IsoFlux™ System
Single-cell analysis of circulating tumor cells using the IsoFlux System

OVERVIEW

- Single-cell analysis of circulating tumor cells (CTCs) helps examine tumor heterogeneity, most notably in the cells linked to metastatic spread of the disease.
- This application note presents validation data for two different single cell analysis approaches following CTC enrichment with the IsoFlux System (Fluxion Biosciences): (1) using manual pipette-based single-cell selection, and (2) using the CellCelector instrument (Automated Lab Solutions) – an automated platform for imaging and isolating single cells.
- Single-cell selection is followed by molecular analysis. The success rate is determined by detecting a known SNP mutation in spiked cancer cells after cell lysis, whole genome amplification (WGA), and qPCR analysis.
- The IsoFlux System provides the necessary level of CTC enrichment to enable efficient single-cell selection and molecular analysis.

BACKGROUND

Molecular profiling of tumor tissue is routinely used as part of the standard of care for cancer patients. Cancer molecular testing has so far largely relied on the use of tissue biopsies from either the primary tumor or, in rare cases, metastatic sites. The challenge for this approach is the paucity of accessible tumor tissue after the first surgical intervention, as well as high cost and risk factors associated with repeated biopsies. Extraction of CTCs from peripheral blood can overcome these difficulties and provide a solution for longitudinal patient monitoring.

The IsoFlux System was introduced to provide high efficiency recovery of CTCs from a routine blood draw. Using an advanced microfluidic separation technology, the platform achieves tumor cell purities on the order of 1-5% using the IsoFlux CTC Enrichment Kit, and purities greater than 10% using the IsoFlux NGS DNA Kit. While these samples are suitable for most molecular assays, isolating single tumor cells from these pre-enriched samples offers two compelling advantages: (1) the ability to analyze circulating tumor material at 100% purity, and (2) the ability to characterize each cell individually, obtaining a profile of the heterogeneity present in the tumor cell population. This application note presents validation results for two single-cell isolation methods, both using the IsoFlux as a necessary starting point for CTC enrichment (Figure 1).

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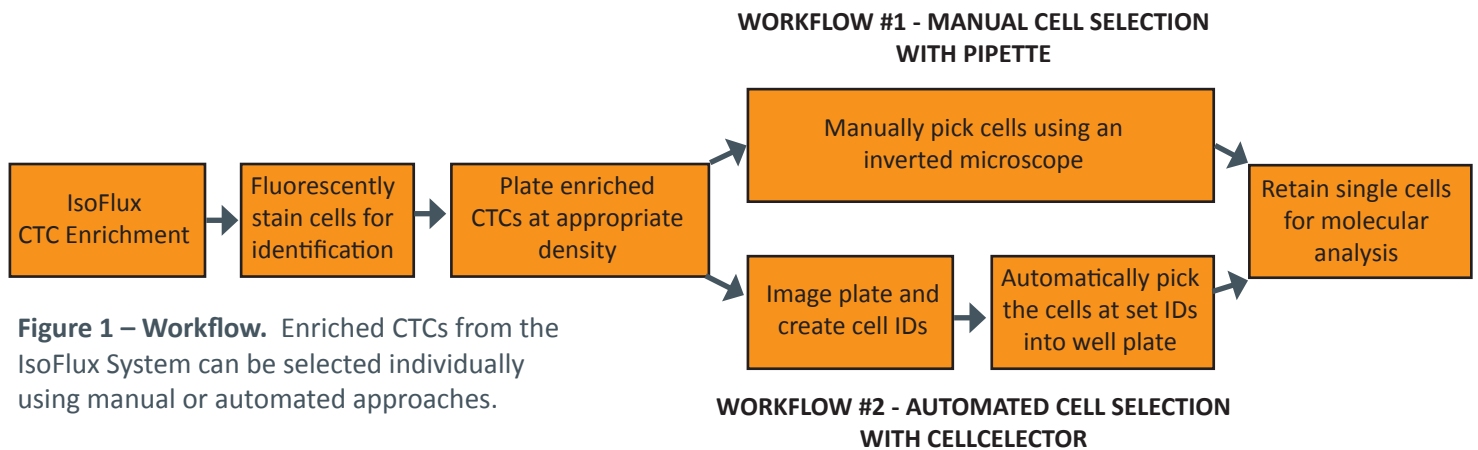**Single-cell analysis of circulating tumor cells using the IsoFlux System**

WORKFLOW #1 - MANUAL CELL SELECTION WITH PIPETTE

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IsoFlux CTC Enrichment → Fluorescently stain cells for identification → Plate enriched CTCs at appropriate density
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Manually pick cells using an inverted microscope → Retain single cells for molecular analysis

Image plate and create cell IDs → Automatically pick the cells at set IDs into well plate →

WORKFLOW #2 - AUTOMATED CELL SELECTION WITH CELLCELECTOR

Figure 1 – Workflow. Enriched CTCs from the IsoFlux System can be selected individually using manual or automated approaches.

METHODS

A. Sample logistics and preparation - Analytical samples were prepared to simulate cancer samples in experiments that rely on knowing the starting concentration of target cells. Healthy donor peripheral blood was obtained from a commercial supplier (AllCells, Emeryville, CA). 7mL blood was drawn into EDTA tubes (Vacutainer®, BD, Franklin Lakes, NJ) and subsequently spiked with a breast cancer cell line: mesenchymal-like MDA-MB-231 (ATCC, Manassas, Virginia). MDA-MB-231 cells were also used for genomic analysis validation since this line harbors a known heterozygous KRAS G13D mutation.

B. IsoFlux Enrichment - Following the magnetic bead coupling step, samples were loaded into the inlet well of the microfluidic cartridge. Samples were flowed through the channel at a flow rate of 20µL/minute. Each sample passed through the channel in less than 45 minutes. The isolated target cells were recovered off of the isolation zone disk via pipetting.

C. Single-Cell Picking, Manual Method - The enriched CTC sample was resuspended in 0.2mL of phosphate buffer saline (PBS) or RPMI 1640 cell culture media and kept on ice during the selection procedure. Sets of cells were dispersed into PBS in a serum-coated 12mm diameter circular area on a microscopy slide mounted on top of an inverted fluorescence microscope. For the validation experiments presented, cells were stained live with anti-CD45-PE and identified based on the absence of CD45 staining, and a bead-decorated appearance indicating EpCAM expression on the cell surface. Using a 10µL micro-pipette with extended tip set to 2µL volume, the tip was placed next to the cell to gently aspirate the cell into the tip. The single cell was transferred by dipping the pipette tip into the PBS in the PCR tube pre-loaded with PBS buffer (liquid to liquid transfer). Picked single-cell tubes were placed on ice or snap frozen and stored at -70°C before batch processing for whole genome amplification. A more detailed version of this protocol is available upon request.

D. CellCelector, Automated Single-Cell Picking and Imaging - Cells were picked from a 24-well plate and delivered to a 96-well PCR plate following a standard CellCelector workflow. Briefly, 100 MDA-MB-231 cells labeled with CellTracker Green were spiked into 7.5mL of healthy donor blood. After standard IsoFlux enrichment, cell pellet was stained with CD45-PE antibody and Hoechst 33342 nucleus stain, resuspended in approximately 30µL PBS, and dispensed into a 24-well plate mounted on top of the inverted microscope stage of the CellCelector instrument (Figure 2). The inverted microscope is equipped with a fluorescence scanning mechanism that images the whole picking area, and identifies particles meeting certain criteria (Figure 3). In this example, FITC and DAPI were used as positive selection channels to identify MDA-MB-231 cells that are CellTracker Green labeled and nucleated. The PE red (TRITC) channel denotes CD45+ contaminating WBCs.

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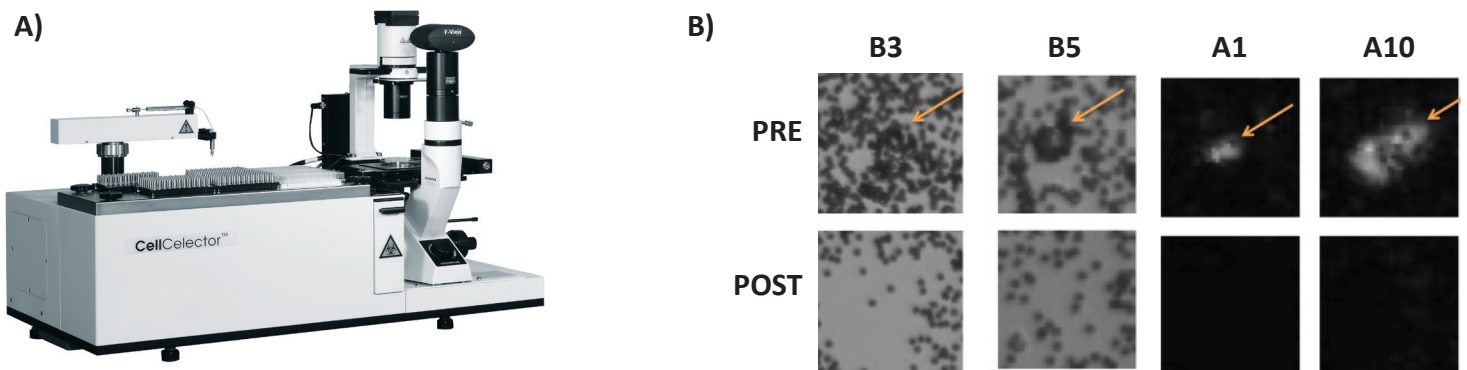## Single-cell analysis of circulating tumor cells using the IsoFlux System

Figure 2 – Single-cell picking using the CellCelector system. The CellCelector (A) consists of a microscopy system and a robotic arm equipped with a glass capillary (left). The microscopy system images the whole source plate and can identify single-cell candidates based on fluorescence signal and morphology. Successful cell aspiration is validated by juxtaposing images before and after cell removal (B). Examples are shown in bright field (particles B3, B5) and FITC fluorescence (A1, A10) for cells selected as fulfilling the criteria of CTCs (Images courtesy of Automated Lab Solutions).

METHODS (cont.)

The cell aspiration parameters were then set. The most important parameters are: the distance between the glass capillary picking tool and source plate substrate during pickup, the aspiration volume, and speed. The loose magnetic beads surrounding the isolated cells serve as a visual confirmation of the area of impact post-aspiration, and greatly facilitate the protocol tuning process (Figure 2B). After the picking parameters were tuned, 2µL of PBS was added into each well of the receiving PCR plate, and automated aspiration/deposition of cells was initiated. Each selected cell was aspirated into the glass capillary mounted on a robotic arm, which then moved over the PCR plate with precision. The aspirated volume (and cell) was deposited into the destination well via fluid-fluid contact.

E. Mutational analysis – Recovered cells were amplified using the REPLI-g Single Cell Kit (Qiagen). Amplified gDNA sample was run with a set of TaqMan® mutant allele assays and a corresponding gene reference assay (castPCR™, Thermo Fisher Scientific) using qRT-PCR instrument (StepOne Plus™, Thermo Fisher Scientific). The dCt value for the mutant allele assay/gene reference assay pair was calculated, and this value was compared to the previously-determined detection dCt cutoff value to identify the cell mutational status. If the dCT of the KRAS mutant allele assay was below cutoff value, the cell was considered to be correctly identified as one of the target cells. If the mutation was not detected, but the reference gene was detected, then the cell was considered to be incorrectly selected (i.e. a white blood cell) but successfully amplified. If neither the reference gene nor the mutant gene is detected, it indicates a breakdown at either single-cell picking/transfer or gDNA amplification steps.

RESULTS AND DISCUSSION

The aim of this study was to establish the feasibility of obtaining genomic mutation signatures from single tumor cells extracted from a blood draw. To that end, analytical validation samples were prepared by spiking MDA-MB-231 cells (a mesenchymal–like breast cancer cell line) into whole blood. This cell line harbors a known KRAS point mutation (G13D), so that the presence of both the reference and mutant genes indicates that all of the steps were performed correctly: (1) single-cell identification, (2) cell aspiration into the pipette and disposition into the PCR plate, (3) cell lysis and DNA amplification, and (4) qPCR for the specific KRAS mutation and reference assays.

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All qPCR experiments were preformed in a blinded fashion. The desired well positions, as set by the single cell manipulation operator, were compared with the qPCR results reported by a second molecular assay operator.

The manual pipette-based method

Manual cell picking is used by numerous laboratories around the world for conducting single-cell assays. This is a skill-dependent technique that can have a good level of success. IsoFlux-enriched samples started out at a CTC purity of approximately 1%. The pellet is delivered by the instrument into a volume of <5µL, but diluted out into 200µL of buffer. Fractional amounts were then dispensed onto a microscope slide, to achieve the optimal density (Figure 3A). Note that the distance between positive cells (orange arrows) is at least 200µm. A typical field of view would only contain 1-2 tumor cells. The gallery of images shown in Figure 3B illustrates cell appearance in bright field. All cells are bead decorated with about 3-10 beads, indicating that they are likely EpCAM-expressing cells. Only CD45-negative cells are selected for picking and dispensing into wells for the qPCR reaction. The images shown are derived from a breast cancer patient sample, whereby the investigators were following this protocol for single-cell analysis. The tumor cells area easily distinguishable from the contaminating WBCs. The images obtained from our analytical validation samples (cell line spike-in) were similar to the ones shown in Figure 3.

For three different batches of cells picked, the reaction loading key and qPCR results are shown in Figure 4. Note that the wells marked 'MDA' signify cells that were picked from a pure MDA-MB-231 cell population, before the spiking of MDA cells into whole blood tube. The characteristic mutation was detected in all of these cells. Out of a total of 20 wells with cells picked, 12 wells (60%) showed amplification of the reference gene. The 8 unamplified wells are likely due to missteps at cell dispensing, cell lysis, or DNA amplification. Of the 12 wells showing reference gene amplification, 11 (92%) also displayed amplification of the G13D mutant. This indicates that the vast majority of cells picked using the microscope visualization protocol were correctly identified to be tumor cells at the molecular level. That adds up to an overall success rate of 55% for single-cell mutational profiling.

ALS CellCelector– the automated method

Where larger numbers of single cells are needed, and to reduce operator dependence, it is desirable to develop automated cell transfer protocols. To this end, we validated the CellCelector instrument from ALS (Automated Lab Solutions). The CellCelector workflow starts with a suspension of post-enrichment cells on the deck of an inverted microscope. An initial imaging phase is used to create a particle map of the entire scanning area (approx. 1 cm in diameter) (Figure 5A). Individual particles are then selected based on their fluorescent characteristics and assembled into a pick list (Figure 5B).
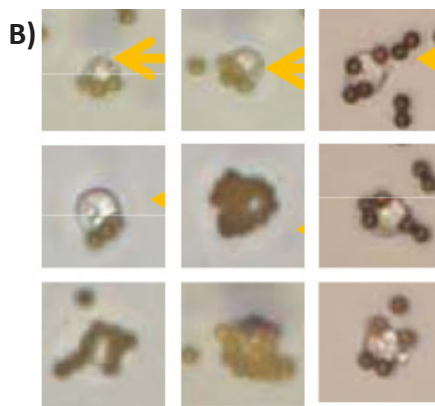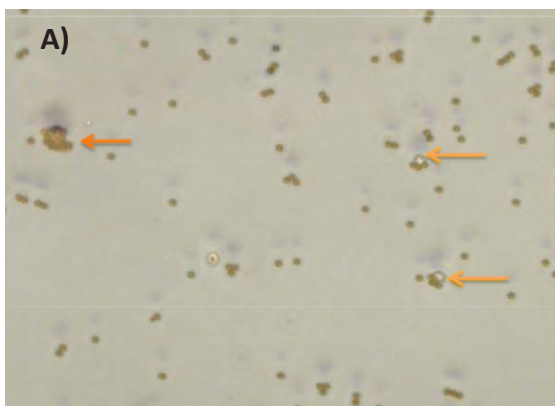

Figure 3 - Images of single breast cancer CTCs plated on slides for manual picking. A) Bright-field view of enriched CTCs from a breast cancer patient on a slide. B) Representative close-up images of single CTCs picked from a breast cancer patient sample. Small dots around CTCs are beads used for CTC enrichment (images courtesy of Houston Methodist University).

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Loading key

Row / Column	1	2	3	4	5	6	7	8	9	10
Batch 1	MDA	MDA	MDA	MDA	CTC	CTC	CTC	CTC	CTC	CTC
Batch 2	MDA	MDA	MDA	MDA	CTC	CTC	CTC	CTC	CTC	CTC
Batch 3	CTC	CTC	CTC	CTC	CTC	CTC	CTC	CTC		

Mutation assay results (qPCR)

Row / Column	1	2	3	4	5	6	7	8	9	10
Batch 1	MUT+	MUT+	MUT+	MUT+	MUT+	WT	MUT+	MUT+	N/A	N/A
Batch 2	MUT+	MUT+	MUT+	MUT+	N/A	N/A	MUT+	N/A	N/A	MUT+
Batch 3	MUT+	MUT+	N/A	MUT+	MUT+	MUT+	N/A	MUT+		

MDA	cell picked from a pure population
CTC	spiked tumor cell selected post separation

N/A	no reference amplification detected (Ct >37)
WT	reference gene amplified, mut sequence not amplified
MUT+	both reference and mutation sequence amplified

qPCR vs Cells Picked	Pure MDA	CTC
No Amplification	0	8
Wild Type	0	1
Mutant +	8	11
Totals	8	20

Results for cells picked manually post separation		
Picking / Amplification success	12/20	60%
Mutation Detection success	11/12	92%

Figure 4 - Single cell mutation detection using the manual method. A comparison between the operator's intended single cell type to be placed into qPCR reaction wells is shown (Loading key) along with the results of the qPCR assay. Detection of specific KRAS mutations (G13D) present in the MDA-MB-231 cell line (MUT+) was used to determine success for all of the steps – cell retrieval, cell placement into the qPCR well, cell lysis, and DNA amplification.
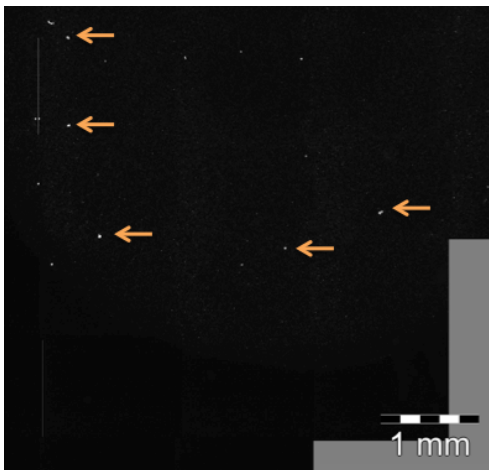
ID	DestinationWell	FITC	DAPI	TRITC
1	B-1	TRUE	TRUE	FALSE
21	B-10	TRUE	TRUE	FALSE
22	B-11	TRUE	TRUE	FALSE
23	B-12	TRUE	TRUE	FALSE
9	B-3	TRUE	TRUE	FALSE
50	B-4	TRUE	FALSE	FALSE
10	B-5	TRUE	TRUE	FALSE
11	B-6	TRUE	TRUE	FALSE
13	B-7	TRUE	TRUE	FALSE
16	B-8	TRUE	TRUE	FALSE
18	B-9	TRUE	TRUE	FALSE
25	C-1	TRUE	TRUE	FALSE
53	C-10	TRUE	FALSE	FALSE
54	C-11	TRUE	FALSE	FALSE
55	C-12	TRUE	FALSE	FALSE

Figure 5 - ALS CellCelector mosaic and particle list. A) Before single cell picking, the system images the source plate containing cells dispersed in a small droplet of buffer, generating a mosaic. B) Image analysis is used to generate a particle list that is selected for picking based on passing threshold for the three different fluorescence channels. The destination wells for each particle are displayed as well.

After the capillary parameters (distance from the surface, aspiration volume and speed, dispensing speed, etc.) are set and validated, automated operation can begin. Each of the identified particles is aspirated into the capillary and dispensed in an automated fashion into sequential wells in the destination well plate, which is mounted on the deck below a robotic arm. Another useful feature of the system is its ability to acquire images both before and after the cell aspiration event (Figure 2). This pair of images can be used as a QC criterion, as they confirm that the aspiration was successful and only one cell was picked. Examples of particles selected for mutational analysis are shown in both bright field and fluorescence modes. Note that the beads surrounding particles B3 and B5 are easily distinguishable in the bright field images. A set of beads is picked up along with the cell of interest, indicating the approximate size of the aspirated region, about 60μm in diameter.

Both target tumor cells, background cells (WBCs) and regions devoid of cellular material were selected for transfer to the qPCR plate (see the Loading key in Fig 6A). A good measure of assay success is the fidelity of the CAST qPCR results obtained in Fig 6A. A perfect result would be MUT+ for all CTC wells, WT for all WBC cells and N/A for 'None' regions. Molecular assays were performed in a blinded fashion, with the resulting Ct values for both KRAS mut (G13D) and KRAS reference gene used to detect both amplification of the reference gene and presence of this particular mutation.

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The results, as summarized in Figure 6, indicate that single-cell picking was successful and DNA amplified in 84% of attempts. Correct selection of a target cell over background should have lead to detection of the mutant KRAS gene. Mutation detection was achieved in 10 out of 14 putative tumor cells selected, or 71%. WBCs were selected and validated in 2 out of 16 total cells, picked, and amplified.

Loading key

Row / Column	1	2	3	4	5	6	7	8	9	10	11	12
A	CTC	None	None	None	CTC	CTC	CTC	CTC	CTC	CTC	CTC	CTC
B	CTC	CTC	CTC	CTC	CTC	WBC	WBC	None	None	WBC	WBC	WBC

qPCR results

Row / Column	1	2	3	4	5	6	7	8	9	10	11	12
A	MUT+	N/A	N/A	N/A	MUT+	MUT+	MUT+	WT	MUT+	MUT+	MUT+	MUT+
B	MUT+	WT	WT	MUT+	WT	N/A	N/A	N/A	N/A	WT	WT	N/A

None	no cell loaded
WBC	white blood cell selected
CTC	tumor cell selected

N/A	no reference amplification detected (Ct >37)
WT	reference gene amplified, mut sequence not amplified
MUT+	both reference and mutation sequence amplified

qPCR vs Cell Loading	None	WBC	CTC
No Amplification	5	3	0
Wild Type	0	2	4
Mutant +	0	0	10
Totals	5	5	14

Picking / Amplification success	16/19	84%
Mutation Detection success	10/14	71%

Figure 6 - Single-cell mutation detection using the CellCelector instrument. The operator's intended single-cell type to be placed into qPCR reaction wells (Loading key) is shown along with corresponding results of the qPCR assay. Detection of specific KRAS mutations (G13D) present in the MDA-MB-231 cell line (MUT+) was used to determine success of all steps – cell retrieval, cell placement into the qPCR well, cell lysis and DNA amplification. The success rates for mutation detection post picking are summarized in the bottom table.

CONCLUSIONS

Tumor analysis at the single-cell level is important due to tumor heterogeneity and the effect of rare mutations that can confer drug resistance to small tumor cell subpopulations. Blood based markers like circulating tumor cells are an attractive sample type for these studies because they enable longitudinal monitoring of tumor genomic changes during treatment. The application data presented here highlights two different workflows that combine the IsoFlux CTC Enrichment Kit with single-cell analysis. For the manual pipette-based approach, 92% of cells that successfully amplified were correctly identified by the characteristic mutation (55% overall success rate including workflow dropouts). For an automated workflow using the CellCelector system from ALS, 71% of experiments resulted in the correct mutation being detected. Both of these workflows demonstrate the capability to start with a routine blood draw and go all the way to a molecular endpoint from single tumor cells.

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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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