LYMPHOCLONAL-C™

Ref: CYT-LC-C

For research use only

INTENDED USE

LYMPHOCLONAL-C™ is a four-color direct immunofluorescence reagent for use in flow cytometry designed to simultaneously determine in peripheral blood, bone marrow and other body fluids the major lymphocyte subpopulations, including the total number of T lymphocytes (CD3+), B lymphocytes (CD19+) and natural killer cells (CD3-CD56+) as well as helper/inducer (CD3+CD4+) and suppressor/cytotoxic (CD3+CD8+) T lymphocyte subsets, B lymphocytes with immunoglobulins bearing kappa light chains (CD19+ Igκ+), and B lymphocytes with immunoglobulins bearing lambda light chains (CD19+ Igλ+).

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 fluorescently-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.
Human lymphocytes may be classified in three main populations according to their biological function and their cell surface antigen expression: T lymphocytes, B lymphocytes and natural killer (NK) cells. T lymphocytes (CD3+), the precursors of which originate in the bone marrow and then migrate and mature in the thymus, can be subdivided as well in functionally different populations. The most clearly defined of these are helper/inducer T cells (CD3+CD4+) and suppressor/cytotoxic T cells (CD3+CD8+). T cells produce no antibodies and are the mediators of cell immunity. B lymphocytes (CD19+) are the producers of antibodies, they mediate humoral immunity particularly effective against toxins, whole bacteria, and free viruses. NK cells (CD3-CD56+) mediate cytotoxicity against certain tumors and virus-infected cells. NK-mediated cytotoxicity does not require class I or class II major histocompatibility complex (MHC) molecules to be present on the target cell.
LYMPHOCLONAL-C™ recognizes the antigens CD3, CD19, CD56, CD4, CD8, kappa light chains and lambda light chains present in the different lymphocyte subsets, and can therefore be used in the characterization studies for immunophenotyping of lymphocytes. These studies are widely applied for monitoring of the immunologic status of post-transplant patients and in the characterization and follow-up of immunodeficiencies, autoimmune diseases, leukemia etc [1-3].

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 reagent is added to the sample, the mixture of fluorochrome-labelled antibodies present in the reagent 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 use of Quicklysis™ (CYT-QL-1) erythrocyte lysing solution is recommended, since it requires no further washing step and contains no fixative, therefore minimizing the handling of the sample and avoiding the cell loss associated to the centrifuge process.[4, 5].
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 leucocytes present in the sample which can itself be determined by FC based on its typical pattern of FSC/SSC (size/granularity or complexity). Because each flow cytometer has different operating characteristics each laboratory must determine its optimal operating procedure.

REAGENT COMPOSITION

LYMPHOCLONAL-C™ is provided in phosphate buffered saline with 0.1% sodium azide. It contains:

- Purified monoclonal CD8 antibody conjugated with fluorescein isothiocyanate (FITC), clone: UCH-T4 , isotype: IgG2a
- Purified polyclonal antibody anti-lambda light chains conjugated with fluorescein isothiocyanate (FITC).
- Purified monoclonal CD56 antibody conjugated with R-phycoerythrin (PE), clone: C5.9, isotype: IgG2b
- Purified polyclonal antibody anti-kappa light chains conjugated with R-phycoerythrin (PE).
- Purified monoclonal CD19 antibody conjugated with the R-phycoerytin protein-Cyanine 5 tandem (PE-Cy5), clone: J4.119, isotype: IgG1
- Purified monoclonal CD4 antibody conjugated with the R-phycoerytin protein-Cyanine 5 tandem (PE-Cy5), clone: 13B8.2, isotype: IgG1
- Purified monoclonal CD3 antibody conjugated with R-phycoerytin protein-Cyanine 7 (PE-Cy7) clone: UCHT1, isotype: IgG1

Purification: Affinity chromatography
Amount per 0.5 ml vial: 20 tests (25 µl LYMPHOCLONAL™ to 10^6 cells)
Reagents are not considered sterile.

STORAGE CONDITIONS

The reagent is stable until the expiration date shown on the label, when stored at 2-8º C. The reagent should not be frozen or exposed to direct light during storage or during incubation with cells. Keep the reagent vial dry. Once opened, the vial must be stored in a vertical position to avoid any possible spillage.

WARNINGS AND RECOMMENDATIONS

1. For in vitro diagnostic use.
2. This product is supplied ready to use. If it is altered by dilution or addition of other components, it will be invalidated for in vitro diagnostic use.
3. The reagent 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 reagent, such as the precipitation or discoloration indicates instability or deterioration. In such cases, the reagent should not be used.
5. It contains 0.1% sodium azide (CAS-Nr. 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 (NaN_3) 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 patient specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection [6], 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 reagent 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

LYMPHOCLONAL-C™ is sufficient for 20 determinations (25 µl reagent to 10^6 cells).

Material required but not included

- 488 nm ion argon laser-equipped flow cytometer and appropriate computer hardware and software.
- Test tubes suitable for obtaining samples in the flow cytometer used. Usually tubes with a rounded bottom for 6 mL, 12x 75 mm are used.
- Automatic pipette (100µL) and tips.
- Micropipette with tips.
- Chronometer
- Vortex Mixer
- Isotypic control reagent
- Quicklysis™ lysing solution
- Wash buffer as phosphate buffered saline (PBS) pH 7.4 containing 0,1% sodium azide.
- Perfect-Count Microspheres™ (CYT-PCM-50) necessary for determining absolute counts.

Preparation

The protocol to process samples with LYMPHOCLONAL-C™ reagent depends on the procedure of the sample:

- Whole blood sample must be taken aseptically by means of a venipuncture[7, 8] in a sterilized tube for blood collection containing an appropriate anticoagulant (use of EDTA is recommended). The analysis requires one hundred (100) µl of the whole blood sample per tube, assuming a normal range of approximately 4 to 10 x 10^3 leucocytes per µl. For samples with a high white blood cell count, dilute samples with PBS to obtain a concentration of cells approximately equal to 1x10^4 cells/µL. Store the blood samples at 18-22ºC until they are to be tested. It is advisable to test blood samples within the 24 hours after their extraction. Hemolyzed samples or samples with suspended cell aggregates should be rejected.
- Bone marrow sample should be pass 3 or 4 times through a syringe in order to disaggregate cell clumps. Perform a white blood cell count of the sample and dilute samples with PBS to obtain a concentration of cells approximately equal to 1 x 10^4 cells/µl.
- Dilution is not required for other body fluid samples such as fine needle aspiration (FNA) or cephalorraquid fluid, which contain low lymphocyte counts.

Before staining samples of peripheral blood and bone marrow, the sample must be washed to remove the soluble serum proteins (step 1). In case of lymph node aspirates, fine needle aspiration or cephalorraquid fluid samples this previous step is not needed.

1. Add 100 µL of the peripheral blood or bone marrow sample to each tube, add 3 ml of PBS pH 7.4 and centrifuge at 300 x g for 5 minutes, then aspirate the supernatant. Wash again using 3 ml of PBS pH 7.4 and centrifuge at 300 x g for 5 minutes, aspirate the supernatant, leaving approximately 100-150 µl of fluid.
2. Mix 100µl of blood or bone marrow sample with 25µl of LYMPHOCLONAL™. In case of working with other body fluids with fewer cells, such as fine needle aspirations, cephalorraquid fluid, bronchoalveolar lavage, gastric lavage, etc, mix 200µl of sample with 25µl of LYMPHOCLONAL™.
   To evaluate the non-specific binding of the antibody, an appropriated isotype control tube can be prepared.
3. Incubate for 10 minutes at room temperature in the dark.
4. Add 2 ml of Quicklysis™* erythrocyte lysing solution and incubate the sample for 10 minutes at room temperature in the dark.
5. Acquire directly on the flow cytometer within the first four hours after finishing the sample preparation. If the samples are not acquired immediately after preparation, they should be stored at 2-8ºC in the dark. Calibration of the instrument must be done according to the manufacturer's advice. Before acquiring samples, adjust the threshold or discriminator to minimize debris and ensure populations of interest are included. Before acquiring the sample on the flow cytometer, mix the cells on the vortex at low speed to reduce aggregation.

*Note: The use of other lysing solutions may require the elimination of the lysed red blood cells. Follow the manufacturer's recommended protocol of the lysing solution used.

If using Perfect-Count Microspheres™ for calculating absolute counts, mix the Perfect-Count Microspheres™ vial manually for 30-40 seconds (do not use Vortex) immediately prior to add to the sample. Using the reverse pipetting technique, add to each tube the same volume of Perfect-Count Microspheres™ as the one used for the sample addition (100 µl in case of blood or bone marrow sample and 200 µl in case of other body fluids sample).

Flow cytometry analysis

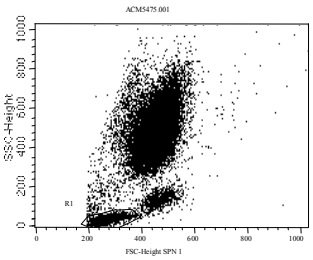
Enable the Red Helium-Neon 633 laser. Check that the cytometer is correctly aligned and standardized for light dispersion (FSC/SSC on linear scale) and fluorescent intensity and that the right color compensation has been set following the instructions of the cytometer manufacturer. Select each fluorochrome FITC, PE, PECy5, PECy7 on logarithmic scale on the corresponding detector and unactivate the ECD detector.

Analysis of the LYMPHOCLONAL-C™ files becomes 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 software INFINICYT. Contact CYTOGNOS or its authorized distributor about the advantages and ways to get this analysis software.

To perform a manual analysis of the results we recommend follow the template shown in the following figures. Data show representative flow cytometry data on peripheral blood (healthy individual) stained with LYMPHOCLONAL-C™.

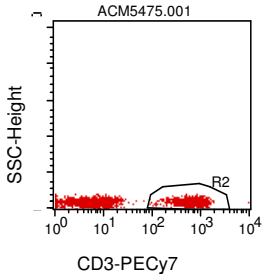
1. Dot plot nº 1: FSC versus SSC, ungated. Drawn a region to select the Lymphoid Area according to its typical low light scatter properties. This region defines all lymphocytes and some contaminants.

1. Gate: UNGATED

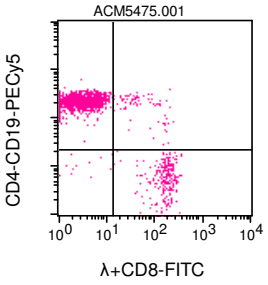


2. Dot plot nº 2: FL5 (CD3-PECy7) versus SSC, gated on region R1. Drawn a region to include all the bright CD3+ T lymphocytes, which should appear as a compact CD3+ cluster with low SSC.
3. Dot plot nº 3: FL1 (CD8-FITC + sIgλ-FITC) versus FL3 (CD4-PE-Cy5 + CD19-PE-Cy5), gated on T lymphocytes defined as combination of lymphoid area and CD3+ in region R2. Set quadrants to distinguish between the T lymphocyte subsets: CD3+CD4+CD8- (upper left: UL), CD3+CD4+CD8+ (upper right: UR), CD3+CD4-CD8- (lower left: LL), CD3+CD4-CD8+ (lower right: LR).

2. Gate: Lymphoid Area

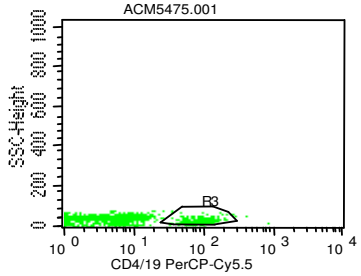


3.Gate: T-lymphs CD3+

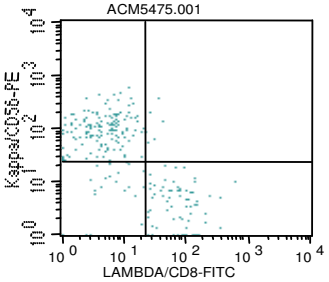


4. Dot plot nº 4: FL3 (CD4-PE-Cy5 + CD19-PE-Cy5) versus SSC, show the lymphocyte subset which are not CD3+T lymphocytes defined as events included on R1 but not on R2. Drawn a region (R3) to include the CD19+ B lymphocytes, which should appear as a compact CD19+ cluster with low SSC.
5. Dot plot nº 5: FL1 (CD8-FITC + sIgλ-FITC) versus FL2 (CD56-PE + sIgκ-PE), gated on B lymphocytes defined as events on lymphoid area which are not CD3+ and they are CD19+ (R3). Set quadrants to distinguish between the B lymphocyte subsets: CD19+sIgκ+ (upper left: UL), CD19+sIgλ+ (lower right: LR).

4. Gate=lymph area AND NOT T-cells

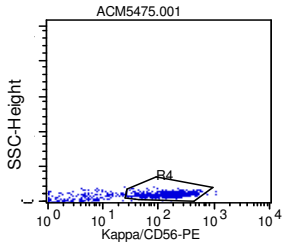


5. Gate : B - Lymphocytes

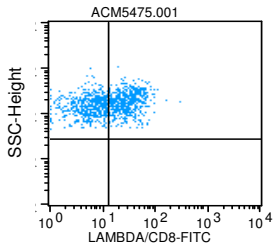


6. Dot plot nº 6: FL2 (CD56-PE + sIgκ-PE) versus SSC, gated on G5 defined as events on lymphoid area which are not T lymphocytes (CD3+) and not B cells (CD19+). Drawn a region (R4) to include the NK cells, which should appear as a CD56+ cluster with low SSC.
7. Dot plot nº 7: FL1 (CD8-FITC + sIgλ-FITC) versus FL2 (CD56-PE + sIgκ-PE), gated on NK cells defined as events on lymphoid area which are not T cells (CD3+) and not B cells (CD19+) and they are CD56+ on R4. Set quadrants to distinguish between the NK cell subsets: CD56+CD8- (upper left: UL), CD56+CD8+ (upper right: LR).

6. Gate: lymphoid area AND NOT T-cells AND NOT B-cells



7. Gate: NK Cells



RESULTS

The different lymphocyte subset counts can be expressed as the percentage of positive cells per lymphocytes or leucocytes present in the sample. Estimation of total lymphocyte percentage must be calculated as the sum of the T-lymphocytes percentage + the B lymphocytes percentage + the NK cells percentage to avoid the contaminants included in the lymphoid area.
The different lymphocyte subsets count can be also expressed as the number of positive cells per microliter of sample (absolute counts). Absolute counts may be determined by two methods:

- The double platform method to calculate absolute counts combines results from haematology analyzer and flow cytometry and uses the following formula:
  Absolute Counts (cells/µl) =Total White Blood Cell Count (cells/ µl obtained from haematology analyzer) x % lymphocytes x % positively-stained cells ÷ 10^4
- The single platform method has emerged as the method of the choice for absolute cell enumeration because comparative laboratory and external quality assessment studies have demonstrated that this methodology offers a lower intra- and inter-laboratory variation [9, 10]. Microbeads-based technology consists of known amounts of fluorescent microbeads are admixed to a known volume of stained sample in a lyse-no-wash technique and the beads are counted along with cells. CYTOGNOS recommends the use of Perfect-Count Microspheres™ (CYT-PCM-50) to determine absolute counts using the following formula:

$$\text{Absolute Count (Cells/µl)} = \frac{\text{Total number of cells of interest counted}}{\text{Total number of Perfect-Count Microspheres}^{TM}\text{ counted}} \times \text{Nº of Perfect–Count Microspheres /µl (value specified by manufacter)}$$

LIMITATIONS

- Blood samples should be stored at 18-22ºC and be tested within the 24 hours after they are obtained.
- It is advisable to acquire stained samples on the cytometer as soon as possible to optimize the results. Nonviable 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 leucocytes.
- 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 using its own test conditions. The data for the reagent's performance have been obtained from whole blood samples collected with EDTA as anticoagulant. The reagent's performance may be affected by the use of other anticoagulants.
- Certain patients may present special problems due to altered or very low number of certain cellular population.
- Cells separated from whole blood by means of density gradients may not have the same relative concentrations of cells as unseparated blood. This may be relatively insignificant for samples from individuals with normal white blood cell counts. In leucopenic patients, the selective loss of specific subsets may affect the accuracy of the determination.
- 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 [11-15]
- Abnormal states of health are not always represented by abnormal percentages of certain leucocyte populations. An individual who may be in an abnormal state of health may show the same leucocyte percentages as a healthy person. For this reason, it is advisable to use the test results in combination with other clinical and diagnosis data.

QUALITY CONTROL

- To obtain optimum results it is advisable to verify the precision of pipettes and that the cytometer is correctly calibrated.
- The fluorochromes fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), R-phycoetrytrin protein-cyanine 5 (PE-Cy5), and R-phycoerytrin protein-cyanine 7 (PECy7) emit in different wavelengths but show a certain spectral overlapping which must be corrected by means of electronic compensation if combinations of different monoclonal antibodies are used conjugated with these fluorochromes. The optimum levels of compensation can be established by analysis in a dot-plot diagram of cells from healthy individuals stained with mutually exclusive monoclonal antibodies conjugated with the fluorochromes to be used in the test.
- To evaluate the non-specific binding of the reagent, an appropriated isotype control tube can be prepared.

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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's sole liability is limited to either replacement of the product or refund of the purchase price.

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104481ENG **Last revision**: ORAN 26/07/2010