Precise Measurement of Viral Copy Number at Single-Cell Resolution for Cell and Gene Therapy Development

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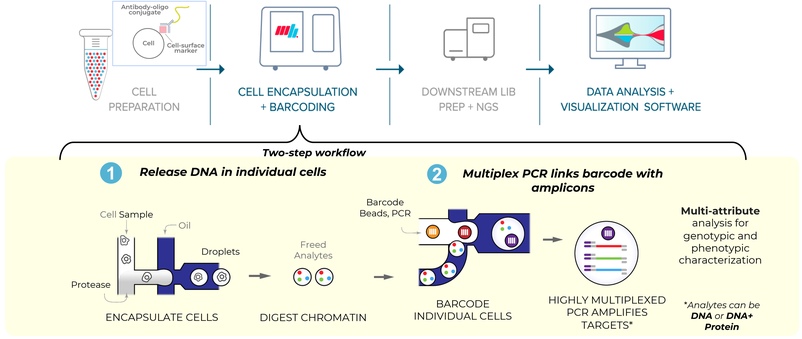
Introduction

Cell and gene therapies are transformative solutions for a host of inherited and acquired diseases for which existing interventions are ineffective. Many such therapies rely on the introduction of transgenes into host cells using viral or non-viral vectors. The accurate measurement of gene transfer is critical to the development of therapeutic agents and is a key attribute for assessing their safety and efficacy. Yet, conventional methods for measuring gene transfer lacks the resolution and representation to truly reflect sample composition and either report a population average (bulk) or involve laborious and time-consuming clonal outgrowth which can take weeks. Mission Bio has developed an end-to-end solution from panel design to data analysis for single-cell targeted DNA sequencing. Here, using the Tapestri platform we demonstrate that single-cell DNA sequencing identifies transduced versus non-transduced cells with exceptional accuracy and precision, as well as measure the single-cell level vector copy number (VCN) for populations of thousands of cells with single nucleotide resolution while reducing sample processing time from weeks to days.

Methods

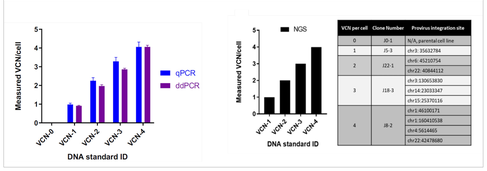
The Tapestri® single cell DNA platform utilizes droplet microfluidic technology to rapidly encapsulate, process, and profile up to 20,000 individual cells for multi-analyte detection. The platform is enabled by a novel two-step microfluidic workflow and a high multiplex PCR biochemistry scheme. The two-step microfluidics allows for efficient access to DNA for downstream genomic reactions and provides flexibility to adapt for additional applications and multi-omics (with oligo conjugated antibody during cell preparation). 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. The final products are sequenced on an Illumina sequencing instrument (Figure 1).

Figure 1: Tapestri workflow

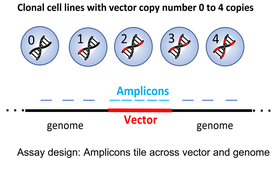


Characterized, experimental, lentiviral transduced Jurkat clonal cell lines with vector copy number of 0, 1, 2, 3 and 4 copies were used in this study [1]. A custom panel of amplicons was designed to enable the vector copy number analysis. Pure clonal cell lines of VCN 0-4 and three (3) mixtures with non-transduced Raji cell spike-in was processed for single-cell sequencing using Mission Bio's Tapestri platform in triplicates. Tapestri data from a control cell line with known VCN, as well as unknown samples, were combined and analyzed with a prototype copy number caller to produce single-cell level VCN calls, as well as a population average VCN and a percent transduction for each sample. A median of 5,116 cells were analyzed for each Tapestri sample.

Figure 2: Lentiviral transduced VCN cell lines and vector assay design[1]



VCN per cell	Clone Number	Province integration site
0	JO-1	N/A, parental cell line
1	J1-3	chr3: 35632784
2	J22-1	chr6: 45202754
		chr22: 40844112
3	J18-3	chr3:130653830
		chr14:23033347
		chr11:25379116
4	J8-2	chr1:46100171
		chr1:160410538
		chr4:5014465
		chr22:42478680



Assay design: Amplicons tile across vector and genome

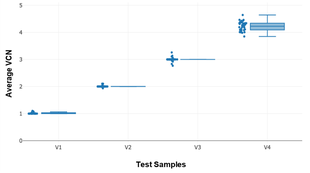
Results

Tapestri vector copy number assessment tool is precise and accurate

Table 1: Combinations of controls and test samples' average VCN

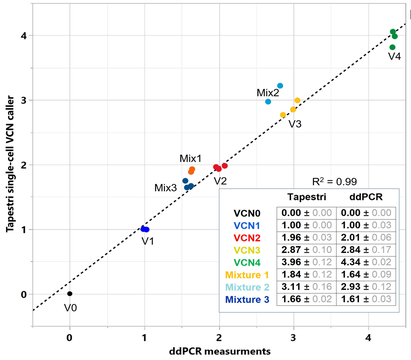
Average copy number for various combinations of controls and test samples		Test Sample											
		VCN1 rep1	VCN1 rep2	VCN1 rep3	VCN2 rep1	VCN2 rep2	VCN2 rep3	VCN3 rep1	VCN3 rep2	VCN3 rep3	VCN4 rep1	VCN4 rep2	VCN4 rep3
Control Sample	VCN1 rep1	1.00	1.00	1.00	2.10	2.00	2.00	3.00	3.00	3.26	4.34	4.46	4.33
	VCN1 rep2	1.00	1.00	1.00	2.00	2.00	2.00	3.00	3.00	3.00	4.12	4.23	4.01
	VCN1 rep3	1.00	1.00	1.00	2.01	2.00	2.00	3.00	3.00	3.05	4.09	4.32	4.10
	VCN2 rep1	1.01	1.03	1.03	2.00	2.00	2.00	3.00	3.00	3.00	4.08	4.27	4.19
	VCN2 rep2	1.02	1.03	1.03	2.01	2.00	2.00	3.00	3.00	3.05	4.18	4.35	4.18
	VCN2 rep3	1.01	1.01	1.02	2.00	2.00	2.00	3.00	3.00	3.00	4.09	4.38	4.34
	VCN3 rep1	1.09	1.08	1.10	2.10	2.08	2.07	3.00	3.11	3.13	4.37	4.64	4.46
	VCN3 rep2	1.03	1.03	1.04	2.00	2.00	2.00	3.00	3.00	3.00	4.18	4.43	4.27
	VCN3 rep3	1.06	1.06	1.06	2.02	2.01	2.00	3.00	3.00	3.00	4.22	4.42	4.29
	VCN4 rep1	1.00	1.01	1.01	2.00	2.00	2.00	2.99	3.00	3.00	4.01	4.34	4.11
	VCN4 rep2	1.00	1.00	1.00	1.94	1.98	1.96	2.77	2.96	2.83	3.85	4.04	3.98
	VCN4 rep3	1.00	1.00	1.01	2.00	2.00	2.00	2.92	3.00	2.96	4.00	4.31	4.03

Figure 3: Control vs. average VCN



Clonal populations of engineered VCN cells (VCN1-4) were single cell sequenced and processed through the Tapestri VCN caller individually (n=3). We demonstrate the single-cell VCN caller can use any cell population with a known number of VCN's to characterize a test sample's VCN with high accuracy and precision (Table 1). Fig 3 is a graphical representation of Table 1, where each dot represents the average VCN of the test sample (x-axis) using various control samples. Using VCN caller model established from VCN 1 to VCN 4 each as reference points for determining average VCN produces coefficient of variation of 2.76%, 1.62%, 2.48% and 4.04%; and percent error of 2.17%, 0.43%, 0.05% and 5.56% respectively across all combinations across runs.

Tapestri VCN results correlates well with orthogonal ddPCR technology



	Tapestri	ddPCR
VCN0	0.00 ± 0.00	0.00 ± 0.00
VCN1	1.00 ± 0.00	1.00 ± 0.03
VCN2	1.96 ± 0.03	2.01 ± 0.06
VCN3	2.87 ± 0.10	2.84 ± 0.17
VCN4	3.96 ± 0.12	4.34 ± 0.02
Mixture 1	1.84 ± 0.12	1.64 ± 0.09
Mixture 2	3.11 ± 0.16	2.93 ± 0.12
Mixture 3	1.66 ± 0.02	1.61 ± 0.03

Figure 4: Tapestri vs. ddPCR average VCN correlation

X-Y scatter plot of the average VCN for each sample called using Tapestri VCN caller vs. ddPCR measurement. Pure cell lines (VCN 0-4) or mixtures of cell lines (Mixes 1-3) were analyzed in triplicate by both Tapestri and ddPCR. The sample's average VCN was then calculate based on single-cell level VCN (y-axis) and plotted against orthogonal ddPCR measurements (Bio Rad QX200, x-axis, n=3). The average copy number of the population estimated using Tapestri correlates well with ddPCR measurements (R^2= 0.99). The average and standard deviation of replicate measurements (n=3) are shown in the bottom right table.

Transduction efficiency can be detected with high sensitivity and specificity

Figure 5: Transduction efficiency sensitivity and specificity

Tapestri also provides vector amplicon specific measurements that can be used to determine transduction efficiency. A mixture of non-transduced Raji and vector containing Jurkat cells was used for the study. The sample transduction efficiency was calculated based on detection of vector amplicon reads and validated using the assignment from SNPs specific to Raji (negative) and Jurkat (positive) cell lines. The Tapestri VCN can detect transduction efficiency with >99.6% and >99.9% median specificity and sensitivity.

Median 99.9% sensitivity 99.6% specificity

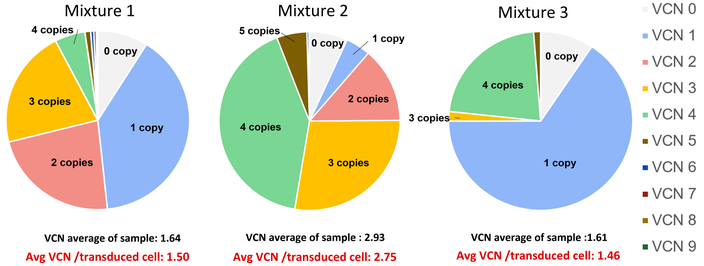
Positive Jurkat cells with ≥ 1 VCN

Negative Raji cells with no vector

	Transduced	Not transduced
Detected transduction	77,654 cells	25 cells
No transduction detected	288 cells	4,136 cells

Single-cell level Vector Copy Number distribution characterization

Figure 6: Single-cell VCN distribution per sample



VCN average of sample: 1.64
Avg VCN /transduced cell: 1.50

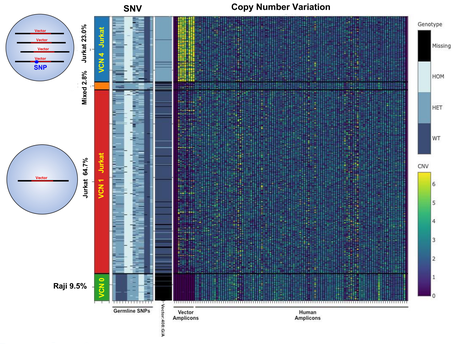
VCN average of sample : 2.93
Avg VCN /transduced cell: 2.75

VCN average of sample :1.61
Avg VCN /transduced cell: 1.46

Besides the measurement of average VCN per sample, Tapestri VCN caller calculates VCN distribution of a particular sample at single-cell level. The pie charts show the VCN distribution calculated for each of the three mixtures, Mix1, Mix2, and Mix3 each with ~10% non-vector Raji cell spiked-in. Note that "Mixture 1" (VCN 1-4's respective proportions = 39.4%, 22.8%, 21.3%, 5.4%) and "Mixture 3" (VCN 1-4's respective proportions = 65.6%, 0%, 1.7%, 22.1%) VCN distributions are drastically different despite having similar average populational VCN value of 1.64 and 1.61. Besides the measurement of average VCN per sample, Tapestri VCN caller calculates VCN distribution of a particular sample at single-cell level. The pie charts show the VCN distribution calculated for each of the three mixtures, Mix1, Mix2, and Mix3 each with ~10% non-vector Raji cell spiked-in. Note that "Mixture 1" (VCN 1-4's respective proportions = 39.4%, 22.8%, 21.3%, 5.4%) and "Mixture 3" (VCN 1-4's respective proportions = 65.6%, 0%, 1.7%, 22.1%) VCN distributions are drastically different despite having similar average populational VCN value of 1.64 and 1.61. Furthermore, Tapestri also provides the average VCN per transduced cell (bottom of each pie chart in red) due to the ability to measure transduction % and VCN distribution.

Single-cell genotype, SNPs, and transgene vector characterization

Figure 9: Heat-map of genotype, VCN and vector sequence



Tapestri amplicon based single-cell DNA sequencing provides the capability to analyze the genomic landscapes of regions of interest at single base resolution. The heat-map to the right shows genotyping of cells (Raji vs. Jurkat) based on unique SNPs using human amplicons. The VCN caller determines the VCN 1 vs. VCN 4 based on the read out from vector amplicons while also providing sequencing data on each amplicon. Here, Tapestri identifies a spontaneous mutation occurring in one out of four copies of the transgene vector at position 408:G/A in the VCN 4 clonal cell line, whereas VCN 1 cells contain only the expected vector sequence. Tapestri not only determines the vector copy number, but also provides sequence integrity information of transduced vector, which directly correlates with the functionality of the transgene.

Conclusion

Using the Mission Bio Tapestri single-cell DNA sequencing platform, this study shows a consistent and reliable assay for in-depth quantification of cell and gene therapy transduction efficiency, single-cell vector copy number distribution, and populational average (or, VCN per transduced cell). The single-cell level VCN distribution, as well as single-cell vector sequence validation provides unprecedented resolution and insight to assess the potential functional efficacy and safety for CGT products. Together, these characteristics and data demonstrate the potential to accelerate and streamline both the development and release testing of cell and gene therapy products.

Reference

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