CYCLOSCOPE B-NHL
REF: CYT-CS-NHL   20 Test

CYCLOSCOPE B-NHL, is a kit used for the flow cytometric analysis of DNA cell contents in B-cell non-Hodgkin's Lymphoma (B-NHL) and B-lineage chronic lymphocytic leukemias (B-CLL). This kit is mainly focused for DNA studies of B-cells from bone marrow or peripheral blood samples of these patients.

INTRODUCTION

In recent years, DNA flow cytometry studies have extended from basic research to clinical laboratories. Thus, flow cytometry analysis of the distribution of the cell nuclei DNA contents is being widely used to estimate the cell cycle distribution and the existence of DNA aneuploidy of either normal and tumour cell populations. Cycloscope kits are based on the combination of DNA cell measurements together with the analysis of tumoral cell antigen expression. This double staining method allows the identification of the neoplastic cells present in the sample in order to performe a DNA analysis separately from that of normal hemopoietic cells, as it is recommended by different consensus reports on DNA analysis by flow cytometry in neoplastic hematopathology.

Previous studies have demonstrated that the percentage of tumor B-cells in S-phase have a clear role as an independent prognostic factor either in non-Hodgkin`s lymphoma or in chronic lymphocytic leukemia. On the other hand, it has been shown that DNA-ploidy correlates well with histopathologic grade in non-Hodgkin's lymphoma patients.

PROCEDURE

This kit has been optimized in order to identify tumoral B-cells in patients diagnosed of B-NHL and B-CLL with the purpose of studying the proliferative rate (proportion of cells in G_0/G_1, S and G_2/M phases of cell cycle) and the existence of DNA aneuploidy of this cell population.

GOALS

1). To study the **cell cycle** distribution of B-cells in patients with B-cell NHL and B-CLL.
2). To detect the presence of **DNA aneuploidy** in B-NHL and B-CLL patients. The criteria for the definition of DNA aneuploidy is defined by the presence of two or more different peaks of cells in the G_0/G_1 cell cycle phases.
3). To detect **minimal residual disease** in B-NHL and B-CLL patients with DNA aneuploid B-cells who are in morphological complete remision.
4) To evaluate disease extent (staging) in B-NHL patients with DNA aneuploid B-cells.

CLINICAL UTILITY

1.- CELL CYCLE
Prognostic evaluation of B-NHL and B-CLL patients. It has been described that the S-phase (S) fraction of tumoral B-cells is an important prognostic indicator in non-Hodgkin lymphomas and B-lineage chronic lymphocytic leukemias.
2.- DNA ANEUPLOIDY
• Monitoring of minimal residual disease (for patients with DNA aneuploidy at diagnosis)
 - Enumeration of tumoral B-cells in samples from B-NHL and B-CLL patients who are in morphologic complete remission, including samples which will be used for autologous transplantation.

- Detection of aneuploid B-cells in peripheral blood and/or apheresis samples which will be used for autologous transplantation.
• Evaluation of disease extent. Detection of aneuploid B-cells in peripheral blood and bone marrow samples in order to assess disease extent.

SENSITIVITY

The lowest level for detection of DNA aneuploid B-cells in samples morphologic complete remission (minimal residual disease) is 1 to 5 DNA aneuploid cells among 10.000 normal cells.

REAGENTS

- **Mixture of primary antibodies:** Vial containing a mixture of purified murine monoclonal antibodies specific for the detection of antigens present in human B-cells. Antibodies are diluted in PBS with 0.1% NaN_3 as preservative. Ready to use. Add 40 µl / test. Volume: 800 µl / vial.
- **Secondary Antibody:** FITC labelled goat anti-mouse IgG F(ab')$_2$. Antibodies are diluted in PBS with 0.1% NaN_3 as preservative. Ready to use. Add 20 µl/test. Volume: 400 µl /vial.
- **Lysing Solution:** vial containing 40 ml of an erythrocyte lysing solution. Add 2 ml / test.
- **DNA Labelling Solution:** vial containing detergent, propidium iodide and RNAse for DNA staining in 20 ml of buffer. Add 1ml / test.

PROTOCOL

1.-Perform a white blood cell count of the sample. In case of using bone marrow samples, prior to cell enumeration pass them 3 or 4 times through a 20 µm garge syringe in order to disintegrate cell clumps. Place 10^6 cells from the sample, in a volume of 100-150 µl.
2.-Labelling of surface antigens characteristic of human B-cells:
   • Add 40 µl of the primary antibody mixture to each tube. Mix gently.
   • Incubate 10' at room temperature (darkness is not necessary).
   • In order to wash out the excess of primary antibodies:
      Fill each tube with PBS (2ml/tube).
      Centrifuge for 5' at 540g.
      Discard the supernatant.
      Resuspend the cell pellet.
   • Add 20 µl of the secondary antibody reagent to each tube. Mix gently.
   • Incubate 10' at room temperature in the dark.
   • In order to wash out the excess of secondary antibody:
      Fill each tube with PBS.
      Centrifuge for 5' at 540g.
      Discard the supernatant.
      Resuspend the cell pellet.
3.-Add 2ml of erythrocyte lysing solution to lyse mature red cells present in the sample. Mix gently and incubate during 10' at room temperature in the dark.
4.-Wash out the lysing solution:
      Centrifuge for 5' at 540g.
      Discard the supernatant and place each tube inverted in a vertical position over a filter paper in order to eliminate the possible remaining lysing buffer from the tube.
      Resuspend the cell pellet.
5.-Add 1ml of DNA labelling solution. Incubate in the dark for 10' at room temperature.

6.-Acquire data in a flow cytometer (low speed position). Data acquisition must be performed within the first three hours after sample preparation is finished. Keep tubes at 4°C until data acquisition in performed.

DATA ADQUISITION

Data acquisition can be performed using two different procedures which are described below:
Procedure 1 (two steps' acquisition):
   -Acquisition of a minimum of 5000 cells from the total cellularity.
   -Acquisition with an activated live gate in which only those cells displaying an intermediate-high FL1-FITC fluorescence intensity (B-cells) are included. Acquire a minimum of 5000 B-cells.
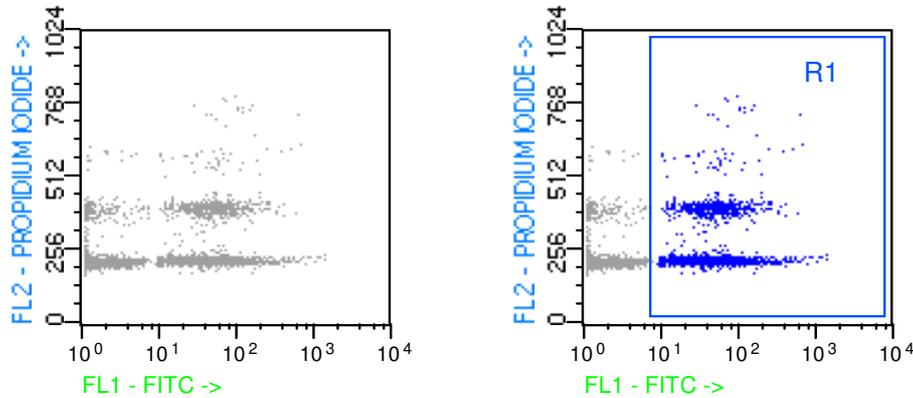

Procedure 2:
   -Acquisition in a single step of a number of cells in which a minimum of 5000 B-cells are included.

DATA ANALYSIS

General Procedure:
1.- Gate singlets in either an FL2-Area/FL2-Width dot plot (in a Becton/Dickinson flow cytometer), or an FL2-Area/FL2-Peak dot plot (instruments from Coulter Corporation and Ortho Diagnostic Systems flow cytometer) following the instructions shown below (dot plots):
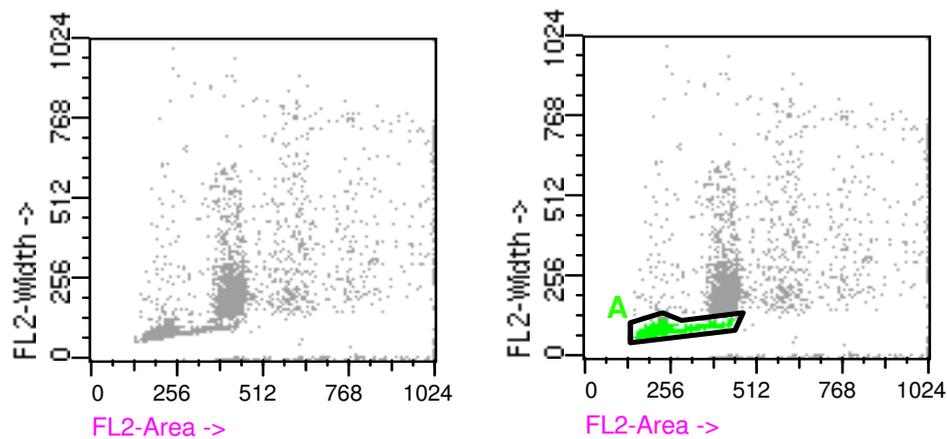
2.-After selecting the singlets, paint a gate on B-cells according to the existence of an intermediate-high FL1-FITC fluorescence intensity. Using two different colors discriminate between those cells displaying intermediate-high intensity for FL1-FITC (B-cells) and the normal residual bone marrow hematopoietic cells (FL1-FITC low/negative) using a SSC/FL1-FITC dot plot or an FL2-Area/FL1-FITC dot plot. The few mature normal residual B-lymphocytes will frequently appear with a higher fluorescence intensity as compared to tumoral B-cells as shown in the following diagrams:
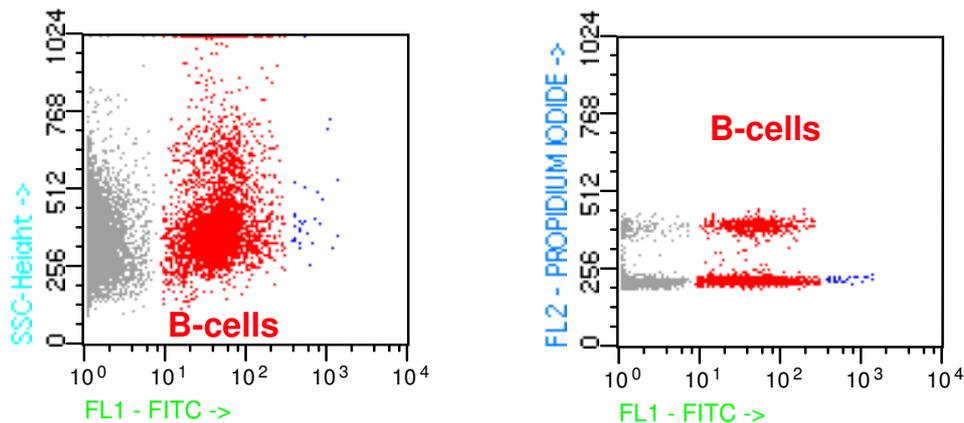
3a.- Explore the possible existence of DNA aneuploidy throught the comparison of the relative distribution of the G_0/G_1 DNA peaks of B-cells and the normal residual hematopoietic cells. If they are aneuploid, calculate the DNA index by dividing the mode of the DNA fluorescence intensity of the G_0/G_1 leukemic B-cells by that of the G_0/G_1 normal residual cells.

3b.- Cell cycle estimation: calculate the percentage of cells in each cell cycle phase using the mathematical models included in the specific software programs available in the laboratory and according to consensus or own laboratory recommendations.

3c.- Calculate the percentage of DNA aneuploid B-cells in those cases in which detection of minimal residual disease is requested.

Note: For a correct calculation of the cell cycle distribution, coefficient of variation of the G_0/G_1 peaks must be lower than 5%.

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