# IMMUNOFLUORESCENCE STAINING – Bridgewater Laboratory Protocol

**DAY 1**

1) Perform **Xylene washes – 3 x 5 mins**
    - Xylene I – 5 mins
    - Xylene II – 5 mins
    - Xylene III – 5 mins
2) Perform **Ethanol** (EtOH) **washes – 2 x 10 mins, 3 x 5 mins**
    - 100% EtOH I – 10 mins
    - 100% EtOH II – 10 mins
    - 95% EtOH – 5 min
    - 70% EtOH – 5 min
    - 50% EtOH – 5 min
3) Perform **PBS** (phosphate-buffered saline) **washes – 2 x 5 mins**
    - Wash 1 – 5 mins
    - Wash 2 – 5 mins
4) Perform Antigen retrieval
        - NOTE: It is best to make the antigen retrieval buffer at the steps for the graded washes (Steps 1-3).
    - Make the antigen retrieval buffer: add **2.94 g of sodium citrate into 1L ddH$_2$O**. Adjust the pH to 6 (**pH=6.00**) with 1M and/or 6M HCl.
    - Heat the buffer in the pressure cooker at **low pressure – 2 mins**
        - NOTE: How to set up pressure cooker:
            1. Make sure *arrow* is aligned before closing.
            2. When closing/turning the lid, there is a 'hiss' sound. This is how it is secured.
            3. Make sure the 'steam release' *arrow* is aligned with the *arrow*.
            4. Set the pressure to 'Low'.
            5. Click 'Start', then 'Start' again, make sure the 'Timer' is set to '02', and then click 'Start' again.
            6. Once the timer goes off, release the steam. Do not open until the steam/pressure has been fully released).
    - Add the slides into the buffer in the pressure cooker and heat at **high pressure – 5 mins**
        - NOTE: How to set up pressure cooker:

1. Make sure *arrow* is aligned before closing.
2. When closing/turning the lid, there is a 'hiss' sound. This is how it is secured.
3. Make sure the 'steam release' *arrow* is aligned with the *arrow*.
4. Set the pressure to 'High'.
5. Click 'Start', then 'Start' again, make sure the 'Timer' is set to '05', and then click 'Start' again.
6. Once the timer goes off, release the steam. Do not open until the steam/pressure has been fully released).

5) Cool the slides down for ~15 mins – take the pot out of the pressure cooker and put it on top of ice in the sink.
    - NOTE: Make sure the slides are not hot/warm anymore (should be either room temperature or a bit colder) before proceeding to Step 6.
    - TIP: While doing this step, wet some paper towels and line a slide box.
6) Dry the areas AROUND the tissues (but NOT the tissues itself) with a Kimwipe. Encircle slides with ImmEdge Pen, then let the circle dry before proceeding.
    - NOTE: 4-5 circles is usually good. Keep the circles' edges a bit close to the edges of the tissue BUT make sure the pen mark DOES NOT touch the tissues.
7) Perform **PBS washes – 2 x 5 mins**
    - Wash 1 – 5 mins
    - Wash 2 – 5 mins
8) Make **Incubation** and **Blocking** buffers.
    - **Incubation Buffer (IB)**: 1500 µl PBS, 400 µl 15% BSA (Bovine Serum Albumin), 100 µl Normal Goat Serum (NGS), 6 µl Tween20.
    - **Blocking Buffer (BB):** 1000 µl IB, 100 µl 15% BSA, 25 µl NGS
9) Apply **Blocking Buffer** onto slides. Incubate for 60 mins in the slide box on the bench top.
10) Prepare **Primary Antibody (/Antibodies) in IB**:
    - Make sure to have the optimal ratio/dilution of the antibody (see company's Antibody datasheet).
    - Dilute in IB, then pipette directly onto samples. Incubate **overnight at 4°C cold room** in the laboratory.
        - NOTE: Remember to put wet paper towels in the slide box before incubating the slides there overnight, to ensure it is humidified and the slides will not dry out.

**DAY 2**

10) Perform **PBS washes – 3 x 10 mins**
    - Wash 1 – 10 mins
    - Wash 2 – 10 mins
    - Wash 3 – 10 mins
11) Prepare **Secondary Antibody (/Antibodies) in IB**:
    - The dilution is *usually* **1:1000**.
        i. Alexa Fluor 488 is Goat Anti-Rabbit and Alexa Fluor OR DyLight 594 is Goat Anti-Mouse.
    - Dilute the Secondary Antibody in Incubation Buffer, then pipette directly onto samples. Incubate for **60 mins** on the bench top.
        i. NOTE: Ensure the slide box is humidified so that the slides will not dry out.
12) Do a **PBS wash – 5 mins**
13) Prepare **DAPI** (4′,6-diamidino-2-phenylindole) and incubate slides in **DAPI** for **5 mins**.
    - NOTE: DAPI is sensitive to light. When preparing this solution, wrap the tube in aluminum foil. This will help block out the natural light from the room.
14) Perform **PBS washes – 3 x 10 mins**
    - Wash 1 – 10 mins
    - Wash 2 – 10 mins
    - Wash 3 – 10 mins
15) Apply **FluoroMount** and **coverslip** on the slides.
    - NOTE: Make sure there are no air bubbles.
16) Let slides **dry** (to make sure Fluoromount is dry) on the bench top. Store in 4°C after.
17) Image slides using the fluorescence microscope (**X-cite Mini**).