Comprehensive On- and Off-target Confirmation Using Integrated rhAmpSeq™ and Targeted DNA Sequencing Single-Cell Technology

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Introduction

Advances in genome editing have enabled precise correction of mutations and engineering of cells with therapeutic functions. A key factor in successful CRISPR editing is assay specificity, where guide RNAs (gRNAs) direct accurate edits with minimal off-target effects. Amplicon-based targeted resequencing is the gold standard for validating edits due to its high sensitivity, multiplexing capability, and cost-effectiveness. Innovations like rhAmpSeq™ improve bulk validation by reducing background noise in multiplexed PCR through optimized primer chemistry. Given that cells are the functional units of gene editing, it is crucial to assess edit outcomes and genotoxicity at the single-cell level. Droplet-based single-cell DNA resequencing offers insights into zygosity, edit co-occurrence, and clonality. In this study, we integrated rhAmpSeq™ with droplet-based single-cell assays (Tapestri platform) to create a flexible workflow for single-cell resolution validation. This approach—encompassing rhAmpSeq™ panel design, single-cell chemistry, and the Tapestri Genome Editing (GE) Pipeline—enables detailed, accurate analysis of gene editing outcomes and potential malignant events.

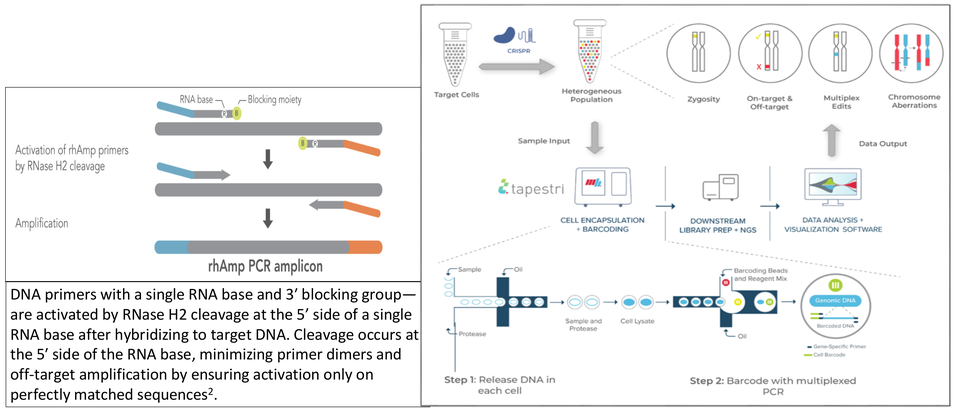
Aim

Proof of concept study of integrating IDT's rhAmpSeq™ targeted sequencing chemistry into Mission Bio Tapestri single-cell DNA sequencing platform

Methods

- Two well-characterized cell lines, GM24385(son, HG002) and GM24149 (father, HG003) with targeting loci that are heterozygous and wild type, respectively, were selected. These have het variants and indels ranging from 1 bp to > 2000 bp.
- The IDT rhAmpSeq™ Design Tool was used to design a 37- plex panel containing a single RNA base and a blocker at the 3'end (rhAmpSeq™ primers) and the corresponding primers without the RNA base and blocker (unblocked primers).
- Cell line mixtures of varying ratios were run through the bulk and Tapestri single-cell system using rhAmpSeq™ primers; RNAse H2 was added to the PCR mix during barcoding step.
- From an average of 10,000 cells per sample captured, raw sequencing data was analyzed using automated Mission Bio proprietary analytical pipeline.
- The number of wt, het and hom variants were determined for each of the cell line mixes.

Integration of rhAmpSeq™ and Single Cell Tapestri Technologies

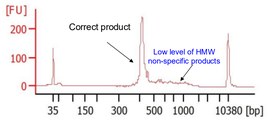


DNA primers with a single RNA base and 3' blocking group—are activated by RNase H2 cleavage at the 5' side of a single RNA base after hybridizing to target DNA. Cleavage occurs at the 5' side of the RNA base, minimizing primer dimers and off-target amplification by ensuring activation only on perfectly matched sequences[2].

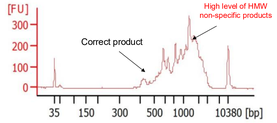
Fig 1: The single-cell rhAmpSeq™ workflow begins with a custom panel design targeting on- and off-target sites via IDT rhAmpSeq™ Design tool. Panels can be used for bulk DNA or single-cell analysis with the Tapestri® platform, which uses droplet microfluidics to process up to 100,000 cells. Data is analyzed with the DNA Variant Pipeline Caller, revealing on/off-target co-occurrence, zygosity, per-cell/allele edits, and clonality

rhAmpSeq™ chemistry minimizes primer dimers and non-specific amplification in bulk and Single-Cell Tapestri PCR

a. BioAs of rhAmpSeq™ libraries w/ rhAmpSeq™ primers (bulk DNA amplification)



b. BioAs of conventional libraries with unblocked primers (bulk DNA amplification)



c. Overlay of BioAs of single-cell Tapestri libraries with rhAmpSeq™ primers and unblocked primers

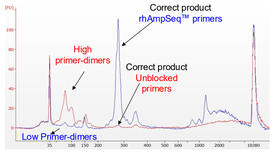
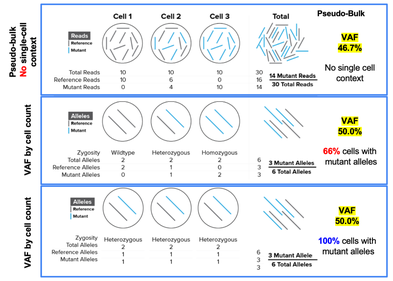


Fig 2: To assess rhAmpSeq™ primer performance, we compared two identical panels—one with rhAmpSeq™ primers and one with unblocked primers—under identical PCR conditions. In bulk DNA assays, rhAmpSeq™ primers greatly reduced long non-specific products (Fig. 2a, 2b). In single-cell droplet reactions, they also minimized short non-specific products like primer dimers (Fig. 2c).

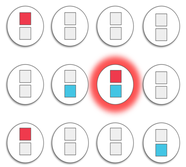
Reference

1. Kalter, Nechama et al. Precise measurement of CRISPR genome editing outcomes through single-cell DNA sequencing. Molecular Therapy Methods & Clinical Development, Volume 33, Issue 2, 101449: https://doi.org/10.1016/j.omtm.2025.101449
2. PCR with RNase H (rhAmp™) PCR: https://www.idtdna.com/pages/technology/qpcr-and-pcr/rhamp-pcr

Tapestri DNA Variant Pipeline determines per cell genotype information



Characterizing single cells – the basic editing biological unit



Example: Total 12 cells edited at two loci

3 cells (25%) harbor Het red allele
3 cells (25%) harbor Het blue allele
7 cells (58%) harbor WT allele
Only 1 cell (8.3%) harbor co-occurring Het red + Het blue alleles

Fig 3: The Tapestri DNA Variant Pipeline Caller enables analysis of editing outcomes at single-cell resolution. The top-left panel shows a "pseudo-bulk" view, aggregating all reads across cells to mimic bulk VAF analysis. In contrast, the bottom panels reveal single-cell genotypes, distinguishing between partially and fully edited populations despite similar overall VAFs. Such single-cell insights are critical in CGT, where understanding zygosity and edit co-occurrence at the cellular level is essential.

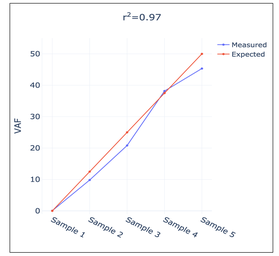
rhAmpSeq™ single-cell workflow characterizes per-cell zygosity in mixed cell study

a) Samples details

Cell mixtures	GM24385 (WT)	GM24149 (WT)	Expected Average VAF	Measured Average VAF*	Expected % cells w/ WT+	Measured % cells w/ WT+
Sample 1	0%	100%	0.0%	0.0%	0%	0%
Sample 2	25%	75%	12.5%	9.8%	25%	18%
Sample 3	50%	50%	25.0%	20.8%	50%	39%
Sample 4	75%	25%	37.5%	38.2%	75%	74%
Sample 5	100%	0%	50.0%	45.3%	100%	85%

* average of all variants

b) Per-variant sample Average VAF



c) Per-variant sample per-cell zygosity

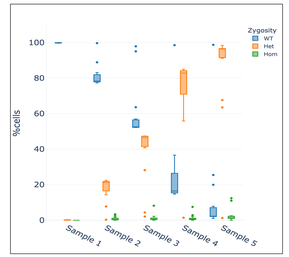


Fig 4: To validate the rhAmpSeq™ single-cell workflow, we used two well-characterized cell lines: GM24385 and GM24149 . Details of the sample mixtures are provided in Fig 4a. Fig 4b shows the average variant allele frequency (VAF) and Fig 4c shows the per-cell zygosity (i.e., the number of cells harboring heterozygous calls) aligning with expectations[2].

Conclusions

- IDT rhAmpSeq™ has been seamlessly integrated with the single-cell Tapestri platform without requiring modifications to either system.
- IDT rhAmpSeq™ primer panels significantly reduced non-specific products and primer-dimers in both bulk and single-cell Tapestri workflows.
- Per-cell zygosity and average VAF closely matched expected values based on known cell mixture proportions.
- Testing and confirmation of large indels with the Tapestri DNA pipeline is in progress to further expand the scope of the rhAmpSeq™ on Tapestri platform.
- This workflow merges rhAmpSeq™ and single-cell sequencing to offer an efficient, flexible approach to enable simultaneous measurement of on-target and off-target edits, including their zygosity, across thousands of cells.