Applied Cells

Rare Cancer Cell Isolation From Whole Blood Using the MARS® Cell Separation System

ABSTRACT

Circulating tumor cells (CTCs) are cancer cells shed from tumors and circulate in the bloodstream. These cells are considered to be a biomarker which potentially has high value in cancer diagnosis and cancer drug development. However, because of the extremely low frequency of CTC in peripheral blood, which is typically as low as less than 1-10 CTC per mL of whole blood [1], the current CTC isolation and analysis methods are not efficient. At Applied Cells, we developed an integrated system MARS® to isolate rare cells from whole blood with high accuracy, recovery and speed. MARS system is built based on multiple physics principles with embedded novel technologies to remove red blood cells and white blood cells to isolate rare cancer cell from blood with automation. We have developed protocols on MARS to isolate rare cancer cells from whole blood for multi-color flow cytometry analysis. In an experimental system, cultured PC3 cells were spiked in whole blood, incubated with fluorescent conjugated antibodies and magnetic particles and then ran through MARS. Enriched PC3 tumor cells were collected and analyzed on a flow cytometer. We demonstrated up to 4 log enrichment of tumor cells with an average recovery of spiked CTC of greater than 90% from the whole blood (R2=0.9855) with a throughput of 10 mL/hour of whole blood sample.

INTRODUCTION

Automation of Cell Isolation Utilizing MARS

MARS, multi-physics automated reconfigurable separation, enables automation of entire cell sample preparation and rare cell isolation from complex biological samples, such as whole blood, apheresis, and bone marrow. It is an integrated system based on multiple physical principles with built-in novel technologies to achieve cell processing and target cell isolation. This platform uses acoustic fluidic module to separate white blood cells (WBC) from lysed red blood cell (RBC) debris and to reduce WBC sample volume. The in-flow magnetic module of MARS immuno-magnetically captures target cells. MARS can do positive or negative separation of target cells. To isolate intact rare cell for better downstream analysis, negative enrichment is preferred. MARS system is operated with built-in software that controls fluidics to automate cell isolation procedures. It significantly reduces the hands-on time and thus human error.

High Speed and High Recovery of Rare Cancer Cells in Blood Isolation Using MARS

Circulating tumor cell (CTC) enumeration in peripheral blood is an FDA-approved prognostic indicator to predict cancer patient outcome[2]. Phenotypic and genomic analyses of CTC have been applied to identify cancer origin and guide therapeutic selection for personalized medicine. The frequency of CTC in blood is extremely low, can be as low as one in a million WBC. The current CTC enumeration technologies often require many steps involving centrifugation that results in poor recovery[3]. Here we present a protocol using MARS to isolate rare cancer cells from whole blood with negative immune-magnetic selection for multi-color flow cytometry analysis and other downstream applications with automation, high speed, and high efficiency. time and thus human error.

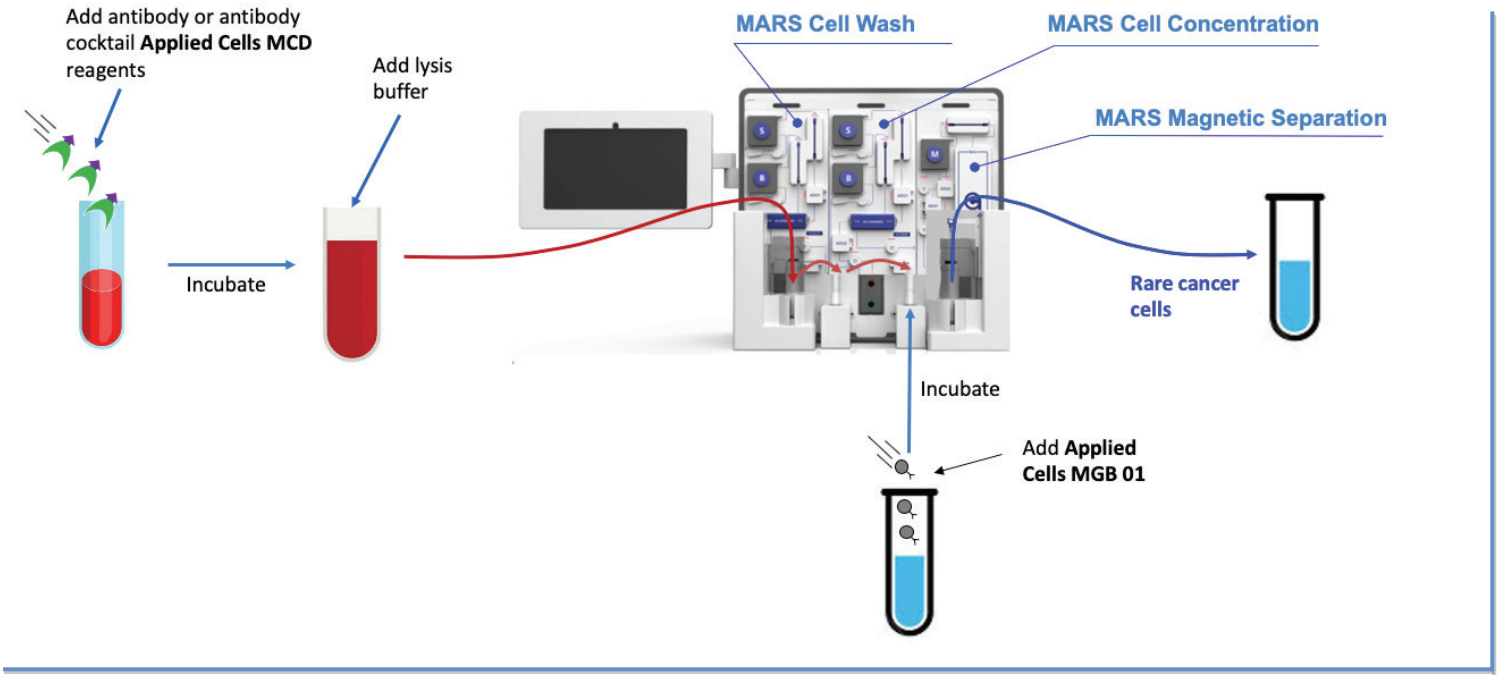


Figure 1. Rare cancer cels in blood isolation workflow.

OBJECTIVE

We designed an experimental system to spike small numbers of cultured PC3 prostate cancer cells into whole blood to develop a protocol to isolate rare cancer cells from whole blood by MARS system. By using MARS system and the protocol developed, we assessed recovery of the spiked rare cancer cells.

METHODS

Cultured PC3 cells were stained with EpCAM PE antibody (5 µL per million cells) and then the number of EpCAM positive PC3 cells were counted by flow cytometer. Determined number of PC3 cells were added to 1 mL of whole blood. Next, EpCAM PE and CD45 AF488 were added to the 1 mL PC3 spiked-in blood and incubated for 10 minutes in the dark at room temperature. Then CD45 biotin (2.5 µg/mL blood) was added to the blood, incubated 20 minutes in the dark at room temperature. After that, RBC lysis buffer (5 mL/mL blood) was added to the blood and incubated for 15 minutes in the dark at room temperature with rotation. Then "MARS WASH" program was run to remove lysed RBC and other debris to isolate WBC. Then "MARS Concentrate" was run to concentrate the WBC into a smaller volume. For removing the WBC, streptavidin conjugated magnetic particles (100 µg/mL blood) were added to the concentrated WBC, incubated for 20 minutes in the dark at room temperature with rotation. Then "MARS MAG" was run to deplete most of the CD45 positive WBC (in the magnetic positive fraction) and recover the rare PC3 cancer cells in the magnetic negative fraction. To analyze the isolated cells and count the PC3 cell number, the entire magnet negative fraction was acquired and recorded by a flow cytometer to get the isolated PC3 cell number. The experimental workflow is shown in Figure 1.

RESULTS

MARS isolates rare cancer cells from whole blood without centrifugation. MARS removes cell debris and non-target cells with three sequential steps, WASH, CONCENTRATE (CON), and Magnetic Separation (MAG). MARS has built-in programs to accomplish the entire workflow with automation. Cell recovery of each cell separation step was assessed separately. The results are shown in Figure 2. Greater than 90% cell recovery from each step were all achieved.

We performed experiments to spike in different numbers (5-80) of PC3 in 1 mL blood with blood cell counts ranging from 2-6 million. The spiked-in samples

were processed by MARS. Debris and most of the WBCs were removed by MARS and PC3 cells were isolated. The numbers of spiked-in cells were counted by a flow cytometer before and after MARS isolation. The recovered PC3 was plotted against the number of cells spiked into the blood (Fig 3A). Average recovery of spiked PC3 was greater than 90% (R2=0.9855) with a throughput of 10 mL per hour of blood.

A bigger range of cancer cells in the whole blood was also assessed. Five cells to 1800 cells were spiked in 1 mL of blood. Good linearity was observed (Figure 3B). Representative flow dot plots of before and after MARS isolation samples were shown in Figure 4, indicating that most of the WBCs and debris but not the rare cancer cells were removed by MARS.

The samples isolated by MARS were further analyzed by imaging analysis. The samples were fixed, permeabilized and stained with DAPI and pan cytokeratin. Fluorescent microscopy images confirmed that most of the isolated cells were cytokeratin-positive cancer cells (Fig 5).

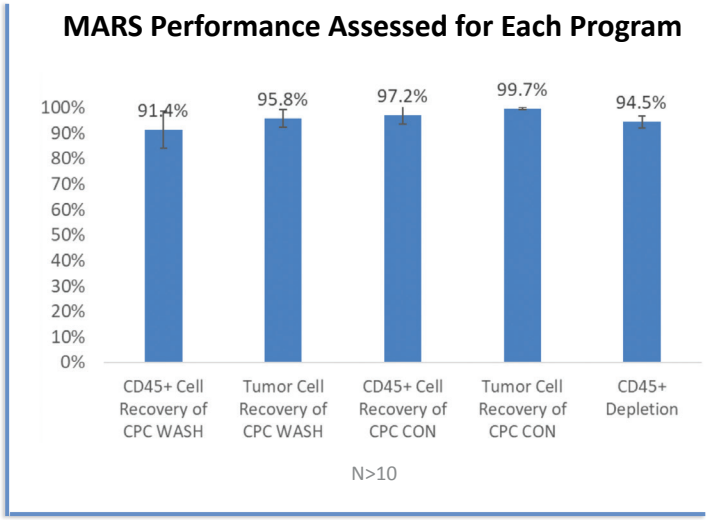


Figure 2. Cancer cell isolation by MARS consists three steps, WASH, CONCENTRATION (CON) and Magnetic white blood cell depletion (CD45+ Depletion). Cell recovery from each step was assessed. Data from more than 10 experiments were compiled. Greater than 90% cell recovery was achieved for each step.

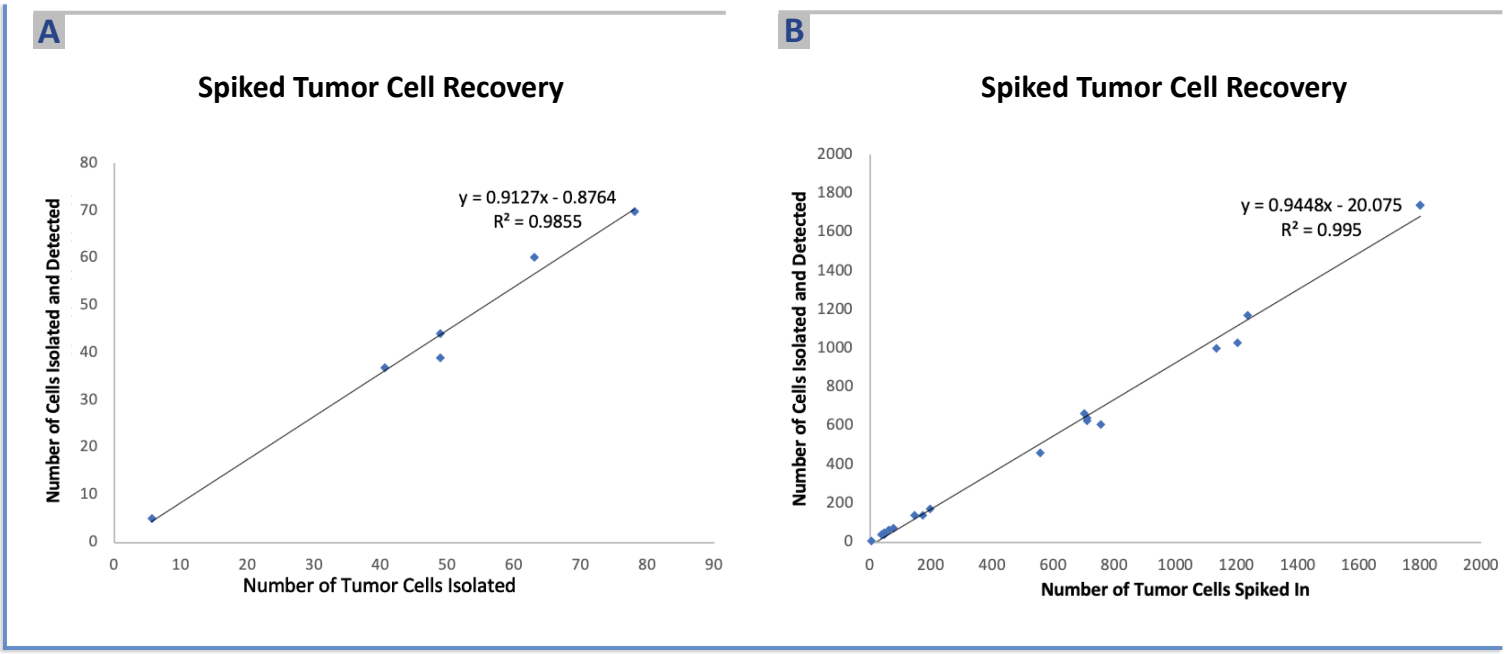
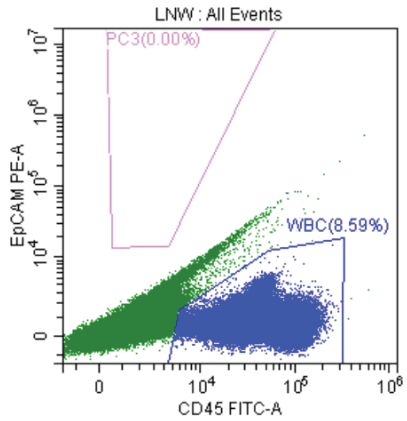


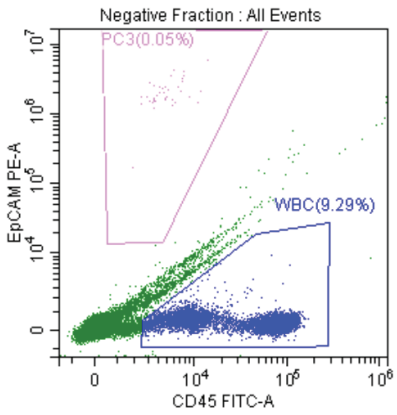
Figure 3. PC3 recovered by MARS isolation was analyzed using linear regression analysis. The samples were processed through MARS and immediately analyzed by a CytoFLEX flow cytometer. **3A** showed the results for spiking 5-80 cells per mL whole blood. **3B** showed the results for spiking 5-1800 cell per mL whole blood.

A **Whole Blood - Lysed No Wash (LNW)**



Population	Events	% Total	% Parent
All Events	2023415	100.00%	100.00%
WBC	173743	8.59%	8.59%
PC3	0	0.00%	0.00%

B **PC3 Spiked Whole Blood - After MARS Workflow**



Population	Events	% Total	% Parent
All Events	81541	100.00%	100.00%
WBC	7578	9.29%	9.29%
PC3	38	0.05%	0.05%

Figure 4. Representative dot plots for spiked PC3 isolation experiments before and after MARS isolation. **4A** showed the lysed no wash (LNW) blood sample before adding PC3 cells. Cells from 33 µL of whole blood were shown, which gave a total of 5,212,290 WBCs in 1 mL of blood. 40 PC3 cells were spiked in 1 mL of blood sample. After the spiked sample being washed, concentrated, white blood cells (WBCs) magnetically depleted on MARS, the entire Negative fraction of the magnetically separated sample from the 1mL PC3 spiked blood sample was run on a CytoFLEX (**4B**). Out of the 40 PC3 cells spiked in, 38 EpCAM positive PC3 cells were captured. Greater than 99% WBCs were depleted. (7,578 WBCs remained in the Negative fraction, from original 5,212,290 WBCs.)

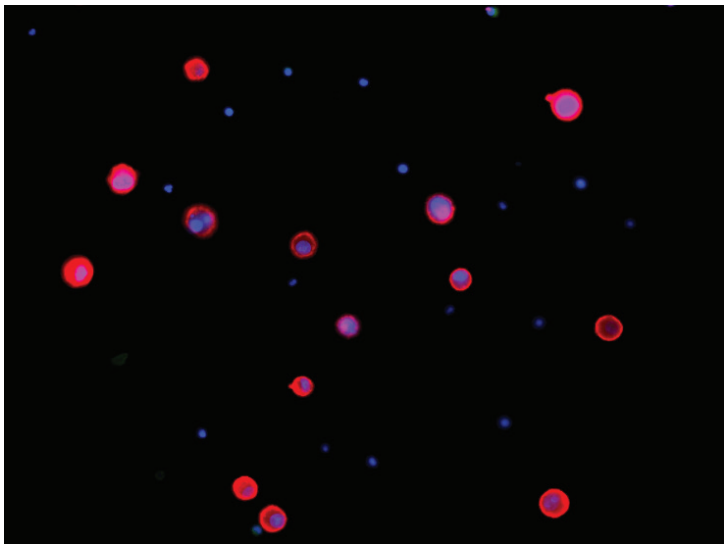


Figure 5. Fluorescent images of spiked PC3 samples after MARS isolation. (Red is pan cytokeratin, blue is DAPI, green is CD45). Notice that most cells recovered were EpCAM positive PC3 cells.

The MARS® System

DISCUSSION

Enumeration and characterization of CTC can guide prognosis in cancer patients, assist in treatment selection, monitor efficacy of therapy, and improve understanding of tumor biology[4]. But one of the challenges for developing clinical CTC applications is isolating limited numbers of CTC from patients' blood without cell loss. Hence, MARS has been developed to enhance efficiency and recovery of rare cancer cell isolation. A significant advantage of this instrument is the ability to perform high- throughput and automated workflow without centrifugation. Research showed that centrifugation and resuspension caused cell loss[5]. MARS utilizes multi-physics cell separation technologies to automate multiple operation steps to minimize cell loss and to achieve high recovery of rare cancer cells in the blood. Another critical feature of our approach is the versatility in terms of the downstream applications. After removing cellular debris and depleting WBCs, intact and enriched cancer cells can be used for FACS sorting, fluorescence analysis, molecular characterization, sterile culture etc. In addition, this workflow provides flexibility to isolate any target cell subset from peripheral blood besides cancer cells just with simple modifications.

CONCLUSIONS

Here, we presented an automated cell separation and sample preparation platform that enables high speed rare cell isolation with high purity and recovery. MARS cell separation system requires no centrifugation to minimize the batch to batch variations. RBC lysed rare cancer patient blood samples can be processed by the automated MARS system through cell washing, concentration, and magnetic depletion process to recover greater than 90% of the rare cancer cells with high throughput while minimizing the hands-on operation and reducing human errors. MARS has combined multiple separation technologies to provide a new solution for rare cancer cell isolation.

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