

Staining Protocol for HC-10 (1/100) or L368 (6ug/ml- dilution depends on lot):**1. Deparaffinize:** (staining dishes, with fresh reagents)

- Preheat slides 20 min in 60° oven or warming tray
- Xylene..... 3 x 5 min
- 100% ETOH..... 1 x 5 min
- 100% ETOH..... 1 x 1 min
- 95% ETOH..... 2 x 1 min
- 70% ETOH..... 1 x 1 min
- H₂O dip
- PBS..... hold

2. Microwave antigen retrieval:..... @30min

- 4.2 ml unmasking sol'n + 450 ml H₂O in glass dish
- Place slides in glass rack
- Bring to boil in microwave
- Boil 10 min
- Remove from microwave and cool down 20 min

3. Wash: PBS + Triton (Staining dish)..... wash**4. H₂O₂** (staining dish)..... 10 min**5. PBS + Triton** (Staining dish)..... wash

* Avidin/ Biotin block (as per instructions)

6. Blocking(10% horse serum)..... 30 min, Room Temp (RT)
(incubation chamber)**7. Primary Ab**.....60 min, RT (or overnight 4°C)
(incubation chamber)
HC-10 at 1/100, L368 at 6ug/ml**8. Wash:** PBS + Triton (Staining dish)..... 3 x 5 min**9. Horse anti-mouse biotinylated secondary Ab**..... 30 min, RT
(incubation chamber)**10. Wash:** PBS + Triton (Staining dish).....3 x 5 min**11. ABC** (incubation chamber)..... 30min, RT**12. Wash:** PBS + Triton (Staining dish)..... 2 x 5 min

METHOD 4

PBS without Triton..... 1 x 5 min

13. **DAB** (incubation chamber) 5 min

Note: all subsequent steps are carried out in the glass staining dishes.

14. Wash gently under running tap water..... 5 min

15. **Hematoxylin** counterstain..... 30 sec

16. Wash gently under running tap water..... 5 min

17. **Dehydrate:**

- 95% ETOH..... 2 x 1 min
- 100% ETOH..... 2 x 1 min
- Xylene..... 2 x 5 min

18. **Mount** in Permount

Immunoperoxidase staining of formalin fixed, paraffin embedded tissues with anti-HLA Class I mAb

Reagents needed:

1. Phosphate buffered saline (PBS)
2. Tris buffered saline (TBS)
3. Trypsin for Antigen retrieval:
 - 0.05% Trypsin
 - 0.05% Triton X-100
 - in TBS pH 7.9
4. Vector Antigen Unmasking Solution (pH 6.0 sodium citrate buffer)
5. 10% Triton in PBS- add Triton slowly and mix with a magnetic stirring bar
6. PBS + Triton for washes:
 - Add 200ul of 10% Triton to 1 liter PBS
7. H₂O₂- *make fresh immediately before use*
 - Add 1ml of 30% H₂O₂ to 300ml H₂O
8. 10% blocking serum: (1 ml will be enough for @ 5 slides)
 - Horse serum..... 100ul
 - PBS..... 900ul
9. Diluent for primary antiserum
 - 0.05% BSA..... 50mg
 - 0.05% Triton X100.. 100ul 10% Triton
 - PBS..... 100ml PBS
10. Biotinylated horse-anti-mouse IgG antibodies from Vector ABC Elite Mouse Kit (1ml will be enough for @ 5 slides)
 - Secondary Ab..... 2ul
 - Horse serum.....15ul 1/500
 - PBS..... 1 ml
11. Avidin-biotin-enzyme complex from Vector ABC Elite Mouse Kit (1 ml). *Note: You must make this 30 min before use.*
 - Sol'n A.....20ul
 - Sol'n B.....20ul
 - PBS 1 ml
12. DAB chromagen: follow directions in kit from Vector. This is a carcinogen and you may wear gloves and dispose of it carefully.
13. Hematoxylin. Richard Allen 1:1 in tap water. Store in covered glass staining dish. (Any type of hematoxylin that your lab routinely uses for counterstaining will be OK)

General points:

1. Slides are handled in one of two ways:
 - Placed all together in a metal staining rack and immersed in @300 ml of sol'n in a covered, square glass staining dish.
 - Treated individually in a humidified incubation chamber. An incubation chamber can be made by placing filter paper on the bottom of a plastic box with a hinged lid and squirting a small amount of dH₂O on the paper. Slides are laid flat on rubber stopper supports and the sections are gently covered with @200ul of sol'n insuring adequate coverage to prevent the sections from drying out.
2. Plastic coverslips, such as the type used in the Apotag Kits, are used to cover sections only during the trypsin incubation which is done at 37°. This prevents the section from drying out at the warm temperature.
3. 175-200ul dispensed by a pipetman is usually adequate to cover the sections on one slide and you
4. **Dilution of HLA-I antibody:** Use 1/100 and 1/200 to optimize the final result.
5. **Control:** Use either mouse IgG or an isotype control such as mAb toCD45 (Zymed)