COOPERATIVE PLATFORM (FACS-NANORT-QPCR): CONTRIBUTION TO THE SUCCESSFUL MANAGEMENT OF PATIENTS WITH MRD LEUKEMIA

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ABSTRACT

t(9;22) Ph translocation is a chromosome abnormality recurrent in human chronic myelogenous leukemia (CML) and cases of childhood acute lymphoblastic leukemia (ALL). Ph is a marker of adverse prognosis significant with remission more frequent and earlier and lower percentage of overall survival. If there are less than 5% blasts in a bone marrow sample, the patient is in "clinical remission". In the light of sensitivity current controls molecular (10-6) are an important role in therapy, although this depends on the amount of cells analyzed. Our goal is to improve the sensitivity by proposing a platforms combination, which allow MRD evaluation with only 10 cells. Flow cytometry sorting (multiparameter immunological detection), nano-PCR (RNA isolation and RT) and qPCR (diagnostic confirmation). The molecular remission has many more probability to be lasting that the cytogenetic remission, both before and after the transplant. We consider this technique as an important step forward towards performed diagnosis, monitoring treatment responses, and identification of relapse in Leukemia patients.

INTRODUCTION

The first chromosomal abnormality associated with malignant disease was the Philadelphia or Ph-chromosome identified in chronic myeloid leukemia (CML) (Nowell & Hungerford, 1960). Ph-chromosome basically refers to the translocation of chromosome 22 (22q-) with a section of chromosome 9 [t (9, 22) (q34, q11)] (Rowley, 1973). t(9;22) Ph translocation is a chromosome abnormality recurrent in human chronic myelogenous leukemia (CML) (Nowell & Hungerford, 1960) and acute lymphoblastic leukemia (ALL) (Kalwinsky et al, 1985). Ph is a marker of adverse prognosis significant with remission more frequent and earlier and lower percentage of overall survival

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(Hoelzer et al, 1988). The minmal residual disease (MRD) is defined as the small number of cancer cells that remain in a patient during or after treatment however clinical and microscopic examinations confirm the complete remission (CR) and the patient shows no symptoms of the disease. MRD is the major cause of disease relapse, its detection gives important feedback about conventional treatment success and helps in selecting therapeutic alternatives. The clinical significance of MRD has been investigated in patients with acute leukemias with approximately 1012 malignant cells at diagnosis. After therapy with a bone marrow normal microscope (CR cytological or hematologic CR), yet hold up to 1010 cancer cells. Flow cytometry is the most commonly used technique for the diagnosis of haematological malignancies. This method is widely used but a high level of expertise is required to interpret the data when it comes to rare event detection such as MRD. The sensitivity for the detection of malignant cells varies according to the type of leukaemia, sensitivities of 1 in 104 up to 1 in 105 (Hauwel & Matthes, 2014). Relative quantification by real time PCR (qPCR) based on detecting a leukaemia-specific messenger RNA (mRNA) sequence is commonly used. That needs to compare changes in different samples even though the samples vary in quality or quantity. In leukemia sensitivity of 106 cells is possible (Brüggemann et al, 2010) however this method is criticized because RNA is a much less stable target for diagnostics and requires careful handling and processing (Hauwel & Matthes, 2014).

In the light of sensitivity current controls molecular are an important role in therapy, although this depends on the amount of cells analyzed. Our goal is to improve the sensitivity by proposing a platforms combination, which allow MRD evaluation with only 10 cells. Flow cytometry sorting (multiparameter immunological detection), nano-PCR (RNA isolation and RT) and qPCR (diagnostic confirmation). This fast protocol greatly reduces the user error. Samples volume from microliters until milliliters allows omits relative quantification, therefore neither calibration curves nor constitutive gen quantification. The molecular remission has many more probability to be lasting that the cytogenetic remission, both before and after the transplant. We consider this technique as an important step forward towards performed diagnosis, monitoring treatment responses, and identification of relapse in Leukemia patients.

MATERIAL AND METHODS

Samples: control K562 cell line and blood samples was provided by laboratory CINVESTAV (Mexico City, Mexico).

Multiparameter Flow cytometry: Flow cytometry is based on identifying cell surface antigen expression using fluorescence-labeled antibodies. A antibody combination was used to distinguish myeloid cells (CD45/CD15 /CD14/CD33 /CD11b) in peripheral blood using MoFlo XDP High-performance cell sorter (Beckman). 10 positives live cells from each sample were sorted directly onto each of the 48 AmpliGrid (Beckman Coulter) reaction sites.

Nucleic acids extraction: After transferring 0.75 µl of cell extraction working solution (Beckman Coulter Cell Extraction Kit) to each reaction site on the AmpliGrid slide, every droplet was covered with 5 µl of sealing solution. The loaded AmpliGrid was placed on the AmpliSpeed slide cycler and the cell extraction program (5' 75oC; 2´95oC) was started.

cDNA synthesis: Nucleic acids (0.75 µL, corresponding to cell extraction volume from 10 cells) was transcribed into cDNA following a LightCycler t(9;22) Quantification Kit TM (Roche) protocol using random hexamer priming and AMV reverse transcriptase. 0.75 µL of RT reaction

mix was added, in the same composition and components proportion recommended for the kit. Discard residual cell components for extraction is no necessary. RT conditions 60' 37o, 10' 65o and final volume of reaction 1.5µl in AmpliSpeed slide cycler. After cDNA synthesis all samples was recovered in 10 µl final volume, 5 µl of the cDNA was used for each of the specific PCRs t(9;22) detection and G6PDH detection.

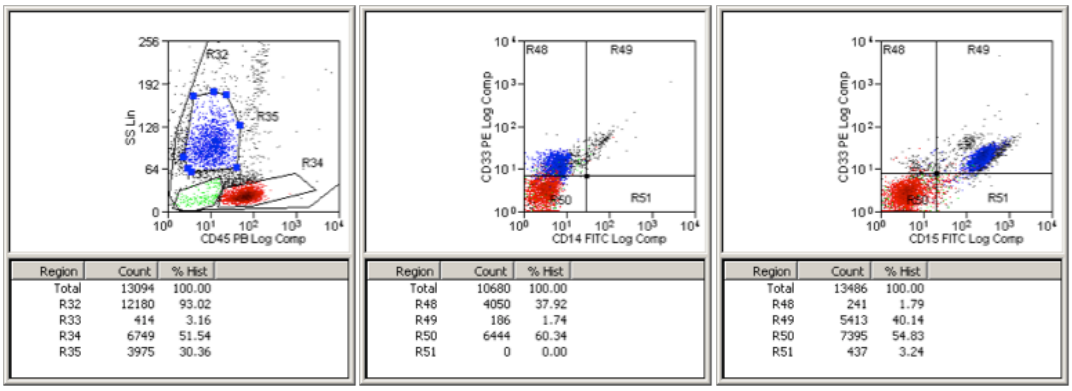
qPCR: Primers and probes: BCR-ABL (BCR-gene, chromosome 22, ABL-gene chromosome 9) breakpoints are most mapped to the major (M-bcr) and minor (m-bcr) breakpoint cluster regions. Three BCR-ABL breakpoints (b2a2, b3a2 [M-bcr] and e1a2 [m-bcr]) represent about 96 % of the described in CML. Those breakpoints are detectable using LightCycler t(9;22) Quantification Kit (fig. 1). qPCR in relative quantification (target/reference ratio), normalization, calibrator and reference gene are no necessary requisites for this protocol coupled to multiparameter flow cytometry and low volume RT. Qualitative real time PCR procedure for BCR-ABL and G6PD mRNA transcripts were performed in duplicates using LightCycler t(9;22) Quantification Kit and the LightCycler 2.0 (Roche) (fig. 5). RNA control from the kit (originally calibrator) was used only like positive control for BCR-ABL and G6PD. PCR amplification was performed according to manufacturer's instruction.

Figure 1. Location of amplification primers and Hybridi-zation Probes for detection of b2a2, b3a2, and e1a2 transcripts.

RESULTS

Multiparameter Flow cytometry

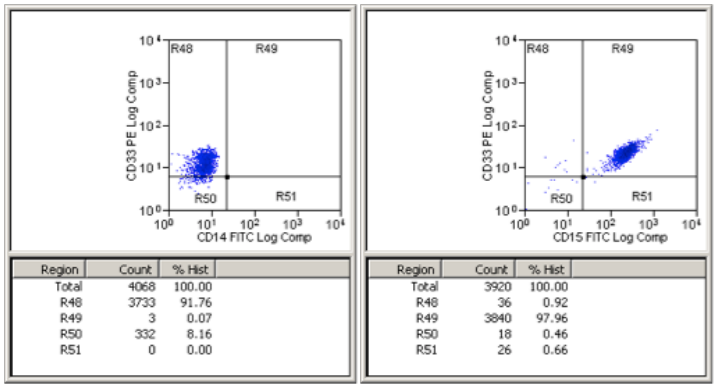
Residual AML panel was used like monitor based in residual AML blasts on pre-treatment phenotype. Markers Included: CD11b, CD11c, CD13, CD14, CD15, CD33, CD79a and MPOx. Figure 2 A shows the analysis of immunophenotype for chronic myeloid leukemia. Figure 2 B shows Myeloid cell panel CD45/CD3/CD79a, Myeloperoxidase in blue The phenotype expensively defined for chronic myeloid leukemia in blue CD45 with CD33+ CD 15 positive and positive



Region	Count	% Hist
Total	13094	100.00
R32	12180	93.02
R33	414	3.16
R34	6749	51.54
R35	3975	30.36

Region	Count	% Hist
Total	10680	100.00
R48	4050	37.92
R49	186	1.74
R50	6444	60.34
R51	0	0.00

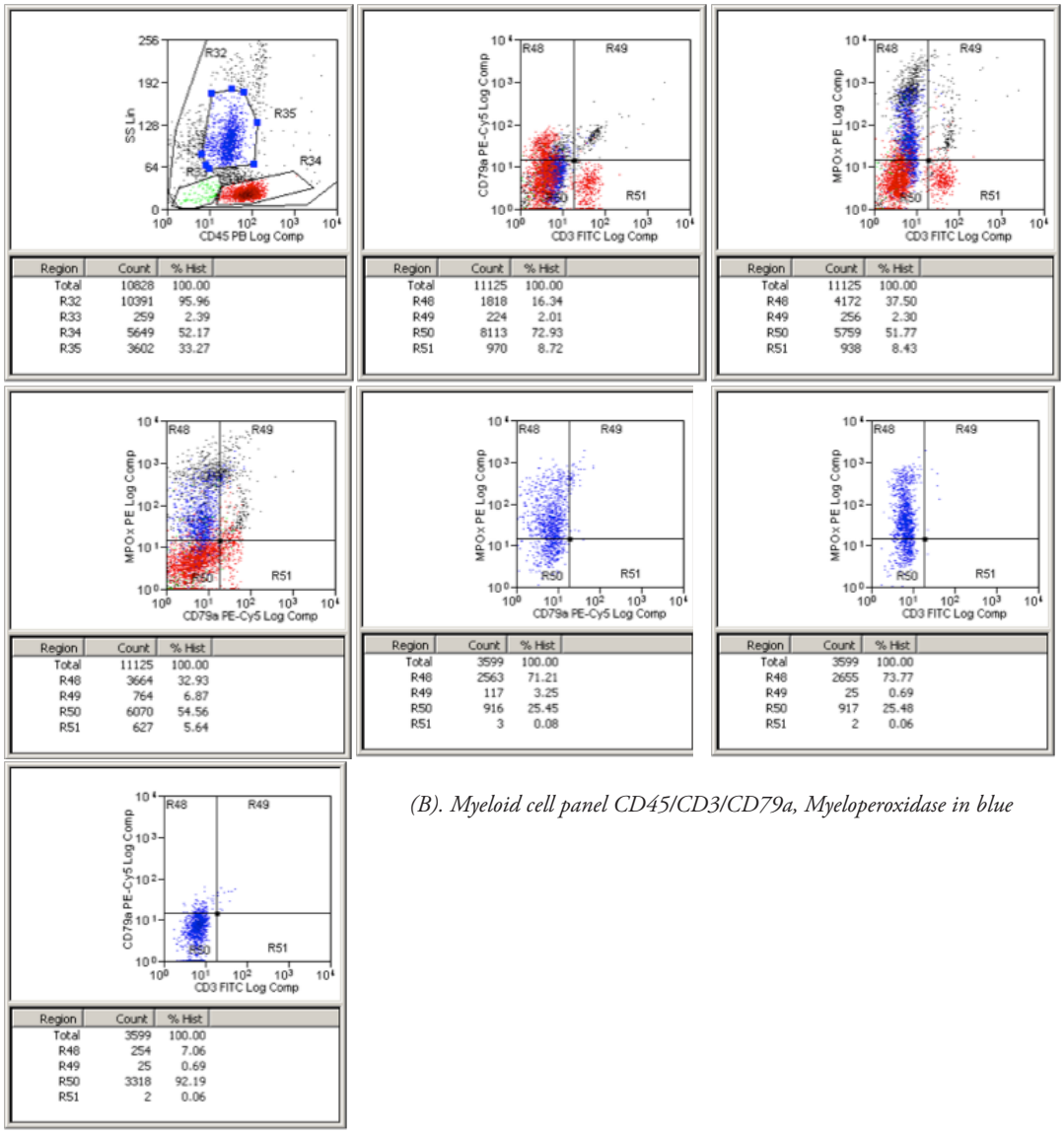
Region	Count	% Hist
Total	13486	100.00
R48	241	1.79
R49	5413	40.14
R50	7395	54.83
R51	437	3.24



Region	Count	% Hist
Total	4068	100.00
R48	3733	91.76
R49	3	0.07
R50	332	8.16
R51	0	0.00

Region	Count	% Hist
Total	3920	100.00
R48	36	0.92
R49	3840	97.96
R50	18	0.46
R51	26	0.66

(A). Myeloid cell panel CD45/CD15 /CD14/CD33 /CD11b



Region	Count	% Hist
Total	10828	100.00
R32	10391	95.96
R33	259	2.39
R34	5649	52.17
R35	3602	33.27

Region	Count	% Hist
Total	11125	100.00
R48	1818	16.34
R49	224	2.01
R50	8113	72.93
R51	970	8.72

Region	Count	% Hist
Total	11125	100.00
R48	4172	37.50
R49	256	2.30
R50	5759	51.77
R51	938	8.43

Region	Count	% Hist
Total	11125	100.00
R48	3664	32.93
R49	764	6.87
R50	6070	54.56
R51	627	5.64

Region	Count	% Hist
Total	3599	100.00
R48	2563	71.21
R49	117	3.25
R50	916	25.45
R51	3	0.08

Region	Count	% Hist
Total	3599	100.00
R48	2655	73.77
R49	25	0.69
R50	917	25.48
R51	2	0.06

Region	Count	% Hist
Total	3599	100.00
R48	254	7.06
R49	25	0.69
R50	3318	92.19
R51	2	0.06

(B). Myeloid cell panel CD45/CD3/CD79a, Myeloperoxidase in blue

MPOx . 10 blood cell was sorted directly lo AmpliGril, from subsequent qPCR.

REAL TIME PCR WITH 10 CELLS

The RNA extracted from the cells 10 were subsequently retro-transcribed and separated to two capillaries in amount equivalent of 5 cells. One capillary of each pair contained probes for amplify G6PDH and the other probes for amplify t(9;22).

For clarity, the amplification curves for the different capillaries in the experiment are shown in different screens. Figure 3 displays the fluorescent signal of the t(9;22) and G6PDH positive control kit (capillary 1 and 2 respectively). Negative cell lines for t(9;22) was positive for G6PDH detection and negative for t(9;22) detection (capillary 3 an 4 respetively). Only G6PDH was detected but with low efficiency, possibly due to excessive cDNA. Positive cell line has positive signal for G6PDH and t(9;22) (capillary 5 and 6 respectively). G6PDH show low efficiency reactions while t(9;22) is very clear. No standard curve was included, G6PDH and BCR-ABL concentrations calculus is no necessary. NTC for both G6PDH and t(9;22) sowed in capillary 7 and 8.

The results clearly show that qPCR amplification from DNAc extracted the equivalent of 5 cells is possible. Even the amount of G6PDH amplification can be so abundant that shows inhibition of the reaction. By other hand t(9;22) amplification had ideal DNAc concentration.

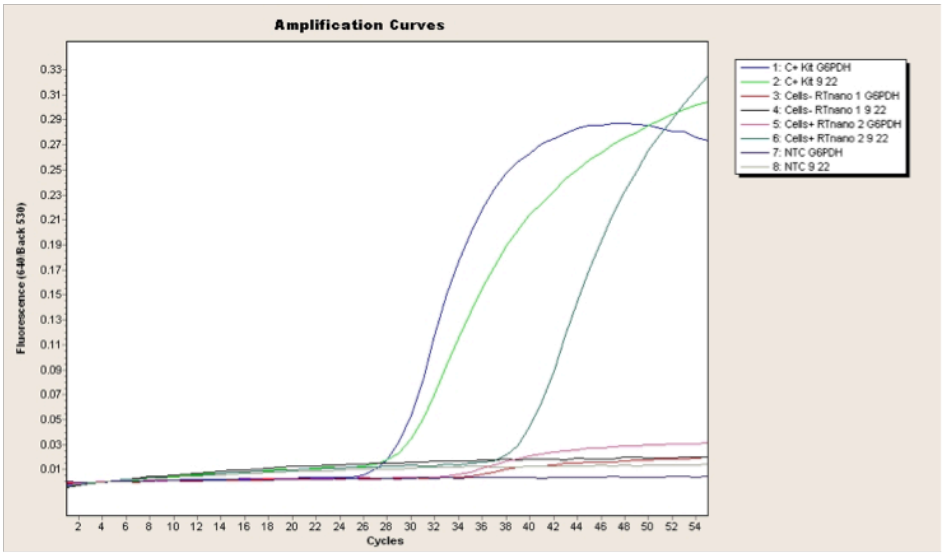


Figure 3. Fluorescent signal of the t(9;22)(capillary 1) and G6PDH positive control kit (capillary 2). Negative cell lines for t(9;22) was analyzed for G6PDH Detection (capillary 3) and t(9;22) Detection (capillary 4). Positive cell line has positive signal for G6PDH and t(9;22) (capillary 5 capillary 6). NTC for both G6PDH and t(9;22) sowed in capillary 7 and 8.

Melting curves analysis (fig. 4) for the same samples, positives and negatives result was confirmed including G6PDH amplification in negative and positive cell control. G6PDH was amplified with low efficiency but however t(9;22) in positive samples has very clear signals. Melting curves are better analysis method for appreciate a comprehensible result. Melting curves in Fig. 5 show a DNAc samples set including t(9;22) negative and positive samples.

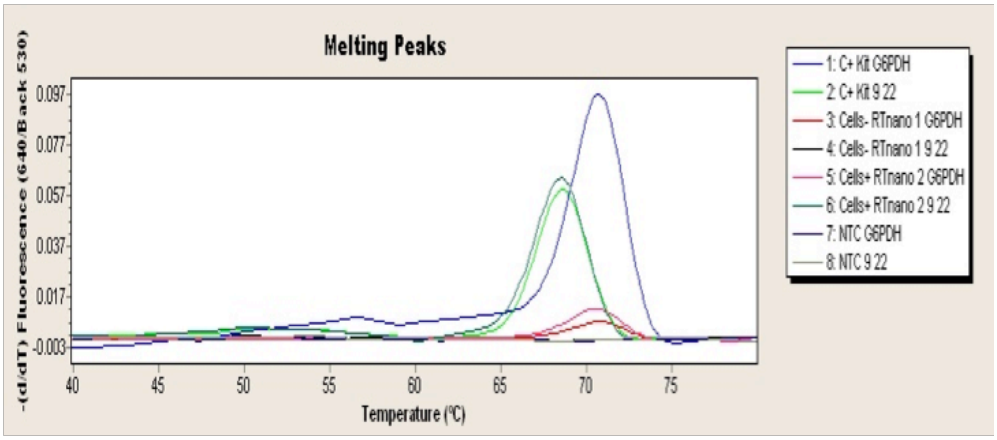


Figure 4. Detection of BCR-ABL fusion and G6PDH control by melting curves. G6PDH Tm= ±70.5, t(9;22) Tm=±70.5. No signal was detected in NTC.

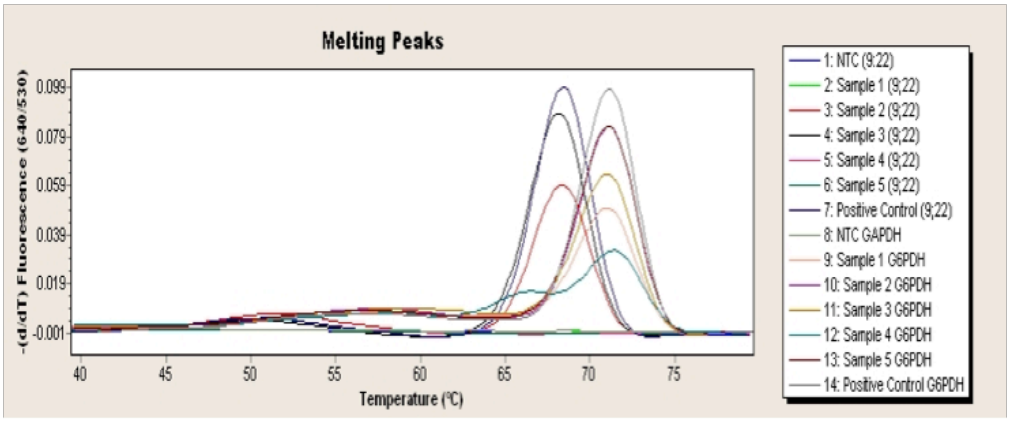


Figure 5: Melting curves form positives and negatives samples sorted onto AmpliGrid. Only samples 2 (capillary 2) an 3 (capillary 4) have positive signal for t(9;22). All samples has positive signal for G6PDH.

CONCLUSIONS

Using Multiparameter Flow cytometry coupled to the low volume cell extraction (AmpliGrid/ Amplispeed technology) and qPCR, a highly sensitive and improved cell analysis can be performed. This protocol can enhanced sensitivity analysis especially important in MRD evaluation. Our goal is to improve the sensitivity by proposing a platforms combination, which allow Minimal Residual Disease evaluation with only 10 cells from samples volume from microliters until milliliters. This fast protocol greatly reduces the user error and allows omits relative quantification, therefore neither calibration curves nor constitutive gen quantification.

The molecular remission has many more probability to be lasting that the cytogenetic remission, both before and after the transplant. We consider this technique as an important step forward towards performed diagnosis, monitoring treatment responses, and identification of relapse in Leukemia patients. We present the Here we are the first steps in the development of this protocol In order to include it in the options for molecular control evaluation for MRD.

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