

## OLOUCHA + HENRY. HRP-TMB PLUS SLOW OSMICATION

Procedure for TMB (Oloucha):

Perfused tissue is cut on a vibratome (50µm) in cold phosphate buffered saline. Keep all tissue and sections in fridge or on ice at all times.

DISSOLVE:

97.5 ml 0.2 M phosphate buffer. Correct pH to 6.0-6.3

97.5 ml distilled water

500 mg ammonium molybdate

DISSOLVE:

10 mg tetramethylbenzidine (TMB)

5 ml absolute alcohol, warmed to 37°C force TMB into solution. DO NOT ALLOW TO BOIL, CHECK TEMP WITH THERMOMETER.

- **NOTE 1.** This amount (200ml) is for large trays we use to process serial sections. If you are reacting in vials you can use smaller volumes.
- **NOTE 2.** For the following steps make sure the solutions are kept cold and avoid exposure to light. I.e. after adding the solutions cover the trays or vials with aluminum foil to avoid full exposure to light. Having the light on while you add or transfer solutions is OK. Prolonged exposure to light drives the reaction and results in higher background.
- **NOTE 3.** To avoid contamination and non specific precipitates the rules are a) use very clean glassware, b) keep all solutions cold, c) avoid prolonged exposure to light.

Combine the above two solutions to form the incubation solution. To keep section in serial order we place sections in a holder that has small compartments and a mesh bottom. This section holder is then placed in dish that contains the TMB solution and this dish is placed in a second dish that contains ice to keep the incubation temperature at approx 4°C. If sections are not to be kept in serial order they can be placed into the solution in a vial but again, keep the temperature at approximately 4°C

**Without waiting** add 8-10 ml of 0.3% hydrogen peroxide.

After 30-60 minutes on a shaker, place entire tray with solution and sections in fridge for 12-18 hrs. The reaction product should appear as dark blue precipitate.

**Slow Osmication (Henry):**

Make solution of 1% Osmium tetroxide in 0.1 M Phosphate buffer corrected to **pH 5.5**

Remove sections from tray, place tray with sections and solution on a tray of ice. Do not allow sections to warm to room temperature as the reaction product will disappear.

At this stage we usually cut out the area of interest from the vibratome section but you can react the whole section if desired. We dissect out areas of interest from the sections one by one, using a dissecting microscope with cold light (fiber optics). Place cut-out EM tissue immediately in individually labeled vials containing the osmium solution. Once the tissue is in the osmium, the reaction product is stabilized. Leave vials 12-18 hrs at room temperature in a dark place (or cover with foil). After this, the tissue can be processed for regular electron microscopy using alcohol dehydration, propylene oxide and Epon embedding. **NOTE.** Even though the osmium stabilizes the reaction product we use short times for the alcohol dehydration as unstabilized TMB dissolves in alcohol and we don't want to take any chances.

Oloucha, F., F.Maninez.Garcia and C. Lopez.Garcia. A new stabilizing agent for the tetramethylbenzidine (TMB) reaction product in the histochemical detection of horseradish peroxidase (HRP). *J. Neurosci. Methods* 13: 131-138, 1985.

Henry, M.A., L.E.Westrum and L.R. Johnson. Enhanced ultrastructural visualization of the horseradish peroxidase-tetramethyl-benzidine reaction product. *J Histochem Cytochem.* 33:1256.1959, 1985.