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Whole blood T cell assay for NHPs, containment protocol

Forked from Immunophenotyping for NHPs, containment protocol

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Protocol status: Working
We use this protocol and it's working

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Disclaimer

Abstract

This is a protocol used to perform T cell assays using whole blood (collected in EDTA tubes) or cells from bronchoalveolar lavage (BAL). We have successfully used this protocol for rhesus macaques, cynomolgus macaques, and African green monkeys (although antibody mix provided was titrated on macaques). It allows the characterization of T cells into Th1 and Th2 (or Tc1 and Tc2) phenotypes. It technically offers 64 different activation profiles (4 cytokines + CD107a + CD69).

The nature and concentration of the stimulant is left unspecified as we have used different ones depending on reagent availability.

This protocol was designed to deal with samples coming from containment labs (> CL2) at our facility. If you are using this protocol at a different facility please ensure that proper testing and approvals are in place. The protocol can be used for experiments completed entirely in CL2, simply go from step 17 directly to 31.

Guidelines

For BAL fluid, make sure to set FSC voltage much lower than for whole blood.

Materials

Human TruStain FcX **BioLegend** **Catalog #**422302

BD FACS Lysing Solution (10X) **Becton Dickinson (BD)** **Catalog #**349202

BD Cytofix/Cytoperm **Becton Dickinson (BD)** **Catalog #**554722

BD Perm/Wash buffer **Becton Dickinson (BD)** **Catalog #**554723

Ghost Dye Red 780 **Tonbo Biosciences** **Catalog #**13-0865-T500

Equipment

NAME	TYPE	BRAND	SKU
FACS Tube	Tube	Falcon	14-959-2A

Equipment

NAME	TYPE	BRAND	SKU
2 ml screw-cap tubes	Microtubes	Sarstedt	72.694.006

PBS

Protocol materials

- Ghost Dye Red 780 **Tonbo Biosciences** **Catalog #**13-0865-T500
- PBS
- Human TruStain FcX **BioLegend** **Catalog #**422302
- BD FACS Lysing Solution (10X) **Becton Dickinson (BD)** **Catalog #**349202
- BD Cytofix/Cytoperm **Becton Dickinson (BD)** **Catalog #**554722
- BD Perm/Wash buffer **Becton Dickinson (BD)** **Catalog #**554723

Safety warnings

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Before start

The antibody mix described in the methods was tested on Cynomolgus macaque whole blood and BAL fluid. All antibodies should cross-react with rhesus macaques and African green monkeys but the panel might need to be re-titrated.

Stimulation

1 Aliquot 100 µL whole blood into a tube or well.

2 Add 200 µL cRPMI containing 1.5 X the desired concentration of stimulant.
(Stimulant(s) and concentration depend on the experiment and can include anti-CD28 and anti-CD49d)

3 Mix and incubate at 37 °C 5% CO2 Overnight .

4 Add monensin and brefeldin A, assuming 1e6 cells.
Anti-CD107a BV421 can be added here as well (5 µL)

5 Mix and incubate at 37 °C 5% CO2 06:00:00 . 6h

Preparations 1h 10m

6 Prepare the staining mix. (for 1 sample:)

	A	B	C	D	E
	Supplier	**Antibody**	**Clone**	**Channel**	**Volume per test**
	BD Biosciences	CD3	SP34-2	Alexa Fluor 700	5
	BD Biosciences	CD8	RPA-T8	BV786	5
	BD Biosciences	CD45RA	5H9	PE-CF594	2.5
	BD Biosciences	CD4	L200	PerCP-Cy5.5	20
	BD Biosciences	CCR7	G043H7	BV711	5
	BD Biosciences	CD69	FN50	BV650	5

	A	B	C	D	E
		Total			42.5
		BD Brilliant Stain			57.5

7 Prepare the 1X FACS Lysing solution by diluting the 10X stock with Milli-Q water. You will need 2 ml of 1X solution for each sample.
BD FACS Lysing Solution (10X) **Becton Dickinson (BD)** **Catalog #**349202

Surface Staining 30m

8 Put 5 µl of TruStain FcX in each tube/well.
Human TruStain FcX **BioLegend** **Catalog #**422302

9 Incubate 10 min at Room Temperature (RT). 00:10:00 Room temperature 10m

10 Add 5 µl of the Ghost Dye Red 780 **Tonbo Biosciences** **Catalog #**13-0865-T500
If staining BAL: Add 5 µl of 1:100 diluted Ghost Dye.

11 Incubate 20 min at RT in the dark. Room temperature 20m
00:20:00

12 Add stain mix. Mix. 100 µL

13 Incubate 20 min at RT in the dark. Room temperature 20m
00:20:00

RBC Lysis

14 Add 2 ml of 1X FACS Lysing solution. Vortex immediately, but gently. 2 mL

15 Incubate no more than 12 min at RT in the dark. 00:10:00 No more than 12 minutes 10m

16 Spin at 300 x g for 5 min. 300 x g, 20°C, 00:05:00

17 Decant supernatant.

18 Add 2 ml of PBS. Vortex. 2 mL
PBS

19 Spin at 300 x g for 5 min. 300 x g, 20°C, 00:05:00

20 Decant supernatant.

Sample Inactivation 30m

21 Resuspend in Cytofix/Cytoperm.
BD Cytofix/Cytoperm **Becton Dickinson (BD) Catalog #**554722

21.1 100 µl per 5 × 10^5 cells.

21.2 Use at least 400 µl for easy decanting.

22 Incubate at least 30 min at RT in the dark. 00:30:00 30m
Room temperature In the dark.

23 Spin at 500 x g for 8-10 min. 500 x g, 20°C, 00:08:00

24 On a clean bench, decant supernatant.

25 Use same volume of Cytofix/Cytoperm as before to resuspend the cells.
BD Cytofix/Cytoperm **Becton Dickinson (BD) Catalog #**554722

26 Transfer in a 2 ml screwcap tube. Shake the tube to cover all surfaces with Cytofix/Cytoperm.

Equipment

2 ml screw-cap tubes	NAME
Microtubes	TYPE
Sarstedt	BRAND
72.694.006	SKU

27 Transfer tubes from containment space to CL2 space according to the facility's approved protocols/SOPs.

28 Tubes can be opened in CL2 (in a BSC) no less than 30 min after the resuspension (step 17).
(Samples are generally processed the next day; keep at 4 C overnight, in the dark)

Intracellular staining 30m

29 Give tubes a quick spin in a tabletop centrifuge.

30 Ensure all tubes have at least a few hundred microliters of Cytofix/Cytoperm.

31 Transfer the samples into 1 ml of BD
BD Perm/Wash buffer **Becton Dickinson (BD) Catalog #**554723 in FACS tubes.

32 Spin at 500 x g for 8-10 min. 500 x g, 20°C, 00:08:00

33 Decant supernatant.

34 Repeat steps 31-33.

35 Prepare the staining mix. (for 1 sample:)
(uses BD Perm/Wash buffer **Becton Dickinson (BD) Catalog #**554723)

	A	B	C	D	E
	Supplier	**Antibody**	**Clone**	**Channel**	**Volume per test**
	BioLegend	IFNgamma	B27	APC	2.5
	BioLegend	TNF	MAb11	PE-Cy7	5
	BioLegend	IL-2	MQ1-17H12	Alexa Fluor 488	0.625
	BioLegend	IL-4	8D4-8	PE	2.5
		Total			10.625
		PermWash			89.375

36 Resuspend in 100 µl of intracellular staining mix.

37 Incubate 4 °C 00:30:00 30m

38 Add 1 mL BD Perm/Wash buffer **Becton Dickinson (BD) Catalog #**554723 .

39 500 x g, 4°C, 00:08:00 8m

40 Decant supernatant and blot tubes/plate.

41 Repeat steps 38-40.

42 Resuspend in PBS 1% formaldehyde.

43 Run on flow cytometer.

44 Gating strategy for whole blood: