1. INTENDED USE
BD Simultest™ CD3/CD8 (Leu-4/2a) is a two-color direct immunofluorescence reagent for enumerating percentages of mature human suppressor/cytotoxic T lymphocytes in erythrocyte-lysed whole blood (LWB).

2. SUMMARY AND EXPLANATION
Human lymphocytes may be divided into three major populations based on their biologic function and cell-surface antigen expression: T lymphocytes, B lymphocytes, and natural killer (NK) lymphocytes. T lymphocytes participate in antigen-specific cell-mediated immunity and regulate the secretion of immunoglobulin by B lymphocytes. T lymphocytes may also be classified based on their functional properties as helper/inducer and suppressor/cytotoxic.

Clinical Applications*
Suppressor/cytotoxic T lymphocytes are a subset of T lymphocytes (CD3+) that are CD8+. Suppressor/cytotoxic T-lymphocyte (CD3+CD8+) percentages may be used to characterize and monitor some forms of immunodeficiency and autoimmune diseases.

The percentage of suppressor/cytotoxic T lymphocytes may lie outside the normal reference range in some autoimmune diseases,¹ and in certain immune reactions such as acute graft-versus-host disease (GVHD)² and transplant rejection.³ The relative percentage of the CD8+ subset is elevated in many patients with either congenital or acquired immune deficiencies, such as severe combined immunodeficiency (SCID)⁴ and acquired

* Not all the studies in this section employed BD reagents.
The relative percentage of the CD8+ cell population is often decreased in active systemic lupus erythematosus (SLE), but can also be increased in SLE patients undergoing steroid therapy.†

**NOTE**  
BD Simultest CD3/CD8 reagent allows suppressor/cytotoxic T lymphocytes to be identified and enumerated separately from contaminating CD3–CD8+ NK lymphocytes.

### 3. PRINCIPLES OF THE PROCEDURE

When monoclonal antibody reagents are added to human whole blood, the fluorochrome-labeled antibodies bind specifically to antigens on the surface of leucocytes. Monoclonal antibodies may be used to identify lymphocyte subpopulations.

An aliquot of the stained patient sample is introduced into the flow cytometer and passed in a narrow stream through the path of a laser beam. The stained cells fluoresce when excited by the laser beam and the emitted light is collected and processed by the flow cytometer.

### 4. REAGENT

**Reagent Provided, Sufficient for 50 Tests**

The BD Simultest CD3/CD8 reagent, sufficient for 50 tests, is provided in 1 mL of buffered saline with gelatin and 0.1% sodium azide. It contains FITC-labeled CD3 (Leu-4), clone SK7,8-11 and PE-labeled CD8 (Leu-2a), clone SK1,12 to identify the suppressor/cytotoxic T-lymphocyte (CD3+CD8+) population. The fluorescein-to-protein ratio (F:P) for BD IgG monoclonal antibody reagents is 2 to 5. The F:P ratio for CD3 (Leu-4) FITC has been optimized for its intended use.

The CD3 (Leu-4) antibody is composed of mouse IgG1 heavy chains and kappa light chains. CD3 reacts with the epsilon chain of the CD3/T-cell antigen receptor (TCR) complex.13 This complex is composed of at least six proteins that range in molecular weight from 20–30 kilodaltons (kDa).15-18 The antigen recognized by CD3 antibodies is noncovalently associated with either αβ or γδ TCR (70–90 kDa).15 The CD3 (Leu-2a) antibody is composed of mouse IgG1 heavy chains and kappa light chains. The CD8 antigen is present on the human suppressor/cytotoxic T-lymphocyte subset 9,16 as well as on a subset of NK lymphocytes.17 The CD8 antigenic determinant interacts with class I major histocompatibility complex (MHC) molecules resulting in increased adhesion between the CD8+ T lymphocytes and the target cells.18-20 Binding of the CD8 antigen to class I MHC molecules enhances the activation of resting T lymphocytes.18-21 The CD8 antigen is expressed as a disulfide-linked bimolecular complex with a 32-kDa α subunit.22 The cytoplasmic domain of the α subunit of the CD8 antigen is associated with the protein tyrosine kinase p56lk.20,21

**Precautions**

- For in vitro diagnostic use.
When stored at 2°C–8°C, the antibody reagent is stable until the expiration date shown on the label. Do not use after the expiration date.

The antibody reagent should not be frozen or exposed to direct light during storage or during incubation with cells. Keep the reagent vial dry.

Alteration in the appearance of the reagent, such as precipitation or discoloration, indicates instability or deterioration. In such cases, the reagent should not be used.

The antibody reagent contains sodium azide as a preservative; however, care should be taken to avoid microbial contamination, which may cause erroneous results.

**WARNING**

All biological specimens and materials coming into contact with them are considered biohazards. Handle as if capable of transmitting infection and dispose of with proper precautions in accordance with federal, state, and local regulations. Never pipette by mouth. Wear suitable protective clothing, eyewear, and gloves.

Concentration values are listed in the following table:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 FITC</td>
<td>25.0</td>
</tr>
<tr>
<td>CD8 PE</td>
<td>6.25</td>
</tr>
</tbody>
</table>

5. **INSTRUMENT**

BD Simulset CD3/CD8 reagent is designed for use on a BD FACS™ brand flow cytometer equipped with appropriate computer hardware, software, and gating electronics. The flow cytometer must be equipped to detect two-color fluorescence, forward scatter (FSC), and side scatter (SSC). We recommend using BD Simulset™ software, version 2.5 or later, for data acquisition and analysis.

All performance characteristics were obtained using a BD FACSscan™ flow cytometer and verified on a BD FACStrak™ flow cytometer. Other systems may have different characteristics and should be verified by the user.

6. **SPECIMEN COLLECTION AND PREPARATION**

Collect blood aseptically by venipuncture into a sterile BD Vacutainer® EDTA blood collection tube. A minimum of 1 mL of whole blood is required for this procedure. Blood should be stained within 6 hours of venipuncture for optimal results. Anticoagulated blood may be stored at room temperature (20°C–25°C) for up to 6 hours until ready for staining. Blood samples refrigerated prior to staining may give aberrant results.

A white blood cell (WBC) count and a differential white-cell count should be obtained from the same sample of whole blood before staining. An acceptable WBC concentration range is from 3.5 x 10³ to 9.4 x 10³ WBC/µL. Samples with counts greater than 9.4 x 10³ WBC/µL may be diluted with 1X phosphate-buffered saline (PBS) containing 0.1% sodium azide. For samples with counts less than 3.5 x 10³ WBC/µL, more blood may be needed and a separation procedure may be required to concentrate the cells.
Interfering Conditions
Previously fixed and stored patient specimens should not be used. Whole blood samples refrigerated prior to staining may give aberrant results. For optimal results, blood samples should be stained within 6 hours of venipuncture. Samples obtained from patients taking immunosuppressive drugs may yield poor resolution. The presence of blast cells or unlysed or nucleated red blood cells (RBCs) may interfere with test results. Hemolysed samples should be rejected. Follow the collection tube manufacturer’s guidelines for the minimum volume of blood to be collected.

CAUTION Use standard precautions when obtaining, handling, and disposing of all human blood samples and potentially carcinogenic reagents.

7. PROCEDURE
Reagent Provided
See Reagent Provided, Sufficient for 50 Tests and Precautions in Section 4, Reagent.

Reagents and Materials Required But Not Provided
- BD Simultest™ Leucogate™ (CD45/CD14) reagent, 1 mL (Catalog No. 340040). For determining a lymphocyte analysis gate, refer to the BD Simultest Leucogate instructions for use (IFU) and the BD Simulset Software User’s Guide. Store at 2°C–8°C.
- BD Simultest Control γ1/γ2a (IgG1 FITC/IgG2a PE) (both keyhole limpet hemocyanin-specific), 1 mL (Catalog No. 340041). Store at 2°C–8°C.
- BD FACS™ lysing solution (10X), 100 mL (Catalog No. 349202). Store at 2°C–25°C. For use, dilute 1:10 with room temperature (20°C–25°C) reagent-grade water. Store in a glass container at room temperature. The prepared solution is stable for 1 month at room temperature. See the BD FACS Lysing Solution IFU.
- BD Calibrite™ beads (Catalog No. 349502). For detailed information on use, please refer to the BD Calibrite Beads IFU.
- BD Vacutainer EDTA blood collection tubes or equivalent.
- Falcon® disposable 12 x 75-mm polystyrene test tubes or equivalent.
- Vortex mixer.
- Low-speed centrifuge (minimum speed 200g) with swinging-bucket rotor and 12 x 75-mm tube carriers.
- Vacuum aspirator with trap.
- Micropipettor with tips.
- Phosphate-buffered saline (PBS) (1X) (Dulbecco’s modified, pH 7.2 ±0.2; 0.01 mol/L PO4; and 0.15 mol/L NaCl). This reagent does not contain calcium, magnesium, phenol red, or sodium azide. Filter PBS through a 0.2-µm filter before use. Store at 2°C–8°C.
- PBS with 0.1% sodium azide.
- BD FACSFlow™ sheath fluid (Catalog No. 342003) or equivalent.

CAUTION Use only BD FACSFlow sheath fluid diluent to dilute BD Calibrite beads.

Falcon is a registered trademark of Corning Incorporated.
Paraformaldehyde (1%) for cell fixation. Dissolve 1 g of paraformaldehyde in 100 mL of freshly prepared PBS by carefully heating at temperatures up to 56°C in a chemical fume hood. Adjust to pH 7.4 ±0.2 with either 0.1 mol/L NaOH or 0.1 mol/L HCl. Filter with a 0.45-µm filter. Store in glass at 2°C–8°C.

Reagent-grade (both distilled and deionized) water.

Staining and Fixing the Cells
Whole blood samples are first stained with BD Simultest Leucogate (tube A), BD Simultest Control (tube B), and the BD Simultest CD3/CD8 (tube C) reagents. Diluted (1X) BD FACS lysing solution is used to lyse RBCs following staining. Use care to protect the tubes from direct light. Perform the procedure at room temperature (20°C–25°C) using room temperature reagents. Refer to Precautions in Section 4, Reagent.

1. For each patient sample, label three 12 x 75-mm tubes A, B, and C. Also label each tube with the sample identification number.

2. Place 20 µL of BD Simultest Leucogate reagent into tube A, 20 µL of BD Simultest Control reagent into tube B, and 20 µL of BD Simultest CD3/CD8 reagent into tube C.

3. For each sample tube, use a fresh micropipettor tip and carefully add 100 µL of well-mixed, anticoagulated whole blood into the bottom of each of the three labeled tubes. The recommended WBC concentration is 3.5 x 10⁴ to 9.4 x 10⁴ WBC/µL. Vortex thoroughly at low speed for 3 seconds and incubate for 15–30 minutes at room temperature (20°C–25°C).

NOTE Protect samples from direct light during this incubation procedure and use care to prevent blood from running down the side of the tube. If whole blood remains on the side of the tube, it will not be stained with the reagent.

4. Dilute 10X BD FACS lysing solution to 1X following the instructions under Reagents and Materials Required But Not Provided in Section 7, Procedure. Add 2 mL of room temperature (20°C–25°C) 1X BD FACS lysing solution to each tube. Immediately vortex thoroughly at low speed for 3 seconds and incubate for 10–12 minutes at room temperature (20°C–25°C) in the dark. Do not exceed 12 minutes.

NOTE Avoid prolonged exposure of the cells to lytic reagents, which may cause white cell destruction.

5. Immediately after incubation, centrifuge tubes at 300 g for 5 minutes at room temperature (20°C–25°C).

6. Aspirate the supernatant leaving approximately 50 µL of residual fluid in the tube to avoid disturbing the pellet.

7. Vortex thoroughly at low speed to resuspend the cell pellet in the residual fluid and then add 2 mL of PBS with 0.1% sodium azide to each tube. Vortex thoroughly at low speed for 3 seconds. Centrifuge at 200g for 5 minutes at room temperature (20°C–25°C).
8. Aspirate the supernatant leaving approximately 50 µL of residual fluid in the tube to avoid disturbing the pellet.

9. Vortex thoroughly at low speed to resuspend the cell pellet in the residual fluid and then add 1% paraformaldehyde to each tube. Vortex thoroughly at low speed for 3 seconds. Make sure the cells are well mixed with the fixing solution.

10. The cells are now ready to be analyzed on the flow cytometer. Cap or cover the prepared tubes and store at 2°C–8°C in the dark until flow cytometric analysis. Analyze the fixed cells within 24 hours after staining. Vortex the cells thoroughly (at low speed) to reduce aggregation before running them through the flow cytometer.

Flow Cytometry
Follow the BD instructions for two-color flow cytometric analysis.

Quality Control
For optimal results, we recommend using BD Calibrite beads and AutoCOMP™ software for setting the photomultiplier tube (PMT) voltages, setting the fluorescence compensation, and checking instrument sensitivity prior to use of BD Simultest CD3/CD8 reagent on a BD FACScan flow cytometer.

We recommend that a control sample from a normal adult subject be run daily to optimize instrument settings and as a quality control check of the system. Correct results for a hematologically normal patient sample are shown in Figure 1.

Figure 1 BD FACScan LWB sample, from a hematologically normal patient, stained with BD Simultest Leucogate (tube A), BD Simultest Control (tube B), and BD Simultest CD3/CD8 (tube C) reagents.

BD Simultest Leucogate reagent was used to reduce debris, monocytes, and granulocytes in the gate shown under tube A. Dot plot displays of FL1 (x-axis) vs FL2 (y-axis) are shown for tubes B and C.

BD Simultest Control reagent is run with each patient sample to set fluorescence 1 (FL1) and fluorescence 2 (FL2) markers between negatively and positively stained lymphocyte clusters and to detect the presence of nonspecific staining that would indicate erroneous patient results.
Visually inspect the dot plot for BD Simultest Control reagent (tube B). If the negative cluster is diffuse and smeared over the FL2 intensity range, the marker may not be set correctly and results may be suspect.

BD Simulset software will automatically inspect the data and alert the operator with a number of possible error messages. Refer to the BD Simulset Software User's Guide for a list of possible messages. Use the following criteria for inspection of the dot plots obtained for each sample to evaluate the quality of the data obtained.

- The operator should reject the results if one or more of the following error messages is received for the normal control: no separation between cellular populations; too few lymphocytes (less than 500); excessive RBC or nucleated RBC contamination and debris (greater than 10%); or excessive monocyte (greater than 3%) or granulocyte (greater than 6%) contamination of the lymphocyte gate.
- If there is no obvious reason for the normal control to fail, a sample from another normal control should be stained and run and the entire staining procedure repeated on all subsequent samples.
- Samples with nucleated RBCs may contain too much debris because of incomplete lysis of nucleated erythrocytes with BD FACS lysing solution. Too much debris may also occur when assaying blood samples from patients with certain hematologic disorders where red cells are difficult to lyse, as in myelofibrosis and spherocytosis. Nucleated erythrocytes will be counted as debris and, if debris exceeds 10%, the software will flag the sample as “too many nonlymphs in gate” and the sample results should be rejected.
- The tube-to-tube difference in gated events must be less than or equal to 500 events for all tubes in the panel except the BD Simultest Leucogate reagent (tube A). (However, if the BD Simultest Control tube is problematic, then the panel is suspect.)

8. RESULTS

Percent Lymphocyte Conversion
When the Percent Lymphocyte Conversion computation is performed, the CD3+CD8+ lymphocyte subset is reported as a percentage of lymphocytes in the lymphocyte analysis gate. If the computation is not performed, results will be reported as a percentage of the gated events.

Three-Part Differential
For lysed whole blood, it is possible to estimate monocytes, lymphocytes, and granulocytes as a percentage of leucocytes using the BD Simultest Leucogate reagent (tube A). BD Simulset software automatically calculates a three-part differential. Refer to the BD Simulset Software User’s Guide for representative data printouts.

NOTE The differential provided by BD Simulset software should be used only for comparison with an independent differential white-cell count for quality control purposes and should not be used in place of an independent laboratory differential white-cell count in patient charts or entered into BD Simulset software to obtain absolute counts.
**Absolute Counts**

An absolute cell count can be computed if a WBC and the lymphocyte percentage from an independent differential white-cell count are obtained using standard laboratory procedures. For instructions on how to calculate absolute counts, refer to the *BD Simulset Software User’s Guide*.

**9. LIMITATIONS**

- Laboratories must establish their own normal reference ranges for the BD Simultest CD3/CD8 reagent parameters that may be affected by sex of patient, age of patient, and preparative technique. Race of patient may also have an effect, although sufficient data is not available to establish this. Age, sex, clinical status, and race of subjects should be known when a reference range is determined.

- Results from BD Simultest CD3/CD8 reagent must be used in conjunction with other information available from clinical evaluation and additional independent diagnostic procedures.

- BD Simultest CD3/CD8 reagent is not intended for screening samples for the presence of leukemic cells or for use in phenotyping samples from leukemia patients. The presence of blast cells may not allow BD Simultest Leucogate reagent to set an adequate lymphocyte analysis gate. The software will flag the sample and results will not be printed.

- Absolute count values of lymphocyte subsets may not be comparable across laboratories due to variations in methods for determining white blood cell counts and/or white-cell differential values.

**10. EXPECTED VALUES**

**Leucocyte Subsets**

The suppressor/cytotoxic T-lymphocyte population present in quadrant 2 (Q2) of tube C is shown in Figure 1 and in the *BD Simulset Software User’s Guide*. The clinically significant results are found in the Physician Report.

BD has investigated the normal reference ranges for BD Simultest CD3/CD8 parameters in 159 normal male and female subjects using the BD FACScan flow cytometer at three sites (two US clinical sites and BD in San Jose, California). The expected normal reference ranges of suppressor/cytotoxic T lymphocytes for LWB are shown in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sex</th>
<th>Age</th>
<th>n</th>
<th>Mean</th>
<th>95% Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>Both</td>
<td>18–70</td>
<td>159</td>
<td>25</td>
<td>11–38</td>
</tr>
</tbody>
</table>

Adult reference ranges should not be used with pediatric blood samples.

Race may be a variable in the establishment of normal reference ranges, although insufficient data was collected by BD to determine this. Expected normal values may vary depending upon age, sex, or race of patient. Because of these differences, each laboratory should establish its own normal reference range for each parameter.
11. PERFORMANCE CHARACTERISTICS

Performance of the BD Simultest CD3/CD8 reagent was established by testing at two US clinical sites and at BD laboratories in San Jose, California.

Within-Sample Reproducibility

Table 2 shows the average within-sample reproducibility obtained for both normal and abnormal subjects.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Parameter</th>
<th>Mean</th>
<th>SDa Within-Stain</th>
<th>dfb</th>
<th>SDb Between-Stain</th>
<th>dfb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>27.2</td>
<td>0.8</td>
<td>12</td>
<td>1.1</td>
<td>11.1</td>
</tr>
<tr>
<td>Abnormal</td>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>31.4</td>
<td>1.4</td>
<td>8</td>
<td>1.6</td>
<td>8.8</td>
</tr>
</tbody>
</table>

a. SD = standard deviation
b. df = degrees of freedom

Between-Instrument Reproducibility

Table 3 shows between-instrument reproducibility results.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Instrument</th>
<th>Mean</th>
<th>SD</th>
<th>CVa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>1</td>
<td>28.9</td>
<td>1.04</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>27.6</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

a. CV = coefficient of variation

Between-Laboratory Reproducibility

Between-laboratory reproducibility is indicated by the ability to pool the normal reference ranges for BD Simultest CD3/CD8 reagent parameters (Table 1).

BD Simultest CD3/CD8 versus Comparative Method

A summary of the results is presented in Table 4.

Table 2 Within-sample reproducibility for BD Simultest CD3/CD8 reagent parameters (six normal subjects and four abnormal subjects) as percentages of lymphocytes (converted)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Parameter</th>
<th>Mean</th>
<th>SDa Within-Stain</th>
<th>dfb</th>
<th>SDb Between-Stain</th>
<th>dfb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>27.2</td>
<td>0.8</td>
<td>12</td>
<td>1.1</td>
<td>11.1</td>
</tr>
<tr>
<td>Abnormal</td>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>31.4</td>
<td>1.4</td>
<td>8</td>
<td>1.6</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Table 3 Between-instrument reproducibility for BD Simultest CD3/CD8 reagent parameter (five normal subjects and two instruments) as percentages of lymphocytes (converted)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Instrument</th>
<th>Mean</th>
<th>SD</th>
<th>CVa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>1</td>
<td>28.9</td>
<td>1.04</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>27.6</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 4 BD Simultest CD3/CD8 reagent vs comparative methoda (BD Simultest™ IMK-Lymphocyte reagent panel and BD Simultest™ IMK Plus reagent panel)b

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Slope</th>
<th>Intercept</th>
<th>r</th>
<th>n</th>
<th>IMK Plus</th>
<th>IMK- Lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressor/cytotoxic T lymphocytes</td>
<td>1.01</td>
<td>–6.03</td>
<td>0.93</td>
<td>84</td>
<td>19–74</td>
<td>12–67</td>
</tr>
</tbody>
</table>

a. This data was collected on a BD FACScan flow cytometer using BD Simultest IMK-Lymphocyte reagents, which included Leucogate, Control γ1/γ2a, and CD3/CD8. Software employed was BD Simultest™ IMK, lymphocyte software, one of the BD Simulset family of software.

b. The two methods differ in that the BD Simultest IMK Plus kit uses CD4/CD8 reagent to identify helper/inducer and suppressor/cytotoxic lymphocytes (including NK lymphocytes), whereas the BD Simultest IMK-Lymphocyte kit uses CD3/CD4 and CD3/CD8 reagents to distinguish true helper/inducer and true suppressor/cytotoxic T lymphocyte subsets.
Stability of Stained Cell Preparations

We recommend analyzing samples within 24 hours of staining.

Cross-Reactivity

The CD8 (Leu-2a) antibody reacts with NK lymphocytes as well as with suppressor/cytotoxic T lymphocytes.

Linearity-Recovery

Results are expected to be linear from $3.5 \times 10^3$ to $9.4 \times 10^3$ WBC/µL.

WARRANTY

Unless otherwise indicated in any applicable BD general conditions of sale for non-US customers, the following warranty applies to the purchase of these products.

The Products sold hereunder are warranted only to conform to the quantity and contents stated on the label or in the product labeling at the time of delivery to the customer. BD DISCLAIMS HEREBY ALL OTHER WARRANTIES, EXPRESSED OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY AND FITNESS FOR ANY PARTICULAR PURPOSE AND NONINFRINGEMENT. BD'S SOLE LIABILITY IS LIMITED TO EITHER REPLACEMENT OF THE PRODUCTS OR REFUND OF THE PURCHASE PRICE. BD IS NOT LIABLE FOR PROPERTY DAMAGE OR ANY INCIDENTAL OR CONSEQUENTIAL DAMAGES, INCLUDING PERSONAL INJURY, OR ECONOMIC LOSS, CAUSED BY THE PRODUCT.

REFERENCES


