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New England Biolabs' Library Prep Kit Finds Favor in Researchers' Analysis of RNA in Circulating Exosomes

by Andrea Anderson

AN ANALYSIS AIMED at establishing a baseline understanding of the small, exosome-bound RNAs present in typical blood samples is also offering insights into the performance of the existing methods used to make libraries of the small RNAs found within the membrane-bound vesicles.

For a study appearing online this month in *BMC Genomics*, researchers from the US and China did small RNA sequencing on more than a dozen exosomal RNA libraries that had been produced using exosomes isolated from the blood of three healthy individuals and commercially available small RNA library preparation kits from New England Biolabs, Bioo Scientific, and Illumina.

The team's comparison of sequence data generated from replicate samples prepared using kits from each of the three companies indicated that the NEB kit topped the heap in the exosomal RNA analysis.

In particular, the study's authors noted that samples prepared with that kit contained a diverse set of RNAs, including those found at low frequency. They also touted the technical replication achieved in those samples, as well as the ability to readily distinguish RNA sequences from sequence adaptors in the NEB kit-prepared samples.

The latter consideration is key when sequencing RNAs from exosomes, given the size range of most RNA found in the vesicles, noted the study's senior author Liang Wang, a pathology and cancer researcher at the Medical College of Wisconsin.

"If you cannot separate the adaptor sequence, what that means is that your sequencing library has a lot of contamination," he told *In Sequence*.

"Based on this preliminary data, we believe [the NEB kit] generally gave us better libraries," Wang said.

More generally, the work offered a peek at the overall small RNA content in circulating exosomes from seemingly healthy individuals, study authors said, laying the foundation for more extensive studies of exosomal RNA in specific disease contexts.

"To our knowledge, this is the first report that applied deep sequencing to discover and characterize profiles of plasma-derived exosomal RNAs," Wang and his co-authors wrote. "Further characterization of these extracellular RNAs in diverse human populations will provide reference profiles and open new doors for the development of blood-based biomarkers for human diseases."

Previous research has shown that cells from several tissue types release membranous exosomes through a fusion process involving vesicles within the cell and the cell's plasma membrane.

These newly liberated, membrane-bound blobs — once thought to contain only waste material — are now believed to contribute to signaling and small molecule transfer between cells located near and far from one another in the body, the researchers noted.

Moreover, there is mounting evidence that the numbers and types of RNAs present within the exosomes can shift during the development of certain diseases, leading more and more researchers to suspect that these exosomal nucleic acids might serve as molecular signposts of disease.

"While exosomes have been shown to play functional roles in

continued on next page

genomeweb In Sequence
The Inside Read on Genome Sequencing

May 28, 2013

recipient cells, the RNA content of the exosomes may provide unique molecular signatures for disease diagnosis and prognosis," the authors of the new study noted.

An added benefit for those on the hunt for biomarkers is the protection that the exosome itself offers against RNA degradation, Wang noted. Whereas RNA that's released directly into the bloodstream or other extracellular sites degrades very quickly, he said, the exosome safeguards RNA over long periods of time, even in harsh conditions.

"This is a very good criteria for biomarkers, because you don't want to get blood [samples] if RNA is degraded immediately," Wang said.

Such features have contributed to the increasing interest in exosomal RNA biomarkers and in relationships between extracellular RNA and disease in general (see, for example, *Gene Silencing News 9/20/2012*).

In addition, several companies have started wading into the burgeoning exosome field. For instance, System Biosciences already offers exosomal RNA sequencing services, while Exosome Diagnostics has been working on potential diagnostic tests for cancer and other conditions that rely on nucleic acid sequences in exosomes found in various biofluids — from blood to urine (*CSN 5/9/2012*).

For the current study, Wang and colleagues set out to better characterize the small RNA content of exosomes in healthy blood samples, while at once assessing available methods for preparing the nucleic acids for sequencing.

To do this, the team first used a reagent called ExoQuick, from System Biosciences, to isolate the exosomes in blood samples from three healthy individuals.

The small RNAs in these samples were subsequently prepared using one of three commercially available kits: the NEBNext multiplex small RNA library preparation kit; the NEXTflex small RNA sequencing kit from Bioo Scientific; and Illumina' TruSeq small RNA sample prep kit.

Specifically, researchers used NEB and Bioo Scientific kits to prepare two replicate small RNA libraries per person for each of the three individuals tested. The pricier Illumina kit was used to prepare two replicate libraries from just one of the participants.

From there, the team sequenced each of the 14 resulting libraries with Illumina's HiSeq 2000, generating nearly 7.3 million reads apiece.

Past studies have found strong miRNA representation within the exosomal RNA, Wang noted, and the current analysis was no exception: some 42 percent of sequence reads in each sample — and more than three-quarters of those that mapped to the known human sequences — came from miRNAs.

All told, the team saw 593 different miRNAs (including 185 new miRNA candidates), with each sample containing between 380 and 474 miRNAs. Five miRNAs were particularly common across the samples, making up nearly half of the mapped miRNA reads.

The exosomal samples also contained a range of other RNA types, from ribosomal RNA and transfer RNA to long, non-coding RNA, piwi-interacting RNA, small nuclear RNA, and small nucleolar RNA.

Bits and pieces of protein-coding messenger RNAs turned up too, though researchers suspect that both the mRNA and lncRNA molecules turning up in exosomes are fragments that may be in the process of degradation — particularly since kits used to produce the sequencing libraries are designed to deal with small RNAs.

"The use of large-scale RNA sequencing will ensure the discovery and characterization of the whole transcriptome (known and unknown RNAs) of the blood-derived exosomes," the study authors noted.

"A fully characterized transcriptome will help gain a better understanding of exosome-mediated molecular mechanisms and will contribute to biomarker discovery," they added. "It is expected that the blood-based sequencing assay described here will find clinical applications as a biomarker discovery tool for disease diagnosis and prognosis."

When they compared and contrasted the three small RNA library preparation methods used in the study, researchers found that sequence data from samples prepared using each of the kits led to roughly comparable numbers of RNA.

But certain RNAs were more or less well represented depending on the individual tested. There were also subtle differences in the number and nature of exosomal RNAs detected depending on the protocol used.

In particular, the NEB-prepared samples had the highest proportion of mappable reads overall, on average. These samples also had greater representation from new and known miR-

May 28, 2013

genomeweb In Sequence
The Inside Read on Genome Sequencing

NAs, the researchers found, particularly those present at relatively low abundance.

"NEB had more consistently detectable miRNAs in the libraries than other kits," Wang said.

Even prior to sequencing, the study's authors noted, quantitative PCR analysis of libraries produced using protocols from all three kit developers) indicated that the samples prepared using the NEB kit had the most robust and reproducible RNA repertoires.

At the moment, Wang said he and his colleagues are generally using the NEB kits for many of their exosomal RNA studies, due to its performance in the head-to-head comparison and its price, which is roughly half that of the Illumina TruSeq kit.

Even so, he noted that other kits appear to be beneficial for studies where researchers are interested in exosomal RNA expression levels rather than in profiling components of the exosomal RNA repertoire.

The group is already applying exosomal RNA sequencing to studies of several cancer types. Wang noted that preliminary, unpublished data suggest that there are detectable differences in exosomal RNA profiles and/or abundance in the blood of individuals with cancer, particularly those with later-stage tumors.

Going forward, though, Wang cautioned that those interested in using exosomal RNA for diagnostics-related purposes are still faced with the challenge of finding the most effective and efficient way of isolating the vesicles.

For the current analysis, his group relied on chemical isolation using ExoQuick. That approach produces predominantly exosomal samples, he said, though recent analyses suggest that the reagent also precipitates some non-exosomal RNA.

At the moment, the most promising method for obtaining pure exosomes appears to be ultra-centrifugation, Wang said. But because that approach is time-consuming, labor intensive, and requires massive amounts of starting material — in this case, blood — it's generally more suitable for biological studies of exosomes, he said.

Still, in the search for biomarkers, he added that some contamination with non-exosomal RNA should be tolerable, at least in the biomarker discovery phase, provided there are significantly different RNA profiles between control and disease samples.