1. Use peripheral blood containing white blood cells of optimal concentration.

2. Phosphate-buffered saline (PBS), 0.01 M phosphate-buffered saline, pH 7.4

3. Monoclonal Mouse Anti-Human CD3/RPE, Clone UCHT1

4. Add distilled water to 1 liter. Filter through a 0.2 µm filter.

5. 5 mL of sterile polyethylene test tubes

6. Transfer pipettes (use pipettes that have non-sticking surfaces)

7. Working Stock Solution

8. 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 for 30 minutes (15).

9. Working solution

10. The lymphocyte population of white blood cells (WBCs) consists essentially of three subpopulations: T cells (thymus derived), B cells (bone marrow derived), and natural killer cells. These subpopulations are identified by cell surface antigens and function. T cells express CD3 which is a T-cell associated antigen.

11. Development of flow cytometry has made it possible to perform immunophenotyping of lymphocytes rapidly and accurately. Immunophenotyping of lymphocytes is widely applied for diagnosis of immunodeficiencies (10, 11). Either Anti-CD3/RPE or Anti-CD3/RPE-Cy5 is one reagent utilized when performing immunophenotyping of lymphocytes.

**SUMMARY AND EXPLANATION**

- The lymphocyte population of WBCs consists of T cells, B cells, and natural killer cells. T cells express CD3 which is a T-cell associated antigen.

- Immunophenotyping of lymphocytes is used for diagnosis of immunodeficiencies.

**SPECKLE COLLECTION AND HANDLING**

- Collect blood specimens by venipuncture into evacuated tubes containing an appropriate anticoagulant, completely expelling the vacuum in the tubes. Mix the blood well with the anticoagulant to prevent clotting. At least 100 µL of whole blood is required for each test. Ideally, blood samples should be processed immediately after collection.

- When this is not possible, each laboratory should validate that its collection and holding methods maintain specimen integrity comparable to freshly processed material.

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- Immunophenotyping of lymphocytes is widely applied for diagnosis of immunodeficiencies (10, 11). Either Anti-CD3/RPE or Anti-CD3/RPE-Cy5 is one reagent utilized when performing immunophenotyping of lymphocytes.
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 a local population of normal, apparently healthy donors.

Interference in the counts of percent positive cells by monocytes and granulocytes with Fc receptor bound antibody may be reduced by proper flow cytometric gating on lymphocytes. An appropriate isotype negative control that is non-human reactive (either IgG1/RPE-Cy5, Dako Code X0928, or IgG1/RPE-Cy5, Dako Code X0955) is used to control for nonspecific antibody binding to lymphocytes in each patient sample. The brightly-stained lymphocyte population is measured within markers set to exclude the low level of nonspecific fluorescence.

Nonspecific fluorescence above the background marker is usually limited to 1–2% in normal individuals. If the background level above the marker for any control sample is greater than 1–2%, the test results may be erroneous.

Blood samples should be stained within 30 hours of collection for optimal results. Retain samples in the original sample tube at room temperature prior to staining and analysis. Do not refrigerate. Refrigerated samples or samples stored longer than 30 hours may give erroneous results. To ensure maximum validity, analyze stained samples promptly. Samples from certain patients may present special problems due to abnormal erythrocyte or leukocyte populations as the result of illness or drug usage.

Peripheral blood mononuclear cells (PBMC's) obtained by means of density gradient separation may not have the same relative percentages of lymphocytes as whole blood preparations. For normal patients, this factor may not be significant. For patients with leukopenia, the selective exclusion of specific lymphocyte subsets may affect the accuracy of the measurement.

Linearity of the Anti-CD3/RPE-Cy5, UCHT1, and Anti-CD3/RPE, UCHT1, monoclonal antibody were performed to determine the linearity of the binding to the CD3 cluster determinate. JM cell line suspended cells (positive epitope for the CD3 antibody) were mixed with Raji cell line suspended cells (negative for the CD3 antibody) at several dilutions to test for binding linearity. Anti-CD3/RPE-Cy5 bound with the JM cells on a linear basis (y = -0.02 + 1.0x, r² = 0.999); with the slope approaching 1.0. Anti-CD3/RPE bound with the JM cells on a linear basis (y = 2.84 + 1.0x, r² = 0.999); with the slope approaching 1.0.

Ten replicates from peripheral blood of three donors were tested for reproducibility of Anti-CD3/RPE-Cy5 and Anti-CD3/RPE run on two flowcytometers from different manufacturers at three concentrations of antigen. Different levels of CD3+ lymphocytes were selected from a population of normal and abnormal peripheral blood samples. Values are expressed as a percent of the total lymphocyte count and are intended as representative values only. Due to unacceptable variance among the different laboratory methods for determining absolute lymphocyte counts, an assessment of the accuracy of the method used is necessary. Each laboratory should establish its own expected values from the local population of normal donors.

EXPECTED VALUES

Blood samples were collected from 153 apparently healthy males and females at three geographically separate locations. The population included adults from a variety of races ranging in age from 20 to 65 years. Samples were stained with Anti-CD3/RPE, Dako code R0810 and Anti-CD3/RPE-Cy5, Dako code C7067 monoclonal antibody. Normal CD3 positive T-cell values were measured by flow cytometry using the Whole Blood lytic 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. Due to unacceptable variance among the different laboratory methods for determining absolute lymphocyte counts, an assessment of the accuracy of the method used is necessary. Each laboratory should establish its own expected values from the local population of normal donors.

QUALITY ASSURANCE

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### FACScan CD3/RPE-Cy5

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean % CD3+</th>
<th>±1 SD</th>
<th>±2 SD</th>
<th>% CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Level</td>
<td>71.52</td>
<td>0.52</td>
<td>1.05</td>
<td>0.01</td>
<td>10</td>
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<tr>
<td>Medium Level</td>
<td>37.52</td>
<td>0.57</td>
<td>1.14</td>
<td>0.02</td>
<td>10</td>
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<tr>
<td>Low Level</td>
<td>10.76</td>
<td>0.42</td>
<td>0.85</td>
<td>0.04</td>
<td>10</td>
</tr>
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</table>

Profile II CD3/RPE-Cy5

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean % CD3+</th>
<th>±1 SD</th>
<th>±2 SD</th>
<th>% CV</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Level</td>
<td>71.44</td>
<td>0.75</td>
<td>1.50</td>
<td>0.01</td>
<td>10</td>
</tr>
<tr>
<td>Medium Level</td>
<td>36.40</td>
<td>0.86</td>
<td>1.72</td>
<td>0.02</td>
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<tr>
<td>Low Level</td>
<td>12.36</td>
<td>0.31</td>
<td>0.63</td>
<td>0.03</td>
<td>10</td>
</tr>
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</table>

Profile CD3/RPE

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean % CD3+</th>
<th>±1 SD</th>
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</tr>
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<tbody>
<tr>
<td>High Level</td>
<td>72.06</td>
<td>1.11</td>
<td>2.22</td>
<td>0.02</td>
<td>10</td>
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<tr>
<td>Medium Level</td>
<td>38.49</td>
<td>0.87</td>
<td>1.74</td>
<td>0.02</td>
<td>10</td>
</tr>
<tr>
<td>Low Level</td>
<td>11.01</td>
<td>0.53</td>
<td>1.06</td>
<td>0.05</td>
<td>10</td>
</tr>
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**Specificity**

Specificity of Anti-CD3/RPE-Cy5, UCHT1, and Anti-CD3/RPE, UCHT1, has been verified by tests performed on five apparently healthy adult donors of various races at Dako.

Cell populations tested were RBCs, granulocytes, monocytes, lymphocytes and platelets. The results indicate that binding of Anti-CD3/RPE-Cy5, UCHT1, Dako Code C7067 and Anti-CD3/RPE, UCHT1, Dako Code R0810 are specific for lymphocytes. Monocyte binding can be excluded from the lymphocyte analysis by proper gating on lymphocytes.

In addition, Anti-CD3/RPE-Cy5, H2D7, is unreactive with other cells in the human hematopoietic system and shows no reaction with non-hematopoietic cells, e.g. in kidney, liver, breast or lung tissues.

### Anti-CD3/RPE-Cy5 Specificity

<table>
<thead>
<tr>
<th></th>
<th>% Positive</th>
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<th>% Positive</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Red Blood Cells</td>
<td>Granulocytes</td>
<td>Monocytes</td>
<td>Lymphocytes</td>
<td>Platelets</td>
</tr>
<tr>
<td>Average (n=5)</td>
<td>0.02</td>
<td>1.50</td>
<td>1.82</td>
<td>71.90</td>
<td>0.12</td>
</tr>
<tr>
<td>(range)</td>
<td>(0.0–0.1)</td>
<td>(0.9–2.2)</td>
<td>(1.1–2.7)</td>
<td>(60.3–78.6)</td>
<td>(0.0–0.3)</td>
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</tbody>
</table>

### Anti-CD3/RPE Specificity

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<tr>
<td>Average (n=5)</td>
<td>0.08</td>
<td>0.60</td>
<td>8.52</td>
<td>69.92</td>
<td>1.08</td>
</tr>
<tr>
<td>(range)</td>
<td>(0.0–0.3)</td>
<td>(0.4–0.7)</td>
<td>(1.6–16.3)</td>
<td>(58.4–75.9)</td>
<td>(0.0–0.8)</td>
</tr>
</tbody>
</table>

### References

15. Lewis, DE. Cytochemistry I: Cell surface immunofluorescence in Clinical Flow Cytometry, KD Bauer, RE Duque, TV Shankey, eds. Williams & Wilkins, Baltimore, MD. 1993;144