General protocol: Chromogenic Immunohistochemistry (IHC)

DAY TWO

1. Prepare three 250mL wash buckets by adding **25mL of 10X PBS** to each bucket, then topping each bucket up to the **250mL line with distilled water**.
    - Add 2.5mL Tween (1%) as well to the middle bucket in the series of three washes.
2. Take slides out of the humidity chamber and check to make sure none of the slides became dry overnight.
    - If this occurred, make a note of which samples this happened to and consider removing from analysis.
3. Move slides to a slide rack while holding the rack above the first wash bucket so that the liquid on the slides is caught by the bucket. Place in this bucket for 5 minutes.
4. Wash in second (Tween-containing) and then third bucket for 5 minutes each.
5. Set a timer for 30 minutes. Blot the first slide and apply minimum necessary amount of drops of **species-appropriate secondary antibody** onto it. Start the timer and apply secondary antibody to each subsequent slide in **regular intervals** (30 seconds, 1 minute, whatever works) keeping the slides in a line in the order they received secondary antibody.
    - Incubate at room temperature.
    - Disperse antibodies with pipette tip if necessary.
    - While secondary antibodies incubate, prepare all materials needed to finish slide prep:

- Make DAB solution by calculating the volume needed to apply **200µL per slide**, then adding **30µL DAB enzyme for every 1000µL of DAB diluent needed.** Invert tube repeatedly to mix
- Fill a 100mL bucket with tap water (NOT distilled this time) and place slide rack into tap water so it is ready.
- Place kimwipes or white piece of paper near this bucket for checking DAB development.
- Empty a wash bucket, rinse, and fill it to the 250mL line with tap water. Place beside hematoxylin in fume hood.
- Fill **2 buckets each with 95% ethanol, 100% ethanol, and xylenes**.

6. Transfer slides into rack and into the first wash bucket at the same time interval as you applied secondary antibody.
    - This ensures that each slide incubates with secondary antibody for a full 30 minutes.
7. When each slide has been placed in the rack in the first wash bucket, perform all 3 washes for 5 minutes each.
8. Reset timer. Choose which slide should receive DAB first; if optimizing, choose the most concentrated antibody prep. Do not use control as first slide. Add 200µL DAB solution to this slide and start the timer.
    - As with secondary antibody application, add DAB solution to each subsequent slide at a **regular time interval**

- Check DAB development as you go! Amount of time needed with DAB is variable but usually within **2-7 minutes**.

9. When DAB development is sufficient, place slides into rack in the tap water bucket. Tap water stops the DAB reaction.
    - Stop DAB reaction after each slide has incubated for the same amount of time by adding them to the bucket at the same time interval DAB was applied.
    - Consider working with a partner if processing large numbers of slides.
10. When all slides have been placed into the tap water bucket, transfer loaded rack into the hematoxylin bucket in the fume hood and incubate for two minutes.
11. Move slides into bucket of water. Bring this bucket to the sink and replace water + wash slides until water is clear.
    - Do not let slides come into contact with the running tap.
12. Dip rack of slides into bucket of bluing buffer **7 times**.
13. Clear slides by dipping them **15 times** each in **2 buckets of 95% ethanol, 2 buckets of 100% ethanol, and 2 buckets of xylenes** in that order. Leave slide rack in final xylene bucket while mounting slides
14. With small pestle put **Permount** on the slide encircling the sample. Squish slide cover on and press to disperse Permount on top of sample and to **push any bubbles away.**
    - Keep in the fume hood
15. Wait **at least 2 hours**, then gently clean slides with kimwipe dipped in xylene.

16. Wait a few hours more until all xylenes have evaporated and mounting media is completely hardened before viewing/imaging