Genetic Profiling of Circulating Tumor Cells using a Label-free Inertial Microfluidic Approach Coupled with Downstream Next Generation Sequencing

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Introduction

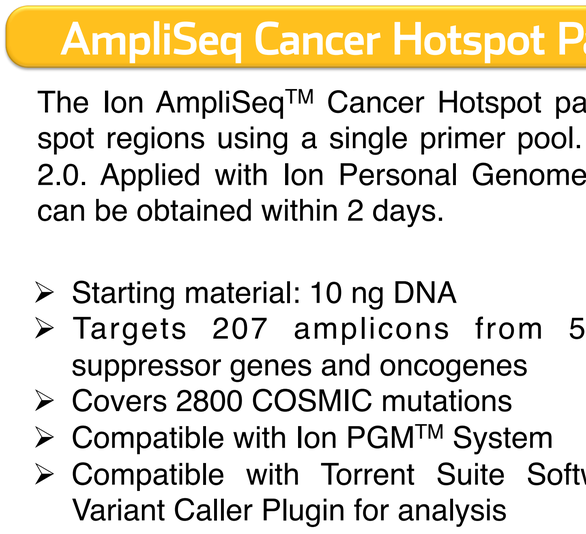
Circulating tumor cells (CTCs) provide significant insights in cancer metastatic events and informed clinical decisions. However, CTCs exist in extreme low numbers in the blood that makes isolation and molecular analysis of CTCs a daunting hurdle. In this study, we adopted a label-free microfluidic system that utilizes Dean Flow Fractionation principle to enrich for CTCs in whole blood. Using lung cancer cell line, H1975 as a mock CTC model, we demonstrated isolation of high purity CTC down to 10 cells/ml spike. The average recovery of H1975 was 44.5% with consistent purity of CTC (<1000 cells). The feasibility of utilizing CTCs for molecular assays was demonstrated using Next Generation Sequencing (NGS), specifically Ion AmpliSeq Cancer Hotspot V2 panel, to confirm known EGFR mutations in H1975 cells. In conclusion, we describe a CTC isolation method compatible with targeted NGS to identify actionable mutations.

CTC Enrichment by ClearCell® FX System

CTChip® FR1

- Label-free isolation method
- Enrichment of CTCs based on *size & inertia*
- Retrieval of wholly *intact* and *viable* cells
- High purity, *5log$_{10}$ depletion of WBCs*
- Process high blood volume of *7.5ml*
- Fully automated CTC enrichment platform
- Small footprint

It is a challenge to identify genetic variants of CTC from an overwhelming background of leukocytes. The ClearCell® FX enriches for CTC in a label-free approach that allows for high purity CTC isolation. The technology utilizes an inertial focusing principle generated within a radial microfluidic chip to subject CTCs and other cell types to both inertial and Dean drag forces to separate CTC from smaller cells in the blood.



Isolation by Size

- Smaller hematologic cells [red blood cells (RBCs) ~8μm; leukocytes ~8-15μm] are affected by the Dean Drag and migrates to outer wall.
- Larger CTCs (~15-20μm) experience strong inertial lift forces as indicated by the red arrows and is focused along the microchannel inner wall.

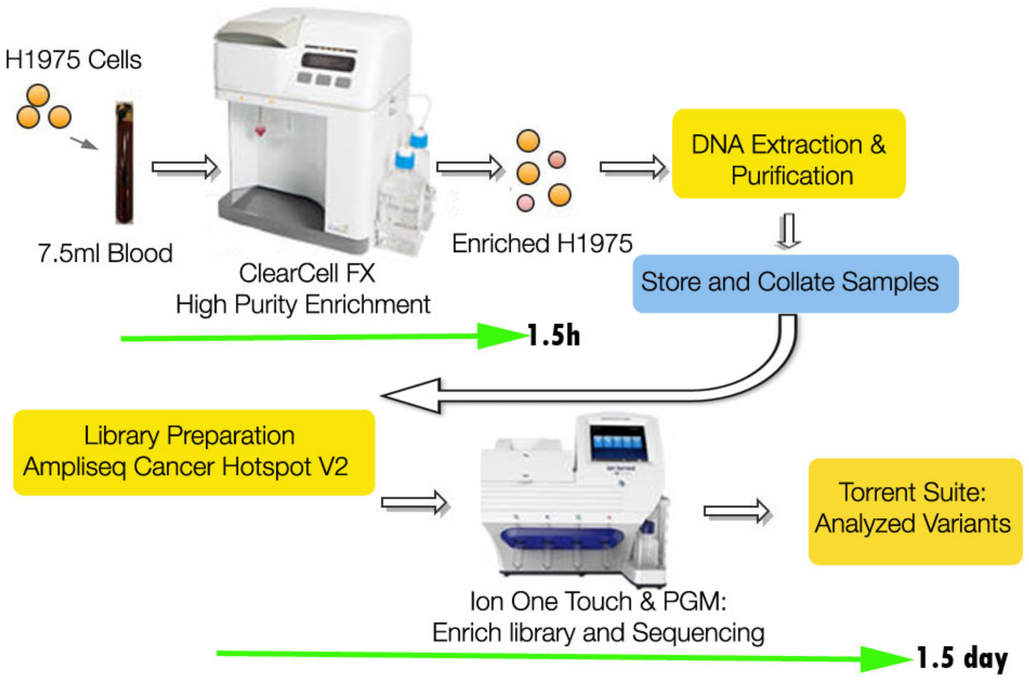
AmpliSeq Cancer Hotspot Panel v2/ Ion Torrent Sequencing

The Ion AmpliSeq™ Cancer Hotspot panel v2 amplifies multiple genomic cancer hot spot regions using a single primer pool. It is used with the Ion AmpliSeq™ Library kit 2.0. Applied with Ion Personal Genome Machine (PGM) system, sequencing results can be obtained within 2 days.

- Starting material: 10 ng DNA
- Targets 207 amplicons from 50 tumor suppressor genes and oncogenes
- Covers 2800 COSMIC mutations
- Compatible with Ion PGM™ System
- Compatible with Torrent Suite Software and Variant Caller Plugin for analysis

Ion PGM™

Workflow

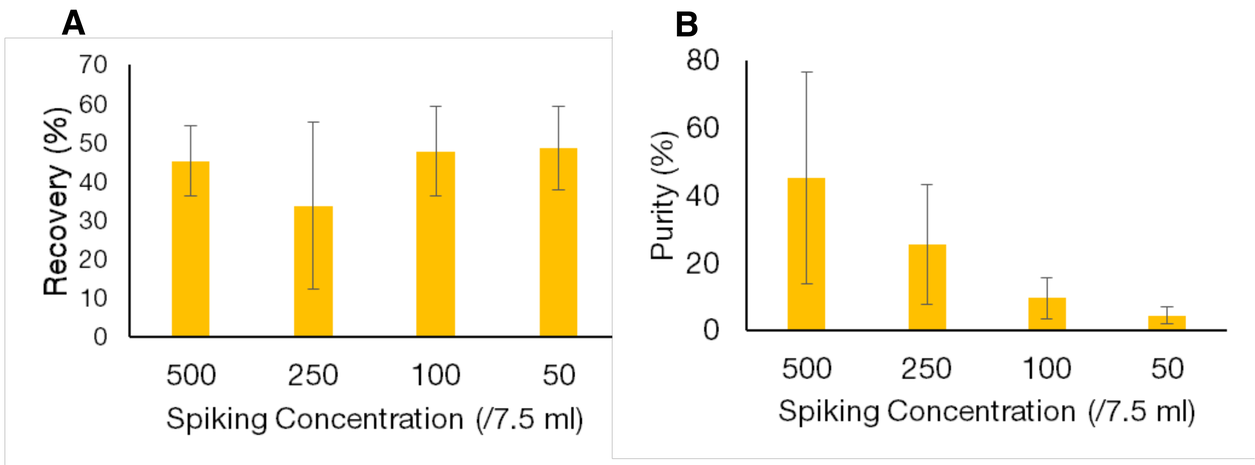


H1975 cells, pre-stained with CellTracker™ Orange, were spiked into 7.5 mL of blood. After RBC lysis, the nucleated cell fraction was pelleted and re-suspended in resuspension buffer. ClearCell® FX system automatically enriched for the cancer cells. DNA was extracted from the output using Qiagen QIAamp DNA Micro Kit. Ion Ampliseq Cancer Hotspot Panel V2 kit amplified the cancer hotspot targets and generated the sequencing library. Template enrichment was carried out using Ion One Touch system and then sequenced on Ion PGM™. The sequencing data was analysed on Torrent Suite™ software. A total of 32 samples were evaluated.

Results

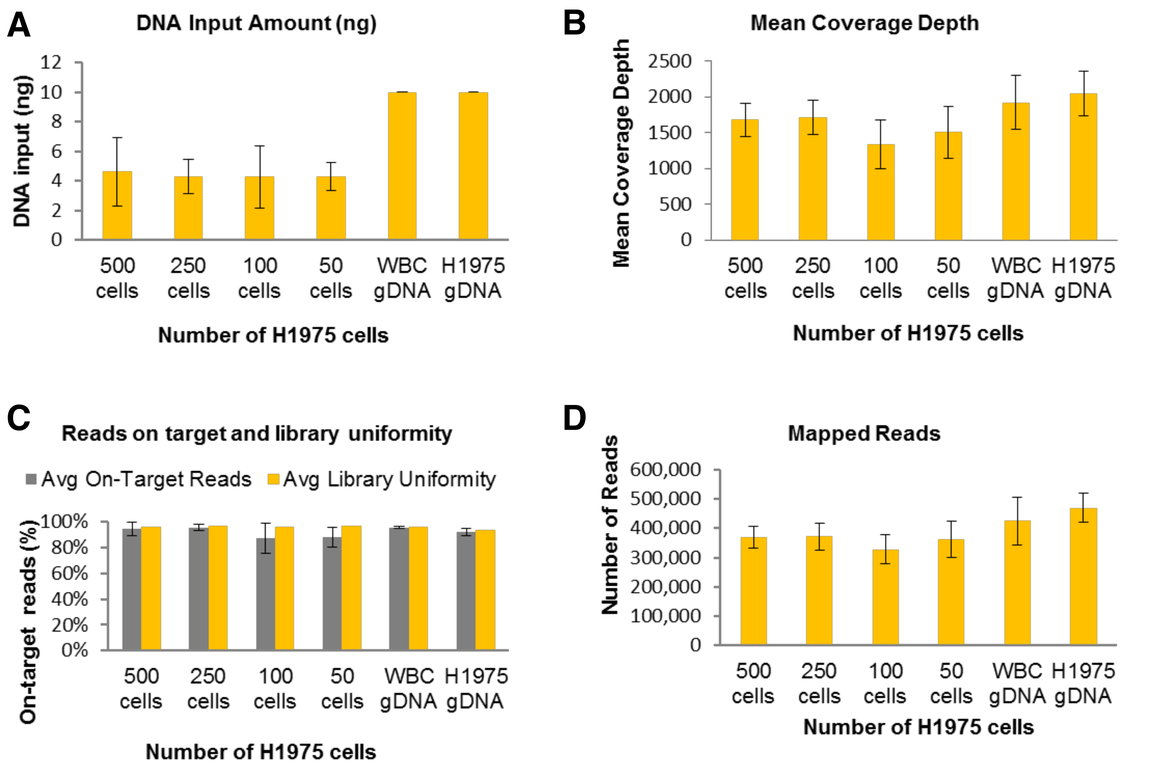
Spiked Cells Recovery and Purity

Pre-stained H1975 cells were spiked at 500 (n=7), 250 (n=7) , 100 (n=9) and 10 (n=9) cells/ 7.5 ml of blood. The blood was processed on ClearCell® FX. The recovered cells were stained with Hoechst and counted. As high as 97% (31/32) of the samples had more than 1% H1975 in the recovered output.



Recovery and purity of spiked cells after ClearCell® FX enrichment. Varying concentrations of NCI-H1975 were spiked into 7.5 ml of blood and processed on ClearCell® FX. (A) Recovery of NCI-H1975 cells was on average 44.5% (s.d. 14.2%). (B) Purity of spiked cells recovered ranged between 0.7% and 83.2%, depending on the number of cells spiked and the number of leukocytes in the output.

Library Preparation Quality Metrics



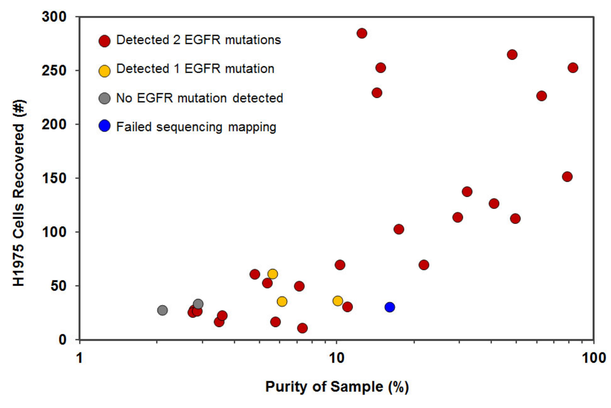
Sequencing library quality metrics. (A) Similar amount of input DNA (<10 ng) was used for the library preparation for each test sample. (B) Mean coverage depth, (C) % on-target reads, library uniformity and (D) mapped reads were similar between the test samples and the control samples.

EGFR Mutations Detected in ClearCell FX-Enriched Samples

Sample Metrics	No. of Cells Spiked/ 7.5mL Blood				Control DNA	
	500	250	100	50	H1975	WBC
No. of Samples Isolated on ClearCell FX	7	7	9	9	NA	NA
No. of Samples Resulting in Successful Library Preparation	7	7	7	9	3	3
Percent of Samples Resulting in Successful Library Preparation	100%	100%	78%	100%	100%	100%
No. of Samples with EGFR L858R Detected	7	6	7	8	3	0
No. of Samples with EGFR T790M Detected	7	6	5	6	3	0
No. of Samples with Both EGFR Mutations Detected	7	6	5	6	3	0
Percent of Samples with Both EGFR Mutations Detected	100%	86%	71%	67%	100%	0

Successful sequencing library preparation and EGFR mutation detected. Table lists the number or percentage of samples successfully prepared in each step of our workflow from cancer cell enrichment to sequenced variant detection.

EGFR mutation detected with respect to H1975 purity and recovery. Plot shows the %recovery and %purity of each sequenced sample and its sequencing outcome. Hotspot EGFR mutations (T790M and L8585R) in H1975 are detected in 90% of the samples (27/30).



Conclusion

High mutant to wild type ratio (>1%) is ideal for NGS and most conventional molecular analysis methods. The new high purity protocol on the ClearCell® FX enables us to isolate sufficient purity of CTC to be integrated with the Ion Torrent Ampliseq platform. Mutations were detected in spiked samples using the Ion Ampliseq Cancer Hotspot Panel V2.