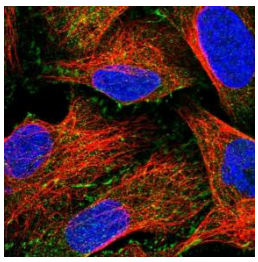
Double staining for Immunofluorescence Microscopy

In order to be able to examine the co-distribution of two (or more) different antigens in the same sample, a double immunofluorescence procedure can be carried out. Primary antibodies raised in different species can be used either in parallel (in a mixture) or in a sequential way.



Preparation of slides and samples

1. Cell lines, cytology smears, cytospin preparations

1.1. Coat coverslips with polyethylineimine or poly-L-lysine for 1 hr at room temperature.

1.2. Rinse coverslips well with sterile H2O (3 times 5 min each).

1.3. Allow coverslips to dry completely and sterilize them under UV light for at least 4 hrs.

1.4. Grow cells on glass coverslips or prepare cytospin or smear preparation.

1.5. Rinse briefly in phosphate-buffered saline (PBS).

2. Frozen (cryostat) sections

2.1. Snap frozen fresh tissue in liquid nitrogen or isopentane pre-cooled in liquid nitrogen. Store frozen blocks at -80°C.

2.2. Cut 4-8 µm thick cryostat sections and mount on superfrost or gelatin coated slides. You can store slides at -80°C until needed.

2.3. Before IF staining, warm up slides at room temperature for 30 minutes.

3. Paraffin-embedded sections

3.1. Deparaffinize sections in xylene 2x5 min.

3.2. Hydrate with 100% ethanol 2x3 min.

3.3. Hydrate with 95% ethanol 1 min.

3.4. Rinse in distilled water and then follow procedure for fixation and antigen retrieval as required (please see IHC protocol

for formalin-fixed paraffin-embedded tissue sections for further details).

Fixation

1. Fix the samples either in ice-cold methanol, acetone (1-10 min) or in 3-4% paraformaldehyde in PBS pH 7.4 for 15 min

at room temperature.

2. Wash the samples twice with ice cold PBS.

Permeabilization

If the target protein is expressed intracellularly, it is very important to permeabilize the cells. Note: acetone fixed samples do not require permeabilization.

1. Incubate the samples for 10 min with PBS containing 0.25% Triton X-100 (or 100 µM digitonin or 0.5% saponin). Triton

X-100 is the most popular detergent for improving the penetration of the antibody. However, it is not appropriate for the use of membrane-associated antigens since it destroys membranes.

2. Wash cells in PBS three times for 5 mins.

Blocking and simultaneous incubation

1. Incubate cells with 1% BSA in PBST for 30 min to block unspecific binding of the antibodies (alternative blocking solutions are 1% gelatin or 10% serum from the species that the secondary antibody was raised in).

2. Incubate cells in the mixture of two primary antibodies (e.g. rabbit against human target-1 and mouse against human target-2, if the targets are human proteins) in 1% BSA in PBST in a humidified chamber for 1 hr at room temperature or overnight at 4°C.

3. Decant the mixture solution and wash the cells three times in PBS, 5 mins each wash.

4. Incubate cells with the mixture of two secondary antibodies which are raised in different species (with two different fluorochromes, i.e. Texas Red-conjugated against rabbit and FITC-conjugated against mouse) in 1% BSA for 1 hr at room temperature in dark.

5. Decant the mixture of the secondary antibody solution and wash three times with PBS for 5 min each in dark.

Counter staining

1. Incubate cells on 0.1-1 µg/ml Hoechst or DAPI (DNA stain) for 1 min.

2. Rinse with PBS.

Mounting

1. Mount coverslip with a drop of mounting medium.

2. Seal coverslip with nail polish to prevent drying and movement under microscope.

3. Store in dark at -20°C or 4°C.

Best Luck to Your Immunofluorescence!