Immunophenotyping for NHPs, containment protocol

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This is a protocol used to perform immunophenotyping of 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 AGM only). It allows the characterization of T cells (CD69/CD25 for activation; CCR7/CD45RA for memory phenotype; α/β vs γ/δ), B cells, Monocytes, Neutrophils, Basophils, Eosinophils, an potentially NK cells (CD56 does not work for AGM).

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.

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flow cytometry, nonhuman primate, immunophenotyping, whole blood, broncho-alveolar lavage

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

Human TruStain FcX **BioLegend Catalog #422302** Step 4

BD FACS Lysing Solution (10X) **BD Biosciences Catalog #349202** Step 2

BD Cytofix/Cytoperm **BD Biosciences Catalog #554722** In 2 steps

Ghost Dye Red 780 **Tonbo Biosciences Catalog #13-0865-T500** In 2 steps

FACS Tube
Tube
Falcon **14-959-2A**

2 ml screw-cap tubes
Microtubes
Sarstedt **72.694.006**

PBS **Contributed by users** In 3 steps

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The antibody mix described in the methods was tested on African green monkey whole blood and BAL fluid. CD56 does not stain AGM NK cells. All antibodies should cross-react with cynomolgus and rhesus macaques but the panel might need to be re-titrated.

Preparations 1h 10m

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

A	B	C	D	E
Supplier	**Antibody**	**Clone**	**Channel**	**Volume per test**
BD Biosciences	CD45	D058-1283	BUV395	1.25
BD Biosciences	CD3	SP34-2	BUV496	5
BD Biosciences	CD8	RPA-T8	BUV563	1.25
BD Biosciences	CD16	3G8	BUV737	5
BD Biosciences	CD45RA	5H9	BV421	0.625
BD Biosciences	CD49d	9F10	BV480	5
BD Biosciences	CD4	L200	BV605	0.625
BD Biosciences	CD14	M5E2	BV650	5
BD Biosciences	CD123	7G3	BV786	1.25
BD Biosciences	CD25	M-A251	BB515	5
Miltenyi Biotec	CD66abce	TET2	PerCP-Vio700	1
BD Biosciences	CD56	MY31 or NCAM16.2	BV711	5
BD Biosciences	CD163	GHI/61	PE	20
BD Biosciences	CCR7	2-L1-A	PE-CF594	0.625
BioLegend	HLA-DR	L243	PE/Fire640	1.25
BD Biosciences	CD69	FN50	PE-Cy7	5
BD Biosciences	TCRgd	B1	APC	5
BD Biosciences	CD20	2H7	Alexa 700	0.625
	Total			68.5
	BD Brilliant Stain			31.5

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2 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) **BD Biosciences Catalog #349202**

3 Prepare a 1:100 dilution of viability dye combining **495 µL PBS** and **5 µL**

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

For whole blood, use the Ghost Dye undiluted.

Surface Staining 30m

4 Put 5 µl of TruStain FcX in FACS tubes (1 tube per sample).

Human TruStain FcX **BioLegend Catalog #422302**

FACS Tube	
Tube	
Falcon	**14-959-2A**

5 Add 100 µl of whole blood (EDTA blood) or BAL cells to the correct tube. Mix.

100 µL Sample

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

7 Add 5 µl of the diluted

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

If staining whole blood: Add 5 µl of undiluted Ghost Dye.

8 Incubate 20 min at RT in the dark. **Room temperature** 20m

00:20:00

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9 Add stain mix. Mix. **100 µL**

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

RBC Lysis

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

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

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

14 Decant supernatant.

15 Add 2 ml of PBS. Vortex. **2 mL**
PBS **Contributed by users**

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

17 Decant supernatant.

Sample Inactivation 30m

18 Resuspend in Cytofix/Cytoperm.

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BD Cytofix/Cytoperm **BD Biosciences Catalog #554722**

18.1 100 µl per 5 x 10^5 cells.

18.2 Use at least 400 µl for easy decanting.

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

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

21 On a clean bench, decant supernatant.

22 Use same volume of Cytofix/Cytoperm as before to resuspend the cells.
BD Cytofix/Cytoperm **BD Biosciences Catalog #554722**

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

2 ml screw-cap tubes	
Microtubes	
Sarstedt	**72.694.006**

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

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25 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)

Final Wash & Run 30m

26 Give tubes a quick spin in a tabletop centrifuge.

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

28 Transfer the samples into 1 ml of PBS in FACS tubes. PBS **Contributed by users**

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

30 Decant supernatant.

31 Resuspend in 200 µl of PBS. PBS **Contributed by users**

32 Run on FACSymphony A5.

33 Gating strategy for whole blood:

Gating_blood.pdf

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