**LEE LAB RESEARCH LABORATORY**

**IMMUNOFLUORESENCE ANTIBODY (IF) STAINING PROTOCOL**

**Purpose:**

This is the basic protocol for antibody staining of formalin fixed paraffin embedded (FFPE) tissue.

**Principle:**

For antibody staining to be successful, most FFPE tissue requires antigen retrieval of some kind. Formalin fixation cross-links proteins during the course of fixation. Antigen retrieval unlinks the proteins and opens up the antigen sites so that the antibody will be able to bind to them.

**Specimen Preparation:**

10% Neutral buffered formalin fixed tissue, or 4% paraformaldhyde fixed tissue, paraffin embedded sections cut at ~5-6 microns and mounted on charged slides.

**Precautions:**

**Personal Protection:** Gloves, lab coat, goggles, fume hood, and use of universal precaution practices.

**Chemical Wastes:** Dispose of alcohols, dyes, and xylene in appropriate labeled waste containers as directed by the University of Minnesota Hazardous Chemical Waste Management Manual 5th Edition.

**Hazards:** Xylene = Flammable, Carcinogen, Skin irritant Eosin & Alcohols = Flammable, Skin irritant Hematoxylin = Skin irritant Avoid strong oxidizers with all listed chemicals

**Materials and Equipment:**

Charged slides

Coverslips

Drying oven, 60 degrees

Fume Hood

Gloves

Microtome

Staining racks

Timer

**Reagents:**

Xylene (**green staining containers only!**)

100% Alcohol

95% Alcohol

80% Alcohol

Dapi nuclear stain

Background Sniper (Biocare Medical)

TRIS with tween 20X (Biolegend,[hydroxymethyl aminomethane])

Secondary antibody, typically one from Jackson Immunoresearch

3% Hydrogen peroxide

True View/True Black Autofluorescent quenching media

Aqueous mounting media with or without DAPI

**Pretreatment reagents:**

Reveal Decloaker (Biocare Medical) preferred

Citrate buffer solution if you don’t use the Reveal

Specific case for Beta-amyloid antibody

70% Formic acid treatment

**DAY 1:**

**Deparaffinize tissue:**

2 options:

1) either place slides on a slide warmer with temperature set to approx. 57 degrees C. Leave the slides on the warming plate until the paraffin looks melted on all of the slides (about 0-15 minutes), or 2) put slides in a 60 degree C oven for about 30 minutes.

Place slides vertically in the gray plastic slide holders & run the slides through the following solution containers located in the fume hood. Check that solutions cover tissue completely.

|  |  |  |
| --- | --- | --- |
| **1.** | **Xylene** | **5 minutes** |
| **2.** | **Xylene** | **5 minutes** |
| **3.** | **Xylene** | **10 minutes** |
| **4.** | **100% Alcohol** | **3 - 5 minutes** |
| **5.** | **100% Alcohol** | **3 - 5 minutes** |
| **6.** | **95% Alcohol x 2** | **3 - 5 minutes** |
| **7.** | **80% Alcohol** | **3 - 5 minutes** |
| **8.** | **Filtered water** | **3 - 5 minutes** |

**Preferred Antigen retrieval steps:**

1) Fill the vegetable steamer with deionized water to the second line in the transparent corner section of the steamer.

2) Add capillary gap slides (same number as the number of slides you are staining) in the slide holder to wet the slides, or if you have a lot of slides, rinse the capillary gap slides separately before use. Fill the plastic “boat” containers with approx. 20 ml of one of the 1X antigen retrieval solutions (Reveal or citrate buffer). Pick up a slide (rough side of the capillary gap slide facing the tissue section slide) and put the slides into one of the troughs in the boat container. Push slides toward each other and allow the fluid to come up slowly between the two slides. Try not to get bubbles between the slides or this will obstruct the antigen retrieval process and give you uneven staining. Put the “boats” into the steamer and set for 35 minutes. This will allow the steamer to come up to temperature and the slides will steam for about 30 minutes.

3) Remove clear basket from the steamer and allow slides to cool for about 20 minutes to room temperature.

4) Transfer the “boats” of slides to the inner steamer container and rinse with running water for about 10-15 minutes. Unpeel the slides from each other and transfer the slides to the gray slide holder for a final rinse (~5 minutes).

Alternative microwave method:

If you don’t have the vegetable steamer you can do antigen retrieval in the microwave by putting the slides in a plastic coplin jar filled with citrate buffer.  Microwave slides/solution for 5 min. on high power (~700 watts). Make sure slides are still covered with retrieval solution or add fresh solution and repeat microwaving. This process can be repeated 2-3 times.

Let slides slowly cool in coplin jar to room temperature for approx. 20 minutes.

**Staining steps:**

1. Dilute or use prediluted TRIS (1x concentration ) for 10 minutes, approx 250-300 microliters per slide.
2. 3% Hydrogen peroxide made in 1X TRIS for 10-20 minutes.
3. Rinse slides in 1X TRIS and add to slides for 5 minutes.
4. 100% Background Sniper for nearly 13 minutes. Do not go longer than 15 minutes. (alternative: 10% normal goat serum made in 1X TRIS or PBS can be substituted for 1 hour) or 1% sodium borohydride made in 1X TRIS soln to decrease autofluoresence.
5. Make up antibody solution or a cocktail of multiple antibodies at desired concentration in a diluent of 5% Sniper in 1X TRIS solution. Using approx. 100 microliters per slide, make up enough antibody to cover all slides.
6. Cover the slides with either a glass coverslip (24 x 60 mm) or parafilm and put in a 4 degree C. refrigerator overnight.

**DAY 2:**

1) Take slides out of the refrigerator and warm up to room temperature about 15-20 minutes. Remove the cover slips and cover the slides with 1X TRIS solution for a few minutes. Rinse off the antibody solution with 1X TRIS 5 minutes x 2. At this stage you will need to use the black staining box to keep the reagents from light exposure.

2) **Secondary reagent/antibody for 1-2 hours.**

Apply the secondary reagent appropriate to the antibody, i.e., if your antibody was raised in a rabbit, you will apply a fluorescent antibody such as Jackson goat anti-rabbit 488 (yellow in tube, excites to green in IF light) using about 250-300 microliter per slide or enough of the reagent to totally cover the tissue. If your antibody was raised in some other animal, you will have to find a secondary to that animal, i.e., donkey anti-mouse, goat anti-rat, etc. Usually a dilution of 1:300 to 1:500 in a diluent of 5% Sniper made in 1X TRIS is a good starting concentration to use.

Often a cocktail of 2 secondary antibodies is used for dual chromogen staining.

In this case, be careful to combine both chromogens in correct ratios with the diluent. If the secondary has glycerol added (which is often the case to keep the solution from freezing), you will have to double the amount of antibody used. Jackson ImmunoResearch sells a host of secondary antibodies. Colors 594 excites red, 488 excites green…

3) Rinse slides with 1X TRIS; 5 minutes x 3

4) If you do not want to use the solutions to block autofluorescence, mount slides in dim light conditions with Prolong gold with DAPI. The dapi will stain the nuclear elements blue. Cover slides in a slide box or tin foil to dry overnight at room temperature to set the mounting media.

5) Blocking Autofluorescence (Optional step) with Vector TrueView

In IHC 4C frig, TrueView kit on top shelf

Add equal volumes of reagents in order, mix A with B and mix together ~10 seconds.

Add C and mix again ~10 seconds. Calculate approx. 100/slide.

Apply to tissue and incubate for 2-5 minutes.

Tissue will stain dark blue. Rinse with 1X Tris.

Stain with DAPI separately as in the following steps:

* DAPI aliquots are in the -20C freezer and should be diluted as follow:
  + 2.1 ul concentrated DAPI in 100ul 1X Tris
  + dilute the above solution 1:1000 in 1X Tris for the working solution of 1X DAPI
  + stain with the working solution of DAPI for 10 minutes
  + Rinse in 1X TRIS a few times and coverslip with VectaShield Vibrance
  + After coverslipping, cover slides and allow media to harden in the dark (room temp)

**Results:**

Nuclei = Blue

488 = Green

594 = Red

647 = Far Red

