

Immunoperoxidase Staining on Cryostat Sections (Single Colour)Reagents:Fixatives: Acetone, absolute methanolBuffers:

- a) PBS + 0.1% NaN₃ (2 ml of a 10% stock/200 ml) + 0.03% H₂O₂ (200 µl of 30% stock soln./200 ml)
 b) TBS (Tris-buffered saline) + 0.05% Tween-20 + 0.25% BSA = **TBS-A**
 c) TBS + 0.05% Tween-2 = **TBS-B**

Stock TBS 10x concentrated pH 7.6:

Tris: MW 121.14 »» 0.5M = 60.57 g/liter

NaCl: MW 58.44 »» 1.5M = 86.1 g/liter

Dissolve 242.28 g Tris and 344.4 g NaCl in 3.5 liter H₂O. Adjust pH to 7.6 with concentrated fuming HCl. Top up to 4 liters with H₂O. Store at +4°C. Do not add azide! - For use dilute 10x with H₂O »» 0.05M Tris in 0.15M NaCl final.

Stock Tween-20 (Fluka 93773)

5% in H₂O : 10 ml/lt = 0.05%

Stock BSA (Sigma A-4503 Fraction V)

20% in EBSS: 12.5ml/lt = 0.25%

Antibodies:

- a) 1st antibody: monoclonal antibodies [mAb] (culture sups or ascites etc)
 b) 2nd antibody: horseradish peroxidase [PO] conjugated antibody directed against 1st antibody: e.g. KPL GAM IgG+IgM(H+L) Cat.No. 14-18-09 **or isotype specific GAM antibodies available from SBA (Southern Biotech)**. Dilute with PBS (above mentioned antibodies work fine at 1:50), add 1-2% fetal lamb serum. Dilute freshly each time!

DAB: (3,3'-Diaminobenzidine-tetrahydrochloride)

Fluka 32750

Take care, this stuff is carcinogenic!

Stock solution: 25mg/ml in PBS. Dissolving is time consuming so do it O/N at +4°C on a rotator, protected from light. Store in aliquots of 200 µl at -20°C. - For use: thaw and dilute in 5 ml PBS. Add 100 µl H₂O₂ 1%. Filter directly on slides. Destroy leftovers with excess H₂O₂.

To avoid having to work with DAB-powder use the Diaminobenzidine Reagent Set from KPL Cat.No. 54-10-00. This kit contains ready - to - use solutions which simply have to be mixed before use.

Counterstain: Giemsa 5% in H₂O. Prepare freshly every 2-3 days.CuSO₄: 0.5M in 0.9% NaCl.

Staining Procedure:

- # Cut cryostat sections (5 - 8 μm). Use gelatine coated slides to enhance sticking of the sections. Air dry the sections well (2 - 18 hrs at room temperature). For storage: freeze at - 20°C in a sealed box. For staining after removing from the freezer let the sealed box warm to room temperature for at least 1 hour before taking out the slides, otherwise condensation takes place on the sections!
- # Fix for 5 min in acetone. Dry with the fan (cold air).
- # Rehydrate in PBS + 0.1% NaN_3 + 0.03% H_2O_2 for 10 min. This step blocks endogenous peroxidase reaction.
- # Change into TBS-A for ~ 5 min.
- # Wipe the slides around the sections, apply mAb (50 - 100 μl) and incubate in a humid chamber for 30 min. [Using a PAP-Pen from SCI Science Services Munich or a DAKO-Pen from DAKOPATTS allows you to omit this wiping step.]
- # Place slides in slide rack allowing excess antibody to flow off, then wash carefully in a tank with TBS-A with 3 changes of medium.
- # Wipe slides and apply 2nd PO-conjugated antibody. Incubate again in a humid chamber for 30 min.
- # Wash carefully, 2x in TBS-A, 1x in TBS-B (which does not contain proteins)
- # Wipe slides and apply DAB
- # Incubate for a few minutes. (Weak staining is not improved by prolonging DAB reaction but background staining could be caused by it)
- # Wash in TBS-B. Get rid of excess fluid, fix in absolute MeOH for 5 min, dry with the fan.
- # Intensify staining in CuSO_4 for 10 min.
- # Wash in deionized or distilled water. Counterstain in 5% Giemsa.(for ca. 20 sec). Check intensity under the microscope. Dry well (preferably O/N), coverslip with Eukitt.

Note: Never let sections get dry except after fixations!

Do not tap or shake slides, the sections are very fragile when moist!

Avoid azide! Peroxidase has a greater affinity for sodium azide than for H_2O_2 »»»» positive staining would be weakened or absent.