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Immunohistochemistry with paraffin sections

I. Material:

Paraffin sections on slides, 2-3µm thick, mounted single or double on appropriate glass slide. For microwave treatment, special slides are recommended (e.g. "Superfrost plus").

Laboratory equipment: Microscope, glass cuvettes, pipettes, paper filters, graduated cylinders, funnels, vacuum pump, heating plate, ventilator, humid chamber, Q-tips, Kleenex, microwave oven (700W), grease pen (Dako-Pen), diamond pencil, cover slip, timer.

Buffers and solutions:

10x PBS:	KCl (m.w. 74.6):	1.6g/l
	KH_2PO_4(m.w. 136):	2.8g/l
	$Na_2HPO_4x2H_2O$ (mw 178):	13.4g/l
	NaCl (mw 58.4):	77.8g/l
	Dilute 1/10 with distilled water before use, check pH and adjust to pH 7.2 if necessary Store PBS at room temperature for up to 2 weeks	
10x Citrate buffer:	42g Citric acid monohydrate (MW 210.14) Dissolve in 1.5 liters distilled water, adjust pH to 6.0 with 10 M NaOH and fill to 2 liters. Store refrigerated. Dilute 1/10 with distilled water before use, check pH and adjust with 10M NaOH if necessary. Final concentration: 10mM.	
Proteinase K	Proteinase K; 20mg/ml (BMA Biomedicals T-3401) Dilute Proteinase K 1:50 in 20mM Tris-HCl, pH 8.0 Trizma Base (Fluka 93304)	121mg/50ml distilled water, adjust pH to 8.0 with 1M HCl
Xylene	Mixture of Isomers (Fluka 95690)	
EtOH (Ethanol):	100% (Fluka 02860)	
96% EtOH:	480ml EtOH + 20ml distilled water	
70% EtOH:	350ml EtOH + 150ml distilled water	
30% H_2O_2:	Hydrogen peroxide (Fluka 95300) Store at 2-8°C	

Acetate buffer: Dissolve 1.4 ml acetic acid (Fluka 45731) in 250 ml A. dest., adjust pH to 4.9 with 10M NaOH.

Store at 2-8°C for up to 2 days.

AEC stock solution: Dissolve 0.4g 3-Amino-9-Ethylcarbazol (Sigma A-5754) in 100ml Dimethylformamid (Fluka 40250).

Store at 2-8°C for up to 4 weeks

AEC substrate: 5ml AEC stock solution
95ml acetate buffer, pH 4.9

Filter through paper filter and add 40µl 30% H_2O_2 (Fluka 95300) immediately before use.

Normal sera (for blocking):

Normal goat serum (NGS), (Gibco BRL)
Normal rat serum ((NRS), (BMA Biomedicals)
Normal mouse serum (NMS), (JacksonImmunoresearch)

Peroxidase labeled antibodies

Peroxidase conjugated Goat-anti-Mouse IgG (H+L)
minimal cross-reaction to human, rat, …
(JacksonImmunoresearch, 115-035-166),
store aliquots at -20°C.

Peroxidase conjugated Goat-anti-Rat IgG (H+L)
minimal cross reaction to human , mouse, …
(JacksonImmunoresearch, 112-035-167),
store aliquots at -20°C.

Peroxidase conjugated Goat-anti-Mouse IgG+IgM (H+L)
minimal cross-reaction to human, …
(JacksonImmunoresearch, 115-035-068),
store aliquots at -20°C.

Peroxidase conjugated Goat-anti-Rat IgG+IgM (H+L)
minimal cross reaction to human, …
(JacksonImmunoresearch, 112-035-068),
store aliquots at -20°C

Mayer's Haematoxylin:

"Mayer's Hemalum" for nuclear staining (Fluka 51275); filter before use.

Royalmount: Royalmount, (BMA Biomedicals T-3402)

DPX-Mountant: Mounting medium for tissue sections (Fluka 44581)

II. Procedure:

1. Mark slide with diamond pen or pencil, depending on slide.

   For improved adhesion of tissue sections slides are silane coated. This is particularly useful with microwave treatment. Commercial products may be used (Superfrost Plus Gold by Menzel)

2. Deparaffinize: 3x10 min. in Xylene
   2x3 min. in 100% Ethanol abs.
   2x3 min. in 95% Ethanol
   2x3min. in 70% Ethanol
   1x30 sec. in distilled water

   Rehydration is important to allow recognition of the antigen by the antibody. Change solutions after using three times.

3. Circle tissue sections with Dako-Pen (keeps antibody solutions apart).

4. Antigen retrieval:

Three different treatments are usually used at BMA:

a. no pretreatment

b. Microwave 700 Watt, 2 x 7 min, slides in holder, in 250ml cuvette in 0.01M citrate, pH 6.0 (add second cuvette with water to compensate for evaporation).Use hot water from second cuvette to replenish first cuvette,if necessary. [fill all spots in holder]. Cool cuvette to room temperatuer for at least 20min.

c. Enzymatic digestion with Proteinase K in 20mM Tris-HCl, pH 8.0

   3 - 12min. depending on antibody at room temperature in humid chamber.

Formalin fixation may lead to chemical modification of the protein epitope recognized by the antibody. This process can be corrected to a variable degree by an antigen retrieval treatment. Thus the antibody may recognize and bind to its specific epitope again.

5. Blocking of endogenous peroxidase: Mix 97ml A. dest and 3ml 30% H_2O_2 in cuvette, place sections in holder, incubate for 15min at room temperature. Rinse 1x with PBS and incubate for 5min in PBS, pH 7.2 (100ml cuvette)

Peroxidase is a naturally occurring enzyme that is found in many tissues. This step serves to suppress peroxidase activity that is not specifically associated with the detection antibody.

6. Blocking with serum: Coat entire tissue sections with 10% NGS in PBS, incubate for 30 minutes at room temperature in a humid chamber.

This step serves to block non-specific binding of the antibody to the tissue.

7. Remove serum by careful aspiration. Do not wash any further.

8. Incubation with primary antibody: Apply primary antibody (dilution according to manufacturer's recommendation). Incubate for 1-16 hours depending on antibody (ab) in humid chamber. Incubation time varies from antibody to antibody.

Specific binding of the antibody may be enhanced by prolonged exposure. On the other hand, non-specific binding may be minimal at short incubation times.

Important: include positive and negative control

9. Remove antibody solution by aspiration and rinse 1x in PBS, then wash for 1x5 minutes in large cuvette with PBS pH 7.2

10. Incubation with peroxidase coupled secondary antibody:

Prepare the following solution:

Peroxidase-conjugated secondary antibody:	according to own titration or manufacturer's suggestion.
Normal Goat Serum:	10%
(Normal Serum of tissue species; only necessary if the secondary antibody serum is not absorbed)	5%
PBS	Add to 100%

Incubate with 100-500µl per section in humid chamber for 60 minutes.

This step detects and enhances the presence of primary antibody.

11. Aspirate antibody solution and rinse 1x in PBS. Wash in PBS for 1x5 minutes in large cuvette (PBS pH 7.2)

12. Incubation with AEC substrate: Incubate with filtered AEC substrate solution (see above) for 12 minutes in cuvette at room temperature in the dark. Check color reaction.

13. Dip slide 3x in PBS, pH 7.2.

Color reaction is terminated as soon as the pH is adjusted to neutral.

14. Hemalum nuclear staining: Filter solution before use. Incubate slide for 1-2 minutes at room temperature, then carefully for 10 minutes under running cold tap water; apply one final rinse in distilled water.

15. Cover sections with Royalmount, 1-3 drops per section. Tissue must still be humid.

16. Polymerization: Put slide on 60°C heating plate for at least 30 minutes.

17. Mount cover slip: Dip slide in xylene (hood), add 1-3 drops of DPX Mountant, dip in xylene again, slip slide on tissue and let dry.

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