SYSY Antibodies

Tailor-made Antibodies
and Tools for Life Science

Protocol for EEA1 Antibody (Cat. No. 237 002)
Western Blot (WB)
AP Detection

In standard Western blot approaches, protein samples are separated according to their molecular weight with denaturing SDS-PAGE (polyacrylamide gel electrophoresis), transferred to a membrane and analyzed by immuno-detection with antibodies. Chromogenic alkaline phosphatase (AP) staining is a cumulative detection system. The color precipitate can easily be observed during development, and the staining reaction can be stopped when the desired signal strength is reached. Compared to enhanced chemiluminescent (ECL) detection, AP staining is less sensitive, but does not require special imaging equipment for visualization of the assay results.

Materials and reagents

- **Membrane staining solution (optional):** suitable staining solution (e.g. Ponceau S)
- **TBST - skimmed milk:** (20 mM Tris, 150 mM NaCl, 5% (w/v) skimmed milk powder, 0.1% Tween 20), pH 7.4
- **Substrate buffer for alkaline phosphatase:** (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$), pH 9.5
- **BCIP staining solution:** 20 mg/ml in 100% di-methyl formamide
- **NBT staining solution:** 50 mg/ml in 70% di-methyl formamide
- **Staining solution complete:** Substrate buffer containing 80 µl BCIP solution and 60 µl NBT solution per 10 ml. Prepare this solution shortly before use.
- **Primary antibody:** EEA1 antibody (cat. no. 237 002)
- **Secondary antibody:** Alkaline phosphatase (AP) conjugated rabbit specific secondary antibody

Procedure

1. Prepare samples (10-20 µg / lane for a 10 lane mini-gel) in reducing sample buffer according to the manufacturer recommendations. If purified proteins or peptides are loaded use 1ng-1µg / lane for a 10 lane mini gel.

2. Incubate samples for 5 min at 95°C.

3. Separate the protein samples to be examined next to a molecular weight standard using SDS-PAGE and transfer to a nitrocellulose membrane by electro-blotting. Follow the manufacturer instructions for your SDS-PAGE and blotting device.

4. *Optional*: *Stain the membrane with membrane staining solution to check protein transfer.*

5. Incubate in **TBST - skimmed milk** for 30 min on an orbital shaker at RT.

6. Incubate in fresh **TBST - skimmed milk** containing the **primary antibody** at a dilution of **1:1000 to 1:5000** for at least 2 h on an orbital shaker at RT or overnight at 4°C.

7. Wash 3-4 times with **TBST - skimmed milk** for 10 min each.

8. Incubate with fresh **TBST - skimmed milk** containing the **secondary antibody** diluted according to the manufacture recommendations for at least 1 h on an orbital shaker.

9. Wash 3 times with **TBST - skimmed milk** for 10 min each.
10. Briefly wash with **substrate buffer.**
11. Replace with fresh **substrate buffer** and equilibrate for 5 min.
12. Replace with freshly prepared **staining solution complete** and develop for 5-30 min. Time can be shortened or extended if signals are extremely strong or weak, resp.
13. Stop staining reaction by washing 3 times with H_2O.

Remarks

For pre-adsorption specificity tests, please refer to our general pre-adsorption protocol.

Note: This protocol has been validated in the SYSY Antibodies laboratories to ensure consistent and reliable staining results. However, for achieving the best specific signal with minimal background, the optimal antibody concentration, incubation temperature, and incubation duration should be optimized for each experiment.