5. Transfer pipettes (use pipettes that have non-stick surfaces)

12. Isotypic Control for Anti-CD19/RPE, HD37 (Dako Code X0928, IgG1/RPE)

Clone HD37

Collect blood specimens by venipuncture (14) into evacuated tubes containing an appropriate anticoagulant, completely expelling the vacuum in the tubes. Mix the blood well when this is not possible, each laboratory should validate that its collection and holding methods maintain specimen integrity comparable to freshly processed material.

When using ACD or heparin anticoagulants, blood samples may be stored at 18-22 °C before testing for up to 30 hours after venipuncture. When using EDTA, blood samples should be processed within 24 hours (15).

SAMPLE PERIMATION

Optimum concentration of white blood cells for each test is (1 ± 0.3) x 10^7 cells/mL. For samples with a high white blood cell count, dilute specimens with autologous plasma to obtain a concentration of cells approximately equal to 1 x 10^7 cells/mL. For samples containing a low white blood cell count (less than 0.7 x 10^7 cells/mL), concentrate cells by centrifuging blood at 18-22°C for 5 minutes at 500 g. Draw off buffy coat with some red cells and plasma to ensure that all white blood cells are collected. Resuspend cells in autologous plasma and adjust white blood cell concentration to approximately 1 x 10^7/mL. Proceed with staining procedure.

STAINING PROCEDURE

1. Use an erythrocyte lysis procedure containing WB’s of optimal concentration.
2. Transfer 10 µL of the anticoagulated blood to the bottom of a polypropylene test tube. Any blood along the sides or top of the test tube must be removed as non WB’s and erythrocytes will be lost. The solution must be mixed gently to avoid disturbing the WB’s.
3. Add 10 µL of Anti-CD19/RPE (Dako Code R098), Mix by gentle tapping
4. Two additional aliquots of samples are needed for controls: One for the WB’s control (Dako Code R097), and one for the gating control, CD34/CD14 (Dako Code RF705). Use steps 1-3 to prepare the two control samples.

5. Incubate in the dark at room temperature (18-22 °C) for 15-30 minutes.

6. If a fixation step is not included with the erythrocyte lysing procedure, resuspend pellet in 0.5 mL of 0.05-1% paraformaldehyde (fixative) in PBS and incubate tubes for 30 minutes.

7. Perform erythrocyte lysing procedure. Follow the reagent manufacturer’s recommendations for time and temperature of incubation. This procedure may require a wash step and may include a lysis step, e.g. Utriprep (Dako D3235 or D3350).

8. If a wash step is required, cells may be centrifuged at 300 x g for 5 minutes at 18-22 °C. Aspirate the supernatant leaving approximately 50 µL of fluid. Add 3 mL PBS. Vortex gently. Centrifuge at 300 x g for 5 minutes at 18-22 °C. In an envelope the supernatant, leaving approximately 50 µL of fluid. Reaspirate in 0.5-0.6 mL PBS.

9. If a fixation step is not included with the erythrocyte lysing procedure, resuspend pellet in 0.5 mL of 0.05-1% paraformaldehyde (fixative) in PBS and incubate tubes for 30 minutes (7).

10. Analyze samples on a flow cytometer within 48 hours. If lysed and fixed samples are held more than 2 hours, store at 4 °C in the dark until analysis (1, 19).

FLOW CYTOMETRIC ANALYSIS

1. Analyze cells on a flow cytometer properly standardized and gated on lymphocytes according to the instrument’s operating manual.
2. Verify gate purity with the gating control tube (CD34/CD14).
3. Set the cursor using the isotype control so that <0.1% events are in the negative cell population.

REPRESENTATIVE HISTOGRAM

Lymphocytes from peripheral blood (healthy individual) labelled with RPE- Conjugated Anti-CD19, HD37 (Dako Code R098), (13% labelled lymphocytes). Clear curve indicates negative control, IgG1/RPE (Dako Code 0928).

EXPECTED VALUES

Blood samples were collected from 150 apparently healthy males and females at three geographically separate locations. The population included adults from a variety of races ranging in age from 19 to 62 years. Samples were stained Anti-CD19/RPE, HD37, monoclonal antibody. Normal CD19 positive B cell values were measured by flow cytometry using the whole blood method, and are presented in the following table. Values are expressed as a percentage of the total lymphocyte count and are intended as representative values only. Each laboratory should establish its own expected values from the local population of normal donors.

<table>
<thead>
<tr>
<th>%B Lymphocytes</th>
<th>N</th>
<th>Min</th>
<th>Max</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>4.47</td>
<td>25.60</td>
<td>12.4±5.38</td>
<td></td>
</tr>
</tbody>
</table>

PHERIPHERAL BLOOD MONONUCLEAR CELLS (SEPARATED CELLS)

Whole Blood

Materials Required, not provided

1. Blood collection tubes containing anticoagulant (K,EDTA, Heparin or ACD)
2. Sodium azide (Na3N3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
3. 1% paraformaldehyde for fixing cells, but avoid their contact with skin and mucous membranes.
4. Architectural good hygiene in the laboratory, avoidance of direct contact with eyes or skin contact with eyes, and skin clothing. If eye or skin contact occurs, immediately flush with water for at least 15 minutes and contact a physician if exposure symptoms develop. Inhalation or ingestion is harmful. If swallowed, induce vomiting and contact a physician immediately.
5. Use prudent laboratory practices when handling reagents. This includes avoiding unnecessary contact, and using protective equipment such as chemical resistant gloves, eye protection, and lab-coat.

Procedure

Materials required, not provided

1. Blood collection tubes containing anticoagulant (K,EDTA, Heparin or ACD)
2. Sodium azide (Na3N3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
3. 1% paraformaldehyde for fixing cells, but avoid their contact with skin and mucous membranes.
4. Architectural good hygiene in the laboratory, avoidance of direct contact with eyes or skin contact with eyes, and skin clothing. If eye or skin contact occurs, immediately flush with water for at least 15 minutes and contact a physician if exposure symptoms develop. Inhalation or ingestion is harmful. If swallowed, induce vomiting and contact a physician immediately.
5. Use prudent laboratory practices when handling reagents. This includes avoiding unnecessary contact, and using protective equipment such as chemical resistant gloves, eye protection, and lab-coat.

Phenol red stock solution

Store the re agent in the dark at 2-8 °C. Do not freeze. Do not use after expiration date stamped on vial.

In the appearance of the reagent, such as precipitation, indicates instability or deterioration. In such cases, the reagent is not to be used.
12. Isotypic Control for Anti-CD19/RPE (Dako Code X0 928, IgG1/RPE)
13. 15 mL centrifuge tubes (tissue culture plasticware or siliconized glassware)
14. Isotonic medium (e.g. PBS with 1% w/v BSA and 0.015 mol/L NaN)

SPECIMEN PREPARATION

1. Carefully layer 8 mL of diluted blood (dilute 1 to 1 with isotonic medium) on top of 4 mL of Ficoll-Paque in a 15 mL centrifuge tube.
2. Centrifuge at 18-22 °C at 400 x g for 30 minutes. Mononuclear cells should form a visible, clean interface between the plasma and Ficoll-Paque.
3. Collect the layer of cells by inverting a plastic pipette through the plasma. Place collected cells in a 15 mL test tube containing 5 mL isotonic medium. Spin at 200 x g for 10 minutes. Aspirate and discard the supernatant.
4. Wash once more with the isotonic medium and centrifuge at 200 x g for 10 minutes.
5. Resuspended cells in the isotonic medium. The cells can then be counted and evaluated for cell viability and further studies.

FLOW CYTOMETRIC ANALYSIS

1. Dissolve Trypan Blue in PBS to a final concentration of 5 g/L.
2. Place a sample of the mixture in a hemocytometer chamber. Do not overfill or underfill the chamber.
3. Count the live and dead (blue) cells. Calculate the percentage of viable cells from the number of live and dead cells.
4. Two additional aliquoted samples are needed for the isotypic control (IgG1/RPE, Code X0298) and one for the gating control (Dako Code FR700, CD45/CD14). Use steps 1-3 to prepare the two control samples.
5. Include in the dark at 4 °C for 30 minutes.
6. Resuspend cells in 1 mL of 1% paraformaldehyde (Fixation) in PBS.
7. Analyze on a flow cytometer within 48 hours.

EXPECTED VALUES

Blood samples were collected from 150 apparently healthy males and females at three geographically separate locations. The population included adults from a variety of races ranging in age from 19 to 62 years. Samples were stained with Anti-CD19/RPE, HD37, monoclonal antibody. Normal CD19 positive B lymphocytes were measured by flow cytometry. As a control, the PEGD3/1 (Positive Control) was used. Values are reported as % of the total lymphocyte count and are intended as representative values only. Each laboratory should determine its own expected values from the population of normal donors.

QUALITY ASSURANCE

Peripheral blood from a normal, apparently healthy donor should be run as a positive control to ensure proper working conditions. Normal ranges should be established within the local population of normal donors.

LIMITATIONS

Blood samples should be stored within 36 hours of collection for optimal results. Retail samples in the original sample tube at room temperature prior to staining and analysis. Do not refrigerate. Refrigerated samples or sample stored longer than 36 hours may give erroneous results. To ensure maximum viability, analyze samples promptly.

REFERENCES

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