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Analytical Performance of a Novel Long-Read RNA Sequencing Assay for Low-Abundance Cancer Transcript Detection from Whole Blood

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Background

- Advances in liquid biopsy technologies have the potential to transform cancer diagnosis and management by enabling minimally invasive and real-time monitoring of tumor biology.
- Circulating tumor nucleic acids, including circulating tumor RNA (ctRNA), provide a rich source of information on gene expression, tumor heterogeneity, and dynamic responses to therapy.
- However, ctRNA molecules are present at very low abundance in blood, exhibit high variability in transcript length and isoform composition, and are masked by a large background of non-tumor RNA, creating substantial technical challenges for their detection and analysis.
- Here, we report the analytical performance of our original method[1], which combines long-read RNA sequencing with proprietary Level-Up technologies to enable sensitive detection of full-length RNA molecules from whole blood.
- We also describe the further development of this method into two dedicated platforms, optimized for either a broad RNA size range or specifically for profiling long RNA molecules.
- These platforms uncover biologically and clinically relevant insights, including protein isoforms in key cancer genes, offering enhanced opportunities for cancer diagnostics and supporting target discovery for precision medicine.

Methods

- Whole blood was used instead of plasma or serum to capture RNA from different cell types and larger extracellular vesicles. 2.5ml of blood was collected in PAXgene Blood RNA Tubes (Qiagen) and stored at -80°C until processing (Fig. 1).
- Total RNA was extracted from the blood and converted into full-length cDNA. Three methods involving different cDNA normalization and selection conditions were used to generate cDNA libraries with desired characteristics. These include our original platform[1] and two new platforms optimised for detecting a wide range of transcript sizes (referred to as “comprehensive” platform hereinafter) and for profiling long RNA molecules (“long-range” platform), respectively.
- Resulting cDNA libraries were sequenced using a long-read sequencing system from Oxford Nanopore Technologies to ensure the accurate annotation of full-length structures of complex transcript variants.
- The data was then analyzed using the TAMA software suite[2] (Wobble Genomics) and adapted pipelines with parameters tuned for the requirements of each platform.
- Analytical performance of the original platform was assessed using 44 replicates derived from three blood tubes per patient across two individuals. Preliminary evaluation of the comprehensive platform was based on six replicates from a single patient. Analytical performance of the long-range platform is under evaluation.
- Spike-in RNAs (SIRV-Set 4; Lexogen), which are synthetic RNA controls of known sequence and concentration, were added to the extracted RNA samples to enable estimation of the limit of detection and measurement precision for RNA molecules.

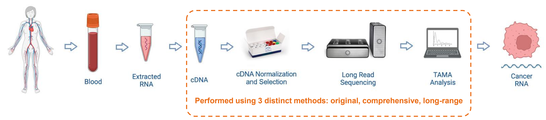

Fig. 1 Overview of the sample analysis platform

Enhanced Liquid Biopsy Platform for Sensitive Detection of Low-Abundance RNA

Limit of Detection (LOD)

- LOD was evaluated for the original and comprehensive platforms, defined as the lowest concentration (measured in parts per million or ppm) at which all replicates yielded a positive result.
- Analyses were performed on transcripts longer than 800 bp, a range inaccessible for isoform-level characterization using short-read sequencing.
- Optimization led to a 10-fold improvement in LOD for the comprehensive platform compared to the original (Fig. 2a).
- Both platforms showed low LODs (original: 0.12 ppm, comprehensive: 0.015 ppm), outperforming many industry-standard assays (typically 1–1000 ppm)[3-6].

Precision

- Precision was assessed by calculating the coefficient of variation (CV) of the observed concentrations of low-abundance (10 ppm) spike-in RNA molecules.
- The comprehensive platform showed improved precision relative to the original across all assessed RNA size ranges (Fig. 2b).
- The precision of both evaluated platforms is comparable to that of industry-standard assays (Fig. 2b)[3,4,7,8].

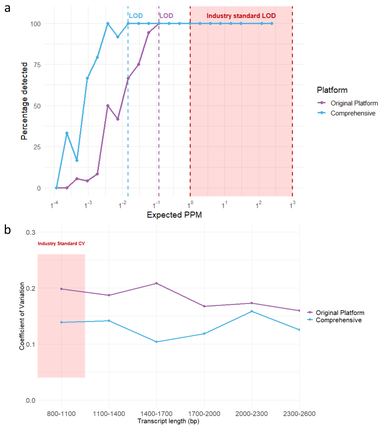

Fig. 2 Platform analytical performance. a) Transcript expression and LOD of transcripts >800bp; b) coefficient of variation by transcript length.

Limit of Blank (LOB)

- In the context of broad and complex gene expression backgrounds in blood derived from whole-body tissues, profiling efforts are ongoing to characterize non-cancerous transcriptomes. Meanwhile, to estimate the LOB for our platforms, we used an all-female sample set (n = 200) and searched for 29 Y-chromosome-specific transcripts as a proxy.
- This set of transcripts is representative of the human transcriptome across multiple characteristics, encompassing a range of transcript lengths (444–7093 bp), protein lengths (106–496 aa), GC content (37–58%), and exon counts (1–15).
- None of the Y-specific transcripts was detected in any of the 200 samples, indicating an LOB of zero using this contrived method.

New Platforms Enable Superior Detection of Clinically Relevant Transcripts

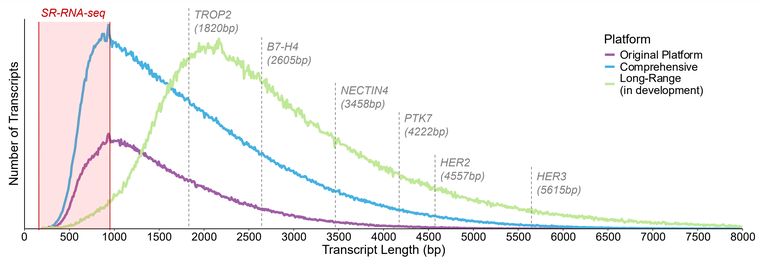

Fig. 3 Transcript length distributions from three platforms, normalized by sequencing depth. Red shaded region shows typical range of transcripts that can be accurately characterized by short-read RNA-seq. Examples of cancer genes relevant to drug development and their canonical transcript lengths are shown in grey.

- Our platforms offer a distinct advantage over short-read RNA-seq methods, which are typically limited to generating reads of 150-300 bp in length and less accurate at detecting and characterizing transcripts greater than 1kb in length.
- Comparison of transcriptome profiles generated using the three platforms from the same sample revealed pronounced differences in transcript detection capability (Fig. 3):
  - The comprehensive platform detected 2.8 times as many transcripts as the original method.
  - The long-range platform demonstrated superior detection performance for transcripts longer than 1.8 kb, including many clinically relevant cancer genes (e.g. *HER2*, *TROP2*, *HER3*, *B7-H4*, *NECTIN4*, *PTK7*).

Long-Range Platform Provides New Insights into Key Cancer Genes

- Antibody drug conjugates (ADC) represent a significant advancement in breast cancer therapy, combining cytotoxic payloads with the target specificity of an antibody.
- However, tumor resistance, off-target toxicity, and drug delivery issues can limit ADC safety and effectiveness[9].
- Our platforms, which enable accurate full-length RNA profiling, provide the capability to more comprehensively elucidate the repertoire of alternative isoforms of ADC target genes.

Example 1: Novel HER2 isoform

- We identified a novel putatively protein-coding isoform of HER2 in breast cancer patients with a unique transcription start site and additional exon (Fig. 4).
- The additional exon introduces a potential new epitope in domain IV that may disrupt the trastuzumab (T-DXd) binding site, while the loss of domain I in the protein structure could further affect drug interactions.

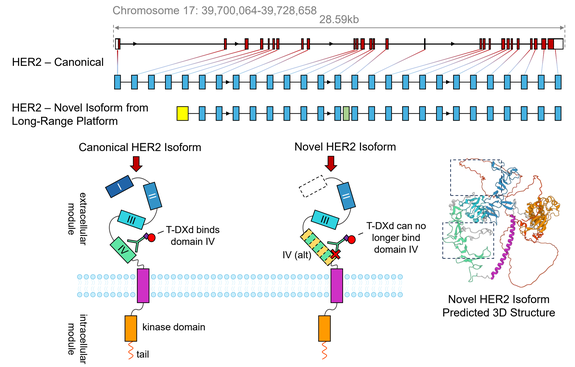

Fig. 4 Example of a novel HER2 isoform containing structural variations affecting the protein structure.

Example 2: Novel TROP2 isoform

- Our platform revealed significant splicing diversity within the TROP2 coding region in breast cancer patients, which could affect reactivity to ADCs such as sacituzumab (Fig. 5).
- Additionally, structural alterations in these novel isoforms may affect antibody binding in immunohistochemistry (IHC) assays, potentially reducing the accuracy of IHC as a predictor of ADC efficacy[10].
- These results highlight the utility of our platform for isoform discovery, enabling more effective ADC development and more accurate patient stratification.

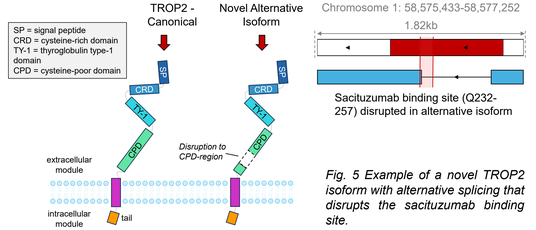

Fig. 5 Example of a novel TROP2 isoform with alternative splicing that disrupts the sacituzumab binding site.

Conclusions

- This study establishes the feasibility and analytical robustness of our method for detecting low-abundance RNA in blood, supporting its application in liquid biopsy.
- The further development of the method into two specialized platforms has allowed for sensitive detection across a broad RNA size spectrum, enabling accurate profiling of both short and long RNA species.
- Collectively, these platforms offer a novel powerful, adaptable tool for transcriptome-level cancer diagnostics, monitoring, and precision medicine development.

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