

Eliminating Bias in the Characterization of Small RNAs Derived from Extracellular Vesicles

Small-RNA Profiling from Cell-free Fluids

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Extracellular vesicles (EV), are membrane-bound compartments which participate in cell-to-cell communication. They are secreted by most cell types and are present in many and perhaps all body fluids, including plasma. The most intensely studied subtype of EV are exosomes. Approximately 50-200 nm in size, the EV transport a cargo of different molecules including small RNAs that are being used as biomarkers in cardiovascular diseases, cancer, neurological disorders, etc¹⁻⁷.

Small RNA sequencing is commonly used to characterize the content of the EV. Despite obvious advantages in relation to qPCR or microarray analysis, sequencing has its own set of challenges. The most notorious challenge is ligation bias which skews small RNA profiles in various sample types and input ranges⁸.

In the publication, Assessment of Methods for Serum Extracellular Vesicle Small RNA Sequencing to Support Biomarker Development⁹, the authors evaluate the value of adding randomized bases at the ends of the adapters, such as those incorporated in the NEXTFLEX[®] small RNA-Seq kit v3. Small RNA-sequencing with fixed ends on the adapter, introduces bias during the ligation step in typical small library prep. The authors went on to show the protocol using the randomized ends “has a more linear range across the input concentrations ... [and] a statistically significant difference in the correlations of output reads ... thus, the NEXTFLEX[®] protocol results were a better fit to the introduced spike-in concentrations” compared to the fixed adapter method. They further compared concordance of expression levels between the sequencing only libraries, and qPCR of the 10 differentially expressed miRNA (the top 5 upregulated and top 5 downregulated). Only the randomized ligation method, incorporated in the NEXTFLEX[®] small RNA-Seq kit v3, detected all 10 miRNAs. The static end ligation method only detected 5 out of 10. None of the missed miRNA molecules were considered low abundance according to the qPCR results, indicating a bias in the method itself rather than a lower limit of detection compared to the randomized ends ligation technology.

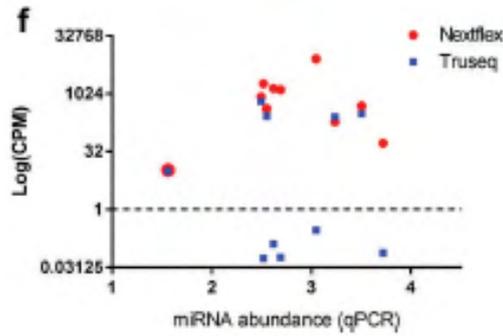
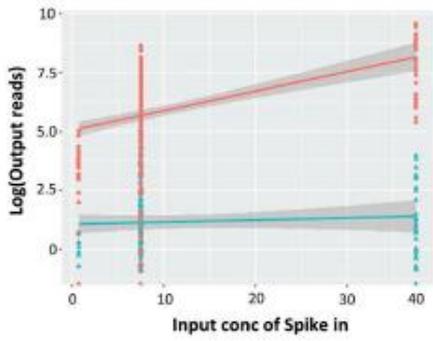


Figure 1: Analysis of spike-in study comparing kits taken from Assessment of methods for serum extracellular vesicle small RNA sequencing to support biomarker development⁹. Spike-ins were added to the 1X sample (2 μ L per mL of serum). These accounting for 1-3% of sequencing reads (left). In the scatter plot of select miRNA expression by sequencing compared to quantitative PCR, only the NEXTFLEX[®] small RNA-seq kit v3 detected all 10 of the miRNAs detected by qPCR (right).

In the publication, A Urinary Extracellular Vesicle microRNA Biomarker Discovery Pipeline; from automated extracellular vesicle enrichment by acoustic trapping to microRNA sequencing¹⁰, the authors highlight the use of a novel automated microfluidic system – termed acoustic trapping – to enrich from a single spot urine sample. This collection yielded 130 pg of total RNA into two different library prep methods. Compared to older methods such as ultracentrifugation for EV-derived small RNA collection, automated acoustic trapping mechanism is more user-friendly and captures a more diverse RNA population. The publication also highlights two different technologies for small RNA sequencing: small RNA-seq with randomized adapter ligation and the “Capture and Amplification by Tailing and Switching” (CATS) method. Results indicate the randomized adapter ligation technology produced data with the largest fraction being miRNAs. This leads to the identification of 393 miRNAs missed by template switching technology. Template switching technology also predominantly mapped to rRNA with the miRNA fractions only accounting for < 5% of reads. Importantly, only 1.3% and 1.7% of the reads from the replicates of acoustic trapping were mappable. The authors noted “investigation into the low mappable reads revealed most of the CATS’ reads harbor cystine or thymidine interspersing the polyadenylated sequence causing misalignment during mapping”.

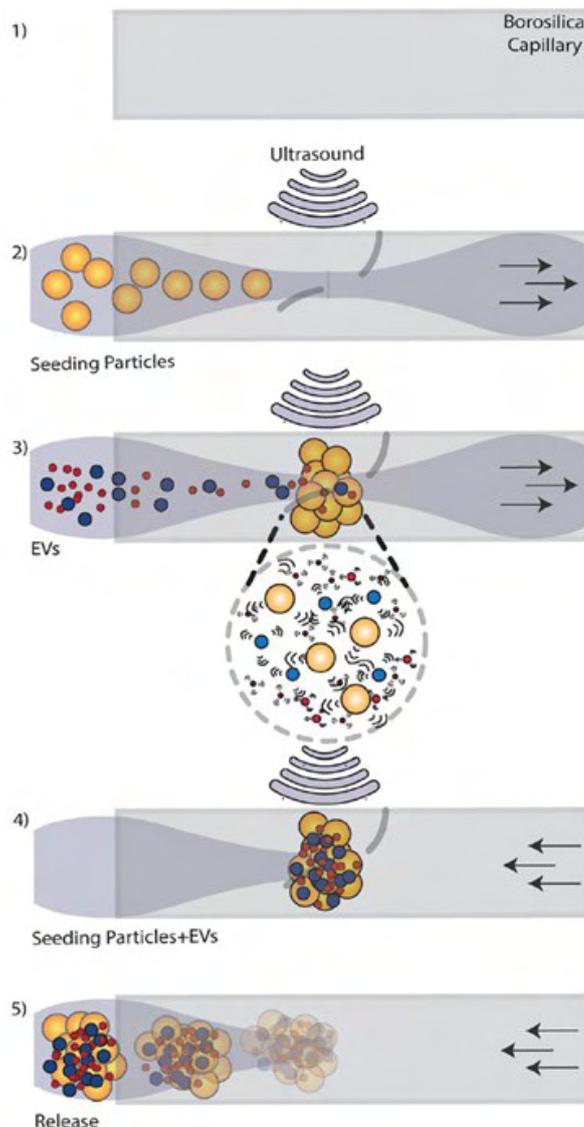


Figure 2: Workflow for acoustic trapping procedure as described in A urinary extracellular vesicle microRNA biomarker discovery pipeline; from automated extracellular vesicle enrichment by acoustic trapping to microRNA sequencing¹⁰.

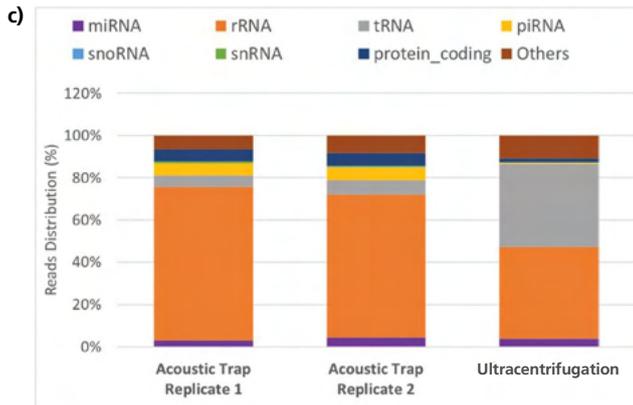
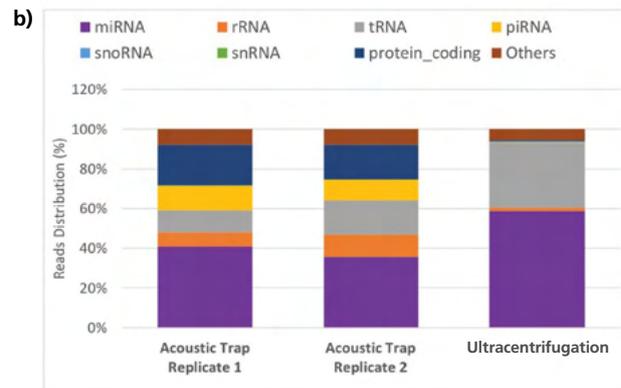
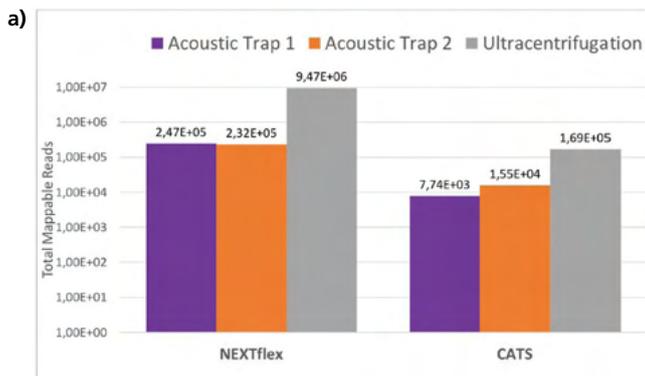


Figure 3: Sequencing data generated using 130 pg of input total RNA from EV-enriched urine samples via acoustic trapping technology into the NEXTFLEX® small RNA-seq kit v3 compared to CATS for total mappable reads (top), percentage read distribution attained using the NEXTFLEX® small RNA-seq kit v3 shows good distribution of small RNAs (middle). CATS small RNA library kit heavily favored rRNAs (bottom).

Small RNAs show great promise in a rapidly progressing, exciting field of biomarker discovery. As biological and technical variability in such a complex molecular assay is anticipated, automation can also be considered when exploring the adoption of new technologies in the laboratory for sensitive applications like small RNA sequencing. Therefore, researchers looking to explore small RNA or miRNA profiles in biofluids should carefully consider the available tools to ensure the best possible route to successfully discovering biomarkers relevant to disease progression and therapy response.

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