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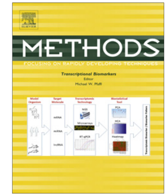

Isolation of circulating tumor cells by immunomagnetic enrichment and fluorescence-activated cell sorting (IE/FACS) for molecular profiling

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ABSTRACT

Circulating tumor cells (CTCs) are cells shed by the primary tumor into the blood stream capable of initiating distant metastasis. In the past decade, numerous assays have been developed to reliably detect these extremely rare cells. However, methods for purification of CTCs with little or no contamination of normal blood cells for molecular profiling are limited. We have developed a novel protocol to isolate CTCs by combining immunomagnetic enrichment and fluorescence-activated cell sorting (IE/FACS). The two-part assay includes (1) immunomagnetic capture using magnetic beads conjugated to monoclonal antibody against an epithelial cell adhesion marker (EpCAM) to enrich for tumor cells; and (2) FACS analysis using EpCAM to purify tumor cells away from mononuclear cells of hematopoietic lineage. Downstream molecular analyses of single and pooled cells confirmed the isolation of highly pure CTCs with characteristics typical that of malignant cells.

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1. Introduction

Currently, very little is known about the biology and molecular characteristics of CTCs. The paucity of information can be largely attributed to the technical hurdles in isolating these rare cells [1]. Despite these challenges, there is a pressing need to elucidate the biology of these tumor cells, including their role in cancer metastasis and progression. CTC based assays can in principle provide easily accessible biomarker information (“liquid biopsy”), and may be more insightful than primary tumor based assays due to greater relevance to metastatic disease, serial sampling ability, and contemporaneous acquisition with cancer progression and treatment response/resistance. Hence, there is a need for an efficient protocol which combines a reliable detection and isolation procedure with availability of cells for further molecular analysis.

A number of approaches have already been developed to detect and count (“enumerate”) tumor cells in blood samples. Initial detection methods included immunocytochemical (ICC) assays as well as nucleic acid–based assays using reverse transcriptase-polymerase chain reaction (RT-PCR). ICC assays for tumor cells typically use monoclonal antibodies (mAbs) directed against antigens associated with epithelial cells but not with hematopoietic cells [2]. For example, Racila and colleagues [3] developed an immunomagnetic enrichment and flow cytometric assay to enumerate CTCs. Subsequently, this approach led to the development of the CellSearch™ assay (Veridex, LLC). This semi-automated image analysis system incorporated sequential immunomagnetic enrichment and ICC [4]. This assay has shown prognostic and predictive significance in multiple cancer types, and is now widely used for the enumeration of CTCs [5,6].

Although ICC- and RT-PCR based methods have been useful in the detection of CTCs, neither approach leads to direct isolation of CTCs. Indeed, true separation of these rare cells from an overwhelming background of hematopoietic cells (~1CTC per billion blood cells) is inherently challenging. With ICC and other slide based methods, efforts to isolate cells by micromanipulation have been proposed but are technically demanding [7,8]. Alternative methods have used epithelial cell capture, thereby greatly enriching the tumor cell component relative to hematopoietic cells [3,9–11]. These enrichment methods, however, still retain a considerable amount of leukocytes, resulting in a heterogeneous admixture which may be difficult to analyze [12,13]. It is also possible that reliance on capture/enrichment methods alone can result in artifactual isolation of non-malignant epithelial cells. It is therefore important to verify that operationally defined circulating epithelial cells are in fact CTCs. Recent reports using a semi-automated dielectrophoretic cell sorting approach (DEPArray system) have also demonstrated the feasibility of this approach for isolation of single and pooled cells and molecular analysis of putative CTCs [14,15].

Our group has developed novel and robust approaches for CTC isolation and molecular analysis [16,17]. We have combined immunomagnetic enrichment and fluorescence-activated cell sorting (IE/FACS) followed by molecular profiling, e.g., array comparative genomic hybridization (aCGH) for genome-wide copy

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number analysis of CTCs. Unlike previous methods, which typically have entailed substantial leukocyte contamination, the IE/FACS approach provides efficient and complete isolation of CTCs, and is highly amenable to molecular profiling of CTCs. We have also demonstrated that the isolated cells are CTCs rather than contaminating hematopoetic or nonmalignant epithelial cells. Here, we describe the IE/FACS method for isolation of CTCs from blood, and describe its utility for downstream molecular analysis.

2. Immunomagnetic enrichment (IE)

2.1. EpCAM capture

The IE/FACS assay was designed to target the cell surface protein EpCAM, also known as tumor-associated calcium signal transducer 1 (TACSTD1), in both immunomagnetic enrichment and flow cytometric steps using two independent mAbs. This eliminates the possibility of missing tumor cells that fail to show adequate expression of two different antigens, such as EpCAM and selected cytokeratins, especially since cancer cells may vary in their cytokeratin expression profile [18]. Note that EpCAM-negative tumors will be missed by any strategy relying upon anti-EpCAM enrichment; however, since up to 95% of epithelial tumors express EpCAM [19], there is only a small possibility of missing cancer tumor cells. Nonetheless, CTCs with low EpCAM expression such as those undergoing epithelial–mesenchymal transition (EMT) may also be missed [20]. It is possible that addition of EMT markers such as vimentin may help detect EpCAM-negative CTCs [20].

Our assay configuration also obviates the need for a permeabilization step to stain for intracellular antigens (e.g., cytokeratins). Since detergent-based permeabilization may affect the suitability of cells for downstream analyses, our assay minimizes such manipulation by direct staining of intact cells prior to acquisition. The need to expose the cells to harsh conditions (e.g., detergents) before analysis may also lead to cell loss during more extensive manipulations.

2.2. Method for immunomagnetic enrichment

The immunomagnetic enrichment step utilizes EpCAM-mAb coated magnetic beads to positively select for EpCAM-expressing cells while reducing the background of hematopoietic cells. IE also involves the reduction of the initial 10–20 mL of blood sample to a volume (0.5 mL) that can be analyzed by FACS for a relatively short period of time, i.e., <30 min/sample (see next section). The setup (Fig. 1), reagents and steps involved in IE are described below. The protocol is designed to process 10 mL of peripheral blood. For other amounts, adjust reagent levels accordingly.

2.2.1. Materials

1. Large magnetic cell separator for 50 mL conical tubes.
2. Small magnetic cell separator for 12 × 75 mm round bottom tubes.
3. 50 mL polypropylene conical tube.
4. 12 × 75 mm round bottom tubes.
5. Peristaltic pump aspirator set up.

2.2.2. Reagents

Note: Reagents were obtained from BD Biosciences and stored long-term at 4 °C. Bring the reagents to room temperature before use. All steps must be performed at room temperature. Reagents for FACS analysis must be RNase- and DNase-free.

Two different monoclonal antibodies against EpCAM, MJ37 and EBA-1, are added simultaneously. MJ37 and EBA-1 recognize distinct epitopes of EpCAM and are non-competing. MJ37 is used for immunomagnetic capture (ferrofluid; proprietary reagent from BD Biosciences), while EBA-1 conjugated to fluorophore is used for detection. The nucleic acid dye (proprietary reagent from BD Biosciences) has an emission maximum that can be detected in the FL-1 (FITC; green) channel. Proprietary BD Biosciences reagents can be requested directly from the company.

1. Cell Buffer [BD Biosciences proprietary formulation; special order].
2. EpCAM (MJ37) mAb-coated magnetic beads (aka ferrofluid) [BD Biosciences proprietary formulation; special order].
3. EpCAM (EBA-1) mAb-conjugated to phycoerythrin (PE) [Catalog #347198].
4. Nucleic acid dye [BD Biosciences proprietary formulation; special order].
5. CD45 (2D1) mAb-conjugated to Cy5-PerCP [Catalog #340953].
6. 1x phosphate buffered saline (PBS) [Catalog #554781].

2.2.3. Protocol

1. Pipette 10 mL peripheral blood into a 50 mL conical tube.
2. Add 5 mL of Cell Buffer.
3. Add 60 µg ferrofluid.
4. Add 400 µl EpCAM (EBA-1) mAb-conjugated to PE [5 µg/mL].
5. Mix by swirling 50 mL conical tube.
6. Incubate for 15 min (mixing at the halfway point and at the end).
7. Place 50 mL conical tube in the large magnetic cell separator for 45 min.
8. Aspirate supernatant at ~15 mL/min using the peristaltic pump aspirator being careful to not disturb the walls of the conical tube.

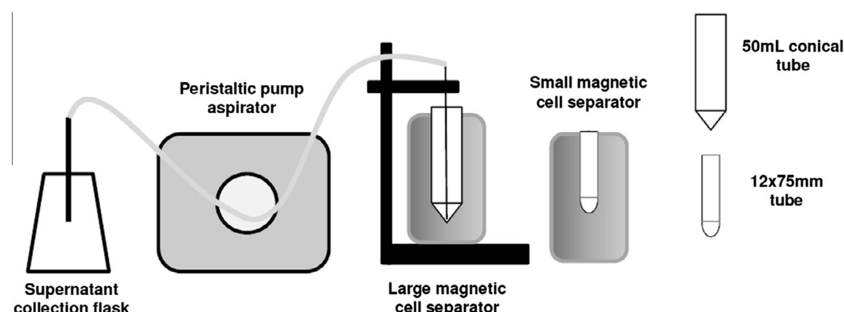

Fig. 1. Immunomagnetic enrichment for circulating tumor cells. Details of the protocol are described in the main text. First, ferrofluids and the EpCAM stain were added to the sample and then exposed to a magnetic field using the large magnetic cell separator. Next, a peristaltic pump system was used to aspirate unbound cells. The bound cells were transferred to a smaller tube and then subjected to another round of enrichment using the small magnetic cell separator. After aspiration of unbound cells, the nucleic acid dye and the CD45 stains were added to the sample and subjected to fluorescence activated cell sorting (FACS).

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