial
mAb anti-phospho-ERK1/2 (T202/Y204) - LA	⩽ -60 °C	1 vial 100 µL/vial	1 vial 1 mL/vial
Positive control lysate	⩽ -60 °C	1 vial 100 µL/vial	2 vials 100 µL/vial
Phosphatase Inhibitor Cocktail (100X)	⩽ -60 °C	1 vial 250 µL/vial	1 bottle 2.5 mL/bottle
Phosphoproteins Lysis Buffer A (5X)	2-8 °C	1 bottle 5 mL/bottle	1 bottle 50 mL/bottle
Phosphoproteins Detection Buffer (10X)	2-8 °C	1 vial 250 µL/vial	1 bottle 2.5 mL/bottle

*3 The tests are sufficient in a 384-well microplate assay format, with 20 µL per well. The details of the protocol refer to "KeyTec® TR-FRET Phosphorylated Protein Assay Kit General Guidebook".

3. Storage Conditions

- Upon receipt, store the kit below -60 °C. Kit components remain stable under appropriate storage conditions as recommended.
- When first thaw, aliquot the components as needed to avoid multiple freeze-thaw cycles
- Up to 1 years from date of receipt, when stored and handled as recommended.

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4. Materials Required But Not Supplied

Materials	Recommended Brand	CAT.
ddH_2O	Multiple Choices	\
Microplates (KeyTec® 384-Well White Flat Low-Volume Microplates)	VKEY-BIO	M2000102N
KeyTec® Fluorescent High-Transparency Microplate Top Seals	VKEY-BIO	M1000102N
Pipettes	Multiple Choices	\
Microplate Reader With TR-FRET	Multiple Choices	\

5. Assay Procedure

5.1 Assay Format

Assay Format	Total Volume (20 µL*4)
Sample or Positive Control	15 µL
Pre-mixed Phospho-(T202/Y204) protein Antibodies	5 µL

*4 The system accommodates 384-well microplates, and assay volumes can be adjusted proportionally to perform in 96- or 1536-well microplates.

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5.2 Procedure [using the 2-plate (transfer) protocol]

Procedure	Stage	Operation
Step 1	Cell Treatment	➢ Seed adherent cells in the culture plate. ➢ Add medium +/- compounds. ➢ Incubate for optimized time.
Step 2	Cell Lysis	➢ Remove the medium. ➢ Add 1X Phosphoproteins Lysis Buffer A supplemented with Phosphatase Inhibitor Cocktail*5. ➢ Shake for 30 min.
Step 3	Detection	➢ Transfer lysate (15 µL) to the detection plate · ➢ Add 4X Pre-mixed Phospho-ERK1/2 (T202/Y204) protein Antibodies (5 µL) ➢ Seal the microplate with "KeyTec® Fluorescent High-Transparency Microplate Top Seals" and incubate for 4 h at room temperature. ➢ (no need to remove the High-Transparency plate sealer) Read on the TR-FRET compatible reader

*5 Depending on cell lines used, volume of lysis should be optimized.

5.3 Data Calculating

- Calculate the ratio of 665 nm/615 nm (TR-FRET Ratio) and the CV for each individual well.

$$\text{TR-FRET Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 615 nm}} \times 10{,}000$$

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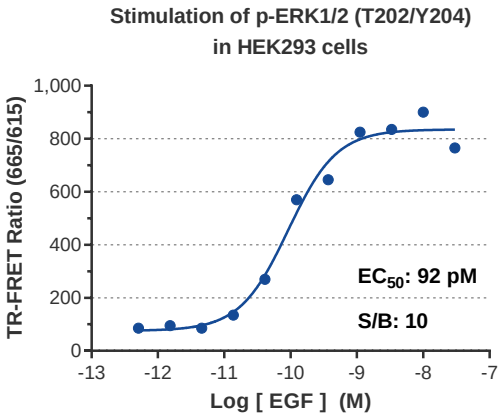
5.4 Performance

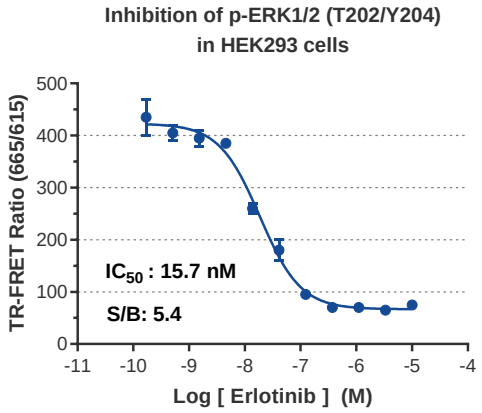
This assay kit has been validated for the relative quantification of phospho-ERK1/2 (T202/Y204) in HEK293 lysates using the 2-plate assay protocol

- Adherent cells were cultured overnight in the 96-well cell culture plate (coated with poly-L-lysine). Using EMEM + 10% FBS as the medium.
- After cell treatment, remove the culture medium, lyse the cells with 50 µL of 1X Phosphoproteins Lysis Buffer A supplemented with Phosphatase Inhibitor Cocktail.
- Shake (400 rpm) at room temperature for 30 min. Transfer 15 µL of the cell lysate to a 384-well plate (Detection plate) then add 5 µL of Pre-mixed Phospho-ERK1/2 (T202/Y204) protein Antibodies (Ab1-Solar Eu, Ab2-LA) for detecting Phospho-ERK1/2 (T202/Y204) protein.
- Seal the microplate with "KeyTec® Fluorescent High-Transparency Microplate Top Seals" and incubate for 4 h at room temperature, then read the TR-FRET signals at 665 nm and 615 nm.

Stimulation of Phospho-ERK1/2 (T202/Y204) in HEK293 cells

HEK293 cells (50K cells/well, in triplicate) were incubated with serial dilutions of EGF for 10 min at room temperature. The data show that treatment of HEK293 cells with EGF stimulates the phosphorylation of ERK1/2 at T202/Y204.





Inhibition of Phospho-ERK1/2 (T202/Y204) in HEK293 cells

HEK293 cells (50K cells/well, in triplicate) were incubated with serial dilutions of the inhibitor Erlotinib for 15 min at room temperature, followed by incubation with 0.5 nM EGF for 10 min at room temperature. The data show that treatment of HEK293 cells with Erlotinib inhibits the stimulated phosphorylation of ERK1/2 at T202/Y204.

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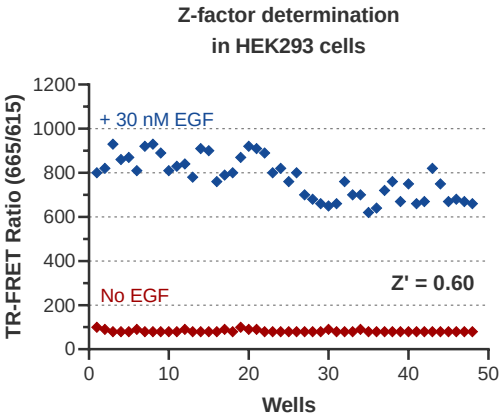
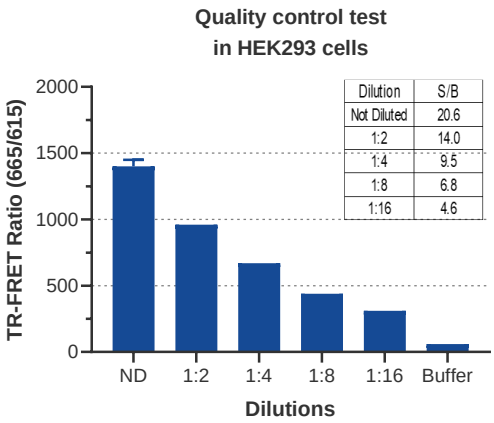
Z-factor determination in HEK293 cells

HEK293 cells (50K cells/well) were incubated with or without 30 nM of EGF for 10 min at room temperature. The Z-factor value was determined using data from a total of 48 wells for each treatment group. The Z-factor value of 0.60 indicates that the assay is robust and suitable for HTS.





Titration of HEK293 cell lysate (Quality Control test)

Quality control: The KeyTec® TR-FRET Phospho-ERK1/2 (T202/Y204) assay kit is routinely tested against EGF-treated HEK293 lysates. HEK293 cells were cultured in a T175 flask to 90% confluence and stimulated with 30 nM of EGF for 10 min at RT. Following cell lysis using 5 mL of 1X Phosphoproteins Lysis Buffer A supplemented with Phosphatase Inhibitor Cocktail, lysates were serial diluted with 1X Phosphoproteins Lysis Buffer A and tested in triplicate. Data show a linear relationship between lysate dilutions and TR-FRET ratio values.

Tip: The data provided above is for reference only. Actual results may vary depended on the performance of the microplate reader used.

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