Positive enrichment of circulating tumor cells (CTCs) on the KingFisher™ Purification System

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ABSTRACT

Circulating tumor cells (CTCs) are an important liquid biopsy analyte and biomarker for the diagnosis, prognosis, and treatment of cancer. However, isolating and detecting these rare CTCs is challenging. Magnetic bead-based technologies have emerged as a ubiquitous method in CTC isolation, but most of them are a manual time-consuming process and lack an effective automation workflow suitable for Thermo Scientific™ KingFisher™ platforms. Here, we have developed a time-saving semi-automation workflow for CTC positive capture from for KingFisher™ instruments.

MATERIALS AND METHODS

We developed streamlined automated workflows for both positive (Figure 1) and negative (Figure 2) CTC isolation. To qualify both the positive and negative workflows and associated automation scripts, we contrived samples by spiking varying concentrations of colon cancer cells into a background of 1 million PBMCs.

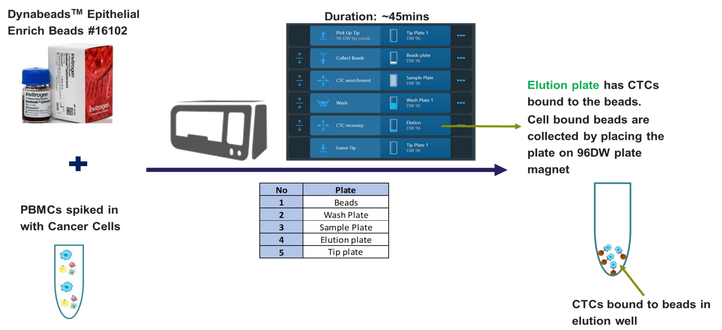
To separate the PBMCs from whole blood, we employed Ficoll-Paque treatment. Two sets of samples were prepared with the purpose of CTC isolation using both positive and negative isolation methods. Colon cancer cells (HCT 116) were spiked into a 1 million PBMC background at three different cell counts: 1000, 100, 10 cells.

The positive isolation method used Dynabeads Epithelial Enrich beads. These beads are designed to selectively target the EpCAM expression on CTCs and attach to the bead. For negative isolation, we utilized Dynabeads CD45. These beads target PBMCs, enabling removal of non-tumor cells. This negative isolation method results in untouched, unbound CTCs, not attached to a bead. The CTCs remain in solution in the sample plate following the PBMC depletion.

To assess the intactness and integrity of EpCam surface markers after both extraction methods, we conducted immunogenetic staining by using EpCam antibodies labeled with FITC dye to stain the EpCam surface markers that are expressed on isolated CTCs. CellTracker™ Red CMTPX was used to stain the isolated CTCs and Hoechst 33342 solution was used as a nuclear stain.

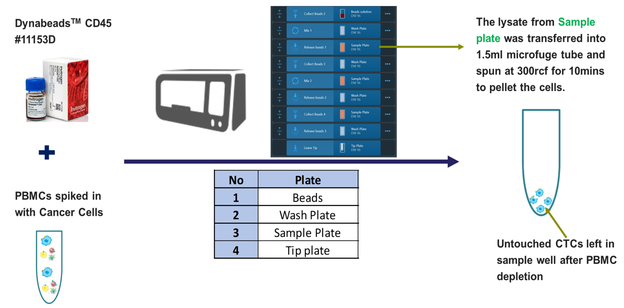
We conducted additional downstream analysis to determine if the isolated cells were viable (Figure 3) and if the cells were suitable for downstream molecular testing. For cell viability evaluation, we cultured the isolated cells in McCoy's 5A Medium (ATCC #30-200) and Fetal Bovine Serum. The cells were cultured for a week, then stained and visualized. To assess compatibility with molecular applications, both captured and untouched cells were lysed using the direct lysis TaqMan™ Cell-to-Ct Express lysis solution (Thermofisher Cat no. A57986) to extract RNA. This was followed by cDNA synthesis (SuperScript IV VILO Master Mix, Thermofisher #111756050), and qPCR using a TaqMan™ Single tube qPCR assay targeting EpCAM with TaqMan™ Fast Advance Master Mix (Thermofisher #4444558). See Figures 4 and 5 for RNA detection Cq values in both workflows.

Figure 1: Positive Isolation workflow and script:



The bead plate was prepared by adding 25xL Dynabeads™ Epithelial Enrich beads to each well. 1 million PBMCs spiked with varying numbers of CTCs (10,100, 1000) were added to the sample plate. The Wash solution and Elution solution consist of 1X PBS (Ca/Mg free) with Bovine serum albumin and 0.5M EDTA. All plates, along with tip comb, were loaded on KingFisher™ Apex instrument to execute positive isolation script. Bead bound CTCs are isolated using this positive isolation method and are in the elution plate when the run completes.

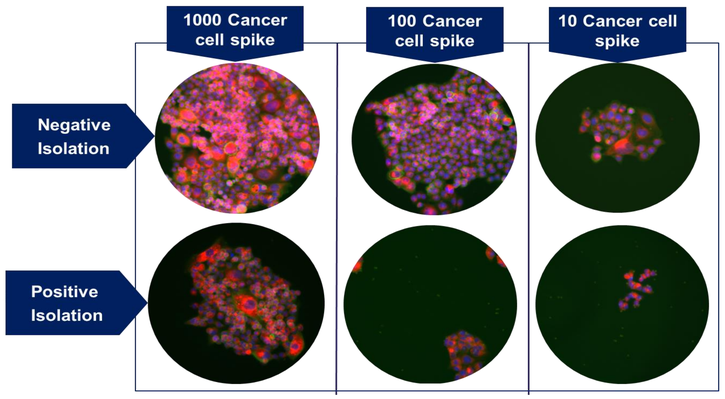
Figure 2: Negative Isolation workflow and script:



The bead plate was prepared by adding 200ul Dyanabeads™ CD45 beads to each well. 1 million PBMCs spiked with varying numbers of CTCs (10,100, 1000) were added to the sample plate. The Wash solution consists of 1X Ca/Mg free PBS with Bovine serum albumin and 0.5M EDTA. All the plates, along with tip comb, were loaded on Kingfisher™ Apex instrument to execute negative isolation script. Untouched CTCs were isolated using this negative isolation method and remain in the sample plate after the run completes.

RESULTS:

Figure 3: Cell culture and Immunostaining



Colon cells at 10, 100, and 1000 cell count were isolated via negative and positive isolation and cultured in cell specific media for one week. Immunostaining was performed using Green - EpCAM Antibody staining for EpCam proteins expressing on CTCs; Red stain - CellTrackerTM Red for cell staining; Blue stain - Hoechst 33342 stain for nuclear staining, For the negative isolation, the tumor cells are untouched, thus unbound to the beads. For the Positive Isolation images, the CTCs are bead bound but still viable. The beads are faintly visible in the images. The beads did not impede culture and began to detach from the cells over time. As growth of the target isolated HCT 116 cells was seen under all conditions, viable cells were recovered for both isolation methods at all 3 initial cell inputs.

Figure 4: EpCam qPCR for Negative Isolation

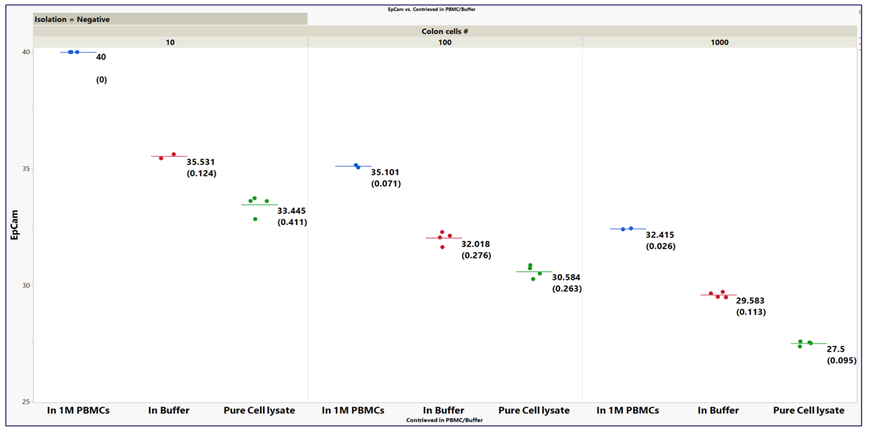
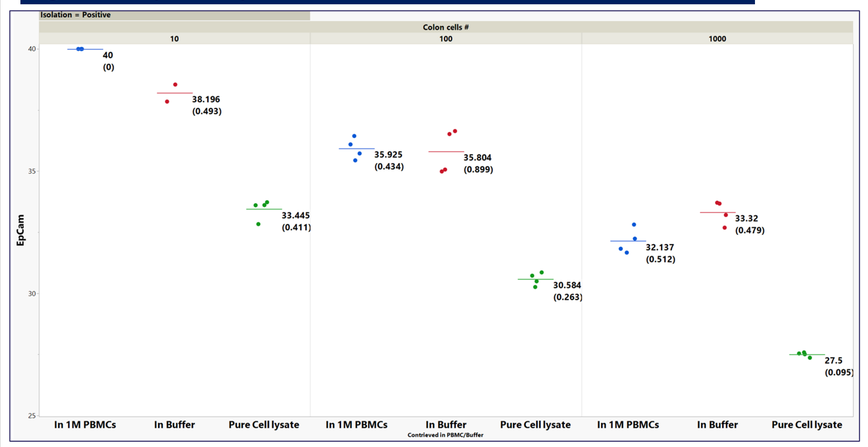


Figure 5: EpCam qPCR for Positive Isolation



Figures 4 (Negative Isolation) and 5 (Positive Isolation): The graphs are divided into 3 columns showing 10, 100 and 1000 HCT116 cell spike in isolated from a 1M PBMC background (Blue), isolated without any PBMC background (Red) and as a pure cell lysate (Green) that did not go through any isolation. EpCam targeted RNA Cq values are displayed on the y-axis.

We were unable to detect the EpCAM RNA from the 10 HCT116 cell input in the 1M PBMC background from either isolation method using the direct lysis RNA extraction method tested in this experiment. Without the PBMC background, we were able to detect EpCam RNA from the 10 HCT116 cell input.

We were able to detect the EpCam target via qPCR for the 100 and 1000 HCT116 cell spikes with and without the PBMC background for both the negative (Figure 4) and positive (Figure 5) workflows. The theoretically possible Cq values for Colon cells that were directly lysed are shown in green for comparison. Though the lowest cell inputs for both methods did not produce detectable Cq values, viable cells were recovered as indicated by the cell culture experiment shown in Figure 3. Based on this initial molecular comparison, recovery and cell lysis methods may be further optimized to increase molecular analysis capabilities from samples with the low target cell counts seen in samples typically utilized for this work.

CONCLUSIONS

In conclusion, we were able to use the KingFisher Apex paired with Dynabeads Epithelial Enrich and Dynabeads CD45 to isolate CTCs using both negative and positive automated methods. The cells isolated from both workflows were intact and still showing expression of the EpCAM marker after isolation. The cells remained viable and were suitable for cell culture after isolation even when still attached to the magnetic bead

Additionally, the CTCs isolated from both methods were suitable for use in downstream molecular assays. Importantly, even though the lowest cell inputs for both methods did not produce detectable Cq values, viable cells were recovered as indicated by the cell culture experiment. With further optimization, particularly by employing methods designed for single cell work, molecular analysis of the lower cell recoveries may be possible.

We have shown that the KingFisher Apex may be used to recover CTCs using both positive and negative recovery methods. The customizability of Dynabeads enables the user to target the cell marker of choice and use the automation workflows described here to further investigate their area of interest. Automation on the KingFisher Apex enables the flexibility of running both positive and negative CTC isolation methods and enables automated nucleic acid extraction using the same magnetic bead-based technology all on the same instrument.

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TRADEMARKS/LICENSING