Quantification of Single Cell Vector Copy Number in CAR T Cell Products Utilizing a Novel Microfluidic Technology

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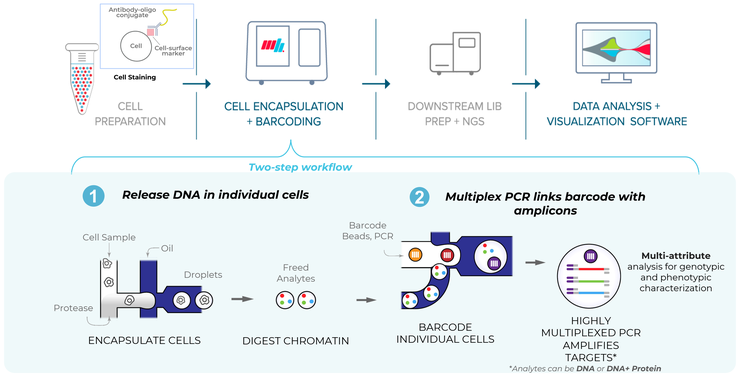
Introduction

Chimeric antigen receptor T-Cell (CAR-T) immuno-therapies have been transformative solutions to treat cancer patients. As most CAR-T therapies rely on the introduction of CAR into host cells using lentiviral vectors followed by re-introducing the modified T-cell back into patients, the quality of CAR-T is extensively regulated. Key safety and efficacy attributes such as transduction efficiency and transgene copy number, or viral vector copy number (VCN), needs to be accurately measured. Yet conventional methods for measuring gene transfer lack the resolution and representation to truly reflect sample composition and either report a population average (bulk) or involve laborious and time-consuming clonal outgrowth. Mission Bio has developed an end-to-end solution from panel design to data analysis for single-cell targeted DNA sequencing to interrogate transgenes. 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 measures 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. In addition, utilizing our oligo conjugated antibody system, we demonstrate **single-cell multi-omics** characterization of protein expression (functional) and transgene (genomic) information of CAR products.

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 process. 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



Validated (ddPCR BioRad Qx200) lentiviral transduced Jurkat clonal cell lines with bi-cistronic CD19/CD22 dual CAR vector copy number of 0 and 2 were used in this study. A custom panel of amplicons was designed to enable the vector copy number analysis (Figure 2). Pure clonal cell lines of VCN 0, and 2, plus two mixtures (50:50 and 25:75 VCN0:VCN2) with non-transduced GM12878 cell (NIST) spike-in was processed for single-cell sequencing using Mission Bio's Tapestri platform in triplicates. For calculating VCN, Tapestri data from one of the VCN2 run was used as control reference (known-truth). All samples were analyzed with Mission Bio's analysis tools to produce percent transduction, single-cell level VCN calls, as well as population VCN average (VCN/cell, or VCN per transduced cell) for each sample. For single-cell multi-omics analysis, PBMC and VCN2 samples were stained with a 45-plex oligo conjugated antibodies (Total Seq-D, Heme Oncology panel, BioLegend) followed by Tapestri workflow. An average of 6,893 cells were analyzed for each Tapestri sample.

CAR vector and assay design background

All validation work was performed on the novel bi-cistronic CD19 X CD22 CAR T cell construct developed in the laboratory of Dr. Terry Fry, Dr. Lindsey Murphy and Dr. Amanda Winters. This construct is currently in Phase I clinical trials for the treatment of relapsed/refractory (R/R) non-Hodgkin lymphomas in the adult population, with Phase I trials opening soon for the treatment of R/R pediatric acute lymphoblastic leukemia. Targeted assays of ~200bp amplicons were designed to interrogate CAR (purple) and the human genome for vector copy number and transgene detection. In addition, SNV information from human amplicons is used for differentiation of cells from different individuals. The orange regions indicate ddPCR assay locations.

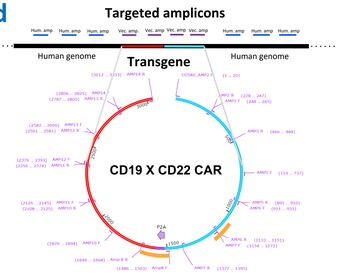


Figure 2: Lentiviral transduced CAR and assay design

High sensitivity, specificity, accuracy and precision

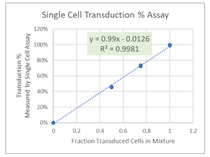
Tapestri provides vector amplicon specific measurements that can be used to determine transduction efficiency. Mixtures of non-transduced GM12878, Jurkat cells (VCN0) and vector containing Jurkat cells (VCN2) were used for this study. Cells were genotyped based on SNVs in human amplicons. Each cell's transduction status was characterized based on the presence or absence of vector amplicons in that cell. The Tapestri-based percent transduction assay has 98.8% specificity and 99.2% sensitivity (Figure 3).

Figure 3: Transduction % Assay Sensitivity and Specificity

99.2% sensivity 98.8% specificity	VCN 2 Jurkat	VCN 0 Jurkat	VCN 0 GM12878
Expected / Measured	Transduced	Not transduced	Not transduced
Detected Transduction	20,093 cells	0 cells	5 cells
No transduction detected	164 cells	9,505 cells	428 cells

Figure 4: Transduction % Assay Precision and Linearity

Cell Mixture			
Non-Transduced	Transduced	Transduction %	%CV
100%	0%	0	nan
100%	0%	0	
100%	0%	0	
50%	50%	45.91%	1.3%
50%	50%	45.19%	
50%	50%	46.34%	
25%	75%	73.48%	0.9%
25%	75%	72.13%	
25%	75%	72.74%	
0%	100%	98.83%	0.4%
0%	100%	99.53%	
0%	100%	98.94%	



The single cell Transduction % Assay was performed on admixtures of non-transduced and transduced (VCN=2) Jurkat cells in triplicate. Figure 4 (left) shows the resulting Transduction % measured for each admixture for each Tapestri run. The %CV is extremely low, demonstrating the excellent precision of this assay. Figure 4 (right) plots the measured Transduction % vs the fraction of transduced cells in the admixture. The R-squared of 0.99 demonstrates the excellent linearity of the assay.

Tapestri VCN correlates with orthogonal ddPCR measurements

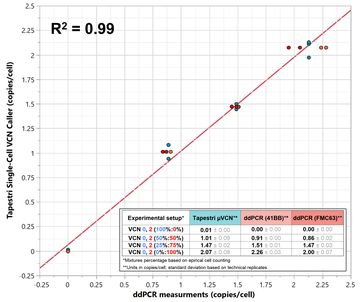
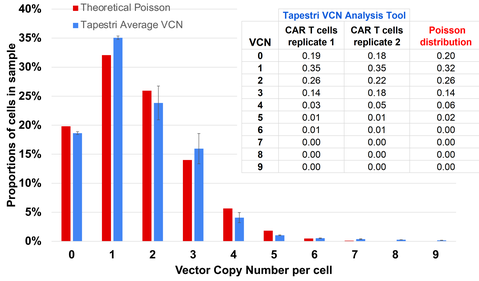


Figure 5: Tapestri vs. ddPCR VCN measurements

Figure 5 shows the X-Y scatter plot of the average VCN for each sample called using Tapestri VCN caller vs. ddPCR measurement (average of two separated ddPCR probe assays targeting 41BB and FMC63 region; albumin as reference; BioRad QX200, n=2 for each) performed on the same admixture samples. The average copy number measured with Tapestri correlates well to ddPCR measurements (R^2= 0.99). The average and standard deviation of replicate measurements (n=2-3) are shown in the bottom right table. Note that the sample average VCN measured by two separate ddPCR assay may not necessarily agree with each other based on assay dependent performance and the degree of optimization.

Single-cell analysis provides vector copy number distribution

Figue 6: VCN distribution in CAR T sample



Tapestri VCN Analysis Tool

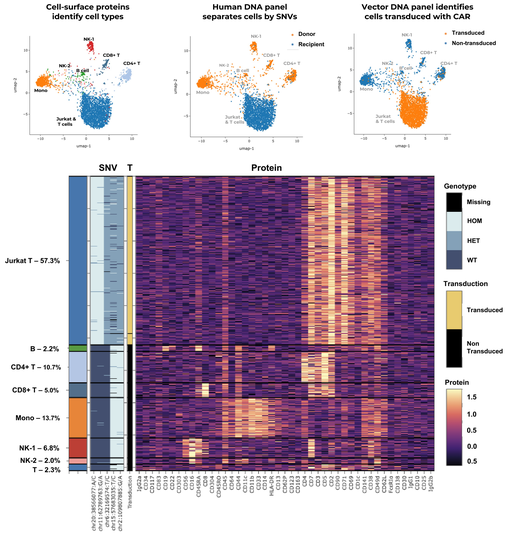
VCN	CAR T cells replicate 1	CAR T cells replicate 2	Poisson distribution
0	0.19	0.18	0.20
1	0.35	0.35	0.32
2	0.26	0.22	0.26
3	0.14	0.18	0.14
4	0.03	0.05	0.06
5	0.01	0.01	0.02
6	0.01	0.01	0.00
7	0.00	0.00	0.00
8	0.00	0.00	0.00
9	0.00	0.00	0.00

The Transduction % and VCN assays were used to analyze lentiviral transduced CAR-T drug product. Average VCN can be reported for both the entire cell population (Avg VCN of **1.62** copies per cell), analogous to the ddPCR population average, or as **VCN per transduced cell** (**1.99** copies per transduced cell). In addition, the Tapestri VCN analysis tool estimates the VCN distribution of a sample at single-cell level (Figure 6). The bar-graph shows the VCN distribution for CAR T product post transduction from VCN 1 to VCN 9. The CAR T product VCN distribution (blue) follows a Poisson distribution (red, μ at **1.62**, correlation R^2 ~ 0.98). The table displays results from two replicates of Tapestri and the theoretical Poisson values.

Single-cell protein + DNA analysis can reveal characteristics of patient vs donor CAR+ Cells

Figure 7: Single-cell Protein + DNA clustering

Protein + DNA multi-omic analysis was performed on a mixture of clonal Jurkat cell line (with CD19 X CD22 CAR vector) plus PBMCs (separate donor, vector null) as background, at 3:2 ratio. Assays include VCN panel (identifies CAR transduction %, VCN); a 99-plex human DNA panel (determines unique SNVs); and a 45-plex cell surface protein panel[1]. Data is clustered based on cells' protein expression (immuno- phenotype). From the UMAP, one can cluster cell types by immunophenotype. The heatmap (each row is a cell) showcases the potential of single-cell multi-omics analysis in combination with VCN assay. SNVs and protein markers infer, quantitatively, the sample makeup of different cell lineages and immuno-phenotypes, as well as identify donor (Jurkat) from recipients (PBMCs). The VCN assay measures critical quality attributes (CQAs) of the drug product (transduction%, VCN), providing both genotypic and functional information. Future studies will evaluate the utility of this technology in measuring both CAR transgene copies and surface expression/density of the CAR at the single cell level to provide much needed single-cell level resolution of CAR T cell drug product attributes.



Conclusion

Here we demonstrate multi-omic, single-cell quantification of transduction efficiency, vector copy number distribution, population average VCN (and avg VCN per transduced cells), and immunophenotyping. The single-cell level VCN analysis provides unprecedented resolution and insights to assess the potential functional efficacy and safety for CAR-T therapy, as well as a way to better understand the biology of these therapies. The Mission Bio Tapestri single-cell multi-omics platform can accelerate and streamline both the development and release testing of engineered cell drug products.

Reference 1. https://missionbio.com/products/panels/totalseq-d-heme-oncology/