Flow cytometry

Technic: Flow Cytometry on human cells

Goal: to measure the fluorescence intensity of cells that have undergone modification (transfection, labeling, etc.) in order to analyze a known parameter (GFP, His-tag, etc.).

Procedure:

1. Cell preparation

It is recommended to work with a 35mm dish at 100% confluence.

- Wash the cells with PBS.
- Detach the cells with trypsin (0.4 ml/35mm dish) or simply detach cells with their growth medium when trypsin is not necessary.
- Neutralize with 2 ml of medium containing 10% FBS.
- Centrifuge for 5 min at 300g at 4°C.

Depending on the experiment, here are the labeling steps used (primary and secondary antibodies if needed).

2. Incubation with the primary antibody

- Add 50µL of serum-free medium + primary antibody at the dilution recommended by the supplier.
- Incubate for 30 min on ice in the dark with gentle agitation.
- Add 200µL of serum-free medium to stop the reaction.
- Centrifuge for 5 min at 400 g at 4°C, discard the supernatant.
- Wash the cells twice with serum-free medium.
- Centrifuge for 5 min at 400 g at 4°C.

3. Incubation with secondary antibody

- Add 50µL of serum-free medium + secondary antibody at the dilution recommended by the supplier.
- Incubate for 30 min on ice in the dark with gentle agitation.
- Add 200µL of serum-free medium to stop the reaction.
- Centrifuge for 5 min at 400 g at 4°C, discard the supernatant.
- Wash the cells twice with serum-free medium.
- Centrifuge for 5 min at 400 g at 4°C.

4. Acquisition using a flow cytometer

Resuspend the cells in serum-free medium at a concentration of 5×10^5 cells/mL (minimum 400 µL).
Transfer them to a flow cytometry tube and store at 4°C away from light until analysis.

Reagents and Solutions:

- PBS (Phosphate Buffered Saline)
- Trypsine-EDTA
- Cell culture medium (ex. DMEM, RPMI… depending on the cell line).
- FBS (Fetal Bovine Serum) to complement the medium
- Serum-free medium
- Primary antibody specific to the target
- Secondary antibody if necessary

Equipment:

- Flow cytometer
- 35 mm culture dishes
- Pipette with appropriate tips
- Tube for flow cytometry
- Centrifuge for 15 ml tubes
- Ice bath

Estimate time:
Cell preparation: 30 min
Cell labeling: 2 hours
Cytometer setup and tube analysis: approximately 2 hours, depending on the number of analyses

Controls and Troubleshooting:

- Unstained cells – to assess autofluorescence and set background levels.
- Single-stained controls – one fluorophore at a time, for compensation setup.
- Isotype control – to evaluate non-specific antibody binding.
- Viability control – using a live/dead dye (e.g. PI) to exclude dead cells.
- Negative control (non-transfected / untreated cells) – to define baseline fluorescence.
- Positive control (transfected / treated cells) – to confirm expected signal.
- Fluorescence Minus One (FMO) – all markers except one, to accurately set gating thresholds.

⚠️Performing flow cytometry on extracellular vesicles will depend mainly on the sensitivity of the device and the threshold you use during your analyses. We used the BD Accuri™ C6 Plus Flow Cytometer at a 10,000 threshold for EVs-only analyses. We used them “pure,” i.e., straight out of the ultracentrifuge and after characterization by NTA.

⚠️In order to test the binding capacity of the GoldenEye protein with CLDN-4 on pancreatic cell lines, we brought the EVs into contact with the latter. You will find the results in the ‘pancreatic cells’ section of the ‘cell biology’ section. More specifically, with flow cytometry.