

Molecular analysis of single- and bulk-enriched circulating tumor cells using the IsoFlux™ System

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ABSTRACT

Circulating tumor cells (CTCs) are rare cells found in the blood of cancer patients with solid tumors and play a key role in cancer dissemination. There has been considerable interest in analyzing these cells as a potential source of clinically-actionable information relating to molecular profile of the patient's disease. One of the current challenges in the field is high recovery and purity of CTCs to enable molecular analysis.

The IsoFlux System (Fluxion Biosciences) provides high recovery of CTCs in a format optimized for downstream analysis. It provides three distinct methodologies for working with the CTCs after collection:

- 1) Analyze the 'bulk' collection of CTCs to look at marker(s) present anywhere in the sample
- 2) Analyze individual CTCs collected one at a time from the enriched fraction to examine cellular heterogeneity
- 3) Analyze pools of individual CTCs to increase the sensitivity of the downstream analysis

Here we present analytical and clinical data using the above mentioned methods. A cohort of Her2+ breast cancer patients was analyzed for gene expression using the 'bulk' method. Single cell analysis was performed using immunofluorescence imaging as an orthogonal approach to measuring gene expression.

To further demonstrate the single cell capability, a series of analytical (spiked) samples were prepared and analyzed using two orthogonal methods for mutational analysis, castPCR (Life Technologies) and targeted Next Generation Sequencing (NGS).

RESULTS

Gene expression in CTCs from Her2+ breast cancer patients

Matched blood tubes from 6 Her2+ breast cancer patients were processed for enumeration and gene expression. The measured CTC values (CTCs/7mL blood) and gene expression levels are presented in the table below. Recovered CTC counts ranged from 5 to 89 cells per sample.

PATIENT ID	HER-2 STATUS	TREATMENT STATUS	TREATMENT	TNM	PATHOLOGY	1 YEAR FOLLOW UP OUTCOME
1	Positive (3+)	Refractory Active	Avastin/Gemzar/Herceptin	T2N0Mx (2011)	Infiltrating Ductal Carcinoma	Stable disease
2	Positive (3+)	Active Tx	Herceptin	T2N3MX (2005)	Invasive Ductal Adenocarcinoma	Stable disease
3	Borderline (2+)	Active Tx	Cyclophosphamide/Methotrexate/5-FU	Unknown	Infiltrating Ductal Carcinoma	Deceased
4	Positive (3+)	Active Tx	Herceptin	T3N3M1 (2013)	Invasive Ductal Adenocarcinoma	No evidence of disease
5	Positive (3+)	Post Tx	Herceptin	T3N1M0 (2013)	Invasive Mammmary Carcinoma	Relapse suspected and being confirmed
6	Positive (3+)	Post Tx	Herceptin	Unknown	Infiltrating Ductal Carcinoma	Deceased

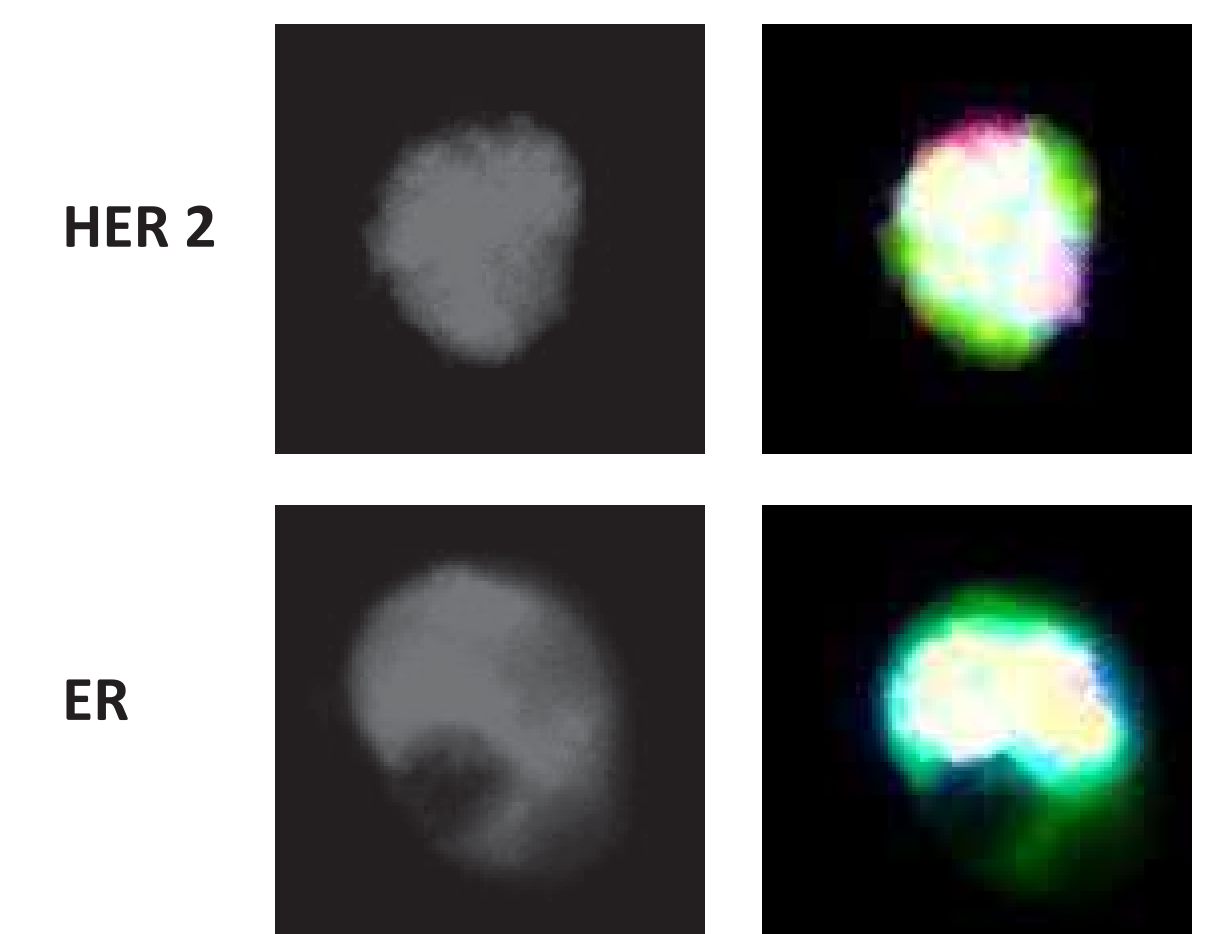
A set of four genes was used for this study, previously shown by Strati, et al. to correlate with disease status. The lower limit of detection for the gene expression data is below 5 cells (analytical data not shown). The expression data is shown using a linear color scale ranging from green (low to no expression) to red (high expression), with all values being normalized to HMBS control. Almost all patients expressed KRT19, MAGE, and TWIST to a level comparable to that of the reference gene used. KRT19 expression was high in all patient samples, with 6/6 (100%) displaying over-expression. In contrast, KRT19 expression was undetected in healthy controls. MAGE-A3 was over-expressed in 4/6 (67%) of patients, and TWIST in 2/6 (33%). None of the patients showed SGGC over-expression. By comparison, Strati, et al. found 54% of patients to be positive for CK, 15% positive for MAGE-A3, and 19% positive for TWIST expression. The comparatively higher detection rate in our patient set is likely due to either more CTCs isolated by the IsoFlux System or a lower WBC background.

PATIENT ID	CTCs	dCt (normalized to HMBS control)			
		KRT19	MAGE-A3	SGCG	TWIST
1	16	-5.9	-3.0	0.3	-3.0
2	89	-4.1	0.3	1.8	-0.6
3	59	-4.5	-2.8	0.9	-1.0
4	5	-3.6	-2.3	2.5	-1.0
5	18	-3.7	-0.5	2.2	-0.4
6	20	-5.2	-3.9	1.6	-1.8

Based on the patients' clinical information, the following trends were observed: Both of the patients with progressive disease (2/6) displayed high CTC counts (177 and 60 respectively) and over-expression of 1 and 2 genes in addition to CK. One of six patients showing no detectable disease and also had the lowest CTC count (15 cells). While the sample size of the pilot study was not large enough to generate statistically-meaningful conclusions, the observed results follow an expected trend.

Single cell immunofluorescence (IF) imaging

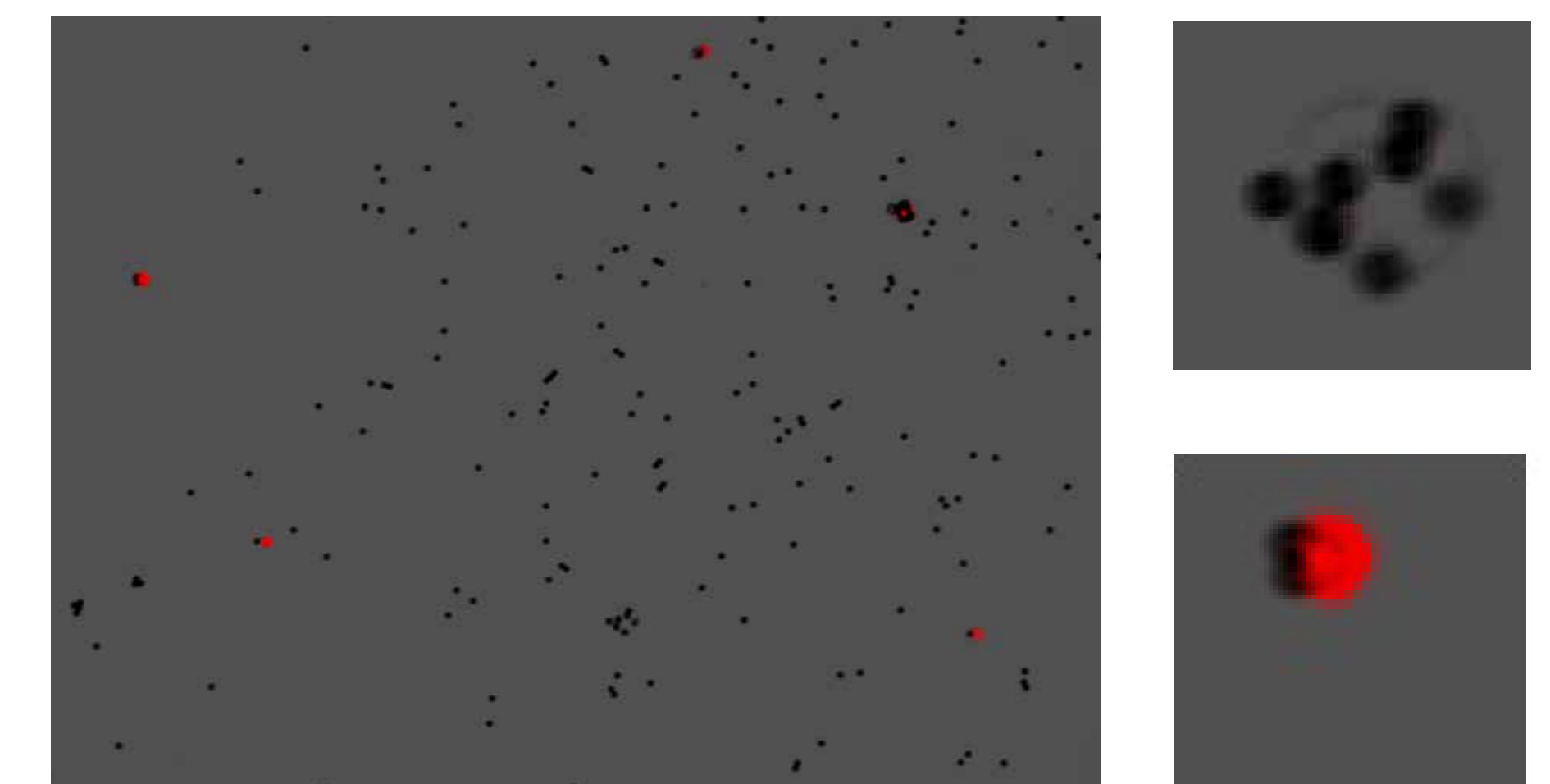
A Her2+ breast cancer patient (235 CTCs detected) was evaluated using IF imaging of Her2 and estrogen receptor (ER). The patient displayed a majority of CTCs with over-expression of both Her2 and ER. Representative cells are shown to the right, with the target (Her2 or ER) shown in monochrome (left), and a merged image with the target (CY3), CK (FITC) and nucleus (DAPI).



A parallel experiment was run for gene expression using blood from the same patient and time point. Six gene expression targets were evaluated: CK(KRT19), ER(ESR1), Her2 (ERBB2), EP-CAM, CD45 and GUSB control. Blood cell samples RNA was used as negative control. Relative gene expression levels were quantified using standard delta Ct method, which is the Ct difference between the control gene (GUS-B, housekeeping gene expressed in all cells) and the target gene (genes expressed more in tumor cells). The dCt of the negative control sample (blood cells) was used as the cut-off dCt for target gene detection in CTC sample. Her2 over-expression was detected in this sample (2 Ct lower than background). ER expression is detected, but not significantly higher than negative control sample (blood cells). CK19 was detected to be higher than negative control (4 Ct below) but the absolute level was low based the Ct.

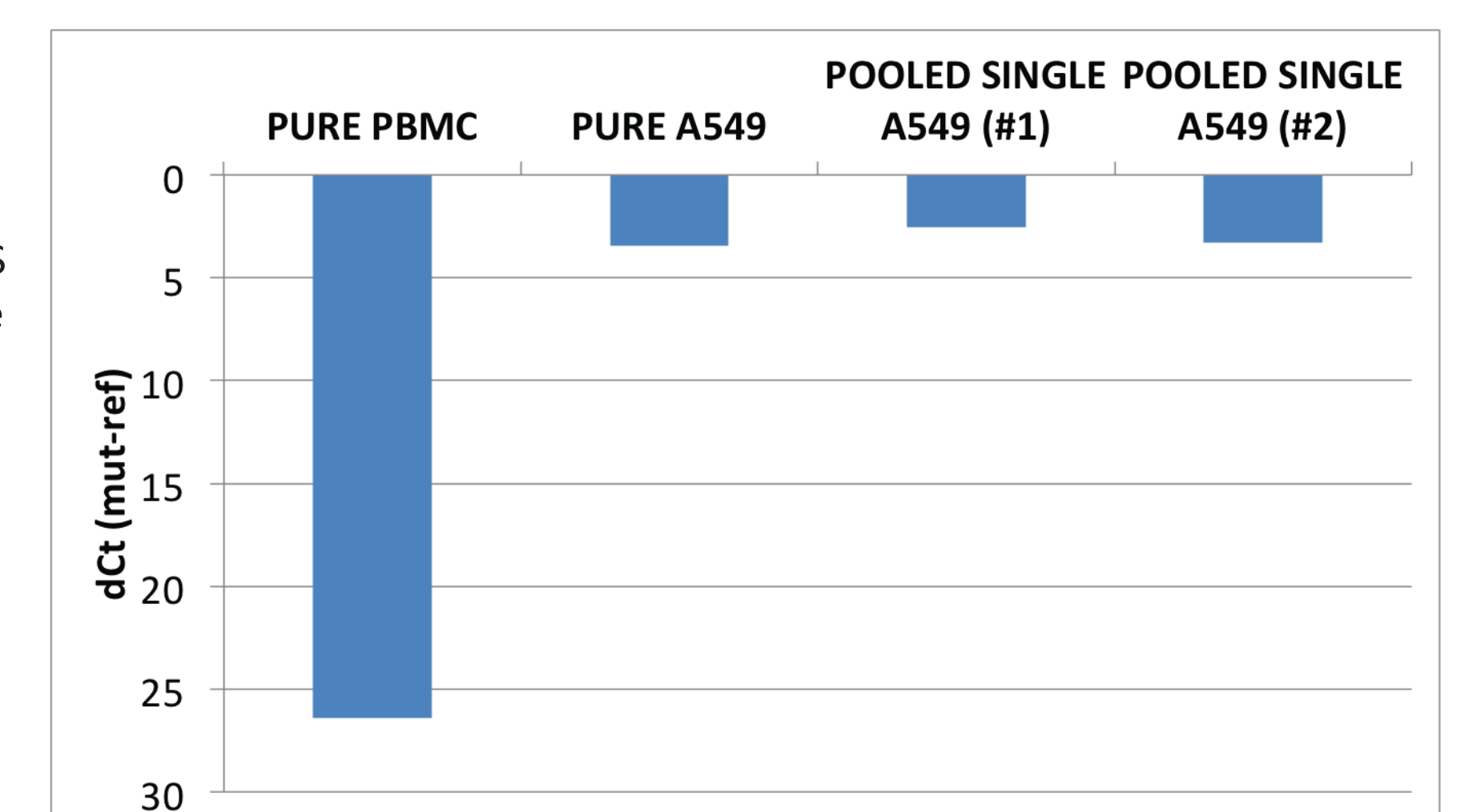
Single cell analysis using mutation detection

Single model CTCs (A549 cell line spiked into healthy blood) were selected from the IsoFlux-enriched samples using a manual pipette under microscopic observation. CTCs were identified based on size, presence of magnetic beads targeted towards cell-surface antigens (EpCAM and EGFR), and lack of CD45 stain (red). A representative field of view (10X objective) is shown on the left, along with a positively selected CTC (top right) and excluded WBC showing CD45 staining in red (bottom right).



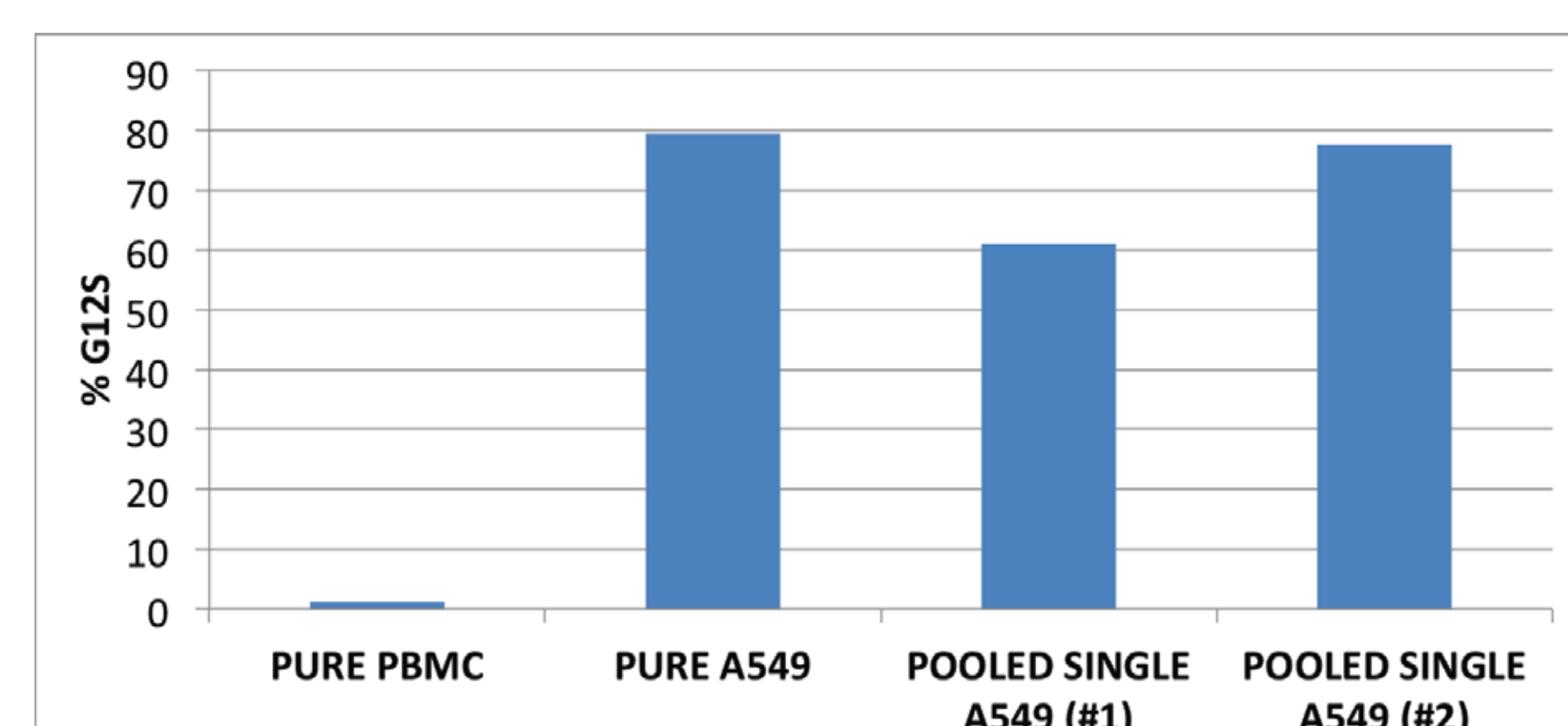
Mutation detection via qPCR (castPCR)

Pooled single CTCs (groups of 10 cells) were lysed and DNA recovered for mutational analysis. The A549 cell line harbors a homozygous G12S mutation. Samples were analyzed using castPCR reagents (competitive allele-specific PCR) and compared against pure populations of the A549 cell line (positive control) and pure PBMC (negative control). Results are reported as dCt (mutation assay Ct minus reference assay Ct) with smaller values corresponding to presence of the mutation. The pooled single CTC fractions showed dCts comparable to that of the pure cell line population, as expected.



Mutation detection via NGS (targeted sequencing)

Pooled single CTCs (groups of 10 cells) were lysed and DNA recovered for mutational analysis. DNA was analyzed using an Illumina HiSeq instrument. Pooled single cell samples showed comparable G12S mutation frequency (61-77%) as the pure A549 cell line controls (79%), as expected.



METHODS AND WORKFLOW

Model CTC System - Model cancer cells (A549, homozygous for KRAS G12S mutation) were spiked into fresh healthy human blood tubes (7mL, EDTA). Ficoll gradient was used to separate the mononuclear fraction. Purified gDNA from A549 (mutant) and healthy PBMC controls was used for analytical validation of the castPCR and NGS assays.

IsoFlux CTC Isolation - Samples were processed on the IsoFlux System using anti-EpCAM immunomagnetic beads. An antibody cocktail of EGFR plus EpCAM was used for A549 cell recovery since these cells are very low EpCAM expressing. Enriched CTCs were eluted in less than 10µL and saved for either enumeration, qPCR, or mutation detection. Enumeration samples were counted using fluorescence microscopy (CK+ / CD45- / nucleated / intact).

Gene expression (qPCR) - Total RNA was extracted from IsoFlux-enriched cells with RNeasy Micro Kit (Qiagen) with 60ng carrier RNA (poly-A RNA, supplied with the kit) and on-column DNAase I treatment following standard protocol. Total RNA was eluted in 14µL nuclease free water with 1U/mL RNase inhibitor (Promega). Reverse transcription was performed with SuperScript VILO cDNA Synthesis Kit (Life Technologies) in 20µL total reaction volume. 2µL RT reaction product was used in each real-time qPCR performed with NuPCR reagents (Illumina) on the Eco real-time qPCR machine with standard protocol. Ct measurements were translated into expression data.

Immunofluorescence imaging - Samples were stained for either Her2 (Her2-Cy3, rabbit polyclonal to Her2/ErbB2 828723 from Abcam and goat anti-rabbit IgG-Cy3) or ER (anti-Estrogen Receptor alpha antibody ab37438 from Abcam and goat anti-rabbit IgG-Cy3). Both samples were also stained for CK (FITC). Samples were analyzed on a Zeiss AxioObserver Z1 inverted microscope.

Single cell mutation detection - After IsoFlux enrichment, single cells were selected using a manual pipette under microscopic observation. Pools of 10 CTCs were analyzed in this experiment. DNA was isolated and amplified using the Qiagen REPLI-g kit. Mutational analysis was performed using two methods: (1) qPCR using castPCR mutation detection assays (Life Technologies), and (2) next-generation sequencing using the Illumina MiSeq.



CONCLUSIONS

- The IsoFlux System delivers high recovery of circulating tumor cells in an optimal format for downstream analysis (high CTC recovery and viability, low elution volume, low background)
- Using the 'bulk recovery' approach provides fast, efficient biomarker analysis across the entire set of CTCs collected
- Using the 'single cell' approach enables analysis of heterogeneity across individual CTCs, as well as provides a means for '100% pure' samples for more efficient NGS and qPCR