MONOSAN® TECHNICAL INSTRUCTIONS FOR IMMUNOHISTOCHEMISTRY

General Technical Instructions for Use

INTRODUCTION:

Monoclonal and polyclonal antibodies are used in a large variety of technical procedures. Methods to be selected are dependent upon the specific demands and materials available. The methods given below may offer a basis, which can be adapted to local demands and available materials. For further technical help see: Current Protocols in Immunology, ed J. E. Coligan *et al*., J. Wiley & sons, New York 1991, ISBN 0-471-52276-7.

A. Membrane Immunofluorescence:

1. Transfer 5 x 10^5 cells in 25 µl to a 10 x 75 mm test tube for staining.
2. Add 25-50 µl of the diluted monoclonal antibody, mix and incubate for 30 min. on ice.
3. Wash twice with PBS containing 1% BSA and 0.02% NaN_3.
4. Add 50 µl of an appropriate FITC- or other fluorochrome- labeled antibody, mix and incubate for 30 min. on ice.
5. Wash twice with PBS containing 1% BSA and 0.02% NaN_3.
6. Resuspend the cells in 100 µl washing buffer and analyze on a fluorescence activated cell sorter (FACS) or mount in 90% glycerol, 10% 1 M Tris/HCl pH 8.0 and analyze by fluorescence microscopy.

B. Microcytotoxicity Testing:

1. Mix 1 µl of a cell suspension (containing 5 x 10^5 - 10^6 cells/ml) with 1 µl of the diluted monoclonal antibody in a Terasaki tray under oil.
2. Incubate for 60 min. at room temperature.
3. Add 5 µl of an appropriate rabbit complement.
4. Incubate for 120 min. at room temperature.
5. Score cell death either by eosin exclusion or propidium iodide staining*.
   *Bruning, J.W.; Claas, F.H.J.; Kardol, M.J.; Lansbergen, Q.; Naipal, A.M.; Tanke, H.J.; Automated reading of HLA-A, B. C-typing and screening. The propidium Iodide (PI) method. Human Immunology 5: 225,1982.

C. Methods for Cells Grown on Coverslips:

a. Indirect Immunofluorescence Microscopy

1. The cells grown on a coverslip are gently washed with PBS and Ca^{++} and Mg^{++} at room temperature.
2. Fix the cells for 30 min. in 1-3% paraformaldehyde in PHEM buffer, pH 6.9.
3. Transfer the coverslips into 0.5% Triton X-100, 3% paraformaldehyde in PHEM buffer for 2 min.
4. Wash 3 times with PHEM buffer for 10 min.
5. Incubate the coverslips 2 times 5 min. each in 50 mM NH_4Cl in PHEM.
6. Wash with PBS 3 times for 15 min.
7. Incubate the coverslips with 100 µl monoclonal antibody diluted in PBS for 60 min. at 37°C.
8. Wash 3 times for 10 min. with PBS.
9. Incubate with FITC or other fluorochrome-labeled second antibody for 60 min. at 37°C.
10. Rinse 3 x 10 min. with PBS.
11. Mount in 90% glycerol, 10% 1 M Tris/HCl pH 8.0.

    Composition PHEM buffer:
    60 mM Pipes
    25 mM Hepes
    10 mM EGTA
    2 mM $MgCl_2$
    pH 6.9

b. Indirect Immunoperoxidase staining

1. 1 to 8 as above
9. Incubate with peroxidase labeled second antibody for 60 min. at 37°C.
10. Wash with PBS 3 x 10 min.
11. Stain with diaminobenzidine (DAB) solution (0.05% DAB, 50 mM Tris/HCl pH 7.4, 0.01% H_20_2 freshly prepared) 10 min. at room temperature.
12. Wash with running tap water, 3 min.
13. Counterstain with Mayer's hematoxylin or OsO_4.

D. and E. Methods for Frozen Sections:

Indirect Immunofluorescence microscopy or Indirect Immunoperoxidase staining of frozen sections

1. 4-6 micron thick sections should be used.
2. Sections are thawed at room temperature and dried for 30 min.
3. Tissue is fixed in acetone.
4. Wash with PBS 3 x 3 minutes. Proceed as described for cells grown on coverslips (C).

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F. Methods for Cells on Coverslips (Acetone):

a. Indirect Immunofluorescence Microscopy

1. The cells grown on coverslips are gently washed with PBS at room temperature.
2. Add cold (-20°C) methanol and transfer to 4°C for 5 min.
3. Transfer the coverslips into cold (-20°C) acetone in an acetone resistant dish for 2 min.
4. Transfer to PBS, rinse a few times.
5. Incubate the coverslips with 100 µl monoclonal antibody diluted in PBS for 60 min. at 37°C.
6. Wash with PBS 3 x 10 min.
7. Incubate with FITC- or other fluorochrome-labeled second antibody directed against mouse IgG at appropriated dilution.
8. Rinse 3 x 10 min. with PBS.
9. Mount in 90% glycerol, 10% 1 M Tris/HCl, pH 8.0.

N.B. Fixation of the cells with formaldehyde or glutaraldehyde is not advisable because of possible destruction of structure and antigenicity.

In order to reduce background staining, dilution of the antibody with PBS supplemented with 0.5% BSA and 0.2% gelatin is recommended.

b. Indirect Immunoperoxidase staining

1. 1 to 6 as above.
7. Incubate with peroxidase labeled second antibody, 30- 60 min. at 37°C.
8. Wash with PBS, 3 x 10 min.
9. Stain with diaminobenzidine (DAB) solution (0.05% DAB, 50 mM Tris/HCl, pH 7.4,0.01% H_20_2 freshly prepared) for 10 min. at room temperature.
10. Wash with running tap water, 3 min.
11. Counterstain with Mayer's hematoxylin or OsO_4.

Fujiwara, K. and Pollard, T.D., 1980, in 'Current topics in Developmental Biology' Vol. 14,271-295, Academic Press.
Osborn, M. and Weber, K.,1982, in "Methods in Cell Biology" 24,997-132, Academic Press.
Lazarides, E., 1982, in "Methods in Cell Biology" 24,313-331, Academic Press.
Wang, K., Ash, J. and Feramisco, 1982, Methods in Enzymology.

G. Indirect Immunoperoxidase Staining on Frozen Sections

1. 4 to 6 micron thick sections should be used.
2. Sections are thawed, 1-2 hours at room temperature.
3. Tissue is fixed in acetone, 10 min.
4. Wash with PBS, 2 x 3 min.
5. Incubate with monoclonal antibody (diluted in PBS), 1-2 hours at room temperature.
6. Wash with PBS, 3 x 3 min.
7. Incubate with peroxidase labeled second antibody, 30- 60 min. at room temperature.
8. Wash with PBS, 3 x 3 min.
9. Stain with diaminobenzidine (DAB) solution 10 min. at room temperature.
10. Wash with running tap water, 3 min.
11. Counterstain with Mayer's hematoxylin, 2 min.
12. Wash with running tap water, 5 min.
13. Dehydrate with increasing grades of ethanol; 50%,70%,96%, absolute, 3 min. each.
14. Clear with xylol, 3 x 3 min.
15. Mount with mounting medium (e.g. malinol).

H. Indirect Immunoperoxidase Staining on Formalin-Fixed and Paraffin Embedded Tissues*

(*also for special fixation as required for anti-PLAP (MON 9002), see below).

1. 4 micron thick sections should be used.
2. Dewax in xylol, 3 x 3 min.
3. Rehydrate in decreasing grades of ethanol: absolute, 96%, 70%, 50%, 3 min. each.
4. Block endogenous peroxidase activity with freshly made 0.3% H_20_2 in methanol, 20 min.
5. Wash with PBS, 3 x 3 min.
   (Only if trypsinization is required (e.g. for anti Vimentin; MON 3005):

5a - Incubate sections with 0.1% Trypsin in 0.1% $CaCl_2$, pH 7.6 for 10 min. at room temperature.
5b - Wash with PBS, 3 x 3 min.

6. Cover the sections with 20% normal rabbit serum in PBS or normal human serum and incubate overnight in a humidity chamber at room temperature to reduce non specific background staining.

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H. Indirect Immunoperoxidase Staining on Formalin-Fixed and Paraffin Embedded Tissues (continued)*

7. Decant 20% normal rabbit serum.
8. Incubate with monoclonal antibody (diluted in PBS), 1- 2 hours at room temperature.
9. Wash with PBS, 3 x 3 min.
10. Incubate with peroxidase-labeled second antibody, 30- 60 min. at room temperature.
11. Wash with PBS, 3 x 3 min.
12. Stain with diaminobenzidine (DAB) solution,10 min. at room temperature. A stock solution of 0.5% DAB in 0.5 M Tris/HCl (pH 7.4) can be made and stored frozen in the dark. Before use a quantity needed for staining can be thawed and diluted 10x with water. The diluted DAB solution should be filtered. Just before use H_20_2 must be added to a final concentration of 0.01 %.
13. Wash with running tap water, 3 min.
14. Counterstain with Mayer's hematoxylin, 2 min.
15. Wash with running tap water, 2 min.
16. Dehydrate with increasing grades of ethanol: 50%, 70%, 96%, absolute, 3 min. each.
17. Clear with xylol, 3 x 3 min.
18. Mount with mounting medium (e.g. malinol).

Recommended fixation procedure for anti PLAP (MON 9002):
Tissue sections (1.5 mm thick) should be fixed for 1.5 hours at room temperature in buffered 4% formaldehyde (0.1 M sodium cacodylate buffer, pH 7.4, containing 1% $CaCl_2$). Fixed tissues should be washed for 15 min. in the same buffer solution without formaldehyde and embedded in Paraplast. Sections (5 micron) should be mounted on goat serum/formaldehyde-coated (or polylysine coated) glass slides, hydrated and treated for 20 min. with 0.003% trypsin in 10 mM Tris/HCl (pH 7.3) containing 0.9% NaCl and 1 mM $CaCl_2$. After equilibration in this buffered saline, specific monoclonal antibody should be applied without washing and incubated overnight (Nouwen et al.,1985). The presence of MAb may be revealed by any commercial PAP or ABC anti-mouse immunoglobulin staining kit.

I. Immunoperoxidase Test on Sections:

1. Frozen sections should have been fixed in acetone for 10 min.
2. Incubation in antisera 40-60 min.

Only if necessary:
2a - 10 min., 20 min. or 30 min. blocking of endogenous PO in methanol, 0.1% H_20_2.
2b - Wash in PBS 2 x 5 min.

3. Incubation in conjugate (e.g. peroxidase conjugated anti-mouse IgG), 30 min.
4. Wash in PBS 2 x 5 min.
5. Incubation in AEC, 0.01% H_20_2, 10 min.

Preparation of substrate:
a. 5 mg AEC (3-amino-9-ethylcarbazole) is solubilized in 0.5 ml DMF (di-methyl-formamide). A glass (or acetone resistant plastic) tube and pipet should be used.
b. add 9.5 ml. 0.05 M NaAc buffer, pH 4.9.
c. add 5 µl 30% H_20_2.

6. Wash in DI water, 2 x 5 min.
7. Slightly counterstain in hematoxylin, e.g. 10 sec.
8. Wash in tap water until sections are blue.
9. Mount in aquamont and examine by microscopy.

J. Membrane Immunofluorescense:

1. Transfer 1 x 10^6 cells to each 10 x 75 mm tube for staining. Pellet cells by centrifuging at 200 g x 5 min. (approx. 1000 r.p.m. in a normal centrifuge).
2. Aspirate supernatant using a fine bore Pasteur pipet leaving the cell pellet as dry as possible.
3. Add 50 µl of the diluted monoclonal antibody to each tube with cells. Mix by tapping the tube and incubate for 20 min. on ice.
4. Wash the cells 2 x in PBS (plus Ca^{++} and Mg^{++}) + 0.2% BSA and 0.1% sodium azide by centrifuging at 200 g x 5 min. After the last wash, aspirate the supernatant using a fine bore Pasteur pipet leaving the cell pellet as dry as possible.
5. Add 50 µl of the appropriate fluorochrome labeled antibody. Mix by tapping the tube and incubate for 20 min. on ice.
6. Wash the cells 2x in PBS + 0.2% BSA and 0.1% azide by centrifuging at 200 g x 5 min. Aspirate the supernatant leaving the cell pellet as dry as possible.
7. Resuspend the cells in 100 µl PBS-glycerol (1:1) and transfer 25 µl of the cell suspension to a frosted microscope slide and cover with coverslip.
8. Examine the slides by fluorescence microscopy.