Receptor Interacting Serine Threonine Kinase 2 Phospho-Ser176 (RIPK2 pS176) Cell ELISA Kit

Catalog No.: abx596663

Size: 96 tests

Detection Range: Qualitative

Sensitivity: Qualitative

Storage: Store all components at 4°C for up to 6 months.

Application: For qualitative detection of RIPK2 pS176 in cell samples.

Principle of the Assay

This kit is based on indirect enzyme-linked immuno-sorbent assay technology. Cells are seeded on to a 96-well plate. The cells are fixed and quenched, and the wells are blocked. The primary antibody specific to RIPK2 pS176, unphosphorylated RIPK2 or GAPDH antibody is added to each well, followed by incubation. HRP-conjugated secondary antibody is used as the detection antibody, which is added to each well, followed by incubation. Unbound conjugates are washed away using wash buffer. TMB substrate is used to visualize HRP activity. TMB is catalyzed by HRP to produce a blue colour product that changes to yellow after adding stop solution. The intensity of the yellow colour is proportional to the RIPK2 pS176 amount bound on the plate. The O.D. absorbance is measured spectrophotometrically at 450 nm in a microplate reader. Optionally, Crystal Violet can be added to measure the number of cells spectrophotometrically at 595 nm in a microplate reader. Results using the GAPDH antibody or the non-phosphorylated form of RIPK2 antibody serve as an internal positive control in normalizing the target absorbance values. Calculation of results does not require the use of both controls – the end user may determine which one to use.

Kit components

1. Uncoated 96-well microplate
2. TBS (10X): 24 ml
3. Quenching Buffer: 24 ml
4. Blocking Buffer: 50 ml
5. RIPK2-pS176 Rabbit polyclonal primary antibody (100X): 60 µl
6. RIPK2 Rabbit polyclonal primary antibody (100X): 60 µl
7. GAPDH Mouse monoclonal primary antibody (100X): 60 µl
8. HRP-conjugated anti-Rabbit IgG antibody: 12 ml
9. HRP-conjugated anti-Mouse IgG antibody: 12 ml
10. Primary Antibody Diluent Buffer: 12 ml
11. Crystal Violet solution: 12 ml
12. TMB substrate: 12 ml
13. Stop solution: 12 ml
14. SDS solution: 24 ml
15. Wash Buffer (15X): 50 ml
16. Plate Sealer: 2

Materials Required But Not Provided

1. 37°C incubator
2. Microplate reader (wavelengths: 450 nm, and 595 nm if using optional Crystal Violet stain)
3. Multi- and single-channel pipettes and sterile pipette tips
4. Squirt bottle or automatic microplate washer
5. ELISA orbital plate shaker
6. Deionised or distilled water
7. Fixing solution (4% or 8% formaldehyde)
8. Poly-L-Lysine (if using cell suspension)
9. 1.5 ml tubes (for sample dilution preparation)
10. Absorbent filter paper
11. 100 ml and 1 L graduated cylinders
12. Parafilm

Protocol

A. Experimental Design and Preparation of Samples and Reagents

1. Experimental Design and Sample Preparation

- The number of cells plated on to the 96-well plate will depend on the level of expression of RIPK2-pS176 in the cells, cell size, treatment conditions and incubation time. Cells should be around 75 – 90% confluent when testing. Typically for HeLa cells, it is recommended to seed between 20,000 and 30,000 cells per well overnight for treatment the following day. This ELISA kit can detect RIPK2-pS176 expression in as little as 5,000 HeLa cells.
- Cells can be treated with inhibitors, activators, stimulators (e.g. chemicals, proteins, peptides) or a combination of these. Cells can be treated with UV and serum starvation if required to meet experimental needs.
- Samples that contain NaN3 cannot be detected as it interferes with HRP.

2. Reagent Preparation

- **Wash Buffer (1X)**
  Dilute the 15X concentrated Wash Buffer 15-fold with Distilled water (i.e. add 50 ml 15X Wash Buffer to 700 ml Distilled water).

- **TBS Solution (1X)**
  Dilute the 10X concentrated TBS 10-fold with Distilled water (i.e. add 24 ml 10X TBS to 216 ml Distilled water).

- **Primary Antibody Dilutions**
  The three primary antibodies (RIPK2-pS176 Rabbit polyclonal primary antibody, RIPK2 Rabbit polyclonal primary antibody, and GAPDH Mouse monoclonal primary antibody) are provided as 100X concentrations. Dilute 100-fold using the Primary Antibody Diluent Buffer as needed. Each well tested will require 50 µl RIPK2-pS176 Rabbit polyclonal primary antibody, or 50 µl of RIPK2 Rabbit polyclonal primary antibody (or, alternatively 50 µl of GAPDH Mouse monoclonal primary antibody).
  Allow for 50 – 100 µl more antibody than is needed. Any unused diluted antibody solutions can be stored for up to 2 weeks at 4°C.

B. Assay Procedure

1. Seed 200 µl of 20,000 adherent cells in culture medium in each well of a sterile 96-well plate. For suspension cells and loosely attached cells, add 100 µl of 10 µg/ml Poly-L-Lysine to each well of the 96-well plate. Incubate at 37°C for 30 min prior to adding cells.
2. Incubate the cells overnight at 37°C, 5% CO2.
3. Treat the cells as desired.
4. Remove the cell culture medium and rinse with 200 µl of 1X TBS. Repeat this again for a total of 2 times.

5. Fix the cells by adding 100 µl of fixing solution and incubating for 20 minutes at room temperature. A solution of 4% formaldehyde is recommended for adherent cells and 8% formaldehyde is recommended for suspension cells and loosely attached cells. Seal the plate with Parafilm during incubation.
6. Remove the Fixing solution and wash the plate 3 times with 1X Wash Buffer. Fill each well completely with 1X Wash Buffer (200 µl) using a multi-channel pipette or autowasher. A 5 minute soaking period with gentle shaking on an orbital shaker is recommended. After the final wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and tap **gently** against clean absorbent paper towels. Complete removal of liquid at each step is essential for good performance.
7. Add 100 µl of Quenching Buffer and incubate for 20 minutes at room temperature.
8. Wash the plate 3 times as directed in Step 6.
9. 200 µl of Blocking buffer and incubate for 1 hour at room temperature.
10. Wash the plate 3 times as directed in Step 6.
11. For each condition tested, mark the position of a test well, positive control well, and negative control well. *It is recommended that each of these is run in duplicate or triplicate*. Add 50 µl of 1X RIPK2-pS176 Rabbit polyclonal primary antibody to the test well. Add 50 µl of RIPK2 Rabbit polyclonal primary antibody (or, alternatively 50 µl of GAPDH Mouse monoclonal primary antibody) to the positive control well. Cover the plate with Parafilm and incubate overnight at 4°C. If the target expression is known to be high, incubate for 2 hours at room temperature with gentle shaking on an orbital shaker.
12. Wash the plate 3 times as directed in Step 6.
13. Add 50 µl of the appropriate 1X secondary antibody to the wells. HRP-conjugated Anti-Rabbit IgG antibody should be added to wells containing RIPK2-pS176 or RIPK2 rabbit polyclonal antibody. HRP-conjugated Anti-Mouse IgG antibody should be added to wells containing GAPDH mouse monoclonal antibody. The negative control wells should now contain just 50 µl of the same secondary antibody used for the test and positive controls. Cover the plate with Parafilm and incubate for 1.5 hours at room temperature with gentle shaking on an orbital shaker.
14. Wash the plate 3 times as directed in Step 6.
15. Add 50 µl of TMB substrate into each well. Cover the plate and incubate at room temperature in the dark for 30 minutes with gentle shaking on an orbital shaker.
16. Add 50 µl of Stop solution into each well. Gently tap the plate to ensure thorough mixing. Ensure that there are no fingerprints or water on the bottom of the plate, and that the fluid in the wells is free of bubbles. Measure the absorbance at 450 nm immediately.

Optional: Crystal Violet Cell Staining

17. After reading the absorbance at 450 nm, wash the plate 2 times with 1X Wash buffer (200 µl), and then 2 times with 1X TBS (200 µl), 5 minutes each time. After the final wash, remove any remaining Wash Buffer by aspirating for 5 minutes or decanting. Invert the plate and tap gently against clean absorbent paper towels. Complete removal of liquid at each step is essential for good performance.
18. Add 50 µl of Crystal Violet solution to each well. Incubate for 30 minutes at room temperature with gentle shaking on an orbital shaker.
19. Remove the Crystal Violet solution by decanting into a beaker. Wash the plate by submerging the plate into a beaker full of water. Carefully rinse the wells in distilled water until no more colour comes off the wells. Allow the plate to dry for 30 minutes.
20. Warm the SDS solution to room temperature, then add 100 µl of SDS solution into each well. Incubate for 1 hour at room temperature with gentle shaking on an orbital shaker.

21. Ensure that there are no fingerprints or water on the bottom of the plate. Measure the absorbance at 595 nm immediately. If the absorbance is very high, the solubilised Crystal Violet solution can be diluted 10X with distilled water on a separate 96-well plate.

C. Precautions

1. Before using the kit, centrifuge the tubes briefly to bring down any contents trapped in the lid.
2. For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.
3. The wash buffer may crystallise and separate. If this happens, please warm the tube and mix gently to dissolve.
4. Avoid foaming or bubbles when mixing or reconstituting components.
5. It is recommended to assay all controls and samples in duplicate or triplicate.
6. Do NOT let the plate dry out completely during the assay as this will inactivate the biological material on the plate.
7. Ensure plates are properly sealed or covered during incubation steps.
8. Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings.
9. To avoid cross contamination do not reuse pipette tips and tubes.
10. Do not use expired components or components from a different kit.
11. The TMB Substrate solution is easily contaminated; work under sterile conditions when handling the TMB substrate solution. The TMB Substrate solution should also be protected from light. Unreacted substrate should be colourless or very light yellow in appearance. Aspirate the dosage needed with sterilized tips and do not dump the residual solution back into the vial.
12. The Fixing solution is volatile and the Stop solution is corrosive. Wear Personal Protective Equipment (PPE) such as lab coats, goggles, mask and gloves when handling these solutions.
13. Vigorous pipetting my cause cells to detach from the plate. Pipette solutions and aspirate gently.

D. Data Normalisation

- **RIPK2 Normalisation**

  OD450 values for the target protein can be normalised using the OD450 values obtained for RIPK2, using the proportion (OD450 RIPK2-pS176 / OD450 RIPK2).

- **GADH Normalisation**

  OD450 values for the target protein, both phosphorylated and un-phosphorylated can be normalised using the OD450 values obtained for GAPDH.

- **Crystal Violet Staining Normalisation**

  The measured OD450 values can be normalised using the OD595 values using (OD450 / OD595).