Introduction

In vitro stimulated proliferation of lymphocyte may provide valuable information in cases of suspected immunodeficiency or impaired lymphocyte function.

In FASCIA (Flow cytometric assay of specific cell-mediated immune response in activated whole blood), whole blood, mixed with complete medium, is stimulated with polyclonal mitogens or antigen-specific stimuli followed by one week incubation and flow cytometric analysis. The stimulation activates lymphocytes to proliferate and start blast formation. Blasts are distinguished from resting non-activated and apoptotic lymphocytes by their side and forward scatter properties in a flow cytometer analysis. Furthermore, using fluorochrome conjugated monoclonal antibodies (e.g., anti-CD4, anti-CD8 or anti-CD19), FASCIA provide answers to the phenotype of the activated proliferating cells. Following seven days of incubation, granulocytes and most monocytes die, while RBCs are lysed before analysis.

Sample material and storage

Peripheral blood (minimum 1 mL) is collected in 5 or 10 mL Heparin vials (EDTA or Citrate preserved blood can’t be used). The samples are stored and transported at room temperature. The analysis should begin within 24 hours of collection. Blood from a healthy donor should also be stimulated as an internal assay control.
**Equipment / supplies**

Laminar air flow bench
75x12 mm 5 mL FACS tubes, sterile with cap (Polypropylene preferred eg. Falcon tubes # 352063).

**Will not work with FacsCalibur.** (Transfer cells after resusp. to regular PS tubes BD # 352054).

50 mL tubes 30x115 mm (eg. Falcon tubes # 352070)
Sterile pipettes 10 ml (eg. Sarsted # 86 1254.001)
Pipettor Eppendorf Easypet® or similar
Calibrated pipettes, 10-1000 µl
Sterile tips
Repeater device (eg. Eppendorf Multipette plus # 4981 000.019) with Combi tips 25 and 2.5 ml
Vortex
Incubator, 95% humidity, 37°C + 5% CO₂
Trucount tubes (BD Biosciences, # 340334)
Flow cytometer
1 mL CryoTubes for freezing of supernatants (if necessary)

**Reagents** (You may need to change country settings to get correct ordering information)

- Phosphate buffered saline (PBS), pH 7.5 supplemented with Albunorm™ 20% (Octapharma). Final concentration ≈ 1% Albunorm™ 20% vol/vol (1 ml + 100 ml PBS).
- RPMI 1640 (Gibco # 21875), N. B.! The media must be L-glutamine supplemented. Store in the dark, in a refrigerator up to one month after opening.
- PeSt 10 000 IU/mL penicillin + 10 000 IU/mL streptomycin mix (Gibco # 15140-122). Store at -80°C.
- L-glutamine (Invitrogen # 25030-024). Store at -80°C.
- Sterile ddH₂O
- IOTest® 3 Lysing Solution, (Beckman Coulter # A07799), diluted (1 + 9) in ddH₂O. Daily fresh prepared.
- anti-human CD3-FITC/CD4-PE Simultest mix (BD Biosciences # 342405)
- anti-human CD19-PC7 (Beckman Coulter # M3628U), CD19-PerCP-Cy™5.5 (BD # 561295) or CD19-APC (BD # 555415)

**Stimuli (mitogens/antigens):**

- PHA-P (Phytohemagglutinin); Stock: 1mg/mL. Final Dilution: 10 µg/mL (Sigma # L8754). Store at -80°C.
- PPD (Tuberculin purified protein derivative), Stock: 1 mg/mL, Final dilution 10 µg/mL (Statens Serum Institute, Copenhagen # 2391). Store at -80°C.
- Con A (Concanavalin A), Stock: 1mg/mL, Final Dilution: 10 µg/mL. (Sigma # C0412). Store at -80°C.
- PWM (Pokeweed mitogen); Stock: 1 mg/mL, Final Dilution: 5 µg/mL. (Sigma # L8777). Store at -80°C.
- Tetanus toxin; use undiluted. Stock: 40 IU/mL (Statens Serum Institute, Copenhagen # 2674). Store dark at 4°C.
- SEA + SEB (Staphylococcal enterotoxin A and B, Sigma # S9399 and # S4881) Stock: 1 mg/mL of each SEA and SEB, Final dilution: 100 ng/mL of each. Store at -80°C.
- Influenza vaccine (GlaxoSmithKline AB, Fluarix or local); Final Dilution 1:100. Store at 4°C.
- Candida albicans; Final Dilution 20µg/mL (Greer; Order to; On the dispatch note stands: XPLM73X1A2 Candida albicans 2.0 ml ACTG 400mcg/vial lyophilized in 5ml) Store at -80°C
- Varizella zoster virus vaccine (GlaxoSmithKline AB, Varilrix) Final Dilution 1:100. Store at 4°C.
- Pneumococcal vaccine (Wyeth, Prevenar 13); Final Dilution 1:100. Store at 4°C.

*Most other antigens can be included after titration.*

**Short panel:** PWM, ConA, Prevenar, Tetanus, SEA/B, Candida

**Culture media (durable 1 week at 4°C):**

49 mL of RPMI 1640
500 µL PeSt
500 µL L-glutamine 2 mM
**Preparations**

For the patient and control sample, label one FACS tube (75x12 mm) for each stimuli and one tube for the assay background (only blood + culture medium). Thaw and prepare 10 x concentrations of the final concentration of mitogens and specific antigens in 50 µL culture medium for each patient and control.

**Procedure**

Day 0 (sterile work):
1. Mix the heparinised blood thoroughly.
2. Dilute the whole blood 1 + 8 in culture medium in a 50 mL Falcon tube.
3. Vortex all prepared stimuli (10 x concentrated) and add 50 µL to the respective tubes. (For example 50 µL of 100 µg/ml PHA in tube labeled as PHA).
4. Add 450 µL of the blood-medium mixture (total volume with antigen 500 µL).
5. Shake gently and check that they contain the same volume and that the tube cap is resting loose.
6. Incubate tubes in 95 % humidity, 37°C + 5 % CO₂ for 7 days (6 days also applicable).

Day 6:
1. If culture supernatants are collected, label one cryo tube for each antigen.
2. Carefully remove supernatant from each sample tube. Store the culture supernatant in -80°C or discard it. It is important to remove the supernatant to decrease assay volume. N.B. If the sample tube is cloudy or bloody centrifuge the tube for 5 min at 350g, before removal of the supernatant.
3. Add 10 µL anti-human CD3-FITC/CD4-PE FACS antibody to all tubes and 10 µL of anti-human CD19-PC7 to the PWM and medium tubes.
4. Incubate tubes in the dark for 10 min.
5. Lyse the erythrocytes by adding 1.5 mL diluted IOTest® 3 Lysing solution/tube.
6. Incubate tubes in the dark for 10 min. Inspect the tubes, they should be transparent after effective lysis.
7. Centrifuge tubes 5 min at 350g.
8. Add 2 mL PBS + 1% albumin and centrifuge tubes 5 min at 350g. Discard the supernatant.
9. Add 450 µL of PBS + 1% Albonorm. All tubes shall have the same volume.
10. Resuspend the beads in TruCount FACS tube in 450 µL of PBS + 1% Albonorm.
11. Analyze the tubes on the flow cytometer within 4 hours!

**Flow analysis of FC500:**
The TruCount FACS tube is used as a reference to determine the volume the flow cytometer absorbs during 80 seconds (s). Use FACS-program CALC.PRO (80 s on medium speed by default). You can run TruCount tube, before, after or between patient samples.
Collect the sample tubes on the same principle but with FACS program FASCIA.PRO (80 s on medium speed by default). Start with the tube stimulated with PHA to set the gates. Make sure the blasts (proliferating cells) are found in region “Blast” and that the CD4+ and CD8+ (CD3+CD4-) cells lie in their respective regions. Blasts are identified by their FSc/SSc properties as larger than resting and dying lymphocytes. In sample tubes containing anti-CD19, the B-cell gate shall also be adjusted.

**Reading/ assessment/ calculation**

Total number beads in Trucount tube (TNB), resuspended in 450 μL PBS + 1% Albunorm.

Number of beads in the gate during the 80 s (NBG); Volume during 80 s (V80s)

The number of proliferating cells/μL blood is calculated using the following formula:

1. \( V_{80s} : \frac{(TNB/450 \text{ } \mu\text{L})}{(NBG/X \text{ number of } \mu\text{L acquired})} \)
   
   eg. \( \frac{(52460/450)}{(8690/X)} \rightarrow X = 75 \mu\text{L} \)

   OR

   \( V_{80s} = \frac{(NBG\times450 \text{ } \mu\text{L})}{TNB} \)
   
   eg. \( \frac{(8690\times450 \text{ } \mu\text{L})}{52460} = 75 \mu\text{L} \)

2. **Number of cells/μL blood:** \( 10 \times\left(\frac{(\text{stimulus blast region} - \text{medium blast region})}{V_{80s}}\right) \)
   
   eg. \( 10\times\left(\frac{(308-31)}{75}\right) = 37 \)

**Reference values**

Small deviations from the reference values should be interpreted with caution. The variation between individuals is large.

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**Precision**
Intra assay for two samples measured five times was 2.2 % (CV) for CD4\(^+\) and CD8\(^+\) T cells. Inter assay precision not applicable.

**Controls**
Blood from a healthy donor should also be stimulated as an internal assay control.

**Safety / Environment**
Contaminated blood
Monoclonal antibodies contain azide, which is hazardous.
Old blood samples
Clotted blood samples

**Publications**