**Immunostaining protocol**

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**Abstract**

This is a protocol that describes how to use antibody staining to detect cellular epitopes by immunofluorescence microscopy.

Keywords: antibody, immunofluorescence, microscopy, staining

**Materials**

Cell culture

25-50% confluent wells of cells growing on coverslips in 24-well plates.

Equipment

* Platform shaker
* Microcentrifuge

Buffers & Reagents

* 4% paraformaldehyde (diluted from 16%; Thermo Fisher Scientific cat. no. 28908) in 1X PBS (diluted from 10X PBS pH 7.4; Thermo Fisher Scientific cat. no. 70011051)
* 1X PBS
* 0.1 % Triton X-100 in PBS
* Blocking Buffer: 5% milk powder (Sucofin), 0.1% Triton X-100 in PBS pH 7.4
* 2 drops/ml NucBlue™ Fixed Cell ReadyProbes™ Reagent (DAPI) (Thermo Fisher Scientific cat. no. R37606)
* Dako Fluorescence Mounting Medium (Agilent cat. no. S3023)
* Appropriate primary and fluorophore-conjugated secondary antibodies
* Microscope slides

**Fixation**

1. Aspirate media and gently wash each well with 1X PBS.
2. Aspirate PBS and place 500 ul of 4% paraformaldehyde onto each well.
3. Fix at room temperature for 10 min.
4. Remove 4% paraformaldehyde and add 1 ml 1X PBS.
   1. Note: at this point, the plate can be stored for several months at 4C.

**Immunostaining**

1. Permeabilize 1 ml 0.1% Triton X-100 and gently shake at room temperature for 5 min.
2. Block in 1 ml Blocking Buffer for 1 hr.
3. Dilute primary antibodies into Blocking Buffer and add 300 ul of diluted antibodies to each well.
4. Shake at 4C overnight.
5. Wash 3x with 1 ml PBS, gently shaking at room temperature each time for 5 min.
6. During washes, centrifuge tubes of secondary antibodies at 16000 x g for 10 min at 4C.
7. Dilute secondary antibodies into Blocking Buffer 1:500.
8. Incubate coverslips in 300 ul diluted secondary antibody for 2-3 hr at room temperature with gently shaking, protected from light.
9. Wash with 1 ml PBS for 5 min with gentle shaking.
10. Add 1 ml diluted NucBlue and incubate with gentle shaking for 5 min, protected from light.
11. Wash 2x with 1 ml PBS for 5 min with gentle shaking.
12. Allow fluorescence mounting medium to come to room temperature.
13. Add one drop of fluorescence mounting medium onto microscope slides for each coverslip.
14. Mount coverslips onto slides.
15. Let slides cure at room temperature overnight, protected from light.