FLUXION

APPLICATION NOTE

Gene Expression Profiling of Circulating Tumor Cells Using the IsoFlux System

OVERVIEW

- Gene expression profiling is frequently used in both clinical research and clinical practice to understand tumor subclass, pathway analysis, and other types of clinically-actionable information.
- Circulating tumor cells (CTCs) provide a means for analyzing cancer gene expression in a longitudinal manner. The IsoFlux System (Fluxion Biosciences) enables routine CTC collections from a standard blood draw that can then be used for molecular analysis, including gene expression studies.
- An analytical limit of detection below 5 cells per blood draw was determined by measuring expression of the CTC specific gene KRT19 in spike-in controls.
- In a pilot clinical study, a panel of genes associated with risk (KRT19, MAGE-A3, SGCG, TWIST) was compared to enumeration data in breast cancer patients. Of the 6 patient blood samples tested, all were CTC positive as measured by enumeration (CTC count >5 cells) and by KRT19 gene expression.
- Patients with progressive disease had higher CTC counts and more genes on the panel over-expressed, as compared to patients with stable disease.

INTRODUCTION

Gene expression profiling of tumor tissue is routinely used in oncology clinical research and treatment. Notable examples of clinical tests include Oncotype Dx (Genomic Health) and the Pervenio lung cancer risk panel (Life Technologies). Expression profiling of enriched circulating tumor cell samples extracted from patient blood has also been studied. In this context, the expression profile of buffy coat samples containing CTCs is used to determine the presence of CTCs (using genes like CK19 and CK7) as well as patient risk (Tewes et al.). Several groups have developed small 3-5 gene panels that can discriminate cancer patients from healthy controls and are predictive of survival (Yu, et al., Sher, et al., Strati, et al.). The sensitivity of expression profiling assays is dependent on both CTC recovery and leukocyte background. Previously described assays employed a variety of simple enrichment strategies encompassing bulk immunomagnetic separation, density centrifugation, and buffy coat isolation.

The IsoFlux System employs immunomagnetic separation in a microfluidic channel to obtain enhanced recovery and sample purity (Harb, et al.). Here, we present a representative work flow for CTC isolation (IsoFlux) followed by gene expression profiling (qPCR) using markers previously shown to have predictive relevance in breast cancer (Strati, et. al.).

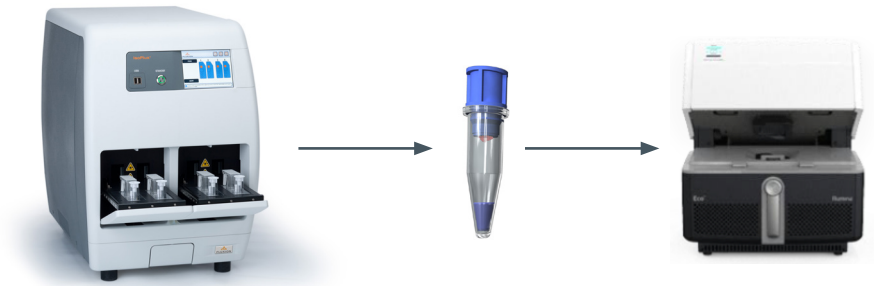


Figure 1: Gene Expression Workflow. The IsoFlux System isolates CTCs and elutes the sample in a low-volume (1-2µL) hanging drop. The enriched sample can be lysed for RNA recovery and analyzed on a conventional qPCR instrument.

METHODS

A. Sample logistics and preparation - Two tubes of 7-10mL whole peripheral blood were collected in EDTA from each patient. Samples were shipped overnight at room temperature in an insulated shipping cooler. All clinical samples were processed the next day within 36 hours of the initial draw. Blood tubes were processed to recover the peripheral blood mononuclear cell (PBMC) fraction. Briefly, Leukosep tubes (Greiner Bio-One) were prepared by adding 15mL of Ficoll-Paque® Plus (GE Healthcare Life Sciences). Blood samples were added and centrifuged for 10 minutes. The PBMC fraction was recovered and resuspended in 600µL of binding buffer (CTC Enrichment Kit, Fluxion Biosciences). Immunomagnetic beads pre-conjugated with anti-EpCAM antibodies (CTC Enrichment Kit, Fluxion Biosciences) were added directly to the sample and incubated at 4°C with passive mixing on a rotator.

B. Separation - Following bead coupling, samples were loaded into the inlet well of the IsoFlux microfluidic cartridge. Samples were flowed through the magnetic separation region at a rate of 40µL per minute; each sample passed through the channel in about 15 minutes. After processing, the isolated target cells were recovered off of the isolation zone disk and dispensed into a microfuge tube for further processing.

C. Enumeration – For analytical samples, the spiked cells were pre-labeled with CellTracker Green (Life Technologies) to provide a more accurate count of recovered cells. For clinical samples, immunofluorescence staining was performed using CK, CD45, and Hoechst (nucleus) following standard protocols (CTC Enumeration Kit, Fluxion Biosciences). Imaging was performed using an inverted epi-fluorescence motorized microscope (Zeiss AxioObserver Z1) and imaging software.

D. mRNA extraction and qPCR measurement methods – Total RNA was extracted from IsoFlux isolated 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.

Gene	Significance
KRT19	epithelial cytoskeletal protein; used as a postoperative biomarker for certain cancers; expressed in CTCs and used for CTC identification
MAGE-A3	over-expressed in lung adenocarcinoma; presence in cancer tissue associated with shorter survival; possible therapeutic target
SGCG (h-MAM)	a breast tissue specific gene of prognostic value in CTCs; used as a biomarker in nodal tissue; overexpressed in breast cancer cell lines
TWIST	overexpressed in metastatic carcinomas; associated w/ chemotherapy resistance; possible therapeutic target
HMBS	reference gene

Table 1: Gene Panel. The genes used to obtain a CTC expression profile were selected as a measure of CTC presence (KRT19) and disease risk as shown in literature data.

RESULTS

Analytical studies were performed to characterize the limits of detection and overall performance characteristics of the assay. The limit of detection was determined using spiked SKBR3 cells, a cell line known to over-express at least two genes of interest, KRT19 and ERBB2. Pure cell line samples (5000 cells) showed a high level of expression for these markers, with Ct values of 19 and 26 for KRT19 and ERBB2 respectively. The limit of detection was determined by spiking increasing numbers of tumor cells into fixed amounts (7mL) of healthy whole blood. Matched samples were processed with one tube used for enumeration of recovered cells and a second tube used for gene expression. Recovered cell counts ranged from 5 to 156 cells per sample (Figure 2). The lower limit of detection is below 5 cells. Amplification of the two genes was detected in the 5-cell samples processed, where the detection limit was set to Ct<35. Amplification was not detected in healthy controls for either gene (Ct >40). Color coded raw data is shown in Fig. 2A, where low expression reflects Ct>39 (black) and high expression Ct<30 (red). A yellow color indicates a Ct of 35. Average Ct data for each spike-in level is shown in Fig. 2B. For samples containing 5 cells and above, the genes were detected in all spiked samples, with Ct values at least 2 below that of reference controls. Some of the variability noticed in spiked samples is due to the combined variability of recovery and amplification efficiency. Samples containing 156 cells by enumeration displayed clear over-expression in all cases, with Cts that were 4 or more below control gene Cts. (Fig. 2B).

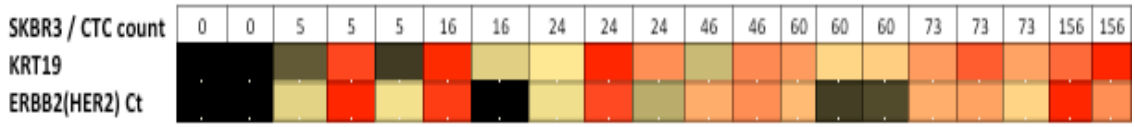
Following the analytical work, we validated a similar gene expression work flow with a pilot clinical study using breast cancer patient samples. A slightly larger set of four genes was used for this study. These targets were previously shown by Strati, et al. to correlate with disease status. Table 1 contains that gene panel and relevance of each gene based on literature reviews. The raw expression data is shown in Figure 3A using a linear color scale ranging from a Ct of 38 (black, low to now expression) to 28 (red, high expression). Almost all patients express KRT19, MAGE, and TWIST to a level comparable to that of the reference gene used. The gene expression levels as normalized to the reference gene HMBS are shown in Figure 3B. Expression levels span from 2 above the reference Ct (black, low expression) to 4 below reference (red, high expression).

Over-expression is defined as having a Ct at least 2 below reference. KRT19 expression was strong in all patient samples, with 6/6 (100%) of breast cancer samples 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 SGCG 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.

Based on the patients' clinical information in Table 2, 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). Aggregate results for the different patient groups are shown in Table 3. Mean CTC counts are 120 for progressive disease (PD) as compared to 44 for the stable disease (SD) group. Similarly, a mean of 2.5/4 risk associated genes are expressed for the PD group as compared 1.75/4 for the SD group. While the sample size of the pilot study was not large enough to generate statistically-meaningful conclusions, the observed results follow an expected trend.

Figure 2: Analytical Data. (A) Raw Data Set: Increasing quantities of the tumor cell line SKBR3 were spiked into whole blood and enriched using the IsoFlux System. KRT19 and ERBB2 expression was measured from the resulting cell pellet via qPCR. A matching sample was counted post-isolation via microscopy and the recovered cell counts are shown in the first row. The expression ranges from undetected (Ct > 40, black) to high expression (Ct < 30, red). (B) Averaged Data Set: The same data presented in (A) was averaged for each cell recovery level and is compared to two different cell lines. Neither gene was detected in healthy controls.

(A)



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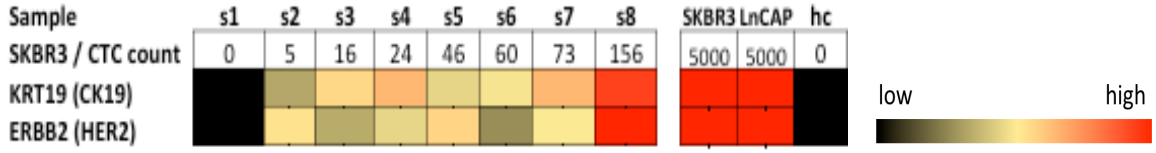
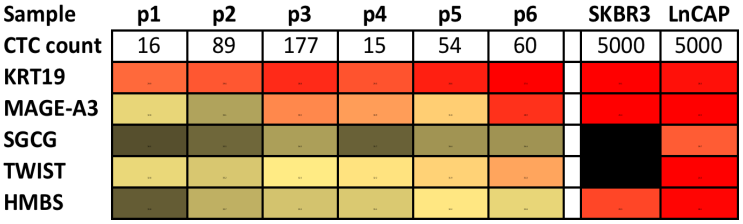


Figure 3: Clinical Pilot Study. (A) Raw Data Set: CTC expression results for a set of six breast cancer patients as compared to two tumor cell lines: SKBR3, a breast cancer tumor cell line and LnCAP, a lung cancer cell line. Color coded Ct data includes the four target genes and HMBS, a reference gene. The color scale varies from a low of Ct = 40 to a high expression level Ct < 29.
(B) Normalized Data Set: The level of expression for a 4 gene panel associated with disease progression and risk normalized to the expression of reference gene HMBS. The color scale ranges from a dCt > +2 with respect to reference (low expression) to dCt < -4 with respect to reference (high expression). The CTC count from a matched sample is also reported for each patient (top row).

(A)



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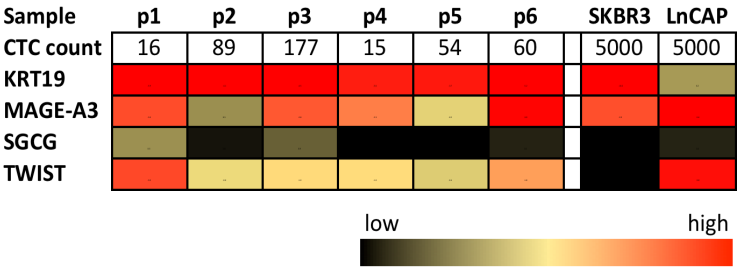


Table 2: Patient Clinical Data. Treatment and disease status of the six patients studied is presented alongside CTC counts and number of gene overexpressed. All patients expressed CK19 in this assay.

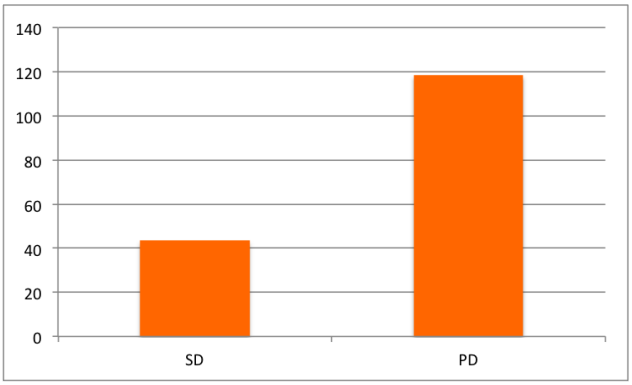
Patient	Her-2 Status	Treatment	Treatment Status	TNM	Pathology Subtype	Outcomes	Disease Status	CTC count	No. Genes overexpressed
1	Positive (3+)	Avastin/ Gemzar/ Herceptin	Refractory Active	T2N0MX	Infiltrating Ductal Carcinoma	Still living, stable disease, being treated with Herceptin, Avastin	SD	16	3
2	Positive (3+)	Herceptin	Active Tx	T2N3MX	Invasive Ductal Adenocarcinoma	Still living, stable disease, being treated with Herceptin	SD	89	1
3	Borderline (2+)		Active Tx	Unknown	Infiltrating Ductal Carcinoma	Now deceased, stage IV, bone and liver lesions and brain metastases; was last on Doxil	PD, deceased	177	2
4	Positive (3+)	Herceptin	Active Tx	T3N3M1	Invasive Ductal Adenocarcinoma	Still living, currently NED (no evidence of disease), being treated with Herceptin and Avastin	NED	15	2
5	Positive (3+)	Herceptin	Post Tx	T3N1M0	Invasive Mammary Carcinoma	Still living, current worry of relapse (waiting for test results), Post Tx (waiting for test results to determine next line of Tx)	SD	54	1
6	Positive (3+)	Herceptin	Post Tx	Unknown	Infiltrating Ductal Carcinoma	Now deceased, had progression of disease, last Tx was Kadcyla	PD, deceased	60	3

SD = stable disease, PD = progressive disease, NED = no evidence of disease

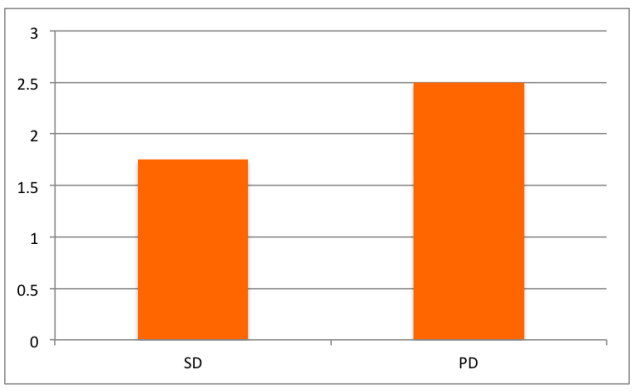
Table 3: Clinical Correlation. Mean CTC counts and numbers of detected genes are shown for the stable disease group (SD) and progressive disease group (PD). PD is characterized by higher CTC counts and overexpression of more risk associated genes.

Disease state	No Patients	Mean CTC count	Genes Overexpressed
SD	4/6	44	1.75
PD	2/6	119	2.5

MEAN CTC COUNTS



MEAN NUMBER OF GENES OVEREXPRESSED



CONCLUSIONS

Fluxion has developed a work flow for gene expression profiling of CTC samples obtained from the IsoFlux System. The IsoFlux System provides high sensitivity CTC recovery in a format optimized for molecular analysis: low 5μL elution volume, viable cells, and minimal background contamination. This enables mRNA extraction and measurement of cancer-specific gene amplification.

Analytical validation data based on spike-in experiments demonstrated the ability to detect less than 5 CTCs per blood tube. Superior CTC recovery in the IsoFlux System yields samples above 5 CTCs per blood tube in 75% of the patients tested (aggregate of Fluxion internal data, 100% of patients in this pilot study had >5CTCs). A pilot study of 6 CTC patients also showed cancer-associated gene over-expression for at least one gene (KRT19) and often 2-3 different risk associated genes from a clinically-focused 4-gene panel. Gene expression data is in agreement with CTC enumeration results showing that the same patients are CTC+.

This approach is amenable to clinical studies where CTC gene expression profiles are correlated to disease progression risk or treatment efficacy. Assays based on gene expression, in conjunction with either bulk immunomagnetic separation or density centrifugation for CTC enrichment, have been previously demonstrated to correlate to disease risk. Here, the use of a microfluidic approach for CTC enrichment enables improved CTC recovery and lower leukocyte backgrounds. This directly translates to gains in sensitivity and specificity. Fluxion offers CTC expression profiling services through its own reference lab.

REFERENCES

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MORE INFORMATION

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About Fluxion Biosciences

Fluxion Biosciences provides cell analysis tools for use in critical life science, drug discovery, and diagnostic applications. Fluxion's proprietary microfluidic platform enables precise functional analysis of individual cells in a multiplexed format. Products include the IsoFlux™ System for circulating tumor cells, the BioFlux™ System for studying cellular interactions, and the IonFlux™ System for high throughput patch clamp measurements. Fluxion's systems are designed to replace laborious and difficult assays by providing intuitive, easy-to-use instruments for cell-based analysis. For more information about Fluxion Biosciences, visit www.fluxionbio.com.

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