

PROTOCOL FOR PRIMARY CULTURE OF LYMPHOCYTES AND GRANULOCYTES FROM WHOLE BLOOD: IN-VITRO STIMULATION WITH TNF-ALPHA, IL-1 BETA, IL-6 AND CpG.

A: Isolation of WBC and culture:

Materials required:

- i) Whole blood from a healthy donor.
- ii) Lympholyte poly (cat# CL5070) from Cedarlane Labs.
- iii) Sterile, 0.45% NaCl.
- iv) RPMI + glutamine+ Penicillin-streptomycin.
- v) Autologous plasma (10%)-from the donor.
- vi) CYTOKINE AND CpG STOCKS and working concentrations:
Reconstituted TNF- α , IL-6 and IL-1 β (from R&D) in sterile PBS+0.1% BSA. Stored aliquots at -70°C .

1. TNF- α :

10 μg supplied. Recommended dilution is at least 10 $\mu\text{g}/\text{ml}$.
Reconstituted as a 10 $\mu\text{g}/0.5 \text{ ml}$. (or 20ng/ μl).
Final concentration required per well = 20ng/ml or 100ng/5ml of culture.
Used 5 μl of stock/well.

2. IL-1 β :

5 μg supplied. Recommended dilution is at least 1 $\mu\text{g}/\text{ml}$.
Reconstituted as a 5 $\mu\text{g}/\text{ml}$. (or 5ng/ μl).
Final concentration required per well = 10ng/ml or 50ng/5ml of culture.
Used 10 μl of stock/well.

3. IL-6:

10 μg supplied. Recommended dilution is at least 1 $\mu\text{g}/\text{ml}$.
Reconstituted as a 10 $\mu\text{g}/\text{ml}$. (or 10ng/ μl).
Final concentration required per well = 15ng/ml or 75ng/5ml of culture.
Used 7.5 μl of stock/well.

4. CpG 1826 (stimulatory oligo):

Total OD=112.7
Concentration=33.90 $\mu\text{g}/\text{OD}$
Total amount=33.90X112.7=3820.53 μg of lyophilized oligo.
Reconstituted the oligo in 0.382053 ml of sterile distilled H₂O to obtain a 10mg/ml Master stock. 20 μl aliquots frozen at -70°C .
Working stock: 10 μl of master stock made up to 100 μl with sterile PBS to give a 1 $\mu\text{g}/\mu\text{l}$ stock. Final concentration required per well = 2 $\mu\text{g}/\text{ml}$ or 10 $\mu\text{g}/5\text{ml}$ of culture.
Used 10 μl of stock/well.

5. MIF:

300 $\mu\text{g}/\text{ml}$ Stock in 20mM Tris-150mM NaCl. Working concentration: 25ng/ml.

Method:

1. Collect 120 ml blood from healthy donor in ACD tubes. Use 12 ml of blood to collect at least 6 ml of autologous plasma for adding to cultures (egs: 6 ml plasma is needed for for 60 ml of medium). Use the remaining blood for isolation of total white cells.
2. Isolate granulocytes and lymphocytes using the lympholyte –poly solution (density gradient centrifugation)-Follow the protocol from the data sheet supplied.

Notes:

- a) Bring all solutions, esp. the lympholyte –poly to RT. Centrifugations should also be done at RT. Brake of centrifuge should be set at zero, so that the layers are not disturbed.
 - b) The protocol is basically optimized for 5mls of blood. For larger volumes of blood (15-20ml), spinning at 500g for 40 mins in 50 ml polypropylene falcon tubes, gives better separation of the layers.
 - c) While lympholyte–poly is ideal for granulocytes; the lymphocyte layer can also be recovered with over 95% viable lymphocytes. Both layers need to be made up with equal volumes of 0.45% NaCl to normalize the tonicity and density, and washed with excess medium to remove the gradient. The 2 layers can be retrieved and washed separately and counted to check the recovery and then, they can be pooled to start the cultures.
3. Keep 20×10^6 cells on ice for Baseline RNA. Also aliquot at least 0.5×10^6 cells per tube, for a total of 9 tubes, for FACS analysis of 0hr culture.
 4. 20×10^6 cells are seeded on to each well of a 6-well plate, in 5 ml of RPMI+glut+Pen-strp+10% autologous plasma, and either vehicle/cytokine is added to the respective well, at required final concentrations. About $0.5-1 \times 10^6$ cells are kept aside to check profile of cell types by FACS and also use 7AAD to confirm viability observed with trypan-blue exclusion.
 6. The cells are cultured for 6 hrs (and/or 24 hrs), at 37deg. C, 5% CO₂.
 7. At the end of 6 hrs (or 24 hrs), perform viability check by trypan blue, 7AAD stain and also check profile of cells using antibodies to pan-cell surface markers.
 8. Save the culture supernatant at –70 deg C for future use (ELISA).
 9. Continue with RNA isolation using Qiagen’s RNA easy mini kit.

Treatment groups:

1. Baseline (without culturing)
2. Vehicle (PBS)
3. Vehicle (PBS+0.1% BSA)
4. 20ng/ml TNF alpha (5 μ l/5ml medium-1 well; from stock 20 μ g/ml in PBS+0.1% BSA)
5. 10 ng/ml IL-1 beta (10 μ l/5ml medium-1 well; from stock 5 μ g/ml in PBS+0.1% BSA)
6. 15 ng/ml IL-6 (7.5 μ l/5ml medium-1 well; from stock 10 μ g/ml in PBS+0.1% BSA)

7. 2 μ g/ml CpG 1826 (Make 1mg/ml WORKING STOCK IN PBS from 10mg/ml MASTER STOCK, in sterile distilled water. Add 10 μ l of working stock to a 5ml culture).
8. MIF: 300 μ g/ml Stock in 20mM Tris-150mM NaCl(15 μ g in 50 μ l sent by Franak). PBS used as vehicle. Final concentration: 25ng/ml.

B. FACS Staining for WBC Subpopulations and for viability check using 7-AAD.

1. Place approximately 0.5×10^6 cells/ FACS tube. Fill FACS tubes to the top with FACS buffer. Keep cells on ice until ready to start procedure
2. Spin cells at 1200 rpm, 10 minutes, 4°C. (program #40)
3. Dump the FACS buffer into the hazardous waste.
4. Re-suspend cells by running end of tube over rack. Add 20 μ l of human Ab serum to each tube.
5. Incubate at room temperature for 20 minutes.
6. Add appropriate antibodies, then slightly vortex or shake the tubes to mix:
NOTE: Tubes 1 through 6 are controls and cells at 0 hr of culture can be used. Tubes 7, 8, 9 have to be prepared for 0hr and at 6 hrs for each condition. Cells that have not been fixed have to be acquired immediately and should be kept on ice.
- i) Unstained cells.
- ii) Iso Tube A: 5 μ l of FITC IgG1 Isotype, 5 μ l of PE IgG1 Isotype, 5 μ l of CyC IgG1 Isotype, 5 μ l of IgG1 APC.
- iii) 10 μ l of CD3-PE
- iv) 10 μ l of CD3-FITC
- v) 10 μ l of CD8-CyC
- vi) 5 μ l of CD3-APC.
- vii) Sample A: 10 μ l of CD4-FITC, 10 μ l of CD3-PE, 10 μ l of CD8-CyC, 5 μ l of CD20-APC.
- viii) 10 μ l of CD66B-FITC, 10 μ l of CD64-CyC
- ix) 7AAD-CyC (1:20 dilution) + 300 μ l of PE-labelled PKH26 reference microbeads.
–DO NOT FIX THIS TUBE-Keep on ice until acquisition.
7. Cover tubes and rack with foil.
8. Incubate for 5 minutes at room temperature.
9. Fill tubes with FACS buffer and spin at 1200 rpm, 10 minutes, 4°C. (program #40)
10. Immediately after spin, dump the FACS buffer into the hazardous waste, add small flick of tube to get rid of excess buffer. Drag tubes over rack to re-suspend cells.
11. Add 0.5 ml of PBS to each tube.
12. Add 100 μ l 4% paraformaldehyde to tubes 1 through 8. Do not fix 7-AAD tubes.
13. Gently vortex tubes; replace caps on tubes or cover with parafilm.
Store in rack covered with foil in 4°C. Label with name and date.

C. RNA isolation:

Column purification using Qiagen's RNA easy kit.
Use 20×10^6 cells/column. Follow protocol in RNAeasy mini handbook.

Store RNA as ethanol precipitate at -70°C until ready to process for probe preparation. Wash RNA twice with 80% EtOH. Resuspend in DEPC water. Do not use up all the RNA for chipping; leave at least 100ng RNA for future use(PCR etc).