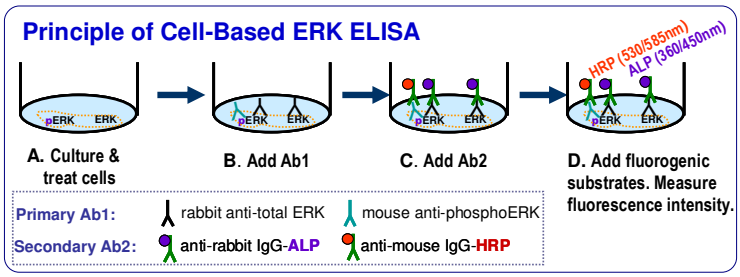
EnzyFluo™ ERK Phosphorylation Assay Kit (EERK-100)

Fluorimetric Cell-Based Assay for ERK Phosphorylation Status

DESCRIPTION

The mitogen-activated protein kinase (MAPK/ERK) pathway plays a key role in cell proliferation, differentiation and migration. Stimulation by mitogens eventually leads to phosphorylation of ERK1 (T202/Y204) and ERK2 (T185/Y187). The MAPK/ERK cascade presents many interesting drug targets for the development of cancer therapies.

BioAssay Systems' cell-based ELISA measures dually phosphorylated ERK1/2 in whole cells. This simple and efficient assay eliminates the need for cell lysate preparation and can be used to study kinase signaling and the effects of kinase inhibitors on cells. In this assay, cells are grown in 96-well plates and treated with ligands or drugs. Cells are then fixed and permeabilized in the wells. ERK1/2 phosphorylation (pERK) is measured using a double immunoenzymatic labeling procedure.



KEY FEATURES

Safe. Non-radioactive assay.
Simple and convenient. Total and pERK can be measured in the same sample.

APPLICATIONS

Determination of ERK phosphorylation status in whole cells.
Evalutation of effects of ligands or drugs on ERK phosphorylation.
Species tested: human, mouse, rat.

KIT CONTENTS

10× Wash Buffer:	25 mL	**Blocking Buffer:**	25 mL
ALP Substrate:	6 mL	**HRP Substrate:**	6 mL
ERK-Ab1:	10 µL	**pERK-Ab1**	10 µL
HRP-Ab2:	10 µL	**ALP-Ab2**	10 µL

Storage conditions: The kit is shipped on ice. Store all reagents at -20°C. Shelf life of 6 months after receipt.

Precautions: reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.

ASSAY PROCEDURE

Important:

1. To avoid cross-contamination, change pipette tips between additions of each reagent or sample. Use separate reservoirs for each reagent. Prior to Assay, dilute 10× Wash Buffer in dH_2O to prepare 250 mL 1× Wash Buffer.
2. It is recommended that assays be run in duplicate. Plan to use four assay wells for each sample: two "Sample Blank" wells in which Blocking Buffer is added for determining Ab2 background fluorescence, and two "Sample" wells in which Ab1 Mixture is added (see Step B2).

A. Culture and Treat Cells

1. Seed 100 µL of 2-4 × 10^4 adherent cells (or 4-10 × 10^4 suspension cells) into each well of a black clear-bottom 96-well plate. Incubate overnight at 37°C in a cell culture incubator.

   Note: The cell number to be used depends on the cell line and ERK1/2 phosphorylation status.

2. Treat the cells as desired (e.g. with ligands or drugs).

3. Prepare formaldehyde solutions (*warning*: formaldehyde is toxic. Use chemical hood and wear appropriate gloves and eye protection):

   For adherent cells, prepare 4% formaldehyde by mixing 1.3 mL of 37% formaldehyde and 10.7 mL of 1× Wash buffer. Simply fix cells in each well by replacing the medium with 100 µL of 4% formaldehyde.
   For suspension cells, prepare 8% formaldehyde by mixing 2.6 mL of 37% formaldehyde and 9.4 mL of 1× Wash buffer. Centrifuge the plate at 500*g* for 15 min at 4°C and carefully remove as much media as possible without disturbing the cell pellet. Fix the cells in each well by adding 100 µL of 8% formaldehyde to cell pellet.

   Cover the plate and incubate for 20 min at room temperature. Alternatively, the plate containing the fixed cells can be sealed and stored for up to 2 weeks at 2-8°C.

4. Remove the formaldehyde solution and wash the cells 3 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 5 min with gentle shaking. For suspension cells centrifuge the plate at 500*g* for 15 min at 4°C before removing the formaldehyde solution. *Note, for all following wash and treatment steps, suspension cells need to be centrifuged before removal of any liquid.*

5. Prepare Quench Buffer by mixing 2.2 mL of 3% H_2O_2 and 8.8 mL of 1× Wash Buffer.

   Remove the Wash Buffer and add 100 µL of Quench Buffer to each assay well. Cover plate and incubate for 20 min at room temperature.

6. Remove the Quench Buffer and wash the cells 3 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 5 min with gentle shaking.

7. Remove the Wash Buffer, and add 100 µL of Blocking Buffer. Cover plate and incubate for 1 hr at room temperature.

B. Add Primary Antibodies (Ab1)

1. Add 100 µL of PBS to the ERK-Ab1 and pERK-Ab1 tubes and mix well. Prepare enough primary antibody Ab1 Mixture for each well by mixing 1 µL diluted ERK-Ab1, 1 µL diluted pERK-Ab1 and 55 µL Blocking Buffer. Unused Ab1 antibodies can be stored at -20°C for up to 45 days.

2. Remove the Blocking Buffer from all assay wells. Add 50 µL of the Blocking Buffer to the Sample Blank wells and 50 µL of Ab1 Mixture to the Sample wells. Cover plate and incubate for 3 hrs at room temperature or overnight at 2-8°C with gentle shaking.

3. Remove the Ab1 Mixture and wash the cells 3 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 5 min with gentle shaking.

C. Add Secondary Antibodies (Ab2)

1. Immediately before use, add 100 µL of PBS to the HRP-Ab2 and ALP-Ab2 tubes and mix well. Prepare enough secondary antibody Ab2 Mixture, for each well, by mixing 1 µL diluted HRP-Ab2, 1 µL diluted ALP-Ab2 and 55 µL Blocking Buffer. Unused Ab2 antibodies can be stored at -20°C for up to 45 days.

2. Remove Wash Buffer and add 50 µL of the Ab2 Mixture to all assay wells. Cover plate and incubate for 2 hrs at room temperature with gentle shaking.

D. Detection

1. Remove the Ab2 Mixture from each well and wash the cells 5 times with 150 µL of 1× Wash Buffer. Each wash step should be performed for 5 min with gentle shaking.

2. Immediately before use, add 6 µL 3% H_2O_2 to the provided 6 mL HRP Substrate (for partial plate assay, adjust the volumes accordingly). Remove the Wash Buffer from the plate and add 50 µL of reconstituted HRP Substrate to each well. Incubate for 20 min at room temperature in the dark.
3. Add 50 µL of ALP Substrate to each well and incubate for an additional 20 min at room temperature in the dark.
4. Read the plate at $\lambda_{ex/em}$ = 530/590nm for phosphorylated ERK (*p*ERK) and at $\lambda_{ex/em}$ =360/450nm for total ERK (ERK).

CALCULATION

Calculate the mean fluorescence intensities for the Sample Blank ("BLK") wells and Sample ("SAMPLE") wells. Subtract the mean fluorescence of the Sample Blank wells from the fluorescence value of the Sample well to yield ΔF values for the phosphorylated ERK (ΔF_{pERK}) at 530/585nm and the total ERK (ΔF_{ERK}) at 360/450nm.

$$\Delta\bar{F}_{pERK} = \bar{F}^{SAMPLE}_{pERK} - \bar{F}^{BLK}_{pERK}; \quad \Delta\bar{F}_{ERK} = \bar{F}^{SAMPLE}_{ERK} - \bar{F}^{BLK}_{ERK}$$

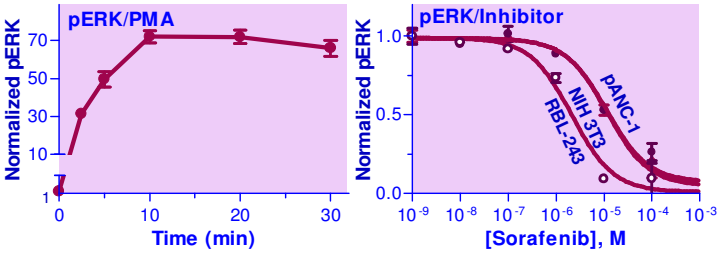
Normalized phosphorylated ERK (pERK) is calculated as,

$$\text{Normalized pERK} = \frac{\Delta\bar{F}_{pERK}/\Delta\bar{F}_{ERK}}{(\Delta\bar{F}_{pERK}/\Delta\bar{F}_{ERK})_o}$$

where $(\Delta F_{pERK} / \Delta F_{ERK})_o$ is the control reference value (e.g. time zero in kinetic studies or untreated wells in drug potency studies.)

MATERIALS REQUIRED BUT NOT PROVIDED

37% formaldehyde (Sigma, cat # F8775); 3% H_2O_2 (Sigma, cat # H1009); black cell culture 96-well plate: available separately at BioAssay Systems (cat# P96BCC) or at Sigma (CLS3603); plate sealers: available separately at BioAssay Systems (cat# AB96SL) or at Sigma (cat# A5596); deionized or distilled water; pipetting devices; cell culture incubators; centrifuge tubes; fluorescence plate reader capable of reading at $\lambda_{ex/em}$ = 530/585nm and at $\lambda_{ex/em}$ =360/450nm.



Left: Kinetics of ERK1/2 phosphorylation in PANC-1 cells on treatment with phorbol myristate acetate (PMA).

Right: inhibition of ERK1/2 phosphorylation by the kinase inhibitor Sorafenib. Cells were treated with drug for 3 hours and then 5 min with PMA. IC_{50} values were 2.1, 11.4 and 11.5 µM respectively, for RBL-243, NIH 3T3 and PANC-1 cell lines.

LITERATURE

1. Cobb MH, et al (1994). The mitogen-activated protein kinases, ERK1 and ERK2. Semin Cancer Biol. 5(4):261-268.
2. Daniluk J, Dabrowski A. (2007). The effect of concomitant stimulation with cholecystokinin and epidermal growth factor on extracellular signal-regulated kinase (ERK) activity in pancreatic acinar cells. J Physiol Pharmacol. 58(3):441-53.
3. Iqbal J, et al (2007). Rapid in vivo effects of estradiol-17beta in ovine pituitary gonadotropes are displayed by phosphorylation of extracellularly regulated kinase, serine/threonine kinase, and 3',5'-cyclic adenosine 5'-monophosphate-responsive element-binding protein. Endocrinology 148 (12): 5794-802.

RELATED PRODUCTS

EnzyFluo™ Direct Phospho-ERK Assay Kit (cat# ERKD-100), for direct fluorimetric cell-based assay for ERK Phosphorylation Status.

96-Well Assay Plate Template