CYCLOSCOPE B-ALL, is a kit used for the flow cytometric analysis of DNA cell contents in B-lineage acute lymphoblastic leukemia (B-ALL). This kit is mainly focused for DNA studies of leukemic blast cells from bone marrow samples of these patients.

INTRODUCTION

Acute lymphoblastic leukemia is a disorder characterized by a clonal expansion of lymphoid progenitor cells arrested at different differentation steps whose progressive accumulation causes bone marrow involvement with more than 30% blast cells at diagnosis. According to the European Group for the Immunological Clasification of Leukemias, assignement of an ALL to the B-lineage is based on the expression on leukemic cells of at least two B lineage associated antigens: CD19, cytoplasmic CD79a (mb-1) or cytoplasmic CD22.

DNA cell content studies by flow cytometry provide relevant information for the prognostic evaluation and follow-up of patients with acute lymphoblastic leukemia. Detection of hyperdiploid leukemic blast cells is an independent prognostic factor strongly associated with favourable clinical and biologic features (1-3); in addition it may be of great utility for the detection of minimal residual disease contributing to relapse prediction in these patients (4, 5). At present the clinical utility of cell cycle studies in B-lineage ALL still remains to be established (6).

PROCEDURE

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

GOALS

1). To study the **cell cycle** distribution of leukemic blast cells in patients with B-progenitor cell acute lymphoblastic leukemia.

2). To detect the presence of **DNA aneuploidy** in B-lineage ALL 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-ALL patients with DNA aneuploid blast cells who are in morphological complete remision.

CLINICAL UTILITY

1.- DNA ANEUPLOIDY

a). PROGNOSTIC EVALUATION AT DIAGNOSIS OF LEUKEMIC BLAST CELLS.

Determination of the DNA cell contents has emerged as an independent prognostic factor in patients with B-lineage ALL. While hipodiploidy is associated to an unfavourable prognosis with a high relapse rate, hyperdiploid cases especially those with a DNA index $\geq$ 1.16, show a favorable prognosis with respect to the response to treatment and both event-free and overall survival (3). At present this parameter is used by several groups in order to classify B-ALL patients into different risk groups and to decide the treatment protocols (1, 7).

b). MONITORING OF MINIMAL RESIDUAL DISEASE (for patients with DNA aneuploidy at diagnosis)

-Enumeration of leukemic blast cells in bone marrow samples from B-lineage ALL patients who are in morphologic complete remission, including samples which will be used for autologous transplantation.

-Detection of aneuploid leukemic cells in peripheral blood and/or apheresis samples which will be used for autologous transplantation.

2.- CELL CYCLE

At present the clinical utility of cell cycle studies in B-lineage ALL still remains to be established.

SENSITIVITY

The lowest level for detection of DNA aneuploid blast cells in bone marrow samples morphologic complete remission (minimal residual disease) is 1-5 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 leukemic blast cells. Antibodies are diluted in PBS with 0.1% NaN_3 as preservative. Ready to use. Add 40 µl/test. Volume: 1ml/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: 0.5ml/vial.
- **Lysing Solution:** vial containing 50 ml of an erytrocyte lysing solution. Add 2ml/test.

- **DNA Labelling Solution:** vial containing detergent, propidium iodide and RNAsa for DNA staining in 30 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 syringe in order to disggregate the sample. Take 10^6 cells from the sample, in a volume of 100-150 µl.

2.-Labelling of surface antigens characteristic of human leukemic B cells with inmature phenotypes:

- Add 40 µl of the primary antibody mixture to each tube. Mix gently.
- Incubate 15' 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 15' 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 in an horizontal position during 10' at room temperature in the dark.

4.-Wash out the lysing solution:

Centrifuge for 5' at 540g.
Discard the supernatant and placed 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 (horizontal position).

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 ºC until data acquisition in performed.

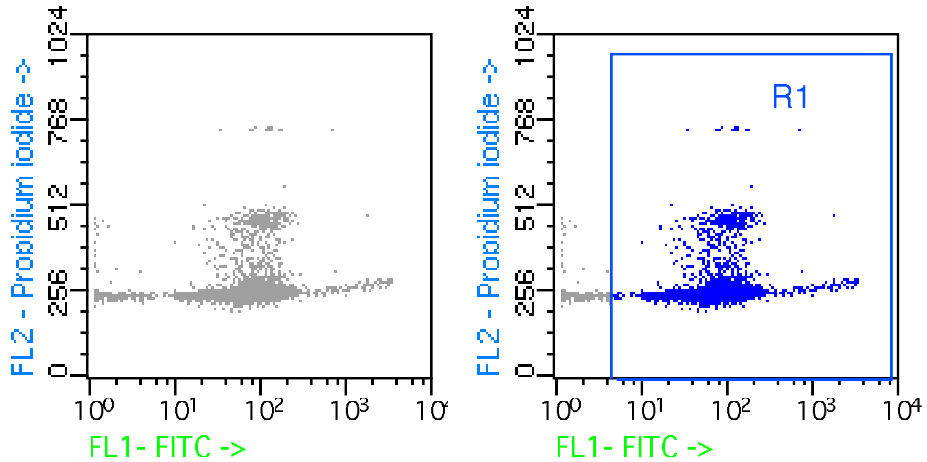
DATA ADQUISITION

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 (leukemic cells) are included. Acquire a minimum of 5000 blast cells.



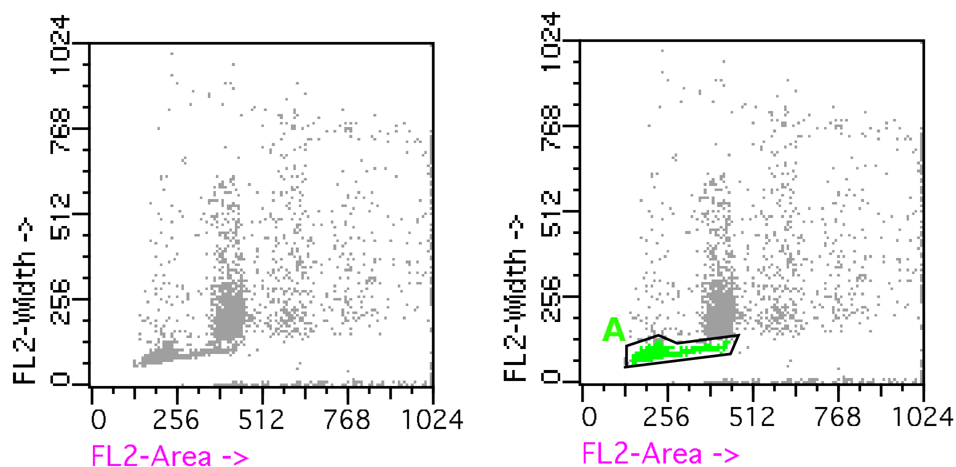
Procedure 2:

-Acquisition in a single step of a number of cells in which a minimum of 5000 blast cells are included.

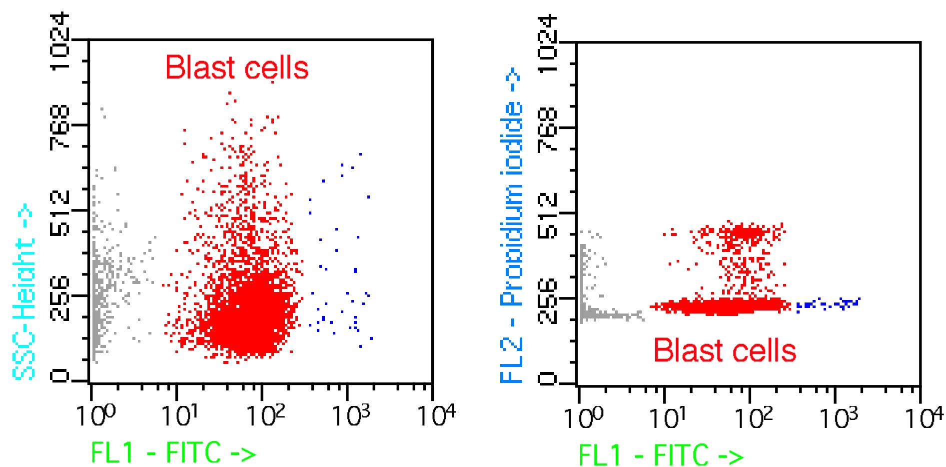
DATA ANALYSIS

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 blast 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-lineage leukemic 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 frecuently appear with a higher fluorescence intensity as compared to blast cells as shown in the following diagrams:



3a.- Explore the possible existence of DNA aneuploid after comparing the relative distribution of the G_0/G_1 DNA peaks of the leukemic blast 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.

3c.- Calculate the percentage of DNA aneuploid leukemic 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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