IsoFlux™ System

Somatic variant detection from circulating tumor cell samples using NGS

OVERVIEW

- The IsoFlux System (Fluxion Biosciences) enables non-invasive circulating tumor cell (CTC) access to provide real-time and frequent monitoring of cancer disease progression while avoiding the risks and burden of tumor biopsies.
- Following CTC enrichment and purification using the IsoFlux System, mutational profiling of 50 oncogenes and tumor suppressor genes was achieved using the Ion AmpliSeq™ Cancer Hotspot Panel v2. Sequencing on the Ion PGM™ System and simple bioinformatics with Ion Reporter™ Software help streamline the analysis of critical cancer variants.
- Variant detection with NGS is possible from as few as 2 CTCs per 1 mL of blood. This level of CTC recovery is routinely achieved with the IsoFlux System (>70% of samples tested to date from multiple solid tumor types, including breast, lung, colorectal, prostate, and pancreatic cancers).
- Using analytical validation samples, CTC enrichment using the IsoFlux NGS DNA Kit resulted in tumor cell purity ranging from 5-22%. Blinded, standardized analysis using a custom variant filter resulted in detection of all cell line variants (single nucleotide variants, SNVs) down to a concentration of 2 cells/mL, and a mutant allelic frequency of 2%, with 95% specificity.
- The results presented here indicate that the IsoFlux System, Ion AmpliSeq™ technology, and the Ion PGM™ System offer a superior workflow for translational oncology studies.

Figure 1. An easy-to-implement, cost-effective, and rapid solution for mutational profiling of circulating tumor cells (CTCs). Up to 12 samples can be processed from sample to results in two days (one day for tumor cell enrichment, one day for NGS). The IsoFlux System and the IsoFlux CTC Enrichment Kit enriches CTCs in 3 hours (up to 4 samples at a time) using a microfluidic immunomagnetic capture technology followed by cell lysis and whole genome amplification (WGA) of the DNA using the IsoFlux NGS DNA Kit. Sequencing libraries are constructed for 50 cancer associated genes (targeting 2856 COSMIC hotspot mutations) using the Ion AmpliSeq™ Cancer Hotspot Panel v2 with a rapid workflow (<11 hours = 3.5 hour library construction, 4 hour template preparation, 2.3 hour sequencing run, and 1 hour data analysis). Following library construction, 200-base sequencing runs are completed in just 2.3 hours for the Ion 314™ Chip and 4.4 hours for the Ion 318™ Chip. Primary data analysis is performed using Torrent Suite™ Software followed by a custom data analysis workflow with variant filtering, annotation, and report generation using Ion Reporter™ Software.

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BACKGROUND

Cancer is a genetic disorder with the disease-causing variants acquired somatically during a person's lifetime. For oncology research, somatic variant detection by next-generation sequencing (NGS) has delivered remarkable insights into the complexity of cancer and identified new genes and biological pathways implicated in tumor initiation, progression, and escape from the site of origin to other tissues and sites (metastasis). A key limitation to our understanding of cancer is the availability of tumor samples that adequately represent the disease at the various stages. Tissue biopsies and fine needle aspiration (FNA) samples have associated risks, costs, and challenges, particularly in difficult to access locations such as lung and pancreatic tumors, and the inability to detect cancer cells in the case of micrometastases. A robust method for profiling the mutational status of circulating tumor cells (CTCs) from a blood sample would enable real-time monitoring of cancer progression that is impractical using standard tissue biopsy.

Mutational profiling of DNA isolated from CTCs as a blood-based biopsy is a valuable research approach towards understanding tumorigenesis, metastasis, as well as cancer treatment response and recurrence. This application note describes a workflow for analyzing somatic variants found in circulating tumor cells (CTCs) isolated with the IsoFlux System using the Ion AmpliSeq™ Cancer Hotspot Panel v2, the Ion PGM™ System, and Ion Reporter™ Software.

IMPORTANCE OF SOMATIC VARIANT DETECTION IN ONCOLOGY

Cancer is multistep progression of genetic changes that corrupt normal physiological processes enabling self-sufficient growth, lack of growth control, avoidance of apoptosis, stimulation of angiogenesis, and metastasis. Cancer progression is further aided by intrinsic genetic instability that generates collateral damage mutations. These variants are also termed 'passenger' mutations since they can be acquired during the error-prone cell replication process of a cancer cell. Unlike 'driver' mutations, these are not selected for during cancer formation nor do they provide adaptive responses such as drug resistance. Additionally, tumors can be heterogeneous with the acquisition of different somatic mutations to form polyclonal tumors with clonal expansion dependent on the specific mutational burden. Molecular profiling using targeted gene panels such as the Ion AmpliSeq™ Cancer Hotspot Panel v2 have helped elucidate the genetic changes, driver mutations, and clonal mutations present in numerous cancer types (Liu et al., 2014; Tabone et al., 2014; Amato et al., 2014; Tan et al., 2013).

Molecular profiling is particularly important since the acquisition of specific somatic mutations is known to parallel the transitional stages of cancer (Hanahan and Weinberg, 2011). This places increased importance on acquiring tumor samples continuously at critical disease inflection points, thus enabling the cancer researcher to track somatic variants over time. Ultimately, this can aid in the understanding of disease progression, relapse, tumor heterogeneity, and clonal expansion.

CHALLENGES IN TUMOR SAMPLE COLLECTION

To date, most of mutational profiling has relied on samples acquired from either tumor tissue that is formalin-fixed, paraffin-embedded (FFPE) for preservation or fine needle aspirate (FNA) biopsies. Although these techniques are the current standard source of nucleic acid for molecular analysis, there are many limitations and challenges that restrict the use FFPE and FNA samples for repeated sampling. This can result in a significant time period in which cancer disease progression proceeds unmonitored. Further, metastatic sites can be difficult to access and not well tolerated for routine sampling. An additional limitation in repeated tumor sample collection is the associated risks and costs for obtaining a tumor biopsy.

Each procedure carries an inherent risk of false positive/negative results due to sampling error, as well risks such as re-seeding of the disease through the inadvertent spread of malignant cells along the biopsy needle track (Goldin et al., 2007). The FFPE preservation process can also pose challenges to the molecular analysis since the gDNA tends to be partially degraded and limited in quantity. The FFPE process can also introduce C:G>T:A deamination mutations. All of these challenges and limitations surrounding tumor tissue samples support the need for a non-invasive tumor-sampling method.

DETERMINING TUMOR MUTATIONAL STATUS USING PERIPHERAL BLOOD SAMPLES

Currently, there are two primary sources of circulating tumor DNA: circulating tumor cells (CTCs) and cell-free DNA (cfDNA). Both sample types can be detected and correlated with disease progression and cancer type (Punnoose et al., 2012). CTCs have the advantage of coming from live, intact cells that are putatively involved in the spread of disease and harbor any resistance mutations that may have arisen from therapeutic intervention. CTCs, when properly collected, can be characterized at the molecular level for both nucleic acid (DNA and RNA) and protein differences (Alix-Panabieres and Pantel, 2013, Lohr et al., 2014). The concentration of CTCs in the circulation is low, on the order of 1 CTC per billion blood cells. Obtaining a CTC sample for molecular analysis requires a combination of high recovery rates, minimal background, and a low level dilution for the final sample volume.

Previous studies using qPCR have shown that mutation detection from both cfDNA and previous CTC enrichment technologies is possible, but the inherent tumor content hovers in the 0.1% range (Figure 2). Filtration approaches to CTC enrichment yield backgrounds of approximately 15,000 WBCs, leading to purities below 0.1% for typical CTC counts (Coumans et al. 2013), while CellSearch CTC isolation results in purities below 0.3% (Punnoose et al, 2012). For cfDNA, the median pre-treatment tumor-derived cfDNA fraction is approximately 1% (Diehl et al. 2005).

These low purities are generally not compatible with most molecular assays based on qPCR or NGS. Modified approaches to molecular analysis have been proposed, such as COLD-PCR and droplet digital PCR, that can offer higher sensitivity. The drawback of such methods is a very limited maximum coverage of 10-100 known somatic mutations (Wiggin et al. 2013). By comparison, the CHPv2 assay covers 2800 known COSMIC mutations and any other mutations in frequently mutated regions spanning 20,000 base pairs.

In order for a sample to be compatible with a targeted sequencing assay that covers thousands of potential variants (e.g. CHPv2), it generally needs to be in the 5% or greater range of tumor content, based on lower limits of allelic frequency sensitivity on NGS around 1-2% and allowing for heterozygosity and sub-clonal distribution.
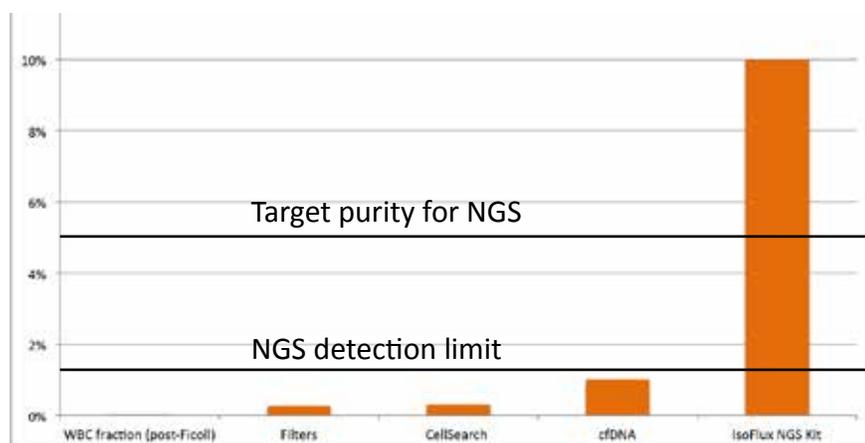
Figure 2: Comparison of typical tumor DNA purity across multiple circulating biomarker approaches. Other methods of accessing tumor DNA from the circulation include CTC recovery (CellSearch, size filtration, etc.) and cfDNA. All of these approaches typically yield DNA purity below 1%. The IsoFlux CTC Enrichment Kit followed by the IsoFlux NGS DNA Kit yields tumor DNA purity above 5%, which exceeds the target amount for the NGS assay.

TUMOR CELL ENRICHMENT WITH THE ISOFLUX SYSTEM

The IsoFlux System and the IsoFlux NGS kit is the first CTC isolation and recovery platform to provide output of >10% tumor cell content for a majority of samples, well beyond the reported purity estimates for comparable CTC approaches (CellSearch, filters) and cfDNA (Figure 2). The IsoFlux System was specifically designed to prepare CTCs for molecular analysis, including NGS and qPCR (Harb et al., 2013). Using an immunomagnetic approach, CTCs are captured in a proprietary microfluidic cartridge at a purity compatible with NGS analysis (Figure 3). Key advantages to the system include:

- **User-defined antibodies** – One or more custom antibodies can be used at the same time for selective capture of CTCs, epithelial–mesenchymal transition (EMT) cells, cancer stem cells, immune cells, or other disease/target-specific cells
- **High target cell recovery** – The unique microfluidic chamber was designed to balance both the flow and gravitational forces for optimal recovery of bead-bound target cells
- **Minimal background cells** – White blood cells (WBC) without bound beads flow out of the chamber into a waste well reducing the overall WBC background
- **Minimal sample dilution** – The final enriched cell pellet is contained in less than 5µL, and can then be transferred into the NGS workflow at the highest concentration possible for optimal results

SAMPLE PREPARATION FOR NGS ANALYSIS

The enriched CTC pellet is collected from the IsoFlux cartridge and further processed using the IsoFlux NGS DNA Kit (Figure 3D). The Purity Enhancement Column in this kit further reduces the background level of WBCs such that the tumor cell purity is ≥10% in the majority of samples, exceeding the analytical sensitivity threshold for AmpliSeq-based mutation detection. The CTCs are then lysed and whole genome amplified (WGA) using the REPLI-g® DNA polymerase that ensures highly uniform and accurate WGA from small samples in just 60-90 minutes. The WGA process results in typical DNA yields of 1-10µg per reaction.

A)

C)

B)

D)
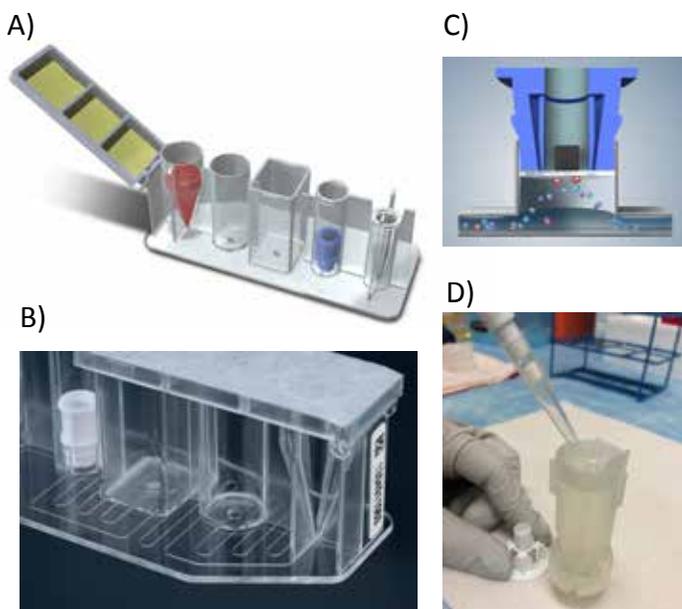
Figure 3. CTC recovery process using the IsoFlux System. A blood sample (7-14 mL) is collected in EDTA tubes and processed within 36 hours. RBCs are removed using Ficoll and the PBMC fraction is mixed with antibody-coated magnetic beads targeted to cell-surface proteins such as EpCAM and EGFR. (A) The sample is then loaded onto the IsoFlux microfluidic cartridge for cell separation inside the instrument. (B) The sample flows through a microfluidic channel that leads up to a capture zone where the CTCs are collected. (C) CTCs are captured on the roof of a removable cap that plugs into the chip. Once the run is complete, the cap is automatically removed inside the instrument and transferred to a holder for subsequent processing. The IsoFlux System retainins the entire enriched cell pellet during transfer. (D) Once collected, the CTCs are processed with the IsoFlux NGS DNA Kit. This kit contains a Purity Enhancement Column that further enhances tumor cell purity and recovers tumor cell DNA for NGS.

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METHODS

Analytical CTC samples were prepared by spiking in a breast cancer cell line MDA-MB-231 (ATCC: HTB-26) into 14 mL of healthy blood (AllCells: WB001-VK2). Two replicates were prepared by adding 0, 3, 6, 12, 24 cells per mL of blood. A third replicate was prepared for enumeration, and served as a proxy for what was actually captured in the other two samples. The mesenchymal-like MDA-MB-231 cell line was selected because it is known to have low epithelial cellular adhesion molecule (EpCAM) expression—one of the cell surface markers used for CTC isolation—and likely better exemplifies a true CTC sample. Samples were enriched for tumor cells using the IsoFlux System using a capture antibody cocktail consisting of anti-EpCAM and anti-epidermal growth factor receptor (EGFR). The resulting tumor cell pellet was processed using the IsoFlux NGS DNA Kit to enhance the tumor cell purity and whole genome amplify (WGA) the isolated gDNA. The resulting DNA product was amplified using the Ion AmpliSeq™ Cancer Hotspot v2 panel, followed by standard library and template preparation with sequencing on the Ion PGM™ using the Ion PGM™ Sequencing 200 Kit v2 and the Ion 318™ Chip at two independent locations (Thermo Fisher Scientific, South San Francisco, CA, and UCSF Genome Center Core Lab, San Francisco, CA). A custom DNA sample developed using AcroMetrix® MegaMix™ technology was used to test analytical sensitivity. This sample contains titrated amounts of DNA that span hundreds of variants represented on the CHPv2, thus serving as a positive control on the NGS process workflow.

VARIANT FILTERING

Data generated on the Ion PGM™ System were first processed by Torrent Suite's variant caller, using parameters loaded from a custom JSON configuration file. These settings result in variant calls down to 0.5% allele ratio, compared to standard settings that only output variant calls with an allele ratio above 5%. The resulting variant file (in the .vcf format) was then uploaded to a hosted or local version of the Ion Reporter™ Software environment for read mapping, filtering of common variants, annotation, and multi-sample analysis. It is critical that variants of interest can be quickly identified and prioritized while common variants and non-deleterious variants (those predicted to have no effect on protein function or expression) can be reliably filtered from targeted sequencing experiments. Using Ion Reporter™ Software, applying stringent filters enables detection of low frequency somatic variants and prioritization of a final list of high-confidence somatic mutations from CTC samples. The following filters are applied directly from Ion Reporter™:

- **ALLELE RATIO:** Low quality calls and likely germline variants are excluded by setting an allele ratio filter of between 0.5-35%. Since the typically CTC purity from the IsoFlux workflow is between 10-20%, any variant with minor allele frequency >35% is likely to be germline (present in both isolated CTCs and normal cells) with variants <0.5% excluded due to baseline noise considerations.
- **CALL CONFIDENCE:** A number of parameters are used to filter variants based on the quality of the call: alternate allele read counts, p-value and homopolymer length.
- **COMMON SNPs:** An additional germline filter automatically excludes common SNPs that appear in the UCSC database.

Ion Reporter™ produces a final report with variant annotation for each detected variant along with the associated sequencing metrics such as read depth and allele ratio. The variant is characterized according to type (nonsense, synonymous, nonsynonymous), and the altered amino acid is indicated for nonsynonymous changes. Further, accompanying content from other databases such as the COSMIC database are associated to detected variants. The final variant list can be exported to other curated genomic databases and interpretation can be enhanced through access to reference content from Oncomine®.

RESULTS

Tumor cell enrichment

Tumor cell enrichment was performed on the analytic samples using the IsoFlux System (Fluxion Biosciences) and the IsoFlux CTC and Rare Cell Enrichment Kits (Fluxion Biosciences). The mean tumor cell recovery was 66% (range: 53-72%) for spike in controls of 0, 3, 6, 12, 24 MDA-MB-231 breast cancer cells per mL of healthy blood (Figure 4). The average tumor cell purity (number of recovered tumor cells/number of total nucleated cells) was 12% (range: 5-22%) for spike in controls of 0, 3, 6, 12, 24 MDA-MB-231 breast cancer cells per mL of healthy blood. The median white blood cell background was 279 cells.

Variant detection and NGS Performance

The MDA-MB-231 breast cancer cell line has three characterized variants that are targeted by amplicons in the Ion AmpliSeq™ Cancer Hotspot v2 panel. Each of these variants should be detected in varying allelic frequencies as a result of zygosity (homozygous or heterozygous) and the effect of copy number changes to the loci for these variants. Each spiked sample was sequenced using the described workflow and analyzed in a blinded fashion. The variant frequencies for all detected variants are reported (Figure 5). As per the analysis workflow, variants passing the stringency filters were called when the allele frequency was greater than 0.5%. Any other variants, excepting the three known cell line variants, were classified as false positives if they passed through all filters. Out of 8 samples (24 potential variant calls), only 1/24 calls was missed. Only 1 false positive call was made in this test group. The custom DNA sample using AcroMetrix® MegaMix™ technology was sequenced as a reference standard. This produced 318 COSMIC variants that passed through all filters, a value that can be referred to as a process control.

Sequencing coverage and depth are primary considerations that impact overall sensitivity and cost of a targeted sequencing workflow. The Ion PGM™ System offers a scalable sequencing solution with experimental fine-tuning possible. Using 200-base sequencing chemistry, there are three expected outputs: the Ion 314™ Chip v2 (30-50 Mb or 400-550 thousand reads) the Ion 316™ Chip v2 (300-600 Mb or 2-3 million reads), and the Ion 318™ Chip v2 (600 Mb-1 Gb or 4-5.5 million reads). For these studies, the Ion 318™ Chip v2 was used in conjunction with the Ion Xpress™ Barcode Adapters to multiplex 12 samples per chip.
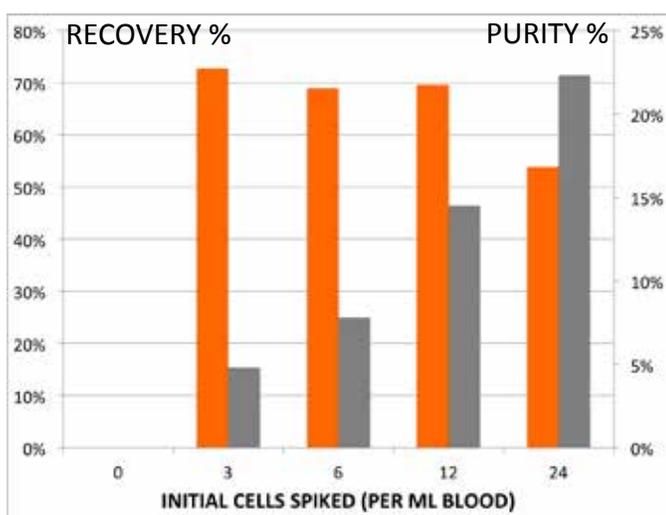
Figure 4. Tumor cell enrichment levels on the IsoFlux System using controlled analytical samples. Simulated CTC samples were prepared by spiking in 0-24 tumor cells (MDA-MB-231 breast cancer cell line, low EpCAM expression) per mL blood. Mean recovery of these cells was 66% (range: 54-73%). CTC purity ranged from 5% to 22% depending on the spike-in concentration, but all samples were above the detection limit for this NGS application.

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MDA CELL NUMBER (per mL blood)	CTC PURITY	SITE 1				SITE 2			
		BRAF	KRAS	TP53	FALSE POS	BRAF	KRAS	TP53	FALSE POS
Pure MDA-MB-231 cell line	100%	58%	68%	99%	NA	58%	68%	99%	NA
12	22%	Not tested				29%	22%	44%	0
						21%	12%	24%	0
8	15%	5.0%	7.0%	11.0%	0	6.0%	6.0%	10.0%	0
		3.0%	3.0%	5.0%	0	3.0%	2.0%	4.0%	0
4	8%	6.0%	6.0%	11.0%	0	7.0%	6.0%	12.0%	0
		ND	6.0%	1.0%	0	ND	5.0%	1.0%	0
2	5%	2.0%	2.0%	6.0%	0	3.0%	2.0%	5.0%	0
		2.0%	3.0%	5.0%	0	2.0%	2.0%	5.0%	0
0	0%	ND	ND	ND	0	ND	ND	ND	1 (TP53)
		ND	ND	ND	0	ND	ND	ND	0

Figure 5. Variants detected when using the MDA-MB-231 mesenchymal-like cell line and the Cancer Hotspot Panel v2. Three variants (BRAS, KRAS, TP53) are present in the cell line used for spiking experiments. Highlighted variant frequencies indicate where the variant was detected and passed all stringency filters. All three variants were detected down to the lowest spike in concentration (2 cells/mL) with a 2% allelic frequency or greater. Any other variant detected was considered as a false positive.

Multi-site validation data

Multi-site concordance for the detection of known variants in the MDA-MB-231 breast cancer cell line was assessed. DNA isolated from analytical spike in controls (0, 3, 6, and 12 MDA-MB-231 breast cancer cells per mL of healthy blood) were blinded and sent to two independent sequencing facilities (Thermo Fisher Scientific, South San Francisco, CA, and UCSF Genome Center Core Lab, San Francisco, CA). Each facility received DNA from the same samples but was independently responsible for targeted sequencing library preparation using the Ion AmpliSeq™ Cancer Hotspot Panel v2, template preparation, and sequencing. Variant calls using the Torrent Suite desktop application at each site were uploaded to the IonReporter cloud analysis tool and analyzed independently. Both sites made the identical variant calls (24/26 calls made) with good concordance between measured allelic frequencies (R2=0.97) (Figure 6).
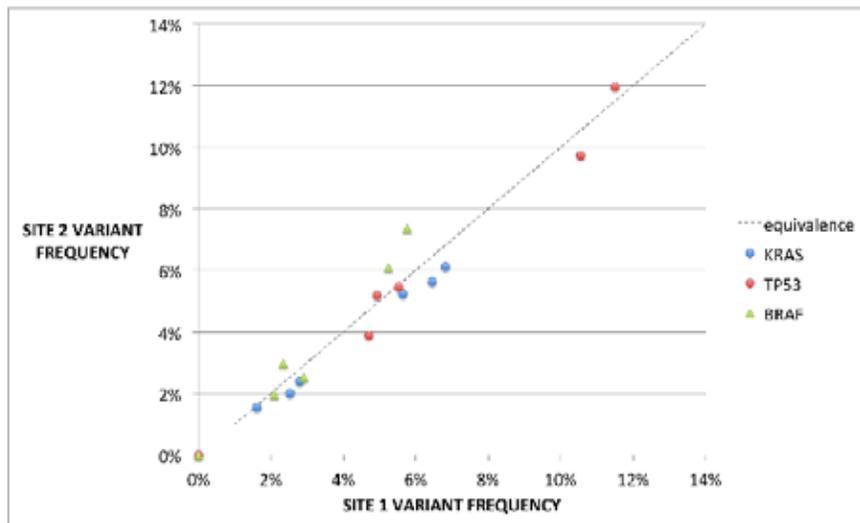

Figure 6. Multi-site concordance for the detection of known variants in the MDA-MB-231 breast cancer cell line targeted by amplicons in the Ion AmpliSeq™ Cancer Hotspot Panel v2. The same analytical samples were sent to two independent sequencing facilities (Thermo Fisher Scientific, South San Francisco, CA, and UCSF Genome Center Core Lab, San Francisco, CA) for a multi-site comparison study. Each facility received gDNA from the same samples but was independently responsible for library and template preparation and sequencing. Samples were processed and analyzed at the two sites in a blinded fashion and resulted in concordant variant calls (R2 = 0.97).

CONCLUSIONS

Somatic variant analysis of CTC samples provides a powerful tool for translational oncology research. The complete workflow presented here enables streamlined processing of blood samples through sequencing and analysis using commercially-available reagents, instruments, and software. The combination of the IsoFlux System, Ion AmpliSeq™ technology, and the Ion PGM™ System enables research into the role of CTCs in cancer disease progression over multiple time points. Through the study of CTCs, it is hoped that in the future cancer can be classified by metastatic potential in which localized tumor growth—that benefits preferentially from surgery—can be differentiated from rapid metastasizing cancer that would benefit from non-surgical intervention. High-resolution sequencing data sets will help ascertain the molecular signature of CTCs and will aid in understanding of recurrent somatic mutations that support a causal role in cancer development and the heterogeneity of tumors present in primary tissue that is difficult or impossible to obtain. Eventually, profiling of CTCs might be used for the identification of "actionable" variants for targeted therapy or patient stratification. This presents an opportunity to explore new possibilities for therapeutic intervention or to correlate mutational status to treatment that early studies suggest can be more effective than a general one (Wheler et al., 2014; Dempke et al., 2010).

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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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