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Whole blood assay-Scrub typhus for flow cytometer

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We use this protocol and it's working

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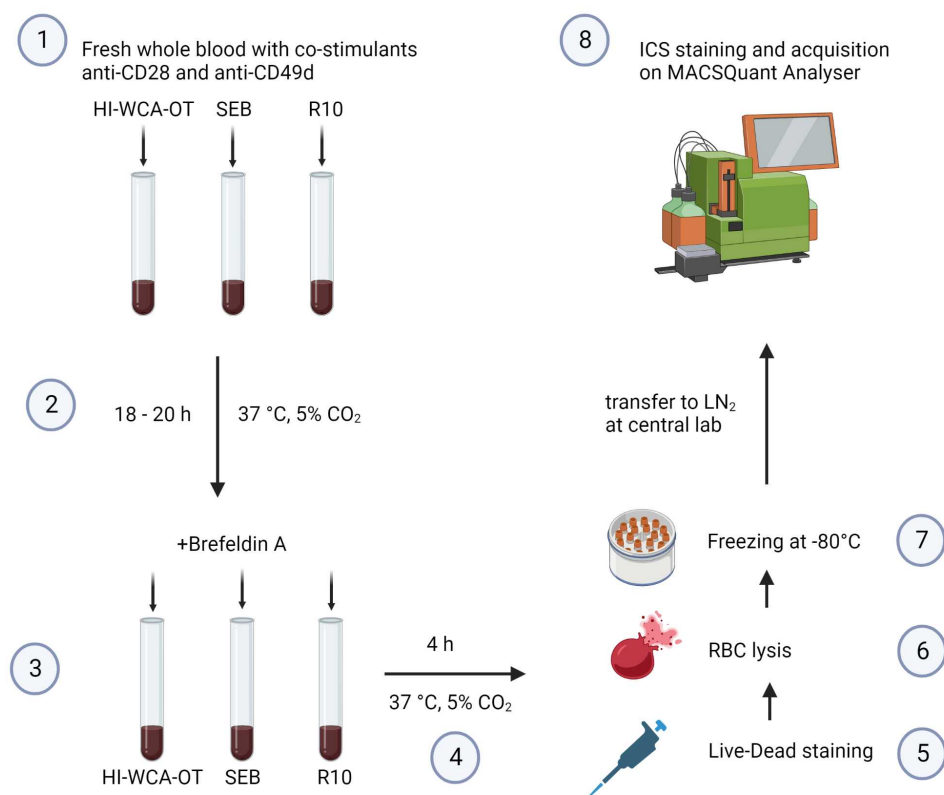
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Abstract

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.



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

Before starting

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 100 ml water.



Protocol materials

 eBioscience® Brefeldin A Solution (1000X) **Thermo Fisher Catalog #00-4506-51**

 LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation **Thermo Fisher Catalog #L10119**

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

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

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

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	<i>prepared from 10E9 copies/ml of Live O. tsutsugamushi</i>
SEB (positive control)	100	Dilute 1: 20 of stock SEB (1 mg/ml) in R10 Add 100 µl of this "SEB WBA" to each tube for final concentration of 10 µg/ml in 400 µl blood
R0 (negative control)	100	

Co-stimulant	Manufacturer	Catalog#	final concentration
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 × 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.

Note

Use lithium heparinised blood collection tube only, Not other anticoagulants



- 4 Incubate at  37 °C for **18-20** hours in CO₂ 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 CO₂ 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

Washing in PBS may help improve lysis

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

- 11 Red blood cell lysis (RBC) was performed by adding 3 ml of 1x FACS lysing solution



BD FACS Lysing Solution (10X) **Becton Dickinson (BD) 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

- 1) Freezing media: 10% DMSO combined with 90% Fetal bovine serum solution
- 2) In the case of field study scrub typhus samples, cells were stored at -80°C for a minimum of 2 weeks, then shipped on dry ice to the central laboratory and stored in Liquid Nitrogen until further analysis

Intracellular cytokine staining (ICS) for Flow cytometry

- 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 Cytotfix (BD Biosciences,  BD Cytotfix/Cytoperm **Becton Dickinson (BD) Catalog #554722**) and Cytoperm/Wash (BD Biosciences  BD Perm/Wash buffer **Becton Dickinson (BD) 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
- 21.6 Remove supernatant by pouring off then vortex to resuspend pellet for 10 s
- 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).