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

In standard Western blot (WB) approaches, denatured protein samples are separated according to their molecular weight with SDS-PAGE (polyacrylamide gel electrophoresis) and transferred to a membrane. The analysis of different organs, cell-types, and subcellular fractions like membranes, versus cytosol or different organelles may also provide useful information about differential protein expression levels. Fluorescent detection systems have a broad linear detection range that is a prerequisite for accurate protein quantification. However, they are less sensitive compared to enhanced chemiluminescent (ECL) detection.

Materials and reagents

- **Membrane staining solution (optional):** suitable staining solution (e.g. Ponceau S)
- **TBS - skimmed milk:** (20 mM Tris, 150 mM NaCl, 5% (w/v) skimmed milk powder), pH 7.4
- **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. 237 002)
- **Secondary antibody:** Fluorophore conjugated rabbit specific secondary antibody compatible with your detection device.
- **Blotting membrane:** nitrocellulose or special low fluorescent PVDF membrane.

Procedure

Standard secondary antibody

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 blotting membrane by electro-blotting. Follow the manufacturer instructions for your SDS-PAGE and blotting device.

   Important: Do not run the blue loading dye running-front out of the gel but stop gel before; then cut it off the gel before transfer. If that is not possible please use a compatible orange loading dye like "Orange Loading Dye" available from LICORbio.
   Clean your blotting device with methanol to remove all remains from previous gel runs. Blue loading front dye may have accumulated in your device and will end up on the membrane leading to high background.
   Use fresh transfer pads/sponges or whatman paper only.
   Do not write with blue or black ink/ball pens on membranes. Use pencils if necessary! If you use pens, the ink will run and cover the whole blot during the antibody incubation steps. This will cause high background in the 700nm channel.

4. *Optional*: *Stain the membrane with membrane staining solution to check protein transfer. PonceauS is not recommended*

since the often contain fluorescent contaminants. If you need to stain after transfer please consider staining with "Revert Total Protein Stain" available from LICORbio.

5. Incubate in **TBS - skimmed milk** for 30 min on an orbital shaker at RT. **Important:** No detergent should be included in this step. Always use clean incubation boxes. Traces of Coomassie or PonceauS can cause high background.

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. The suggsted dilution has been determined for AP staining. The ideal antibody concentration for fluorescent detection has 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 instructions 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 let equilibrate for 5 min.

11. Scan your membrane.

FluoTag based secondary antibodies

NanoTag's unique FluoTags coupled to fluorescent dyes are ideal for time saving and quantitative western blotting with high precision.
Since single domain antibodies (sdAbs) are monovalent, they can be directly incubated with purified primary antibodies without forming non-functional clusters. Unpurified formats like antiserum, culture supernatants and ascites are not recommended.

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 sample to be examined and a molecular weight standard using SDS-PAGE and transfer to blotting membrane by electro-blotting. Follow the manufacturer instructions for your SDS-PAGE and blotting device.

   Important: Do not run the blue loading dye running-front out of the gel but stop gel before; then cut it off the gel before transfer. If that is not possible please use a compatible orange loading dye like "Orange Loading Dye" available from LICORbio.
   Clean your blotting device with methanol to remove all remains from previous gel runs. Blue loading front dye may have accumulated in your device and will end up on the membrane leading to high background.
   Use fresh transfer pads/sponges or whatman paper only
   Do not write with blue or black ink/ball pens on membranes. Use pencils if necessary! If you use pens, the ink will run and cover the whole blot during the antibody incubation steps. This will cause high background in the 700nm channel.

4. *Optional*: *Stain the membrane with membrane staining solution to check protein transfer. PonceauS is not recommended since the often contain fluorescent contaminants. If you need to stain after transfer please consider staining with "Revert Total Protein Stain" available from LICORbio.*

5. Incubate in **TBS - skimmed milk** for 30 min on an orbital shaker at RT. **Important:** No detergent should be included in this step. Always use clean incubation boxes. Traces of Coomassie or PonceauS can cause high background.

6. While blocking the membrane, pre-form complexes comprising primary antibodies and the secondary FluoTag reagent in a separate tube. For this, incubate 1 µg of your **primary antibody** with 4 µl of your FluoTag in 50 µl **TBST - skimmed milk** for 30 min in the dark.

7. Incubate your membrane in fresh **TBST - skimmed milk** containing the primary antibody fluoTag complex at a dilution corresponding to a **1:1000 to 1:5000** of the primary antibody for at least 2 h on an orbital shaker at RT or overnight at 4°C. The suggsted dilution has been determined for AP staining. The ideal antibody concentration for fluorescent detection has to be adjusted individually.

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

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

10. Scan the membrane.

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.