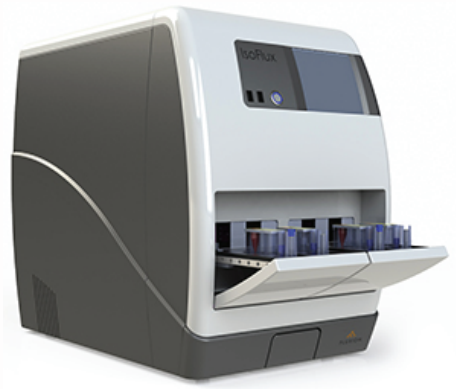
FLUXION

APPLICATION NOTE

Mutation detection in circulating tumor cell samples obtained from the IsoFlux™ System

Overview



- DNA mutations are frequently found in human cancers, such as colorectal cancers and non-small cell lung cancers.
- KRAS is an oncogene involved in the epidermal growth factor receptor (EGFR) pathway that controls cell proliferation and differentiation. The EGFR pathway is a common target for cancer therapies.
- Mutations in the KRAS gene have been correlated with poor response to EGFR-targeted compounds such as Erbitux (cetuximab).Clinical testing for KRAS mutations is now routine.
- Routine tissue biopsies to monitor mutational status are not clinically feasible. Circulating tumor cells (CTCs) offer this capability from a simple blood draw. The IsoFlux System provides a robust method of isolating CTCs for downstream molecular assays.
- A KRAS mutation detection assay was developed for CTCs utilizing the IsoFlux System and Competitive Allele-Specific TaqMan® PCR (castPCR) assays from Life Technologies.

Introduction

Cancer progression in humans stems from acquired DNA mutations in a specific group of cells. While the causes of these mutations are still not fully understood, their role in malignancy has become evident. As such, the detection of mutations in cancer causing oncogenes has become increasingly important in the clinical management of disease.

The KRAS oncogene is a notable example of a mutation site with clinical utility. KRAS is involved in the epidermal growth factor receptor (EGFR) pathway that controls cell proliferation and differentiation. KRAS mutations are frequently found in human cancers such as colorectal cancer (CRC) and non-small cell lung cancer. About 40% of colorectal cancer patients have a KRAS mutation, typically found on codons 12/13. Many modern cancer therapies, such as Erbitux (cetuximab), are used to treat colorectal cancer by targeting the EGFR pathway. When the KRAS gene is mutated, it continues to send growth signals even if the EGFR is inhibited by therapy. As such, patients with KRAS mutations have been shown not to respond to drugs like cetuximab. A KRAS mutation detection test was cleared by the FDA in 2009 and is routinely used for CRC patients about to go on therapy.

Although mutational testing is routinely performed in tissue biopsies at the time of diagnosis, the limited availability of these specimens makes it difficult or impossible to track mutational status longitudinally. In most cases, taking repeat biopsies over time is not clinically feasible due to trauma caused to the patient, excessive costs, and lack of tissue availability in cases where the primary tumor has been resected.

Circulating tumor cells (CTCs) have shown great promise to offer longitudinal insight to metastatic disease. CTCs can be obtained from a simple blood draw that overcomes previously mentioned challenges in obtaining tissue specimens. Additionally, it is likely that the CTCs better represent the metastatic disease and the cells most likely to contribute to cancer progression.

Utilizing CTCs to obtain mutational data is not without challenges. There is far less cellular material available as compared to tissue biopsies. This requires the CTC isolation platform to be as efficient as possible in recovering CTCs for analysis. The downstream detection methodology must also be sensitive to detect mutations even in the presence of background blood cells.

Here we present a novel KRAS mutation detection assay that can be run on CTCs obtained from the IsoFlux System (Fluxion Biosciences, South San Francisco, CA). The IsoFlux System is a benchtop instrument and microfluidic chip that efficiently recovers rare CTCs from blood samples and passes them off for further testing.

For the downstream KRAS mutational assay, a sensitive allele-specific Taqman based qPCR approach was utilized (castPCR, Life Technologies). The combination of these two technologies provides a complete, 'sample-to-answer' workflow for mutation detection.

Utilizing CTCs for mutational characterization

Historically, it has been challenging to utilize CTCs for anything but counting the cells on a microscope. Many CTC recovery approaches fail to deliver CTCs in sufficient quantity or quality (e.g. viability, elution volume, purity) for molecular analysis. Downstream of the CTC isolation, the genetic assay must also be sufficiently sensitive to detect potentially rare mutations.

Target cell recovery

CTCs are found in low concentration in the peripheral circulation on the order of 1 in a billion cells. The CTC isolation process must recover sufficient quantities of cells from a significant portion of the patient population for a mutational test to be clinically viable. The IsoFlux System utilizes magnetic field focusing in a microfluidic environment to greatly improve the capture efficiency of immunomagnetic separation. This leads to larger numbers of CTCs collected in the vast majority of patients across many tumor types. Figure 1 highlights CTC recovery data across multiple pathologies, and shows greater than 5 CTCs collected in 75% of the patients tested.

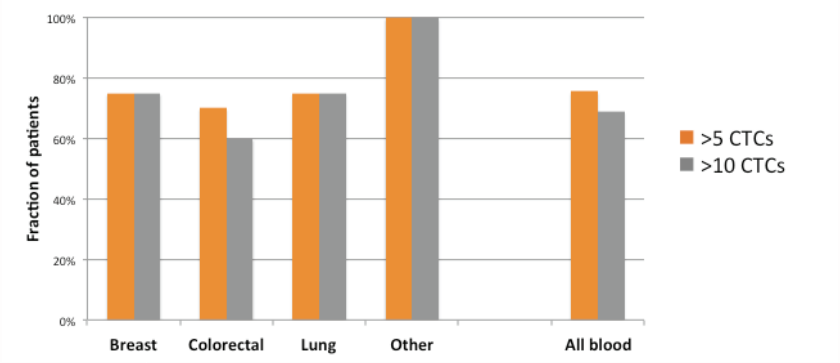


Figure 1 - CTC recovery: A series of 7mL whole blood samples (N=45) from multiple pathologies were processed on the IsoFlux System. On average, 75% of patients presented >5CTCs (defined with immufluorescence, CK+ / CD45- / nucleated). This number is above the limit of detection for mutational analysis using the assay described below.

In addition to isolation efficiency, off-chip recovery is important as well. The CellSpot technology included in the IsoFlux System enables lossless recovery of cells from the microfluidic flow channels. Cells are deposited in a small area of the CellSpot substrate and extracted in very low dead volumes (2-5µL).

Insufficient assay sensitivity

The low amount of target cells requires an assay with a limit of detection down to a few copies of target DNA. Additionally, the assay format must tolerate some presence of wild type background. In collaboration with Life Technologies, we validated their Competitive Allele-Specific TaqMan (CAST) assays for use with IsoFlux CTC samples. The CAST assay has very high sensitivity, with data previously presented by Life Technologies showing sensitivities down to 1 copy of mutant DNA. Most importantly, the assay is designed to be insensitive to high wild type background DNA copy numbers (hundreds to thousands). gDNA samples extracted from cell lines were used in order to validate assay performance for KRAS mutations. Using the CAST assay format, levels down to 3 copies of target DNA are detectable in samples that contained very high numbers of background wild type copies (20k).

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 Isolation Kit, Fluxion Biosciences). Immunomagnetic beads pre-conjugated with anti-EpCAM antibodies (CTC Isolation 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. Imaging was performed using an inverted epi-fluorescence motorized microscope (Zeiss AxioObserver Z1) and imaging software (BioFlux Montage, Fluxion Biosciences).

D. Mutational analysis – Recovered cells were amplified using the REPLI-g UltraFast Mini Kit (Qiagen). Amplified gDNA was isolated using QIAamp DNA Micro Kit (Qiagen)and run with a set of TaqMan® mutant allele assays and a corresponding gene reference assay (castPCR, Life technologies) using a qRT-PCR instrument (StepOne Plus™, Life technologies). The dCt value for the mutant allele assay and gene reference assay pair was calculated. This value was compared to the previously determined detection dCt cutoff value to determine the sample mutation status (Figure 2).

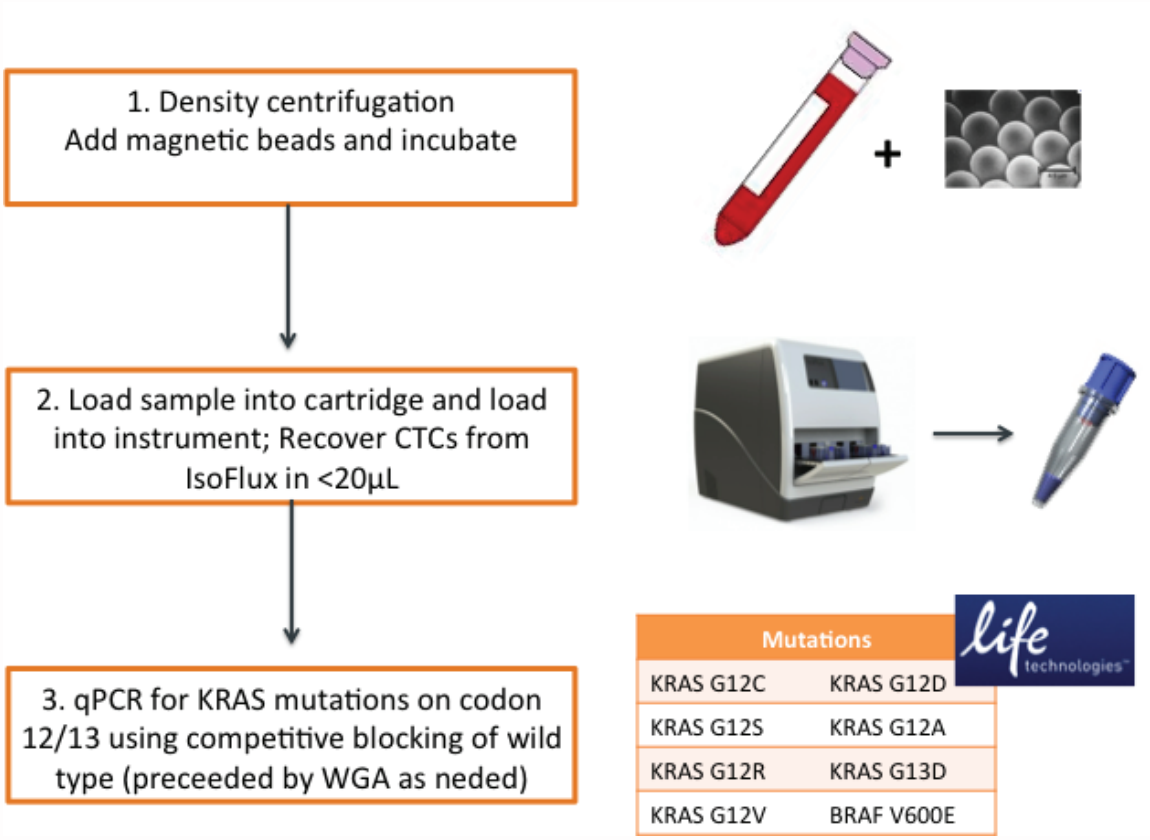


Figure 2 – Workflow: Mutation detection experiments consist of three process steps: 1 – Sample collection in an EDTA blood tube and separation of the white blood cell fraction via FICOLL centrifugation; magnetic bead coupling. 2 – CTC enrichment using the IsoFlux instrument; the positive fraction is recovered in an elution volume below 20µL. 3 - Mutation detection for key oncogenes using a standard qPCR instrument and the CAST reagents kits from Life Technologies.

Results

Assay sensitivity and limits of detection: gDNA equivalents

As a first step, we determined the sensitivity of the CAST assay for a KRAS point mutation in a high wild type background (one that is much higher than backgrounds obtained using the IsoFlux System). For the mutant DNA we used gDNA extracted from a cancer cell line (MDA-MB-231) presenting a known heterozygous mutation (G13D) of the KRAS gene. Between 0-16 mutant gDNA copies were spiked into a background of 20,000 wild type gDNA copies (10k cell equivalents) isolated from a Jurkat cell line. The mutation detection assay results are presented in Figure 3. The mutation detection dCt threshold was set to 2 Ct below the median wild type controls, or at a dCt = 21. The positive control (heterozygous mutant gDNA only) presented a dCt of 7.5. All negative controls tested appeared well above the cutoff. Copy numbers of 4 cell equivalents and above tested positive for the mutation. For 2 cell equivalents, 2 out of 3 samples tested positive for the mutation. This variability may be due to either true assay variability or due to inaccuracies of the serial dilution at low copy numbers. In either case, the established limit of detection for this study was 4 cell equivalents.

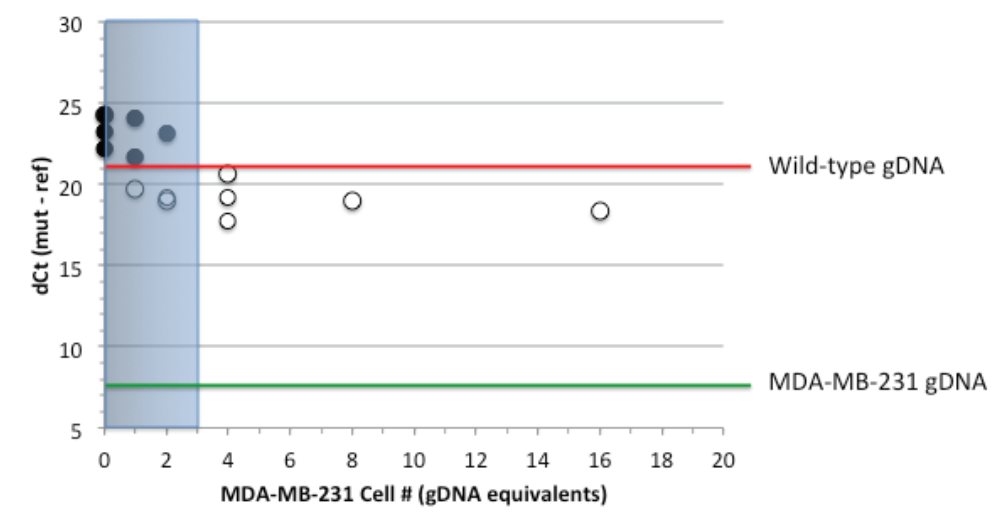


Figure 3 - Mutational assay sensitivity: A dCt cutoff was established at 21 Ct's by running a number of negative controls (red line) while the positive control (gDNA from G13D mutant cell line, green line) yielded a dCt of 7. Mutations are called correctly for all samples above n=4 copies (or n=4 heterozygous cell equivalents)

Assay sensitivity: full separation workflow

Spike-ins of the same cancer cell line were used to characterize the true ability to capture a small number of cells from a blood tube and accurately determine the mutational status. The same cell line was used (MDA-MB-231) and samples were processed through the entire IsoFlux workflow. This included CTC recovery, DNA isolation, whole genome amplification, and qPCR using the CAST assay from Life Technologies. Matched samples were processed through the CTC recovery step and enumerated based on target cell immunofluorescence staining. The results are presented in a plot of dCt values (and therefore mutation status) against matched sample enumeration results in Figure 4. CTC positive samples are clearly distinguished from CTC negative controls. We obtained a median dCt value of 15 for samples with CTC counts of 9 cells. Using the same cutoff as gDNA samples (dCt = 21) results in a separation of 6 Ct's. By comparison, the gDNA samples only showed a separation of approximately 4 Cts for a 16-cell equivalent spiking experiment. The lower separation in gDNA spiking experiments may be due to the fact that a larger background (10k cell equivalents) was used as compared to the actual leukocyte backgrounds in patient blood.

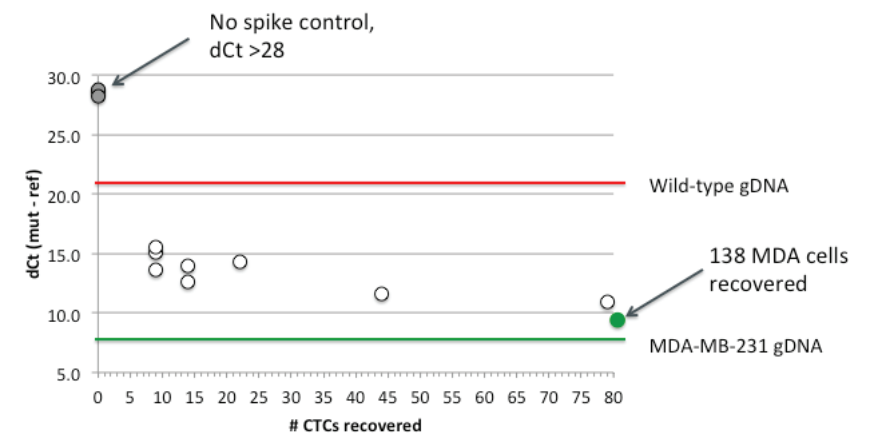


Figure 4 - Analytical validation using spiked samples: MDA-231 cells were spiked into whole blood in a 7.5 mL tube in increasing quantity. Each sample analyzed had a matching sample for which target MDA cells were separated using the IsoFlux system and enumerated. The results indicate a very clear mutation signal above the noise floor (dCt = 21) for all samples that contained target cells. There is a clear indication that for samples presenting 5 CTCs and above the mutational status will be easy to determine using this assay format.

We conclude from this data that the assay is in line with the gDNA titrations and similar mutations will be detectable for patients presenting at least 5CTCs in matched enumeration assays. A 5 CTC limit of detection (LOD) was later confirmed by working with clinical samples, where mutations were called for enumerations results down to 7 cells whereas this was the lowest number of cells detected in a CTC-positive patient.

Clinical validation and workflow

A group of twenty colorectal cancer patient samples were evaluated using the previously described KRAS mutation detection assay. Four additional healthy donor blood samples were used as negative controls. The CTC enumeration values and mutation detection status are shown in Figure 5. All four of the healthy donor samples had dCT well above the established cutoff. Ten of these CTC samples were shown to have a KRAS mutation (these appear below the dCt cutoff in the green zone).

These clinical samples confirm the ability to detect KRAS mutations using the IsoFlux System and KRAS mutational detection panel. Samples containing as few as 7 CTCs were shown to have KRAS mutations which is consistent with the analytical validation.

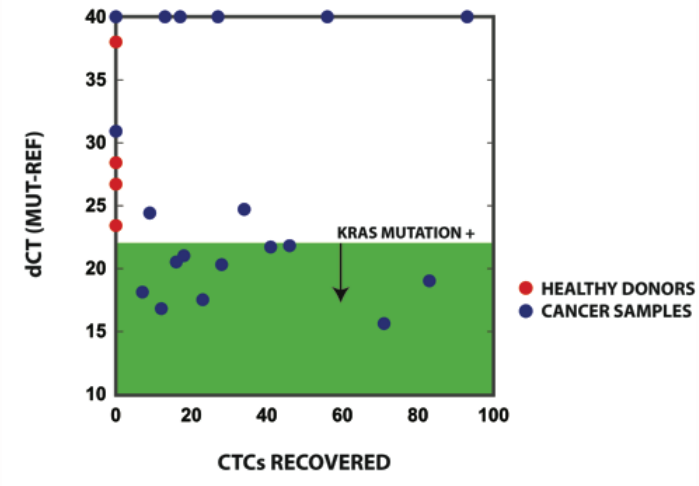


Figure 5 – Clinical evaluation of KRAS mutation assay: Four healthy donors and 20 metastatic cancer patients were tested for KRAS mutational status. Mutations detected on the IsoFlux KRAS panel appear in the green zone if detected, and otherwise appear above the cutoff if wild-type.

Conclusions

Fluxion has developed a complete 'sample to answer' workflow for mutation detection in CTC samples obtained from the IsoFlux System. The IsoFlux System provides high recovery of CTCs in an optimal format for downstream analysis: low elution volume, intact viable cells and minimal background contamination. The CAST-PCR mutation detection assays provide a complimentary approach to detecing mutations from IsoFlux CTC samples. Analytical and clinical validation data demonstrates the high sensitivity of this assay and ability to detect point mutations from cancer patient blood samples. Superior recovery yields CTC quantities above the assay's limit of detection for 75% of the patients tested.

This approach is amenable to a number of clinical applications where identification of mutational status may be beneficiary. This includes clinical trial patient stratification, identification of therapeutic responders, and prognosis information about disease progression.

While this assay focused primarily on KRAS mutations, the CAST-PCR mutation detection assays have been designed for over 200 mutations including ones of BRAF, EGFR, NRAS, PIK3 and other cancer genes of interest. Any of these assays can be run using the IsoFlux System and the CAST-PCR reagents on a standard qPCR instrument. Additionally, Fluxion offers mutational testing services on CTCs through its own reference lab in South San Francisco, CA.

Please visit www.fluxionbio.com/isoflux for more information or contact support@fluxionbio.com