INTENDED USE
Small Sample Tube (SST) is a kit with 11 conjugated antibodies designed for the detection of normal and aberrant lymphocyte populations of B, T and NK lineage by flow cytometry. This 8-color panel can be used for evaluation of “small” samples and samples with (very) low cell counts, such as fine needle aspirates (FNA), cerebrospinal fluid (CSF), vitreous humor, etc. with a clinical suspicion of primary lymphoma. SST is designed as part of the EuroFlow SST tube.  

SUMMARY AND EXPLANATION
Flow Cytometry is a powerful tool for the analytical and quantitative characterization of cells which provides rapid, quantitative and multiparametric analysis of heterogeneous cell populations on a cell-by-cell basis. Flow cytometry is performed on cells in liquid suspension that have been incubated with fluorescence-labeled antibodies directed against specific cellular proteins. The relative fluorescence intensity of the positive cells indicates the amount of antibody bound to specific binding sites on the cells, and therefore provides a relative measure of antigen expression.

SST kit recognizes by flow cytometry the antigens CD20, CD45, CD8, CD4, CD19, CD3,CD14, CD38, kappa light chains and lambda light chains present in the different lymphocyte subsets and plasma cells, and can therefore be used in the characterization studies for immunophenotyping. In small samples and samples with low cell counts, these studies are applied in the characterization and follow-up of primary lymphoma.  

The use of 8 color panels in flow cytometry involves the use of new fluorochromes here described:

- **Pacific Blue™** and Orange Cytognos 515 are fluorochromes excited with the violet laser (405nm). Pacific Blue™ emits at 455nm and Orange Cytognos 515 emits at 515nm. These fluorochromes provide maximum resolution and narrow emission peaks, which results in little spectral overlap and minimal compensation requirements.
- **APC-C750** is a tandem dye with a maximum emission peak at 779 nm, which grants bright signal, low unspecific noise and high photostability. When excited by light from a red laser, the APC fluorochrome can transfer energy to C750 molecule, which then emits at a longer wavelength. It is recommended to use a 780/60 nm bandpass filter along with a red sensitive detector to use in conjunction antibodies conjugated with APC and APC-C750.

PRINCIPLES OF THE PROCEDURE
Flow cytometry (FC) is an innovative technology by means of which different cell characteristics are simultaneously analyzed on a single cell basis. This is achieved by means of hydrodynamic focusing of cells that pass aligned one by one in front of a set of light detectors; at the same time they are illuminated by a laser beam. The interaction of the cells with the laser beam generates signals of two different kinds: those generated by dispersed light (FSC/SSC), which mainly reflects the size of the cell and its internal complexity, and those related to the emission of light by the fluorochromes present in the cell. These signals become electric impulses which are amplified and registered as digital signals to be processed by a computer.

When the reagents are added to the sample, the mixture of fluorochrome-labeled antibodies present in the reagents bind specifically to the antigens they are directed against, allowing the detection by FC of the different lymphoid subsets. The erythrocyte population, which could hinder the detection of the target population, is eliminated by the use of a red blood cell lysing solution previous to acquire the sample on the cytometer. The different lymphocyte subsets count is generally expressed as the number of positive cells per microliter of sample (absolute counts), or as the percentage of positive cells per lymphocytes or leukocytes present in the sample.

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REAGENT COMPOSITION
CTY-SST contains sufficient volume for 25 tests. It includes the following reagents:

1. 5 Lyophilized vials with the following pre-mixed cocktail of 11 conjugated antibodies. Each lyophilized vial contains sufficient amount for 5 tests.

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Pacific Blue™</th>
<th>OC515</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP- Cyanine5.5</th>
<th>PE- Cyanine7</th>
<th>APC</th>
<th>APC-C750</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker</td>
<td>CD20</td>
<td>CD45</td>
<td>CD8</td>
<td>Smlgκ</td>
<td>CD56</td>
<td>Smlgκ</td>
<td>CD4</td>
<td>CD19</td>
</tr>
<tr>
<td>Clone</td>
<td>2H7</td>
<td>GA90</td>
<td>UCHT-4</td>
<td>Polyclonal</td>
<td>CS-9</td>
<td>Polyclonal</td>
<td>SK3</td>
<td>J3-119</td>
</tr>
<tr>
<td>Isotype</td>
<td>IgG2b</td>
<td>IgG2a</td>
<td>IgG2a</td>
<td>Lambda light chain</td>
<td>IgG2b</td>
<td>IgG1</td>
<td>IgG1</td>
<td>IgG1</td>
</tr>
<tr>
<td>Reactivity</td>
<td>B cells</td>
<td>Leukocytes</td>
<td>Cytotoxic T cells</td>
<td>Lambda light chain</td>
<td>NK cells</td>
<td>Kappa light chain</td>
<td>Helper T cells</td>
<td>B cells</td>
</tr>
</tbody>
</table>

2. Additionally a vial of CD45-OC515, of other of CD19-PE-Cyanine7 and another of CD38-APC-C750 are included for compensation purposes.

All components contain < 0.1% sodium azide (NaN₃). Reagents are not considered sterile.

STORAGE CONDITIONS
70-SST is stable until the expiration date shown on the label, when are stored at 2-8º C. The expiration date applies to the lyophilized product. Vials with the pre-mixed cocktail of 11 conjugated antibodies are stable one month from date of reconstitution. Components should not be frozen or exposed to direct light during storage or during incubation with cells. Keep vials dry. Once opened, vials must be stored in a vertical position to avoid any possible spillage.
RECONSTITUTION
Reconstitute each lyophilized vial containing the pre-mixed cocktail of 11 conjugated antibodies with 300 μL of distilled water. It will be necessary to use 50 μL of this solution per determination. Unused volume of reconstituted vial is stable during one month from date of reconstitution if it is stored at 2-8º C.

WARNINGS AND RECOMMENDATIONS
1. For research use only. Not for use in diagnostic procedures.
2. If components of this kit are altered by addition of other components, such conditions must be validated by the user.
3. The kit is stable until the expiration date shown on the label if it is properly stored. Do not use after the expiration date shown on the label. If the reagents are stored in conditions different from those recommended, such conditions must be validated by the user.
4. Alteration in the appearance of the reagents, such as the precipitation or discoloration indicates instability or deterioration. In such cases, the reagents should not be used.
5. It contains < 0.1% sodium azide (CAS-No. 26628-22-8) as a preservative, but even so care should be taken to avoid microbial contamination of reagent or incorrect results may occur.
   - Sodium azide (NaN₃) is harmful if swallowed (R22), if swallowed, seek medical advice immediately and show this container or label (S46).
   - Wear suitable protecting clothing (S36).
   - Contact with acids liberates very toxic gas (R32).
   - Azide compounds should be flushed with large volumes of water during disposal to avoid deposits in metal drains where explosive conditions may develop.
6. All subject specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection (12), and disposed according to the legal precautions established for this type of product. Also recommended is handling of the product with appropriate protective gloves and clothing, and its use by personnel sufficiently qualified for the procedures described. Avoid contact of samples with skin and mucous membranes. After contact with skin, wash immediately with plenty of water.
7. Use of the reagents with incubation times or temperatures different from those recommended may cause erroneous results. Any such changes must be validated by the user.

PROCEDURE
Material included
70-SST is sufficient for 25 determinations. It includes the following reagents:
   - 5 Lyophilized vials with a pre-mixed cocktail of 11 conjugated antibodies.
   - Additionally a vial of CD45-OC515, other of CD19-PE-Cyanine7 and other of CD38-APC-C750 are included for compensation purposes. Compensation requirements for OC515 and APC-C750 are similar to Pacific Orange™ and APC-H7 respectively.

Material required but not included
   - Flow cytometer equipped with 405 nm violet laser, 488 nm ion argon laser, 633 red laser, 780/60 nm bandpass filter, and appropriate computer hardware and software associated.
   - Test tubes suitable for obtaining samples in the flow cytometer used. Usually tubes with a rounded bottom for 6 mL, 12x75 mm are used.
   - Automatic pipette (100 μL) and tips.
   - Micropipette with tips.
   - Vortex Mixer.
   - Chronometer.
   - Centrifuge.
   - Pasteur pipette or vacuum system.
   - Distilled water.
   - Isotypic control reagent.
   - Erythrocyte lysing solution.
   - Wash buffer as phosphate buffered saline (PBS) + 0.09% of NaN₃ + 0.5% of Bovine Serum Albumin (BSA).

Preparation
Small sample must be taken aseptically by means of lumbar puncture (10) in case of CSF sample, vitrectomy or fine needle aspirate in a sterilized tube. Store samples at 4-8ºC and processed within 1 hour after their extraction; otherwise they should be stabilized to avoid deterioration of cells.

1. The SST panel includes surface membrane (Sm) immunoglobulins (Ig) staining and immunoglobulins (Ig) staining, therefore samples must be washed twice to remove the soluble serum proteins (steps 1a-1j). Be careful with volumes after discarding supernatants.
   a. Spin down total volume of the small sample (i.e. CSF, vitreous aspirates) during 5 min at 540 g. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet.
   b. Add 2-5 mL of filtered PBS + 0.09% of NaN₃ + 0.5% of BSA. Mix well.
   c. Centrifuge for 5 min at 540 g.
   e. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet.
   f. Add 2-5 mL of filtered PBS + 0.09% of NaN₃ + 0.5% of BSA. Mix well.
   g. Centrifuge for 5 min at 540 g.
   i. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet.
   j. Resuspend cell pellet in 150 μL of PBS + 0.09% of NaN₃ + 0.5% of BSA.

2. Pipette 50 μL of this sample in a new tube and add 50 μL of the pre-mixed cocktail of 11 conjugated antibodies from a reconstituted vial.
3. Mix well.
4. Incubate for 15 min at room temperature (RT) protected from light.
5. Add 2 mL of an erythrocyte lysing solution containing fixatives.
6. Mix well.
7. Incubate for 10 min at room temperature protected from light.
8. Centrifuge for 5 min at 540g.
9. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 50 µL residual volume in each tube.
10. Wash by adding 2 mL of PBS + 0.09% of NaN₃ + 0.5% of BSA to the cell pellet.
11. Mix well.
12. Centrifuge for 5 min at 540g.
13. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet, leaving approximately 50 µL residual volume in each tube.
14. Resuspend the cell pellet in 200 µL of PBS + 0.5% of BSA (without NaN₃).
15. Acquire the cells after staining or (if not immediately acquired) store at 4ºC for maximally 1 hour until measured in the flow cytometer.

This is the detailed EuroFlow Standard Operating Procedure for sample preparation and staining. Other staining protocols must be validated for the use of this reagent.

Important recommendations:
It is recommended follow the Calibration EuroFlow Standard Operating Protocol for Cytometer Setup (11). You will find a complete guide (Cytometer Setup SOP) on the web site www.euroflow.org, which includes recommendations for fix instrument configuration, FSC and SSC setting, target channel PMT setting, compensation setting and instrument performance monitoring. An appropriated compensation setting is required for the acquisition of this tube. Most fluorochromes emit also in surrounding inappropriate channels but this spillover can be mathematically corrected. Single stained tubes are used for compensation settings. For this purpose a sample of CD45-OC515 (positive target population: lymphocytes), other of CD19-PE-Cyanine7 (positive target population: B-cells) and another of CD38-APC-C750 (positive target population: CD38 hi lymphocytes population) are included in the kit. Use 5µl of each of these reagents in order to prepare the single stained tubes.

Flow cytometry analysis
Analysis of the SST files could become complicated with a manual definition of gates and regions, because different cell populations are present in the same fluorescence. Cytognos recommends the use of the analysis software Infinicyt™, which is capable to use pattern recognition and store analysis strategies to apply in batch to other samples using always the same criteria. You will find complete information about Infinicyt™ on the web site: www.infinicyt.com.

To analyze the results of a SST tube we recommend follow these indications:
1. Exclude debris and non-leukocyte events from analysis by gating on forward light scatter (FSC-A and FSC-H), side light scatter (SSC) and preferably CD45. Plasma cells may have CD45 negative/dim expression; therefore it is recommended to include also CD45 low positive cells within the leukocyte gate.
2. Select cells of interest using a lineage-specific marker and side scatter (SSC) and preferably a dual-anchor gating strategy using CD45 versus SSC. Analyze un-gated data as well.
3. Use normal lymphocytes, monocytes, and neutrophils within the sample as an internal control for negative or positive antigen expression.
   a) Analyze B cell data for abnormal patterns of antigen expression and/or light scatter characteristics (large-cell lymphoma) using CD19 and CD20 (in some occasions CD19 is weak or negative so use of CD20 is advisable). Analyze light-chain expressions within the abnormal population in combination with CD19 and CD20. Populations are classified as monoclonal (or showing clonal excess) when a surface immunoglobulin (smIg) Kappa/Lambda ratio below 0.25 or above 4 is observed, provided that enough (n > 100) smIg+ B cells have been measured. Some cases, i.e., diffuse large B cell lymphoma, may be light-chain negative.
   b) Analyze T cell data looking for abnormal patterns of antigen expression as well as light-scatter characteristics.
4. Sensitivity and minimum number of events to define leukocyte infiltration in the sample vary depending on the number of events simultaneously assessed.
RESULTS
The different lymphocyte subset counts can be expressed as the percentage of positive cells per lymphocytes or leukocytes present in the sample.

LIMITATIONS
- Small samples should be stored at 4-8°C and processed within 1 hour after their extraction; otherwise they should be stabilized to avoid deterioration of cells.
- It is advisable to acquire stained samples on the cytometer as soon as possible to optimize the results. Non viable cells may stain nonspecifically. Prolonged exposure of whole blood samples to lytic reagents may cause white cell destruction and loss of cells from the target population.
- When using whole blood procedures, all red blood cells may not lyse under following conditions: nucleated red blood cells, abnormal protein concentration or hemoglobinopathies. This may cause falsely decreased results due to unlysed red blood cells being counted as leukocytes.
- Results obtained by flow cytometry may be erroneous if the cytometer laser is misaligned or the gates are improperly set.
- Each laboratory should establish a normal range for lymphocyte subsets and plasma cells using its own test conditions. We recommend follow the EuroFlow antibody panels 1-10 together with the EuroFlow instrument set-up, sample preparation and data analysis procedures (11).
- It is important to understand the normal pattern of expression of these antigens and its relation to the expression of other relevant antigens to carry out an adequate analysis 11-30.

QUALITY CONTROL
- To obtain optimum results it is advisable to verify the precision of pipettes and that the cytometer is correctly calibrated.
- It is recommended follow the Calibration EuroFlow Standard Operating Protocol for Cytometer Setup. You will find a complete guide (Cytometer Setup SOP) on the web site www.euroflow.org, which includes recommendations for fix instrument configuration, FSC and SSC setting, target channel PMT setting, compensation setting and instrument performance monitoring.
- To evaluate the non-specific binding of the reagent, an appropriated isotype control tube can be prepared.

REFERENCES

WARRANTY
This product is warranted only to conform to the quantity and contents stated on the label. There are no warranties that extend beyond the description on the label of the product. Cytognos' sole liability is limited to either replacement of the product or refund of the purchase price.