

IMMUNOFLUORESCENCE PROTOCOL

The following outlines an ImmunoFluourescence (IF) protocol, using Alexa Fluor conjugated secondary antibodies on paraffin-embedded tissues, to allow optimal fluorescent detection markers for IHC.

Materials needed:

1. Fluroescent-tagged Secondary Antibody
2. Serum from secondary antibody's host (only if background is an issue).
3. Xylene
4. Ethanol
5. Coverslips
6. Slide racks and holders
7. ddH₂O
8. Anti-Fade Solution (e.g. Prolong Gold from Invitrogen Catalog # S36936, or Prolong Gold Anti-Fade Solution plus DAPI from Invitrogen Catalog # P36931)
9. A standard laboratory rocker
10. Pipettors and tips

1. Place slides in a glass slide holder and place in the following solutions serially (at room temperature):

- a. Xylene: 5 minutes swishing every 2 minutes (in fume hood).
- b. Xylene: 5 minutes, swishing every 2 minutes (in fume hood).
- c. 100% Ethanol: 2 minutes, swishing every 30 seconds.
- d. 95% Ethanol: 2 minutes, rocking
- e. 80% Ethanol, 2 minutes rocking.
- f. 70% Ethanol, 2 minutes rocking.
- g. 50% Ethanol, 2 minutes rocking.
- h. ddH₂O: 3 minutes, rocking. Repeat one time.

2. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes, rocking at room temperature. Repeat once.

3. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) another 2 times, but for three minutes each this time.

4. Transfer slide holder to plastic, autoclavable container and submerge in 1X Epitope Unmasking Solution (Catalog # AA5).

5. Cover in foil and autoclave for 20-30 minutes.

6. Retrieve sample from autoclave and place on counter to let cool at room temperature for 20 minutes (Continuing submersion in Epitope Unmasking Solution (Catalog # AA5).

NOTE: For fatty tissue, such as brain or breast, use these directions for antigen retrieval:

Place slides in slide rack. Lower slide rack into 600 mL gently boiling 1X Epitope Unmasking Solution (Catalog # AA5). Boil gently for exactly 8 minutes, and then remove from the heat source (a large beaker filled with 1x Epitope Unmasking Solution (Catalog # AA5), with boiling chips on the bottom and sitting on a heat block works well). Slowly add 500 mLs of ddH₂O to bring buffer temperature down. Quickly put slide rack in glass slide holder and wash twice with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes each times.

7. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes, rocking at room temperature. Repeat once.

8. *Optional to reduce background fluorescence:* Block for 30 minutes at room temperature using serum from secondary antibody's host.

9. Put slides back into slide carrier and wash in 1X Amplifying IHC Wash Buffer (Catalog # AA4) for three minutes. Repeat twice.

10. Dilute primary antibody in Amplifying Antibody Dilution Buffer (Catalog # AA3). Use a minimum of 3 mLs per slide to completely submerge in the Antibody Amplifier™. The suggested dilution will vary for each antibody, and you will have to titrate out. We typically initially titrate from 1 in 1000 to 1 in 1 million. Place lid onto the Antibody Amplifier™ and place on a standard laboratory rocker. Rock overnight at 4°C. After use, store primary antibodies at -20°C. The antibodies can generally be re-used up to 4X without compromising sensitivity.

11. Put slides back into glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat once.

12. Prepare dilution of fluorescent anti-mouse secondary antibody. Secondary antibody dilution is 1:5000 in 1X PBS MADE IN THE DARK, but optimal dilution is at the discrepancy of the end-user (the use of the Antibody Amplifier Eclipse™ rocking on a standard laboratory rocker often allows the user to significantly titrate out antibodies from that which is recommended by the supplier). Incubate slides rocking at room temperature for one hour in Antibody Amplifier Eclipse™. Remember that each slide needs 3 mLs for full submersion. If you wish to store and re-use the secondary antibody, make the dilution buffer as follows: 0.01 M PBS pH 7.2, 0.05% thimerosal. This solution can be stored at 4°C wrapped in tinfoil for 6 months, do not expose secondary antibody to light.

13. Incubate slides rocking at room temperature for 1-2 hours in the Antibody Amplifier Eclipse™. Remember that each slide needs 3 mLs.

- 14.** Prepare Prolong Gold Anti-fade Solution. Take out of freezer and let thaw at room temperature for 1 hour. Do not artificially speed up warming.
- 15.** Put slides in tinfoil wrapped glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Keep slides in the dark- do not expose to light. Repeat once.
- 16.** Wash slides in ddH₂O for 3 minutes.
- 17.** Prepare fluorescent microscope according to manufacturer's instructions.
- 18.** Under dim lighting, use a kim-wipe to dry most of the slide, keeping the mounted tissue moist. Add 1 drop of room temperature Prolong Gold Anti-Fade Solution to the center of tissue. Cover with a coverslip, gently removing air bubbles.
- 19.** To view immediately, tack down the corners of the coverslip with clear nail polish. After viewing, to permanently seal the slide, paint the edges of the coverslip with the clear nail polish and let dry. Put the slides into the Antibody Amplifier Eclipse™, and allow the slides to cure in total darkness for 2-24 hours depending on the thickness of the tissue and the relative humidity of the environment.
- 20.** To keep slides in optimal condition indefinitely, store them upright in a covered slide box containing a dessicant at -20°C.