Leveraging Single-cell DNA Sequencing for In-depth Characterization of Cell and Gene Therapies

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Abstract

The development of cell and gene therapies is rapidly transforming the treatment landscape for a host of intractable diseases. CRISPR continues to be a powerful tool for the development of novel therapies. Despite its ability to precisely alter the genome, CRISPR yields heterogeneous editing outcomes. Cell-to-cell variation in on- and off-target edits and the generation of rare chromosomal alterations can impact the safety and efficacy of therapeutic agents. For this reason, protocol optimization and in-depth characterization are critical to successfully bringing new products to market. Conventional sequencing technologies that analyze CRISPR-edited cells often report bulk measurements, an approach that fails to measure the zygosity and co-occurrence of alterations within individual cells. Analysis of clonal populations provides this information but can add weeks to experimental timelines. Here, we demonstrate that single-cell DNA sequencing on Mission Bio's Tapestri Platform provides sensitive and nuanced quantification of genetic edits made to up to 10,000 individual cells with a simple workflow.

Introduction

Gene editing tools like CRISPR have revolutionized how we manipulate DNA and have subsequently led to advances in cell and gene therapies. Yet, gene editing yields heterogeneous populations where some cells may have all desired edits while others do not. Moreover, cells may vary in undesirable outcomes like off-targets and chromosomal aberrations.

The heterogeneity of gene-modified cells is important to understand in order to ensure the efficacy and safety of therapeutic products. For instance, if a small percentage of cells have an off-target effect that is oncogenic, even a few cells could potentially cause cancer in a recipient patient.

Most conventional methods of measuring gene editing outcomes are bulk analyses that report only population averages. Moreover, often analytical analyses require multiple assays that are conducted separately on different instruments, requiring data to be integrated from different cell samples.

Mission Bio's Tapestri Platform, however, can measure **multiple genotypic attributes simultaneously in individual cells in a single assay (Fig 1)**. This allows for comprehensive characterization of cells as well as the rapid optimization of gene editing protocols.

Methodology

Genome Editing:

1: Three genes (HBB, CLTA and RAB11A) were edited with CRISPR/Cas9 in a cancer cell line in partnership with Agilent Technologies. Single-cell DNA sequencing quantified all on- and predicted off-target events for the three genes in the multiplexing CRISPR experiment.

2: Stimulation of human PBMCs, followed by lentiviral transduction (Cas9) and electroporation (sgRNA targeting 3 loci), was performed using standard protocols. After culturing cells for at least 5 days post-editing, cells were enriched for primary T cells (>95%), cryopreserved, and then analyzed on Tapestri.

Tapestri Analysis:

The Tapestri Platform is an end-to-end solution for single-cell DNA sequencings (Fig 2). Cells are first encapsulated with a protease, which exposes the DNA. Following lysate preparation, the proteases are inactivated via heat denaturation on a thermal cycler. Individual cell lysates are returned to the same cartridge on Tapestri and then partitioned with barcoded beads, multiplex-PCR primers, and reagents to target specific regions of the genome.

A Custom Tapestri Single-Cell DNA Panel with 20 amplicons that are designed to target 3 on-target sites and 3 known off-target sites was used for each experiment. Four negative control sites are also included to determine false positive rates and are defined as neither on-target nor known off-target sites.

Additionally, the panel includes 10 amplicons to assess allele dropout rate. These amplicons target SNVs known to be highly polymorphic across a variety of populations. After barcode incorporation and target amplification were complete, library amplification, purification, and quantification was performed prior to sequencing on Illumina platforms.

Fastq files were processed through Tapestri Pipeline Software to group reads by the cell barcode, thereby reconstructing the mutational profile for each cell. Thereafter, custom analysis was performed to precisely determine the distribution and frequency of on-/off-target edits, zygosity, co-occurrence, and translocations in the pool of cells.

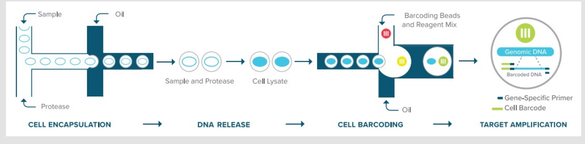
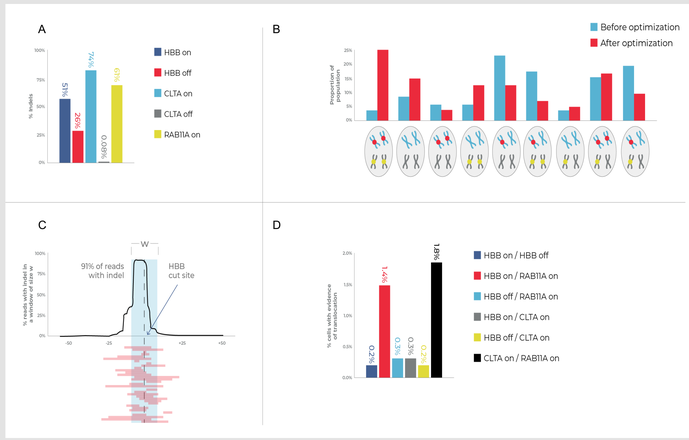


Figure 2. The Tapestri microfluidic workflow involves cell encapsulation, the release of DNA, and barcoding and amplification of genetic targets.

Results

1: Single-cell analysis also revealed proportions of cells with different editing frequencies across the HBB, CLTA and RAB11A on- target sites as well as predicted off-target sites (Fig 3A). For instance, predicted off-target edits for CLTA were low (0.08%), whereas they were higher for HBB (26%). multiple loci (Fig 3B).



Quantifying multiple editing events for several genes in the same assay allows researchers to select gRNAs and optimize conditions that increase the proportion of on-to-off-target edits for all the genetic targets simultaneously. These data also enabled the optimization of editing protocols to increase the frequency of homozygous edits (Fig 3B).The indels for each edit were identified (Fig 3C), as well as rare (<0.5-1.5%) translocations across on- and off-target editing sites (Fig 3D).

Figure 3. (A) Simultaneous measurement of on- and predicted off-target edits, (B) co-occurrence/ zygosity, (C), indels, and (D) translocations in cells with co-edited targets: HBB, CLTA and RAB11A.

2: On-target editing ranged from ~12% to 61% (Fig 4A). With 3 on-target sites, each with 3 possible edit results, there were 26 edit combinations and 1 without any edits. In a single experiment, Tapestri revealed the distribution of the zygosity and co-occurrence of the edits and resolved every possible on-target edit combination (Fig 4B).

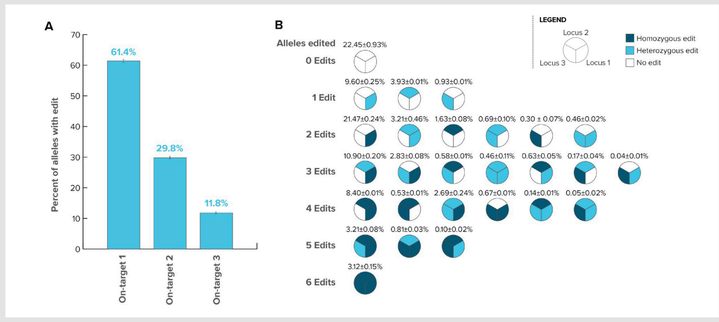


Figure 4. (A) Pseudo-bulk measurement of 3 on-target editing frequencies, (B) frequencies of all 27 editing outcomes assessed by Tapestri. Note that ~3% of cells had all 6 edits (homozygous edits at all 3 loci)

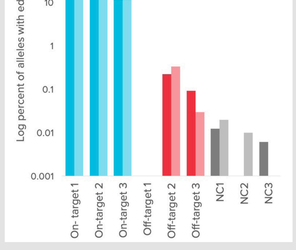


Figure 5. Frequencies of edits at known off-target sites, negative control sites, and on-target sites in the same experiment. NC= Negative control. 1 of 4 NCs not shown.

Tapestri revealed known off-target edits at ~0.1% and mutations at negative control sites at ~0.01% while simultaneously detecting high on-target frequencies (Fig 5). The single-cell analysis of edited cells enabled the optimization of CRISPR protocols to bias homozygous editing at all 3 target loci. The frequency of cells with desired homozygous edits at all 3 loci was increased from 3% to ~50% (Fig 6A-B, respectively).

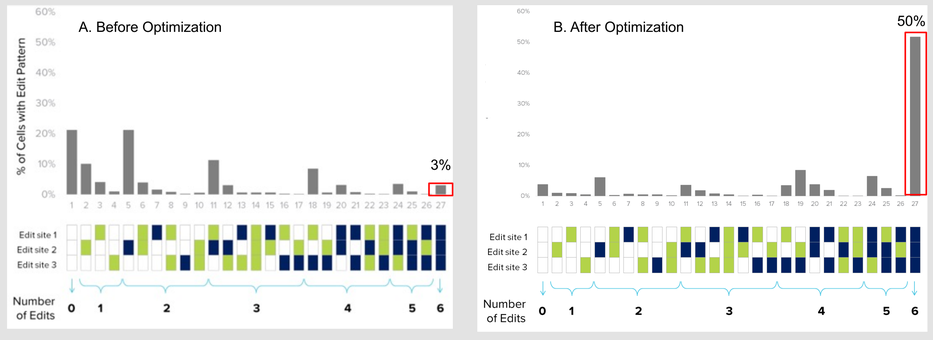


Figure 6. Percent cells with different editing patters before (A) and after (B) optimization. Red boxes denote the frequency of cells with homozygous edits at all 3 targeted loci.

Objectives

Here, we demonstrate several capabilities of the Tapestri Platform:

1: The ability to co-measure on-target editing (3 targets), predicted off-target editing, zygosity of edits, and translocations in the same cells.

2: Assess all combinations of multiplex editing (3 targets) at a single-cell level so that CRISPR protocols can be optimized.

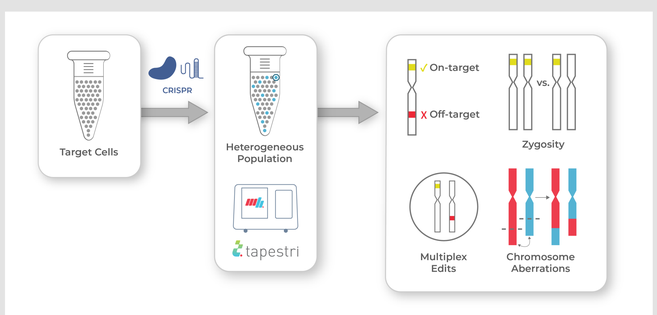


Figure 1. Tapestri can be used to characterize the heterogeneity of 1000s of gene-edited cells, including the simultaneous measurement of multiple attributes at single-cell resolution.

Conclusion

- The Tapestri Platform enables high-throughput analysis of gene-edited cells, reporting cell-specific attributes that conventional bulk NGS assays do not.
- We demonstrate that Tapestri reliably co-measures on-/ off-targets, zygosity, edit co-occurrence, and translocations at single-cell resolution in a single assay.
- Single- cell analysis enables the rapid optimization of CRISPR protocols and provides rich characterization of cell-based therapeutics to ensure safety and efficacy.

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