abbexa®

AMPKα (pT172) Cell ELISA Kit

Catalog No.: abx595976

Size: 96T

Range: Qualitative

Sensitivity: Qualitative

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

Application: For qualitative detection of pT172 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 pT172, AMPKα antibody or GAPDH antibody is added to each well, followed by incubation. Fluorescent dye-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. The RFU of each well is measured at excitation/emission wavelengths 651/667 nm (Dye 1) or at 495/521 nm (Dye 2).

Kit components

1. 2 × 96-well black cell culture microplate
2. TBS (10X): 24 ml
3. Quenching buffer: 24 ml
4. Blocking buffer: 2 × 24 ml
5. GAPDH mouse monoclonal primary antibody (100X): 110 µl
6. pT172 rabbit polyclonal primary antibody (100X): 60 µl
7. AMPKα rabbit polyclonal primary antibody (100X): 60 µl
8. Dye 1-conjugated Anti-Rabbit IgG Antibody: 6 ml
9. Dye 2-conjugated Anti-Mouse IgG Antibody: 6 ml
10. Primary antibody diluent buffer: 12 ml
11. Wash buffer (30X): 30 ml
12. Plate Sealer: 2

Material Required But Not Provided

1. 37°C incubator
2. Fluorescent plate reader (two channels at Ex/Em: 651/667 & 495/521 nm)
3. Multi and single channel pipettes and sterile pipette tips
4. Squirt bottle or automated microplate washer
5. ELISA orbital shaker
6. Deionised or distilled water
7. Fixing solution (4% or 8% formaldehyde)
8. Poly-L-Lysine (if using suspension cells)
9. 1.5 ml tubes to prepare standard/sample dilutions
10. Absorbent filter papers
11. 100 ml and 1 liter graduated cylinders
12. Parafilm

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Protocol

A. Experimentatal design and Preparation of sample 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 pT172 in the cells, cell size, treatment conditions and incubation time. Cells should be around 75-90% confluent when testing.
- Cells can be treated with inhibitors, activators, stimulators (e.g. chemicals, proteins, peptides) or a combination of these. Stimulation of cells should be controlled.
- Positive control: Mouse Anti-GAPDH Antibody is used as an internal positive control to normalize the RFU values of the target protein in each well.
- Negative control: Add 50 µl of primary antibody diluent buffer instead of the primary antibody mixture solution. Add the 50 µl secondary antibody mixture solution as described in the assay procedure (i.e. only the secondary antibody mixture solution is incubated).
- Blank control: Add 50 µl of primary antibody diluent buffer instead of the primary antibody mixture solution or the secondary antibody mixture solution (i.e. no antibody mixture solution is incubated).

2. Wash buffer and TBS

- Dilute the concentrated Wash buffer 30-fold (1/30) with distilled water (i.e. add 5 ml of concentrated wash buffer into 145 ml of distilled water).
- Dilute the concentrated TBS 10-fold (1/10) with distilled water (i.e. add 10 ml of concentrated TBS into 90 ml of distilled water).

3. Preparation of non-phosphorylated primary antibody mixture solution: prepare no more than 15 min. before the experiment.
Add 50 µl of 100X AMPKα antibody and 50 µl of 100X GAPDH antibody into 4900 µl of Primary antibody diluent buffer, and mix thoroughly.

4. Preparation of phosphorylated primary antibody mixture solution: prepare no more than 15 min. before the experiment.
Add 50 µl of 100X pT172 antibody and 50 µl of 100X GAPDH antibody into 4900 µl of Primary antibody diluent buffer, and mix thoroughly.

5. Preparation of secondary antibody mixture solution: prepare no more than 15 min. before the experiment.
Add 3 ml of Dye 1-conjugated Anti-Rabbit IgG Antibody and 3 ml of Dye 2-conjugated Anti-Mouse IgG Antibody together, and mix thoroughly. This solution is light sensitive and should be kept in the dark.

B. Assay Procedure

1. Seed 200 µl of the desired cell concentration 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% CO_2.
3. Treat the cells as desired. Please consider the treatment method as cell treatment may result in cell death.
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.

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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 3 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. Add 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. Add 50 µl of non-phosphorylated primary antibody mixture solution to the appropriate wells for AMPKα detection. Add 50 µl of phosphorylated primary antibody mixture solution to the appropriate wells for pT172 detection. Cover the plate with Parafilm and incubate for 16 hours (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 secondary antibody mixture solution antibody to the appropriate wells. Cover the plate with Parafilm and incubate for 1.5 hours at room temperature with gentle shaking on an orbital shaker.
14. Keep the plate in the dark during this step. Wash the plate 3 times as directed in Step 6. Afterwards, rinse with 200 µl of 1X TBS.
15. Read the plate at Ex/Em 651/667 nm (Dye 1) and 495/521 nm (Dye 2). Avoid direct light exposure.

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. Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings.
7. To avoid cross contamination do not reuse pipette tips and tubes.
8. Do not use expired components or components from a different kit.
9. 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.
10. Vigorous pipetting my cause cells to detatch from the plate. Pipette solutions and aspirate gently.

D. Data Normalisation

GAPDH Normalisation: RFU values for the target protein can be normalised using the RFU values obtained for GAPDH.

Antibody Normalisation: RFU values for the phosphorylated and non-phopsphorylated target protein can be normalised using (RFU Phosphorylated Antibody / RFU Non-Phosphorylated Antibody), assuming that the RFUs were derived from the same read under the same excitation and emission wavelengths.

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Antibody Normalisation: The measured OD_{450} values can be normalised using the OD_{595} values using (OD_{450} / OD_{595}).

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