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Standard Operating Procedure

Title	***Whole blood assay for cytokine production***
Subtitle	
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Contents

1 Purpose

The purpose of this standard operating procedure (SOP) is to describe the method for assessing cytokine production from a small amount of whole blood (250 µl per sample) stimulated with a known concentration (10 ng/ml) of bacterial lipopolysaccharide (LPS). Various substances can be tested in this assay, using LPS as positive control and medium alone as negative control.

2 Principle

Whole blood aliquots of 250 µl are cultured in RPMI medium. An untreated sample (negative control) will be compared to an LPS-stimulated sample. After 3, 8, and 24 h of culture, cells are harvested. Settled blood cells and supernatant can be collected separately and subsequently tested by ELISA to determine internal cytokine production (cell lysates) and secreted cytokines (supernatants). Alternatively, total cytokine production can be measured in the whole sample.

3 Materials

	Reagents	**Equipment**
Blood collection		BD Vacutainer® EDTA tube (purple top); butterfly needle; syringe; tourniquet; disinfectant; band aid
Cell counting	Tryphan blue; Red Blood cell lysing buffer; Diff-Quik staining set	light microscope, Neubauer (cell counting) chamber; Cytospin-2 with cytofunnel, metal holder, filter cards and object slides (in alternative, automatic blood counting instrument)
Cell culture	RPMI medium; LPS stock	1.5 ml cryotubes; racks; pipettes; tips; incubator (37°C); laminar-flow cell culture cabinet
Harvesting	Triton-X-100 (5% in dd or Milli-Q H_2O)	Pipettes; tips; vortex; freezer (-80°C)

4 Procedure

4.1 Blood collection

- Blood is collected by an authorized person and mixed well in the purple Vacutainer by inverting at least 5x (blood can now be stored for several hours at RT before proceeding with the experiment; for shipment and longer periods blood should possibly be stored at +4°C)
- 250 µl of whole blood are needed per tube

4.2 Culturing blood cells

- Cryotubes are prepared with RPMI medium under sterile conditions. Depending on blood availability, prepare one or two replicate tubes per condition.
  Tubes A: 750 µl RPMI (negative control), prepare two replicate tubes

  Tubes B: 700 µl RPMI + 50 µl LPS solution [0.2 µg/ml in RPMI] (positive control), prepare two replicate tubes

  Tubes C on: 700 µl RPMI + 50 µl test agent/drug [20x stock in RPMI] (experimental samples), prepare 1-2 replicate tubes

  N.B. tubes can be prepared the previous day and stored at +4°C. Keep the tubes at room temperature for at least 1 h before adding blood. Prepare one rack of tubes for each experimental time point.
- Mix blood by inverting the tube 5x before pipetting it into the cryotubes
- Pipet 250 µl blood with P1000 to each cryotube
- Close tightly; invert tubes 5x to mix well
- Incubate at 37°C for the experimental time (3, 8, and 24 h)

4.3 Counting blood cells

If total and differential cell counts are not provided by the physician in charge, it is necessary to proceed to cell counting. This is necessary for expressing the cytokine production data in relation with the number of cells (or cell type) in samples from different donors. The procedure below is for manual counting, in the case an automated counter is not available.

- Lyse red blood cells as described in the Red Blood Cell Lysing Buffer instructions
- Stain with appropriate dilution of PBS/trypan blue (1:2) and count cells in Neubauer chamber within 3 minutes (trypan blue is toxic for cells after this time). Calculate the concentration of viable cells and the percentage of dead cells (blue).
- In alternative, since the cell viability is always very high, dilute whole blood 1:20 with Türk's solution (0.5 ml glacial acetic acid, 1.5 ml gentian violet 1%, dd H_2O up to 150 ml) and count WBC (red cells will be lysed, and the WBC fixed, differential counting is possible for an expert observer).
- Normal range: 6-8x10^6 WBC/ml in adults; 14-16x10^6 WBC/ml in young children.

For differential counts, Wright-Giemsa stained smears are used. The procedure described hereafter implies the use of a cytocentrifuge and a modified rapid Wright-Giemsa stain.

- Collect 1x10^5 cells and dilute them with PBS to a final volume of 200 µl
- Prepare cytospin slides, place them in the cytospin and add the cell suspension into the cytofunnel, then centrifuge at 700 rpm for 10 min
- Remove slides and allow them to air-dry (about 1 min)
- Dip slides 3 times for 3 sec into each Diff-Quik staining solution (1. Fixative, 2. Solution I, 3. Solution II), then rinse in dd H_2O
- When dry, perform the differential count under light microscope using 40x magnification.

4.4 Harvesting

- Prepare 5% Triton-X solution (in dd H_2O) beforehand (ideally one day before it is needed); cut the tip before pipetting the viscous Triton-X and mix very thoroughly by vortexing.
- Prepare Eppendorf tubes for sample collection (two tubes for each cryovial), label them carefully, *e.g.*, A1s, A2s, A1c, A2c, meaning replicates 1 and 2 of condition A, supernatant (s) or cell pellet (c).
- Very carefully take the cryovials out of the incubator. Make sure to avoid mixing the sedimented cells with the supernatant!
- Collect clear supernatant (700 µl) without touching cell pellet, transfer to eppendorf vial (*e.g.*, A1s) and fill up the volume with 400 µl RPMI medium to final volume of 1.1 ml
- Collect the blood cells (300 µl), transfer to corresponding eppendorf tube (A1c), and wash the cryovial with 700 µl medium, and add to the cryotube. Add 100 µl of 5% Triton-X to the sample (final volume 1.1 ml) and vortex
- Freeze samples at -80°C (this step is essential for complete lysis)
- Before using blood cell samples for ELISA, centrifuge samples at 5000 rpm for 5 min and use clear supernatant

ALTERNATIVE METHOD: TOTAL CYTOKINES

- Prepare 5% Triton-X solution (in dd H_2O) as described above
- Take the vials out of the incubator
- Add 100 µl of 5% Triton-X to each vial (final volume 1.1 ml) and vortex
- Freeze samples at -80°C (this step is essential for complete lysis)
- Before using blood cell samples for ELISA, centrifuge samples at 5000 rpm for 5 min and use clear supernatant

5 Potential hazards and waste disposal procedures:

Before using any reagent for this protocol, please read the material safety data sheets (MSDS). This allows you to be aware, of any potential hazard associated with the product and correct waste disposal.

All reagents should be disposed in appropriate liquid waste containers (e.g., a beaker or bottle) containing bleach and according to MSDS indications. Sharps are designated to specific waste containers. Plastic ware is disposed of in bins for laboratory solid waste. All biological waste must be sterilised.

Equipment must be handled with care for personal safety (e.g., make sure to close lid of centrifuge) and to avoid damage/contamination caused by spills (to avoid this make sure tubes are closed properly and working area is clear).

6 Personal Protective Equipment

For personal safety, wear gloves and lab coats at all times when handling the reagents and equipment. The use of gloves requires particular attention, to avoid to accidentally contaminating equipment and surfaces by touching them with gloves that were in contact with reagents.