General IHC Protocol for Formalin-fixed Paraffin Embedded Staining
(Steps 16-18: follow only if using biotinylated secondary Ab)

Materials

1. Histoclear
2. Ethanol (100%, 95%, 70%)
3. Albumin from bovine serum (BSA): Sigma A7906-500g
4. Primary Antibody
5. Anti-primary (Secondary) Antibody
6. Permount mounting medium
7. Triton X-100: Sigma T8787-100ml
8. Modified Meyer's Hematoxylin
9. Phosphate Buffered Saline 1x
10. H_2O_2 30% solution
11. Coplin jars (plastic for Ag retrieval)
12. Pressure cooker
13. Dako pen
14. DAB substrate reagents
15. Slide Coverslips

Solutions

1. 5% BSA/0.3% Triton X-100 (should be made fresh every 1-2 months)
   a. 25 g of BSA into 500 ml of 1x PBS
   b. Add 1.5 ml of 100% Triton X-100
   c. Filter and store at 4°C
2. 1% BSA/0.3% Triton (should be made fresh every 1-2 months)
   a. 5 g of BSA into 500 ml of 1x PBS
   b. Add 1.5 ml of 100% Triton X-100
   c. Filter and store at 4°C
3. Sodium Citrate pH 6.0 (Genentech Media Prep), or DAKO Antigen Retrieval Buffer (see step 6a)

Procedure

1. Fix tissues in formalin immediately after collection 1hr/mm of tissue (perfused tissue is best)
2. Wash in PBS 1x for 4x15min and store in 70% EtOH
3. Embed in paraffin and section

Day 1 (~6 hours)

5. Deparaffinize slides 60min:

Done using 250 ml of solution in staining buckets with slide rack

Note – never let your slides dry out!!!!!! Throughout this whole procedure

Deparaffinize
- 1.) Histoclear x 10min
- 2.) Histoclear x10min

Rehydrate
- 3.) 100% EtOH (or Flex 100) x 3-5 min
- 4.) 100% EtOH (or Flex 100) x 3-5min
- 5.) 95% EtOH (or Flex 95) x 3-5min
- 6.) 95% EtOH (or Flex 95) x 3-5min
- 7.) 70% EtOH x 3-5min
- 8.) MQ H20 x 5 min

Note all of the above steps do not have to be exact… you can leave for longer

6. Antigen retrieval (Heat Induced Epitope Retrieval) 30min:
    a. In **Plastic** Coplin jar(s), place slides in **Dako Antigen Retrieval Buffer** (To prep: in fridge, make excess of 1:10 dilution with distilled water, pH to 6.0-6.2, optimal is pH 6.1, using HCl if necessary.) (Alternatively, may use 10 mM **sodium citrate buffer**); antigen retrieve for 10 min at low pressure in the pressure cooker (filled between the 6 and 8 mark with MQ water).
        i. When pressure cooker is done *(takes roughly 20 minutes)* – quick release the steam, allowing pressure to normalize
        ii. Take off lid, remove Coplin jar(s) – careful! They'll be hot, use oven mitt and set them on bench, remove lids and allow them to cool to ~tepid RT. Wait for water to cool before discarding from the cooker.
        iii. Pull Coplin jar out and let cool to warmish RT – 20min maybe?

7. Wash in PBS 1x5 min in Coplin jar, on bench, RT
8. Incubate in 3% H2O2 10 min RT – (25mls 30% H202 up to 250mls MQH20) in Coplin jar, on bench
9. Wash with PBS 5 min in Coplin jar, on bench
10. Block for 1-3 hours at room temperature 5% BSA/0.3% Triton X-100 (If having background issues, 3-6hrs works well). Block in the Coplin jars either on bench top or (if background issues) on the Belly-dance shaker
11. Primary antibodies overnight (>10 hrs) at 4°C in 1% BSA/0.3% Triton X-100. Use 200ul of primary antibody solution per slide (if smaller tissues, use ~50ul). Best if in a level, moist container (blue slide containers, holds 20 slides each, or slide boxes with 1" MQ water in bottom).

Primary Antibody	Concentration
Casp3	1:400
pH3	1:300
Pax 5	1:200

****Do NOT add Ab to your unstained control here!**
Instead, incubate in 1% BSA/0.3% Triton X-100 o/n

Day 2(~5.5 hrs-6.5 hrs)

12. Save primary Abs label with concentration and date (store at 4C for 2wks-1mo)
13. Rinse in PBS 4 x 15 min in Coplin jars on Belly-dance shaker at room temperature (60min)
14. Secondary antibodies (1:500) for 2 hrs at room temperature in 1% BSA/0.3% Triton X-100. [250ul (or~50ul) per slide] Use blue slide containers or slide boxes.

Primary Antibody	Secondary Antibody (1:500)
Casp3	Biotinylated anti-rabbit
pH3	Anti-rabbit
Pax 5	Biotinylated anti-goat

15. Wash in PBS 4 x 15min (60 min) in Coplin jar on Belly-dance shaker.
    → Don't need to save Secondary Abs

STEPS 16-18 ONLY APPLY TO RXNS WITH BIOTIN

16. Secondary antibodies – Biotin conjugated 2° AB
17. Incubate for 30min in ABC reagent (use blue slide containers/ slide boxes)
18. Wash for 2x5min in PBS

19. Perform **DAB reaction**
    Make DAB reagent (400ul per slide)
    Make sure you have a bucket filled with MilliQ H_20 ready
        Cover 15ml falcon tube in foil – reagent needs to be kept in dark and used within 30min
        1 drop Vector A per 1ml MQ H20 - mix
        2 drops Vector B per 1 ml
        1 drop Vector C per 1 ml
        Mix by inversion, wait 2 mins before adding to samples.
    Incubate slides for 10 min sharp – in the dark (foil) – used inverted pipet tip boxes to keep in the dark.
        Usually do slides 2 by 2 so that it is more precisely 10 min
20. Place immediately in the H2O after 5min of DAB – leave there until all slides have finished the DAB incubation
21. Change H_2O to fresh H_2O – wash 5min – if slides look particularly brown do this again
22. Counterstain with modified Meyer's Hematoxylin 10 min
    Put in warm tap water bucket and run warm tap water over it for 8 min

23. Dehydrate:
    70%EtOH x 3min
    95%EtOH x 3min
    95%EtOH x 3min
    100%EtOH x 3min
    100% EtOH x 3min
    Histoclear x 10min
    Histoclear x 10min

    Coverslip with Permount
        Use about 100ul Permount per slide
        Wipe off excess
        Let dry at least 1 hour before looking at on scope