

APPENDIX C

Cell Isolation

Purpose: Peripheral blood mononuclear cells (PBMC), consisting of lymphocytes and monocytes, and polymorphonuclear cells (PMNC) consisting of granulocytes were isolated and purified using a double density Ficoll separation methods (Toth et al., 1992). To maintain sterile conditions for lymphocyte (cell culture) proliferation, cell separation should be performed under a laminar flow hood.

Materials (all must be sterile):

Histopaque 1.077 (Sigma Chemical Co., St. Louis, MO)

Histopaque 1.119 (Sigma Chemical Co., St. Louis, MO)

15 ml conical tube (Fisher Scientific Co., Norcross, GA)

20 gauge needle

whole blood

automatic pipette

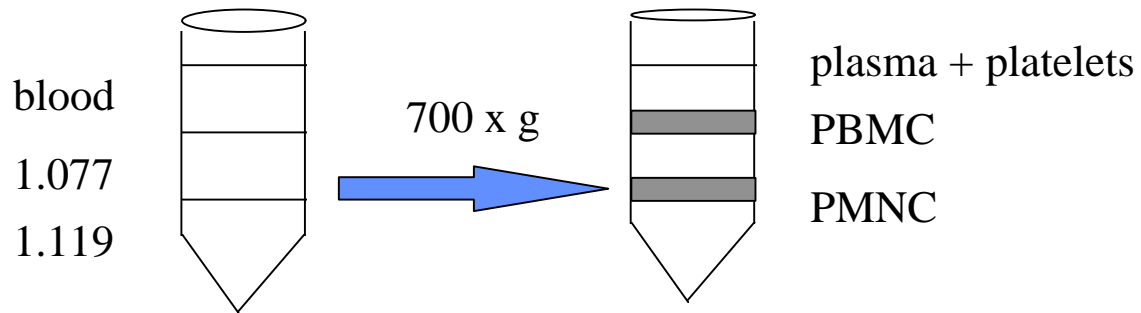
pastuer pipettes

HBSS (Hank's Balanced Salt Solution; Gibco Laboratories, Burlington, Ontario)

Methods:

1. Add 3 ml of Histopaque 1.119 to a 15 ml conical tube with a 20 gauge needle.
2. Using a clean 20 gauge needle and syringe, slowly layer 3 ml of Histopaque 1.077 over the Histopaque 1.119 layer. (Careful not to mix.)
3. Using an automatic pipette, slowly layer 6 ml of whole blood (collected in EDTA Vacutainer® tube) over the Histopaque 1.077 layer using an automatic pipette. (There should now be 3 distinct layers.)
4. Centrifuge the 15 ml conical tube at 700 x g for 30 minutes (min) with no brake at room temperature.
5. After centrifugation, separation of the cells will lead to two opaque bands of interfaces, peripheral blood mononuclear cells (PBMC) which includes the lymphocytes and polymorphonuclear cells (PMNC). The top layer (PBMC) of each sample should be aspirated with a pasteur pipette and transferred to a 50 ml conical centrifuge

tube containing 15 ml HBSS for cell washing. (Remember to use different pipettes for plasma (top) and cell layer.)



Reference:

Toth TE, B Smith, H Pyle. Simultaneous separation and purification of mononuclear and polymorphonuclear cells from the peripheral blood of cats. *J Virol Methods*. 1992; 36:1185-91.

APPENDIX D

Cell Washing

Purpose: PBMC need to be purified to remove excess Histopaque and platelets following the double density Ficoll separation.

Materials (all need to be sterile):

50 ml conical tubes (Fisher Scientific Co., Norcross, GA)

Hank's Balanced Salt Solution (HBSS; Gibco Laboratories, Burlington, Ontario)

complete media [100 ml complete media = 87 ml RPMI 1640, 10 ml of 10% fetal bovine serum (FBS), 1 ml L-glutamine, 1 ml sodium pyruvate, 1 ml pen-strep; Sigma Chemicals, St. Louis, MO]

hemocytometer

Methods:

1. Centrifuge the 50 ml conical tube containing the PBMC interface and 15 ml HBSS at 200 x g for 10 min with the brake on.
2. Discard the supernatant and repeat the procedure again. (Add 15 ml HBSS to pellet, vortex and centrifuge to mix up pellet).
3. Discard the supernatant and resuspended pellet in 10 ml complete media.
4. Centrifuge cell suspensions at 400 x g for 20 min with the brake on to remove any platelets.
5. Discard supernatant and resuspended in 2-4 ml HBSS (depending on pellet size) for cell counting on the hemocytometer. Adjust cells to 1×10^6 cells/ml with HBSS for each CD4 and CD8 quantification, 1×10^6 cells/ml for lymphocyte proliferation (for each set of wells, i.e. 3×10^6 cells for triplicates), 1×10^6 cells/ml for each CD4 and CD8 72 hours quantification, and 1.0×10^6 cells/ml for calcium flux, using the following equation (next page):

$$\frac{\text{cells counted}}{\text{ml HBSS}} = \frac{\text{cells needed}}{x}$$

x = ml HBSS needed to adjust cells to appropriate volume

Note: Compare appropriate volume with needed volume to make sure there is enough.

APPENDIX E

CD4/CD8 Quantification - Time Zero

Purpose: Identification of T lymphocyte surface markers using flow cytometric analysis.

Materials:

12 x 73 mm tubes

Hank's Balanced Salt Solution (HBSS; Gibco Laboratories, Burlington, Ontario)

Fluorescein Isothiocyanate (FITC) - conjugated rabbit antimouse IgG secondary antibody
(Southern Biotechnology, Inc., Birmingham, AL)

CD4 monoclonal antibody (Southern Biotechnology, Inc., Birmingham, AL)

CD8 monoclonal antibody (Southern Biotechnology, Inc., Birmingham, AL)

paraformaldehyde (2%) - (10 g paraformaldehyde, 5 g FA Bactobuffer, 400 ml distilled
H₂O)

Methods:

1. Label 3 tubes as FITC, CD4 and CD8 (for each sample).
2. Add 500 µl of adjusted cells (10⁶ cells/ml) (explained in cell washing) to each labeled tube.
3. Add 25 µl of 1:25 dilution of primary mouse anti-feline unlabeled purified CD4 monoclonal antibody to CD4 tube or CD8 monoclonal antibody to CD8 tube.
(Nothing is added to FITC tube).
4. Incubate tubes for 30 min at 4° C.
5. Centrifuge for 10 min at room temperature at 200 x g with brake on.
6. Discard supernatant and resuspend the pellet in 500 µl HBSS.
7. Add 2 µl of FITC-conjugated rabbit anti-mouse IgG (H + L) secondary antibody to all tubes.
8. Incubate for 30 min at 4° C.
9. Centrifuge for 10 min at room temperature at 200 x g with brake on.
10. Discard the supernatant and resuspend cell pellets in 500 µl of paraformaldehyde (2%).

11. Analyze on the flow cytometer. CD4⁺ and CD8⁺ expression of PBMC is calculated by subtracting the value of the negative control (FITC) sample from the value obtained from the sample that has been incubated with both primary (CD4 or CD8) and secondary (FITC) antibodies.

Reference:

Ackley CD, Hoover EA, Cooper MD. Identification of a CD4 homologue in the cat. *Tissue Antigens*. 1990; 35:92-8.

APPENDIX F

Lymphocyte Proliferation Assays

Purpose: To determine lymphocyte proliferation *in vitro*. Perform under laminar flow hood to maintain sterile conditions.

Materials:

2 sterile 96-well round-bottom microplates (Corning Co., Corning, NY)
Concanavalin-A (Con-A, 5 µg/ml complete media; Sigma Chemicals Co., St. Louis, MO)
complete media [100 ml complete media = 87 ml RPMI 1640, 10 ml of 10% fetal bovine serum (FBS), 1 ml L-glutamine, 1 ml sodium pyruvate, 1 ml pen-strep; Sigma Chemicals, St. Louis, MO]
Alamar Blue™ (Accumed International, Inc., Westlake, OH)
12 x 73 mm tubes
Fluorescein Isothiocyanate (FITC) - conjugated rabbit antimouse IgG secondary antibody (Southern Biotechnology, Inc., Birmingham, AL)
CD4 monoclonal antibody (Southern Biotechnology, Inc., Birmingham, AL)
CD8 monoclonal antibody (Southern Biotechnology, Inc., Birmingham, AL)
paraformaldehyde (2%) - (10 g paraformaldehyde, 5 g FA Bactobuffer, 400 ml distilled H₂O)

Methods:

1. Adjust cells to 1.0×10^6 cells/ml in complete media for each sample. [(Centrifuge aliquoted cells for proliferation in centrifuge at 200 x g for 10 min. Discard supernatant and add 1300 µl complete media (for triplicates, 3×10^6 cells, 100 µl per cell well (total 12) with 100µl leeway).
2. To two sterile 96-well round-bottom plates, add 100 µl of adjusted cells to 6 wells see template on next page).
3. Add 100 µl of complete media alone to 3 of the wells (label them media only).
4. Add 100 µl of complete media + Con-A (label them Con-A)
5. Incubate both plates in a humidified incubator at 37°C at 5% CO₂ for 72 hours.

Plate 1 - Alamar Blue™

Media	O	O	O
Con-A	O	O	O
Media	O	O	O
Con-A	O	O	O
Media	O	O	O
Con-A	O	O	O

Plate 2 - CD4/ CD8 72 Hours

	F	4	8	F	4	8
media	O	O	O	O	O	O
Con-A	O	O	O	O	O	O
Media	O	O	O	O	O	O
Con-A	O	O	O	O	O	O
Media	O	O	O	O	O	O
Con-A	O	O	O	O	O	O

CD4/CD8 72 hours

Purpose: To quantify CD4 and CD8 lymphocyte marker expression *in vivo*.

Methods:

1. After 72 hours incubation, take 1 plate out of the incubator.
2. Label 6 tubes (for each sample) CD4, CD8, FITC, CD4 Con-A, CD8 Con-A, and FITC Con-A.
3. Aspirate cells with a pastuer pipette with HBSS to break up pellet. Put cells from each control well (3 wells, 1 well per tube) into each of the labeled tubes CD4, CD8 and FITC. Put cells from each Con-A well (3 wells, 1 well per tube) into each of the labeled tubes CD4 Con-A, CD8 Con-A and FITC Con-A.
4. Add enough HBSS for centrifugation (~ 500 μ l), and centrifuge 10 min at 200 x g with brake on.
5. Discard supernatant and add 500 μ l HBSS.
6. Add 25 μ l of 1:25 dilution of primary mouse anti-feline unlabeled purified CD4 monoclonal antibody to CD4 tube or CD8 monoclonal antibody to CD8 tube. (Nothing is added to FITC-labeled tube).
7. Incubate tubes for 30 min at 4° C.
8. Centrifuge for 10 min at room temperature at 200 x g with brake on.
9. Discard supernatant and resuspend the pellet in 500 μ l HBSS.
10. Add 2 μ l of FITC-conjugated rabbit anti-mouse IgG (H + L) secondary antibody to all tubes.
11. Incubate for 30 min at 4° C.
12. Centrifuge for 10 min at room temperature at 200 x g with brake on.
13. Discard the supernatant and resuspend cell pellets in 500 μ l of paraformaldehyde (2%).
14. Analyze on the flow cytometer. CD4+ and CD8+ expression of PBMC is calculated by subtracting the value of the negative control (FITC) sample from the value

obtained from the sample that has been incubated with both primary (CD4 or CD8) and secondary (FITC) antibodies.

Lymphocyte Proliferation - Alamar Blue

Purpose: To determine total lymphocyte proliferation. **Note:** Must be performed under a laminar flow hood to maintain sterile conditions. When Alamar Blue is added to the cell cultures, it is in an oxidized (blue color) form. As cells proliferate, the dye is reduced (red color). The absorbance read at 570nm and 600nm determines optical density (OD) of reduced and oxidized forms of Alamar Blue. Subtraction of OD at 600 nm from OD at 570 nm accurately determines the true absorbance (specific absorbance), which reflects proliferation.

Methods:

1. After 48 hours incubation, take plate out of incubator.
2. Under a laminar flow hood, add 20 μ l of Alamar Blue™ to all wells in the plate and return to the incubator.
3. After an additional 24 hours incubation, read plate on a microplate reader (Molecular Devices, Menlo Park, CA), with absorbances set at 570 nm and 600 nm.
4. The specific absorbance of unstimulated cells (media alone) should be subtracted from the specific absorbance of the cells incubated with Con-A to yield a Δ -specific absorbance.
i.e. $.467 - .167 = .300$ $.300 = \Delta$ - specific absorbance
5. The mean Δ -specific absorbance of triplicates should then be calculated.

Reference:

Gogal RM, Ahmed SA, Larson CT. Analysis of Avian Lymphocyte Proliferation by a New, Simple, Nonradioactive Assay (Lympho-pro). Avian Diseases. 1997; 41:714-25.

APPENDIX G

Intracellular Calcium

Purpose: To determine intracellular calcium concentration of lymphocytes using Fluo-3 and SNARF-1. Fluo-3 is a long wavelength indicator for measurement of $[Ca^{2+}]_i$, and SNARF-1 is a pH indicator used to calibrate Fluo-3.

Materials:

1mM stock solution of Fluo-3 (Molecular Probes, Eugene, OR)
2mM stock solution of SNARF-1 (Molecular Probes, Eugene, OR) in DMSO (dimethylsulphoxide) (Sigma Chemical Co., St. Louis, MO).
RPMI 1640 and 1% FBS (99 ml RPMI 1640 and 1 ml FBS; Sigma Chemical Co., St. Louis, MO).
Hank's Balanced Salt Solution (HBSS; Gibco Laboratories, Burlington, Ontario)

Methods:

1. Make a 1:10 dilution of each of two fluorescent probes, Fluo-3 and SNARF 1, dissolved in RPMI 1640 and 1% FBS.
2. Resuspend PBMC cells to a concentration of 1.0×10^6 cells/ml in RPMI 1640 and 1% FBS. (Do this by centrifuging aliquoted cells and adding 1 ml RPMI 1640 and 1% FBS).
3. Load cells with 10 μ l of each Fluo-3 and SNARF-1 working solutions and incubate for 30 min in 37° C water bath.
4. Centrifuge cells at 200 x g for 10 min.
5. Pour off supernatant and resuspend cells at 1.0×10^6 cells/ml in RPMI 1640 and 1% FBS.
6. Centrifuge cells at 200 x g for 10 min.
7. Pour off supernatant and resuspend cells at 1.0×10^6 cells/ml in RPMI 1640 and 1% FBS.

8. Centrifuge cells at 200 x g for 10 min.
9. Resuspended cells in calcium-free (Ca^{2+}) HBSS at a final concentration of 1.0×10^6 cells/ml.
10. Analyze cells on a flow cytometer by measuring the Fluo-3/SNARF-1 ratio over time. Allow cells to equilibrate for 15 seconds, pause instrument, add the Ca^{2+} ionophore Ionomycin, at a final concentration of 10 μM , continue collecting information for a total of 120 seconds.
11. Intracellular calcium flux ($[\text{Ca}^{2+}]_i$) is expressed as a mean ratio (fluo-3 ratio of activated vs. resting population).

Reference:

Rijkers GT, Justement LB, Griffioen AW, Cambier JC. Improved Method for Measuring Intracellular Ca^{2+} With Fluo-3. *Cytometry*. 1990; 11:923-27.