

DOUBLE IMMUNOFLUORESCENCE- SEQUENTIAL PROTOCOL

Fixation and permeabilization, are the same as for part XIII.

Blocking and Sequential Incubation:

1. **First blocking step:** incubate cells with the first serum (10% serum from the species that the secondary antibody was raised in) for 30 min to block unspecific binding of the antibodies (alternative blocking solutions are 1% gelatin or 1% BSA) at room temperature.
2. Incubate cells with **the first primary antibody** in 1% BSA or 1% serum in PBST in a humidified chamber for 1 hr at room temperature or overnight at 4°C depending on the concentration of the antibody and the accessibility of the antigen.
3. Decant the first primary antibody solution and wash the cells three times in PBS, 5 min each wash.
4. Incubate cells with **first secondary antibody (labelled with Fluorochrome-1)** in 1% BSA in PBST for 1 hr at room temperature in dark.
5. Decant the first secondary antibody solution and wash three times with PBS for 5 min each in dark.
6. **Second blocking step:** incubate cells with the second serum (10% serum from the species that the secondary antibody was raised in) for 30 min to block unspecific binding of the antibodies (alternative blocking solutions are 1% gelatin or 1% BSA) at room temperature in the dark.
7. Incubate cells with the **second primary antibody** in 1% BSA in PBST in a humidified chamber in the dark for 1 hr at room temperature or overnight at 4°C depending on the concentration of the antibody and the accessibility of the antigen.
8. Decant the second primary antibody solution and wash the cells three times in PBS, 5 min each wash in dark.
9. Incubate cells with **second secondary antibody (labelled with Fluorochrome-2)** in 1% BSA for 1 hr at room temperature in dark.
10. Decant the second 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 in dark.
2. Rinse with PBS in dark.

Mounting:

Mount coverslip with a drop of mounting medium.

1. Seal coverslip with nail polish to prevent drying and movement under microscope.
2. Store in dark -20°C or 4°C.