WEBSITE www.immunoway.com
E-MAIL order@ immunoway.com
tech@ immunoway.com

CELL-BASED COLORIMETRIC ELISA PROTOCOL - FOR ACETYL-SPECIFIC PROTEIN

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 Acetyl-Specific and total-protein respectively. Required materials but not provided are listed on the data sheet.

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 20X Washing Buffer to 19 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 2X buffer. Simply dilute with 1XPBS. A small amount of white precipitate may appear if thawed in a warm bath. This doesn't interfere with performance.

Diluted acetyl-specific antibody The acetyl-specific antibody recognizes relative molecule acetylated at specific site. Reconstitute primary acetyl-specific antibody by dissolving lyophilized antibody into 5ml of antibody diluent. Each well contains 50ul of diluted primary antibody. This provided antibody can be diluted 1:400 in Primary Antibody Dilution Buffer (see Quick Chart for Preparing Buffers).

Diluted total-protein antibody This total-protein antibody recognized both the non- acetylated and the

acetylated forms of protein. Reconstitute the anti-total protein antibody by dissolving the lyophilized antibody in 5ml of antibody diluent. Each well contains 50ul of diluted primary antibody. The supplied antibody can be diluted 1:400 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).

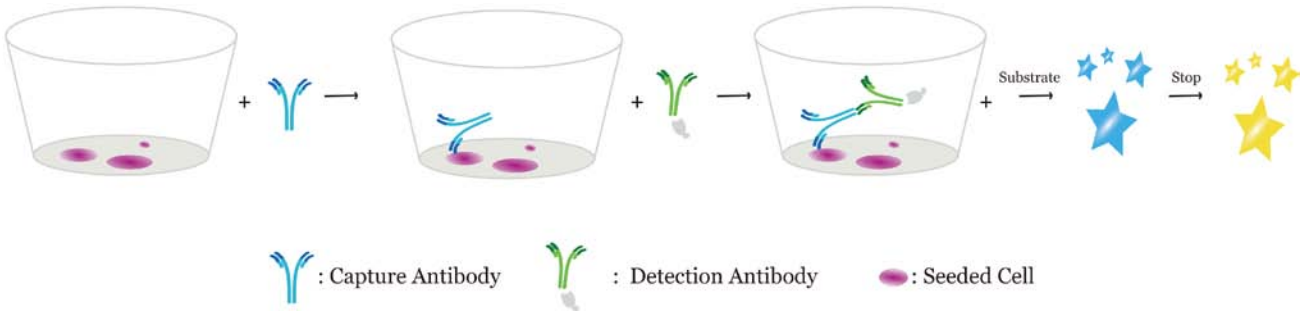
1%SDS Solution 1% SDS Solution is used in the Crystal Violet procedure to solubilize cells and release the dye for subsequence quantification at 595nm. This buffer is supplied as ready-to-use.

Crystal Violet Solution This is supplied ready-to-use. Crystal violet is used to determine the relative number of cells in each well. Crystal violet binds to DNA in cell nuclei. OD595 value is proportional to cell numbers.

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

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 25-30mins 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 acetyl-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 20-25mins 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 5mins 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 5-25mins 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 5mins 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 30mins.
3. Flick the plate to remove crystal violet solution, rinse the plate by filling the wells with tap running water, each wash 5mins 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

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

1. Pre-treat the 96-well plate with 10ug/ml poly-L-Lysine for 30mins 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 5mins.
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 20mins fixation incubation at room temperature.

4. Follow the rest of steps written in Adherent Cell Protocol.