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ImmunoFACS

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Protocol to isolate distinct cell populations by immunostaining followed by FAC-sorting.

Florian Noack, Silvia Vangelisti, Boyan Bonev 2021. ImmunoFACS. **protocols.io** https://protocols.io/view/immunofacs-b2a2qage

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2% Formaldehyde solution (8ml)
Dilute 1ml of 16% Formaldehyde solution (ThermoFisher, Cat. N.: 28908) with 7ml PBS

2M Glycine solution (200ml)
Mix 30.024g of Ultrapure Glycine (Invitrogen, Cat. N.: 15527013) with 200ml of PBS

10% Saponin Solution (2.5ml)
Mix 0.25g Saponin (Sigma, Cat. N.: 47036-50G-F) with 2.5ml Nuclease-free water.

PBS with 1% BSA (7.5ml)
1% BSA (ThermoFisher, Cat. N.: AM2618)
1:100 RNAsin plus RNase inhibitor (Promega, Cat. N.: N261A)
PBS
(scale the volume accordingly to how many samples you have)

For 7.5ml
- 1.5ml Ultrapure BSA 5%
- 5.925ml PBS
- 75µl RNAsin plus RNase inhibitor

Wash Buffer (15ml)

0.1% Saponin (10% stock solution, freshly prepared)
0.2% BSA Ultrapure BSA 5% (ThermoFisher, Cat. N.: AM2618)
1:100 RNAsin plus RNase inhibitor (Promega, Cat. N.: N261A)
PBS
(scale the volume accordingly to how many samples you have)

For 15ml

- 150µl 10% Saponin
- 600µl Ultrapure BSA 5%
- 14.1ml PBS
- 150µl RNAsin plus RNase inhibitor

Staining Buffer (5ml)(100 µl reaction volume for maximum 1x10^6 cells)

0.1% Saponin (10% stock solution, freshly prepared)
1% BSA Ultrapure BSA 5% (ThermoFisher, Cat. N.: AM2618)
1:25 RNAsin plus RNase inhibitor (Promega, Cat. N.: N261A)
PBS
(scale the volume accordingly to how many samples you have)

For 5ml

- 50µl 10% Saponin
- 1ml Ultrapure BSA 5%
- 3.75ml PBS
- 200µl RNAsin plus RNase inhibitor

DAPI Buffer (5ml)

1:1000 DAPI 5mg/ml (ThermoFisher, Cat. N.: D1306)
0.5% Ultrapure BSA 5% (ThermoFisher, Cat. N.: AM2618)
1:100 RNAsin plus RNase inhibitor (Promega, Cat. N.: N261A)
PBS
(scale the volume accordingly to how many samples you have)

For 5ml

- 5µl DAPI
- 500µl Ultrapure BSA 5%
- 4.445ml PBS
- 50µl RNAsin plus RNase inhibitor

Resuspension Buffer (5ml)

0.5% Ultrapure BSA 5% (ThermoFisher, Cat. N.: AM2618)
1:25 RNAsin plus RNase inhibitor (Promega, Cat. N.: N261A)
PBS
(scale the volume accordingly to how many samples you have)

For 7.5ml

- 750µl Ultrapure BSA 5%
- 6.45ml PBS

- 300µl RNAsin plus RNase inhibitor

Antibodies dilutions optimal for ~1x10^6 cells:
Pax6-A488 (BD, Cat. N.: 561664) 2,5µl in 100µl
Eomes-PE (BD, Cat. N.: 566749) 3µl in 100µl
bTUBIII-A647 (BD, Cat. N.: 560394) 7.5µl in 100µl

1 Prepare a single cell solution using the Milteny Dissociation Kit. Count cells and resuspend in PBS to a concentration of 1 million cells per milliliter.

2 Add freshly prepared 2% Formaldehyde (from a new vial) in PBS to a final concentration of 1% and incubate **for 10 minutes** at room temperature with slow! rotation

3 Add 2M glycine solution to a final concentration of 0.2M to quench the reaction and incubate 5 minutes at room temperature with slow! rotation

4 Centrifuge cells for 5 minutes at 500xg at 4C.

5 Wash with cold PBS with 1% BSA.

6 Resuspend in Wash Buffer.

7 Wash for 10 minutes in the cold room, on the rocker.

8 Split aliquot for negative control – wash again (5 minutes).

9 Spin at 2500xg for 3 minutes at 4˚C.

10 Remove supernatant and add 100µl per 1 million cells of Stain Buffer with primary antibodies. Incubate 1 hour on the rocker in the cold room.

11 Wash cells 2x5 minutes in Wash Buffer and pellet with 2500xg for 3 minutes @ 4˚C.

12 Resuspend in DAPI Buffer and incubate for 10-15 minutes in the cold room. Wash with Resuspension Buffer.

13 Resuspend the cells in Resuspension Buffer at a concentration of ~10 million cells per ml*, pass the cell suspension through a 40µm cell strainer and proceed immediately to FACS-sorting (check staining on slides to test the protocol)

* the optimal concentration depends on the used FACS

14 Sort the cells into ~20µl of cold PBS with 1% BSA. Sorting should not exceed more than 3-4 hours. Wash the sorting tubes with PBS with 1% BSA beforehand to prevent cells from sticking to the sides.

15 After sorting cells can be used immediately or pelleted (5 minutes at 2500xg at 4˚C) and snap-frozen in liquid nitrogen for storage at -80˚C.