

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 polyethyleneimine or poly-L-lysine for 1 hr at room temperature.
- 1.2. Rinse coverslips well with sterile H<sub>2</sub>O (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.

3. Incubate the samples for 10 min with PBS containing 0.25% Triton X-100 (or 100  $\mu$ M 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.
4. Wash cells in PBS three times for 5 mins.

## Blocking and simultaneous incubation

5. 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).
6. 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.
7. Decant the mixture solution and wash the cells three times in PBS, 5 mins each wash.
8. 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.
9. Decant the mixture of the secondary antibody solution and wash three times with PBS for 5 min each in dark.

## Counter staining

10. Incubate cells on 0.1-1  $\mu$ g/ml Hoechst or DAPI (DNA stain) for 1 min.
11. Rinse with PBS.

## Mounting

12. Mount coverslip with a drop of mounting medium.
13. Seal coverslip with nail polish to prevent drying and movement under microscope.
14. Store in dark at -20°C or 4°C.