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Immunomagnetic separation of B type acute lymphoblastic leukemia cells from bone marrow with flow cytometry validation and microfluidic chip measurements

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ABSTRACT

In order to detect the blast cells from bone marrow of patients, one strategy is to first isolate the cells using immunomagnetic beads. The aim of this study was to report the experimental results of the immunomagnetic separation efficiency of the blast cells from bone marrow of pediatric leukemia patients. To test the efficiency of immunomagnetic separation, flow cytometry measurements at critical steps were performed. We here reported 94.5% capture efficiency for CD10 nano beads. Patients samples were also analyzed with a microfluidic chip to test the feasibility for further developments.

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B type acute lymphoblastic leukemia; immunomagnetic separation; flow cytometry; bone marrow; hematogone; microfluidic chip

Introduction

Chemotherapy is the generally used treatment for leukemia patients, the persistent low level of blast cells can cause relapses known as Minimal Residual Disease (MRD). The patients have to be monitored for MRD to detect the risk of relapse and predict long term prognosis.[1] According to Berlin-Frankfurt-Münster (BFM) 2009 protocol,[2] the 15th day of chemotherapy is an important check-point to evaluate the response of the patient to the chemotherapy. It was reported that long term monitoring of MRD assess the risk groups and allows do determine the more or less intensification of the therapy.[3] The morphological evaluation, immunophenotyping, fluorescence in situ hybridization (FISH), and next-generation sequencing-based screening of bone marrow are the common techniques that are performed to assess the risk groups and determine the next steps of the therapy.[4] However, these methods are costly and are not accessible in low-income countries. Flow cytometry (FC) is able to screen multiple surface markers on the cell membrane and FC can detect 1 leukemic cell among 10000 normal cells, and thus it is one of the most referenced technique for MRD monitoring.[5] Immunophenotypic characteristics of blast cells can differ from patient to patient and there are different combinations of antigens such as CD10, CD19, CD45, CD38, CD58, CD66c, CD34, CD22, used for FC based monitoring MRD.[6,7]

The technological advancements in micro/nano-technology motivated researchers to develop low-cost systems alternative to the conventional methods. As a part of these efforts, there are significant number of microfluidic systems that have been developed for cancer research such as detection, sorting, and characterization of tumor cells.[8,9] In order to detect MRD in patients with acute myeloid leukemia (AML), a microfluidic system incorporating sinusoidal flow channels and fluorescent labels was reported.[10]

Immunomagnetic separation has been extensively used to isolate target biomolecules or cells from blood,[11] serum,[12] bone marrow,[13] and food samples.[14] The immunomagnetic separation method also integrated with microfluidics to detect cancer markers.[15] The immunomagnetic beads are incorporated with non-magnetic beads to screen two surface markers of tumor initiating cells, thus, only immunomagnetic separation of $CD44^{+}/CD24^{-}$ cells were attained.[16]

Screening cells for one surface marker is not sufficient for MRD monitoring. Recent studies reported that 8-color FC panel including CD19, CD45, CD34, CD10, and CD20 was standardized to monitor MRD.[17,18] To test the feasibility of an alternative microfluidic platform to monitor the diseases like MRD, first part of our strategy was to immunomagnetically capture target cells having two different surface markers (CD19 and CD10, double immunomagnetic sorting), the second part of the strategy

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to capture and immobilize the CD19+ CD10+ cells in a microfluidic platform functionalized with a third antibody. Immobilized cells were quantified and overall this strategy allows screening cells for three different antibodies. Previously we reported the capture efficiency of single and double sorting of B type leukemia cells with various immunomagnetic beads[19] and recently we reported an immunomagnetic bead-assisted microfluidic system to monitor the comparative response of patients to the treatment.[20] Micro- and nano-size immunomagnetic beads coated with various antibodies are readily available on the market. Contrary to nano-size beads, micro-size particles are visible under bright-field optical microscope and these particles can be automatically detected and quantified using image processing algorithms.[21]

The ultimate goal is to develop a low-cost and easily accessible microfluidic system which allows personalized adjustment of the dosing. It is known that not all patients show the same response to chemotherapy where some patients need aggressive therapy. On the other hand, for some patients, unnecessary intense dose can be toxic. To further develop the microfluidic platform reported in recent study[20] and to gain insight into how the immunomagnetic beads effect the FC measurements, we performed experiments using patient samples. To the best of our knowledge, the impact of the micro- and nano-size immunomagnetic beads on FC measurements have not been reported before.

In this study, we investigated the separation efficiency of immunomagnetic beads to capture blast cells from the bone marrow of leukemia patients and explored the capture efficiencies using flow cytometry.

For this purpose, we performed two groups of experiments, the first group is depicted in Fig. 1. The main goal of the first group of experiments was to better understand the impact of micro- and nano-size immunomagnetic beads on FC measurements. For that purpose, we analyzed patient samples with FC at different steps of double sorting.

The bone marrow samples at diagnosis of leukemia patients were double sorted using two types of magnetic beads, 120 nm size (CD10) and 4.5 µm size (CD19), coated with different antibodies. FC measurements were performed at different steps of the double sorting procedure to investigate the separation efficiency and the impact of the immunomagnetic beads on the FC measurements. The patients were treated with chemotherapy according to ALL Intercontinental (IC)-BFM 2009 protocol[2] and for long term MRD monitoring, patient samples analyzed again with FC as the second group of experiments. In the second group of experiments, patient samples at diagnosis, on the 90th day and 150th day were double sorted with immunomagnetic beads and samples were analyzed with FC. Also, patient samples were examined with the microfluidic platform to show potential for further developments.

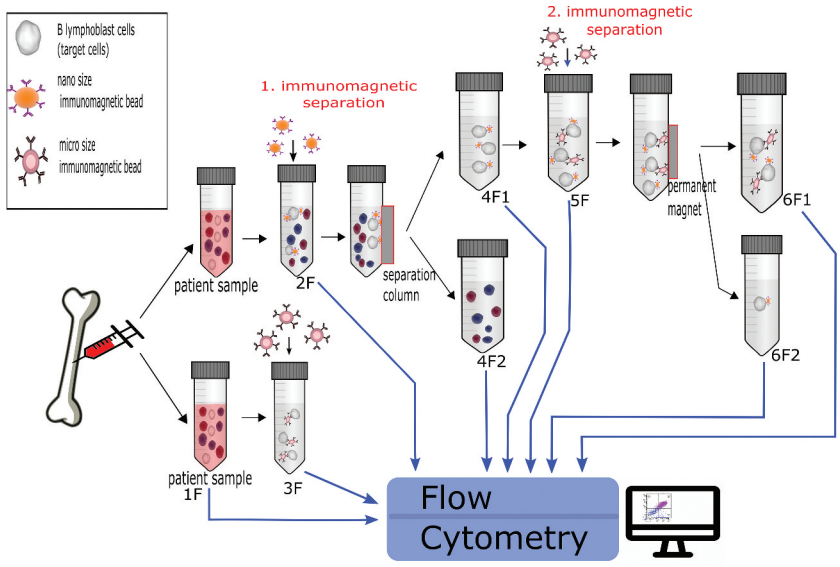

Figure 1. Illustration of double sorting experiments (CD10 nano-size beads, CD19 micro-size beads); immunomagnetic separation of target cells from bone marrow of patients and flow cytometry measurements at critical steps. 1 F: patient sample (without any bead incubation), 2 F: patient sample incubated with CD10 nano-size beads, 3 F: patient sample incubated with CD19 micro-size beads, 4F1: separated cells with CD10 nano-size beads, 4F2: the unconjugated cells after the first separation, 5 F: cells incubated with CD19 micro beads after the first separation, 6F1: after second separation of target cells (double sorting), 6F2: the unlabeled, missing cells of the second separation.

In the next sections, we explain the materials, methods, and the experimental results and finally conclude with the discussion.

Materials and methods

Immunomagnetic beads of 120 nm in diameter coupled with monoclonal anti-human antibody were purchased from Miltenyi Biotec (Auburn, CA). Immunomagnetic beads of 4.5 μm in diameter coupled with monoclonal anti-human antibody (Dynabeads) were purchased from Thermo-Fisher (Waltham, MA). B lymphoblast cells CCRF-SB were acquired from ATCC (Rockville, MD), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Nano- and micro-size immunomagnetic bead separation

We followed the manufacturer recommendations and same experimental procedure that was reported earlier in Ref.[19] Briefly patient samples were divided into vials of 1.5 mL, superparamagnetic immunomagnetic beads were incubated with cells in a buffer solution and specific magnetic separation racks were used to isolate the captured cells.[20]

Cell counting

Cell counting was performed manually by using a Neubauer Chamber (Sigma-Aldrich, St. Louis, MO). For each experiment initial number of cells, the number of cells (noncaptured cells) in the washing solutions of immunomagnetic separation and the cells captured by immunomagnetic beads after the separation (single and double sorting) were counted.

Flow cytometry measurements

For flow cytometry, cells from bone marrow were isolated and surface markers stained with anti-human CD19, CD10, CD45, and CD38 antibodies which were purchased from Biolegend (Biolegend, San Diego CA). Cell -Surface staining protocol of the manufacturer was followed, briefly cells were washed once with the Staining Buffer (PBS supplemented with 2% Fetal Bovine Serum (FBS)). Antibodies were added in 100 μl of Staining Buffer in accord with the recommended dilution and incubated for 20–30 min at 4°C in dark. Then, cells were washed twice with staining buffer and resuspended in 200 μl Staining Buffer and run on a FACSArialIII (BD Biosciences, San Jose, CA). Data analysis was performed using FlowJo and FACSDiva softwares (BD Biosciences, San Jose, CA).

Microfluidic chip analysis

The details of the microfluidic chip and counting methods were reported in .[20,22] Briefly Polymethyl methacrylate (PMMA) (Mc Master Carr, Elmhurst, IL) material was used to form the side walls and the upper cover of the microfluidics, the bottom cover was a standard microscope slide and a glass slide containing square-shaped micro-size (15 μm × 15 μm) gold pads were inserted in the chamber. Double sided tape was used to seal the layers. The gold pads were functionalized with first 11-Mercaptoundecanoic acid (MUA), then N-Ethyl-N' -(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) linker layers followed by protein G and CD45 antibody immobilization.

Ethical approval and statistics

Pediatric patients with acute lymphoblastic leukemia were treated according to the international Berlin-Frankfurt-Münster 2009 (BFM 2009) protocol[2] at the Pediatric Hematology Department of Erciyes University. The sample collection procedure followed in this study was approved by the Clinical Research Ethics Committee of the Erciyes University Faculty of Medicine (Approval date: 09/01/2015, Decision no:2015/21, Kayseri, Turkey). The Declaration of Helsinki was followed throughout the study. Written informed consent was obtained from all parents or legally authorized representatives of the pediatric patients. Six patient samples were analyzed and compared using non-parametric Mann-Whitney test, there was no significant statistical difference ($p = .05$).

Results and discussion

Flow cytometry acquisition of patient samples incubated with immunomagnetic beads

Immunomagnetic beads coated with CD10 antibody and CD19 antibody were tested to capture B lymphoblast cells. Flow cytometry measurements were performed to investigate the capture efficiency and the impact of immunomagnetic beads on flow cytometry acquisitions. The FC measurements indicated in Fig. 1, are shown in Figs. 2 and 3.

As expected, many cells were stained positive for both CD10 and CD19 antigen which indicated the presence of many blast cells (Figs. 1f, 2a). When the CD10 nano-size beads bound to CD10 antigen on the cell membrane

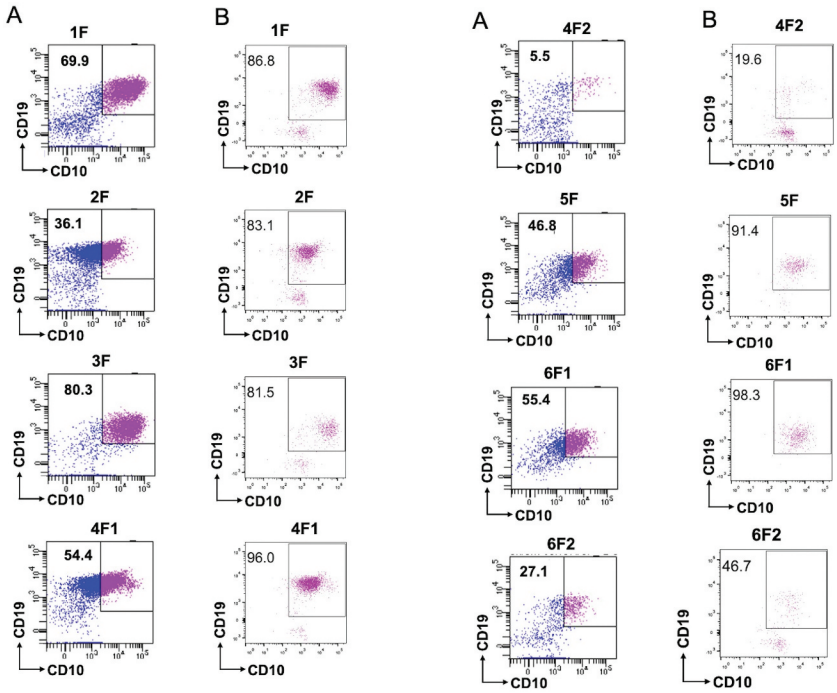

Figure 2. Flow cytometry results of the samples depicted in Fig. 1. (A) before adjusting the gating, (B) after adjusting the gating. 1 F: patient sample (without any bead incubation), 2 F: patient sample incubated with CD10 nano-size beads, 3 F: patient sample incubated with CD19 micro-size beads, 4F1: separated cells with CD10 nano-size beads, 4F2: the unconjugated cells after the first separation, 5 F: cells incubated with CD19 micro beads after the first separation, 6F1: after second separation of target cells (double sorting), 6F2: the unlabeled, missing cells of the second separation.

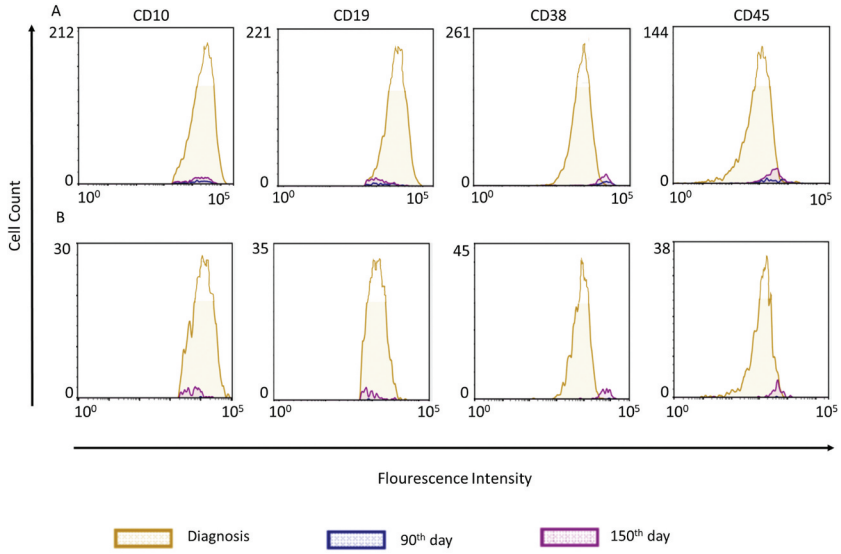

Figure 3. The mean fluorescence intensity of CD10, CD19, CD38, and CD45, Yellow at diagnosis, blue on the 90th day, pink on the 150th day (A) before and (B) after double sorting (CD10+ CD19+ isolate).

and blocked surface antigens, this caused the decrease of binding sites for fluorescent labeled anti-CD10, thus a shift toward to left was observed (Fig. 2a,f). However, when the CD19 micro-size beads bound to CD19 antigen on the cell membrane, beads only partially blocked the surface antigens thus fluorescent labeled anti-CD19 were able to bind the other uncovered CD19 antigens (Figs. 2a, 3f). In Figs. 2a, 4f, the separated sample included many CD10 (+) and CD19 (+) cells as expected, the impact of the nano beads on FC reading was the shift toward left. The wash out solution was investigated with FC and only few cells were CD19(+)

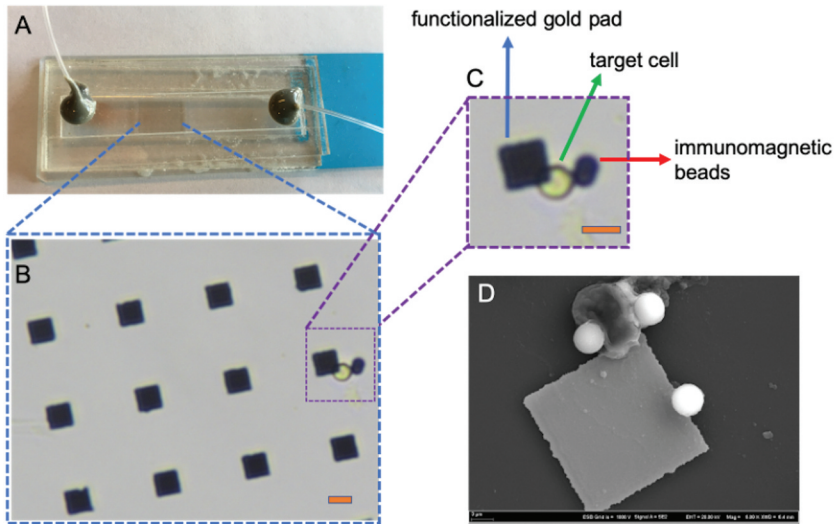

Figure 4. A, B, C Bright-field optical micrographs of the microfluidic system and inside, gold pads, and a captured target cell. Scale bar 10 µm. D SEM image of another target cell captured by two microbeads and functionalized gold pad.

and CD10(+), this measurement showed the capture and separation efficiency of CD10 nano beads (5.5%, Fig. 2a, 4f2). After the first sorting cells were incubated with CD19 micro beads and as expected many cells were CD10(+) and CD19(+). The incubation with CD19 micro-size beads does not have significant impact on flow cytometry acquisitions (Figs. 2a, 5f). The Figs. 2a, 6f1 represents that many cells were captured with CD19 and CD10 immunomagnetic beads. Not only the pink dots but also some blue dots (as a result of previous measurements) were expected to be CD10(+) and CD19 (+), the few cells missed by immunomagnetic beads were shown in Figs. 2a, 6f2.

The flow cytometry measurements showed the impact of immunomagnetic beads on the flow cytometry acquisitions. To determine the separation efficiency, the shift due to the immunomagnetic beads had to be considered. The data analyzed in the light of the results in Fig. 2a and gating parameter was adjusted (Fig. 2b columns).

As seen from figs. 2b, 1f, 86.8% of the cells from the patient sample were CD19 and CD10 positive. Compared to Fig. 2a, adjustment of the gating parameter revealed that the CD19 and CD10 positive cells were 83% (Fig. 2b, f) which was close to reading of patient sample without any incubation. The impact of the CD19 micro-size bead incubation and adjustment of the gating parameter revealed that the CD19 and CD10 positive cells were 81.5%. Figures 2b, 4f1,2 show that the CD10 nano-size beads separated 96% of the cells and only 19.6% of the cells in the negative fraction were CD19+ CD10 + . After the first sorting cells were incubated with CD19 micro beads and 91.4% of the cells separated were CD19 and CD10 positive. The double sorting (CD10 nano-size beads and CD19 micro-size beads) separated 98.3% of the cells and only 46.7% of the cells in the negative fraction after double sorting were $CD19^{+}$ $CD10^{+}$ as compared with the positively selected cells (98.3%), which showed the impact of FC gating and enrichment efficacy.

Long term measurements

After analyzing the impact of immunomagnetic bead bound cells on flow cytometry measurements at diagnosis, a similar investigation was performed on patient samples at the time of diagnosis, 90^{th} and 150^{th} day of the chemotherapy (Table 1). As stated earlier for MRD monitoring, the FC methods rely on screening combinations of multiple antigens. For this study, we chose screening CD38 and CD45 antigens in addition to CD10 and CD19 antigens. Table 1 shows the cell population (CD10+ CD19+ as blast or hematogone) percentages and its Mean Fluorescent Intensities (MFI) values of the different steps of the immunomagnetic separation.

Patient samples, CD10- cells (cells left after the first immunomagnetic separation), CD10+ CD19+ were analyzed with flow cytometry. At the diagnosis 85% of the cells are double positive for CD10 and CD19 positive. The cells, left after the first immunomagnetic separation, was measured with the flow cytometry and only 0.81% of the cells were CD10+ CD19+ that means 99.19% CD10- and immunomagnetic separation has high yield of capturing cells. The FC reading of 70.42% for the CD10 + CD19+ cells could be due to the impact of saturating cell surface markers by the nano-size immunomagnetic beads, so that binding of fluorescent labels to the cell surface was decreased compared to 85% of intact cell.

As a result of the chemotherapy, the blast and hematogone cell numbers decrease on the 90th and 150th days as expected and observed from the FC measurements (5.24% and 6.23%). The increase in the mean florescence intensity (MFI) of CD45 and CD38 values on the 90th and 150th days (Fig. 3) are expected and shows the increase of hematogones.[23]

Microfluidic chip measurements

Another set of experiments were performed using the microfluidic platform developed in Ref.[20] The platform included gold pads functionalized with anti-CD45 antibody to capture the cells on the pads in the microfluidic platform. The target cells after the two immunomagnetic separation steps were introduced to the microfluidic platform. The cells captured in the microfluidics were counted (Fig. 4). To visualize the cells and immunomagnetic beads, the captured cells were imaged using an optical microscope and scanning electron microscope (SEM) (Fig. 4). Contrary to the nano beads which were not visible under optical microscope, micro beads were apparent under optical microscope with uniform shape and size, and this was an important reason to use micro-size beads. The recorded images of microfluidics including micro-size beads, pads, and cells were analyzed and the amount cells were quantified using an image processing algorithm reported previously.[22]

The microfluidic platform is low-cost and easy to use compared to the FC; however, it can only screen for three different antigens. Figure 5 shows the number of cells captured in the microfluidic platform for the samples from two different pediatric leukemia patients. The number of cells in the patient sample on the 15th day (Fig. 5a) was not enough to measure with the microfluidics so excluded from the graph.

The variations of the number of cells were as expected; the number of CD19+ CD10+ and CD45 + cells were high at diagnosis for a leukemia patient, on 15th day as a result of chemotherapy the number of blast cells significantly reduced and on long term the number of hematogones started increasing (health cells with CD45+).

FC is a standard technique for monitoring MRD, a 8-color FC antibody panel can provide sensitivity $\leq 10^{-}$[5] which is comparable to PCR based methods.[17] Today FC instruments does not only provide information on cell populations but also florescent intensities are also measured as an indication of marker expression and thus subpopulations of the cells, *e.g.* MFI of CD38 from hematogones is considerably higher than MFI of CD38 from leukemic cells.[24] During the chemotherapy the regeneration pattern of normal hematogones and leukemic cells present different responses and it is a challenge in MRD monitoring to distinguish hematogones and leukemic cells.[24] It is utmost important in FC measurements on 33rd, 79th, or later days to detect hematogones and leukemic cells. After the 15th day of intensive chemotherapy, the bone marrow regenerates and the number of hematogones start increasing (Fig. 5b). The hematogones and leukemic cells may express same markers but the number of markers on a cell may differ. For the patient sample presented in Table 1, the comparison of 90th day and 150th day measurements with respect to the measurements at diagnosis, CD19 and CD10 expressions were decreased, while CD38 and CD45 expressions were increased. The high amount of CD19+ and CD10+ cells indicate the leukemic blasts at the diagnosis. As a result of chemotherapy, CD19 and CD10 expression was reduced while CD38 and CD45 expression was increased which indicates the generation of hematogones. We reported in[20] the microfluidic chip can be used to perform comparative analysis between diagnosis and 15th day thus detect early relapses. For the patient sample investigated, CD45 antibody is immobilized on the gold micropads to capture cells and the Fig. 5b shows the increase in the number of cells on the 90th day which is a result of increased number of hematogones. The FC and microchip measurements show similar qualitative results, but the sensitivity level of the microfluidic system needs improvement to perform MRD monitoring for late relapses which requires detecting few leukemic cells.

Table 1. Flow cytometry analysis of patient samples showing the MFI before and after immunomagnetic separations at diagnosis, 90th day and 150th day.

			CD10+ CD19+ p6 (blast/hematogone)				
Time	Immunomagnetic Separation		%	MFI cd10	MFI cd19	MFI cd38	MFI cd45
Diagnosis	SNG 1	Bone Marrow	85.05	30318	2870	10180	569
	SNG 1_A	CD10neg	0.81	5105	2426	9532	12573
	SNG 1_B	CD10+ CD19+	70.42	1580	1091	7059	1884
90th day	SNG 2	Bone Marrow	5.24	18696	1779	66668	4442
	SNG 2_A	CD10neg	0.23	3397	2594	26411	13569
	SNG 2_B	CD10+ CD19+	33.33	10272	843	91224	4123
150th day	SNG 3	Bone Marrow	6.23	20701	1455	54225	3559
	SNG 3_A	CD10neg	1.11	4681	2325	6340	573
	SNG 3_B	CD10+ CD19+	4.74	8499	1368	69191	4395

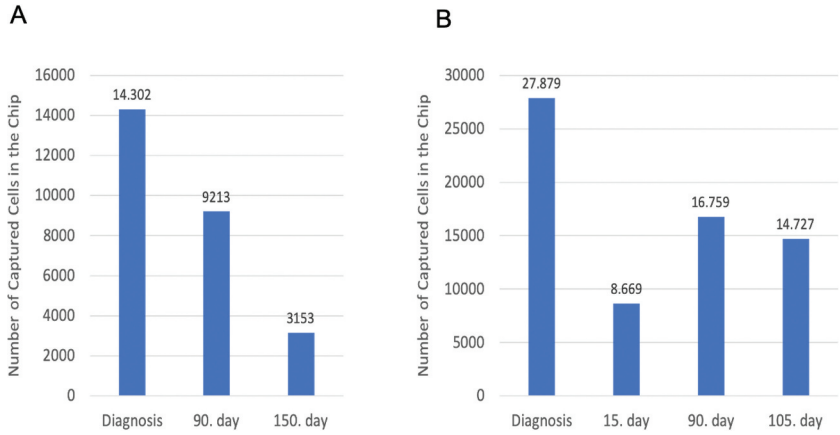

Figure 5. The cell counting of captured cells in the microfluidics for two pediatric patients.[22] (A) Patient samples available only at diagnosis and long term. (B) Patient samples available at diagnosis, 15th day and long term.

Conclusion

Previously we reported the double-sorting cell capture efficiency for blast cells from cell culture.[19] In this study, we examined the capture efficiency for blast cells from bone marrow samples. When we performed FC measurements, we observed that the presence of nano-size immunomagnetic beads effect the FC acquisitions. We anticipated that nano-size beads bind to cell surface antigens and block the binding of some of the fluorescent labels. Micro-size beads did not show a similar impact because of their size. As presented in Figs. 1 and 2a, to measure the cell populations in the presence of nano beads, the gating should be adjusted. However, this was valid for analyzing the cells incubated with nano-size beads. When analyzing the remaining cells from the immunomagnetic separation, with the original gating (Figs. 1, 4f2) the CD10 + cell ratio was 5.5 which represents the missing cells, the capture efficiency of CD10 beads could be determined as 94.5%. However, when the gating was adjusted to compensate the shift due to the CD10 nanobeads, the missing cell ratio was increased to 19.6%.

The double-sorting efficiency at the diagnosis was high around 95%; however for the samples collected in long term after the chemotherapy started, we could not reach high capture efficiencies. The number of CD10 + CD19+ cells decreased significantly, and we observed that the optimized immunomagnetic separation protocols for the diagnosis need reoptimization for analyzing few cells. Even though the immunomagnetic separation did not perform well for long term samples, we were still able to detect the increase in the number of hematogones in FC measurements and in microfluidic chip measurements.

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Author contributions

K.I., E.U., S.O. designed the experiments, A.M., S.O., E.U. and K.I conducted the experiments, S.C., A.E. G.D. conducted FC measurements and analyzed the data, all authors interpreted the data and wrote the manuscript.

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