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WORKS FOR ME 1

Whole blood assay-Scrub typhus for flow cytometer

COMMENTS 0

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ABSTRACT

This is an optimized new whole blood assay for studying scrub typhus-specific cellular responses specifically suited for work in remote areas. This assay is based on stimulation with a whole cell *O. tsutsugamushi* antigen (HI-WCA-OT) in order to study broad antigen-specific cellular immune responses.

We provide the preparation method (Heat inactivation) and the adequate concentration for the antigen (10^9 of *O. tsutsugamushi* DNA copies/ml) to obtain optimal measureable specific cellular immune responses in whole blood samples for use in a Biosafety level (BSL) 2 laboratory.

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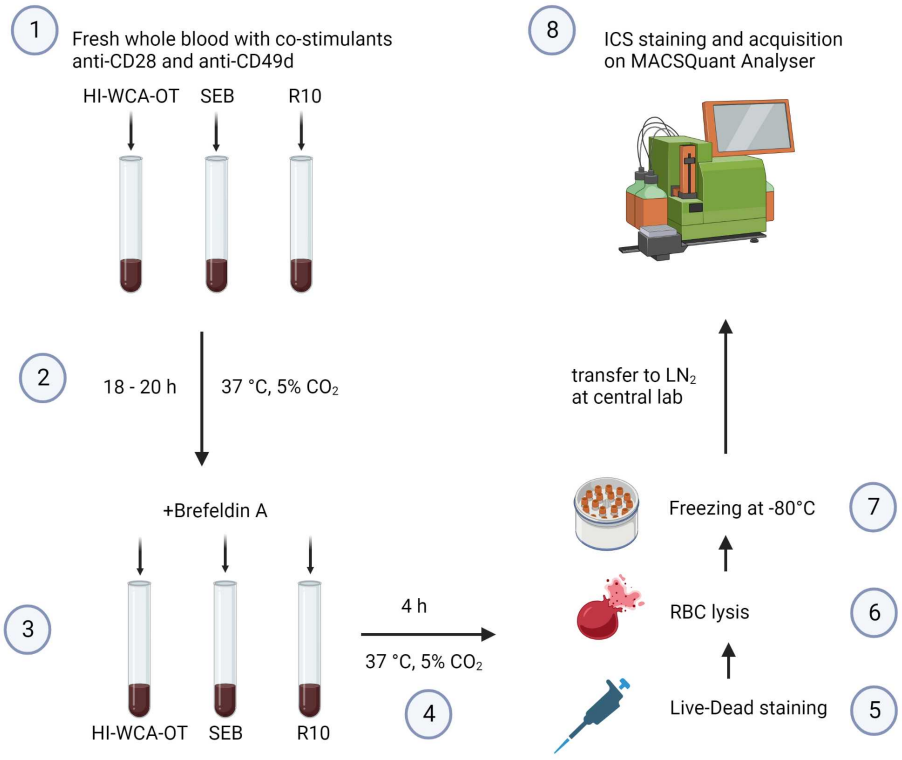

Procedure to perform the optimised WBA for patients with scrub typhus at a field site laboratory

Before starring

Solutions to be made up

R0·490 ml RPMI 1640 (without glutamine)

·5 ml 10000 U/ml penicillin/streptomycin from aliquot.

·5 ml 200 mM L-Glutamine from aliquot.

·Label “R0” and write date, initials, and write PS and LG lot numbers on the bottle. Store at 4°C and replace after 1 month or if any concern about sterility

PBS

·Made with PBS tablets from SIGMA, and dH2O.1 tablet in 100ml water.

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Day 1. Whole blood stimulation for scrub typhus study

1 Prepare pre-labelled 5 ml tubes to each contain **5** µl of "**COCKTAIL**" (co-stimulant) and **NO Brefeldin A.** Add the stimulants as follows to respective tubes as below

Stimulant	Volume of stimulant (µl)	Concentration of Stimulant
HI-WCA-OT	100	prepared from 10E9 copies/ml of Live O. tsutsugan
SEB (positive c	100	Dilute 1: 20 of stock SEB (1 mg/ml) in R10 Add 100
R0 (negative cc	100	

Co-stimulant	Manufacturer	Catalog#	final concentrat
CD28	BD Bioscience	340975	1 µg/ml
CD49d	BD Bioscience	340976	1 µg/ml

COCKTAIL (co-stimulant)
Each provided as 200 µg in 0.01% azide in PBS; **1** mg/mL. Store at 4°C in the dark.
Dilute 1: 10 of each in a 1 ml tube, combine
3 µl anti-CD28
3 µl anti-CD49d
24 µl PBS. Label "COCKTAIL"
For 500 µL blood; add **5** µl of the above "COCKTAIL" solution for a final concentration of 1 µg/mL each.

2 Each subject in the study needs 3 x 5 ml tubes labelled with their ID number and stimulant (antigen or control) i.e. "HI-WCA-OT"(Antigen of interest, Heat inactivated whole cell antigen of *Orientia tsutsugamushi*) "SEB" (Positive control) or "R0" (Negative control)

3 Add 400 µL of lithium heparinised blood to each tube, cap tightly, vortex.

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Note

4 Incubate at 37 °C for **18-20** hours in CO_2 incubator or in a water bath (optional).

Day 2. Harvest stimulated white blood cells and cryopreservation

5 Add **25** µL Brefeldin A (10 µg/mL)
eBioscience™ Brefeldin A Solution (1000X) **Thermo Fisher Catalog #00-4506-51**

6 Incubate at 37 °C for further 4 hours in CO_2 incubator or in a water bath (optional).

7 Stain with Live-Dead Staining dye (Near IR) 1 µl which enables discrimination of live from dead cells in subsequent flow cytometry analysis
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation **Thermo Fisher Catalog #L10119**

8 Incubate on ice for 20 min.

9 Wash the sample once with Phosphate buffered saline (PBS) and centrifuge at 500 g for 5 min.

Note

10 Remove supernatant by carefully pipetting without disturbing the pellet (RBC)

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11 Red blood cell lysis (RBC) was performed by adding 3 ml of 1x FACS lysing solution
BD FACS Lysing Solution (10X) **BD Biosciences Catalog #349202**

12 Incubate at room temperature for 10 min. (to help RBC lysis), vortex for 30 s.

13 Spin in centrifuge for 5 min at 500 rcf

14 Remove supernatant by pouring off then vortex to resuspend pellet for 10 s

15 Adding 3 ml of 1x FACS lysing solution, vortex for 30 s.

16 Spin in centrifuge for 5 min at 500 rcf

17 Remove supernatant by pouring off then vortex to resuspend pellet for 10 s

18 Add 1 ml freezing mix to resuspended pellet and transfer 500 µl each into 2 labelled cryotubes (so that there is two tubes for each condition for freezing) followed by stepwise freezing to -80°C

Note

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19 To do ICS, cryopreserved stimulated whole blood samples were slowly thawed in a 37°C water bath

20 The cells are then undergo fixation and permeabilization using Cytofix (BD Biosciences, BD Cytofix/Cytoperm **BD Biosciences Catalog #554722**) and Cytoperm/Wash (BD Biosciences BD Perm/Wash buffer **BD Biosciences Catalog #554723**) according to the manufacturer's instructions

21 Cells were stained with the following fluorochrome conjugated antibodies to assess polyfunctionality and memory:
Surface staining: CD8 V450 (clone RPA-T8, Cat 560347), CD3 V500 (clone SP34-2, Cat 560770), CD4 FITC (clone RPA-T4, Cat 555346 and clone M-T477, Cat 556615), CD45RA-PE (clone HI100, Cat 555489) all from BD Biosciences.
Intracellular staining: IFN-gamma APC (clone B27, BD Biosciences, Cat 554702), IL-2 PE (clone N7.48A, Beckman, Cat IM2718U) and TNF PCP-Cy5.5 (clone MAb11, eBioscience, Cat 45-7349-42).

21.1 After adding antibody for surface staining, the cells are incubated for 30 min at 4 °C

21.2 Washing the cells in centrifuge for 5 min at 500 rcf

21.3 Remove supernatant by pouring off then vortex to resuspend pellet for 10 s

21.4 After adding antibody for intracellular staining, the cells are incubated for 30 min at 4 °C

21.5 Washing the cells in centrifuge for 5 min at 500 rcf

Remove supernatant by pouring off then vortex to resuspend pellet for 10 s

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21.6

21.7 Resuspend cells with 200 µl fixation buffer (BD Bioscience, Cat 554655) for subsequent flow cytometric analysis.

22 Acquisition of a minimum of 100,000 cells per sample was performed on a MACSQuant 10 analyzer (Miltenyi Biotec).

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