Original Article

Optimization of EGFR High Positive Cell Isolation Procedure by Design of Experiments Methodology

Ofer Levi,[1] Baruch Tal,[1] Sagi Hileli,[2] Assaf Shapira,[3] Itai Benhar,[3] Pavel Grabov,[2] and Noam Eliaz[1]*

[1]Biomaterials and Corrosion Lab, Department of Materials Science and Engineering, Tel-Aviv University, Ramat Aviv 6997801, Israel

[2]Advanced Logistics Development Ltd, Tel Aviv 6713701, Israel

[3]Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Ramat Aviv, Tel-Aviv 6997801, Israel

Background: Circulating tumor cells (CTCs) in blood circulation may play a role in monitoring and even in early detection of metastasis patients. Due to the limited presence of CTCs in blood circulation, viable CTCs isolation technology must supply a very high recovery rate.

Methods: Here, we implement design of experiments (DOE) methodology in order to optimize the Bio-Ferrography (BF) immunomagnetic isolation (IMI) procedure for the EGFR high positive CTCs application. All consequent DOE phases such as screening design, optimization experiments and validation experiments were used.

Results: A significant recovery rate of more than 95% was achieved while isolating 100 EGFR high positive CTCs from 1 mL human whole blood.

Conclusions: The recovery achievement in this research positions BF technology as one of the most efficient IMI technologies, which is ready to be challenged with patients' blood samples.

Key terms: bio-ferrography; carcinoma; design of experiments; EGFR; flow cytometry; immunomagnetic isolation

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Circulating tumor cells (CTCs) in blood play a role in spreading metastases to secondary tumor sites. Detecting the presence of CTCs in the circulating blood outside the primary tumor can serve the purposes of monitoring treatment in patients, metastasis prognosis, and even early detection (1,2).

There are various technologies for isolating CTCs from patients' blood samples (3). An ideal technology should be able to detect a small number of antigen-positive cells from a background of human whole blood (HWB). Immunomagnetic isolation (IMI) technologies are based on the overexpression of proteins on the cell surface. Few IMI-based technologies with high recovery rates have been reported. Yet, the only FDA-approved IMI technology is the CellSearch™ (4).

Bio-Ferrography (BF) allows the magnetic isolation of target cells and tissues on a microscope slide (ferrogram) and their microscopic, chemical, and biological characterization while preserving their original shape (5–8). BF has been evaluated only three times in cancer research. Fang et al. isolated rare MCF-7 breast carcinoma cells from a background of human peripheral leukocytes (9). The samples were spiked with a constant number of 30 target cells, and the recovery rate of the MCF-7 cells was about 30%. In another work (10), CD4

*Correspondence to: Noam Eliaz, Biomaterials and Corrosion Lab, Department of Materials Science and Engineering, Tel-Aviv University, Ramat Aviv 6997801, Israel. E-mail: neliaz@eng.tau.ac.il

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cells were isolated from leukocytes and murine lymphoma cells in human peripheral blood. No recovery rate values were reported.

Recently, we have demonstrated (11) the ability of BF to isolate rare epidermal growth factor receptor (EGFR) high positive tumor cells from HWB. Recovery rates of $49 \pm 11\%$ were achieved, predominantly due to improvement of certain immunological aspects. The effect of process variables on the recovery rate was primarily determined based on previous works and protocols.

Allard et al. (4) used CellSearch™ to isolate CTCs from various types of cancer patients in numbers ranging from only a few to approximately fifty. Thus, it is assumed that only few CTCs can be found in 1 mL human blood and that the recovery rate must be maximized toward 100%.

This study implements design of experiments (DOE) methodology in order to maximize the recovery rate (compared to 49% that has already been demonstrated in Ref [11]). DOE plays an important role in science and engineering (12,13). In an experiment, some input x's transform into an output that has at least one observable response variable, y. The approximation of the response function $y = f(x_1, x_2, \ldots, x_q)$ can be derived using DOE by systematically studying the effects of experimental factors on the response variable of interest. The number of the experiments required depends on the number of involved factors (i.e., independent variables affecting the analyzed response variable) as well as on the number of levels per factor (i.e., particular settings of each factor). Since a large number of experiments is both time consuming and costly, the so-called *fractional factorial design* was used in this study to carry out a realistic number of experiments.

Here, we optimize the process by implementing DOE methodology in order to isolate a very small number of 100 target cells from a small volume (1 mL) of HWB with a recovery value approaching 100%.

MATERIALS AND METHODS

A431Cell Line

In this study, cells of the A431 epidemoid carcinoma cell line were used as EGFR overexpressing target cells. The A431 cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 12.5 U/mL nystatin (Biological Industries, Israel) in a humidified 5% CO_2 incubator at 37°C. The A431 cells were transfected to stably express a red (mCherry) fluorescent protein. The A431 target cells were fixed with 4% formaldehyde before any isolation procedure.

Human Whole Blood Samples

HWB samples were drawn from blood doses of healthy donors that were tested and supplied by MDA Israel blood services. The use of human blood for this study was approved by the Tel-Aviv University Ethics Committee. To simulate blood containing CTCs, the HWB samples were spiked with 100 A431 target cells at the beginning of each isolation procedure.

Isolation Procedure

The isolation procedure is comprised of an immunomagnetic labeling stage and an isolation by BF stage.

The immunomagnetic labeling stage is as follows:

The capture Ab EGFR (R-1) mouse-anti-human IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 0.2 mg/mL) was mixed with Miltenyi MicroBeads conjugated to monoclonal rat-anti-mouse IgG antibodies (Miltenyi Biotec, Inc., Auborn, CA) and incubated at 7°C for 20 minutes. This mixture will hereafter be referred to as the *cocktail suspension*. Subsequently, 1 mL of HWB samples, spiked with 100 target cells, (A431) were prepared. At least three drops were drawn to a slide prior any experimentation in order to verify the inlet amount of 100 spiking target cells. The target cells were counted for each drop and averaged. The average value (i.e., "the inlet amount of target cells") was used for the recovery rate calculation in every run. The standard-deviation (STDV) of the three drops counting results remained very low in all runs. For example, in the validation phase experiments, the STDV's average and median were 1.5 cells at the inlet amount of spiking cells.

The blood samples were washed (no red blood cell lysis procedure was applied on the HWB samples), incubated with the cocktail suspension at −20°C using an orbital shaker (for mild shaking), followed by washing steps. This stage may be divided into two parts: (1) cocktail incubation, and (2) blood sample incubation.

The isolation by BF was conducted as follows, using a Bio-Ferrograph 2100 (Guilfoyle Inc., Belmont, MA) (6), see Figure 1.

All samples were washed before BF separation and filled with phosphate-buffered solution (PBS) to a fixed volume of 0.5 mL. During the BF separation process, the capture cell and reservoir were filled with 0.5 mL PBS at a flow rate of 0.1 mL/minute. This was followed by inserting the sample with the target cells into the reservoir, and isolating the target cells on the capture band at a defined flow rate. Finally, the chambers were washed with PBS at the same flow rate. The slide with the isolated cells was separated from the BF and examined under an inverted microscope (Olympus IX71) using the fluorescence mode for identifying and counting the isolated cells. The recovery rate (response variable) was calculated according to Eq. (1):

$$\text{Recovery } [\%] = \frac{\text{Amount of cells observed at the capture field [cells]}}{\text{Amount of cells counted at the inlet of the process [cells]}} \quad (1)$$

At least three replicates were made for each run. Each replicate was divided by the inlet amount of target cells that was averaged before that specific run in order to

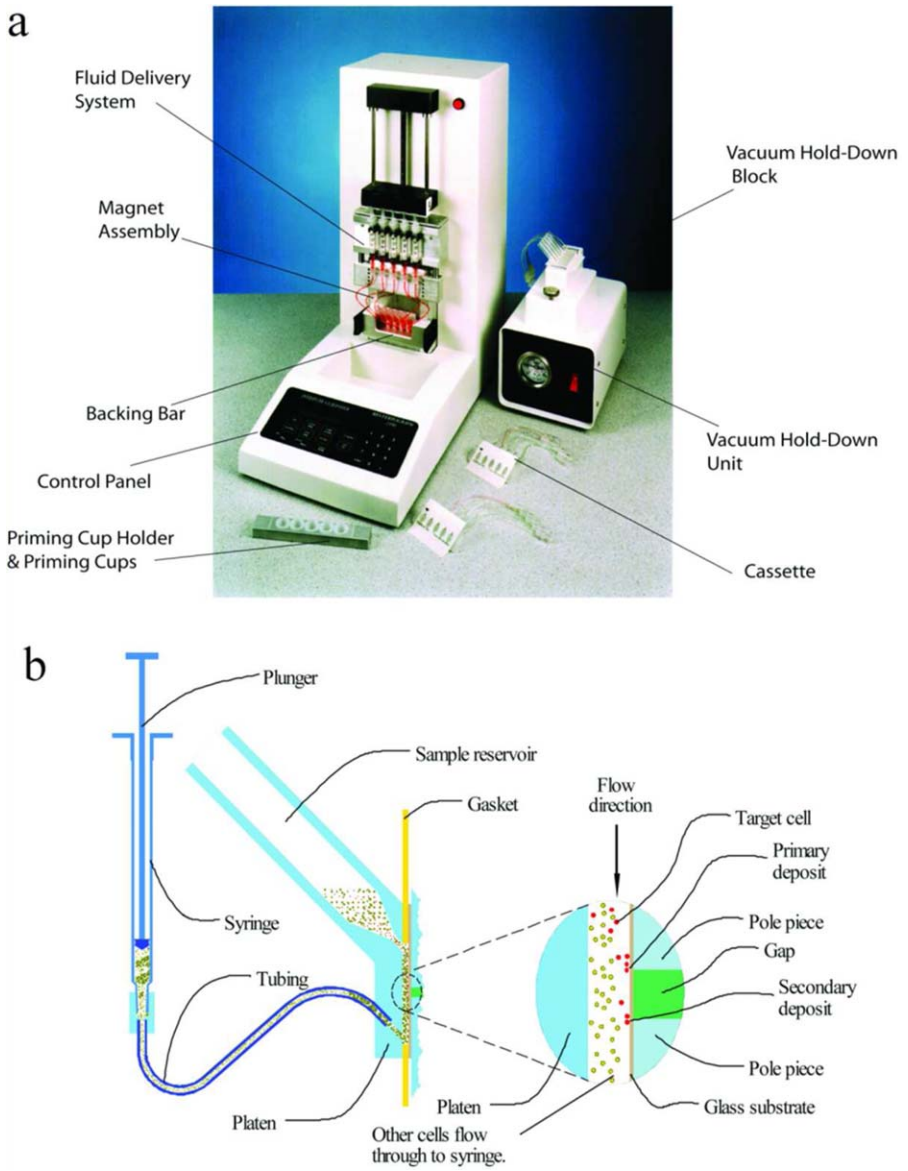

FIG. 1. (**a**) The components of the Bio-Ferrograph 2100 (Guilfoyle, Inc.); (**b**) The deposition scheme of captured particles on the slide (5,6). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

obtain the recovery rate in Eq. (1). The mean recovery rate values reported in this manuscript are the averages of the three calculated recovery rates.

Optimization Methods

Understanding the potential effects of independent variables (factors) on the dependent variable recovery rate (response variable) is one of the most critical parts in the optimization process. A preliminary phase of minimizing the number of factors was applied. The preliminary phase was based on: (1) learning the previous studies, (2) conducting experiments such that only one factor was changed in each experiment, keeping all other factors constant, (3) dividing the whole process to two sections that could be optimized independently. The two sections were (a) the application-specific cocktail suspension preparation, and (b) the labeling and isolation by BF. Section a is specific to each application, while section b is generic and will be the same for all applications.

The application-specific optimization

This section includes four factors: the concentration of capture Ab (EGFR(R-1)), the volume of the microbeads suspension (the concentration values are Miltenyi's proprietary), the incubation volume, and the duration. In accordance with the microbeads' manufacturer instructions, the duration of the incubation was set to 20 minutes at 2–7°C. Referring to preliminary data, the incubation volume was set at 0.3 mL. The concentration

Table 1
The Results of the Experiments in the Specific Section Screening Phase (Run #1–#7) and the Optimization Phase (Run #8–#11).

Run #	x_1 level	x_2 level	x_1 value [μg/μL]	x_2 value [μL]	y	STDV
1	−	−	0.4	2	44.3%	3.0%
2	+	−	1	2	33.8%	2.9%
3	−	+	0.4	11	63.3%	3.7%
4	+	+	1	11	**72.5%**	1.2%
5	0	0	0.7	6.5	63.6%	1.6%
6	0	0	0.7	6.5	59.5%	0.8%
7	0	0	0.7	6.5	63.6%	1.6%
8			0.6	11	**77.7%**	8.5%
9			0.6	15	64.3%	4.6%
10			0.6	20	55.3%	6.7%
11			0.6	25	46.8%	1.8%

of the capture antibody and the volume of the microbeads suspension remained the main factors to be optimized.

A set of experiments was designed according to first-order type of response surface methodology (RSM). Table 1 shows the levels that were chosen to be tested. x_1 is defined as the 'half interval' of the concentration of capture Ab at upper ("+") and lower ("-") levels of 0.4 μg/μL and 1 μg/μL, respectively. x_2 is defined as the 'half interval' of the volume of microbeads suspension at upper and lower levels (2 μL and 11 μL, respectively). y is the recovery rate.

Eq. (2) defines the surface lines for every x_1-x_2 couple. The resulting y from Eq. (2) can then be used to confirm Eq. (3). The coefficients of the surface lines can be determined by Eq. (4).

$$y = \beta_0 + \beta_1 x1 + \beta_2 x2 + \varepsilon \tag{2}$$

$$\hat{y}_i = b_0 + b_1 x_{1i} + b_2 x_{2i} \tag{3}$$

$$b_0 = \frac{1}{7}\sum\nolimits_i y\mathrm{i}; b_1 = \frac{1}{4}\sum\nolimits_i y_\mathrm{i} \times x_{1\mathrm{i}}; b_2 = \frac{1}{4}\sum\nolimits_i \mathrm{y_i} \times x_{2\mathrm{i}} \tag{4}$$

Given b_0, b_1 and b_2, the slope for every $\{y = \text{const.}\}$ can be calculated by Eq. (5):

$$\tan\theta = -\frac{b_1}{b_2} \tag{5}$$

According to the RSM, to maximize y (recovery rate), the x_1 and x_2 values should fall on a line perpendicular to the surface lines. This line should intersect the set point (0,0) in intervals ratio that can be determined by Eq. (6):

$$x_2 = -\frac{1}{\tan\theta} x_1 \tag{6}$$

Additional experimental tests of x_1 and x_2 values according to Eq. (6) allowed finding the optimized values for the highest recovery value.

The effect of each factor and its variance can be determined by Eqs. (7) and (8), respectively:

$$\text{Effect} = \frac{1}{2^{k-1}}\left[\sum_i \delta_i \times y_i\right] \tag{7}$$

$$\text{Var}[\text{Effect}] = \frac{s^2}{m \times 2^{k-2}},\ s^2 = \frac{\left[\sum_i (m_i - 1) s_i^2\right]}{\left[\sum_i (m_i - 1)\right]} \tag{8}$$

The generic section optimization

This part of the study included three phases: (1) a screening phase to examine the effect of each of the suspected eight factors and to choose the most critical ones, (2) an optimization phase to find the optimal values for the most critical factors, and (3) a validation phase to prove the optimized recovery values under different uncontrolled operational conditions.

The screening phase. The so-called L27 Taguchi plan of fractional factorial design (14) was used to reduce the number of experiments from the original 6,561 (required to cover all possible combinations of the involved eight factors) to a mere 27 experiments. L27 allows for the evaluation of both linear and non-linear effects of all factors. After model analysis performance, using the desirability function approach (14), the optimal values of each factor were found.

Optimization phase. The optimization phase focused on the three critical factors identified by the screening phase. The levels chosen were in the vicinity of the optimal values for these factors (the so-called "best of screening"). L9 Taguchi plan of fractional factorial design (14) was used in these experiments, reducing the number of experiments required from 27 to 9. It was decided that additional experiments (corresponding to the Box–Behnken (14) design plan for response surface modeling) would be added, if it would be found necessary after execution and analysis of the experiments. This flexible strategy allowed for the reduction of the number of experiments, while at the same time maintaining high experimental effectiveness. The optimal values were found using the desirability function approach (14).

Validation phase. The main goal of the validation phase was: (1) to determine the recovery rate at optimal values of the critical controlled factors in the presence of uncontrolled external factors (noise), and (2) to determine the sensitivity of the optimized process to these external factors. Three uncontrolled external factors were chosen: patient blood type, the time passed since the patient was sampled until the BF process was run, and the target cells that each sample contained. Three different patients and two levels of target cell numbers were examined within the range of 24 to 72 hours from patient sampling. As a complimentary step, seven different people and five different values of target cells were examined in a "One-Factor-at-a-Time" method.

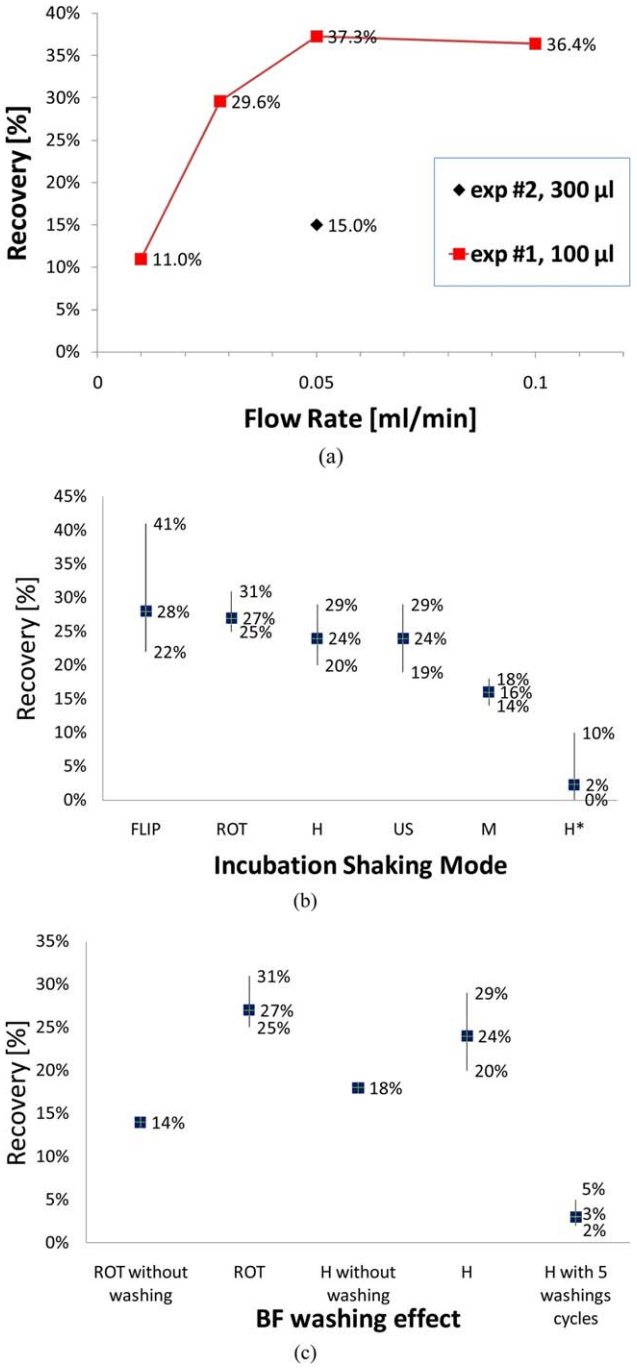

FIG. 2. The preliminary experiments results for factors number reduction: (**a**) the dependence of the recovery rate on the capture flow rate during isolation by BF ($n = 1$). The effect of sample volume is evident when comparing the data points in exp #1 and exp #2 for the same flow rate of 0.05 mL/minute ($n = 1$). (**b**) The effect of incubation shaking mode on the recovery rate. The squares indicate the averages of the results. FLIP—flip ($n = 4$), ROT—rotation ($n = 4$), H—manually, 6 times in 1 h ($n = 5$), US—ultrasonic ($n = 3$), M—motorized vortex ($n = 2$), H* - manually, coincidentally, 1–3 times in 1 h ($n > 3$). (**c**) The effect of the washing stage during BF on the recovery rate. It can be noticed that both no washing and excessive cycles of washings lower the recovery value ($n = 2$, except $n = 1$ for 18% and 14%). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS

Preliminary Factors Reduction in the Isolation Procedure

Analysis of the BF process as a whole can lead to the suspected factors that may affect the recovery rate. The following potential factors were considered.

The immunomagnetic labeling stage (14 factors). Ab concentration, microbead suspension volume, cocktail and blood samples incubation conditions (volume, duration, temperature, shaking mode and frequency), and washing conditions (number of steps, volume, duration, temperature, centrifuge force).

The BF isolation stage (seven factors). Priming conditions (volume and flow rate), isolating conditions (sample volume and flow rate), and washing conditions (volume, number of steps, and flow rate).

Altogether, 21 factors were thus considered for the whole procedure. Assuming that each factor should be examined at two levels, for min and max boundaries only, a total amount of 2^{21} (= 2,097,152) experiments are required. Since each experiment lasts for more than 4 h, this number of experiments is not realistic.

Previous studies demonstrated the necessity to develop a work procedure for each application, in particular optimizing the ratios between target cells/bacteria, antibodies and magnetic microbeads (15). The capture flow rate was adjusted in a previous application (8). The BF manual recommends a capture flow rate of 0.05 mL/min, but it is noted that this factor should be optimized for every new application. Differences in the washing step can be found in the literature. Evans et al. (16) applied three washing cycles at a centrifugation force of 3000*g*, while Fang et al. (9) applied only two washing cycles at a force of 300*g*. Preliminary experiments were conducted to evaluate the effect of the aforementioned factors. Figure 2 summarizes these preliminary experiments. Finally, the whole process was divided to two sections: (1) a specific section, and (2) a generic section.

The following factors were chosen to be optimized:

- For the specific section: Ab and microbead concentrations (two factors).
- For the generic section: blood sample labeling incubation conditions (volume, duration and shaking frequency), washing conditions (number of steps and centrifuge force), isolation conditions (sample volume and flow rate), and the volume of the final washing at the BF (8 factors).

Specific Section Optimization

The chosen factors of Ab concentration and microbeads suspention volume were tested at two levels as shown in Table 1. The optimization process is divided into two phases: a screening phase (Table 1, Runs #1–7) and an optimization phase (Table 1, Runs #8–11). The surface lines comply with the following relation:

$$y = 57.2 - 0.33x_1 + 14.4x_2 \quad (9)$$

where x_1 and x_2 are the 'half intervals' between the upper and the lower levels of the factors. The steepest slope that allows for the parameters to be extracted to the highest recovery rate is associated with the following interval factors ratio:

$$x_2 = -44.4x_1 \quad (10)$$

Evaluating the effects of the factors and the variance, one may conclude that the effect of the microbeads suspension volume is 28.8, whereas the effect of the concentration of the capture Ab is −0.66, with variance of 2.8 for both effects. These results show that the microbeads suspension volume affects the recovery rate significantly, while the effect of the Ab's concentration is both in the opposite direction, and negligible.

Theoretically, for every reduction in the concentration of the Ab's 'half interval', the microbeads suspension volume should be increased by 44.4-times its own 'half interval'. The interval ratio of 44.4 is very large due to the neglected value of the Ab effect. Thus, progression toward the optimal values required lowering the Ab concentration values by a third of the half interval, and increasing the microbeads suspension volume gradually, as shown in Table 1. The results of the optimization phase (Table 1, Runs #8–11) revealed a maximal value of recovery rate (77.7%) with a STDV of 8.5% for concentrations of 0.6 μg/μL capture Ab and 11 μL microbeads suspension volume.

Generic Section Optimization

Screening phase

The screening phase aimed to evaluate the effect of each factor on the recovery rate. Twenty-seven fractional factorial experiments were designed for the eight factors, at three levels each, with at least three repetitions for every experiment. The rationale behind choosing three levels was to check possible non-linear effects of the factors on the response variable.

The following eight factors were evaluated: washing steps ("# Wash"), incubation duration ("Inc T"), incubation volume ("Inc V"), sample volume ("Samp V"), washing volume ("Wash V"), centrifuge force ("g"), incubation r.p.m. ("RPM"), and BF flow rate ("Flow Rate"). Tables 2 and 3 present each factor, its levels, the set of experiments, and their results

Three critical factors were identified from the screening experiments: the flow rate, the sample volume, and the centrifuge force. The effects of both the flow rate and the sample volume are characterized by linear dependence, whereas the effect of the centrifuge force is quadratic. A stepwise regression setting was used for the model construction, while the choice of predictive variables was carried out by an automatic procedure taking the form of a sequence of *t*-tests and adjusted *R*-square criteria (14). Equation 11 represents the model describing the dependence of the response rate on the three aforementioned factors.

Table 2
The Generic Screening Phase Levels

Factor	("−") Level	("0") Level	("+") Level
# Wash [#]	1	2	3
Inc T [min]	25	105	185
Inc V [mL]	0.9	1	1.5
Samp V [mL]	0.5	1	2
Wash V [mL]	0.1	0.5	1.5
g [rcf]	100	300	600
RPM [rpm]	50	100	150
Flow Rate [mL/min]	0.01	0.05	0.25

$$y = -0.279388236 + 0.183196196[Samp\ V] + 0.00307853[g] - 0.94155346\ [Flow\ rate] - 0.00000357[g]^2 \quad (11)$$

The coefficient of determination, *R*-squared, was very high (0.87), reflecting the strong predictive power of the model. After model analysis, the optimal values of each factor were found: 431 g, sample volume of 2 mL and a flow rate of 0.01 mL/min. These levels were subsequently chosen as a basis for the optimization phase (i.e., "the best of screening").

Optimization phase

The optimization phase focused on the three critical factors identified by the screening phase. Tables 4 and 5 present the factors, levels and experimental results. These results were used to derive Eq. (12) (using the standard procedure of the least square method, LSM), which shows the dependence of the recovery rate on the three critical factors:

$$y = -3.3538682 + 1.7855983[\text{Samp } V] + 0.0113244[g] + 26.485736[\text{Flow Rate}] - 0.12419227[\text{Sample } V]^2 - 0.0000078[g]^2 - 1216.6266314[\text{Flow Rate}]^2 - 0.0032221[\text{Sample } V] \times [g] + 0.0615314[\text{Flow Rate}] \times [g] \quad (12)$$

The coefficient of determination, *R*-squared, for this model was very high (0.996), reflecting the high predictive power of the model. In a comparison between this model and the model from the screening phase, the optimal values deduced from the screening model (431 g, sample volume of 2 mL, and flow rate of 0.01 mL/min) were applied to the optimization model.

The comparison revealed high similarity between the results: recovery rate of 81% according to the screening model, and 80% according to the optimization model.

Table 3
The Output of Experiments in the Generic Screening Phase

#	Pattern	Mean #wash	Mean Inc *T*	Mean Inc *V*	Mean samp *V*	Mean wash *V*	Mean *g*	Mean RPM	Mean flow rate	Mean Recov.
1	−−−−−−−−	1	25	0.9	0.5	0.1	100	50	0.01	15.60%
2	−+++−−−+	1	185	1.5	2	0.1	100	50	0.25	8.20%
3	−−−−++++	1	25	0.9	0.5	1.5	600	150	0.25	19.00%
4	−++++++0	1	185	1.5	2	1.5	600	150	0.05	54.50%
5	−+++000−	1	185	1.5	2	0.5	300	100	0.01	**79.40%**
6	+−+0−+0−	3	25	1.5	1	0.1	600	100	0.01	38.00%
7	++0−−+0+	3	185	1	0.5	0.1	600	100	0.25	10.00%
8	+−+0+0−+	3	25	1.5	1	1.5	300	50	0.25	21.00%
9	++0−+0−0	3	185	1	0.5	1.5	300	50	0.05	79.10%
10	++0−0−+−	3	185	1	0.5	0.5	100	150	0.01	5.40%
11	+−+00−+0	3	25	1.5	1	0.5	100	150	0.05	3.70%
12	+0−++0−−	3	105	0.9	2	1.5	300	50	0.01	59.90%
13	+0−+−+00	3	105	0.9	2	0.1	600	100	0.05	73.60%
14	+0−+0−++	3	105	0.9	2	0.5	100	150	0.25	3.80%
15	0+−0+−00	2	185	0.9	1	1.5	100	100	0.05	18.20%
16	0+−00+−−	2	185	0.9	1	0.5	600	50	0.01	41.30%
17	0+−0−0++	2	185	0.9	1	0.1	300	150	0.25	41.20%
18	00+−+−0−	2	105	1.5	0.5	1.5	100	100	0.01	7.50%
19	0−0++−0+	2	25	1	2	1.5	100	100	0.25	5.00%
20	0−0+−0+−	2	25	1	2	0.1	300	150	0.01	26.20%
21	00+−0+−+	2	105	1.5	0.5	0.5	600	50	0.25	17.90%
22	00+−−0+0	2	105	1.5	0.5	0.1	300	150	0.05	29.90%
23	0−0+0+−0	2	25	1	2	0.5	600	50	0.05	59.30%
24	−000+++−	1	105	1	1	1.5	600	150	0.01	42.90%
25	−000−−−0	1	105	1	1	0.1	100	50	0.05	28.80%
26	−−−−0000	1	25	0.9	0.5	0.5	300	100	0.05	26.20%
27	−000000+	1	105	1	1	0.5	300	100	0.25	34.10%

An ultimate recovery rate of 97% can be achieved according the optimization model when substituting values of 400 g, sample volume of 2 mL, and flow rate of 0.021 mL/min. Figure 3 presents the target cells on a slide that underwent the optimal procedure.

Validation Phase

Tables 6 and 7 list the results of the validation experiments for each uncontrolled factor. From Table 7 it is evident that the average recovery rate at optimal values of critical controlled factors in the presence of the uncontrolled external factors is 97%. The *P*-values indicate that the time since sampling is an insignificant factor (0.76), while both the number of target cells (0.02) and the blood type (0.01) should be further analyzed at various levels. An additional seven blood samples from different patients were thus examined. The mean recovery rate value was 91.3%, with a STDV of 6.3%. The number of target cells was also examined at various values. Figure 4 shows the linear behavior ("calibration curve") of the recovery rate, with *R*-square = 0.994 and a mean value of 100%.

Table 4
The Phase Levels at the Generic Phase of Optimization

Factor	("−") level	("0") level	("+") level
Samp V [mL]	1.5	2	2.5
g [rcf]	300	400	500
Flow rate [mL/min]	0.008	0.02	0.035

DISCUSSION

The most significant achievement in this study is the outstanding recovery rates for the isolation of a very small number of target cells suspended in a small volume (1 mL) of HWB. This achievement is due to the careful implementation of DOE methodology. The first major contributor to the improvement of the recovery rate was the idea to optimize the specific section factors using the RSM approach. This then led to an additional significant improvement of 29% in the recovery rate. The RSM focused on two variables only and their influence on the entire process: the concentration of the capture antibody and the magnetic microbeads

Table 5
Experiments and Results of the Generic Optimization Phase

#	Pattern	Mean Samp *V* [mL]	Mean *g* [rcf]	Mean flow rate [mL/min]	Mean recov.
1	−−−−−−−−	1.5	300	0.008	58.10%
2	−+++−−−+	1.5	500	0.035	87.70%
3	−−−−++++	2.5	300	0.035	70.59%
4	−++++++0	2.5	500	0.02	70.14%
5	−+++000−	2	500	0.008	61.11%
6	−000+++−	2.5	400	0.008	72.18%
7	−000−−−0	1.5	400	0.02	94.96%
8	−−−−0000	2	300	0.02	89.43%
9	−000000+	2	400	0.035	74.54%

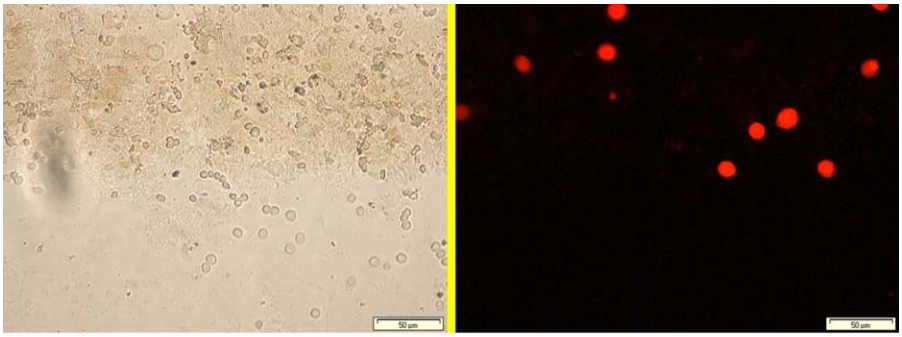

FIG. 3. Target cells in the capture band on a microscope slide (ferrogram) after isolation by the optimal procedure. The well-preserved condition of the cells can be noticed, both in a bright-field image (**a**) and in a red fluorescence image (**b**). Scale bars equal 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

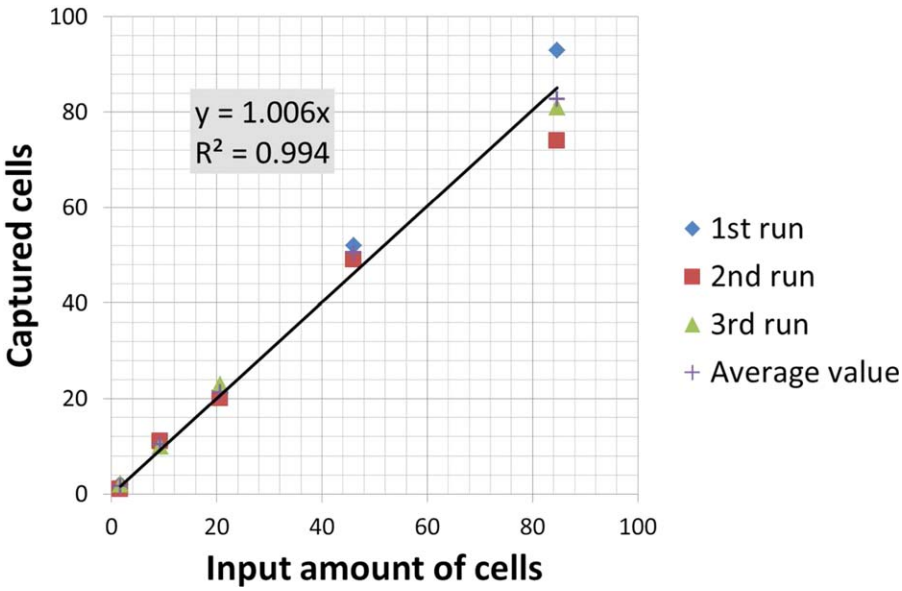

FIG. 4. The "calibration curve." Note the high value of R^2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

suspension volume. Our study showed that both variables should be matched. Low values of magnetic microbeads suspension volume will not utilize the full potential of conjugations with the antibodies. In contrast, high values of magnetic microbeads suspension volume might exceed the potential conjugations with the Ab, so that the excessive microbeads float unconjugated in the suspension during the whole process. Their exposure to the capture field on the BF might lead to interruptions, disorders, and even to partial saturation of the capture field, and thus the reduction of the effective magnetic force that acts on the target cells. Low concentrations of antibody will not utilize the full potential EGFR conjugations, and thus the capability of the cell to be magnetized will not be optimal. On the other hand, high concentrations of Ab's might lead to EGFR saturation on the cell surface, and to unwanted conjugations of microbeads with the excess Ab's thereby preventing them from binding to the cell membrane. Without matching the right quantities of both variables, the optimal recovery value cannot be reached.

Implementation of the DOE methodology allowed for the achievement of extraordinarily high recovery rate values, and at the same time significant reduction in the number of experiments performed.

Table 6
The Levels of the Factors at the Validation Phase

Factor	"0" Level	"+" Level
Time since sampling [h]	72	Up to 24
Number of target cells	100	50
Blood type	A/B	C

Table 7
The Results of the Validation Phase Experiments

#	Pattern	Time passed since sampling [h]	Number of target cells	Blood type	Mean Recov.
1	00+	Up to 24	100	A	89.4%
2	00+	Up to 24	100	B	98.0%
3	0+0	Up to 24	50	C	100.1%
4	+00	72	100	C	93.8%
5	+++	72	50	A	94.6%
6	+++	72	50	B	102.8%
7	000	Up to 24	100	C	95.6%

The optimization of the generic section revealed three critical factors: the centrifugation force, the sample volume and the flow rate. The significance of the first factor may be explained in its relation to the effectiveness of the serum, excessive microbeads, and excessive conjugate Ab's removal. The last two factors may be related to one another. Both deal with the amount of target cells simultaneously exposed to the capture field. The third factor also deals with the amount of background cells simultaneously exposed to the capture field per target cell. A flow rate of 0.021 mL/min for 2 mL sample volume gives an isolation time of 95 minutes. In addition to the incubation times of 2 h and 20 minutes, the total duration of isolation can reach almost 4 h. In comparison, the only FDA-approved IMI technique, CellSearch™, requires 4 h for a complete run (17), hence no significant abnormalities are expected.

Figure 5 presents the sequence of all phases in the course of this study. One can see that at the starting point, the recovery rate was <50%. The optimization of the specific section resulted in a recovery rate of 78% (not shown at Fig. 5). The screening experiments started with eight subspecies factors possibly affecting the response variable. Using fractional factorial design with 27 experiments it was shown that in reality only three factors affect the recovery rate, the value of which was 81% at the optimal values of these three factors. Optimization experiments planned and performed in the vicinity of the "best of screening" area allowed for further improvement in the recovery rate to 95%. Only nine experiments were performed, using the reduced version of the Box–Behnken design plan for response surface modeling. Finally, validation experiments at optimal values of the critical controlled factors were performed in the presence of the uncontrolled external factors (noise). A high recovery rate of 97% was achieved.

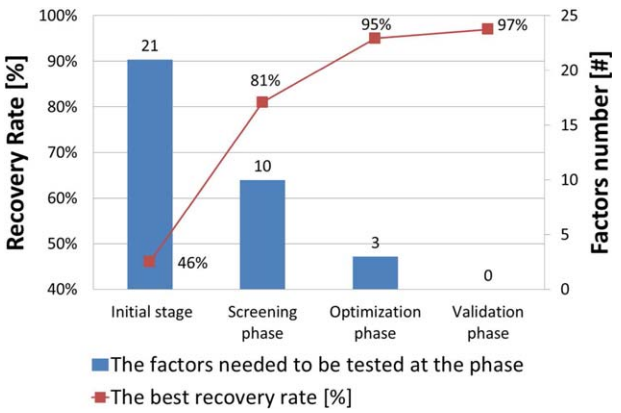

FIG. 5. The evolution of recovery rate values in different phases of this study. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

BF was proven to be an efficient isolation technique for EGFR high positive CTC's from small volume (1 mL) of HWB. The high recovery rate that was achieved in this study, as well as the successful approval procedure that was conducted in our recent study (11), favor the application of this technology for blood samples from patients, either as a monitoring tool or for early detection of cancer.

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