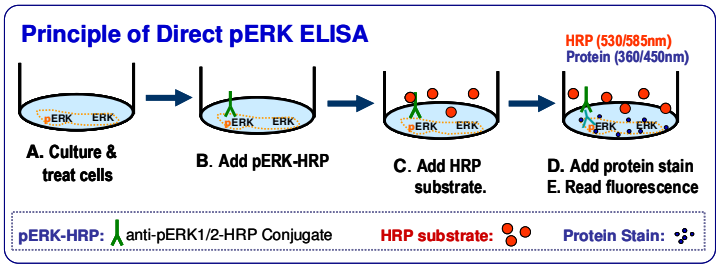
EnzyFluo™ Direct Phospho-ERK Assay Kit (ERKD-100)

Direct 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 normalizes the signal to the total protein content. The assay is a useful tool to study the MAP kinase signaling pathway. 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 HRP-conjugated antibody specific for phosphorylated ERK1/2.



KEY FEATURES

Safe. Non-radioactive assay.
Simple and convenient. Saves time, direct one-step reaction, no secondary antibody.

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
Protein Stain	6 mL	**HRP Substrate:**	6 mL
pERK-HRP:	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, prepare 250 mL 1× Wash Buffer by diluting 25 mL 10× Wash Buffer in 225 mL dH_2O.
2. It is recommended that assays be run in duplicate. Include in every experiment two "Sample Blank" wells with no cells but with the same volume of culture medium. The Sample Blank wells are treated the same way as with the "Sample Wells" for background fluorescence determination.

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 flat-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. 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 pERK antibody-HRP Conjugate (pERK-HRP)

1. Add 100 µL of PBS to the pERK-HRP tube and mix well. Prepare enough pERK-HRP mixture for each well by mixing 1 µL diluted pERK-HRP and 55 µL Blocking Buffer. Unused pERK-HRP 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 50 µL of pERK-HRP mixture to the Sample and Sample Blank wells. Cover plate and incubate for 3 hrs at room temperature or overnight at 2-8°C with gentle shaking.

3. Remove the pERK-HRP mixture 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.

D. Detection

1. Remove pERK-HRP 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 30 min at room temperature in the dark.

3. Add 50 µL of Protein Stain to each well and incubate for an additional 3 min at room temperature in the dark.

4. Read the plate at $\lambda_{ex/em}$ = 530/585 nm for pERK and at $\lambda_{ex/em}$ =360/450nm for total protein.

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 ERK1/2 (ΔF_{pERK}) at 530/585nm and the total Protein (ΔF_{PROT}) at 360/450nm.

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

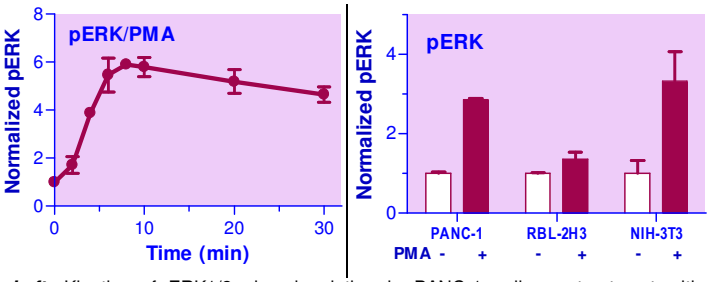
Normalized phosphorylated ERK (pERK) is calculated as,

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

where $(\Delta F_{pERK} / \Delta F_{PROT})_o$ 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 System (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 $\lambda_{ex/em}$ = 530/585 nm and at $\lambda_{ex/em}$ =360/450 nm.



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

Right: Species reactivity. Human (PANC-1), rat (RBL-2H3) and mouse (NIH-3T3) cells were grown to confluency in 96 well plates. 10 ng/L PMA or vehicle was added for 10 minutes before cells were fixed and processed.

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™ ERK Phosphorylation Assay Kit (cat# EERK-100), for fluorimetric cell-based assay for ERK phosphorylation status.

96-Well Assay Plate Template