Immune Tolerance Network

Biomarker & Discovery Research

STANDARD OPERATING PROCEDURE

Title: **Whole Blood Basophil Activation Assay**	SOP No.: 001	Version: 1.2
	Effective Date: 23April2014	Page: **1 of 6**
Trial Number (if applicable):ITN050AD		

Document Approval

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1 Purpose

To describe the procedure for executing the basophil activation assay using whole blood samples to support clinical trial ITN050AD at the Stanford Nadeau laboratory.

2 Definitions and Abbreviations

RPMI-1640	Roswell Park Memorial Institute medium, is a general purpose media with a broad range of applications for mammalian cells, especially hematopoietic cells
BD Pharmlyse	A buffered, concentrated (10X) ammonium chloride-based lysing reagent.
PBS	Phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid – chelating agent
FACS tube	Tubes compatible with flow cytometer
WB	Whole Blood
BSA	Bovine Serum Albumin
PE	Peanut extract

3 Equipment and Materials

Reagents:

- RPMI 1640
- BD Pharmlyse (made from 10X stock with dH_2O)
- PBS + 20 mM EDTA
- Staining Buffer: PBS + 2 mM EDTA + 0.5% BSA

- Monoclonal antibodies:

Vendor	Reagent	Ab Clone Name	Catalog Number(s)
eBiosciences	aCD123-FITC	6H6	11-1239-42
BioLegend	aCD203c-PE	NP4D6	324606 (100 test)
BioLegend	aHLADR-PerCP	L243	307628 (100 test)
BioLegend	aCD63-APC	MEM-259	312010 (100 test)

- Stimulants (prepared in RPMI immediately before use)
- 2 µg/mL Anti-IgE (final concentration 1 µg/mL)
- 2 µM fMLP (final concentration 1 µM)
- 5 µg/mL Anti-FceRI (final concentration 2.5 µg/mL)
- Peanut extract from the Golden Peanut Company (final top concentration 10 µg/ml, prepare 6 dilutions starting at 20 µg/mL and serially diluting 10-fold). Note that this preparation is from the same supplier as that used to dose participants in the IMPACT ITN050AD trial.

Equipment

- Bench top refrigerated centrifuge capable of 800 x g
- LSR II flow cytometer

4 Procedures

1. Label the required number of round-bottom, polypropylene FACS tubes.

Tube	Cells	Stimulant (100µl)	Antibodies
1	100µl WB	RPMI	aCD123-FITC aCD203c-PE aHLADR-PerCP aCD63-APC
2		PE 0.2 ng/mL	
3		PE 2 ng/mL	
4		PE 20 ng/mL	
5		PE 200 ng/mL	
6		PE 2000 ng/mL	
7		PE 20000 ng/mL	
8		aIgE 2 µg/mL	
9		fMLP 2µM	
10		aFcεRI 5µg/mL	
Isotype control		RPMI	aCD123-FITC, aHLADR-PerCP IgG-PE, IgG-APC

2. Dilute stimulants to the appropriate concentration (above) in RPMI (Peanut protein fMLP, anti-IgE and aFcεRI)

3. Transfer 100 µL of RPMI to tube 1 and isotype control

4. Transfer 100 µL of appropriate stimulant to tubes 2-10 as above.

5. Transfer 100 µL of patient whole blood to each tube (total volume = 200 µL).

6. Incubate for 30 minutes at 37°C (5% CO2) in an incubator.

7. During the incubation, prepare ice bucket and mAb staining cocktail: 2.5µl CD123FITC + 5µL HLADR-PerCP + 10µl CD203c-PE + 5µl CD63-APC per condition/tube.

8. Add 20 µL cold (4°C) 20 mM EDTA to each tube and put the tubes on ice to stop degranulation.

9. Stain cells by adding 22.5µL of the prepared Ab cocktail to tubes 1-10

10. For isotype control, add 2.5µl CD123-FITC + 5µl HLADR-PerCP + 2.5µl PE-isotype + 10µL APC-isotype

11. Incubate all tubes at 4°C for 30 minutes in the dark

12. Add 3 mL cold (4°C) staining buffer to each tube & centrifuge @ 300xg for 5 minutes at 4°C.

13. Decant supernatant very carefully as the pellet will be loose.

14. Add 4 mL of 1x lysis buffer (BD Pharmlyse) with serological pipette while vortexing at a low speed.

15. Incubate at room temperature in the dark for 15 minutes.

16. After incubation, centrifuge @ 800 x g for 10 minutes at 4°C.

17. Decant supernatants carefully, add 3 mL of cold staining buffer to each tube and centrifuge @ 500xg for 5 minutes at 4°C.

18. Decant supernatants carefully and resuspend in 200 µL of staining buffer. Transfer to a 96 well plate for acquisition on the LSR-II.

19. Store at 4°C in the dark until ready to acquire (same day is optimal, acquisition within 24 hours is acceptable).

5 Attachments and References

6 History of Revision

Version	Effective Date	Section	Description of Revisions/Justifications
1.0	*20May2013*	*3 Reagents*	*Updated source of peanut flour from Byrd to* Golden Peanut Co