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.
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.

Important: Some proteins have special requirements for good separation (e.g. unboiled samples or special gel systems). Please refer to the **remarks** sections for western blotting on the respective data sheet.

Materials and reagents

- **Ponceau S staining solution:** 5% acetic acid, 0.1% Ponceau S
- **5% skimmed milk in Tris buffered saline with Tween 20 (5% skimmed milk-TBST):** 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% (w/v) skimmed milk powder, 0.02% sodium azide, 0.1% Tween 20
- **Substrate buffer for alkaline phosphatase:** 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$
- **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
- Alkaline phosphatase (AP) conjugated secondary detection reagent

Procedure

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

1. Stain the membrane with **Ponceau S staining solution** for several minutes at room temperature to check the efficiency of transfer.
2. Rinse the membrane in water to remove the Ponceau S staining solution and incubate in **5% skimmed milk-TBST** for 30 min on a lab shaker at RT.
3. Incubate in fresh **5% skimmed milk-TBST** containing the primary antibody at the appropriate dilution for at least 2 h on a lab shaker at RT or over-night at 4°C.
4. Wash 3-4 times with **5% skimmed milk-TBST** for 10 min each time.
5. Incubate with fresh **5% skimmed milk-TBST** containing the recommended AP-conjugated secondary antibody (anti-mouse IgG, anti-rabbit IgG, resp.) at the appropriate dilution for at least 1 h on a lab shaker.
6. Wash 3 times with **5% skimmed milk-TBST** for 10 min each time.
7. Wash with **substrate buffer** and equilibrate for 5 min.
8. Replace with fresh **staining solution complete** and develop for 15-30 min. Time can be shortened or extended if signals are extremely strong or weak, resp.
9. Stop staining reaction by washing 3 times with H_2O.

Note: *The SYSY standard protocol generates good results in the SYSY labs and may be used as a reference. However, to achieve the highest specific signal and lowest non-specific background signal, the best antibody concentration, incubation temperature, and incubation time must be determined individually.*

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