Supporting Text

Blood and Tissue Processing. Blood and tissue samples were processed immediately after collection. Detailed descriptions for all of these protocols and specific laboratory methodologies (Standard Operating Procedures; SOPs) can be obtained from published reports [Lapointe, J., Li, C., Higgins, J. P., van de Rijn, M., Bair, E., Montgomery, K., Ferrari, M., Egevad, L., Rayford, W., Bergerheim, U., *et al.* (2004) *Proc. Natl. Acad. Sci. USA* **101,** 811-816], and the Inflammation and Host Response to Injury web site, www.gluegrant.org. For RNA samples isolated from whole blood, blood specimens were collected by using the PaxGene Blood RNA System (PreAnalytiX GmbH, Hombrechtikon, Switzerland) to stabilize total RNA. See Standard Operating Protocol G003.03 (below). Simultaneously, aliquots of whole blood were processed to isolate total leukocyte populations using one of two techniques. The techniques have been referred to as a "buffy coat isolation" and a "lysis isolation," and are provided in Standard Operating Protocols G001.03 and G002.06, respectively. In both cases (i.e., "buffy coat" and "lysis"), erythrocytes were removed by isotonic lysis, and the remaining constituents recovered by centrifugation. In the former case, the whole blood sample was first centrifuged, the interfacial band containing leukocytes was removed, contaminating erythrocytes were lysed, and the leukocytes were recovered after centrifugation. In the latter, all of the erythrocytes were removed first by isotonic lysis, and the remaining leukocytes recovered by centrifugation.

In addition, a negative isolation technique feasible for use in clinical settings was also developed for the isolation of enriched T cell and monocyte populations, based on the RosetteSep technology (StemCell Technologies, Vancouver). The protocol for isolating enriched blood T cells and monocytes is described in Standard Operating Protocol G029 and is also given below. Waste skeletal muscle specimens were also obtained at reconstructive surgery in patients with severe burns; specimens were immediately stabilized by immersion in either liquid nitrogen, ice-cold 70% ethanol, or RNA*later* (Ambion, Austin TX). The protocol for stabilizing the tissue in the three reagents is provided in Standard Operating Procedure B001.03, and the processing of these tissues to total cellular RNA is identified in Standard Operating Procedure G026.01.

Flow Cytometric Analysis. Flow cytometry was used to determine the phenotype of leukocyte populations (details are provided in Standard Operating Procedure G029, see appendices below). Briefly, isolated cells were suspended in PBS without Ca^{2+} and Mg^{2+} supplemented with 10% autologous serum, 0.5% bovine serum, and 2 mM EDTA and immediately blocked for 15 min with human IgG (ICN, Irvine, CA) to minimize nonspecific binding. Then, the cells (5×10^5 per stain) were incubated with pretitrated panels of antibodies (CD2, CD3, CD14, CD19, CD33, CD56, CD66b) conjugated with fluorochromes (FITC, PE, allophycocyanin) as described [Cobb, J. P. & O'Keefe, G. E. (2004) *Lancet* **363,** 2076-2083]. After incubation, cells were washed twice with FACS buffer and subjected to flow cytometry. Samples were acquired and analyzed on a six-parameter FACSCalibur machine with CELLQUEST software (Becton Dickinson). At least 5×10^4 gated events were collected per sample.

Microarray Procedures. Total cellular RNA was extracted from the leukocyte cell pellets or from the muscle specimens using a commercial RNA purification kit (RNeasy, Qiagen, Valencia, CA), according to Standard Operating Procedure G001.03, G002.06, G003.03, or G026.01. Biotinylated cRNA was generated from 4 μg of total cellular RNA based on the protocol outlined by Affymetrix (Santa Clara, CA), with some modifications (detailed descriptions for the RNA isolation and cRNA synthesis procedure can be obtained from Standard Operating Procedure G007.07 and www.gluegrant.org). cRNA was hybridized onto U133A or U133^{+} oligonucleotide GeneChips (Affymetrix), stained, and washed according to the Affymetrix protocol.

Statistical Analyses. GeneChip expression signal normalization was performed on all microarrays of a given chip type (U133A or U133^{+}) with DNA Chip Analyzer (DCHIP v1.3, www.dchip.org) using the perfect match algorithms. Probe sets whose apparent expression differed among groups were analyzed by Significance Analysis of Microarrays (SAM), using a false discovery rate of less than 0.001 [Feezor, R. J., Baker, H. V., Mindrinos, M., Hayden, D., Tannahill, C. L., Brownstein, B. H., Fay, A., Macmillan, S., Laramie, J., Xiao, W., *et al.* (2004) *Physiol. Genomics* **19,** 247-254]. Hybridization signal intensities for those probe sets that differed among the groups by SAM analysis were examined by hierarchical cluster and principal component analysis.

Pearson's product moment correlation between all of the expression values for pairs of microarrays was used as a measure of variability within and between groups of microarrays. For groups of independent microarrays, all pairwise correlations were averaged to estimate a single mean within group correlation. A population standard deviation was then estimated from all pairs of independent correlations. The correlation between groups of microarrays was estimated from the within subject correlation between groups. For the analysis of within subject replicates, all pairwise correlations within each subject were averaged. The mean and sample standard deviation of the within subject averages are reported. For selected groups of microarrays, the coefficient of variation (COV) for each probe set was computed as an additional measure of variability. To estimate the within subject coefficient of variation, the coefficient of variation was computed within each subject separately and then averaged over subjects.

Appendices Containing Standard Operating Procedures

BUFFY COAT ISOLATION FROM HUMAN WHOLE BLOOD FOR TOTAL CELLULAR RNA EXTRACTION

Laboratory methodologies/standard operating procedures

Genomics Core/PACB Core, Protocol G001.03

University of Florida College of Medicine

created August, 2001, modified June, 2002

-.01 modification, addition of on-column DNasing

-.02 modification, modified at the St. Louis meeting, May 15, 2002

-.03 modification, elimination of cold storage of whole blood, June 19, 2002

PRINCIPLE

Trauma and sepsis alter the patterns of gene expression in leukocyte populations from whole blood. The purpose of this procedure is to obtain total cellular RNA from a buffy coat preparation obtained from human whole blood for subsequent analysis of mRNA populations, either by the PCR (after reverse transcription) or by microarray analyses. The initial separation of the white blood cells (buffy coat) takes advantage of the relative differences in density between erythrocytes and leukocytes, and the sensitivity of erythrocytes to hypotonic lysis. Once the enriched leukocyte population is isolated, total cellular RNA is extracted using a commercial kit (RNeasy, Qiagen). Very briefly, the buffy coat preparation is lysed with guanidine isothiocyanate, which immediately inactivates RNases. Ethanol is added to alter the binding properties, and the solution is applied to an RNeasy column that retains the RNA and washes away the contaminants. RNA is then eluted with distilled water.

A more detailed description of the principle and protocols can be found in the accompanying documentation to "*RNeasy Midi/Maxi Handbook (1st Edition)*", Qiagen, Valencia, CA)

METHODS

1. Venous or arterial whole blood is collected with a 21-gauge needle into three 7-ml draw green top blood collection tubes (Becton Dickinson Vacutainer, catalog no. 367676) which contain sodium heparin as an anticoagulant. The collection of blood should be obtained from an existing arterial or venous line, or by venipuncture, and should be performed by someone experienced in the technique and familiar with infectious precautions. The blood should be processed further immediately.

2. Centrifuge the samples in a desktop, refrigerated centrifuge (Beckman Model GPR or equivalent) at 4°C for 10 min at 400 × *g*, with the brake OFF.

3. Using a disposable plastic pipette (Fisher Scientific catalog no. 13-678-11E), aliquot off the plasma and separate into three 1.5-ml Eppendorff tubes (for cytokine measurements) and store at -80°C.

4. Using a clean plastic pipette, remove the buffy coat from each of the three tubes, ensuring all of its removal. (This will necessitate some erythrocyte contamination). Transfer the buffy coat from the three collection tubes to a 50-ml conical centrifugation tube (Corning catalog no. 430828), and store at 4°C.

5. Add 35 ml of Erythrocyte Lysis (EL) Buffer (Qiagen, catalog no. 79217) to the conical tube, and allow to sit on ice for 15 min, vortexing the sample every 5 min during that period.

6. Centrifuge the sample in a desktop, refrigerated centrifuge (Beckman Model GPR) at 4°C for 10 min at 400 × *g*, with the brake OFF.

7. Discard supernatant, and resuspend the pellet in 15 ml of EL buffer, vortexing.

8. Repeat step 6, and discard supernatant.

9. Loosen cell pellet by gently tapping the side of the tube on a hard surface. At room temperature, add 8 ml of RLT Buffer (Qiagen, RNeasy Midi kit, catalog no. 75142) to which 80 µl of β-mercaptoethanol (βME) (Sigma catalog no. M6250) has been freshly added.

10. Homogenize the pellet in the RLT/βME at room temperature by aspirating and forcing the sample repeatedly through a 10- or 12-ml syringe (Monoject, catalog no. 512878) with an 18-gauge needle (Becton Dickinson catalog no. 305199) (a minimum of 10 times).

11. Add an equal volume (8 ml) of 70% ethanol, and shake vigorously.

12. Add 4 ml (≈1/4 the volume in each tube) onto two RNeasy columns and centrifuge at room temperature at 3,300 × *g* for 10 min. Discard the flow-through and reload the two columns with the remaining 4 ml from each sample.

13. Discard the flow-through, and add 2.0 ml of RW1 Buffer (Qiagen) to each column. Centrifuge at room temperature at 3,300 × *g* for 5 min. Discard the flow-through.

14. When using the RNase-Free DNase Set (Qiagen, catalog no. 79254) for the first time, prepare the DNase stock solution by dissolving the solid DNase I (1500 Kunitz units) in 550 µl of RNase-free water (1500 Kuntz units/0.55 ml). Mix by inversion and store at -20°C. For each RNeasy column, add 20 µl of DNase I stock solution to 140 µl of Buffer RDD. Mix by gently flicking the tube (do not vortex), and centrifuge briefly to collect residual liquid from the sides of the tube.

15. Pipet the DNase I incubation mix (160 µl) directly onto the spin-column membrane, and place it upright at room temperature for 15 min.

16. Add 2.0 ml of Buffer RW1 to the RNeasy column, and place at room temperature for 5 min. Then centrifuge at room temperature (20-25°C) at 3,300 × *g* for 5 min.

17. Discard the flow-through and add 2.5 ml of RPE Buffer (Qiagen) to each column. Centrifuge at room temperature (20-25°C) at 3,300 × *g* for 2.5 min.

18. Repeat step 17, and centrifuge at room temperature (20-25°C) at 3,300 × *g* for 5 min.

19. Transfer the column to a new 15-ml collection tube (Qiagen).

20. Pipette 150 µl of RNase-free water onto the column and allow to stand at room temperature for ~1 min. Centrifuge at room temperature (20-25°C) at 3,300 × *g* for 3 min. Repeat the procedure with an additional 150 µl of RNase-free water, and collect into the same tube.

21. Transfer the eluate to a sterile RNase-free 1.5-ml microcentrifuge tube. Add 0.1 volume of 3 M sodium acetate pH 5.2 to the eluate and then add 2.2 volumes of ice-cold absolute ethanol. Vortex and store overnight at -20°C.

22. Centrifuge at 16,000 × *g* (maximum) at 4°C for 30 min. Decant the ethanol, and SpeedVac on low heat the remaining ethanol to dryness (≈5 min).

23. Resuspend the RNA pellet in 20 µl of RNase-free water. Combine the two samples into a single Eppendorf tube with an Eppendorf pipette.

24. Label and store sample at -80°C until RNA analysis.

Reagents

Seven-milliliter, Green top blood collection tubes (Becton Dickinson Vacutainer catalog no. 367676)

EL buffer (Qiagen, catalog no. 79217)

Qiagen, RNeasy Midi kit catalog no. 75142

β-mercaptoethanol (βME) (Sigma catalog no. M6250)

12-ml syringe (Monoject, catalog no. 512878)

18-gauge 1 1/2 inch needle (Becton Dickinson catalog no. 305199)

RNase-free DNase Set (Qiagen, catalog no. 79254)

LYSIS ISOLATION OF TOTAL LEUKOCYTES FROM HUMAN WHOLE BLOOD FOR TOTAL CELLULAR RNA

Laboratory methodologies/standard operating procedures
Genomics Core/PACB Core, Protocol #G002.06
UMDNJ-Robert Wood Johnson Medical Center
modified 8 March 2005

Principle

Trauma and sepsis alter the patterns of gene expression in leukocyte populations from whole blood. The purpose of this procedure is to obtain total cellular RNA from a buffy coat preparation obtained from human whole blood for subsequent analysis of mRNA populations, either by the polymerase chain reaction (following reverse transcription) or by microarray analyses. The initial separation of the white blood cells (buffy coat) involves a lysis of the erythrocytes in the whole blood with an ionic stress (ammonium chloride). After lysis, the remaining leukocytes are collected by centrifugation. The leukocyte pellet is subsequently washed, and total cellular RNA is subsequently isolated and purified using a commercial kit (RNeasy, Qiagen). A more detailed description of the principle and protocols can be found in the accompanying documentation to "*RNeasy Midi/Maxi Handbook (1st Edition)"*, Qiagen)

Methods

1. Venous or arterial whole blood is collected into three seven ml draw purple top blood collection tubes (Becton-Dickinson Vacutainer catalog no. 16852) which contains the chelator EDTA as an anticoagulant. The collection of blood should be obtained from an existing arterial or venous line, or venipuncture should be performed by someone experienced in the technique, and familiar with infectious precautions. The blood should be processed further immediately.

2. Transfer two ml of whole blood immediately to each of 10 fifty ml polystyrene (clear) conical tubes (Corning catalog no. 430304) and add 40 ml of room-temperature, sterile, pyrogen free lysis buffer (8.26 g/L ammonium chloride, 1.0 g/L potassium bicarbonate, 0.037 g/L tetrasodium EDTA) to each tube.

(Note: the sterile, pyrogen-free lysis buffer is generally made up in 1 L lots. After being sterile filtered using Corning filtration units, the solution is transferred to 100 ml containers that must be tightly capped. Upon prolonged exposure to room air, the bicarbonate in the solution will equilibrate with room air and the pH will progressively increase. The pH of lysis solution should be routinely tested with pH strips.)

3. Mix the samples thoroughly and allow to stand at room temperature for 5 min to lyse the erythrocytes.

4. Centrifuge the samples in a desk-top, refrigerated swinging bucket centrifuge (Beckman Model GPR or similar) at 4^{o}C for 10 min at 400 x g, with the brake OFF.

5. Remove the supernatant and discard. Loosen cell pellet by tapping the side of the tube on a hard surface, then vortexing gently.

To each tube, add 4 ml of ice-cold , calcium-free, magnesium-free, phosphate buffered saline (Mediatech catalog no. 21-031-CV) and vortex gently. Combine all resuspended

pellets from the same sample into a single tube. The total volume should be 40 ml. Repeat steps 4 and 5, then proceed to step 6.

6. At room temperature, add 8 ml of RLT Buffer (Qiagen, RNeasy Midi kit catalog no. 75142) to which 80 µl of β-mercaptoethanol (βME) (Sigma catalog no. M6250) has been freshly added.

7. Homogenize the pellet in the RLT/βME at room temperature by aspirating and forcing the sample repeatedly through a 12 ml syringe (Monoject, catalog no. 512878) with an 18 g needle (Becton-Dickinson catalog no. 305199) (a minimum of ten times).

8. Add an equal volume (8 ml) of 70% ethanol, and shake vigorously.

9. Add 4 ml (~1/4 the volume in each tube) onto two RNeasy columns and centrifuge at room temperature (20-25^{o} C) at 3,300 x g for 10 min. Discard flow-through and reload the two columns with the remaining 4 ml from each sample.

10. Discard the flow-through, and add 2.0 ml of RW1 Buffer (Qiagen) to each column. Centrifuge at room temperature at 3,300 x g for 5 min. Discard the flow-through.

11. When using the RNase-Free DNase Set (Qiagen, catalog no. 79254) for the first time, prepare the DNase stock solution by dissolving the solid DNase I (1500 Kunitz units) in 550 µl of RNase-free water (1500 Kuntz units/0.55 ml). Mix by inversion and store at –20^{o} C. For each RNeasy column, add 20 µl DNase I stock solution to 140 µl Buffer RDD. Mix by gently flicking the tube (do not vortex), and centrifuge briefly to collect residual liquid from the sides of the tube.

12. Pipet the DNase I incubation mix (160 µl) directly onto the spin-column membrane, and place it upright at room temperature for fifteen min.

13. Add 2.0 ml Buffer RW1 to the RNeasy column, and place at room temperature for five min. Then centrifuge at room temperature (20-25^{o} C) at 3,300 x g for five min.

14. Discard the flow-through and add 2.5 ml of RPE Buffer (Qiagen) to each column. Centrifuge at room temperature (20-25^{o} C) at 3,300 x g for 2.5 min.

15. Repeat step 14, and centrifuge at room temperature (20-25^{o} C) at 3,300 x g for five min.

16. Transfer the column to a new 15 ml collection tube (Qiagen).

17. Pipette 150 µl of RNase-free water onto the column and allow to stand at room temperature for ~ 1 min. Centrifuge at room temperature (20-25^{o} C) at 3,300 x g for 3 min. Repeat the procedure with an additional 150 µl of RNase-free water, and collect into the same tube.

18. Transfer the eluate to a sterile RNase-free 1.5 ml microcentrifuge tube. Add 0.1 volumes of 3 M sodium acetate pH 5.2 to the eluate and then add 2.2 volumes of ice-cold absolute ethanol. Vortex and store overnight at -20° C.

19. Centrifuge at 16,000 x g (maximum) at 4° C for 30 min. Decant the ethanol, and SpeedVac™ on low heat the remaining ethanol to dryness (~5 min).

20. Resuspend the RNA pellet in 20 µl of RNase-free water. Combine the two samples into a single Eppendorf tube with an Eppendorf pipette.

21. Label and store sample at -80^{o} C until RNA analysis.

Reagents

Seven ml, Purple top Blood Collection Tube (Vacutainer, Becton-Dickinson catalog no. 16852)

Sterile, Pyrogen-free Lysis Buffer

-dissolve 8.26 g ammonium chloride (Fisher Scientific catalog no. A649, USP grade), 1.0 g potassium bicarbonate (Fisher Scientific catalog no. P235, USP grade), and 0.037 g tetrasodium EDTA (Fisher Scientific catalog no. S312, USP grade) in 800 ml of double-distilled water, and q.s. to one liter. Sterile filter (0.22 micron) the solution into a 1 liter plastic container (Corning 1Liter filter system catalog no.430511) and store for up to one month at 4°C.

Dulbecco's Calcium and Magnesium-free, Phosphate Buffered Saline. (Mediatech, Inc.-Cellgro catalog no. 21-031-CV)

Qiagen, RNeasy Midi kit catalog no. 75142

β-mercaptoethanol (βME) (Sigma catalog no. M6250)

12-ml syringe (Monoject, catalog no. 512878)

18 g 1 1/2 inch needle (Becton-Dickinson catalog no. 305199)

RNA ISOLATION FROM HUMAN WHOLE BLOOD USING PAXgene BLOOD RNA SYSTEM

Laboratory methodologies/standard operating procedures

Genomics Core/PACB Core, Protocol G003.03 including Stanford and PAXGene modifications

University of Florida College of Medicine

created November, 2001, modified 30 May 2002

-*.01 Stanford modifications*

-.02 PreAnalytiX modifications, including on-column Dnasing

-.03 modified at the St. Louis meetings of May 15, 2002

Principle

Trauma and sepsis alter the patterns of gene expression in leukocyte populations from whole blood. The purpose of this procedure is to obtain total cellular RNA from human whole blood for the subsequent analysis of mRNA populations, either by the PCR (after reverse transcription) or by microarray analyses. This procedure does not contain an initial separation of leukocytes, but rather relies on the denaturing of proteins and precipitation of RNA/DNA from whole blood directly. Following lysis, total cellular RNA is removed from DNA and proteins by precipitation and chromatographic separation. A more detailed description of the principle and protocols can be found in the accompanying documentation from the *PAXgene Blood RNA Tube* C*ircular* and the *PAXgene Blood RNA kit Handbook.*

Methods

1. Arterial or venous whole blood is collected into a room temperature (18-25ºC) PAXgene Blood RNA Tube (PAXgene, PreAnalytiX, Hombrehtikon, Switzerland, distributed by Qiagen, catalog no. 762115). The collection of blood should be obtained from an existing arterial or venous line, or venipuncture should be performed by someone experienced in the technique and familiar with infectious precautions. The PAXgene Blood RNA tube is held vertically, below the donor's arm, during blood collection. Allow at least 10 seconds for a complete blood draw to take place. If the PAXgene Blood RNA tube is the only tube to be collected, draw into a "discard tube" before using the PAXgene Blood RNA tube. Otherwise, the PAXgene Blood RNA tube should be the last tube drawn. It is essential that, before the use of the PAXgene system for collecting blood, the operator has fully read the PAXgene Blood RNA Handbook and understands the procedures and their potential risks to the operator and the patient.

2. After blood collection, gently invert the PAXgene Blood RNA tube 8-10 times. Store the PAXgene Blood RNA tube at room temperature until the sample is processed.

3. After collection of the blood sample, incubate the PAXgene Blood RNA tube for at least 2 h at room temperature to ensure complete lysis of the blood cellular constituents.

4. Centrifuge the PAXgene Blood RNA tube for 10 min at 3,300 × *g* in a swinging-bucket centrifuge (in a Beckman Model GPR centrifuge, or equivalent). All

centrifugation steps in this protocol are carried out at room temperature, unless otherwise specified.

5. Remove and decant/discard the supernatant. Dry the rim of the tube with a clean Kimwipe. Add 5 ml RNase-free water (provided in the PAXgene kit) to the pellet and close the tube using a fresh secondary Hemogard closure (provided in the PAXgene kit).

6. Vortex thoroughly to resuspend the pellet, then centrifuge for 10 min at 3300 × *g* in a swinging bucket centrifuge. Decant and discard the entire supernatant.

7. Thoroughly resuspend the pellet in 360 µl Buffer BR1 (PAXgene kit) by vortexing.

8. Using a micropipette, transfer the sample (usually 500-1,200 µl) into a 1.5-ml microcentrifuge tube (USA Scientific catalog no. 1415-2600). Add 300 µl of Buffer BR2 (PAXgene) and 40 µl Proteinase K solution (PAXgene). Mix by vortexing, and incubate for 20 min at 55ºC using a shaker-incubator, heating block, or water bath. If using a heating block or water bath, vortex each sample once during the incubation. Do not allow the temperature of the sample to decrease during vortexing.

9. Centrifuge for 20 min at maximum speed in a microcentrifuge (Eppendorf model 5415C, maximum speed 14,000 rpm or 16,000 × *g*). Transfer the supernatant to a new 1.5-ml microcentrifuge tube.

10. Add 350 µl of 100% ethanol. Mix by vortexing and centrifuge briefly (1-2 s, 1,000 × *g*) to remove drops from the inside of the tube lid. Do not centrifuge for longer than 1-2 s, as this may result in pelleting of the nucleic acids and reduced RNA yield.

11. Apply 700 µl of sample to the PAXgene column sitting in a 2-ml processing tube (all supplied in the PAXgene kit). Centrifuge for 1 min at 8,000 × *g*. Place the PAXgene column in a new 2-ml processing tube and discard the old processing tube containing the flow-through.

12. Apply the remaining sample to the PAXgene column and centrifuge for 1 min at 8,000 × *g*. Again, place the PAXgene column in a new 2 ml processing tube and discard the old processing tube containing flow-through.

13. Pipet 350 µl of Buffer BR3 to the PAXgene column and centrifuge for 1 min at 8,000 × *g*. Place the PAXgene column in a new 2-ml processing tube and discard the old processing tube containing flow-through.

14. Prepare DNase I stock by dissolving solid DNase I (1500 Kunitz units; Qiagen, catalog no. 79254) in 550 µl of RNase free water and mix by inversion (1500 Kuntz units/0.55 ml). Pipet 10 µl of DNase I stock solution into 70 µl of Buffer RDD. Mix by gently flicking the tube (do not vortex) and centrifuge briefly.

15. Pipet DNase I incubation mix (80 µl) directly onto PAXgene column and place upright at room temperature for 15 min.

16. Pipet 350 µl of Buffer BR3 to the PAXgene column and centrifuge for 1 min at 8,000 × *g*. Place the PAXgene column in a new 2-ml processing tube and discard the old processing tube containing flow-through.

17. Apply 500 µl of buffer BR4 to the PAXgene column and centrifuge for 1 min at 8,000 × *g*. Place the PAXgene column in a new 2-ml processing tube and discard the old processing tube containing flow-through. Note that Buffer BR4 is supplied as a concentrate. Ensure that the ethanol is added to Buffer BR4 before use.

18. Add another 500 µl of buffer BR4 to the PAXgene column. Centrifuge for 3 min at maximum speed to dry the PAXgene column membrane.

19. To eliminate residual Buffer BR4, discard the tube containing the flow-through, place the PAXgene column in a 2 ml processing tube (USA Scientific catalog no. 1620-2700), and centrifuge for 1 min at full speed.

20. Discard the tube containing the flow-through and transfer the PAXgene column to a 1.5 ml elution tube (supplied as part of the PAXgene kit). Pipet 40 µl of Buffer BR5 directly on to the PAXgene column membrane (without touching the membrane with the pipet tip) and centrifuge for 1 min at 8,000 × *g*.

21. Repeat the elution step (step 20) as described, using 40 µl Buffer BR5.

22. Incubate the eluate for 5 min at 65ºC in a heating block or water bath. After incubation, chill immediately on ice.

23. Add 0.1 volumes of 3 M sodium acetate pH 5.2 to the eluate and then add 2.2 volumes of ice-cold absolute ethanol. Vortex and store overnight at -20°C.

24. Centrifuge at 16,000 × *g* (maximum) at 4°C for 30 min. Decant the ethanol, and Speed-Vac on low heat the remaining ethanol to dryness (≈5 min).

25. Resuspend the RNA pellet in 20 µl of RNase-free water. Combine the two samples into a single Eppendorf tube with an Eppendorf pipette.

26. Label and store sample at -80ºC until RNA analysis.

Reagents

PAXgene Blood RNA Tube (PreAnalytiX, A Qiagen/BD Company) catalog no. 762115

Absolute ethanol, USP grade (AAPER Alcohol and Chemical, Shelbyville, KY).

DOUBLE-STRANDED cRNA SYNTHESIS, *IN VITRO* TRANSCRIPTION, AND AFFYMETRIX GENECHIP HYBRIDIZATION OF ISOLATED TOTAL HUMAN BLOOD RNA: 4 μg of Starting Material

Laboratory methodologies/standard operating procedures

Genomics Core/PACB Core, Protocol G007.07

University of Florida College of Medicine

created February 2002

-modified at the St. Louis meetings, May 15, 2002

last revised October 26, 2003bb

Principle

Trauma and sepsis alter the patterns of gene expression in leukocyte populations from whole blood. One of the principal goals of the Large Scale Collaborative Research Program is to explore gene expression patterns from whole blood of patients with trauma and burn injury. The purpose of this procedure is to prepare biotinylated complementary RNA (cRNA) from human whole blood total RNA for the subsequent analysis of mRNA populations by microarray analyses. This procedure involves first the reverse transcription of poly(A)$^+$ RNA to a single and then double stranded cDNA. Subsequently, an *in vitro* transcription is performed to generate a biotinylated cRNA strand which is then fragmented and hybridized to the Affymetrix GeneChip. A more detailed description of the unmodified version of this procedure and a complete list of required reagents can be found in the Affymetrix GeneChip Expression Analysis Technical Manual.

Methods

I. RNA isolation. The RNA isolation technique starting from whole blood has been described in other protocols (Specifically G001 and G003, and their derivatives). It is presently recommended to start with 10 μg of total cellular RNA from each sample. Recent data has suggested that adequate cRNA yields can be obtained starting with as little as 1.0 μg of total cellular RNA, with good gene expression correlation (R^2) from chips loaded with cRNA synthesized from 10 μg of starting RNA and cRNA synthesized starting with 1.0 μg of RNA. The amounts of the reagents will vary depending on the amount of starting cellular RNA, but those included here are for 4.0 μg of starting material.

II. Synthesis of Double Stranded cDNA from total RNA.

A. First Strand cDNA synthesis.

1. The evening before cDNA synthesis, aliquot 4.0 μg of total cellular RNA into a sterile RNase free 1.5-ml tube (USA Scientific, catalog no. 1415-2600). Lyophilize the RNA to dryness (with a Speedvac). Store at -20°C overnight.

2. The next day, resuspend the sample(s) in RNase free water (Ambion, catalog no. 9939) to a final volume of 7.5 μl. Heat for 5 min at 37°C in a heating block to dissolve RNA completely. Vortex and spin briefly. Transfer the RNA to either a clean 0.2 ml PCR tube (Dot Scientific Incorporated, catalog no. 501-8PCR) or clean 0.6-ml tube (Continental Lab Products, catalog no. 3435.S, or similar).

3. Make a mixture of 3 μl RNase free water and 1 μl of diluted (20 μM) T7-(dT)24 primer (Affymetrix, catalog no. 900375) per sample. For multiple samples, make a "master mix" of water and T7-(dT)24 primer. Add 4 μl of the master mix to each PCR tube (Dot Scientific Incorporated, catalog no. 501-8PCR), then add 7 μl of RNA. Mix by pipetting and spin briefly. Incubate at 70°C for 10 min in a PCR thermal cycler, place on ice for 1 min, then spin briefly.

Note: All subsequent temperature-dependent steps are performed in the PCR thermal cycler (PCR Express Thermohybaid or equivalent) or Eppendorf Thermomixer unless otherwise specified.

4. While incubating the RNA with the T7-(dT)24 primer, assemble the first strand master mix with the following reagents in the following order:

	Master Mix Multiplier____x
4 μl of First Strand cDNA buffer	____
2 μl of 0.1 M DTT	____
1 μl of 10 mM dNTP (Invitrogen, catalog no. 18427-088)	____
2 μl of Superscript II RT (Invitrogen, catalog no. 18064-014)	____
9 μl Total	

(If more than one reaction is being processed, create a master reaction mixture with ≈10% excess of each reagent.) First strand cDNA buffer and DTT are supplied with the Superscript II RT.

5. Add 9 μl of Master mix to each tube. Mix the sample by pipetting (do not vortex) and centrifuge briefly. Incubate for 1.5 h at 42°C. Place the reaction mixture on ice for 1 min, then centrifuge briefly.

B. Second Strand cDNA synthesis

1. Add the following reagents in the order listed:

	Master Mix Multiplier____x
91 μl RNase-free water.	____
30 μl 5X Second Strand Reaction Buffer (Invitrogen, catalog no. 10812-014)	____

3 µl 10 mM dNTP mix ____
1 µl (10 U) of *E. coli* DNA ligase (NEB, catalog no. M0205L) ____
4 µl (40 U) of *E. coli* DNA polymerase I (NEB, catalog no. M0209L) ____
0.5 µl (1 U) of *E. coli* RNase H (NEB, catalog no. M0297L) ____
130 µl Total

(If more than one reaction is being processed, create a master reaction mixture with ≈10% excess of each reagent.)

2. Add 130 µl to each tube, mix by pipetting, and centrifuge briefly. The total volume of each is now 150 µl. Incubate at 16°C for 2 h.

3. Add 2 µl (6 U) of T4 DNA Polymerase (NEB, M0203L), mix by pipetting with a P200 Eppendorf pipette, spin briefly, and incubate for 15 min at 16°C.

4. Add 10 µl of 0.5 M EDTA (Sigma, catalog no. E-7889). Store at -20°C or proceed with III.

III. Extraction and purification of double-stranded (ds) cDNA.

Note: All reagents used in the cDNA cleanup, except for 100% ethanol, are supplied in the GeneChip Sample Cleanup Module (Affymetrix catalog no. 900371). Before beginning the cleanup procedure, ensure that the cDNA Wash Buffer has been diluted with ethanol as indicated on the bottle.

1. Transfer the 162-µl cDNA synthesis preparation to a sterile RNase-free 1.5 ml tube.

2. Add 600 µl cDNA Binding Buffer to the 162-µl cDNA sample. Vortex for 3 s.

3. Check that the mixture is yellow in color (similar to the cDNA Binding Buffer without the cDNA added). Note: If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

4. Apply 500 µl of sample to the cDNA Cleanup Spin Column in a 2 ml collection tube. Centrifuge for 1 min at ≥8,000 × *g* (≥ 10,000 rpm). Discard flow-through.

5. Reload the spin column with the remaining sample (262 µl) and centrifuge for 1 min at ≥8,000 × *g* (≥ 10,000 rpm). Discard flow-through and collection tube.

6. Transfer spin column to a new 2-ml collection tube (supplied). Add 750 µl of cDNA Wash Buffer to the column. Centrifuge for 1 min at ≥8,000 × *g* (≥ 10,000 rpm). Discard flow-through.

Note: cDNA Wash Buffer is supplied as a concentrate. Ensure that ethanol has been added to the cDNA Wash Buffer before use.

7. Open the cap of the spin column and centrifuge for 5 min at maximum speed (≤ 25,000 × *g*). Discard flow-through and collection tube.

8. Transfer spin column to a 1.5-ml Collection Tube and pipet 24 µl of cDNA Elution Buffer directly onto the spin column membrane. Incubate for 1 min at room temperature and centrifuge for 1 min at maximum speed (≤ 25,000 × *g*) to elute.

9. Proceed to IVT reaction setup.

IV. Synthesis of Biotin-Labeled cRNA

1. Use 11 µl of the cDNA sample in the IVT reaction. Store the remainder of the cDNA at -80°C for later use. At room temperature, assemble a Master Mix with the reagents from the BioArray HighYield RNA Transcript Labeling kit (Enzo Diagnostics, catalog no. 42655-10):

		Master Mix Multiplier____x
2 µl	10X HY reaction buffer (solution 1)	____
2 µl	10X Biotin labeled ribonucleotides (solution 2)	____
2 µl	10X DTT (solution 3)	____
2 µl	10X RNase inhibitor mix (solution 4)	____
1 µl	20X T7 RNA polymerase (solution 5)	____
9 µl	TOTAL	

Add 9 µl of Master Mix and 11 µl of cDNA to a PCR tube. Mix by pipetting and centrifuge briefly.

2. Incubate the tubes at 37°C for 8.5 h. After the incubation, keep samples at 4°C until further processing.

V. Cleanup of Biotin-Labeled cRNA:

Note: Unless otherwise specified, all reagents used in the cRNA cleanup are from the GeneChip Sample Cleanup Module. All steps of the cRNA cleanup should be performed at room temperature. Before beginning the cleanup procedure, ensure that the IVT cRNA Wash Buffer has been diluted with ethanol as indicated on the bottle.

1. Remove samples from thermocycler and leave them at room temperature while preparing the 1.5-ml tubes.

2. Label 1.5-ml RNase-free microcentrifuge tubes. Add 350 µl of IVT cRNA binding buffer to each tube.

3. Add 80 µl of RNase-free water to the IVT sample to adjust the sample volume to 100 µl. Mix well.

4. Transfer the 100-µl solution of cRNA to the 1.5-ml tube and mix thoroughly by pipetting.

5. Pipet 100 µl of the solution in the 1.5-ml tube back into the IVT sample tube to "wash" out any remaining sample. Pipet all remaining liquid from the IVT tube back into the 1.5-ml tube. Vortex for 3 s to mix.

6. Add 250 µl of 100% ethanol (not supplied in the Cleanup kit) and mix thoroughly by pipetting. Do not centrifuge.

7. Apply entire sample (~700 µl) to the IVT cRNA Cleanup Spin Column in a 2-ml collection tube. Centrifuge for 1 min at ≥8,000 × *g* (≥10,000 rpm). Discard flow-through and collection tube.

8. Transfer column to new 2-ml collection tube (supplied). Pipet 500 µl IVT cRNA Wash Buffer onto column and centrifuge for 1 min at ≥8000 × *g* (≥10,000 rpm). Discard flow-through. Note: Ensure that the IVT cRNA Wash Buffer has been properly diluted with ethanol before use.

9. Pipet 500 µl of 80% ethanol onto column. Centrifuge for 1 min at ≥8,000 × *g* (≥10,000 rpm). Discard flow-through.

10. Open the cap of the spin column and centrifuge at full speed for 5 min (≤ 25,000 × *g*). Discard flow-through and collection tube.

Note: Orient opened caps in the opposite direction to the centrifuge rotation to avoid damaging the caps.

11. Transfer column to new 1.5-ml collection tube (supplied) and pipet 11 µl of RNase-free water onto center of membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge for 1 min at maximum speed (≤ 25,000 × *g*) to elute.

12. Pipet 10 µl of RNase-free water onto center of membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge for 1 min at maximum speed (≤25,000 × *g*) to elute.

13. Check the A_{260} of a diluted sample to quantify yield from reaction.

VI. Fragmentation and hybridization

Note: Ensure that a heat block is preheated to 99°C for use in step.

1. Aliquot 15 µg of IVT product into a new 1.5 ml RNase-free microcentrifuge tube. Lyophilize. Resuspend in 32 µl RNase-free water (Ambion).

(At this point, you can take the GeneChips out of the refrigerator or cold room and allow them to warm to room temperature on the bench)

2. To a PCR tube, add the 32 µl of cRNA and 8 µl of 5× Fragmentation buffer.

3. Incubate at 94°C for 35 min in the thermocycler. Proceed with hybridization or store samples at -20°C until ready to perform hybridization.

4. Add the following components to the hybridization Master Mix:

		Master Mix Multiplier____x
7 µl diluted Oligo B2 (Affymetrix catalog no. 900301)		____
3 µl Herring Sperm DNA (Promega cat D1811)	____	
3 µl Acetylated BSA (50 mg/ml) (Invitrogen catalog no. 15561-020)		____
150 µl 2X Hybridization Buffer	____	
97 µl RNase-free Water (Ambion)	____	
260 µl Total		

Note: To dilute the Oligo B2, tranfer the 180 µl of 3 nM Oligo B2 stock to a clean 1.5-ml tube. Add 720 µl of nuclease free water (1:5 dilution). Mix thoroughly.

5. Prepare 300 µl of 1× Hybridization Buffer per chip (150 µl water plus 150 µl 2× Hybridization Buffer)

6. Label chips on both sides. Pierce top septa with a pipet tip.

7. Turn on hybridization oven, set to 45°C.

8. Label microcentrifuge tubes. Add 260 µl of Master Mix to each tube. Add 40 µl of fragmented cRNA. Vortex.

9. Heat the hybridization mixture (master mix + fragmented cRNA) to 99°C for 5 min in a heat block. Meanwhile, add 300 µl of 1× Hybridization Buffer to each chip and shake the chip a little to distribute buffer on the chip.

10. Centrifuge the hybridization mixture for 5 min in a microcentrifuge at maximum speed.

11. Remove the 1× hybridization buffer from the chip and add the sample to the chip, leaving a small air bubble. If you wish to standardize the “small air bubble”, use 250 µl each time.

12. Hybridize for 16 h in the rotisserie box oven at 60 rpm.

VII. Eukaryotic Arrays: Washing, Staining, and Scanning

Probe Array Wash and Stain

1. After 16 h of hyb, remove hybridization mixture from probe array and place in micro centrifuge tube. Fill probe array completely with **Buffer A**, non-stringent buffer. Ensure no bubbles are present.

2. Store hybridization mixture in ice while working and at -80°C for long term storage.

3. If necessary probe array may be stored at 4C for up to 8 h as per Affymetrix. (Personal communication with Stan Trask). Equilibrate the probe array at room temperature before washing and staining

4. Prepare the stain solutions. For EukGE4-WS2 you will need two SAPE stains and one Antibody stain. Stains will be made and used the day they will be used)

SAPE Stain (make master mix and then aliquot, you will need two SAPE stains per chip, aliquot in amber tubes, and shelter from light)

Components	Volume	Final Concentration	Vol needed for
2X MES Stain Buffer	300µl	1x	_____
50mg/ml acetylated bsa	24µl	2mg/ml	_____
1mg/ml streptavidin_phycoerythrin	6µl	10ug/ml	_____
distilled water	270µl	----	_____

Antibody Stain (make master mix and then aliquot)

Components	Volume	Final Concentration	Vol needed for
2X MES Stain buffer	300µl	1X	_____
50mg/ml acetylated BSA	24µl	2mg/ml	_____
10mg/ml Normal Goat IgG	6µl	0.1mg/ml	_____
0.5mg/ml biotinylated antibody	3.6µl	3µg/ml	_____
Distilled water	266.4µl	----	_____

Washing and Staining

1. Fluidics needs to be primed before the staining and washing steps. Please refer to the GeneChip Expression Analysis Manual, Chapter 4, Section 2 for instructions if needed.

2. In the Fluidics Station dialogue box on the workstation, select the correct experiment name from the drop-down **EXPERIMENT** list.

3. In the **PROTOCOL** drop down list, select the appropriate protocol to control the washing and staining of the probe array format being used.

4. Choose **RUN** in the fluidics station.

5. IF you are unfamiliar with the fluidics station please refer to the manual for detailed explanations.

6. Insert appropriate probe array into the designated module of the fluidics station while the leveler is in the **EJECT** position. When finished, return the leveler to the **ENGAGE** position.

7. Three-stain protocols require the user to replace stain solutions as directed by the LCD window during staining steps.

8. Remove any microcentrifuge tube remaining in the sample holder of the fluidics station module(s) being used.

9. Place microcentrifuge containing SAPE stain 1. Follow instructions as directed by the LCD window.

10. The procedure takes ≈80 min to complete.

Break out of times

start ➔ Insert 2nd stain 45 min

2nd stain ➔ Insert 3rd stain 11 min

3rd stain ➔ end of protocol 30 min

All these times are approximate and will vary from Fluidics to Fluidics and from Module to Module.

11. When the protocol is complete, the LCD window displays the message **EJECT CARTRIDGE**.

12. Remove probe arrays and inspect for bubbles, if large bubbles are present return to fluidics station.

13. Keep the probe arrays in the dark until ready for scanning.

14. If necessary, clean the glass surface of probe array with a non-abrasive tissue. Do not use alcohol to clean the glass.

Reagents:

BioArray HighYield RNA Transcript Labeling kit (Enzo Diagnostics) catalog no. 42655-10 or (Affymetrix) catalog no. 900182

GeneChip Sample Cleanup Module (Affymetrix) catalog no. 900371.

Absolute ethanol, USP grade (AAPER Alcohol and Chemical Corp, Shelbyville, Kentucky).

EVALUATION OF RNA YIELDS FROM HUMAN SKELETAL MUSCLE OBTAINED FROM BURN PATIENTS: A COMPARISON OF DIFFERENT TISSUE STABILIZATION PROTOCOLS

Laboratory methodologies/standard operating procedures

Genomics Core/PACB Core, Protocol B001.03

University of Texas Medical Branch/University of Florida College of Medicine

Created December 2002/ modified December 2002

Principle

The Large Scale Collaborative Research Program (Glue Grant) requires the collection of human tissues from burned and traumatized patients for the isolation of total cellular RNA. Total cellular RNA will be surveyed for genome wide expression profiling using the U133A Affymetrix GeneChip, in an effort to predict outcome in the burned patients. At the present time, the optimal technique for the stabilization of the tissue samples after their harvesting, and the extraction of their RNA is unknown. Furthermore, both theoretical and practical concerns exist regarding which technique will be optimal in a clinical setting performed by a number of sites with varying degrees of proficiency. The optimal technique will be one that meets the following criteria:

1. The technique can be performed reproducibly by research nurses or research technicians at the clinical sites following an adequate training period.

2. The technique poses no significant occupational risk to the research nurses or technicians at the clinical sites when the individuals are adequately trained.

3. The quantities of RNA obtained from the tissues are sufficient to complete the proposed expression studies.

4. The quality of the RNA obtained from the tissues is sufficient to complete the proposed expression studies.

5. The technique is cost-effective in terms of reagents and operator time.

We propose an analysis of tissue using protocols similar to those tested in rodent liver, and summarized in G006.01. Very briefly, freshly obtained samples of skin, adipose

tissue, and muscle will be obtained in the operating room and immediately placed on ice. The samples will be immediately dissected to remove contaminating tissues and will then be sectioned appropriately. One aliquot will be snap frozen in liquid nitrogen, a second will be fixed in greater than 10 wt/vol of 70% ethanol, and the other will be immersed in Ambion's RNA*later*. Samples will then be transported to a Genomics Core analytical site where the total cellular RNA will be isolated. The quantity and quality of the RNA will be determined.

Although preliminary studies conducted with rodent liver suggest that the two techniques of snap freezing and RNA*later* are equivalent, these protocols have not been performed on human tissue samples obtained from acutely ill patients after burn injury. Furthermore, there have been recent recommendations from some investigators that fixation of the samples in 70% ethanol not only preserves the RNA and protein, but also permits subsequent histological analyses. Preliminary studies conducted at analytical sites with a high degree of skill and capability have suggested that results obtained to date with both the snap-freezing and the RNA*later* techniques appear equivalent. However, and importantly, these same studies have not been performed by research nurses or technicians under conditions that will closely approximate the actual conditions of the Large Scale Collaborative Research Agreement. Under these conditions, it remains unclear whether the advantages associated with the 70% ethanol or the RNA*later* are maintained when performed in these real world settings.

The purposes of this study are:

1. to obtain and distribute to a selected burn clinical site the necessary reagents to conduct the proposed blood collection and RNA isolation study

2. to determine the relative feasibility of the three sample preparation protocols by the individual analytical site when tested on human tissue samples obtained from burned patients.

These goals will be achieved through a coordinated effort initially by Dr. David Herndon's laboratory to conduct the proposed protocol on tissues obtained from burned patients (using entry criteria, as determined by the Clinical Studies Core). Tissues will be fixed and shipped to the Sample Collection and Coordination site, and then distributed to the Genomics Core for sample analysis.

The Sample Collection and Coordination Site (University of Florida) will be responsible for providing the analytical sites with the required proprietary reagents, unique identifier and instructions for the processing and shipping of the samples. The Clinical Site will be responsible for obtaining the samples in a timely manner, processing the samples as required, and forwarding the samples to the Sample Collection and Coordination site. The Sample Collection and Coordination Site will be responsible for forwarding the appropriate samples to the Genomics Core Analytical Site for subsequent RNA isolation, evaluation of its quality, and subsequent microarray analyses.

Methods

1. The Sample Collection and Coordination Site will provide D.H. with a listing of required supplies and reagents that he must obtain to complete the protocol.

2. The Sample Collection and Coordination Site will provide D.H. with "Tissue Sample Collection kits" containing sample collection tubes, 70% ethanol, RNA*later*, bar-coded labels, sample collection sheets, collection and shipping instructions.

3. After IRB approval is granted, samples of waste skeletal muscle will be obtained at surgery. The samples will immediately be placed on ice, and within 15 min, will be dissected free of contaminating tissue and cut into three sections of ≈30 mg of grossly identical samples which will be individually placed 1) into sample collection tubes and immersed in liquid nitrogen, and then stored at –80°C, 2) into sample collection tubes containing 2 ml of RNAlater and stored at room temperature for up to 12 h, and then stored at –70°C, or 3) into sample collection tubes containing 2 ml of ice-cold 70% ethanol, and stored at –70°C. After processing, appropriate bar-coded labels will be attached, sample collection forms completed, and the samples will be stored appropriately until they can be shipped to the Sample Collection and Coordination Site. Detailed standard operating procedures will be completed for each of the three collection protocols.

4. The Sample Collection and Coordination Site will then transfer all of the samples to the individual Genomics Core for analysis of RNA quality (Agilent 2100) and assessment of selected gene expressions (if recommended).

Endpoint Analyses

The overall null hypothesis of this experiment is that the three methods, when performed by clinical personnel on samples from burned patients, will yield equivalent amounts of total cellular RNA, and the quality of the RNA product, as well as the gene expression patterns that are determined from the RNA will be equivalent.

The following will be the primary endpoints:

1. Total RNA yields (μg, μg/mg of tissue)

2. RNA quality, as determined by

a. $Abs_{260/280}$

b. Agilent 2100 chromatographic analysis

c. cRNA yield from RNA source

3. gene expression patterns (U133A)

Statistical Analyses

Continuous parametric variables such as RNA yield and $Abs_{260/280}$ ratios will be analyzed by one-way analysis of variance with replications (for individuals). Post hoc comparisons among the three treatment groups will be performed with a multiple range test, such as Tukey's or the Student-Newman-Keul's. Nonparametric or complex data (such as the Agilent 2100 readouts will not be analyzed statistically. Microarray data will be evaluated by several approaches simultaneously. Initially, microarray data will be normalized according to MAS v5 or DChip, and the data set will be filtered to remove probe sets whose expression in all of the samples is not above background. As a first iteration, the data will be variance normalized and the top half of the sample set with the greatest variance will be further analyzed by hierarchical cluster analysis using the SAM and BRB analytical programs. Additionally, principal component analysis will be performed.

TOTAL RNA ISOLATION FROM HUMAN SKIN, MUSCLE AND SOLID ORGANS

Laboratory methodologies/standard operating procedures

Genomics Core/PACB Core, Protocol #G026.01
University of Florida College of Medicine
Created March 2003/last modified 12/23/2003 / MCL

Principle

Trauma and sepsis alter the patterns of gene expression in tissues and solid organs. The purpose of this procedure is to obtain total cellular RNA from human skin, muscle and solid organs for subsequent analysis of mRNA populations, either by the polymerase chain reaction (following reverse transcription) or by microarray analyses. The protocol is based on a commercial preparation, RNeasy® Fibrous Tissue Mini Kit (Qiagen, Cat# 74704), and is conceptually similar to the blood leukocyte RNA isolation procedures. The initial processing of the tissues requires a mechanical homogenization in the presence of a chaotrope (RLT buffer). A proteinase K digestion follows and nucleic acids are removed by the addition of ethanol, followed by application to an RNeasy column that retains the RNA and washes away the contaminants. RNA is then eluted with distilled water.

A more detailed description of the principle and protocols can be found in the accompanying documentation to RNeasy® Fibrous Tissue Mini Kit (Qiagen, Cat# 74704) Qiagen, Valencia, California.

Things to do before starting

1. Heat a water bath or heating block to 55°C for proteinase K digestion in step 6
2. β-mercaptoethanol (β-ME) (Sigma catalog no. M6250) must be added to Buffer RLT before use. Dispense in a fume hood and wear appropriate protective

clothing. Add 10 µl β-ME per 1ml Buffer RLT. Buffer RLT is stable for 1 month after addition of β-ME.

3. Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96-100%), as indicated on the bottle, to obtain a working solution.

Methods

All reagents are from the RNeasy® Fibrous Tissue Mini Kit (Qiagen, catalog no. 74704) unless otherwise specified.

1. Remove tissue from storage. If flash-flash frozen tissue is to be used, transfer the samples from the freezer to liquid nitrogen. Do not let the samples thaw before processing. RNA*later*™ stabilized tissues and tissue saved in 70% Ethanol can be thawed before processing. Begin with approximately 30 mg of skin or muscle tissue. Record weight.

2. Pipet 300 µl RLT buffer to which β-mercaptoethanol (β-ME) (Sigma catalog no. M6250) has been freshly added into a sterile 1.5 ml Microcentrifuge tube (Fisher catalog no. 14-959-11B). Add the tissue to the tube.

3. Disrupt tissue and homogenize lysate using a rotor-stator homogenizer (IKA UltraTurrax T25, Tissue-Tearor, or similar) until sample is uniformly homogeneous, usually 45-60 seconds. Ensure that all the tissue has been disrupted.

4. Add 590 µl RNase-free water to the homogenate. Then add 10 µl proteinase K solution and mix thoroughly by pipetting.

5. Incubate at 55°C for 10 min.

6. Centrifuge for 3 min at 10,000 x *g* at 20-25°C.
   Note: FYI A small pellet of tissue debris will form, sometimes accompanied by a thin layer or film on top of the supernatant.

7. Pipet the supernatant (~900 µl) into a new sterile 1.5 ml Microcentrifuge tube (Fisher catalog no. 14-959-11B).

Note: Avoid transferring either the pellet or the filmy layer with the supernatant. Carryover of a small amount of pelleted debris will not affect the RNeasy procedure, but care should be taken to minimize this. Hold the pipet tip under the thin layer or film on top of the supernatant, if present. This layer will usually adhere to the outside of the pipet tip and should not be transferred.

8. Add 0.5 volumes (usually 450 µl) of 100% ethanol to the cleared lysate. Mix well by pipetting. **DO NOT** centrifuge.

9. Pipet 700 µl of the sample, including any precipitate that may have formed, into an RNeasy Mini Spin Column in a 2 ml collection tube. Centrifuge for 15 s at ≥8,000 x *g*. (≥10,000 rpm). Discard the flow-through.

10. Repeat step 9, using the remainder of the sample. Discard the flow-through.

11. Pipet 350 µl Buffer RW1 into the RNeasy Mini Spin Column, and centrifuge for 15 s at ≥8,000 x *g* to wash. Discard flow-through.

12. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube**. Do not vortex.**
Note: If using the DNase for the first time, prepare DNase I stock by dissolving solid DNase I (1500 Kunitz units) in 550 µl of RNase free water and mix by inversion (1500 Kuntz Units/0.55 ml). Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. Do NOT vortex.

13. Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy silica-gel membrane, and place on the benchtop at room temperature for 15 min.

14. Pipet 350 µl Buffer RW1 into the RNeasy Spin Column, and centrifuge for 15 s at ≥ 8000 x *g* to wash. Discard flow-through and collection tube.

15. Transfer the RNeasy Spin Column into a new 2 ml collection tube (supplied). Pipet 500 µl Buffer RPE onto the RNeasy Spin Column. Close the tube gently and centrifuge for 15 s at ≥ 8000 x *g* to wash. Discard flow-through.
Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol has been added to Buffer RPE before use.

16. Add another 500 µl Buffer RPE to the RNeasy Spin Column. Close the tube gently and centrifuge for 1 min at ≥8,000 x *g* to wash. Discard flow-through. Centrifuge for 2 min at full speed .
Note: Following the centrifugation, remove the RNeasy Mini Spin Column from the collection tube carefully so the column does not contact the flow-through as this will result in carryover of ethanol.

17. To elute, transfer the RNeasy Mini Spin Column to a new 1.5 ml collection tube (supplied). Pipet 35 µl RNase free water directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 min, centrifuge for 1 min at ≥8000 x *g* to elute.

18. Repeat the elution step as described by pipetting another 35µl of RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 min, centrifuge for 1 min at ≥8000 x *g* to elute. Elute into the same collection tube.

Materials

All reagents are from the RNeasy® Fibrous Tissue Mini Kit (Qiagen, catalog no. 74704) unless otherwise specified

G029 GENE EXPRESSION PROFILING FROM WHOLE BLOOD LEUKOCYTES AND ENRICHED T CELLS AND ENRICHED MONOCYTE POPULATIONS ISOLATED FROM HEALTHY HUMAN VOLUNTEERS

Laboratory methodologies/standard operating procedures

Genomics Core/PACB Core, Protocol G029.02

University of Rochester School of Medicine/University of Florida College of

Medicine

Created January 30, 2003. Updated August 1, 2003 at 3:19:02 p.m.

Principle

Groundwork studies supported by the Large Scale Collaborative Research Agreement have examined the effects of RNA isolation methods on gene expression analysis, both from healthy human subjects (G004), healthy human subjects receiving endotoxin (CR001), and from traumatized patients (G017). The preliminary results suggest that there are significant and distinct variations in whole blood leukocyte expression patterns depending upon the method of RNA isolation. Furthermore, endotoxin administration to healthy subjects induces reproducible changes in the global pattern of gene expression. One possible explanation for these changes in gene expression in whole blood is that variances in leukocyte subpopulations within samples may influence the observed expression patterns in response to endotoxin administration. Therefore, there is a compelling need to identify the contribution of individual leukocyte subpopulations to the gene expression patterns seen in the entire leukocytes population.

One of the major future goals of the program is to determine the effects of traumatic or burn injury on the gene expression and the proteomic patterns of individual leukocyte subpopulations. These studies will require an easy and reproducible protocol for the isolation of enriched leukocyte populations, which can be adapted to a multicenter study. The PACB Core has developed protocols (P003), which have been shown to result in the isolation of enriched monocyte and T cell populations, based on negative selection. Using blood obtained from healthy subjects, the monocyte and T cell enrichments approach 80-95% purity.

The question that immediately arises is whether this degree of monocyte and T cell enrichment obtained with P003 will be adequate to obtain and interpret gene expression

data from traumatized and burned patients. Our concern is that contamination of enriched leukocyte populations with modest amounts of contaminating cells (5-20%) may introduce sufficient variation in the gene expression profile to make interpretation of the gene expression patterns of the predominant cell population inadequate. To test this hypothesis, we propose to examine gene expression profiles from T cell and monocyte enriched populations, based on P003, from healthy subjects

Experimental Design

Aim. The primary goal of this experiment is to determine patterns of gene expression in T cell and monocyte enriched populations (80-95%), as proposed in P003, from healthy human volunteers. The null hypothesis is that gene expression patterns seen in T cell and monocyte-enriched populations will not be different from the expression patterns from total leukocyte populations when obtained from healthy human volunteers. Secondary goals for this experiment are to determine whether gene expression patterns from mixed leukocyte population obtained from blood after RBC lysis (G001.03) can be predicted from the gene expression patterns obtained from T cell and monocyte pure populations, when the differential count of the buffy coat is known. There has been speculation that algorithms could be developed which can predict the contribution of individual leukocyte populations to the gene expression pattern of whole leukocytes population, when the differential count is known. The present studies will provide experimental data to validate these algorithms.

Locations and subjects. Healthy subjects will be studied at the University of Rochester School of Medicine. Signed informed consent to donate 90 ml of venous blood will be obtained from healthy volunteers. Blood will be processed immediately after collection.

Experimental Setting. As shown in Fig. 1 from each normal subject 90 mls, 4 ml of blood will be removed, and processed according G025 protocol. Plasma sample will be preserved. After performing a differential analysis by flow cytometry on G025 samples,

leukocytes will be subjected to RLT buffer scheduled for RNA isolation in accordance with G001.03 (Shredder column will be applied). Remaining sample from normal subjects will be split for further purification according to Standard Operating Protocol P003. The monocyte and T cells will be phenotyped by flow cytometry, and 7×10^6 cells will be lysed in RLT buffer according to G001.03.

Lysis of the samples in RLT buffer will take in room temperature after pushing samples through Shredder column. Such prepared samples will be frozen at -80°C and processed next day for RNA isolation (G001.03). Total cellular RNA will be isolated using RNeasy RNA isolation system (Qiagen) according to G001.03, A small sample (200 ng per sample) will be taken from each RNA isolate to evaluate quality of RNA. RNA yields are determined by absorbance at 260/280 nm. The quality of the RNA is judged by Agilent 2100 analysis as performed previously. Gene expression profiling will be performed by using Affymetrix U133a GeneChips.

To determine the phenotype of the isolated cell populations, flow cytometry will be used. Each cell subpopulation (Lysis, Monocytes, T cells) will be stained with set of antibody (CD2, CD3, CD14, CD19, C56,CD66b) according to the attached flow cytometry protocol.

More detailed description of sample processing is described in Table 1, Fig. 1, flow cytometry protocol, and blood separation procedure below

Blood Separation Procedure

1. 90 ml of blood will be collected into the plastic syringes filled with anticoagulant (EDTA; concentration according P003) after venipuncture from healthy normal subjects.

2. 4 ml of the blood will be removed and processed according to G025.01

protocol with flow cytometry evaluation of the purity. The cells will be counted and ≈20 $\times 10^6$ (or all of them) will be lysed in RLT buffer and stored at -80°C.

3. The remaining 86 ml (normal) of blood will be spun down (1,500 × *g* for 10 min) to separate plasma from cellular elements. The plasma will be saved for further cytokine profiling and cells separation (It is also necessary to store some plasma to prepare buffer for Miltenyi Column).

4. 30 ml of blood from normal subjects will be processed for T cells separation, and 56 ml of blood from normal subjects will be processed for separation of the monocytes. Both separations will be conducted in accordance with P003 protocol.

5. The cells will be counted.

6. The purity of the sample after P003 protocol will be assessed with flow cytometry

7. A total of 7×10^6 cells will be lysed in RLT buffer and frozen at -80°C.

8. The RNA isolation from frozen RLT samples will be conducted according to G001.03 protocol.

G025 - Slightly modified for G029.

1. Venous or arterial whole blood is collected into 30-ml syringes filled with EDTA (1 mM final concentration, 300 µl of 100 mM EDTA stock solution) The collection of blood should be obtained from an existing arterial or venous line, or venipuncture should be performed by someone experienced in the technique, and familiar with infectious precautions. The blood should be processed further immediately.

2. Separate 4 ml of sample into separate 50-ml Falcon tubes.

3. Centrifuge the samples in a swinging bucket refrigerated centrifuge at 4°C for 10 min at 400 × *g*, with the brake OFF. Remove the plasma (taking care not to disturb the cellular constituents)

4. Label the samples with the attached bar codes and store at -80°C.

5. Transfer the remaining packed cells immediately to one 50-ml conical tubes (Corning Labs. catalog no. 430828) and add 45 ml of lysis buffer at room temperature. The lysis buffer is provided to you in a single-use container. Do not use if the seal has been broken or the container has exceeded its expiration date.

6. Mix the samples thoroughly and allow to stand at room temperature for 5-10 min, vortexing the samples gently every 5 min during that period. As a rule of thumb, lysis is complete when you can read black type on white paper through the tube.

7. Centrifuge the samples in a swinging bucket, refrigerated centrifuge at 4°C for 10 min at 400 × *g*, with the brake OFF.

8. Remove the supernatant and discard. Loosen cell pellet by gently tapping the side of the tube on a hard surface, then vortex gently.

9. To each tube add 4 ml of ice-cold, calcium-free, magnesium-free, PBS (catalog no. 21-031-CV) and vortex gently.

10. Repeat steps 7 and 8.

11. At the end at room temperature, add 600 µl of RLT Buffer (Qiagen, RNeasy Mini kit catalog no. 74104) for each 10^7 cells.

12. Homogenize the pellet in the RLT Buffer by pushing the sample through Shredder column.

13. Place a barcode label on the tube and store at -70°C until ready for RNA isolation.

P003.06 Modified for G029

1. The collection of blood should be obtained from an existing arterial or venous line, or venipuncture by someone experienced in the techniques, and familiar with infectious (universal) precautions. Blood can be collected from an existing arterial or venous line, or by venipuncture using a Vacutainer (Becton Dickinson) blood collection system. In this case, venous whole blood is collected at room temperature (18-25°C) into two 7-ml lavender blood collection tubes (Becton Dickinson Vacutainer catalog no. 367669). The lavender top tubes are gently inverted several times to mix the blood with the contained anticoagulant.

2. Transfer by decanting the blood from the two lavender blood collection tubes to a 15 ml centrifuge tube (Fisher catalog no. 05-538-59A, or Becton Dickinson catalog no. 35-2097).

3. Centrifuge the samples in a desktop, refrigerated centrifuge (Beckman Model GPR, or equivalent) with swinging buckets at room temperature for 10 min at 300 × g, with the brake OFF. Administrative note: It would be optimal if two centrifuges with swinging bucket rotors were available to maintain one at room temperature, and one at 4°C.

4. Using a permanent lab marker, draw a line on the centrifuge tube indicating the upper level of the plasma inside the tube. Then, using a disposable plastic pipette (Fisher Scientific catalog no. 13-678-11E) or sterile glass pipette (VWR catalog no. 14672-410, rubber bulb VWR catalog no. 56311-062), carefully transfer the plasma to a new 15-ml conical tube being sure not to withdraw any of the erythrocyte layer, and centrifuge at 1,500 × *g* for 10 min at room temperature. Administrative note: only collect the plasma fraction to within 0.5 ml of the erythrocyte layer. Do not contaminate the plasma fraction with the erythrocytes.

5. Remove plasma supernatant using a disposable plastic pipette (Fisher Scientific catalog no. 13-678-11E) without disturbing the pellet, and aliquot plasma into three or four 2.0 ml yellow-capped USA Scientific microcentrifuge tubes (catalog no. 1420-9706). Label the tubes with a bar-coded label, complete the Sample Collection Form, and store at -80°C, until shipment to the Sample Collection and Coordination Site (see protocols P002 and P005).

6. To the blood (from step 4), replace the plasma with an equal volume of Wash/Dilution Buffer **(**identified as Solution B or Wash/Dilution Buffer). The addition of the Wash/Dilution Buffer should restore the volume of the sample to the line marked in Step 4.

7. Add 140 µl of heparin (1,000 units/ml) (Elkins-Sinn catalog no. 2440-45) and mix gently (identified as Solution C).

8. Divide the diluted blood into two new 15 ml Corning centrifuge tubes. Label one tube as “Monocyte separation” and the other as “T cell separation”. There should be approximately 7 ml of blood in each tube.

9. To the tube marked “Monocyte separation” add 350 µl of the “Human Monocyte Enrichment Mixture” (RosettSep, catalog no. 15068, Stem Cell Technologies) (identified as Solution D). To the tube marked “T cell separation,” add 350 µl of “T cell Enrichment Mixture” (RosettSep, catalog no. 15061, Stem Cell Technologies) (identified as Solution E). Mix gently by inversion and incubate the tubes at room temperature for 20 min.

10. Using a sterile, ten ml serological pipette (VWR catalog no. 53283-740), add seven ml of the Wash/Dilution Buffer (Solution B) at room temperature to each tube and mix gently.

11. Prepare two 50-ml centrifuge tubes (Becton-Dickinson catalog no. 35-2098) for the density centrifugation. Label one "Monocyte separation" and one "T cell separation," . Gently mix the Ficoll before use. Ficoll preparations must be at room temperature. Using a new serological pipette, add 15 ml of Ficoll-Paque Plus (catalog no. 07957, StemCell Technologies) (Solution F) to the tube marked "Monocyte separation " and 15 ml of DM-L Ficoll (catalog no. 15705, StemCell Technologies) (Solution G) to the tube marked "T cell separation".

12. Using a sterile glass or plastic transfer pipette (Fisher Scientific catalog no. 13-678-11E), gently layer blood from each tube on top of its corresponding Ficoll very carefully to avoid mixing. Put the layered blood into the desk-top centrifuge (Beckman Model GPR), and centrifuge the blood at 1,200 × *g* at room temperature for 20 min with the brake OFF. Administrative note: while the blood is layered over the Ficoll preparations, it cannot be allowed to stand for extended periods of time (>5 min) without the blood settling through the Ficoll preparation. This settling of blood is to be avoided.

13. Using a plastic transfer pipette (Fisher Scientific catalog no. 13-678-11E), discard the top layer to within one cm of the white interfacial layer. Using a new pipette, gently transfer the interfacial layer from each tube to two new 50-ml conical centrifuge tubes containing 25 ml of *ice-cold* Wash/Dilution Buffer **(**Solution B). Label tubes accordingly as "Monocyte separation." and "T cell separation". From this point onward, the tubes should be kept at 4°C.

14. Fill the centrifuge tubes to a volume of 50 ml with ice-cold Wash/Dilution Buffer (Solution B), invert gently several times, and centrifuge at 500 × *g* for 10 min at 4°C.

15. Gently decant the supernatant to the cell pellet and resuspend cells in two ml of Wash/Dilution Buffer **(**Solution B**)**. Using a clean transfer pipette, release the pellet and mix by gently aspirating the cells with the pipette. Subsequently, fill the tubes with 50 ml of ice-cold Wash/Dilution Buffer (Solution B), and centrifuge at 500 × *g* for 10 min at 4°C.

16. Decant the supernatant from each of the two cell pellets (from "Monocyte separation." and "T cell separation") and resuspend each of the cell pellets in one ml of 4°C Freezing solution (10% DMSO (Sigma catalog no. D2650) in 90% FCS) (Solution H). Transfer the monocytes to a blue-capped 2.0 ml microcentrifuge tube (USA Scientific catalog no. 1420-9701) and transfer the T cells to an amber 2.0 ml microcentrifuge tube (USA Scientific catalog no. 1420-9707). Place a bar-coded label on each tube, complete a Sample Collection Form, and place the tubes in a styrofoam box (VWR catalog no. 15713-539) (provided by the Sample Collection and Coordination Site). Place the box in the -80°C freezer at least overnight. Sample is ready to be shipped to the Sample Collection and Coordination Site, according to standard operating protocols P002 and P006.

Reagents

Solution A – Not being used in current iteration.

Solution B. Wash/Dilution buffer - 2% FCS (heat inactivated) (Mediatech catalog no. MT-35-011-CI, or HyClone catalog no. SH30070.03) and 100 units/ml polymixin B (Sigma, catalog no. P1004 or Calbiochem catalog no. 5291), dissolved in Dulbecco's Calcium and Magnesium-free, Phosphate Buffered Saline. (Mediatech/Cellgro catalog no. 21-030-CV or GIBCO catalog no. 14040-133)

Solution C. Sodium Heparin (1,000 units/ml) (Elkins-Sinn catalog no. 2440-45)

Solution D. Monocyte Enrichment Mixture (RosettSep, catalog no. 15068, Stem Cell Technologies)

Solution E. T Cell Enrichment Mixture (RosettSep, catalog no. 15061, StemCell Technologies)

Solution F. Ficoll-Paque Plus (catalog no. 07957, Stem Cell Technologies)

Solution G. DM-L Ficoll (catalog no. 15705, Stem Cell Technologies)

Solution H. Freezing solution: 10% DMSO (Sigma catalog no. D2650) in 90% Fetal Bovine Serum (Mediatech catalog no. MT-35-011-CI, or HyClone catalog no. SH30070.03)

G001.03 for G029.02

1. Homogenize the pellet in the RLT/BME at room temperature by using Shredder Column.

2. Add an equal volume of 70% ethanol, and shake vigorously.

3. Apply mixture onto RNeasy Mini column.

4. Centrifuge at room temperature at ≥8,000 × *g* for 15 s. Discard the flow-through and reload the column with any remaining samples (max twice).

5. Discard the flow-through, and add 350 µl of RW1 Buffer (Qiagen) to the column. Centrifuge at room temperature at ≥8,000 × *g* for 15 s. Discard the flow-through.

6. When using the RNase-Free DNase Set (Qiagen, catalog 79254) for the first time, prepare the DNase stock solution by dissolving the solid DNase I (1,500 Kunitz units) in 550 µl of RNase-free water (1,500 Kuntz units/0.55 ml). Mix by inversion and store at -20°C. For each RNeasy column, add 10 µl of DNase I stock solution to 70 µl of Buffer RDD. Mix by gently flicking the tube (do not vortex), and centrifuge briefly to collect residual liquid from the sides of the tube.

7. Pipet the DNase I incubation mix (80 µl) directly onto the spin-column membrane, and place it upright at room temperature for 15 min.

8. Add 350 µl Buffer RW1 to the RNeasy column, and place at room temperature for 5 min. Then centrifuge at room temperature (20-25°C) at ≥8,000 × *g* for 15 s.

9. Discard the flow-through and add 500 µl of RPE Buffer (Qiagen) to each column. Centrifuge at room temperature (20-25°C) at ≥8,000 × *g* for 15 s.

10. Repeat step 9, and centrifuge at room temperature (20-25°C) at ≥8,000 × *g* for 2 min.

11. Transfer the column to a new 1.5-ml collection tube (Qiagen).

12. Pipette 30 µl of RNase-free water onto the column and allow to stand at room temperature for ~1 min. Centrifuge at room temperature (20-25°C) at ≥8,000 × *g* for 1 min. Repeat the procedure with an additional 30 µl of RNase-free water, and collect into the same tube.

Flow Cytometry Protocol

1. Use the cells at 10^7 cells per ml.

2. Calculate appropriate number of cells for all staining and dissolve the cells in 1:1 ratio in FACS blocking buffer.

3. Incubate for 15 min at 4°C.

4. Add appropriate amount of either antibody mixture or positive antibody or isotype control to real staining and control tube respectively.

5. Equalize volume to 80 ml in total with FACS wash buffer.

6. Vortex cells.

7. Incubate 5 min in the dark at 4°C.

8. Vortex cells.

9. Incubate 10 min in the dark at 4°C.

10. Add 3 ml of FACS buffer and centrifuge cells at 500 × *g* for 5 min at 4°C.

11. Discard supernatants.

12. Repeat steps from 4 to 12 skipping step 6 if secondary staining is necessary.

13. Resuspend the cells in 150 ml of FACS buffer.

14. Run the sample on flow cytometer.

15. Perform analysis.

Following panels are used

1. Murine anti-human CD3PE/murine anti-human CD2 Biotinylated APC - T cells identification

2. Murine anti-human CD14FITC/murine anti-human CD33APC - MØ identification

3. Murine anti-humanCD19PE/murine anti-human CD33APC - B cells identification

4. Murine anti-human CD56PE/murine anti-human CD33APC - NK identification

5. Murine anti-human CD66bFITC/murine anti-human CD14APC - Granulocyte and MØ identification

A FACSCalibur flow cytometer is used for collecting the data. The cells were gated on FS/SS scatter after setting FS threshold below T cell population. For color analysis, all cells were gated after adjusting voltages to visualize histograms during acquisition of the cells stained with isotype control. Single staining data are computed by substracting % positive gated cells from previously established isotype control. The performance of the flow is checked weekly with Calibration Beads and FACSCOMP software.

FACS Blocking Buffer

Reagent	Company	catalogue no.	Final concentration	Stock	How much for 10mls
FCS	Hyclone	#SH30070.03	0.05mM	10mM	1ml
Human IgG	ICN	55838	0.1mg/ml	1mg/ml	1ml
PBS noCa/Mg	Cellgro	21-040-CV	N/A	N/A	8mls

FACS WASH

Reagent	Company	catalogue no.	Final concentration	Stock	How much for 500mls
FBS	Hyclone	#SH30070.03	2.5%	100%	10 mls.
Sodium azide	VWR	VW 3465-2	0.05%	5%	10mls.
PBS noCa/Mg	Cellgro	21-040-CV	N/A	N/A	q.s 500mls

Antibodies Employed

Ab	Vendor;catalogue no.	Amount	Corresp. isotype	Vendor;catalog no.	Amount
CD3 PE	BD;555333	3ml	m IgG1 PE	BD;555749	3ml
CD2 Bio	BD;555325	1ml	m IgG1 Bio	BD:550615	1ml
CD14 FITC	BD;555399	3ml	m IgG2a FITC	BD;555573	3ml

CD33 APC	BD;340474	3ml	m IgG1 APC	BD;340442	3ml
CD19 PE	BD;555413	3ml	m IgG1 PE	BD;555749	3ml
CD56 PE	Immunotech;2073	3ml	m IgG1 PE	BD;555749	3ml
CD66b FITC	Coulter;IM0531	3ml	m IgG1 FITC	BD555748	3ml