Enrichment of rare cancer cells through depletion of normal cells using density and flow-through, immunomagnetic cell separation

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(Received 6 November 2003; revised 6 July 2004; accepted 12 July 2004)

Objective. To develop a reliable technique to enrich for rare cells in blood suspensions using only negative selection steps including a flow-through immunomagnetic cell separations system and by optimizing variables normally encountered during such enrichment processes.

Methods. A human breast cancer cell line was cultivated and spiked at a ratio of 1 cancer cell to $10^5$ total leukocytes in buffy coat or 1 cancer cell to $10^8$ total cells in whole blood samples. The final, optimized process consisted of: a red cell lysis step, immunomagnetically staining leukocytes with an anti-CD45 PE, anti-MACS sandwich, immunomagnetic sorting using a flow-through system (QMS), and a final cell analysis step using either an automated cell counter, filtration, and visual counting or a cytospin analysis.

Results. The final, optimized process produced a final enrichment of the rare cancer cells of 5.17 log\textsubscript{10} and an average, final recovery of 46%. It should be noted that a negative depletion protocol was used (i.e., no labeling of the rare cancer cells was used).

Conclusions. To the authors’ knowledge, no examples in the literature exist of a 5.17 log\textsubscript{10} enrichment of cancer cells in human blood using a negative depletion protocol. The closest example is a 4 log\textsubscript{10} enrichment in which two positive magnetic cell separation steps were used (none were used in this study). Ongoing studies are investigating further modifications of the precommercial, prototype flow-through immunomagnetic separation system to increase both the enrichment and recovery rate. However, even at current performance levels, the presented process could significantly improve visual and molecular analysis of rare cells in blood.
Table 1. Some examples of rare Cancer cell enrichment in blood

<table>
<thead>
<tr>
<th>Target cell</th>
<th>Cell sample</th>
<th>Selection</th>
<th>Antigen for IMS</th>
<th>Detection method</th>
<th>Initial purity (# target cells / total cells)</th>
<th>Recovery of target cells</th>
<th>Enrichment</th>
<th>Sensitivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunomagnetic: MACS (Miltenyi Biotec, Germany)</td>
<td></td>
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<tr>
<td>Melanoma CL</td>
<td>SK-Mel-30 in PB</td>
<td>Positive</td>
<td>MCSP</td>
<td>ICC</td>
<td>50–1000 per 50 mL PB</td>
<td>11.4%</td>
<td>—</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Renal carcinoma CL</td>
<td>Caki-1 in PBL</td>
<td>Positive</td>
<td>CK</td>
<td>ICC</td>
<td>1/10–1/10^3</td>
<td>73.6%</td>
<td>—</td>
<td>—</td>
<td>12</td>
</tr>
<tr>
<td>Breast cancer patients</td>
<td>PB, BM, PBPC</td>
<td>Positive</td>
<td>HEA-125</td>
<td>ICC</td>
<td>6/10^6</td>
<td>24.4%</td>
<td>6.8</td>
<td>—</td>
<td>21</td>
</tr>
<tr>
<td>Breast cancer CL</td>
<td>BT474 in PBL</td>
<td>Positive</td>
<td>CK-8</td>
<td>ICC, FC</td>
<td>4/10^6–4/10^5</td>
<td>57.7%</td>
<td>10477</td>
<td>—</td>
<td>22</td>
</tr>
<tr>
<td>Renal carcinoma CL</td>
<td>Caki-1 in PBL</td>
<td>Positive</td>
<td>CD45</td>
<td>ICC</td>
<td>1/10–1/10^3</td>
<td>84.3%</td>
<td>—</td>
<td>—</td>
<td>12</td>
</tr>
<tr>
<td>Gastric cancer CL</td>
<td>MKN-45 in PBL</td>
<td>Negative</td>
<td>CD45</td>
<td>FC</td>
<td>1/10–1/10^6</td>
<td>—</td>
<td>9</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>Colorectal cancer CL</td>
<td>SW-480 in PBL</td>
<td>Positive</td>
<td>CD45</td>
<td>RT-PCR</td>
<td>1/10–1/10^6</td>
<td>—</td>
<td>1/10^6 MMC</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>Immunomagnetic: Dynabeads (Dynal, Norway)</td>
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<tr>
<td>Cancer CL</td>
<td>T47D in PBL</td>
<td>Positive</td>
<td>MUC-1</td>
<td>RT-PCR</td>
<td>1/10^3–1/10^5</td>
<td>—</td>
<td>—</td>
<td>1/10^5 MMC</td>
<td>10</td>
</tr>
<tr>
<td>Urological malignant CL</td>
<td>5 CLs in PBL</td>
<td>Positive</td>
<td>Ber-EP4</td>
<td>ICC</td>
<td>4/10^3–4/10^5</td>
<td>—</td>
<td>15.3</td>
<td>—</td>
<td>11</td>
</tr>
<tr>
<td>Colorectal cancer CL</td>
<td>SW-480 in PBL</td>
<td>Positive</td>
<td>Ber-EP4</td>
<td>RT-PCR</td>
<td>1/10^3–1/10^5</td>
<td>—</td>
<td>—</td>
<td>1/10^5 MMC</td>
<td>15</td>
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<tr>
<td>Colon cancer CL</td>
<td>SNPC4 in PB</td>
<td>Positive</td>
<td>CEA</td>
<td>RT-PCR</td>
<td>1–10^7/mL PB</td>
<td>—</td>
<td>—</td>
<td>10^1/mL PB</td>
<td>18</td>
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<tr>
<td>Head and neck SCC</td>
<td>PB, BM</td>
<td>Positive</td>
<td>Ber-EP4</td>
<td>ICC</td>
<td>1–25/10^6</td>
<td>22–32%</td>
<td>—</td>
<td>—</td>
<td>23</td>
</tr>
<tr>
<td>Cancer CL</td>
<td>5 CLs in PBL</td>
<td>Positive</td>
<td>Ber-EP4</td>
<td>ICC</td>
<td>—</td>
<td>9–45%</td>
<td>—</td>
<td>—</td>
<td>24</td>
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<tr>
<td>Urological malignant CL</td>
<td>5 CLs in PBL</td>
<td>Negative</td>
<td>CD45</td>
<td>ICC</td>
<td>4/10^3–4/10^4</td>
<td>—</td>
<td>57.3%</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>Head and neck SCC</td>
<td>PB, BM</td>
<td>Negative</td>
<td>CD45</td>
<td>ICC</td>
<td>1–25/10^6</td>
<td>—</td>
<td>36–62%</td>
<td>—</td>
<td>23</td>
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<td>Immunomagnetic: Immunicon (USA)</td>
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<tr>
<td>Breast cancer CL</td>
<td>SKBr3 in PB</td>
<td>Positive</td>
<td>EpCAM</td>
<td>ICC, FC</td>
<td>50–4500 per 5 mL PB</td>
<td>76%</td>
<td>—</td>
<td>1/1 mL Blood</td>
<td>25</td>
</tr>
<tr>
<td>Prostate cancer CL</td>
<td>PC3 in PB</td>
<td>Positive</td>
<td>EpCAM</td>
<td>FC</td>
<td>25–800 per 7 mL PB</td>
<td>73.7%</td>
<td>—</td>
<td>—</td>
<td>26</td>
</tr>
<tr>
<td>Breast cancer CL</td>
<td>Colo-205 in PB</td>
<td>Positive</td>
<td>EpCAM</td>
<td>ICC, FC</td>
<td>1–200 per 5 mL PB</td>
<td>60–90%</td>
<td>—</td>
<td>—</td>
<td>27</td>
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<tr>
<td>Prostate cancer</td>
<td>PB</td>
<td>Positive</td>
<td>EpCAM</td>
<td>FC</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1/7.5 mL Blood</td>
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<tr>
<td>Immunomagnetic: Magnetic deposition (CCF, USA)</td>
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<tr>
<td>Breast cancer CL</td>
<td>MCF-7 in PBL</td>
<td>Positive</td>
<td>HER-2/neu</td>
<td>ICC, FC</td>
<td>1/10^3–1/10^5</td>
<td>—</td>
<td>20–60%</td>
<td>—</td>
<td>1/10^6 MMC</td>
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<tr>
<td>Immunomagnetic: QMS (OSU, USA)</td>
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<tr>
<td>Breast cancer CL</td>
<td>HCC1954 in PBL</td>
<td>Positive</td>
<td>HER-2/neu</td>
<td>FC</td>
<td>1/10^5</td>
<td>—</td>
<td>66–89%</td>
<td>5–50</td>
<td>1/10^6 MMC</td>
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<tr>
<td>Immunoaffinity Column (CellPro, USA)</td>
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</tr>
<tr>
<td>Breast cancer patients</td>
<td>PB, BM</td>
<td>Positive</td>
<td>ICC, FC</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>—</td>
<td>—</td>
<td>31</td>
</tr>
<tr>
<td>Filtration</td>
<td></td>
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<tr>
<td>Tumor CL</td>
<td>5 CLs in PB</td>
<td>Positive</td>
<td>—</td>
<td>ICC</td>
<td>1–3/1 mL PB</td>
<td>—</td>
<td>—</td>
<td>1/1 mL Blood</td>
<td>32</td>
</tr>
<tr>
<td>Breast cancer CL</td>
<td>MCF-7 in PBL</td>
<td>Positive</td>
<td>Ber-EP4</td>
<td>FC</td>
<td>2/10^5–1/10^6</td>
<td>50–60%</td>
<td>—</td>
<td>—</td>
<td>33</td>
</tr>
<tr>
<td>Density-gradient centrifugation</td>
<td></td>
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</tr>
<tr>
<td>MDA-361</td>
<td>PB</td>
<td>Positive</td>
<td>ICC, FC</td>
<td>0.73/10^3</td>
<td>55.2%</td>
<td>303</td>
<td>—</td>
<td>—</td>
<td>34</td>
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</table>
principle is based on the deflection, under the influence of a magnetic energy gradient, of immunomagnetically labeled cells into one stream and the unlabeled cells into a second stream [37]. Results showing 99% purity and 86% recovery have been achieved using QMS in progenitor cell isolation [38] and CD4+ and CD8+ cell sorting. Additionally, Nakamura et al. [30] reported the positive separation of a spiked breast cancer cell line from human blood with a throughput of $3.29 \times 10^5$ cells/s and a recovery of 89% of the cancer cells.

A photograph of the complete QMS system, including syringe pumps, separation element, and magnet, is shown in Figure 1. The schematic drawing of the QMS separation element is also shown in Figure 1. Cell suspension is fed to inlet $a$ while carrier or sheath fluid is fed to inlet $b$. Ideally, immunomagnetically labeled cells deflected due to the magnetic energy gradient are collected from outlet $b$ and nonmagnetic cells are collected from outlet $a$.

The QMS has several advantages over fluorescence-activated cell sorting (FACS) and batch immunomagnetic cell sorting systems in applications to rare cell separations. In addition to the high throughput, demonstrated to be as high as $10^6$ to $10^7$ cells/s (positive selection mode of operation) [38], performance of this sorter can be tuned. Specifically, the performance of the QMS can be predicted using a computer model, experimental determination of the magnetophoretic mobility of labeled cells, and QMS operating conditions. These model predictions of the QMS performance have been experimentally verified for several systems including magnetic microbeads, human hematopoietic stem cells, and immunomagnetically labeled cancer cells spiked into human blood [30,38–40]. To measure the magnetophoretic mobility of cells, our laboratory has developed an instrument, referred to as a cell tracking velocimeter (CTV), which is capable of measuring the magnetically induced velocity on a particle-to-particle basis [41].

While positive selection has proved effective for enrichment and isolation of rare, or not so rare, cells in a cell suspension of various types, there are significant limitations. One of these major limitations is the lack of information about the phenotype of the target cell. In the case of a rare, circulating cancer cell, one is assuming that the label, typically an antibody conjugate, can tag the cancer cell sufficiently, and specifically, to allow an acceptable separation. A study has recently been published which addresses the labeling conditions and performance of a number of commercial cancer cell immunolabels on two human cancer cell lines [42]. A second limitation of a positive selection mode of operation is the fact that one is labeling the very cell that one wishes to analyze further. While in many cases it has been demonstrated that binding of antibody magnetic nanoparticle-conjugates to target cells does not have effects on cell function, it is more desirable to have an enrichment step that does not modify and/or bind the target cell. This would then allow further molecular analysis of a nonmanipulated cell. Third, for some immunomagnetic cell separation systems, a negative depletion of the nontargeted cells potentially provides overall superior operational performance.

In addition to a magnetic separation technique, many of the reported processes to enrich for a rare cancer cell include an initial depletion of the red blood cells, RBC. Two commonly used techniques are reported: depletion of RBC using a Ficoll-Hypaque cushion and the use of a red cell lysis buffer. Despite its popularity, the Ficoll-Hypaque cushion performance has been linked to the cell load and differences in individual harvesting techniques. The Becton-Dickinson web page (www.bd.com/vacutainer/products/molecular/heparin/results.asp) states that when Ficoll-Hypaque separation gradient is used, the expected recovery of mononuclear cells is around 68% of the original number of cells present.

Partridge et al. [23] reported that epithelial tumor cells sediment preferentially with the mononuclear cell fraction, although the authors also state that in some cases it was possible to find more cancer cells in the granulocyte fraction than in the mononuclear fraction. Krüger et al. [21] stated that the detection of epithelial tumor cells is influenced by the erythrocyte depletion method. The authors report further that more cells are detected when red cell lysis is used instead of Ficoll-Hypaque centrifugation.

Based on the research and potential clinical need to enrich for very rare cells, such as cancer cells in blood, and the limitations of the approaches outlined above, we are developing and optimizing a process, based solely on negative selection, to significantly enrich a blood sample for a rare cell of interest. The process presented in this paper has three separation stages: a density-gradient separation or lysis to remove erythrocytes, an immunomagnetic separation step to remove leukocytes, and a filtration step to concentrate, collect, and evaluate the final cell suspension. Alternatively, the final step can be modified and replaced with a centrifugation and/or direct cell lysis step for further molecular evaluations of the final, enriched cancer cell suspension.

Material and methods

Cell sources

A breast carcinoma cell line (MCF-7) was grown to confluence on Eagle medium (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA) in a 75 cm² tissue culture flask (Corning, Corning, NY, USA) and incubated at 37°C in 5% CO₂. MCF-7 cells were harvested using Accutase (Innovative Cell Technologies, USA) in a 75 cm² tissue culture flask. Before the enrichment experiments, MCF-7 cells were filtered through a 20-μm nylon mesh in order to obtain a single cell suspension.

Fresh blood (whole blood) was provided by healthy donors before each enrichment experiment. Buffy coats of peripheral blood
from healthy donors were obtained from American Red Cross, Central Ohio Region. The concentration of leukocytes in blood was determined by hemocytometer using Unopette Microcollection system (BD Biosciences, San Jose, CA, USA). The total cell concentration in blood, including erythrocytes and white blood cells, was measured using an automated particle counter (Multisizer II, Beckmann Coulter, Fullerton, CA, USA).

**Density separation**
For some experiments, density separation was conducted to remove erythrocytes using Ficoll-Hypaque (Accurate Chemical and Scientific Corporation, Westbury, NY, USA) with a gradient density of 1.077 g/mL. Prior determination of MCF-7 cell density, 1.066 g/mL, using a continuous Percoll gradient, indicates that cancer cells will preferentially sediment with the leukocytes at the interface separating plasma and the neutrophile fraction. Accordingly, isolating peripheral blood leukocytes (PBL) from the interface will theoretically yield the spiked MCF-7 cells in the leukocyte fraction.

**Lysis of erythrocytes**
Following the basic protocol shown on the manufacturer’s website (http://www.zellbiophysik.fh-aachen.de/methoden/protocol1.html),
typically 2 mL of fresh blood at the concentration of 1 MCF-7 cell per 10^6 total cells (except for studies when a high concentration of MCF-7 cells was used) was rapidly added to 40 mL fresh lysing buffer (154 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA), and incubated for 5 minutes at room temperature with occasional shaking. After 5 minutes of centrifugation at 300g, the cell pellets were washed twice using the labeling buffer, and resuspended in the buffer to obtain the PBL suspension. In several runs, the lysis protocol was repeated in an attempt to remove more RBC.

**Immunological labeling**

**Cell preparation.** Blood suspensions spiked with MCF-7 cells, from either density separation or red cell lysis, were washed twice using phosphate-buffered saline (PBS; JRH Biosciences, Lenexa, KS, USA) supplemented with 2 mM EDTA (Invitrogen Corporation, Carlsbad, CA, USA) and 0.5% bovine serum albumin (BSA; Invitrogen Corporation, Carlsbad, CA, USA). After the final washing, cells were resuspended to a final concentration of 40 × 10^6 cells/mL.

**Antibodies used and labeling optimization studies.** A double-step immunomagnetic staining technique was used to target the PBL for the immunomagnetic separation step. The primary antibody used was a mouse anti CD45 PE (Cat# IM 2078, Immunotech, Marseilles, France) and the secondary antibody was an anti-PE MACS microbeads (Miltenyi Biotec, Auburn, CA, USA). A third reagent, FcR blocking reagent (Miltenyi Biotec), was also tested. Flow cytometry measurements were made on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and magnetophoretic mobility measurements were made on our previously developed, and reported, cell tracking velocimetry instrument [42].

**Final cancer cell staining.** To visually assist in the detection of cancer cells after the final filtration step for some of the studies conducted, an antibody specific to a human epithelial marker, mouse anti-human HEA FITC (Miltenyi Biotec, Auburn, CA, USA) was used. The labeling of this marker, at a concentration of 10 μL of antibody per 10^6 cells, occurred when cells were directly on the filtration membrane. While cells stained with the anti-HEA FITC can be easily identified under a fluorescence microscope, an antibody-enzyme-dye procedure was used to further facilitate visualization with a light microscope. Specifically, a rabbit-anti-FITC phosphatase alkaline conjugate was used as the secondary antibody (Cat # A4843, Sigma, St. Louis, MO, USA) and Fast Red™ TR-Naphthol AS-MX (Cat # F – 71, Sigma, USA) as the substrate for the enzyme. The action of the alkaline phosphatase and Fast Red creates an intense red-color dye that is easily detected using a light microscope.

**Immunomagnetic cell separation step**

The design and construction of the QMS system has been described previously [40]. The characteristics of the magnetic system include: a maximum magnetic field strength of the quadrupole (B₀) of 1.37 T and a mean force field strength (S_m) of 2.382 × 10⁶ (T-A)/m². In contrast to the previous studies, however, a precommercial separation column (prototype # 10597) manufactured by SHOT INC. (Greenville, IN, USA) was used. This separation column had a length of 15.24 cm, an internal diameter of 0.6922 cm, and a maximum diameter of the core rod of 0.4661 cm. The inlet splitter outer diameter was 0.5128 cm and outlet splitter outer diameter was 0.6058 cm.

Two modes of operation were evaluated: continuous mode, in which both the cell suspension (a’) and sheath flow (b’) flowed continuously through the channel during the separation; and semi-continuous mode, in which the cell suspension was continuous and the sheath flow was zero. To operate the QMS in the continuous mode, total flow rates at the inlet and outlet remained constant using three syringe pumps. Two pumps were used at the inlet: the first pump injected the sample and sheath carrier at the beginning of the operation until the sample has been injected into the column (a’ inlet). Upon completion of sample injection, flow entering the a’ inlet was switched to the second pump using a three-way valve which injected sheath fluid into the a’ inlet to flush remaining sample from the system and to maintain a constant flow rate at the inlet. A third pump was connected to the outlets a and b to collect sorted fractions. This third pump creates the transport lamina thickness required for sorting while maintaining the total flow rate constant. For the semi-continuous mode of operation, the system was prepared as above, and when the cell suspension sample was injected, both b and b’ flow was stopped.

Before performing an actual separation, the QMS column is filled with degassed PBS buffer (PBS, 0.5 mM EDTA, and 0.5% BSA) so that no bubbles are trapped inside the device. Labeled cells are diluted to a final concentration in the range of 1 to 3 × 10^6 cells/mL using PBS buffer before injection. Syringes with sample, carrier, and empty syringes to collect sorted fractions are mounted and separation performed. After the sample has been completely injected, the three-way valve is switched to allow carrier from the second pump to enter the system and push the remaining sample within the tubing through the column.

**Operating parameters of the QMS**

Cell separations in the continuous mode in the QMS are governed by three adjustable parameters: total flow rate (Q_t), inlet flow rate ratio (Q_a/Q_t), and outlet flow rate ratio (Q_b/Q_t). The first parameter, total flow ratio (Q_t), is responsible for the residence time of the cell inside the column; therefore, (Q_t) is directly responsible for the radial displacement of an immunomagnetically labeled cell. The larger the flow rate, the less time the cell is subjected to the magnetic field and the less distance traveled in the radial direction as a result of the magnetic force. The remaining two parameters, inlet flow ratio and outlet flow ratio (Q_a/Q_t, Q_b/Q_t, respectively), are responsible for creating the transport lamina that an immunomagnetically labeled cell must cross radially to reach elution port b [43].

**Final processing step**

The enriched fraction from the nonmagnetic outlet, stream a (Fig. 1), was processed in one of three ways: a) total cell recovery and distribution with Coulter Multisizer II measurements and total recovered volume, b) total cell recovery and analysis by filtration, and c) cytospin. Figure 2 presents the size distribution of the peripheral blood lymphocytes and MCF-7 cells, as measured by Coulter Multisizer II, clearly indicating significant difference in cell size. Cell samples analyzed by filtration were filtered using a syringe pump at a flow rate of 1 mL/min through a 5-μm pore size diameter polycarbonate membrane (Whatman, Maidstone, Kent, UK). These retained cells were subsequently stained with anti-FITC alkaline phosphatase antibody and Fast Red substrate to assist in visually counting through a light microscope (Model BX60F, Olympus, Tokyo, Japan). Cytospin analysis was accomplished following a typical protocol. Specifically, the enriched fraction from
the QMS a outlet was centrifuged for 5 minutes at 300g, the pellet resuspended in 50 µL labeling buffer, and added to the cytospin funnel for 2 minutes at 2000 rpm. The cells were then layered evenly onto the glass slide, stained with Diff-Quik (DADE Behring AG, Dudingen, Switzerland) and air-dried for subsequent microscopic evaluation.

Results
A commonly understood principle in chemical synthesis or separation processes is the negative, multiplicative effect of nonperfect recoveries and/or yields. For example, a five-step separation process in which each step has a 90% recovery of the targeted cell will result in an overall recovery at the end of the process of only 59%. Consequently, considerable effort was placed on minimizing the number of steps and maximizing the recovery of the spiked cancer cells in each step used.

Since centrifugation is a processing step that is used a number of times to prepare samples for labeling and removal of specific agents (such as lysis buffer), a set of experiments were conducted to determine the recovery of a typical centrifugation step. Specifically, nine experiments were conducted in which 2.00 × 10^6 MCF-7 cells were placed in a 50-mL conical tube, resuspended in 40 mL fresh labeling buffer (PBS, 2 mM EDTA, and 0.5% BSA), and centrifuged for 5 minutes at 300g. The supernatant was then removed, and the cell pellet was resuspended in 2 mL labeling buffer. The cell concentration of this resuspended cell suspension was then determined with the Coulter Multisizer II. The average recovery of these nine experiments was 93.4% with a relative standard deviation of 0.04.

Density-gradient separation optimization studies
To assess the performance of the Ficoll-Hypaque density-gradient separation step, DGS, MCF-7 cells were seeded into aliquots of blood from buffy coats, DGS was performed, and the number of MCF-7 cells recovered was counted with the Coulter Multisizer II using a gate setting of 5.64 to 12.43 µm for PBL and 12.95 to 40.18 µm for MCF-7 cells.

Figure 3 presents the recovery of MCF-7 cells, seeded at a fractional concentration of 0.13%, as a function of percent cell volume (hematocrit). This curve clearly shows that there is a maximum in MCF-7 cell recovery, which was used as the optimum point to perform density-gradient separation. MCF-7 cell recovery peaks at 73% when a cell suspension with percent cell volume concentration of 22% is layered on top of the high-density cushion.

Red cell lysis studies
To replace the DGS step which, as shown previously, produces suboptimal recovery of the spiked cancer cells, studies were conducted to measure the recovery of spiked MCF-7 cells in human blood and to determine the effect, if any, that the red cell lysis step has on the viability of the desired cells as well as the binding of antibodies to specific cell-surface markers.

To test the performance of the red cell lysis step with respect to the recovery of MCF-7 cells, aliquots of whole, human blood were seeded at a concentration of 1 MCF-7 cell per 1.00 × 10^4 total cells. After lysis, the number of MCF-7 cells recovered was counted using the Coulter Multisizer II. The results of 10 experiments demonstrated an average recovery of MCF-7 cells of 89.4%, with a relative standard deviation of 0.04.

In general, no adverse effect was detected with respect to antibody binding to lymphocytes enriched with Ficoll

Figure 3. Recovery of MCF-7 cells seeded at a concentration of 0.13% in buffy coat samples as a function of hematocrit. Samples were loaded on a Ficoll-Hypaque gradient at the concentration specified by the experimental point.
DGS versus red cell lysis. Specifically, 99% of the leukocytes were observed to bind anti-CD45–PE antibodies after Red cell lysis, vs 96% after Ficoll separation (relative to total leukocytes identified by forward and side scatter). Based on trypan blue staining, 90% of the MCF-7 and 95% of the leukocytes were observed to be viable after the lysis step. However, a slight decrease in absolute fluorescence intensity, FI, between labeled leukocytes obtained from red cell lysis when compared to labeled leukocytes obtained from Ficoll separation was observed. (It should be noted that these comparisons were made sequentially on the flow cytometer so direct comparisons of the FI could be made.)

Labeling saturation studies

Previous studies in our laboratory indicate that the magnetophoretic mobility of an immunomagnetically labeled cell significantly affects the performance of the magnetic separation process [30,36,44]. We have also demonstrated that the magnetophoretic mobility is a function of the number of antibody binding sites on a cell, the degree to which the binding sites are saturated, the field interaction parameter of the magnetic nanoparticle, and the number of magnetic nanoparticles per site [42,45] (the amplification).

Of these four variables, once a cell type and antibody are chosen, the remaining variable to optimize is the labeling concentration, which has been shown to have a significant effect on the saturation of the binding sites, and correspondingly, the magnetophoretic mobility of the labeled cells [42].

Figure 4A is a saturation-style plot of the normalized fluorescence intensity as a function of the concentration of the primary, anti-CD45 PE antibody, in units of µg of antibody per mL of cell suspension with a total of 1.0 × 10^6 cells present. The normalization process consisted of dividing the fluorescence intensity at a given condition to the maximum value obtained in the given set of experimental flow cytometry analysis experiments. Figure 4B is a saturation plot of the magnetophoretic mobility of the PBL cells, previously labeled with anti-CD45 PE (15 µg/mL), as a function of the number of µL of MACS reagent per total µL of suspension (note Miltenyi Biotec does not provide concentration of their reagents in units of µg). Finally, Figure 4C is a bar graph demonstrating the effect, or lack thereof, of the use of FcR blocking reagent on PBL cells labeled with 2.82 µg/mL of anti-CD45 PE and 50 µL of MACS reagent.

Since the cell suspension consists of RBC, leukocytes, and rare MCF-7 cells, and the overall goal is to recover all of the MCF-7 cells in the desired final fraction, nonspecific binding of the anti-CD45 PE to the MCF-7 cells is to be avoided. Figure 5 presents flow cytometry plots of PBL and MCF-7 cell samples stained with anti-CD45 PE and anti-HEA FITC antibodies. Plots show that when PBL is stained with anti-HEA FITC or MCF-7 cells are stained with anti-CD45 PE antibody, no nonspecific binding is detected (Fig. 5C and E). However, when a sample containing PBL and MCF-7 cells in a 1:1 ratio is used, flow cytometry detects some cross binding (Fig. 5F). It is suggested that this effect is an analysis artifact due to the gating technique of the analysis region (i.e., the gating region for the MCF-7 cells is more ambiguous than for the PBL); however, such ambiguities in flow cytometry analysis contributed toward the motivation to use a direct staining and visual counting procedure of the final, separated product.

Figure 6A and B present representative histograms, in linear and log format, of the magnetophoretic mobility of PBL labeled with antiCD45 PE antibodies (0.225 µg/10^6 cells) and 15 µL of secondary antibody/10^6 cells. For comparison, a histogram for the magnetophoretic mobility distribution of unlabeled MCF-7 and MCF-7 cells only labeled with the secondary antibody is presented in Figure 6C.

Results of overall process using optimized operating parameters

Based on the previously presented optimization studies, three sets of experiments were conducted using slightly different methodologies to enrich spiked cancer cells in human blood. These three sets of experiments are presented in Tables 2, 3, and 4.

In the three separation methodologies, MCF-7 cells were added to fresh, whole human blood and subsequently subjected to the red cell lysis protocol (an exception was in three runs in Table 2, buffy coat of human blood was used and in several experiments the lysis step was performed twice). The cell suspension was subsequently labeled with optimized amounts of antibodies (2.82 µg/mL of anti-CD3 PE, and 100 µL of MACS beads) and pumped through the QMS separation system using the flow rates presented in the footnotes of the tables. For the first two sets of experiments, the QMS was operated in the complete flow-through mode (two exit streams a and b) while in the last set of experiments (Table 4) the sheath flow (b' and b) was turned off during the actual experiment and the system was operated in a deposition mode of operation. A final difference was in the first set of experiments (Table 2), a significantly higher cancer cell concentration was used (namely, 1 in 10^5 or 10^6 total cells) while in the last two sets of experiments (Tables 3 and 4) the concentration of cancer cells was significantly lower, on the order of 1 in 10^8 total cells. This higher level of spiked cancer cells allowed the final concentration (number) of cancer cells obtained after the QMS separation to be evaluated using the Coulter Multisizer II; therefore, the final number of cells recovered, and final purity, was determined from knowing the final volume collected and the final concentration from the Coulter Multisizer II. The final number of cancer cells recovered in the last two sets of experiments (Tables 3 and 4) was determined by visually counting the cells under a microscope. In all of these experimental runs, the nonmagnetic flow stream from the QMS sorter (stream a) was split into two samples, one of which was filtered, stained, and visually counted, while the other was...
Figure 4. Saturation curve of CD45 surface receptor on PBL (A) with the independent variable the concentration of antibody (µg of Ab/mL-10⁶ cells) and dependent variable is normalized fluorescence intensity. (B) is saturation plot of the magnetophoretic mobility of the PBL cells, previously labeled with anti-CD45 PE (15 µg/mL), as a function of the number of µL of MACS reagent per total µL of suspension. (C) is a bar graph demonstrating the effect of the use of FcR blocking reagent on PBL cells labeled with 2.82 µg/mL of anti-CD45 PE and 50 µL of MACS reagent. The abbreviations in the y-axis of panel B refer to the SI units of Tesla (T), ampere (A), seconds (s).

The results of these three sets of experiments are presented in Tables 2, 3, and 4. Specifically, for the set of experiments presented in Table 2, the average purity increased from an initial purity of 3.15 × 10⁻⁶ to 1.87 × 10⁻², which represents an average 3.38 log₁₀ enrichment with an average recovery of the spiked cancer cells of 46%. At a lower initial purity, an average of 9.7 × 10⁻⁹ (Table 3), an increase of purity to 5.9 × 10⁻⁵ was obtained, a similar 3.75 log₁₀ enrichment. The average recovery of cancer cells using this methodology was 47.5%. However, when the mode of operation of the QMS is changed from completely flow-through to a partial deposition mode of operation (sheath fluid flow is...
stopped), the average log_{10} enrichment increased to 5.17, which translates into taking an average initial purity of 1.43 × 10^{-8} and increasing it to an average of 1.95 × 10^{-3}; yet the average recovery was maintained at 46%. Photographs of final, separated samples of stained cells on the filtration membrane and after cytopsin are presented in Figures 7 and 8, respectively.

A final point with respect to this separation methodology is time. The current process takes approximately two hours from the beginning of the lysis of RBC to final analysis, this time including the incubation of antibodies with the cells. Further optimization should shorten this time.

**Discussion**

Obviously, a 7 to 8 log_{10} enrichment, with 100% recovery, is ideal. Nevertheless, an average log_{10} enrichment of 5.17 and average recovery of 46% of the spiked cancer cells in whole blood using a completely negative selection process is encouraging. This result is especially encouraging when one considers that the best reported enrichment we have been able to find (either positive or negative mode of operation) was a 4 log_{10} positive enrichment using two positive selection steps [22].

While it is always important to increase the overall recovery of the target cell or molecule in a separation process, a 100% recovery of the spiked cancer cells in the experiments presented in Table 4 would only increase the enrichment from 5.17 to 5.47 log_{10}. The most obvious increase in enrichment performance would result from an increase in the removal of unwanted cells from the final sample. This is clearly demonstrated by comparing the results in Tables 2 and 3. Specifically, despite lowering the initial purity from 3.2 × 10^{-6} to 9.7 × 10^{-9}, the removal of RBCs in the final step through filtration allowed the log enrichment to stay the same. Finally, the higher level of PBL removal using the partial deposition mode of operation further reduced the number of PBLs in the final product, resulting in a further two-log increase in enrichment (Table 3 vs Table 4).
As with all separation and analysis technology, improvement of the resolving power improves the overall performance of the technology. This same concept has been demonstrated theoretically and experimentally with immunomagnetic cell separation in the QMS system [42,45,46]. In the current application, an increase in resolving power would theoretically allow a further improvement in the removal of unwanted cells in the final product. Figure 6A and B illustrates this resolving power as the difference in mean magnetophoretic mobility between labeled and unlabeled PBL, when compared to dispersion of the mobility distribution [47]. As can be observed, despite a significant difference in the mean magnetophoretic mobility between the labeled and unlabeled cells (a factor of $10^4$) there still is some overlap in the magnetophoretic mobility between the labeled and unlabeled cells. It is possible that it is this overlap that accounts for some of the PBL in the final product that results in a lower, final enrichment. Previous and ongoing research in our laboratories has focused on developing methods to increase the magnetophoretic mobility of target cells without increasing nonspecific binding of nontargeted cells [36,45].

With respect to recovery, the average of recovery of 46%, approximately the same using the three separation methodologies, is respectable considering that in our laboratory, we only obtain an average recovery of approximately 93% per centrifuge step. Thus, with six centrifuge steps (typically used in this study) at 93% yield and one lysis step at 89% yield, one would expect an overall recovery of 57%, surprisingly close to the actual recoveries obtained. This is especially surprising since this simple calculation assumes 100% recovery in the QMS step.

The desire to obtain the highest recovery per step led to the replacement of the Ficoll density sedimentation step with the red cell lysis step. Figure 3 suggests that the recovery of cancer cells sedimenting with the mononuclear fraction is a function of the percentage of cell volume concentration initially present in the suspension to be separated. The lack of increase in recovery at high initial concentrations ($>30\%$) suggests a potential overloading of the density gradient.
Table 2. Experimental data of rare cancer cell enrichment using a MCF-7 concentration of $10^{-5}$–$10^{-6}$ (cells/total cells) and the complete flow-through mode of operation

<table>
<thead>
<tr>
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<th>3$^{a}$</th>
<th>4$^{a}$</th>
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<td></td>
</tr>
<tr>
<td>Number of total blood cell added</td>
<td>$3.90 \times 10^{10}$</td>
<td>$1.39 \times 10^{10}$</td>
<td>$2.88 \times 10^{10}$</td>
<td>$4.01 \times 10^{10}$</td>
<td>$2.17 \times 10^{10}$</td>
<td>$1.76 \times 10^{10}$</td>
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<tr>
<td>Number of PBL added</td>
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<td></td>
<td></td>
<td></td>
<td>$1.75 \times 10^{7}$</td>
</tr>
<tr>
<td>Number of total MCF-7 added</td>
<td>552,000</td>
<td>13,851</td>
<td>26,400</td>
<td>40,100</td>
<td>21,700</td>
<td>17,570</td>
</tr>
<tr>
<td>MCF-7 concentration (cells/total cells)</td>
<td>$1.4 \times 10^{-5}$</td>
<td>$1.0 \times 10^{-6}$</td>
<td>$0.92 \times 10^{-6}$</td>
<td>$1.0 \times 10^{-6}$</td>
<td>$1.0 \times 10^{-6}$</td>
<td>$1.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>MCF-7 concentration (cells/PBL)</td>
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<td></td>
<td></td>
<td></td>
<td>$3.15 \times 10^{-6}$</td>
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<td>Cell numbers after lysis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Number of total PBL obtained</td>
<td>$1.17 \times 10^{7}$</td>
<td>$3.5 \times 10^{7}$</td>
<td>$2.60 \times 10^{6}$</td>
<td>$3.00 \times 10^{7}$</td>
<td>$8.40 \times 10^{7}$</td>
<td>$7.20 \times 10^{7}$</td>
</tr>
<tr>
<td>Number of RBC obtained</td>
<td>$3.60 \times 10^{7}$</td>
<td>$3.04 \times 10^{7}$</td>
<td>$3.35 \times 10^{7}$</td>
<td>$8.40 \times 10^{7}$</td>
<td>$1.75 \times 10^{7}$</td>
<td>$2.12 \times 10^{7}$</td>
</tr>
<tr>
<td>Cell numbers after QMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of PBL in the outlet a</td>
<td>$0.81 \times 10^{6}$</td>
<td>$7.30 \times 10^{6}$</td>
<td>$1.26 \times 10^{6}$</td>
<td>$1.00 \times 10^{6}$</td>
<td>$0.40 \times 10^{6}$</td>
<td>$0.40 \times 10^{6}$</td>
</tr>
<tr>
<td>Number of RBC in the outlet a</td>
<td>$4.14 \times 10^{6}$</td>
<td>$1.81 \times 10^{7}$</td>
<td>$7.80 \times 10^{5}$</td>
<td>$1.49 \times 10^{6}$</td>
<td>$2.64 \times 10^{6}$</td>
<td>$6.96 \times 10^{6}$</td>
</tr>
<tr>
<td>Number of MCF-7 in the outlet a</td>
<td>317,458</td>
<td>9,081</td>
<td>15,660</td>
<td>6,140</td>
<td>4,148</td>
<td>10,500</td>
</tr>
<tr>
<td>Final purity, number MCF-7</td>
<td>$6.41 \times 10^{-2}$</td>
<td>$3.58 \times 10^{-4}$</td>
<td>$7.62 \times 10^{-3}$</td>
<td>$2.46 \times 10^{-3}$</td>
<td>$1.36 \times 10^{-3}$</td>
<td>$1.08 \times 10^{-3}$</td>
</tr>
<tr>
<td>concentration (cells/total cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$1.87 \times 10^{-2}$</td>
</tr>
<tr>
<td>MCF-7 recovery in the outlet a (%)</td>
<td>57.4</td>
<td>65.6</td>
<td>59.3</td>
<td>15.3</td>
<td>19.2</td>
<td>59.8</td>
</tr>
<tr>
<td>Log enrichment (-)</td>
<td>3.66</td>
<td>2.55</td>
<td>3.92</td>
<td>3.39</td>
<td>3.13</td>
<td>3.03</td>
</tr>
</tbody>
</table>

$^{a, b, c}$ Magnetophoretic mobility is about $1.12 \times 10^{-4}$ mm$^2$/T.A.s. $Q_i = 10$ mL/min, $Q_1/Q_i = 0.15$. $Q_1/Q_i = 0.5$, transport lamina thickness = 586 µm. Cell feed volume is 15 mL. The number of MCF-7 after enrichment was determined using Coulter Counter and microscope.

$^{a}$ Magnetophoretic mobility = $1.24 \times 10^{-4}$ mm$^2$/T.A.s. $Q_i = 8$ mL/min, $Q_1/Q_i = 0.2$. $Q_1/Q_i = 0.5$, transport lamina thickness = 486 µm. Cell feed volume is 15 mL. The number of MCF-7 after enrichment was determined using Coulter Counter and microscope.

$^{a, c}$ Fresh blood was used in the experiments.

A concern can be raised with respect to how representative these presented studies are to actually separating cancer cells from the blood of cancer patients. Several observations can be made with respect to this concern. First, in all but a few of the experimental results presented in Tables 2–4, fresh human peripheral blood from normal donors was used for the spiking studies (in the few other studies, buffy coat purchased from the American Red Cross was used).

Second, how representative are MCF-7 cells of actual cancer cells circulating in a cancer patient’s blood? In the

Table 3. Experimental data of rare cancer cell enrichment using a MCF-7 concentration of $10^{-8}$ (cells/total cells) and the complete flow-through mode of operation

<table>
<thead>
<tr>
<th>Run</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average</th>
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</thead>
<tbody>
<tr>
<td>Feedstock</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of total blood cell added</td>
<td>$3.84 \times 10^{10}$</td>
<td>$3.93 \times 10^{10}$</td>
<td>$1.76 \times 10^{10}$</td>
<td>$1.58 \times 10^{10}$</td>
<td>$1.21 \times 10^{10}$</td>
<td>$1.21 \times 10^{10}$</td>
</tr>
<tr>
<td>Number of PBL added</td>
<td>$12.23 \times 10^{7}$</td>
<td>$14.22 \times 10^{7}$</td>
<td>$1.75 \times 10^{7}$</td>
<td>$1.25 \times 10^{7}$</td>
<td>$1.75 \times 10^{7}$</td>
<td>$1.75 \times 10^{7}$</td>
</tr>
<tr>
<td>Number of total MCF-7 added</td>
<td>389</td>
<td>335</td>
<td>176</td>
<td>158</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>MCF-7 concentration (cells/total cells)</td>
<td>$1.0 \times 10^{-8}$</td>
<td>$0.85 \times 10^{-8}$</td>
<td>$1.0 \times 10^{-8}$</td>
<td>$1.0 \times 10^{-8}$</td>
<td>$1.0 \times 10^{-8}$</td>
<td>$1.0 \times 10^{-8}$</td>
</tr>
<tr>
<td>MCF-7 concentration (cells/PBL)</td>
<td>$0.32 \times 10^{-5}$</td>
<td>$0.25 \times 10^{-5}$</td>
<td>$1.0 \times 10^{-5}$</td>
<td>$1.26 \times 10^{-5}$</td>
<td>$0.69 \times 10^{-5}$</td>
<td>$9.7 \times 10^{-9}$</td>
</tr>
<tr>
<td>Cell numbers after lysis</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Number of total PBL obtained</td>
<td>$5.69 \times 10^{7}$</td>
<td>$5.13 \times 10^{7}$</td>
<td>$7.2 \times 10^{6}$</td>
<td>$8.60 \times 10^{7}$</td>
<td>$6.99 \times 10^{7}$</td>
<td>$6.99 \times 10^{7}$</td>
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<tr>
<td>Number of RBC obtained</td>
<td>$2.35 \times 10^{7}$</td>
<td>$1.79 \times 10^{7}$</td>
<td>$2.12 \times 10^{7}$</td>
<td>$1.45 \times 10^{7}$</td>
<td>$3.83 \times 10^{7}$</td>
<td>$3.83 \times 10^{7}$</td>
</tr>
<tr>
<td>Cell numbers after QMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of PBL recovered in the outlet a</td>
<td>$3.55 \times 10^{6}$</td>
<td>$5.9 \times 10^{6}$</td>
<td>$2.77 \times 10^{6}$</td>
<td>$0.74 \times 10^{6}$</td>
<td>$0.35 \times 10^{6}$</td>
<td>$0.35 \times 10^{6}$</td>
</tr>
<tr>
<td>Number of RBC recovered in the outlet a</td>
<td>$1.06 \times 10^{7}$</td>
<td>$1.81 \times 10^{7}$</td>
<td>$7.00 \times 10^{5}$</td>
<td>$6.60 \times 10^{6}$</td>
<td>$4.29 \times 10^{6}$</td>
<td>$4.29 \times 10^{6}$</td>
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<tr>
<td>Final recovery after filtration</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Number of PBL recovered in the outlet a</td>
<td>$3.55 \times 10^{6}$</td>
<td>$5.9 \times 10^{6}$</td>
<td>$2.77 \times 10^{6}$</td>
<td>$7.40 \times 10^{5}$</td>
<td>$3.50 \times 10^{5}$</td>
<td>$3.50 \times 10^{5}$</td>
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<tr>
<td>Number of RBC recovered in the outlet a</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Number of MCF-7 recovered in the outlet a</td>
<td>164</td>
<td>208</td>
<td>120</td>
<td>66</td>
<td>28</td>
<td>28</td>
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<tr>
<td>Final purity, number MCF-7</td>
<td>$4.62 \times 10^{-5}$</td>
<td>$3.53 \times 10^{-5}$</td>
<td>$4.33 \times 10^{-5}$</td>
<td>$8.92 \times 10^{-5}$</td>
<td>$8.00 \times 10^{-5}$</td>
<td>$5.88 \times 10^{-5}$</td>
</tr>
<tr>
<td>concentration (cells/total cells)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7 recovery in the outlet a (%)</td>
<td>42.2</td>
<td>62.1</td>
<td>68.2</td>
<td>41.8</td>
<td>23.3</td>
<td>47.5</td>
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<tr>
<td>Log enrichment (-)</td>
<td>3.66</td>
<td>3.62</td>
<td>3.64</td>
<td>3.95</td>
<td>3.90</td>
<td>3.75</td>
</tr>
</tbody>
</table>

$^{a}$ Magnetophoretic mobility was approximately $1.12 \times 10^{-4}$ mm$^2$/T.A.s. $Q_i = 10$ mL/min, $Q_1/Q_i = 0.15$. $Q_1/Q_i = 0.5$, transport lamina thickness = 586 µm. Cell feed volume was 10 mL. The number of MCF-7 after enrichment was determined using Cytospin or membrane stain. Fresh blood was used in the experiments.
Table 4. Experimental data of rare cancer cell enrichment using a MCF-7 concentration of $10^{-8}$ (cells/total cells) and the partial deposition mode of operation

<table>
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<th>Cycle</th>
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<th>4</th>
<th>5</th>
<th>6&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Average</th>
<th>7&lt;sup&gt;b&lt;/sup&gt;</th>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of total blood cell added</td>
<td>$1.58 \times 10^{10}$</td>
<td>$1.21 \times 10^{10}$</td>
<td>$3.57 \times 10^{10}$</td>
<td>$5.62 \times 10^9$</td>
<td>$1.28 \times 10^{10}$</td>
<td>$1.74 \times 10^{10}$</td>
<td>$1.28 \times 10^{10}$</td>
<td>$1.16 \times 10^{10}$</td>
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</tr>
<tr>
<td>Number of PBL added</td>
<td>$1.25 \times 10^7$</td>
<td>$1.75 \times 10^7$</td>
<td>$3.84 \times 10^7$</td>
<td>$4.24 \times 10^7$</td>
<td>$0.78 \times 10^7$</td>
<td>$3.77 \times 10^7$</td>
<td>$0.78 \times 10^7$</td>
<td>$0.25 \times 10^7$</td>
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</tr>
<tr>
<td>Number of total MCF-7 added</td>
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<td>120</td>
<td>360</td>
<td>212</td>
<td>128</td>
<td>150</td>
<td>1,160,000</td>
<td>500,000</td>
<td></td>
</tr>
<tr>
<td>MCF-7 concentration (cells/total cells)</td>
<td>$1.0 \times 10^{-8}$</td>
<td>$1.0 \times 10^{-8}$</td>
<td>$1.0 \times 10^{-8}$</td>
<td>$3.77 \times 10^{-8}$</td>
<td>$1.0 \times 10^{-8}$</td>
<td>$0.86 \times 10^{-8}$</td>
<td>$1.43 \times 10^{-8}$</td>
<td>$9.09 \times 10^{-9}$</td>
<td>$4.31 \times 10^{-5}$</td>
</tr>
<tr>
<td>MCF-7 concentration (cells/PBL)</td>
<td>$1.26 \times 10^{-5}$</td>
<td>$0.69 \times 10^{-5}$</td>
<td>$0.94 \times 10^{-5}$</td>
<td>$0.5 \times 10^{-5}$</td>
<td>$1.64 \times 10^{-5}$</td>
<td>$0.40 \times 10^{-5}$</td>
<td>$1.49 \times 10^{-5}$</td>
<td>$1.99 \times 10^{-1}$</td>
<td></td>
</tr>
<tr>
<td>Cell numbers after lysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of total PBL obtained</td>
<td>$8.60 \times 10^6$</td>
<td>$6.99 \times 10^6$</td>
<td>$2.70 \times 10^7$</td>
<td>$14.70 \times 10^6$</td>
<td>$12.77 \times 10^6$</td>
<td>$18.99 \times 10^6$</td>
<td>$12.77 \times 10^6$</td>
<td>$25.00 \times 10^6$</td>
<td></td>
</tr>
<tr>
<td>Number of RBC obtained</td>
<td>$4.40 \times 10^7$</td>
<td>$1.16 \times 10^7$</td>
<td>$5.79 \times 10^7$</td>
<td>$1.40 \times 10^7$</td>
<td>$9.07 \times 10^7$</td>
<td>$8.3 \times 10^7$</td>
<td>$9.07 \times 10^7$</td>
<td>$14.28 \times 10^6$</td>
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</tr>
<tr>
<td>Cell numbers after QMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of PBL recovered in the outlet a</td>
<td>45,454</td>
<td>32,567</td>
<td>130,000</td>
<td>46,165</td>
<td>45,964</td>
<td>18,286</td>
<td>69,669</td>
<td>38,897</td>
<td></td>
</tr>
<tr>
<td>Number of RBC recovered in the outlet a</td>
<td>$1.58 \times 10^7$</td>
<td>$0.91 \times 10^6$</td>
<td>$0.80 \times 10^6$</td>
<td>$1.10 \times 10^6$</td>
<td>$13.88 \times 10^6$</td>
<td>$0.73 \times 10^6$</td>
<td>$31.78 \times 10^6$</td>
<td>$0.54 \times 10^6$</td>
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<tr>
<td>Final recovery after filtration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of PBL recovered in the outlet a</td>
<td>45,454</td>
<td>32,567</td>
<td>130,000</td>
<td>46,165</td>
<td>45,964</td>
<td>18,286</td>
<td>69,669</td>
<td>38,897</td>
<td></td>
</tr>
<tr>
<td>Number of RBC recovered in the outlet a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Number of MCF-7 recovered in the outlet a</td>
<td>76</td>
<td>61</td>
<td>160</td>
<td>80</td>
<td>65</td>
<td>69</td>
<td>500,000</td>
<td>186,224</td>
<td></td>
</tr>
<tr>
<td>Final purity, number MCF-7 concentration (cells/total cells)</td>
<td>$1.67 \times 10^{-3}$</td>
<td>$1.87 \times 10^{-3}$</td>
<td>$1.23 \times 10^{-3}$</td>
<td>$1.73 \times 10^{-3}$</td>
<td>$1.41 \times 10^{-3}$</td>
<td>$3.77 \times 10^{-3}$</td>
<td>$1.95 \times 10^{-3}$</td>
<td>$0.877$</td>
<td>$0.827$</td>
</tr>
<tr>
<td>MCF-7 recovery in the outlet a (%)</td>
<td>48.1</td>
<td>50.8</td>
<td>44.4</td>
<td>37.7</td>
<td>50.8</td>
<td>46.0</td>
<td>46</td>
<td>43.1</td>
<td>37.2</td>
</tr>
<tr>
<td>Log enrichment (-)</td>
<td>5.22</td>
<td>5.27</td>
<td>5.09</td>
<td>4.66</td>
<td>5.15</td>
<td>5.64</td>
<td>5.17</td>
<td>4.90</td>
<td>5.05</td>
</tr>
</tbody>
</table>

<sup>a</sup>Magnetophoretic mobility is in the range of $1.8$–$3.0 \times 10^{-4}$ mm$^2$/T.A.s Qa = Qa' = 15 mL/min. Cell feed concentration is in the range of $1$–$3 \times 10^6$ cells/mL. The number of MCF-7 after QMS separation was determined using cyto spin or membrane stain.

<sup>b</sup>Lysis twice.
overall process used in this study, no specific markers or assumptions on the cancer cell density (w/v) were used for the separation/isolation of the cancer cell, except to make it appear red on the filter to facilitate counting in the final quantification step. (This dye was not used in the cytospin procedure.) With respect to the size of the cell, informal discussions with cancer researchers at the Ohio State University Comprehensive Cancer Center indicated that all the human cancer cells currently being grown in the researchers’ labs are significantly larger than PBL, and typically on the order of the MCF-7 used in this study. The only exception are blood-based cancer cell lines which, in many cases, probably express CD45, which would then defeat the negative selection used in this study. While filtration was used for half of the cell suspension from the QMS separation step, this filtration was not necessary as reported since the other half was centrifuged and subjected to a cytospin. In addition, the filter medium used allowed red blood cells to pass, but not PBL; hence the pore size of the membrane was smaller than six microns; considerably smaller than any other cancer cells (of which the authors are aware).

In conclusion, it has been experimentally demonstrated that it is possible to obtain an average $5.17 \log_{10}$ enrichment of rare cells using a negative cell selection process. Ongoing studies are currently focused on further improvement of both the enrichment and recovery of the target cells, and shortening the time of the overall separation. Also, studies using more clinically relevant cell lines with the ultimate goal of testing and use on human cancer patients is currently being conducted.

Acknowledgments

This work has been supported by the National Science Foundation (BES-9731059 and BES-0124897 to J.J.C.; NSF SBIR 02-056 awarded to SHOT INC.) and the National Cancer Institute (R01 CA62349 to M.Z., R33 CA81662-01, R01 CA97391-01A1 to J.J.C.), and P30CA16058.

References


Figure 7. Photographs of MCF-7 cells recovered in the nonmagnetic fraction stained with anti-HEA FITC as primary antibody, anti-FITC phosphatase alkaline as secondary antibody, and enzyme substrate (FastRed). Cells appear as a bright red color body in the microscope field.

Figure 8. Representative photograph of cytospin of pure MCF-7 cells, pure PBL, and two samples from the QMS after magnetic separation (A, B, C, and D, respectively).