Collagen Type IV Alpha 2 (COL4A2) Cell ELISA Kit

Catalog No.: abx595136

Size: 96T

Range: Qualitative

Sensitivity: Qualitative

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

Application: For qualitative detection of COL4A2 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 COL4A2 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 catalysed 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 COL4A2 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 serve as an internal positive control in normalising the target absorbance values.

Kit components

1. Uncoated 96-well microplate
2. TBS (10X): 30 ml
3. Quenching buffer: 24 ml
4. Blocking buffer: 2 × 25 ml
5. GAPDH mouse monoclonal primary antibody (100X): 60 µl
6. COL4A2 rabbit polyclonal primary antibody (100X): 60 µl
7. HRP-conjugated Anti-Rabbit IgG Antibody: 6 ml
8. HRP-conjugated Anti-Mouse IgG Antibody: 6 ml
9. Crystal Violet solution: 6 ml
10. Primary antibody diluent buffer: 12 ml
11. TMB substrate: 12 ml
12. Stop solution: 12 ml
13. SDS solution: 24 ml
14. Wash buffer (30X): 25 ml
15. Plate Sealer: 2

Material Required But Not Provided

1. 37°C incubator
2. Microplate reader (wavelength: 450 nm and 595 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%, 8% or 37% paraformaldehyde)
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

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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 COL4A2 in the cells, cell size, treatment conditions and incubation time.
- 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 NaN_3 cannot be detected as it interferes with HRP.

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 GAPDH primary antibody working solution: prepare no more than 30 min. before the experiment.
a.) Calculate the total volume of 1X GAPDH antibody required: 50 µl / well × quantity of wells. Allow 50-100 µl more than the total volume.
b.) Dilute the 100X GAPDH antibody with Primary antibody diluent buffer at 1/100 and mix thoroughly (i.e. Add 25 µl of 100X GAPDH antibody into 2475 µl of Primary antibody diluent buffer).

4. Preparation of COL4A2 primary antibody working solution: prepare no more than 30 min. before the experiment.
a.) Calculate the total volume of 1X COL4A2 antibody required: 50 µl / well × quantity of wells. Allow 50-100 µl more than the total volume.
b.) Dilute the 100X COL4A2 antibody with Primary antibody diluent buffer at 1/100 and mix thoroughly (i.e. Add 25 µl of 100X COL4A2 antibody into 2475 µl of Primary antibody diluent buffer).

B. Assay Procedure

1. Seed 100 µl of 20,000 adherent cells in each well of a sterile 96-well plate. Add solutions slowly from the side wall of the wells. The optimal seeding concentration should be determined by the end user.
   For suspension cells and loosely attached cells: Add 20 µl of Poly-L-Lysine to each well of the 96-well plate and allow to stand for 5 minutes. Remove the solution by aspiration and rinse once with tissue culture grade water. Allow to dry for 2 hours before seeding cells. If the cell line is known to digest Poly-L-Lysine, Poly-D-Lysine may be used as an alternative.
2. Incubate the cells for at least 6 hours (or 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. Remove the cell culture medium gently.
4. For loosely attached cells, skip this step and move to Step 5. Rinse each well twice with 200 µl of 1X TBS. Remove the 1X TBS gently.
5. Fix the cells by adding 100 µl of Fixing solution and incubating for 20 minutes at room temperature. A solution of 4% paraformaldehyde is recommended for adherent cells and 8% paraformaldehyde is recommended for suspension cells and loosely attached cells. If 37% paraformaldehyde is used, dilute to the desired percentage using 1X PBS.
6. Remove the Fixing solution and wash the plate 3 times with 1X Wash buffer. Fill each well completely with 1X Wash buffer (300 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.

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7. Add 100 µl of Quenching buffer into each well 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 into each well and incubate for 1 hour at room temperature.
10. Wash the plate 3 times as directed in Step 6.
11. Add 50 µl of 1X primary antibody (COL4A2 antibody and/or GAPDH antibody) to the appropriate wells. Cover the plate 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 1X secondary antibody to the appropriate wells. HRP-conjugated Anti-Rabbit IgG antibody should be added to wells containing COL4A2 rabbit polyclonal antibody. HRP-conjugated Anti-Mouse IgG antibody should be added to wells containing GAPDH mouse monoclonal antibody. Cover the plate 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 with the plate sealer. Gently tap the plate to mix thoroughly. Incubate at room temperature for 15-20 minutes with gentle shaking on an orbital shaker. The incubation time is for reference only, the optimal time should be determined by end user. Avoid exposure to light.
16. Add 50 µl of Stop solution into each well. The colour should change to yellow. 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.
17. After reading the absorbance at 450 nm, wash the plate 3 times as directed in Step 6. Allow the plate to air dry for 5 minutes at room temperature.
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.

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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 colorless 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, the Stop solution is corrosive, and the Crystal Violet solution is an intense stain. Wear Personal Protective Equipment (PPE) such as lab coats, goggles, mask and gloves when handling these solutions.
13. Vigorous pipetting my cause cells to detatch from the plate. Pipette solutions and aspirate gently.

D. Data Normalisation

GAPDH Normalisation: OD_{450} values for the target protein can be normalised using the OD_{450} values obtained for GAPDH.

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

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