Phospho-IRAK-1 (T100) Cell-Based Colorimetric ELISA Kit

Catalog No. KA1109C

Detection and Quantification of Phospho-IRAK-1 (T100) Protein Concentration in Cell.

Research Purposes Only. Not Intended for Diagnostic or Clinical Procedures.

Cell-Based Phosphorylation ELISA Kit

Cell-Based ELISAs for measuring relative protein expression and phosphorylation levels

ImmunoWay Biotechnology provides cell-based ELISA kit. Traditional Western blot analysis is time consuming and only semi-quantitative, the Cell-Based Phosphorylation ELISA is an accurate method to determine relative protein levels and degree of phosphorylation among various cell types. Cell-based ELISAs are performed in 96-well microplates, are scalable and conserve cell culture and treatment reagents. Because assay data are measured using a standard ELISA plate reader, the results are readily available for analysis.

Simple, Fast, High Throughput and Cost Efficient

Product Description

The Cell-based Phospho-Specific ELISA kit enables the level of different target phosphorylated proteins or degree of different phosphorylation stimulation in different cell types. The relative amount of phospho-specific or total protein is determined using target-specific primary antibody and HRP conjugated secondary antibody detection agent. Following the colorimetric measurement of HRP activity, the crystal violet provided whole cell staining is used for cell number counts. After staining, the results can be analyzed by normalizing the OD values to cell accounts, by which the plating difference can be adjusted.

Kit Components and Storage—Colorimetric Assay

Cell-Based Phospho-Specific ELISA Colorimetric Kit components can be stored at -20 prior to first use. Recommend storage conditions are indicated in the tale below.

Reagent	Volume	Container Color	Storage
Phospho-Specific antibodies	60ul	Colorless	4°C
Total-Protein antibody	60ul	Yellow Cap	4°C
HRP-conjugated anti-rabbit IgG	10ml	Colorless	4°C
Blocking Buffer	22ml	Colorless	4°C
Antibody Dilution Buffer (1X)	25ml	Colorless	4°C
Washing Buffer (10X)	50ml	Colorless	4°C
One-Step TMB Substrate	11ml	Brown Bottle	4°C
Stop Solution	11ml	Colorless	4°C
1% SDS	11ml	Colorless	4°C

Kit Performance and Benefits

Cell-Based Phosphorylation ELISA Kits are for research use only. Not for diagnosis purpose.

Antibody specificity: Phospho-specific antibodies were raised against synthetic phospho-peptide

corresponding the residues surrounding the exact phosphorylation site in human proteins molecules and don't cross-react with other sites. Accordingly, total antibodies or total site specific antibodies were raised against synthetic peptide corresponding to the residues surrounding either general antigenic domain or the specific site. All the peptides are designed followed the same rules and criteria. The total-protein antibody recognizes the corresponding protein regardless of the status of phosphorylation.

Cross-reactivity: Cell-Based Phospho-Specific ELISA Kits detect phosphorylated proteins and relative total proteins from human, mouse and rat. No cross-reactivity is measured among different phosphorylated sites or species.

Assay time: Duration process varies from 5hours to 24hours. <3 hours of hands-on time.

Assay sensitivity: HeLa, MD-MAB-231 and HCT-116 cells have been tested. Cell-based Phospho-Specific ELISA kit can detect as low as 5000 cells.

Note on data interpretation: The phospho-specific antibodies and total-protein antibodies can be used on equivalent cell cultures to determine the effects of various cell treatments on the ratio of phosphorylated vs total protein. However, if the signals obtained with phospho-specific antibodies and total-protein antibodies are identical, you cannot make a conclusion such that treatment resulted in phosphorylation of 100% of protein of interest.

Cell-Based Phosphorylation Experimental Design

Cell-based Phospho-Specific ELISA is a high volume method for quantifying cellular level of protein molecule of interest and its phosphorylated form. ELISA kits can be used for the types of cells that have been shown to contain readily detectable levels of protein molecules, and under appropriate condition, relative phosphorylated forms. Prior to starting Cell-Based Phospho-Specific ELISA, it is important to determine the experimental conditions and designation of each well of 96-well plate to maximize the information to be obtained.

Points to be considered:

1. What type of cells do you work with, adherent or suspension cells? Modifications and optimization on protocol for the use of suspension cells are provided.
2. You can prepare multiple plates simultaneously and then perform the Cell-Based assay when desired. If you don't use the fixed cells plates immediately, fixed cells should be stored in formaldehyde solution in the refrigerator and plated should be sealed with parafilm, stored in sealed plastic bag and refrigerated.
3. After planning the experiment, you should determine the amount of each buffer/reagents required and prepare accordingly. Multi-channel pipettors and pipettor reservoirs should be used when appropriate.

Material required not provided:

1. Multi-channel pipettor
2. Multi-channel pipettor reservoirs
3. Electronic shaker

4. Parafilm
5. Microplate reader with wavelength of 595nm and 450nm as detection wavelength
6. 30% hydrogen peroxide (H_2O_2)
7. 37-38% Formaldehyde
8. 10ug/ml poly-L-Lysine
9. 10XPBS
10. 96-well plates
11. Adhesive Plate sealer

Warning: Formaldehyde is highly toxic reagents. Personal protection equipments are strongly recommended while working with these chemicals. In addition, formaldehyde should be only used in ventilated hood due to potential inhalation contaminations.

Protocols-Colorimetric Assay

Buffer Preparation and Recommendation

We provide an excess of buffer components for you in order to perform two plates 96-well Cell-Based ELISA assay with each of Phospho-Specific and total-protein respectively. Required materials but not provided are listed on the previous page.

Preparation of 1XPBS 1 X PBS is prepared by adding 1 volume of 10XPBS to 9 volume of distilled water and mixing thoroughly.

Preparation of Quenching Buffer Quenching buffer is used to consume and to remove the endogenous peroxidase activity. It is normally fresh prepared by adding hydrogen peroxide to washing buffer. 1% hydrogen peroxide is used for this purpose. The calculation recipe for 1% hydrogen peroxide solution is based on 30% stock solution and written in Quick Chart for Preparing Buffer.

Preparation of Fixation Buffer Fixation buffer is used to fix cell after culture and treatment. It is prepared by adding formaldehyde to 1XPBS and mixing well. 4% formaldehyde is used with adherent cells and 8% formaldehyde is used with suspension cells. The calculation for 4% and 8% formaldehyde solution is based on 37% formaldehyde stock and written Quick Chart for Preparing Buffer.

Preparation of 1X Washing Buffer Washing buffer is used throughout the whole Cell-Based ELISA protocol and prepared by adding 1 volume of 10X Washing Buffer to 9 volume of distilled water and mixing well.

Blocking Buffer This item is ready-to-use. A small amount of white precipitate may appear if thawed in a warm bath. This doesn’t interfere with performance.

Antibody Dilution Buffer This is 1X buffer. A small amount of white precipitate may appear if thawed in a warm bath. This doesn’t interfere with performance.

Diluted Phospho-specific Antibody The phospho-specific antibody recognizes relative molecule phosphorylated at specific site. Reconstitute primary phospho-specific antibody by dissolving lyophilized antibody into 5ml of antibody diluent. Each well contains 60ul of diluted primary antibody. This provided antibody can be diluted 1:200 in Primary Antibody Dilution Buffer (see Quick Chart for Preparing Buffers).

Diluted Total-protein Antibody This total-protein antibody recognized both the non-phosphorylated and the phosphorylated forms of protein. Reconstitute the anti-total protein antibody by dissolving the lyophilized antibody in 5ml of antibody diluent. Each well contains 60ul of diluted primary antibody. The supplied antibody can be diluted 1:00 in Primary Antibody Dilution (see Quick Chart for Preparing Buffers).

Diluted HRP-Conjugated anti-rabbit IgG Secondary Antibody HRP-conjugated anti-rabbit IgGis used as the secondary antibody to detect bound primary antibodies. Each well contains 50ul of diluted secondary antibody. The supplied antibody will be diluted 1:10,000 in Antibody Dilution Buffer (see Quick Chart for Preparing Buffers).

1%SDS Solution 1% SDS Solution is used in the Crystal Violet procedure to solubilize cells and release the dye for subsequence quantification at 595nm. This buffer is supplied as ready-to-use.

One-Step TMB Substrate This is supplied as ready-to-use. Substrate solution should be warmed to room temperature before use. This solution is light sensitive and should be protected from direction exposure to intense light during storage. The amount of Substrate Solution required for the assay should be transferred to secondary container.

Stop Solution This is supplied as ready-to-use. Prior to each use, transfer the amount of Stop Solution required for the assay into a secondary container. After use, discard any remaining Stop Solution in the secondary container.

Warning: The stop solution is corrosive. Wear personal protection equipment when handling, i.e. labcoat, gloves and eye protection.

Adherent Cell Protocol-Colorimetric Assay

Please read the entire protocol before starting.

Cell Preparation of Adherent Cells (including cell culture and cell fixation)

1. Seed cells onto the 96-well plates at the different density depending on the size of the cell and desired treatments and incubation time. The cells for testing should be around 75-90% confluent. The plates included in the Kit are sterile and treated for cell culture.
2. Culture and treat the cells as desired.
3. Fix cells by removing the cell growth culture medium, following with twice PBS rinse, and a final incubation with 100ul of 4% formaldehyde in PBS. The incubation can be kept around 25-30 minutes at room temperature. To minimize the vaporization of formaldehyde, the plates are sealed with parafilm. Note: Formaldehyde is very volatile, Wear appropriate personal protection equipments (mask, gloves and glasses) when using this chemical.
4. Remove the formaldehyde solution and rinse the cells three times with wash buffer. Each wash step should be minimum five minutes with gentle shaking on the shaker.
5. Add 50ul of PBS into each well of 96-well plate if no ELISA is performed right away and store the plate in the refrigerator for a short of period of time. If long-term storage desired, keep 4% formaldehyde solution and store the plates following the previously mentioned methods.

Cell-Based ELISA: Binding of Primary Antibody and Secondary Antibody

Note: Based on the experiment design, primary antibody incubation can be performed with different phospho-specific antibodies and total. Secondary antibody incubation can be performed with no primary antibody incubation as ELISA negative control.

1. Remove the final Wash buffer, add 100ul of Quench buffer and incubate for 20-25 minutes at room temperature, and seal, cover the plate with parafilm.
2. Remove the Quench buffer and rinse the cells three times for 5 minutes each with 200ul of wash buffer on the shaker.
3. Remove the Wash buffer and add 100ul of Blocking Buffer and incubate for 1-2 hours at room temperature. After blocking, wash the plates three times with Wash buffer for 5 minutes each wash.
4. Dilute the primary antibody with antibody dilution buffer according to the label on the antibody tube.
5. Add 50ul of primary antibody into each well on 96-well plate. Incubate the plate overnight. Seal the plate with parafilm or incubate the plate in a humid-box in refrigerator, and make sure the plate plated at the even level.
6. Remove the primary antibody, wash the wells three times for 5 minutes each with 200ul of Wash buffer with gentle shaking on the shaker.
7. Remove the Wash buffer, add 50ul of secondary antibody diluted in antibody dilution buffer each well, except the empty blank wells. Cover and seal the plates with parafilm, gentle shaking on the shaker for 1-2 hour at the room temperature.
8. During the incubation, prepare Substrate Developing Solution. Transfer the solution to another container and bring the solution to the room temperature.

Colorimetric Reaction

1. Remove the secondary antibody, wash cells 3-4 times for 5 minutes each time with 200ul of Wash buffer.
2. Add 50ul of Substrate Developing Solution to each well on the 96-well plate.
3. Incubate the plate for 5-25 minutes at room temperature protected from direct light. Closely monitor the blue color development. Don't over develop.
4. Add 50ul of Stop solution. The acidic solution turns blue color into yellow. Make sure that each well develops for the same amount of time.
   Note: Stop solution is corrosive, Wear personal protection equipment while using this solution.
5. Read the absorbance under a micro-plate reader at 450nm with optional reference wavelength of 665nm.

Optional: Crystal Violet Cell Staining

Crystal violet binds to cell nuclei and give absorbance reading at 595nm that is proportional to cell numbers. If normalization OD value from cell numbers is desired, follow the protocol listed below.

1. After finishing reading the absorbance at 450nm, wash the plate twice with 200ul of wash buffer and twice 1X PBS for 5 minutes each. Tap the plates on paper towel to remove the excess liquid. Let plate air dry.
2. Add 100ul of crystal violet solution to each well, incubate on the shaker at room temperature for 30 minutes.
3. Flick the plate to remove crystal violet solution, rinse the plate by filling the wells with tap running water, each wash 5 minutes for 3 times.
4. Add 100ul of 1% SDS into each well and incubate on the shaker at room temperature for 1 hour.
5. Read absorbance at 595nm under microplate reader. You can also dilute the supernatant from each well

if absorbance is greater than range.

6. OD450 reading can be normalized by cell number with this formula of OD450/OD595.

Suspension cell Protocol-Colorimetric Assay

The protocol for suspension cell based Cell-Based Phospho-Specific ELISA can be modified from adherent cell based Cell-Based Phospho-Specific ELISA by seeding and fixing the cells as follows.

1. Pre-treat the 96-well plate with 10ug/ml poly-L-Lysine for 30 minutes at 37 °C by adding 100ul of 10ug/ml poly-L-Lysine into each well on 96-well plate. Rinse the plate twice with PBS, each rinse for 5 minutes.
2. Seed the desired amount of cells for your experimental cells. Grow and treat the cells as your study requires.
3. Remove the cell culture medium and rinse with pre-warmed 1XPBS before formaldehyde fixation. Add 100ul of 8% formaldehyde solution for 20 minutes fixation incubation at room temperature.
4. Follow the rest of steps written in Adherent Cell Protocol.

Quick Chart for Buffer Preparation

Name of Buffer	How to make
Quench Buffer	1ml of 30% hydrogen peroxide in 29ml wash buffer
4% formaldehyde	3ml of 36% formaldehyde in 24ml 1XPBS
1XPBS	3ml of 10X PBS in 27ml distilled water
8% formaldehyde	6ml of 36% formaldehyde in 21ml 1XPBS
Secondary Antibody Work Solution	1ul of secondary antibody in 10ml of antibody dilution buffer
Primary Antibody Work Solution	25ul of primary antibody in 5ml of antibody dilution