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Capture and Analysis of Cells Using Magnetic Beads

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Capture and Analysis of Cells using Magnetic Beads

A project

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To

Governors State University

By

Alexandro Dominguez

In the partial fulfillment of the

Requirement for the Degree

of

Master of Science

In

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DEDICATED TO MY PARENTS

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Abstract

Magnetic separation of cells and bacterium is a fascinating field of study. There are tremendous capabilities in separation, selectivity, and sensitivity.¹ Magnetic separation reduces complex techniques, time consuming preparation, and complicated multistep methods of separation.² The separation of cells, bacterium, and macromolecules can be extremely challenging. Fortunately, magnetically tagged cells, bacterium and macromolecules can be easily separated without great expertise. Antibody magnetic micro beads are specific to a cell, bacterium, or macromolecules. Separating a mixture can lead to a better, more accurate analysis of samples that can be crucial in the medical field, therapeutic drug delivery systems, and in some circumstances lifesaving.³ The ability to positively identify a disease in hours instead of days results in faster recovery times and minimizes the spread of the disease.⁴ Faster recovery time can be lifesaving in many countries because then the disease can be treated quickly.

Introduction

Magnetic cell sorting can lead to a better and more comprehensive understanding of the human body. A better understanding of the human body would lead to better medical treatments, smarter prevention, and new medical intervention for diseases. A variety of cells can be more thoroughly studied with fluorochromes incorporated with magnetic separation.⁵ Magnetic separation purity can be increased by various methods. One method is uses a double column where the second column further purifies and separates the macromolecules from the solution. Having several micro bead antigens can also increase the purity of the sample. Finally, stronger magnets can polarize the molecules to a better separation due to magnetization.⁶

The ability to be exceptionally selective is a concept that is important to the scientific community. Cells, bacterium, and macromolecules are composed of very unique molecules. These molecules polymerize into macromolecules, and macromolecules have unique binding sites that can be utilized in magnetic separation. Magnetic micro beads attach to the binding sites and are magnetically separated from the solution. Magnetic micro beads can be purchased from several pharmaceutical companies. There is a huge demand for synthesizing specific magnetic beads because of their capabilities in separation. It is important to note that separation techniques are based on several factors such as affinity, size, binding, stereochemistry, van der waal forces, etcetera.⁷ Micro magnetic beads are prepared based on what is being separated, and the best method of separation. Different samples have different physical characteristics. Though there is a strong inclination for the magnetic bead to only attach to the target cells, physical forces from non-target cells can also influence magnetic interaction.⁸

Background

There are two methods of magnetic labeling; direct and indirect magnetic labeling. Direct labeling is a one-step process and is usually the simplest to perform. The specific antibody micro bead is directly labeled to the cell. There are numerous antibody micro beads such as biotinylated antibody, streptavidin micro bead, and fluorochrome-conjugated antibodies available in the market. Indirect magnetic labeling is usually completed when no direct micro beads are available for the cell. A cocktail of antibodies can be used to remove undesired cells from the desired cell.⁹ A pharmaceutical company can produce an antibody micro bead solution that is specific to a particular antigen on the cell where several antibody micro beads will attach to the cell. Magnetically labeled cells can then be separated with magnetization. There are two methods of separation, one being positive selection where the target cells are magnetically labeled and are

magnetically retained in cell fraction. This strategy is the easiest method resulting in outstanding purity, excellent recovery, and fast results. The second method is the untouched isolation, where the undesired cells are removed. This method works well if there is no specific antibody available for target cells, and if binding to the target cell is not desired.¹⁰

There are several methods of micro magnetic separation. One piece of ground-breaking technology that can be seen here are magnetic-activated cell sorting (MACS) separators. The MACS high gradient magnetic separation columns can separate large cells up to 50 μm in diameter. The capacity max is 2×10^8 total cells and 1×10^7 magnetically labeled cells.¹¹ The large cell separation columns are engineered for positive selection of human and animal cells. There is a wide range of applications from small proteins to cells. The column itself has a hydrophilic coating. The buffers suggested for rinsing are PBS, EDTA, or BSA.¹¹ The column is washed to remove unlabeled cells, and the cell-magnet complex is separated. Then the flow resistor is removed, and the target cells elute from the column. The MAC column demonstrates how routine the separation really is. There are several automatic magnetic separator instruments, manual magnetic separators, magnetic separator kits, and micro beads available in the market.¹¹

Examples of Experiments

Magnetic Capture of *Mycobacterium Avium* subsp. *Paratuberculosis*

Magnetic separation allows enormous specificity, sensitivity, and separation of bacterium. *Mycobacterium avium* subsp. *paratuberculosis* is an example of a bacterium that was captured effectively with magnetic separation. The immunomagnetic (IMS)-phage assay yields excellent detection of *M. avium* subsp. *paratuberculosis*. The recovery was ≤ 10 CFU/ml from both spiked broth and milk.¹² Table one lists several different paramagnetic beads and their

corresponding vendors (the paramagnetic beads are coated in-house with the appropriate antigen.)¹²

TABLE 1. In-house-prepared paramagnetic-bead-coating-antigen combinations evaluated.

Paramagnetic beads	Coating antigen ^a				
	Polyclonal antibody S624	aMp3 peptide	aMptD peptide	Biotinylated aMp3 peptide	Biotinylated aMptD peptide
Dynabeads, M280 sheep anti-rabbit IgG ^b	+	-	-	-	-
Magnabind carboxyl derivatized beads ^c	-	+	+	-	-
Amine-coated magnetic hollow glass microspheres ^d	+	+	+	-	-
Dynabeads, MyOne Carboxylic acid ^b	+	+	+	-	-
Dynabeads, MyOne Tosylactivated ^b	+	+	+	+	+
Dynabeads, MyOne Streptavidin-T1 ^b	-	-	-	+	+
Dynabeads, M280	-	-	-	+	+

Coating antigen^a

Paramagnetic beads

Polyclonal	aMp3	aMptD	Biotinylated	Biotinylated
antibody S624	peptide	peptide	aMp3 peptide	aMptD peptide

Streptavidin^b

^a+, tested; -, not tested.

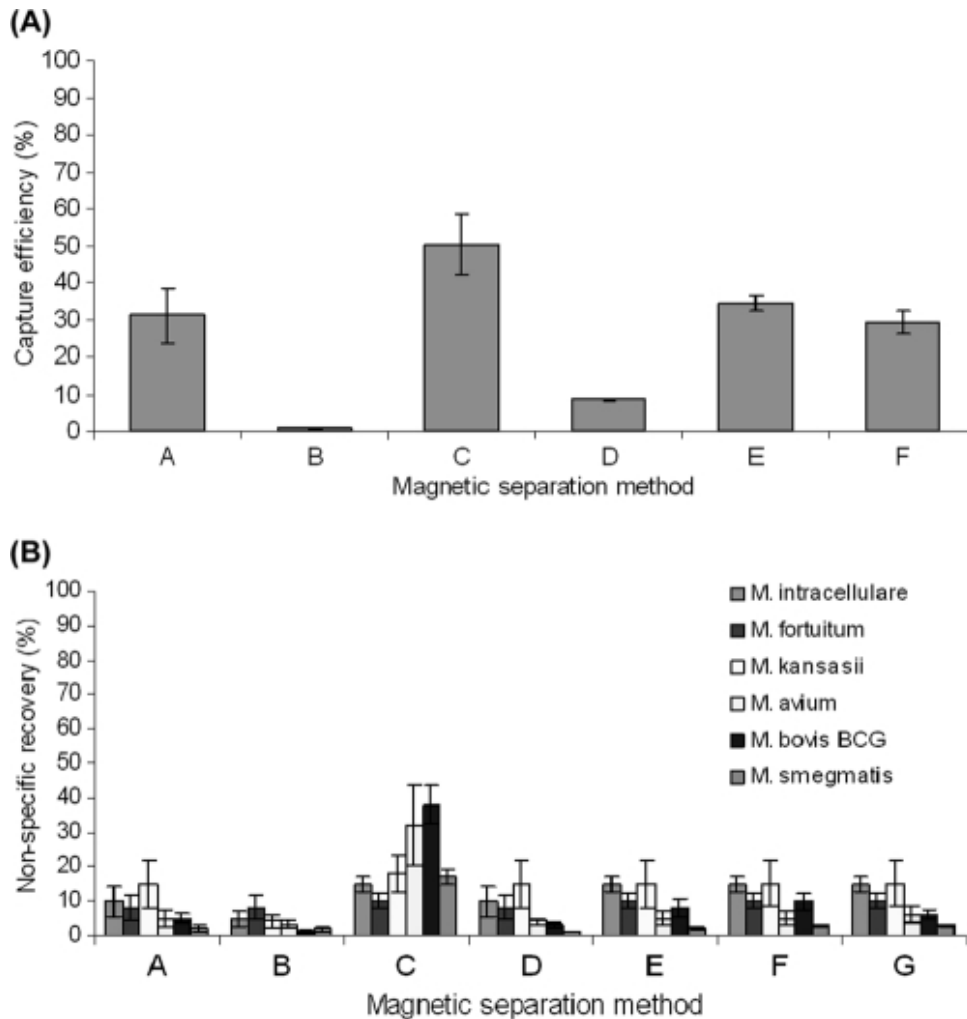
^bFrom Invitrogen, Life Technologies Corporation.

^cFrom Pierce Protein Research Products, Thermo Scientific.

^dFrom Microsphere Technology Limited, Adare, County Limerick, Republic of Ireland.

There are numerous paramagnetic beads and the coating antigens are specifically synthesized for the target bacterium. Bacterial species have abundant binding sites that can be selected for optimal binding and separation. Binding sites are based on abundance, accessibility, and cost effective generation of the respective antibody. There is an undesired effect of having antibody micro beads attached to several bacteria for a positive selection. A false positive/negative can lead to massive recalls of milk, food, and the health of the consumer being compromised. Therefore, it is important to know the medium/interferences, and use the micro beads that specifically bind to the target bacterium. The immunomagnetic (IMS)-phage assay plays an important role because there is minimal nonspecific binding from other mycobacteria.

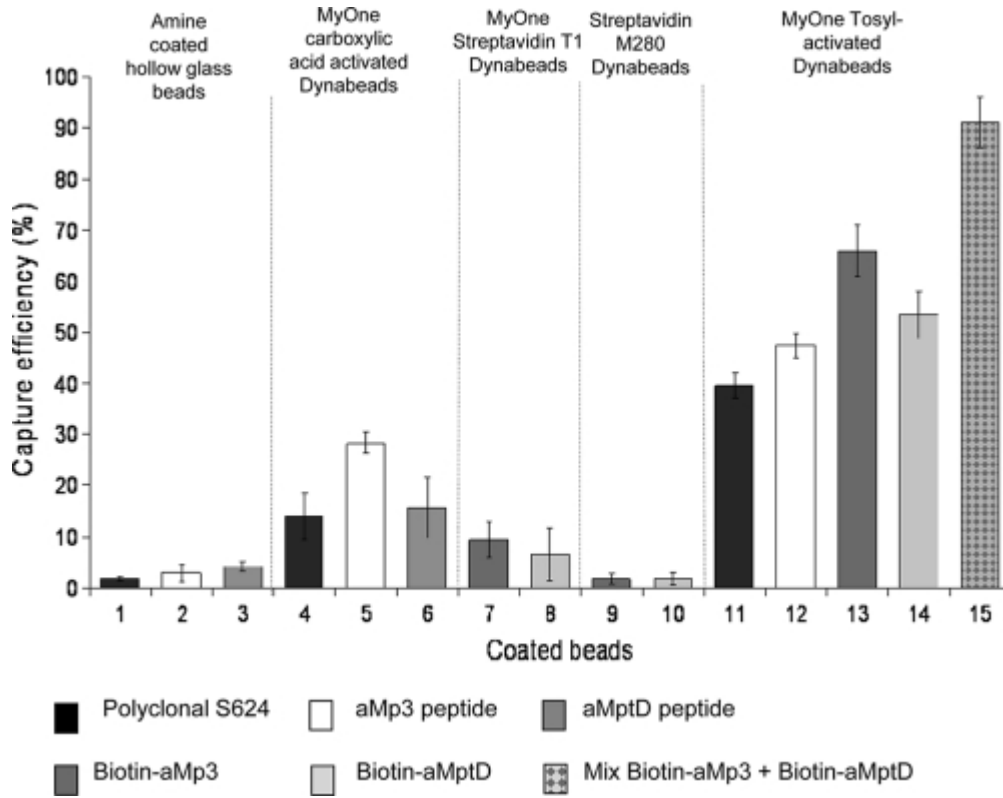
Figure 1. Magnetic separation method capture efficiency (%) graph A and nonspecific recovery (%) graph B.



Initial evaluation of the performance of six different in-house-coated or commercially available paramagnetic beads for magnetic separation applied to 1-ml aliquots of Middlebrook 7H9-OADC broth containing 10^3 to 10^4 CFU *Mycobacterium* sp./ml in terms of the mean efficiency of capture (expressed as a percentage) of *M. avium* subsp. *paratuberculosis* (A) and the mean percentage of nonspecific recovery of other *Mycobacterium* spp. (B). Methods A and B, M280 sheep anti-rabbit IgG Dynabeads coated with polyclonal antibody S624 and a 1:10 dilution of polyclonal antibody S624, respectively; method C, Pathatrix PM50 beads coated with a polyclonal antibody (Matrix Microscience, Newmarket, United Kingdom); method D, AnDiaTec beads coated with a monoclonal antibody (AnDiaTec GmbH, Kornwestheim, Germany); methods E and F, Pierce MagnaBind carboxyl derivatized beads carbodiimide linked with aMp3 and aMptD, respectively; method G, uncoated Pierce MagnaBind carboxyl derivatized beads.¹²

(See Figure 1) Graph A displays the commercially available Pathatrix PM50 beads coated with polyclonal antibody. Method C has the most efficiency capture of *M. avium* subsp. *paratuberculosis* at a mean of 50.3% with a deviation of $\pm 8.4\%$. Method A has the M289 Dynabeads coated with polyclonal antibody S624 has a mean of 29.2% to 34.2%. The graphs revealed reasonable capture for method E, A and F. Poor capture was demonstrated in methods B and D. (See Figure 1) Graph B displays Pathatrix PM50 beads coated with polyclonal antibody (method C) with the most nonspecific recovery percentage at $22\% \pm 11\%$. The antibody micro beads interact with various bacteria. Graph B displayed less than 10% nonspecific recovery for all groups except group C. Remember, life is composed of the same building blocks. Hence, the different bacteria can display the same polypeptide or a similar polypeptide with the same physical forces. There was no *M. avium* subsp. *paratuberculosis* capture at 100%. It is of utmost importance to be as close as possible to 100% capture efficiency.

Figure 2. Improved capture efficiency with combination of paramagnetic bead-coating antigen.



(See Figure 2) The addition of extra paramagnetic bead coating antigen significantly increased capture efficiency. The most efficient capture of *M. avium* subsp. *paratuberculosis* is 91.5% ± 5.0% by PMS with a 50:50 ratio of MyOne Tosylactivated Dynabeads coated with biotinylated aMp3 and biotinylated aMptD. The MyOne Tosylactivated Dynabeads, bars 11 through 15, exhibit impressive improved capture efficiency. The amine-coated magnetic hollow glass beads coated with polyclonal antibody S624, aMp3, and aMptD presented capture efficiency below five percent. Group nine and ten with M280 streptavidin Dynabeads coated with biotinylated aMp3 and biotinylated aMptD had less than three percent capture efficiency. Different characteristics that determine the selectivity of capture are the coating antigen

polyclonal, monoclonal, antibody, peptide or biotinylated. In addition, the bead characteristics such as composition, size concentration and surface play a role in capture efficiency.¹²

In the use of a combination of several paramagnetic bead coating antigens, the automated and manual PMS did not have significant variance in efficient magnetic capture, however, the mean recovery of nonspecific recovery by *M. bovis* BCG by automated IMS (AIMS) was noted as less than the manual IMS. It is suggested that AIMS moves the beads from tube to tube during processing. That movement of beads most likely leaves non target mycobacterium behind on surface of tubes versus the manual process which leaves beads in the same tube while processing.¹²

Immunomagnetic separation of pathogenic mycobacterium was accomplished. Magnetic beads with genus specific polyclonal and mouse monoclonal antibodies complexes were tagged with anti-mouse biotinylated antibody. The addition of quantum dots resulted in a fluorescent detection. The limit of detection was 10^4 bacteria/ml and 10^3 bacteria/ml with the usage of a spectrofluorometer.¹³ Immunomagnetic isolation of $CD4^+CD25^+FoxP3^+$ natural T regulatory lymphocytes is a more complicated and longer process. The T regulatory cells were isolated from leukapheresis products via double negative selection of anti-CD8 and anti-CD19 monoclonal antibody continued with positive selection of anti-CD25 monoclonal antibody. The final cell fraction, $CD4^+/CD25^+$, resulted in a mean purity of 93.6% with a standard deviation of ± 1.1 . The recovery efficiency was $81.52\% \pm 7.4\%$.¹⁴ Immunomagnetic separation can be a series of steps that finally leads to a purified product.

Magnetic Cell Sorting of Parasitized Erythrocytes

Magnetic cell sorting is a fast and accurate method of analyzing diseases. Magnetic sorting depends on interaction between cell surface antigens, antibodies, and magnetic particles. Magnetic deposition microscopy (MDM) (See **Figure 3**) captures parasitized erythrocytes in a magnetic field and the sample is placed on a slide. The sample on the slide can then be stained and viewed immediately.¹⁵

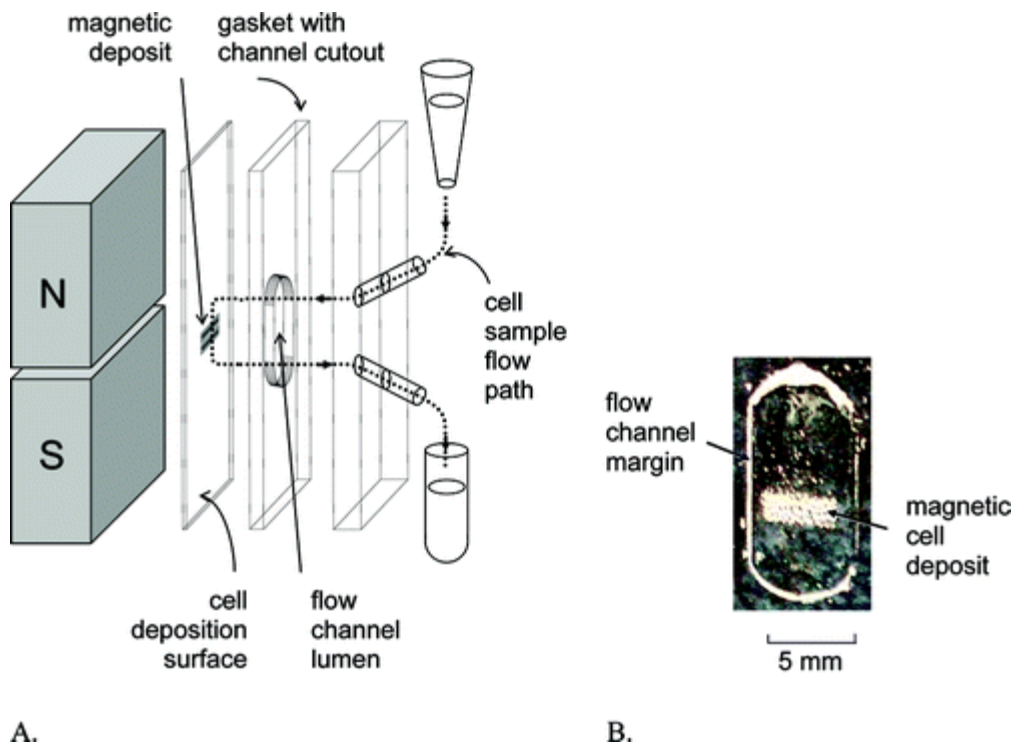


Figure 3. (A) Components of the malaria MDM device and the sample flow path. The location of the expected magnetic cell deposit band next to the magnet pole piece tips. (B) An unaided eye appearance of the magnetic deposition, collected in the interpolar gap area (Panel A and B), from a *P. falciparum* parasitized blood sample.¹⁵

High Gradient Magnetic Separation of Infected Red Blood Cells

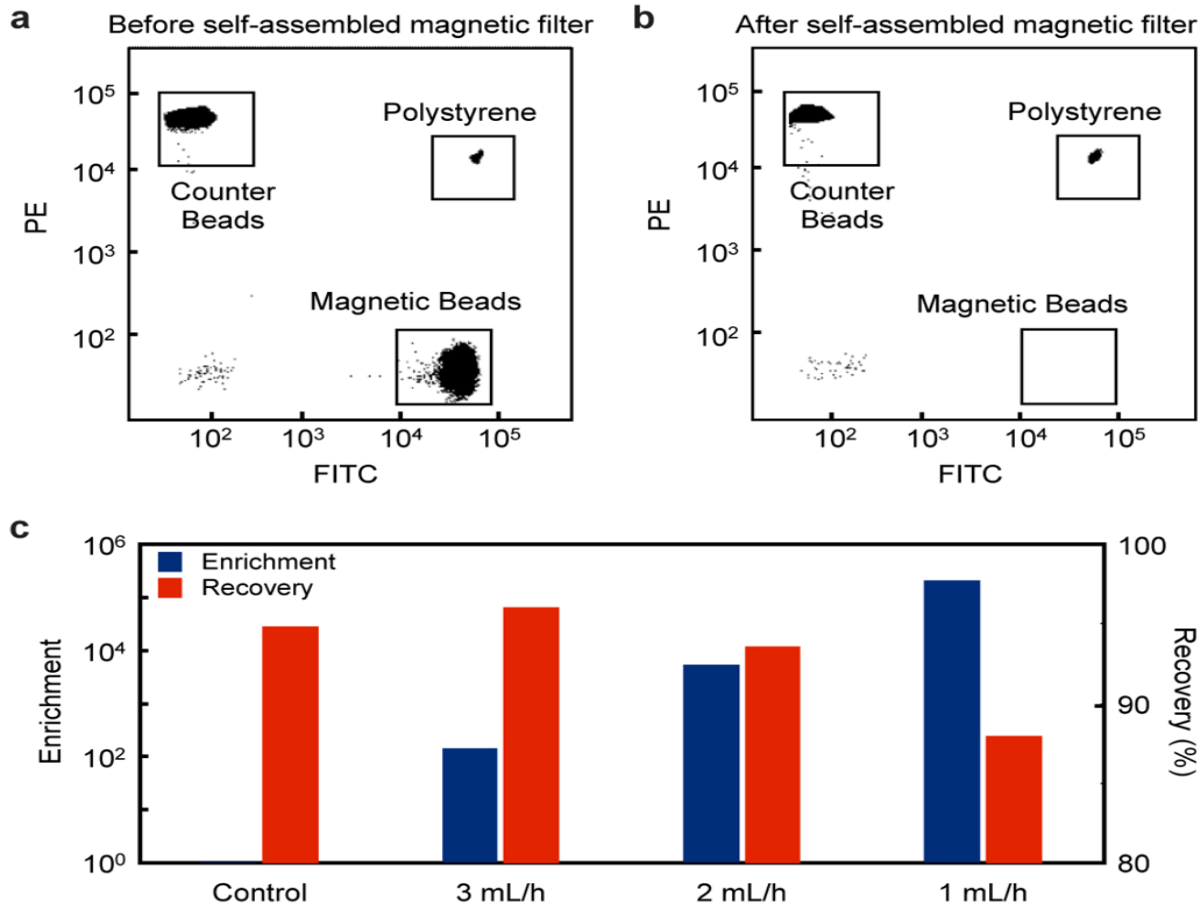
High gradient magnetic separation has been utilized for concentrating or eliminating malaria from infected red blood cells (IRBCs) via blood magnetic properties (Fe content). The

column was loaded with 5×10^8 RBCs. The average yield for the six experiments was $12.1 \times 10^6 \pm 2.6 \times 10^6$ IRBC with a purity of $95.74\% \pm 1.38\%$. Purities ranged from 94.23% to 98.26%.¹⁶ Any particle with a higher magnetic capacity will replace a particle with lower magnetic capacity. Optimizing the column load can significantly increase the purity on the eluted sample. In addition, using a second column can further purifying the product.¹⁶

Magnetic Filter of Leukocytes from Tumor Cells

Another approach to immunomagnetic separation is the use of a magnetic filter. The magnetic filter attracts magnetically tagged cells from solution with alternating magnetic dipoles. The magnetic filter was first tested by sorting magnetic beads from nonmagnetic beads. (See **Figure 4**) High capture yields were attained with approximately 90% of nonmagnetic beads eluted from the filtration assembly whereas magnetic beads were preferentially captured. The magnetic filter enhanced the removal of nonmagnetic beads by a factor greater than 10^5 . A strong magnetic force can achieve high capture efficiency on a moderate flow rate of 1 ml/hr. The magnetic field decays rapidly with distance creating large gradients. The length of the magnetic field can be adjusted with the size of the grains.¹⁷ Therefore, much of the separation occurs rapidly and the separation rate progressively approaches zero with time. The filtration assembly was tested on the basis of sorting magnetic beads from non-magnetic polystyrene beads. The self-assembled magnetic filter also demonstrated proficient sorting of cells.

Figure 4. The graphs below represent flow cytometry before and after self-assembled magnetic filter with the enrichment recovery ratio of polystyrene and magnetic beads.



The self-assembled magnetic filter was examined by filtering a population of magnetic beads from polystyrene beads. Flow cytometry quantified the bead population before and after the filtration. The enrichment and recovery ratio were measured at several flow rates.

Another example uses negatively enriching tumor cells from leukocytes solution that were tagged with CD-45 magnetic beads (MACS, Miltenyi Biotec).¹⁷ (See **Figure 5**) The self-assembled filtration enriched the population of tumor cells to leukocytes by a factor greater than 10^3 . The fraction of tumor cells that passed through the system was approximately 90%. The leukocytes were stained with green dye. Tumor cells were incubated with magnetic beads labeled

with anti-CD45 antibodies and fluorescent antibodies. (See Figure 6) The cells were observed through fluorescence micrographs depicting ratios of 1/10, 1/100 and 1/1000 (tumor/leukocytes).¹⁷ The solution filtered through self-assembled magnetic and concentrated on an integrated micropore filter. Fluorescence micrographs of the solution before the filter and after the micropore filter display a significant difference. After filtration there is a minimal amount of green fluorescent that is visible.¹⁷

Figure 5. Magnetic filtration of leukocytes from tumor cells.

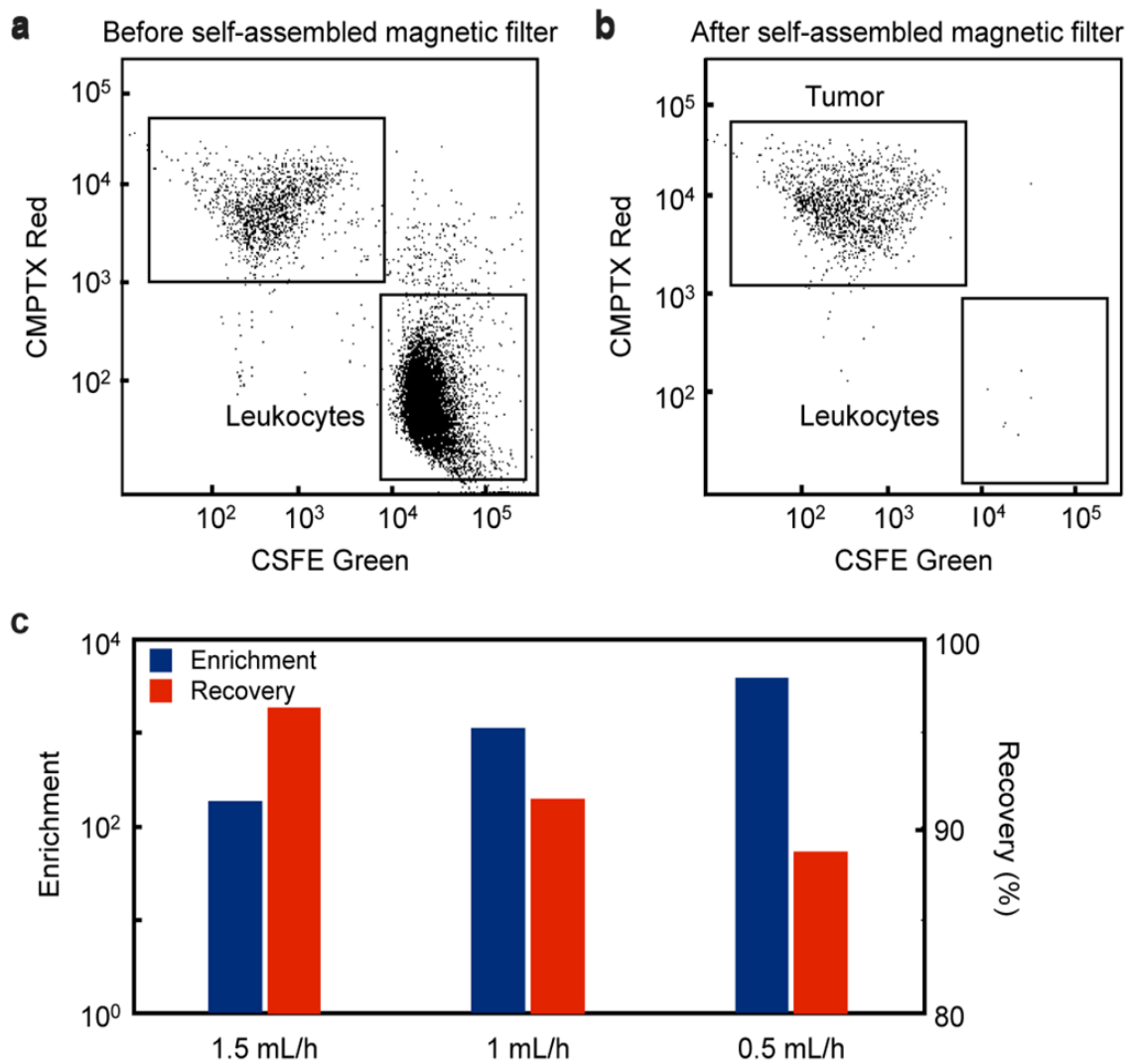
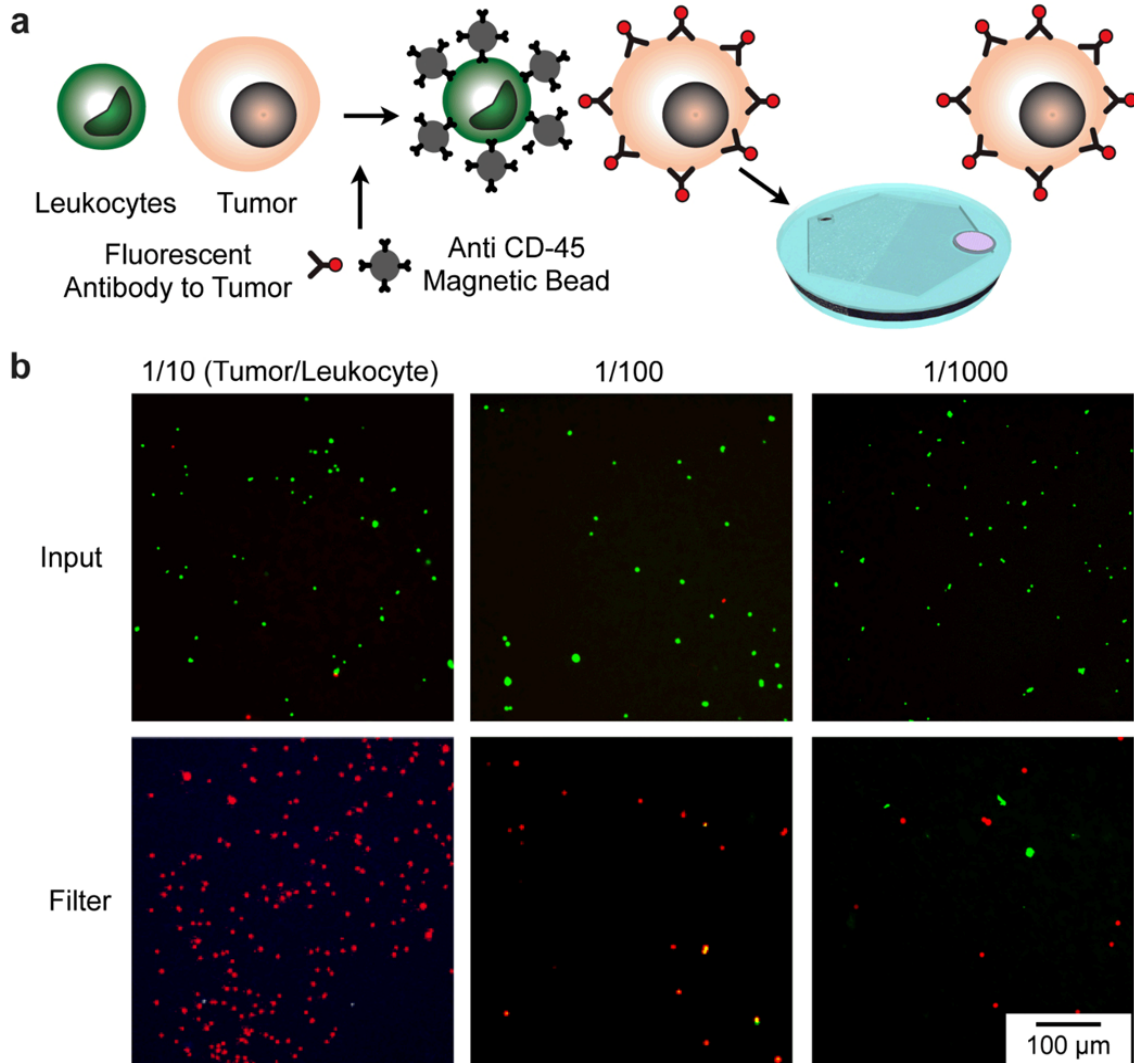


Figure 6. The graphs below illustrate direct labeling and fluorescent micrographs.



Self-Assembled Magnetic Arrays

The concept noted as “Ephesia,” is another method for immunomagnetic separation. Super paramagnetic beads are assembled in microfluidic channel on an array of magnetic traps. The magnetic beads are injected into the microfluidic channel and the beads align together when the magnetic field is switched on. (See **Figure 7**) The array is divided into a series of rows with magnetic ink that attracts the beads within a magnetic field.¹⁸

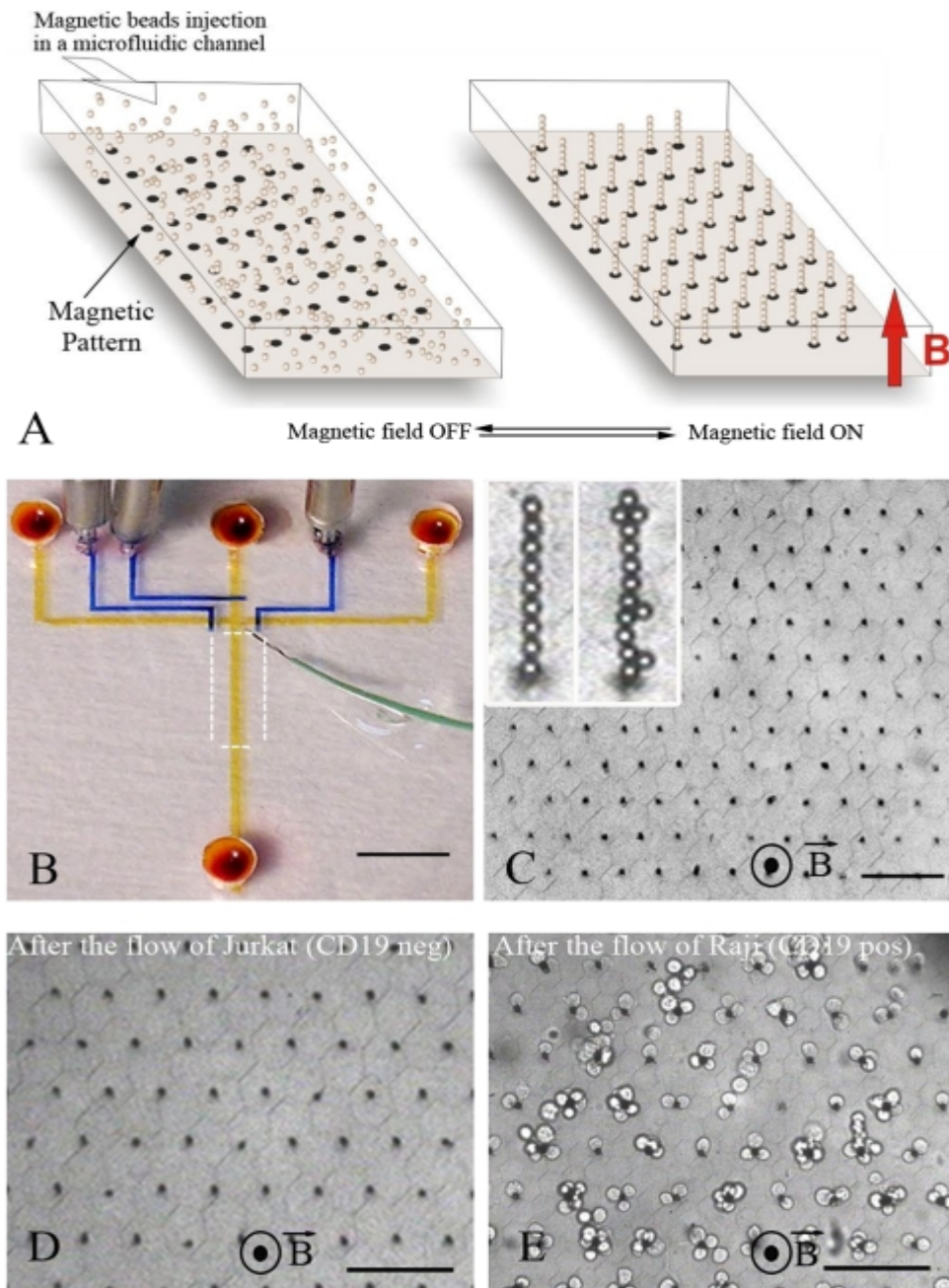


Figure 7. A) Principle of magnetic self-assembly. A hexagonal array of magnetic ink is patterned at the bottom of a microfluidic channel. Beads coated with an antibody are injected in the channel. Beads undergo to Brownian motion. The application of an external vertical magnetic field induces the formation of a regular array of bead columns localized on top of the ink dots. (B) Two levels PDMS integrated microchip. Channels were filled with colored water. Delivery and separation channels for the cells appear in *yellow*. Inlets ports appear in *orange*. The separation channel is the longer vertical branch. The area bearing magnetic posts is marked by the *dotted white box*. Channels in the upper PDMS layer, controlling the opening and closing of the inlet channels, appear

in *blue*. The green wire is a thermocouple for in situ control of the temperature in the system. (Scale bar: 0.5 cm.) (C) Magnetically assembled array of columns of 4.5 μm beads coated with anti-CD19 mAb (specifically retaining Raji B-Lymphocytes). Typical column shapes are shown in the insets. (Scale bar: 80 μm .) (D) Optical micrograph of the columns after the passage of 1,000 Jurkat cells. No cell can be seen. (E) After the passage of 400 Raji cells, numerous ones are captured and rosetted on the columns (Scale bar: 80 μm .)¹⁸

The cell capture correlates to a function of flow rate. The flow rate can increase or decrease the amount of beads that are retained. A channel with the width of 500 μm , a channel height of 50 μm and a 100 $\mu\text{m}/\text{s}$ flow correlates to a flow rate of a few $\mu\text{l}/\text{min}$ with a throughput in the vicinity of ten to hundred cells. Positive and negative cell sorting yielded $97 \pm 2\%$ Raji cells and over 98% Jurkat cells eluted out. There were 612 cells analyzed, 31% Raji and 69% Jurkat that yielded a capture of 97% Raji and 0.02% capture of Jurkat. Resulting in 97% in purity of Raji.¹⁸ Magnetic cell sorting of circulating tumor cells (CTCs) was completed by removing normal blood cells by erythrocyte lysis and pan-leukocyte marker (CD45) antibody tagging.¹⁵

Immunomagnetic nano beads were implemented for detection of circulating tumor cells in several patients. The characterization of CTCs was the underlying task. The purity and recovery of spiked SW620 was analyzed with three enrichment methods. (See **Table 2**) The method was CD45 depletion, positive enrichment and CD45 depletion with positive enrichment. The results of the performance enrichment after spiking 100 SW620 cells in 5ml of peripheral blood are displayed in table two. The CD45 depletion displays the highest recovery at 58%.¹⁹

Table 2. The CD45 depletion, positive enrichment and CD45 depletion with positive enrichment.

Method	Total number of leukocytes		Recovery		Purity
	Before enrichment	After enrichment	Average (%)	Range (%)	Average (%)
CD45 depletion	3×10^7	6.0×10^3	58	50-66	0.97%
Positive enrichment	3×10^7	2.0×10^3	25	24-26	1.25%
CD45 depletion + positive enrichment	3×10^7	1.5×10^3	22.5	20-25	1.50%

Table 3. The detection rate of CTCs in 84 blood samples from 48 epithelial cancer patients and 30 samples from 22 melanoma patients.

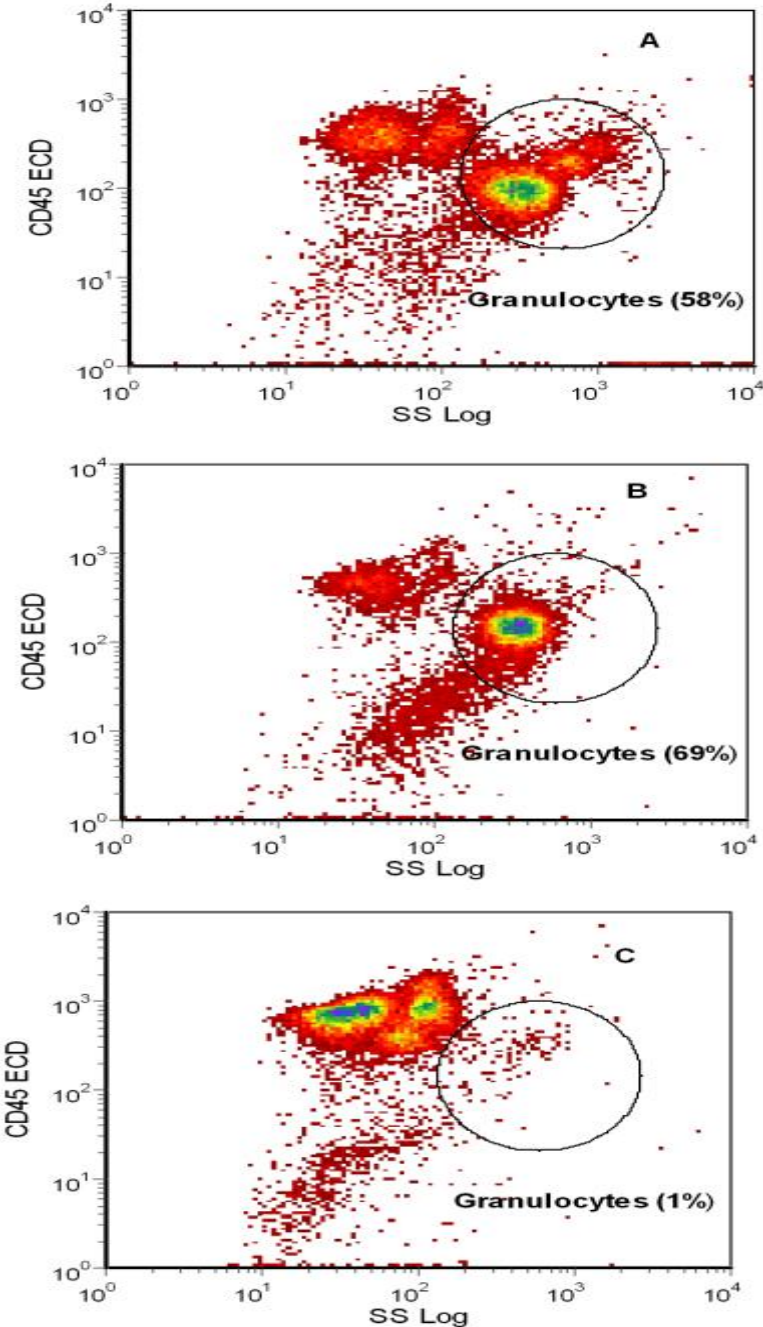
Carcinoma	Number of blood samples	Number of patients	Positivity of blood samples	Positivity of patients
	Gastric	5	3	80% (4/5)
Colon	25	11	44% (11/25)	64% (7/11)
Ovarian	8	6	50% (4/8)	50% (3/6)
Breast	21	10	52% (11/21)	60% (6/10)

Carcinoma	Number of blood samples	Number of patients	Positivity of blood samples	Positivity of patients
Cervix	11	7	64% (7/11)	86% (6/7)
NSCLC	4	3	75% (3/4)	100% (3/3)
SCCHN	10	8	70% (7/10)	75% (6/8)
Melanoma	32	22	53% (17/32)	64% (14/22)

(See Table 3) The detection rate of CTCs ranged from 44% to 80%. It can be challenging and difficult to detect rare CTCs. Immunomagnetic separation is providing an alternative to identification of CTCs and can eventually lead to a more accurate estimation of CTCs.¹⁹

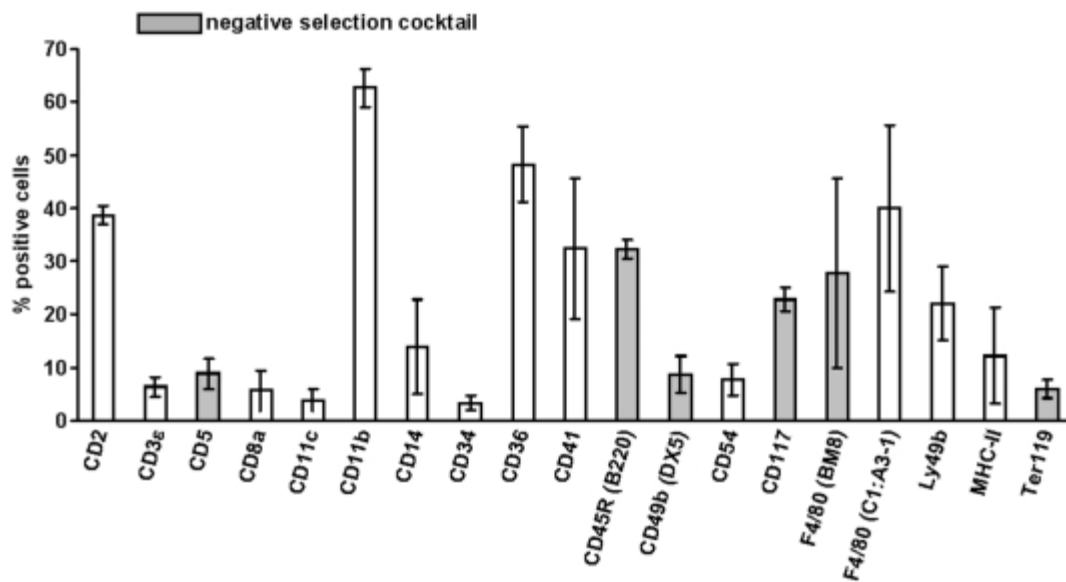
Immunomagnetic bead separation of mononuclear cells contaminating granulocytes in blood samples was also accomplished. The anti-CD15 micro beads were effective due to increased numbers of CD15 binding sites. (See Figure 8) Histogram A with anti-CD-ECD antibody displays 58% granulocytes, histogram B with histopaque displays 69% and histogram C displays 1%.²⁰ Histogram C is after magnetic separation. The histopaque procedure does not remove granulocytes. A high level of separation was attained with magnetic beads.

Figure 8. The Histograms with magnetic separation and without magnetic separation are displayed.



Immunomagnetic negative enrichment of neutrophil granulocyte from bone marrow was accomplished. Polymorphonuclear neutrophils (PMN) mediate early immunity infection and bone marrow is a known greater source of PMN. (See Figure 9) A negative cocktail was implemented to eliminate any direct binding to PMN. Positive selection of PMN was not completed due to previously recorded data of low detection of PMN complex.²¹

Figure 9. Represents a negative antibody cocktail versus percent of positive cells.



Conclusion

The underlying task of magnetic separation relies on novel separation techniques, purification, and analytical analyses, understanding what needs to be isolated is the first step in choosing a method of magnetic separation. There are a variety of magnetic separation techniques that can be chosen to optimize separation efficiency. Immunomagnetic sorting has broad and near limitless application across a spectrum of scientific fields. There are numerous complex cells, bacterium, and macromolecules that can be separated via magnetic separation. Magnetic separation can also purify small molecules. Otherwise, a cell separation would be time

consuming, complex and a multistep process. Reducing the time of a test and having accurate results is imperative especially for disease detection. Future progress with fabrication of complex magnetic structures and magnetic molds will ultimately aid in the development of new techniques and the improvement of existing methods. The molds can be shaped to a specific shape and filled with magnetic material. It is only a matter of magnetic interaction interwoven with selectivity that is the basis for these methods.

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