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Intracellular Cytokine (ICS) Staining Protocol

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ABSTRACT

This protocol details about intracellular cytokine (ICS) staining.

ATTACHMENTS

400-867.pdf

MATERIALS

Materials

- Human Fc Block (BD/564220)
- 10% BSA
- Iono (1mg/ml) and PMA (100ug/ml)
- FACS buffer
- Golgi Plug & Golgi Stop
- Blocking Buffer
- Sodium azide at permeabilization step with saponin

Prepare Individual Peptides of Peptide Pools

	A	B	C
	Stimuli	Stock Concentration	Final Concentration
	Peptide Pool	1 mg/mL	Antigen Dependent
	DMSO (Negative Control)		Same concentration as peptide
	PMA + Ionomycin	100 ug/mL (PMA)1 mg/mL (Iono)	0.1ug/mL(PMA)0.5ug/mL(Iono)

Golgi Plug

Protein Transport Inhibitor (Containing Brefeldin A) **Becton Dickinson (BD) Catalog #555029**

Golgi Stop

Protocol status: Working

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- Protein Transport Inhibitor (Containing Monensin) **Becton Dickinson (BD) Catalog #554724**
- LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit, for UV excitation **Thermo Fisher Catalog #L23105**
- Human BD Fc Block™ **Becton Dickinson (BD) Catalog #564219**
- BUV395 Mouse Anti-Human CD3 **Becton Dickinson (BD) Catalog #563548**
- Brilliant Violet 510™ anti-human CD16 Antibody **BioLegend Catalog #302048**
- Brilliant Violet 510™ anti-human CD14 Antibody **BioLegend Catalog #367124**
- Brilliant Violet 510™ anti-human CD20 Antibody **BioLegend Catalog #302340**
- Brilliant Violet 570™ anti-human CD45RA Antibody **BioLegend Catalog #304132**
- BV711 Mouse Anti-Human CD4 **Becton Dickinson (BD) Catalog #740769**
- PE/Cyanine7 anti-human CD197 (CCR7) Antibody **BioLegend Catalog #353226**
- Brilliant Stain Buffer Plus **Becton Dickinson (BD) Catalog #566385**
- Brilliant Violet 785™ anti-human IL-17A Antibody **BioLegend Catalog #512338**
- BUV737 Rat Anti-Human IL-4 Clone MP4-25D2 **Becton Dickinson (BD) Catalo #612835**
- TNF alpha Monoclonal Antibody (MAb11) eFluor™ 450 eBioscience™ **Thermo Fisher Scientific Catalog #48-7349-42**
- IFN gamma Monoclonal Antibody (4S.B3) FITC eBioscience™ **Thermo Fisher Scientific Catalog #11-7319-82**
- BB700 Rat Anti-Human IL-2 Clone MQ1-17H12 **Becton Dickinson (BD) Catalo #566405**
- PE/Dazzle™ 594 anti-human IL-10 Antibody **BioLegend Catalog #506812**

Stimulus Solution

1 Label U-bottom plate with donor, stimulation solution, name and date.

2 Prepare PMA+Ionomycin and DMSO mix separately.

3 Prepare and arrange the remaining stimulation solution. Mix thoroughly by pipetting up and down

before adding to the experimental plate.

4 Add appropriate stimulus solution to each well in 96-well U-bottom plates.

5 Store stimuli-loaded plates in 37 °C incubator while thaw cells in next step.

	A	B	C
	anti-CD40		
	Antibody	Clone/vendor/catalog	
	anti-CD40 1.5 ug/mL per 10million cells	RF8B2/BD/740266	
	HR5		
	Total Volume		

PBMC Counting and Stimulus Preparation

7h 32m

6 Obtain indicated number of vial(s) of PBMCs.

7 For each donor, prepare sterile 50ml tubes with 10 mL HR5 and 20 µL Benzonase per vial to be thawed.

8 Thaw PBMC vials.

9 Centrifuge @ 1200 rpm, 00:07:00 .

7m

10 Resuspend cells in HR5 and determine cell number.

11 Centrifuge @ 1200 rpm, 00:07:00 . 7m

12 While sample(s) spinning, prepare the CD40 antibody solution the stock for all donors.

13 Resuspend each donor at 1.5 undetermined per 10 million cells per ml in prepared 1.5 undetermined CD40 antibody solution.

14 Add CD40 antibody solution to all wells already containing stimulus.

15 Keep plate in incubator at 37 °C while thawing/preparing cells in the following steps.

16 Obtain indicated number of vial(s) of PBMCs.

17 For each donor, prepare sterile 50ml tubes with 10 mL HR5 and 20 µL Benzonase per vial to be thawed.

18 Thaw PBMC vials.

19 Centrifuge @ 1200 rpm, 00:07:00 . 7m

20 Resuspend cells in HR5 and determine cell number.

21 Centrifuge @ 1200 rpm, 00:07:00 . 7m

22 While sample(s) spinning, prepare the CD40 antibody solution the stock for all donors.

23 Resuspend each donor at 10 million cells per ml in prepared 1.5 undetermined CD40 antibody solution.

24 Incubate the tube for 00:15:00 at 37 °C /5% CO_2. 15m

25 Add 100 µL of CD40 antibody-treated PMBCs to each well already containing stimulus.

26 Incubate plate for a total of 20-24 hours at 37 °C /5% CO_2.

27 After 20-24 HR, add 50 µL Intracellular Transport Blocking solution to each well and incubate for an additional 04:00:00 at 37 °C /5% CO_2. 4h

	A	B	C	D
	Intracellular Transport Blocking Solution			
	Reagent	Vendor/catalog	Minimum (µl)	
	Golgi Plug	BD/#555029	4	
	Golgi Stop	BD/#554724	4	
	HR5		992	
	Total Volume		1000	

28 After incubation, spin plate at 1400 rpm, 4°C, 00:02:00. 2m

29 Wash plate by adding 200 µL PBS and spinning at 1400 rpm, 4°C, 00:02:00. 2m

30 Resuspend cells in 100 µL of LIVE/DEAD and FC block mix. Prepare as follows:

	A	B	C
	Reagent	Clone/Vendor/Catalog/Peak	Amount per well (uL)
	Fixable Live/Dead Blue	Thermo/L23105/UV6	0.2
	Human FC Block	BD/564220	5
	PBS		94.8
	*Total Volume		100

31 Incubate at 4 °C for 00:30:00, protected from light. Wrap plate in aluminum foil and place in fridge. 30m

32 After incubation, add 100 µL PBS buffer and spin plate at 1400 rpm, 4°C, 00:02:00. Decant. 2m

33 Resuspend cells in 100 µL of surface antibody mix and incubate at 4 °C for 00:30:00, protected from light. 30m

	A	B	C	D	E
	Surface Stain (100µl per well)				
	Membrane Antibody	Fluorochrome	Clone/vendor/catalog	Amount per well (uL)	
	CD3	BUV395	UCHT1/BD/563546	1	
	CD8	BUV661	RPA-T8/BD/750699	0.5	
	CD16	BV510	3G8/Biolegend/302048	0.5	
	CD14	BV510	63D3/Biolegend/367124	0.5	
	CD20	BV510	2H7/Biolegend/302340	0.5	
	CD45RA	BV570	HI100/Biolegend/304132	2	
	CD4	BV711	RPA-T4/BD/740769	1	
	CCR7	PE-Cy7	G043H7/Biolegend/353226	1	
	FACS Buffer			83	
	BSB plus		BD Horizon/566385	10	
	*Total Volume			100	

34 After incubation, add 100 µL FACS buffer and spin plate at 2000 rpm, 4°C, 00:01:00. 1m

35 Wash plate.

35.1 Wash plate using 200 µL FACs buffer at 2000 rpm, 4°C, 00:01:00. (1/2) 1m

35.2 Wash plate using 200 µL FACs buffer at 2000 rpm, 4°C, 00:01:00. (2/2) 1m

36 Add 200 µL 4% PFA to each well, pipette to mix, cover and incubate at 4 °C for 00:10:00. 10m

37 After incubation spin plate at 2000 rpm, Room temperature, 00:05:00. 5m

38 Wash 1X with 200 µL PBS at 2000 rpm, Room temperature, 00:05:00. Meanwhile, prepare saponin buffer as follows: 5m

	A	B	C	D	E	F	G
		Saponin Buffer					Blocking Buffer

	A	B	C	D	E	F	G
		Saponin powder	10% BSA	0.01% azide	Vol. PBS	Total Vol.	Blocking buffer (10% Human serum in SB)
	Example	0.05 g	1 mL	100µL of 1% azide	8.9 mL	10 mL	100 µL+ 900 µL SB

39 Wash 1X with 200 µL of saponin buffer at 2000 rpm, Room temperature, 00:05:00. 5m

Meanwhile, prepare blocking buffer.

Note

Note: Use the rule 50 µL blocking buffer per well + 3ml excess.

40 Add 50 µL blocking buffer to each well and incubate protected from light at Room temperature for 00:05:00. 5m

41 Add 50 µL of prepared intracellular stain to each well. Incubate protected from light at Room temperature for 00:30:00. 30m

	A	B	C	D	E
	Intracellular Stain (50µl per well)				
	IC Antibody	Fluorochrome	Clone/vendor/catalog	Amount per Well (µL)	
	IL-4	BUV737	MP4-25D2/BD/612835	0.5	
	IL-17	BV785	BL168/Biolegend/512338	1	
	TNFa	eFluor450	Mab11/eBioscience/48-7349-42	0.2	
	IFNg	FITC	4S.B3/eBioscience/11-7319-82	0.2	
	IL-2	BB700	MQ1-17H12/BD/566405	0.5	

	A	B	C	D	E
	IL-10	PE-Dazzle594	JES3-19F1/BioLegend/506812	1	
	CD40L	APC-ef780 Changed from percp-ef710	24-31/LifeTech/47-1548-42	2	
	PBS			34.6	
	BSB plus		BD Horizon/566385	10	
	*Total Volume			50	

42 Wash 1X with 100 µL saponin buffer at 2000 rpm, Room temperature, 00:05:00. 5m

43 Wash 1X with 200 µL PBS at 2000 rpm, Room temperature, 00:05:00. 5m

44 To store plate Overnight, add 200 µL FACS buffer. Wrap in foil and store at 4 °C until analysis. 30m