Activation Induced Marker (AIM) Staining Protocol

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ABSTRACT

This protocol details about activation induced marker staining.

ATTACHMENTS

399-866.pdf

MATERIALS

Materials

- Brilliant Stain Buffer Plus
- CD40 Antibody, anti-human, pure functional grade

Flow Antibodies Needed:

	A	B	C	D
	Membrane Antibody	Fluorochrome	Clone/Vendor/Catalog#	Vol. Per Test (µl)
	CXCR5	BUV395	RF8B2/BD/740266	1
	CD8	BUV496	RPA-T8/BD/612942	2
	CD3	BUV805	UCHT1/BD/612895	2
	CD45RA	BV421	HI100/Biolegend/304130	2
	LIVE/DEAD	eFluor 506	eBioscience/65-0866-18	0.5
	CD14	V500	M5E2/BD/561391	2
	CD19	V500	HIB19/BD/561121	2
	CD4	BV605	RPA-T4/BD/562658	4
	CD38	BV786	HIT2/BD/563964	4
	CCR7	FITC	G043H7/Biolegend/353216	2

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	A	B	C	D
	CD40L	PerCP-ef710	24-31/eBioscience/46-1548-42	4
	CD69	PE	FN50/BD/555531	10
	PD-1	PE-Dazzle594	EH12.2H7 /Biolegend/329940	2
	OX40	PE-Cy7	Ber-ACT35/Biolegend/350012	2
	CD137	APC	4B4-1/Biolegend/309810	4
	HLA-DR	AF700	LN3/ebioscience/56-9956-42	4
	CD40	X (unconjugated)	(Miltenyi Biotech, 130-094-133)	1.5
	Brilliant Stain Buffer Plus		BD Horizon/566385	10

Prepared Individual Peptides or Peptide Pools

	A	B	C
	Stimuli	Stock Concentration	Final Concentration
	Peptide Pool	1 mg/mL	Assay Dependent
	DMSO (Negative Control)		Same concentration as peptide
	PHA (Positive Control)	1 mg/mL	1 ug-20 ug/mL

BUV395 Rat Anti-Human CXCR5 (CD185) **Becton Dickinson (BD) Catalog #740266**

BUV496 Mouse Anti-Human CD8 **Becton Dickinson (BD) Catalog #612942**

BUV805 Mouse Anti-Human CD3 **Becton Dickinson (BD) Catalog #612895**

Brilliant Violet 421™ anti-human CD45RA Antibody **BioLegend Catalog #304130**

eBioscience™ Fixable Viability Dye eFluor™ 506 **Thermo Fisher Catalog #65-0866-18**

V500 Mouse Anti-Human CD14 **Becton Dickinson (BD) Catalog #561391**

V500 Mouse anti-Human CD19 **Becton Dickinson (BD) Catalog #561121**

BD Horizon™ BV605 Mouse Anti-Human CD4 **Becton Dickinson (BD) Catalog #562658**

BV786 Mouse Anti-Human CD38 **Becton Dickinson (BD) Catalog #563964**

FITC anti-human CD197 (CCR7) Antibody **BioLegend Catalog #353216**

CD154 (CD40 Ligand) Monoclonal Antibody (24-31) PerCP-eFluor 710 eBioscience™ **Thermo Fisher Scientific Catalog #46-1548-42**

PE Mouse Anti-Human CD69 **Becton Dickinson (BD) Catalog #555531**

PE/Dazzle™ 594 anti-human CD279 (PD-1) Antibody **BioLegend Catalog #329940**

PE/Cyanine7 anti-human CD134 (OX40) Antibody **BioLegend Catalog #35001**

APC anti-human CD137 (4-1BB) Antibody **BioLegend Catalog #309810**

HLA-DR Monoclonal Antibody (LN3) Alexa Fluor™ 700 eBioscience™ **Thermo Fisher Scientific Catalog #56-9956-42**

Peptide Stimulation Solution

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

2 Prepare PHA 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 After adding stimulation solutions, prepare an additional solution of anti-CXCR5 antibody as described in below table.

	A	B	C	D
	Antibody	Fluorochrome	Clone/vendor/catalog	Amount per well(50ul) (uL)
	CXCR5	BUV395	RF8B2/BD/740266	1
	HR5			49

6 Add anti-CXCR5 antibody solution to all wells already containing stimulus.

7 Keep plate in incubator at 37 °C until cells are ready to be added.

PBMC Counting and Stimulus Preparation

1h 9m

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

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

10 Thaw PBMC vials.

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

12 Resuspend cells in HR5 and determine cell number.

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

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

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

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

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

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

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

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

21 Resuspend cells in 100 µL of antibody mix and incubate at 4 °C for 00:30:00, protected from light. Wrap plate in aluminum foil and place in fridge. 30m

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

23 Wash 1X plate using 200 µL MACs buffer at 1400 rpm, 4°C, 00:02:00.. 2m

24 Wash 1X plate using PBS µL MACs buffer at 1400 rpm, 4°C, 00:02:00. 2m

25 Resuspend in 120 µL PBS.

26 Wrap in foil and store at 4 °C until analysis.