# Protocol for EEA1 Antibody (Cat. No. 237 105)
# Western Blot (WB)
# ECL Detection

In standard Western blot (WB) 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. Enhanced chemiluminescent (ECL) detection systems and substrates have different sensitivities and have a narrow linear detection range that can be used for protein quantification. In general, the experiment has to be carefully optimized for reliable 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
- **Washing solution A:** (20 mM Tris, 150 mM NaCl, 0.1% Tween 20), pH 7.4
- **Washing solution B:** (20 mM Tris, 150 mM NaCl), pH 7.4
- **Primary antibody:** EEA1 antibody (cat. no. 237 105)
- **Secondary antibody:** Horseradish conjugated Guinea pig specific secondary antibody.
- **Substrate solution:** Western Lightning® Plus-ECL PerkinElmer, Inc. or comparable product

## Procedure

1. Prepare samples (10-20 µg / lane for a 10 lane mini-gel) in reducing sample buffer according to the manufacturer recommendation. 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** for at least 2 h on an orbital shaker at RT or overnight at 4°C. The suggested dilution has been determined for AP staining. The ideal antibody concentration for ECL detection needs to be adjusted individually.

7. Wash 3-4 times with **washing solution A** for 10 min each.

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

9. Wash 3 times with **washing solution A** for 10 min each.

10. Replace **washing solution A** with **washing solution B** and equilibrate for 5 min.

11. Replace with fresh **substrate solution** and develop (X-ray film or ECL-reader). Exposure time can be shortened or extended, if signals are extremely strong or weak, resp.

## Remarks

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

A very weak signal may be caused by the primary and/or secondary antibody concentration being too high. The ECL substrate solution has a limited capacity, and high amounts of local peroxidase can use up all the substrate within seconds before the picture is taken in your ECL reader.
Please try a lower concentration of primary and secondary antibodies in this case.

*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.*