LYMPHOCLONAL 9

(CD8 + smIgλ -FITC / CD56 + smIgκ –PE / CD4 + CD19 –PerCP-Cyanine5.5 / CD3-PE-Cyanine7 / CD20 –APC / CD45-APC-C750)

Ref: CYT-LC9

For research use only

INTENDED USE

Lymphoclonal 9 is a 6-colour 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+CD20+) 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+). This 6-colour panel can be used for initial evaluation of several suspected clinical conditions, such as lymphocytosis, lymph node enlargement, unexplained cytopenias, etc.

SUMMARY AND EXPLANATION

Detection of phenotypically aberrant and clonal mature lymphocytes is the diagnostic hallmark of chronic lymphoproliferative disorders (CLPD). Clonogenic events lead to the expansion and accumulation of mature-appearing lymphocytes, which carry a proliferative and/or survival advantage over their normal counterparts.
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-labelled 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.
Lymphoclonal 9 recognizes by flow cytometry the antigens CD45, CD3, CD56, CD4, CD8, CD20, CD19, 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 in the characterization and follow-up of different hematological malignancies [1-5].
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 longpass 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 analysed 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 to be processed by a computer.
When the reagents are added to the sample, the mixture of fluorochrome-labelled 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 leucocytes present in the sample.

REAGENT COMPOSITION

CYT-LC9 contains sufficient volume for 25 tests (50µL reagent to 10^6 cells). It includes the following reagents:

1. 5 lyophilized vials with the following pre-mixed cocktail of 9 conjugated antibodies. Each lyophilized vial contains sufficient amount for 5 tests.

Fluorochr.	FITC		PE		PerCP-Cyanine5.5		PE-Cyanine7	APC	APC-C750
Marker	**CD8**	**smIgλ**	**CD56**	**smIgκ**	**CD4**	**CD19**	**CD3**	**CD20**	**CD45**
Clone	UCHT-4	Polyclonal	C5.9	Polyclonal	SK3	HIB19	UCHT-1	2H7	HI30
Isotype	IgG2a		IgG2b		IgG1	IgG1	IgG1	IgG2b	IgG1
Reactivity	Suppressor / Cytotoxic T cells	Lambda Ig light chain	NK cells	Kappa Ig light chain	Helper / inducer T cells	B cells	T cells	B cells	Leucocytes

2. Additionally vials of anti human CD45-APC-C750 and CD3-PE-Cyanine7 antibody are included for compensation purposes.

All components contain 0.1% sodium azide (NaN_3). Reagents are not considered sterile.

STORAGE CONDITIONS

Lymphoclonal 9 kit is stable until the expiration date shown on the label, when is stored at 2-8º C. The expiration date applies to the lyophilized product. Vials with the pre-mixed cocktail of 9 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 9 conjugated antibodies with 300µL of distilled water. It will be necessary to use 50µL 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.
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 ≤0.09% (m/v) 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.

   Indication(s) of danger:

H302	Harmful if swallowed

   Safety advice:

P264	Wash thoroughly after handling.
P270	Do not eat, drink or smoke when using this product.
P301+P312	If swallowed, call a poison center or doctor/physician if you feel unwell.
P301+P330	If swallowed, rinse mouth.
P501	Dispose of contents/container in accordance with local/regional/national/international regulation.

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 [7], 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

CYT-LC9 is sufficient for 25 determinations (50µL reagent to 10^6 cells). It includes the following reagents:

- 5 lyophilized vials with a pre-mixed cocktail of 9 conjugated antibodies.
- Additionally a vial of CD45-APC-C750 and CD3-PE-Cyanine7 are included for compensation purposes. Compensation requirements for APC-C750 are similar to APC-H7.

Material required but not included

- Flow cytometer equipped with 488 nm ion argon laser, 633 red laser, 780/60 nm longpass 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.
- 10 mL tubes to perform a bulk wash procedure.
- Automatic pipette (100µL) 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 NaN_3 + 0.5% of Bovine Serum Albumin (BSA).

Preparation

Whole blood sample must be taken aseptically by means of a venipuncture [8, 9] in a sterilized tube for blood collection containing an appropriate anticoagulant (use of EDTA is recommended). 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.

1. **The LC-9 panel includes surface membrane (Sm) immunoglobulins (Ig) staining, therefore samples to be studied must be washed twice to remove the soluble serum proteins (steps 1a-1i). Be careful with volumes after discarding supernatants.**
   a. Pipette 100µL of sample into a test tube. For small samples (i.e. CSF, vitreous aspirates) spin down the total volume (5 min at 540g), discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet.
   b. Add 2 mL of filtered PBS + 0.09% of NaN_3 + 0.5% of BSA.
   c. Mix well.
   d. Centrifuge for 5 min at 540g.
   e. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet.
   f. Add 2 mL PBS + 0.09% of NaN_3 + 0.5% of BSA to the cell pellet.
   g. Mix well.
   h. Centrifuge for 5 min at 540g.
   i. Discard the supernatant using a Pasteur pipette or vacuum system without disturbing the cell pellet.

2. Use 100µL of this sample in a new tube and add 50µL of this reagent.
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 50µL residual volume in each tube.
10. Wash by adding 2 mL of PBS + 0.09% of NaN_3 + 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 50µL residual volume in each tube.
14. Resuspend the cell pellet in 200µL of PBS (without NaN_3)
15. Acquire directly on the flow cytometer within the first hour after finishing the sample preparation. If the samples are not acquired immediately after preparation, they should be stored in the dark at 4-8 ºC for maximum 1 hour.

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 and for this purpose a sample of CD45-APC-C750 and CD3-PE-Cyanine7 are included in the kit.

Flow cytometry analysis

Analysis of Lymphoclonal 9 FCS 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 Lymphoclonal 9 tube we recommend follow these indications:

1. Select leucocytes population based on their CD45 positive expression. Show only these cells on the screen and continue with the analysis.
2. Select T cells (CD3+) as the first population to identify. This mixture of antibodies include CD3-PE-Cyanine7 alone in this fluorochrome, therefore a selection of T cells is clear to gate.
3. Gating T cell population, select and classify T cell subsets using the other T cell markers included in the mixture of antibodies (CD8-FITC and CD4-PerCP-Cyanine5.5).
4. Once T cells are classified, it is recommended not show these cells on the screen and continue with the analysis of B cells.
5. B cell population can be then clearly identified based on their CD20 and CD19 positive expression since other markers in this fluorochrome are not show if T cells are not visible in the two dimensional DotPlot (2DDotPlot).
6. Gating B cell population, select and classify B cell subsets using the other B cell markers included in the mixture of antibodies (smIgλ-FITC and smIgκ-PE).
7. Once T and B cells are classified, it is recommended not show these cells on the screen and continue with the analysis of NK cells.
8. NK cell population can be then clearly identified based on their CD56 positive expression since smIgκ-PE positive cells are not show if B cells are not visible in the 2D DotPlot.

RESULTS

The different lymphocyte subset counts can be expressed as the percentage of positive cells per lymphocytes or leucocytes present in the sample.

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. 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 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.
- 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 [1-5, 10, 11]
- 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.

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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.

EXPLANATION OF SYMBOLS

Symbol	Meaning
	Use by (YYYY-MM)
2°C / 8°C	Storage temperature limitation (ºC)
	Consult instructions for use
RUO	*For Research Use Only*
LOT	Batch code
REF	Catalogue number
	Keep away from (sun)light
Xn	Caution: Sodium azide included
	Manufacter

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