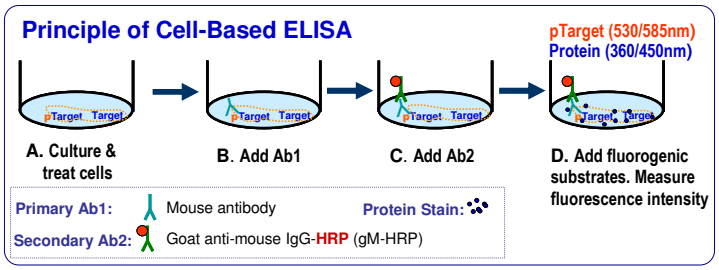
EnzyFluo™ Custom Cell-Based ELISA Kit (EFC1M-100)

Rapid Convenient Fluorimetric ELISA for Any Protein or Signal Pathways

DESCRIPTION

Many biological processes are regulated by changing the concentration and/or activity of proteins in the cell. A rapid assay capable of monitoring such changes is highly desirable in life science research and drug discovery. BioAssay Systems' Custom Cell-based ELISA Kit provides a rapid and sensitive starter tool for measuring the relative content of any target protein in cultured cells. Only a target-specific, mouse antibody (Ab1) for the protein of interest is needed to run the assay.

This simple and efficient assay eliminates the need for cell lysate preparation and can be used to determine a particular protein content, or signaling pathway activity and effects of treatments by e.g. drugs, inhibitors, siRNA or activators. In this assay, cells grown in a 96-well plate are fixed and permeabilized. The target protein is measured using your own mouse Ab1 followed by a HRP-conjugated secondary antibody with a fluorogenic substrate, and a fluorescent reagent that measures the total protein in the same well. This convenient kit provides all necessary reagents for the user to develop their own assay. Upon request, BioAssay Systems can also custom develop it for the user.



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. Target protein 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 relative protein content and signal pathway (e.g. phosphorylation status) in whole cells.

Evaluation of target modulation by drugs, activators, inhibitors, siRNA etc.

Species: human, mouse, rat and more, depending on choice of primary antibody.

KIT CONTENTS

Stock Wash Buffer:	25 mL	**Blocking Buffer:**	25 mL
Protein Stain:	6 mL	**Dye Reagent:**	120 µL
Ab1 (mouse, target dependent):	not provided	**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 the Assay, prepare *1x Wash Buffer* by diluting Stock Wash Buffer 15-fold with dH_2O, e.g. mix 20 mL Stock Wash Buffer and 280 mL dH_2O. *Note: 1. If less stringent wash conditions are required, dilute the Stock Wash Buffer more. 2. 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 target protein, 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 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 a 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. Perform a pilot titration to determine optimal Ab1 dilution factor: prepare Ab1 in Blocking Buffer at different dilutions (e.g. 500 - 5000). Choose the dilution factor that gives the highest response for future assays.

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 diluted Ab1 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 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 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 to all assay wells. Cover plate and incubate for 90 min at room temperature with gentle shaking.

D. Detection

1. Remove the Ab2 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 and add 50 µL of the 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 $\lambda_{ex/em}$ = 530/585nm for target protein and at $\lambda_{ex/em}$ =360/450nm for total protein.

CALCULATION

Calculate the mean Target fluorescence intensity at 530/585nm for the Sample Blank ("No Ab1" wells, $\mathbf{F}_{Target.Blank}$) and Sample wells ($\mathbf{F}_{Target.Sample}$). Calculate the mean total protein fluorescence intensity 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:

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

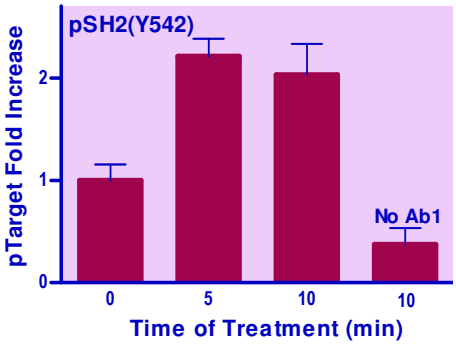
Normalized pTarget is calculated as,

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

where $(\Delta\mathbf{F}_{Target}/\Delta\mathbf{F}_{Protein})_o$ 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.



EXAMPLE

Induction of SHP2(Y542) 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. SHP2(Y542) phosphorylation was measured using the ESHP2-100 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