ELISA: Sandwich ELISA Protocol - Membrane Proteins

Materials

- Maxisorb 96-well plate
- Goat anti-mouse IgG, unconjugated
- Goat anti-rabbit IgG, peroxidase conjugated
- Specific monoclonal mouse antibody as capture antibody
- Specific polyclonal rabbit antibody as detector antibody
- Protease inhibitors (suggested: 1 mM PMSF, 1 µg/ml Aprotinin, 1.5 µM Pepstatin A)
- Microplate absorbance reader with filters at 450 nm and a reference wavelength (e.g. 620-650 nm)

Reagents

- **Coating buffer:** 0.1 M Na-carbonate, pH 9.6 (store 0.5 M stock at -20°C)
- **Blocking buffer A:** 1% tryptone/peptone from casein (TP) in 0.1 M Na-carbonate, pH 9.6
- **Washing buffer:** Tris buffered saline (TBS) with 0.05% Tween 20 (TBST)
- **Solubilization buffer A:** 10% sodium dodecyl sulfate (SDS) in PBS
- **Solubilization buffer B:** 1.2% Triton X-100 in PBS
- **Antigen buffer A:** 0.2% Triton X-100/0.05% TP in TBS
- **Antigen buffer B:** 0.05% TP in TBS
- **Blocking buffer B:** 0.5% TP/0.5% BSA/0.5% gelatin in TBST
- **Substrate solution:** Tetramethylbenzidine (TMB) reagent for development
- **Stop solution:** 0.25 M H_2SO_4 to stop development

Procedure

1. Coat 96-well microplate with 100 µl goat anti-mouse IgG (1 µg/ml) in **coating buffer** and incubate for 3 h at RT and 700 rpm.
2. Block the surface with **blocking buffer A** for 1 h at RT and 700 rpm.
3. Wash the plate three times with **washing buffer** (at least 5 min per wash) and transfer them to 4°C.
4. Apply **monoclonal capture antibody** diluted in **washing buffer** and incubate overnight at 4°C. Dilute ascites 1:4000, purified antibody 1:2000 (50-75 ng/well).
5. **Antigen solubilization**: Adjust protein standard and samples to 3 mg/ml total protein and a final SDS concentration of 1.2% with **solubilization buffer A** and rotate 15 min at RT. Add 5 volumes of ice-cold **solubilization buffer B** to each sample and rotate 15 min at 4°C. Pellet the insoluble fraction at 100,000x g for 30 min (acceptable alternative: 13,000 rpm for 30 min at 4°C in a tabletop centrifuge) and transfer the supernatant to a new tube. Dilute the supernatant in antigen buffer B to 0.2% Triton X-100 concentration.
   Note: If complete tissue samples are used, DNase should be added as 0.1 µg/µl together with protease inhibitors, and SDS should be added as last component after mixing everything else.
6. Wash the plate once with **washing buffer**, twice with **antigen buffer A** at RT.
7. Apply antigen diluted in **antigen buffer A** and incubate for 2 h at RT and 700 rpm.
8. Wash twice with **antigen buffer A**, once with **blocking buffer B**.
9. Incubate with **blocking buffer B** for 30 min at RT.
10. Apply **polyclonal detector antibody** diluted in **blocking buffer B** (dilution 1:1000 up to 1:8000) and incubate for 1 h at RT and 700 rpm.
11. Wash three times with **blocking buffer B**.
12. Incubate with **HRP-coupled goat anti-rabbit antibody**, diluted 1:10,000 in **blocking buffer B**, for 1 h at RT and 700 rpm.
13. Wash three times with **washing buffer**.
14. Add 100 µl **substrate solution** for development.
15. Stop the reaction after 30 min with 100 µl **stop solution** and read the absorbance at 450 nm (ref: 620-650 nm).

Reference

Geumann C, Grønborg M, Hellwig M, Martens H & Jahn R (2010). A sandwich enzyme-linked immunosorbent assay for the quantification of insoluble membrane and scaffold proteins. Analytical Biochemistry 402: 161-9.