APPLICATION NOTE

Improved detection of circulating transcripts

- Circulating RNA biomarker analysis can potentially monitor health of tissues throughout the body
- Tagmentation with enrichment protocol for NGS library preparation enables robust sequencing for low-input samples like circulating RNA
- Illumina RNA Prep with Enrichment offers a streamlined workflow for preparing libraries from circulating RNA

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Introduction

Nucleic acids in the bloodstream can serve as biomarkers to monitor human health.[1] Circulating RNA (C-RNA; also referred to as cell-free RNA or cfRNA) is released by many tissues into the circulation via cellular processes of apoptosis, microvesicle shedding, and exosome signaling. Because of these diverse origins, C-RNA measurements reflect tissue-specific changes in gene expression, inter-cellular signaling, and the degree of cell death occurring within different tissues throughout the body.[2]

Accessible from a simple blood draw, C-RNA holds promise for various biomedical applications, including use as predictive biomarkers for cancer or pregnancy health monitoring.[3-6] This application note compares methods for creating next-generation sequencing (NGS) libraries from circulating transcripts.

Challenges of working with C-RNA

C-RNA is comprised of full-length RNA and fragmented RNA, and is stable due to its containment within vesicles protecting it from degradation by nucleases. However, it is of low abundance and warrants careful processing for effective sequencing library preparation. C-RNA also predominantly consists of ribosomal RNA, which is not informative. Each milliliter of plasma yields only a few nanograms of C-RNA, which copurifies with cell-free DNA (cfDNA).

The C-RNA sequencing workflow involves extracting nucleic acids from plasma, followed by DNase treatment to remove cfDNA from the sample. Because the input amounts are low, ribosomal RNA is retained in the sample and acts as a carrier through the library preparation. This ribosomal RNA is later removed through exome enrichment.

Demonstrated value of C-RNA analysis

To demonstrate the utility of C-RNA sequencing, Illumina scientists identified a C-RNA signature that can classify pregnancies affected by preeclampsia.[1] For this proof-of-concept study, the authors used a ligation-based library preparation approach with probe-assisted enrichment for C-RNA detection. This ligation protocol involved multiday workflows for library preparation and a two-step exome enrichment, which introduced opportunity for technical variation when using very low-input samples. For optimal conversion efficiency, the RNA needed to be chemically fragmented prior to library prep. To overcome these limitations and improve the quality and yield of the C-RNA library, Illumina scientists sought to identify a more robust C-RNA sequencing workflow.

Advantages of tagmentation

Tagmentation-based library preparation and enrichment workflows provide consistently high-quality results for low-input samples like C-RNA. Tagmentation uses bead-linked transposomes (BLT) to fragment and add adapter sequences in a single reaction step, avoiding ligation steps. For enrichment assays, tagmentation-based methods show high library conversion with few primer dimers.

The streamlined workflow for tagmentation-based protocols, including library preparation and one-step enrichment, can be completed in fewer than nine hours (Figure 1). Reducing processing time and the number of experimental steps helps improve sensitivity and replicability of the assay, while making the workflow more efficient.

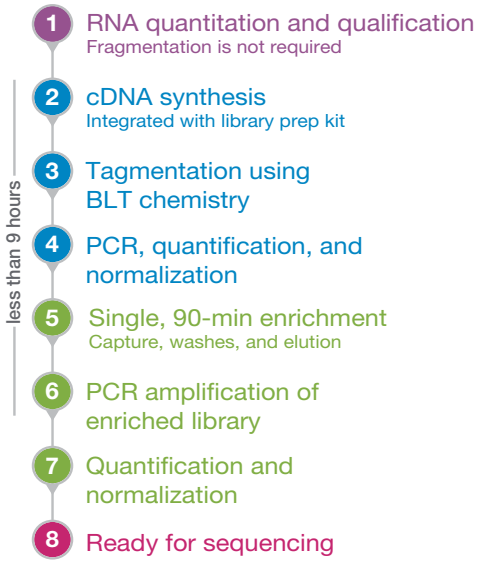

Figure 1: Efficient workflow for tagmentation-based library prep—Illumina RNA Prep with Enrichment uses on-bead tagmentation technology followed by a single 90-minute hybridization step for a < 9-hour protocol.

Broad improvements in C-RNA detection

To validate the tagmentation approach for C-RNA sequencing, Illumina scientists compared blood samples from five pregnant and five nonpregnant women. C-RNA was isolated from two milliliters of plasma and library preparation was performed in parallel using a tagmentation-based method and a ligation-based enrichment method, with three technical replicates per sample.

C-RNA libraries created via tagmentation showed significant improvements in library yield and quality compared to libraries prepared via ligation.* The tagmentation C-RNA library preparation method demonstrated high conversion efficiency with 30% fewer duplicate reads and detection of over 300 additional genes (Figure 2).

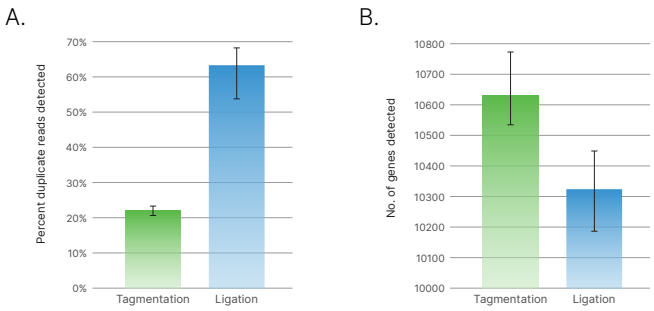

Figure 2: Broad improvements in C-RNA library diversity—C-RNA libraries prepared by the tagmentation method showed (A) 30% fewer duplicate reads, and (B) over 300 additional genes detected compared to ligation-prepared libraries.

Tagmentation-based library preparation also showed minimal noise between technical replicates† and reduced biological variation for low-abundance transcripts among samples within a cohort (Figure 3). This increased sensitivity allowed researchers to detect 67% more differentially abundant genes between pregnancy and nonpregnancy C-RNA (Figure 4). The tagmentation approach identified 29 genes of increased abundance (including 16 of the 18 genes detected using the ligation method) and one gene of decreased abundance. All 14 additional transcripts of differential abundance detected with tagmentation were relevant to pregnancy. Many had low-fold changes, illustrating the benefit of reduced noise when using the tagmentation method.

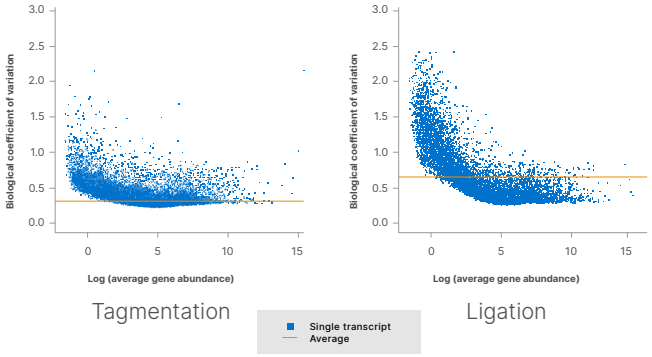

Figure 3: Reduced biological variation for low-abundance C-RNA transcripts—Biological coefficient of variation quantifies variation among samples within a cohort; high values can obscure differentially abundant transcripts. Tagmentation C-RNA libraries exhibit lower biological variation (reduced noise) for transcripts with low abundance, versus ligation-based C-RNA libraries.

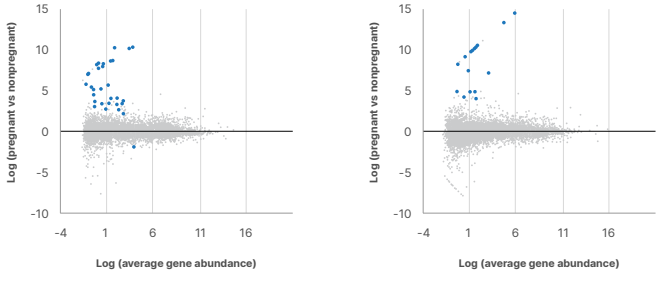

Figure 4: Increased detection of differentially abundant genes—Average C-RNA gene abundance for pregnant vs nonpregnant cohorts. Blue dots indicate $P < 0.05$ and fold-change greater than two. Tagmentation identified 29 upregulated genes, and one downregulated gene. Ligation detected only 18 upregulated genes, including 16 genes also detected by the tagmentation method.

* Data not shown. PicoGreen quantification of library yield before enrichment. Quality determined on Bioanalyzer system.

† Data not shown. $R^2 = 0.97$ for linear regression of FPKM (fragments per kilobase of transcript per million mapped reads) correlation plots for tagmentation technical replicates.

Tagmentation method for low-input RNA

Based on this data, tagmentation-based RNA enrichment provides a robust solution for C-RNA analysis in experimental studies. Illumina offers complete kits for tagmentation-based RNA library preparation, ideal for low-input applications like C-RNA detection. Illumina RNA Prep with Enrichment provides an efficient workflow for RNA library preparation and enrichment, starting with cDNA conversion and on-bead tagmentation, followed by a simplified, single-step hybridization (Figure 1). Illumina RNA Prep with Enrichment provides exceptional capture efficiency and coverage uniformity, minimizing required sequencing depth for accurate C-RNA detection without bias.

Conclusion

Circulating transcript detection represents an opportunity to provide noninvasive insights into disease states, with the potential of early detection and prediction of disease outcomes. Tagmentation-based library preparation with enrichment approaches deliver excellent yield and data quality for robust sequencing of low-abundance samples like C-RNA. Illumina RNA Prep with Enrichment is an easy-to-use, complete RNA library preparation solution that can help accelerate research looking at C-RNA for identification and monitoring of disease biomarkers.

Learn more

To learn more about Illumina RNA Prep with Enrichment, visit illumina.com/products/by-type/sequencing-kits/library-prep-kits/rna-prep-enrichment.html

References

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