Serum Incubation of Minicells

Materials:

- **Human Serum from human male AB plasma, USA origin, sterile filtered**
- **Phosphate-buffered saline (PBS) 1X solution**
  1 L volume, store at room temperature
  - NaCl (8 g)
  - KCl (0.2 g)
  - Na2HPO4 (1.44 g)
  - KH2PO4 (0.24 g)
    Dissolve reagents in 800 mL of H2O, adjust pH to 7.4 with HCl, and then add H2O up to 1L.
- **SDS sample buffer**
  30 mL volume, store at 4°C
  - 1M Tris pH 6.8 (0.72 mL)
  - 100% glycerol (3 mL)
  - 10% SDS (6 mL)
  - β-mercaptoethanol (1.5 mL)
  - 1% bromophenol blue (3 mL)
  - ddH2O (15.78 mL)

Directions:

1. When the plasma ships in, freeze in small aliquots (5 mL each) in the -80 freezer. Don't freeze-thaw these too many times, or they will lose activity.
2. Setting up the control: Heat inactivate a small portion (one or two samples, where each sample ~500 uL) of serum for ~30 minutes at 56°C.
3. Incubate minicell with % serum 0-100% (by volume with buffer = phosphate buffered saline (PBS)) for 20 minutes at 37°C, mixing every 5 minutes.
4. Pellet minicells with Kay's centrifuge at 10,000xg. Keep supernatant as control- run gel immediately- freeze if gel won't be run immediately.
5. Wash minicells in PBS twice- 5 minutes each- let it resuspend, pellet again, resuspend pellet in PBS, add equal volume of 2x SDS sample buffer and boil for 5 minutes, freeze, then boil again.
6. Before loading gel, thaw samples, boil again for 3 minute, microfuge for 1 minute, take sample out from top Run an SDS page gel with solubilized washed minicells. Cut gel in half: stain one half for visualization with Coomassie blue, perform a western blot on the other half with C3 antibodies to check for complement activation.

Western Blot (standard)

Transferring to Nitrocellulose Membrane:

Materials:

- Transfer Buffer- Tris-glycine buffer (stored in -20)
  - 10% 10X Tris-glycine stock solution
  - 20% Methanol

- 70% water

Need about 1.4 L per box (that means 140 mL Tris-glycine, 280 mL of methanol, and 980 mL water)

Directions:

1. Soak membrane in methanol for 15 seconds
2. Rehydrate membrane in water for 1 minute
3. Soak membrane in transfer buffer for 5 minutes
4. Soak Blotting Paper(s) in transfer buffer
5. Cut out gel
6. Assemble sandwich in the following order
    a. Clear plate
    b. Sponge
    c. Blot paper (roll out bubbles after placement)
    d. Wet gel with transfer buffer *prevents tearing* (roll out bubbles after placement)
    e. Nitrocellulose membrane (roll out bubbles after placement)
    f. Second piece of blot paper (roll out bubbles after placement)
    g. Sponge
    h. Clear Plate
7. Close sandwich and add ice
8. Run at 200 milliamps for 2 hrs. or 30-100 milliamps overnight
   Runs to red(+)

Blocking:

Materials:

- Blocking Buffer- 5% non fat milk in 1xPBS at a 1:1 ratio (you need at least 50 mL to cover the whole thing)
- PBST- 1X PBS + 0.05% Tween: recommended to make about 500 mL (500 mL PBS with 250uL Tween)

Directions:

1. Wash 2-3 times in deionized water and mark lanes and markers with a ballpoint pen
2. Typically block overnight at 4 degree or 2 hrs. at room temperature
3. Dilute primary antibody solution with blocking buffer to appropriate concentration
   **use 1:1000 dilution*
4. Add 50 mL to membrane- just enough to cover it (or do it in a bag to save antibodies)
5. Leave in the 4 degree for 2-3 hours
    a. If in fridge, take out every 30 minutes and shake back and forth
    b. If in cold room, leave on bellydancer
6. Wash twice quickly, twice for 5 minutes and twice for 10 minutes
7. Dilute secondary antibody with blocking buffer
   **use 1:5000 dilution*
8. Incubate in room temperature (on belly dancer if possible) for 30 min- 1 hr (longer incubation time leads to longer band visibility)
9. Wash 4 times, 5-10 minutes each with PBST
10. Wash 2 times with PBS for 5-10 minutes