

Standard operating procedure FASCIA

Introduction	1
Sample material and storage	1
Equipment/ supplies.....	2
Reagents.....	2
Preparations.....	3
Procedure	3
Reading/ assessment/ calculation.....	4
Reference values	4
Precision	5
Controls.....	5
Safety / Environment.....	5
Publications.....	5

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 (eg. 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 Multipipette 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 μ L culture medium for each patient and control.

Procedure

Day 0 (sterile work):

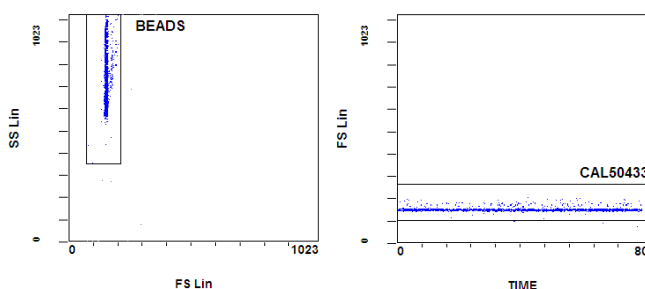
1. Mix the heparinised blood thoroughly.
2. Dilute the whole blood 1 + 8 in culture medium in a 50 mL Falcon tube.
3. Vortex all prepared stimuli (10 x concentrated) and add 50 μ L to the respective tubes. (For example 50 μ L of 100 μ g/ml PHA in tube labeled as PHA).
4. Add 450 μ L of the blood-medium mixture (total volume with antigen 500 μ L).
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:

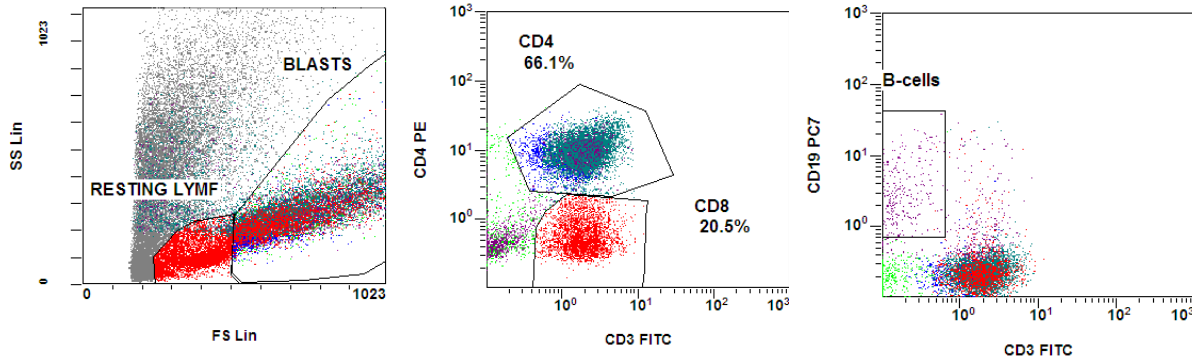
1. If culture supernatants are collected, label one cryo tube for each antigen.
2. Carefully remove supernatant from each sample tube. Store the culture supernatant in -80°C or discard it. It is important to remove the supernatant to decrease assay volume. N.B.! If the sample tube is cloudy or bloody centrifuge the tube for 5 min at 350g, before removal of the supernatant.
3. Add 10 μ L anti-human CD3-FITC/CD4-PE FACS antibody to all tubes and 10 μ L of anti-human CD19-PC7 to the PWM and medium tubes.
4. Incubate tubes in the dark in RT for 10 min.
5. Lyse the erythrocytes by adding 1.5 mL diluted IOTest® 3 Lysing solution/tube.
6. Incubate tubes in the dark in RT 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 μ L of PBS + 1% Albuorm. All tubes shall have the same volume.
10. Resuspend the beads in TruCount FACS tube in 450 μ L of PBS + 1% Albuorm.
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 μ L PBS + 1% AlbuNorm
 Number of beads in the gate during the 80 s (**NBG**); Volume during 80 s (**V_{80s}**)

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

$$1. \quad V_{80s} : (TNB/450 \mu L) = (NBG/X \text{ number of } \mu L \text{ acquired})$$

eg. $(52460/450) = (8690/X) \rightarrow X = 75 \mu L$

OR

$$V_{80s} = (NBG \times 450 \mu L) / TNB$$

eg. $(8690 \times 450 \mu L) / 52460 = 75 \mu L$

$$2. \quad \text{Number of cells}/\mu L \text{ blood: } 10 \times ((\text{stimulus}_{\text{blast region}} - \text{medium}_{\text{blast region}}) / V_{80s})$$

eg. $10 \times ((308 - 31) / 75) = 37$

Reference values

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

		n	Mean	stdev	5.th	95.th
SEA	CD4	76	3525	2077	426	7400
	CD8	76	1112	886	43	2534
PHA	CD4	108	1461	1023	181	3469
	CD8	108	1230	1093	83	3416
PWM	CD4	109	1282	782	307	2688
	CD8	109	269	265	59	602
	CD19	105	216	214	38	734
ConA	CD4	105	1814	1000	497	3728
	CD8	105	819	703	152	1928
PPD	CD4	94	493	653	7	1884
	CD8	94	16	53	0	51
Candida	CD4	74	440	666	41	1230
	CD8	74	16	35	0	51
Pneumococ	CD4	89	37	155	0	164
	CD8	89	2	6	0	12
Tetanus	CD4	102	76	220	0	293
	CD8	102	3	9	0	14
Flu/Fluari	CD4	86	227	330	16	902
	CD8	86	46	230	0	177
VZV	CD4	49	61	116	2	202
	CD8	49	7	11	0	30

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

H Gaines, L Andersson and G Biberfeld: "A new method for measuring lymphoproliferation at the single cell level in whole blood cultures by flow cytometry", Journal of immunological Methods 1996; 195: 63-72.

A Svahn, A Linde, R Thorstensson, K Karlén, L Andersson and H Gaines: "Development and evaluation of a flow cytometric assay of specific cell-mediated immune response in activated whole blood for the detection of cell-mediated immunity Against Varicella- zoster virus", Journal of immunological Methods 2003; 277: 17-25.