Agarose-Acrylamide Composite Gel Electrophoresis (SDS-AgPAGE)

- Steve Fernandes

Materials & Reagents:

- Life Technologies gel cassettes (#NC2015)
- 15-ml conical tubes
- Laboratory utility oven set to 65°C
- Agarose (Lonza Seakem Gold #50152)
- Freshly prepared, 40% ammonium persulfate (APS)
- Biorad "30% Acrylamide/Bis Solution" (#161-0158, 37.5:1 acrylamide:bis-acrylamide)
- 2 M Tris-HCl (pH 8.1)
- 8 M urea in water
- TEMED
- Glycerol
- Loading Buffer: 3 ml NuPAGE LDS sample buffer (LifeTechnologies NP0007) plus 2 ml 1 M dithiothreitol (DTT)
- Gel electrophoresis apparatus with power supply
- iBlot Gel Transfer Device and iBlot Transfer Stacks (LifeTechnologies, or equivalent)
- Running Buffer : To 850 ml water add 96 ml of 2 M Tris-Borate pH 7.6, 10 ml 100 mM EDTA and 10 ml 10% SDS. Bring to 1 L. Final pH = 8.2
- Phosphate-buffered saline/0.1% Tween 20 (PBST): 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1% Tween 20
- Blocking Solution: 5% w/v Blotto (nonfat dry milk) in PBST

Cast gel:

1. Place in the empty gel cassettes in the 65°C oven.
2. Pipette 10.3 ml 2 M Tris-HCl (pH 8.1), 8.9 ml water and 27.5 ml 8 M urea into the bottom of a 125-ml Erlenmeyer flask being careful not to wet the sides of the flask. Swirl gently but thoroughly.
3. Add 1.1 g of agarose, swirl gently, wait 2-3 min, microwave on "high" for 30sec. Swirl to dissolve agarose completely (microwave additional 15 sec if necessary).
4. Place the thoroughly mixed agarose solution in the oven. Add 2.8 ml of 30% Acrylamide and swirl.
5. Add 5.5 ml glycerol and thoroughly pipette for homogeneous mixing.
6. Add 18.5 µL TEMED. Swirl and immediately distribute equally into four 15-ml conical tubes (~13 ml each)
7. To an individual tube add 24 µl of freshly prepared 40% APS. Pipet twice to mix and transfer immediately into gel cassettes at ambient temperature (RT).
8. Immediately insert the comb. After 30 min at RT, transfer the cassette to 4°C for 1 h.

9. Wrap with absorbent paper moistened with 10 ml of Running Buffer per gel cassette.

NOTE:

- Gels stored at 4°C can be used within 1 week of preparation for optimal band separation, resolution and protein detection.

Run Sample (Electrophoresis & Western Blot):

1. Dilute samples 1:1 with Loading Buffer in a 500 µl Eppendorf tube. Heat 10 min at 75° C. Vortex and centrifuge briefly to collect liquid to the bottom of the tube.
2. Load 10 µl of each sample or markers. Note: Load 10 µl of 1:1 diluted Loading Buffer into any empty well.
3. Run at 80 V for 130 min in Running Buffer.
4. Open the cassette and immerse the gel in water
5. Transfer the gel to the iBlot apparatus
    a. Place "Bottom" iBlot Stack plate into the iBlot
    b. Place the gel on top of the "Bottom" plate
    c. Wet the iBlot Stack filter paper with water and place on top of gel; flatten with roller to remove bubbles.
    d. Place the "Top" iBlot Stack on top of the filter paper and roll to flatten
    e. Place the sponge into the iBlot lid
    f. Close and run using Program 3 (P3) for 7 min
6. Remove the PVDF membrane and immerse in Blocking Solution for 30 min at RT.
7. Pre-complex Siglecs (concurrent with step 6, volumes are per blot)
    a. For **Siglec-8**, add 6 µg of Siglec-8-Fc to 300 µl containing 4.4 µg (equivalent to 1:500 dilution) of HRP-human IgG-Fc (Sigma A0170) in a 1.5-ml low-bind Eppendorf tube; For **Siglec-9** add 1.25 µg Siglec-9-Fc to 60 µl containing 0.89 µg (equivalent to 1:500 dilution) of the above secondary antibody; For **Siglec-F** add 3 µg Siglec-F-Fc to 134 µl containing 0.89 µg (equivalent to 1:500 dilution) of the above secondary antibody
    b. Incubate on ice for 30 min
    c. Dilute the pre-complex mixtures to 6 ml with PBST
8. Wash membrane with PBST and then overlay with 6 ml/blot of pre-complexed Siglec-Fc
9. Incubate overnight at 4° C (or 2h at RT).
10. Wash with PBST twice for 5 min each
11. Overlay with 1.6 ml of freshly prepared ECL Prime Detection Reagent (GE Healthcare RPN2232).

Reference: Samah M. A. Issa et al. *Electrophoresis* 2011, *32*, 3554–3563.