Detection of phospho IRF3 and p65 in PBMCs by flow cytometry V.2

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[1]In Situ Therapy

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ABSTRACT

This protocol describes a method to detect phosphorylated IRF3 and phosphorylated p65 (subunit of NFkB) in PBMCs by flow cytometry. An extracellular staining to identify different immune subsets is followed by an intracellular staining steps for the detection of phospho proteins

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PROTOCOL CITATION

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pIRF3, phosphoFlow, pP65, IRF3, p65

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Before start

1 Prepare DC Medium and pre-warm to 37 °C:

RPMI 1640 Medium, GlutaMAX™ Supplement **Thermo Fisher Catalog #61870036**
+ 5% Human Serum
+ 1% MEM Non-Essential Amino Acids Solution (100X) **Thermo Fisher Catalog #11140050**
+ 1% Sodium Pyruvate (100 mM) **Thermo Fisher Scientific Catalog #11360070**

Prepare Flow Buffer and cool to 4 °C:

DPBS no calcium no magnesium **Gibco - Thermo Fisher Catalog #14190169**
+2% heat inactivated FBS
+ 2mM
Ethylenediaminetetraacetic acid disodium salt solution BioUltra for molecular biology pH 8.0 ~0. **Merck Millipore Sigma Catalog #03690**

Put an aliquot of Methanol in the freezer and an aliquot of
ROTI®Histofix 4 % 500ml **Carl Roth Catalog #P087.4** and PBS in the fridge
Cool centrifuge to 4 °C

2h

Preparation and stimulation of cells

2 Thaw PBMCs and adjust concentration to [M] 1.11*10^7 cells/ml in DC Medium

3 Seed 90 µL cell suspension in 96-well round bottom plate (1x10^6 cells per well).

4 Prepare a 10x stock solution of your stimulus.

Note

A 1 µM diABZI STING agonist-1 **MedChemExpress Catalog #HY-112921A** solution (final concentration in well) can be used as positive control

5 Add 10µl of your prepared stimulus to your cells

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6 Incubate at 37C, 5%CO2 for the desired time

Note

Maximum phosphorylation was detected after 1h for pIRF3 and after 30min for pP65 when stimulated with STING agonists

1h

Extracellular Staining

7 All following steps have to be performed On ice using ice cold buffers. All centrifugation steps need to be performed at 4 °C
Put an Aliquot of PBS, Flow Buffer and HistoFix on ice

10m

8 Spin cells down 300 x g, 4°C, 00:05:00
Discard supernatant
Resuspend in 200µl Flow Buffer
Spin cells down 300 x g, 4°C, 00:05:00
Discard supernatant

9 Prepare Human BD Fc Block™ **Becton-Dickinson Catalog #564220** and staining mixes. Keep in the fridge until use.

9.1 Fc Block: Dilute 1:20 in Flow Buffer. Volume needed: 50 µL per sample

9.2 Master Mix:
Prepare in BrilliantStainBuffer:

Master Mix for 1 sample:

A	B	C	D	E
Fluoroch	Markers	Dilut	µl/test	µl Ab
BV421	CD3	50	1	1
BV510	CD14	20	2.5	2.5
BV605	HLA-DR	50	1	1

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A	B	C	D	E
BV711	CD56	50	1	1
BV785	CD123	50	1	1
FITC	CD19	5	10	10
AF647	CD11c	100	0.5	0.5
eFluor78	Via eF78	500	0.1	0.1
			µl Brilliant	32.9

9.3 Prepare intracellular staining mix
Dilute pIRF3 or pP65 antibody 1:50 in Flow Buffer
Volume needed: 50µl per sample

9.4 Prepare staining mixes for controls
e.g. Viability Dye Single Staining, FMO controls

15m

10 Resuspend in 50 µL FcBlock (1:20 pre-dilution in Flow Buffer)
Incubate 00:15:00 On ice

5m

11 300 x g, 4°C, 00:05:00
Discard supernatant

20m

12 Resuspend cells in 50µl MasterMix
Incubate for 00:20:00 On ice in the dark (cover with aluminum foil to protect from light)

10m

13 Add 150µl of Flow Buffer per well
Spin cells down 300 x g, 4°C, 00:05:00
Discard supernatant
Resuspend in 200µl Flow Buffer
Spin cells down 300 x g, 4°C, 00:05:00
Discard supernatant

1h 5m

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Fixation and permeabilization

1h 5m

10m

14 Resuspend cells in 100µl of FlowBuffer
Add 100µl of ice cold 4% HistoFix
Incubate On ice for 00:10:00 in the dark

10m

15 Spin cells down 500 x g, 4°C, 00:05:00
Discard supernatant
Resuspend in 200µl ice cold PBS
Spin cells down 500 x g, 4°C, 00:05:00
Discard supernatant

30m

16 Resuspend cells slowly in 180µl ice cold Methanol
Incubate for 00:30:00 - 60min at 4 °C in the fridge

15m

17 Spin cells down 500 x g, 4°C, 00:05:00
Discard supernatant
Resuspend in 200µl ice cold FACS Buffer
Spin cells down 500 x g, 4°C, 00:05:00
Discard supernatant
Resuspend in 200µl ice cold FACS Buffer
Spin cells down 500 x g, 4°C, 00:05:00
Discard supernatant

Intracellular Staining

40m

30m

18 Resuspend cells in 50µl intracellular staining mix
Incubate 00:30:00 at 4C

10m

19 Add 150µl of Flow Buffer per well
Spin cells down 500 x g, 4°C, 00:05:00
Discard supernatant
Resuspend in 200µl Flow Buffer
Spin cells down 500 x g, 4°C, 00:05:00
Discard supernatant

20 Resuspend in 100µl of Flow Buffer and keep at 4C protected from light until acquisition

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