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FACS Staining of phosphorylated antigens

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Protocol status: Working

We use this protocol and it's working



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Abstract

This is a protocol for the detection of phosphorylated antigens via flow cytometry without the use of commercially available kits. It is adapted from the publication linked after the description

Materials

MATERIALS

- ☒ Fetal Bovine Serum **Gibco - Thermo Fisher Scientific Catalog #10270106**
- ☒ Methanol **J.T. Baker Catalog #9093-03**
- ☒ Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P6148**
- ☒ Phorbol 12-myristate 13-acetate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P8139**
- ☒ Dulbecco's Phosphate Buffered Saline **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D5652**
- ☒ Dimethyl sulfoxide (DMSO) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D2650**
- ☒ IMDM **Gibco - Thermo Fisher Scientific Catalog #12440053**
- ☒ Gentamicin **Gibco - Thermo Fisher Scientific Catalog #15750037**
- ☒ 2-Mercaptoethanol (50 mM) **Gibco - Thermo Fisher Scientific Catalog #31350010**
- ☒ L-Glutamine (200 mM) **Gibco - Thermo Fisher Scientific Catalog #25030081**
- ☒ Ionomycin from Streptomyces conglobatus **Merck MilliporeSigma (Sigma-Aldrich) Catalog #I9657**
- ☒ Albumin Bovine Fraction V **Serva, Germany Catalog #11924**

Troubleshooting

Safety warnings

- ! Wear gloves and safety goggles while handling 8%PFA and methanol. Incubation with methanol only under a fume hood.



Before start

Prepare IMDM (complete):

Add to the 500 ml IMDM Bottle:

25 ml fetal bovine serum (final concentration: 5%)

5 ml L-Glutamine (final concentration 2 mM)

500 µl Gentamicin (final concentration 50 µg/ml)

500 µl 2-Mercaptoethanol (final concentration 50 µM)

Prepare PBS with 5% BSA. Dilute part of it to 0,5%.

Prepare 1mM Ionomycin stocks in DMSO.

Prepare 10 µg/ml PMA Stocks in DMSO.

Prepare 8% PFA Solution in 1xPBS.

Cool methanol to 4 °C.

Prepare ice bath under the fume hood for incubation with methanol.

Cool down the centrifuge.

Incubation

- 1 Resuspend cell suspension at a concentration of 5×10^6 cells/ml. Incubate for 2 h at 37 °C and 5 %CO₂.

 02:00:00 First Incubation

 10 mL IMDM (complete) per Sample

 37 °C and 5% CO₂

- 2 Pipette 1 ml of single cell suspension in each of 6 FACS-Tubes. Label them accordingly (0 Minutes, 5 Minutes, 15 Minutes, 30 Minutes, 60 Minutes, 120 Minutes).

 02:00:00 longest incubation

 37 °C and 5% CO₂

- 3 Incubate the cells for the allotted time. Immediately after incubation take the samples out and add 200 µl 8% PFA (final concentration 1,5% PFA). Incubate at room temperature for 10 minutes.

 00:10:00 for each sample

 200 µL 8% PFA

 20 °C

Permeabilization and Staining

- 4 Centrifuge for 5 minutes at 520 g and 4 °C. Decant supernatant. Resuspend samples in the return flow.

 00:05:00 centrifugation

 4 °C

- 5 Add 1 ml of ice cold methanol. Incubate for 30 minutes on ice.

 1 mL Methanol per sample

 00:30:00 Incubation on ice

 4 °C on ice

- 6 Add 3 ml PBS/0,5% BSA to each sample. Centrifuge for 5 minutes at 520 g and 4 °C. Decant supernatant and Resuspend in 300 µl PBS/5% BSA.

 300 µL PBS/5% BSA per sample

 00:05:00 centrifugation

 4 °C



🧪 3 mL PBS/0,5% BSA per sample

- 7 Transfer 75 µl of sample into a new FACS-Tube. Add 25 µl antibody mastermix.

Example for 10 stainings:

	Antigen	Dye	Clone	Volume/ Sample	Volume in mastermi x
	CD8a	PerCP	53-6.7	0.5	5
	CD4	APC	RM4-5	0.5	5
	TCRgd	FITC	eBioGL3	0.5	5
	STAT3P	v450	4/P-STAT3	20	200
				PBS/5 % BSA	40

Incubate for 30 minutes at room temperature in the dark.

🧪 25 µL Mastermix per sample

🌡️ 20 °C

⌚ 00:30:00 RT in the dark

- 8 Add 3 ml PBS/ 0.5% BSA. Centrifuge for 5 Minutes at 520 g and 4 °C. Decant supernatant.

🧪 3 mL PBS/0,5% BSA

🌡️ 4 °C

⌚ 00:05:00 centrifugation

- 9 Repeat Step 8. Add 200 µl of PBS/0,5% BSA for measuring.

➡️ go to step #8 once

🧪 200 µL PBS/0,5% BSA