Single-Cell Lentiviral Vector Integration Sites and Clonal Tracking Assay for Cell and Gene Therapy

mission bio

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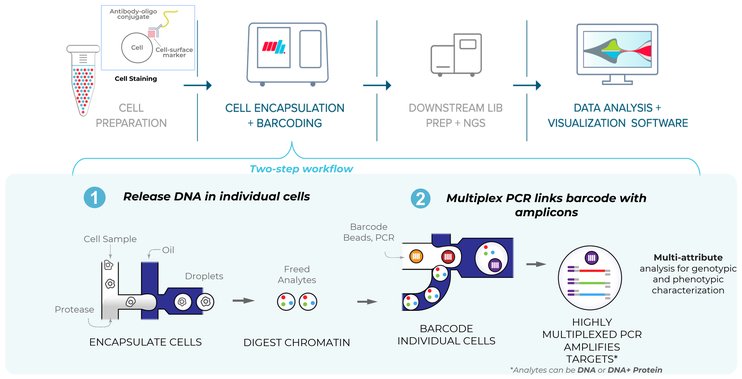
Introduction

Lentiviral vector (LV) has been widely adopted as an efficient vehicle to deliver transgenes into cells due to its long-term efficacy. However, the semi-random integration of LV has raised safety concerns due to its potential to trigger tumorigenesis during CAR-T therapy. To mitigate this issue, characterization of vector integration on clonal expansion after gene therapy has become a crucial practice to monitor the activity of viral vectors on *in vivo* selection of patient clones. Here, we developed a single-cell resolution, lentiviral vector integration site assay to survey the **co-occurrence of specific integration sites** and somatic genomic variants. Based on a set of LV transduced cell lines with known integration sites validated by orthogonal data, a targeted panel was designed to cover both the 5′ and 3′ ends of each integration site with predefined integration orientations. Samples include negative control and LV transduced cell lines with known vector copy numbers ranging from one to four copies. Using chemistry optimized for LTR junctions, we demonstrate the capability to quantitatively detect individual cells harboring specific vector integration sites and longitudinal tracking of cell clones with different vector copy numbers. Furthermore, besides amplicons targeting integration sites, in the same reaction, another set of internal vector amplicons (10-plex) and human genome amplicons (99-plex) are included to estimate vector copy number (VCN) and characterize somatic variants in the cell population. Taken together, a high throughput single-cell multi-omics platform enabled us to simultaneously identify somatic variants along with vector integration events in individual cells, providing both potential functional mutation identification and clonal tracking capabilities. The development of single-cell lentiviral vector integration sites and clonal tracking assay provides a unique opportunity to better study longitudinally CAR-T cell clonal dynamics and lead to a more effective therapeutic agent.

Methods

The Tapestri® Platform utilizes droplet microfluidic technology to rapidly encapsulate, process, and profile up to 20,000 individual cells for multi-analyte detection. The platform is enabled by a novel two-step microfluidic workflow and a high multiplex PCR biochemistry scheme. The two-step microfluidics allows for efficient access to DNA for downstream genomic reactions and provides flexibility to adapt for additional applications and multi-omics. The multiplex PCR chemistry is developed and co-optimized with an AI-powered panel design pipeline and enables direct and efficient amplification of targeted genomic regions within barcoded individual cells. Taken together, the platform produces high genomic coverage, low allele dropout rate, highly uniform amplification in thousands of cells from a single run, is compatible with diverse and difficult samples, and is easily deployable for custom content. The final products are sequenced on an Illumina sequencing instrument (Figure 1).

Figure 1: Tapestri workflow



Vector insertion site identification is accurate at single-nucleotide resolution

Figure 2: VIS assay design and IGV view of sequence

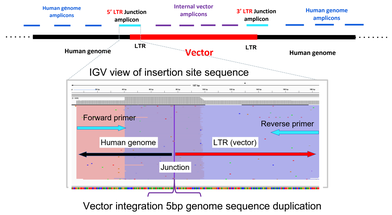


Table 1: Tapestri detected integration coordinates and sequence

Vector insertion sites at single cell resolution by Tapestri

Clonal cell line	Insertion site at 5′ LTR (hg38)*	Insertion Site at 3′ LTR (hg38)*	Vector insertion orientation**	Insertion duplication (fully closed, hg38)***	Insertion duplication sequence
Vector copy 1	chr3:45632785	chr3:45632789	Reverse	chr3:45632785-chr3:45632789	GTTAT
Vector copy 2	chr6:55210830	chr6:55210835	Reverse	chr6:55210830-chr6:55210835	GTCTG
	chr22:50844216	chr22:50844212	Forward	chr22:50844212-chr22:50844216	GATAC
Vector copy 3	chr3:230934991	chr3:230934987	Forward	chr3:230934987-chr3:230934991	CACCC
	chr14:32564447	chr14:32564451	Reverse	chr14:32564447-chr14:32564451	ATTTG
	chr15:35124969	chr15:35124973	Reverse	chr15:35124969-chr15:35124973	ATATG
Vector copy 4	chr1:55634499	chr1:55634495	Forward	chr1:55634499-chr1:55634495	GTTTG
	chr1:260440776	chr1:260440780	Reverse	chr1:260440776-chr1:260440780	GTACA
	chr16:3259702	chr16:3259698	Reverse	chr16:3259698-chr16:3259702	GCTAA
	chr22:52082676	chr22:52082671	Forward	chr22:52082671-chr22:52082676	GTTTT

* Exact insertion sites are slightly modified for this poster due to manuscript under preparation.
* Vector orientation: Forward = vector orientation (5′LTR - transgene - 3′LTR) matches genome orientation. Reverse = vector orientation (3′LTR - transgene - 5′LTR) matches genome orientation.
**Duplication sequence matched to genome coordinates shown in matched color.

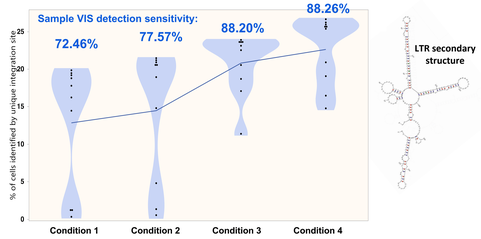
Characterized, experimental, lentiviral transduced Jurkat clonal cell lines with vector copy number of 0, 1, 2, 3 and 4 copies were used in this study [1]. In Figure 2, top, a custom panel of amplicons was designed to survey both ends of known vector junctions (light blue), internal vector amplicons (purple) to enable vector copy number analysis, as well as human amplicons (99-plex) to survey somatic variants in each cell. Figure 2, bottom, shows the IGV view of one of the VIS amplicons. Since the assay is designed to survey both ends of the vector junctions, we can also accurately identify the 5 bp duplication of genomic sequence associated with viral integration mechanism. Table 1 shows a summary of vector integration sites validated by Tapestri is accurate, as well as identifying vector integration sites at single-nucleotide resolution.

VIS detection assay optimized for LTR-human junction amplification

Table 2: Clonal co-occurring VIS detection and overall sensitivity

Clones	Unique insertion sites	% of cells assigned with detected integration: Condition 1	Condition 2	Condition 3	Condition 4
VCN 1	Ins. site 1	14.46	14.81	17.08	14.78
VCN 2	Ins. site 2	19.36	20.63	23.59	20.91
VCN 2	Ins. site 3	1.23	1.35	20.55	19.07
VCN 3	Ins. site 4	18.79	20.99	23.7	26.65
VCN 3	Ins. site 5	19.19	20.91	22.53	26.08
VCN 3	Ins. site 6	1.22	4.81	18.7	25.6
VCN 4	Ins. site 7	19.85	21.22	23.83	25.38
VCN 4	Ins. site 8	17.79	18.94	23.57	25.41
VCN 4	Ins. site 9	0.33	0.55	11.39	16.47
VCN 4	Ins. site 10	16.23	20.49	23.12	25.92
Sample detection sensitivity:		72.46%	77.57%	88.20%	88.26%

Figure 3: Violin plot of VIS detection sensitivity vs. conditions



Tapestri's single-cell VIS detection assay is optimized to interrogate sequence that has high degrees of secondary structures such as LV's LTR regions (Figure 3, right side). Here, we use a sample that consists of a mixture of the four clones VCN 1, 2, 3, and 4 (each mixed ~20-25% each, cell counted by Invitrogen Countess) to test four different assay chemistries that target parameters surrounding primer hybridization kinetics, and alleviating template secondary structure formation to improve VIS amplification, and hence detection, efficiency. Table 2 shows the percentage of cells in the sample with specific vector insertion sites detected. We expect each clone's unique insertion sites that co-occur to have equal % of cells detected in a sample (i.e. VCN 4 cells has four co-occurring integration sites in each cell, ins. site 7, 8, 9 and 10, in table 2, purple. If VCN 4 represents 25% of the sample, each insertion sites should be detected in 25% of the cells in the sample). As seen in table 2, with improved conditions from 1 to 4, the integration sites detection sensitivity improved (i.e. VCN3, insertion site 6, improved sensitivity from 1.22% to 25.6%). Furthermore, the clonal specific, co-occurring VIS are more uniformly detected (i.e. VCN3, Insertion site 4, 5, and 6 each detected at in 26.6%, 26.08% and 25.6% of the sample). As individual VIS assay's sensitivity improves, the overall VIS detection sensitivity also improved from 72.46% to 88.26%. Figure 3 is a graphical representation of the data in Table 2, with each dot representing one of the 10 unique integration site detections.

Single-cell VIS co-occurrence assay identifies clones with high specificity

In this experiment, various mixtures of VCN 1, 2, 3, 4 Jurkat cell lines are spiked in a background of Raji cells. Using the cell line specific SNVs (detected by the 99-plex human amplicons, Figure 2), we assign cells in the Tapestri runs to either Jurkat (vector positive) and Raji (vector negative). At the same time, clonal identity (VCN1, 2, 3 or, 4) can be assigned through analysis of clonal specific VIS co-occurrence in a cell. Using the combined information, we assess the VIS assay's clonal identification accuracy, sensitivity and specificity. In Figure 4, we show an example of a VCN1, 2, 3, and 4 (randomly mixed) mixture and its clonal distribution (bar graph) identified by vector integration site analysis (Jurkat cells only) and the VIS sites clonal co-occurrence detection (bottom table). Table 3 shows the analytical results from combined 20,307 cells and the expected vs. measured results. The Tapestri VIS assay has an average of 86.59% ± 0.06% sensitivity, 96.49% ± 0.15% specificity and 97.29 % ± 0. 015% clonal identification accuracy.

Figure 4: Clonal identity assigned via clonal specific VIS detection

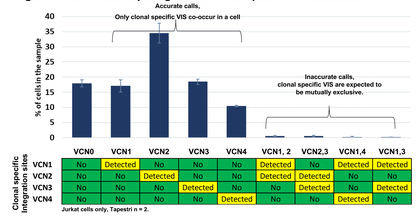


Table 3: Clonal identification via VIS sensitivity and specificity

20,307 cells analyzed — Positive Jurkat cells with VCN1, 2, 3, 4. Each with unique sets of VIS — Negative Raji cells with no vector

Expected / Measured	Cells with vector transduced	Cells with no vector
Cells with expected clonal specific VIS	84.75%	3.43%
Cells with incorrect mix of clonal specific VIS	2.39%	0.08%
No VIS detected	12.86%	96.47%

86.59% ± 0.06% sensitivity
96.49% ± 0.15% specificity
97.29 % ± 0. 015% cells with VIS accurately called

VIS assay enables quantitative longitudinal tracking of clonal progression

Figure 5: VCN analysis vs. VIS clonal calls

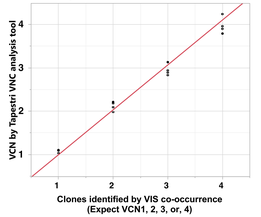
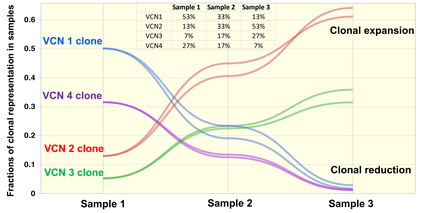


Figure 6: Quantitative longitudinal tracking of clones by VIS analysis example



As an orthogonal method to assess the accuracy of clonal (VCN 1, 2, 3, or 4) identification by VIS, we use the "internal vector amplicons" (Figure 2) and their relative read value to a reference to identify each clone's VCN value. For example, all the cells in the mixture that are identified to be VCN 4 cells via VIS assay is analyzed for their vector copy number value. The results shows that the vector copy number analysis correlates well with the expected VCN value of clones identified by VIS (Figure 5, R^2 = 98.94% ± 0.15%, n=4). Using Tapestri's single-cell assay to identify co-occurrence of clonal specific VIS, one can quantitatively track lineages longitudinally to evaluate clonal evolution and clonal functional expansion or reduction. Figure 6 shows a mock temporal data set example by mixing the VCN1, 2, 3, and 4 clones at various ratio (table in Figure 6). The plot displays 2 replicates for sample 2 and 3, demonstrating Tapestri VIS assay can robustly quantitatively track longitudinal clonal dynamics.

Conclusion

Mission bio has developed a single-cell lentiviral vector integration site assay (based on known VIS sites) to survey the co-occurrence of integration sites in a single cell. For demonstration of assay performance, using a set of known VCN cell lines, we show the Tapestri VIS assay has high specificity, sensitivity and can accurately assign clonal identity based on VIS co-occurrence measurements. The VIS assay can be applied to better study longitudinally CAR-T cell clonal dynamics, understanding CART biology, which lead to a more effective therapeutic agent.

Reference 1.https://doi.org/10.1038/s41598-020-79698-w