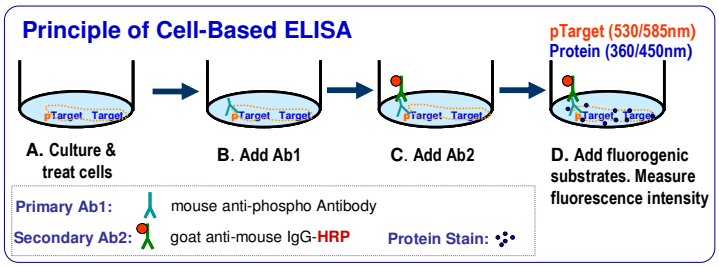
EnzyFluo™ Human/Mouse RB(T821/826) Phosphorylation ELISA Kit (ERBT-100)

Fluorimetric Cell-Based Assay for RB(T821/826) Phosphorylation Status

DESCRIPTION

PROTEIN PHOSPHORYLATION plays key roles in regulating protein activity within cells. A large number of protein kinases and phosphatases are known to be involved in a variety of signal transduction pathways. A rapid assay capable of monitoring such signal transduction pathways, target protein phosphorylation status and drug effects is highly desirable in life science research and drug discovery.

The *RetinoBlastoma* tumor suppressor protein (Rb, RB1, pRb, retinoblastoma 1, OSRC, PPP1R130, p105-Rb, pp110, retinoblastoma protein, RB transcriptional corepressor 1, p110-RB1) regulates cell differentiation, apoptosis and cell cycle control. BioAssay Systems' RB(T821/826) Phosphorylation ELISA Kit provides a rapid and sensitive assay for measuring the phosphorylated protein pRB(T821/826) in cultured cells and normalizes the level of phosphorylation to the total protein content in the same well. This simple and efficient assay eliminates the need for cell lysate preparation and can be used to study the underlying signal pathway and effects of inhibitors, siRNA or activators on the pathway. In this assay, cells grown in a 96-well plate are fixed and permeabilized. The phosphorylation of RB(T821/826) is measured using a specific primary antibody followed by a HRP-conjugated secondary antibody with a fluorogenic substrate, and a fluorescent reagent that measures the total protein in the same well.



KEY FEATURES

New and improved. Total assay time reduced from the standard 21 hours to 6.5 hours (hands-on time 2.5 hrs).

Simple and convenient. Cells are directly cultured in 96-well plates. No cell lysis necessary.

Accurate and high-throughput. Protein phosphorylation is normalized to total cellular protein in the same well, greatly minimizing well-to-well variations. Can be readily automated as a high-throughput 96-well plate assay for thousands of samples per day.

APPLICATIONS

Determination of RB(T821/826) phosphorylation status in whole cells.
Evaluation of pathway modulation by activators, inhibitors, siRNA etc.
Species tested: Human, mouse, rat.

KIT CONTENTS

Stock Wash Buffer:	25 mL	**Blocking Buffer:**	25 mL
Protein Stain:	6 mL	**Dye Reagent:**	120 µL
Ab1:	10 µL	**Ab2 (gM-HRP):**	10 µL

Storage conditions: This kit is shipped on ice. Upon delivery, store all reagents at -20°C. Shelf life of these reagents is about 6 months after receipt.

Precautions: Reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using these reagents. Please refer to the 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. We recommend the use of a multi-channel pipette. Use separate reservoirs for each reagent. Prior to Assay, prepare *1x Wash Buffer* by diluting Stock Wash Buffer 40-fold with dH_2O, e.g. mix 10 mL Stock Wash Buffer and 390 mL dH_2O.

   Important: reserve 6 mL 1x Wash Buffer for Detection Step 2 below.
2. It is recommended that samples be assayed in triplicate or more.
3. Two different blanks in triplicate are required, a Protein Blank (no cells) and a Sample Blank (cells with No Ab1 added, only Ab2). The blanks are used to determine background fluorescence for total protein and pRB(T821/826), respectively.

A. Culture and Treat Cells

1. Seed 100 µL of 1-3×10^4 adherent cells (or 4-10×10^4 suspension cells) into each well of a black 96-well culture plate. Add 100 µL of cell-free culture media into three separate wells for the Protein Blank. Incubate overnight at 37°C in a cell culture incubator.

   Note: The cell number to be used depends on the cell line and the phosphorylation level of the target protein.
2. Treat the cells as desired (e.g. with ligands or drugs).
3. Prepare formaldehyde solution with caution (*warning*: formaldehyde is toxic. Use chemical hood and wear appropriate gloves and eye protection):

   For adherent cells, prepare 4 wt% formaldehyde solution 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 media with 100 µL of 4% formaldehyde solution.

   For suspension cells, prepare 8 wt% formaldehyde solution by mixing 2.6 mL of 37% formaldehyde and 9.4 mL of 1× Wash buffer. Centrifuge the plate at 500x*g* for 15 min at 4°C and carefully remove as much media as possible without disturbing the cell pellet (repeat this step for suspension cells with each wash step below). Fix the cells in each well by adding 100 µL of 8% formaldehyde solution to the 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 2 times with 200 µL of 1× Wash Buffer. Each wash step should be performed with gentle shaking for 3 min.
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. 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 200 µL of 1× Wash Buffer. Each wash step should be performed with gentle shaking for 3 min.
7. Remove the Wash Buffer. Add 100 µL of Blocking Buffer. Cover plate and incubate for 1 hr at room temperature.

B. Add Primary Antibodies (Ab1)

1. Prepare 55 µL of primary antibody Ab1 Mixture for each well by mixing Ab1 and Blocking Buffer in a 1:1000 dilution.
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 90 min at room temperature or overnight at 2-8°C with gentle shaking.
3. Remove the Ab1 Mixture and wash the cells 3 times with 200 µL of 1× Wash Buffer. Each wash step should be performed with gentle shaking for 3 min.

C. Add Secondary Antibodies (Ab2)

1. Prepare 55 µL of secondary antibody Ab2 Mixture for each well by mixing Ab2 into Blocking Buffer in a 1:1000 dilution.
2. Remove Wash Buffer and add 50 µL of the Ab2 Mixture to all assay wells. Cover plate and incubate for 90 min at room temperature with gentle shaking.

D. Detection

1. Remove the Ab2 Mixture from each well and thoroughly wash the cells 4 times with 200 µL of 1× Wash Buffer. Each wash step should be performed with gentle shaking for 3 min.
2. Immediately before use, prepare HRP Substrate by mixing 60 µL Dye Reagent with 6 mL 1x Wash Buffer and 6 µL 3% H_2O_2 (for partial plate assay, adjust the volumes accordingly).

   Remove the Wash Buffer from the plate. Using a multi-channel pipette, quickly add 50 µL of mixed HRP Substrate to each well. Incubate for 30 min at room temperature in the dark.
3. Using a multi-channel pipette, quickly add 50 µL of Protein Stain to each well and incubate for an additional 5 min at room temperature in the dark.
4. Read the plate at $\lambda_{ex/em}$ = 530/585 nm for pRB(T821/826) and at $\lambda_{ex/em}$ =360/450 nm for total protein.

CALCULATION

Calculate the mean pTarget for pRB(T821/826) fluorescence intensities at 530/585nm for the Sample Blank ("No Ab1" wells, $\mathbf{F}_{pTarget.Blank}$) and Sample wells ($\mathbf{F}_{pTarget.Sample}$). Also calculate the mean protein fluorescence intensities at 360/450nm for the Protein Blank (no cells well, $\mathbf{F}_{Protein.Blank}$) and Sample wells ($\mathbf{F}_{Protein.Sample}$). Calculate the specific fluorescence values for the pTarget and total protein:

$$\Delta\mathbf{F}_{pTarget} = \mathbf{F}_{pTarget.Sample} - \mathbf{F}_{pTarget.Blank}$$
$$\Delta\mathbf{F}_{Protein} = \mathbf{F}_{Protein.Sample} - \mathbf{F}_{Protein.Blank}$$

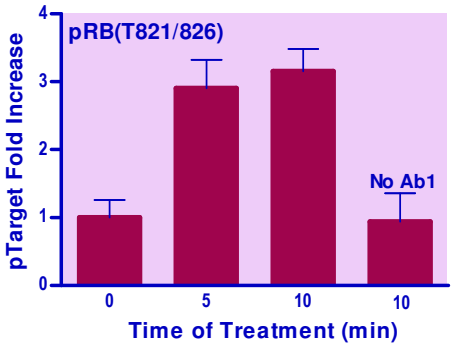
Normalized pTarget is calculated as,

$$\text{Normalized } p\mathbf{Target} = \frac{\Delta\mathbf{F}_{pTarget} / \Delta\mathbf{F}_{Protein}}{(\Delta\mathbf{F}_{pTarget} / \Delta\mathbf{F}_{Protein})_o}$$

where $(\Delta\mathbf{F}_{pTarget} / \Delta\mathbf{F}_{Protein})_o$ 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 # 323381); Black clear-bottom 96-well cell culture plate: available separately at BioAssay Systems (cat# P96BCC) or at Sigma (cat# 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.



Induction of RB(T821/826) Phosphorylation in NIH 3T3 Cells

3 x 10^4 NIH 3T3 cells in 100 µL culture media were plated into a 96 well plate. After overnight incubation at 37ºC, cells were treated with 50 ng/mL PDGFbb in serum-free media. RB(T821/826) phosphorylation was measured using the ERBT-100 kit.

LITERATURE

1. Cohen, P. (2002). The origins of protein phosphorylation. Nature Cell Biology. 4 (5): E127–130.
2. Smoly, I. et al (2017). An asymmetrically balanced organization of kinases versus phosphatases across Eukaryotes determines their distinct impacts". PLOS Computational Biology. 13 (1): e1005221.
3. Vlastaridis, P. et al (2017). Estimating the total number of phosphoproteins and phosphorylation sites in eukaryotic proteomes. GigaScience. 6 (2): 1–11