[Letter to the Editor]

Comparison of plasma extracellular RNA isolation kits reveals kit-dependent biases

Extracellular RNAs (exRNAs) are present in biofluids such as breast milk, cerebrospinal fluid, plasma, saliva, and urine, and comprise a wide spectrum of RNA species, including microRNAs (miRNAs), messenger RNAs (mRNAs), ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), and long non-coding RNAs (lncRNAs) (1). Successful detection of organ-specific and disease-associated exRNAs from plasma or serum samples by PCR, microarray and, more recently, RNA-seq has positioned circulating exRNAs as promising candidates for non-invasive biomarker discovery (2–6). However, exRNA isolation from plasma or serum is challenging, partly because of the low abundance of exRNAs but also because contaminants from blood such as heme and immunoglobulin G (IgG) can inhibit PCR (7–8). In addition, RNA species are carried in the plasma or serum via different mechanisms, such as within RNA-protein complexes or extracellular vesicles (4,9–12). Different forms of stable exRNAs might therefore possess different levels of resistance and vulnerability to particular isolation methods.

Commercially available kits that simplify and accelerate the process of exRNA isolation have become frequently used tools in circulating exRNA research. Whether isolation kits differ in their overall ability to isolate exRNA and remove contaminants

from plasma samples, as well as their potential preferences for specific RNA species, has not been directly evaluated. Such testing is critical since systemic biases would complicate interpretation of results and comparisons between experiments in which different exRNA isolation kits are used or when no information is provided regarding the RNA isolation method used (1).

Here, we evaluated seven widely available and commonly used exRNA isolation kits (Table 1) on their overall recovery rate of synthetic RNA, ability to recover synthetic RNA of different sizes, plasma exRNA yield and purity, and amplification efficiency of plasma exRNA samples purified using these kits.

To assess the overall recovery rate and size bias of the kits, we used 6 synthetic RNAs ranging in length from 200 to 6000 nucleotides (Agilent RNA 6000 Nano Ladder; Agilent Technologies, Waldbronn, Germany). As shown in Figure 1A, all kits achieved $\geq 50\%$ recovery of RNA input. The Quick-RNA Mini Prep and DirectZol RNA Prep kits manufactured by Zymo Research (Irvine, CA) obtained the highest recovery of $\geq 80\%$, followed by RNAdvance (Agencourt Bioscience, Beckman Coulter, Beverly, MA), mirVana PARIS (Life Technologies, Thermo Fisher Scientific, Waltham, MA), miRNeasy (Qiagen, Hilden, Germany), and MagMAX (Life Technologies, Thermo Fisher Scientific)

kits with recovery rates of 60%–80%. The miRCURY-Biofluids (Exiqon, Vedbaek, Denmark) kit recovered the least amount of RNA input ($54.3 \pm 5.4\%$) (mean $\pm$ SEM).

While the MagMAX, Quick-RNA, DirectZol, miRNeasy, and mirVana kits showed comparable recovery of RNA of all lengths (Figure 1B), there was a significant loss of 4000 and 6000 nucleotide RNAs from RNA samples isolated by the miRCURY-Biofluids kit. This is consistent with the kit's specification for isolating RNA <1000 nucleotides in length, though the 2000 nucleotide band was still present in the recovered RNA. Notably, RNA bands ≥ 2000 nucleotides were largely absent from RNA samples recovered by the RNAdvance kit.

To compare kit performance on plasma exRNA purification, three biological replicates were performed with each kit using plasma from a single blood draw. RNA isolation was performed using input and elution volumes and protocols recommended by the manufacturers (Table 1). Purified RNA samples were quantified using the Qubit RNA HS Assay (Life Technologies, Thermo Fisher Scientific). exRNA yield per prep ranged from 1.65 ± 0.26 ng (mean $\pm$ SEM) for the MagMAX kit to 35.17 ± 22.09 ng for the RNAdvance kit (Supplementary Table S1). exRNA yield per 1 mL plasma (Figure 1C) was 175.8 ± 110.5 ng for the RNAdvance kit, 30–40 ng for the miRCURY-Biofluids, Quick-RNA, and mirVana kits, and

Table 1. Plasma extracellular RNA (exRNA) isolation kit specifications.

Purification method	Kit	Manufacturer	RNA size range	Input volume (μ L)	Elution volume (μ L)
Magnetic bead	Agencourt RNAdvance Blood Kit	Agencourt Bioscience, Beckman Coulter (Beverly, MA)	All	200	50
	MagMAX Viral RNA Isolation Kit	Life Technologies, Thermo Fisher Scientific (Waltham, MA)	All	400	50
Protein precipitation and column	miRCURY RNA Isolation Kit -Biofluids	Exiqon (Vedbaek, Denmark)	<1000 bp	200	50
Proteinase K and column	Quick-RNA Mini Prep	Zymo Research (Irvine, CA)	All	200	50
Phenol (no phase separation) and column	DirectZol RNA Prep Kit	Zymo Research	All	200	50
Phenol (phase separation) and column	miRNeasy Serum/Plasma Kit	Qiagen (Hilden, Germany)	All	200	14
	mirVana PARIS Kit	Life Technologies, Thermo Fisher Scientific	All	400	100

Commercial kits we tested are grouped according to purification method. Manufacturer information and RNA size purified in manufacturers' specifications are indicated. Plasma input and elution volumes used in this study are also shown.

Figure 1. Comparison of extracellular RNA (exRNA) isolation kit performance. (A) Bar graph of average recovery rates of RNA ladder expressed as percent of input for indicated kits. (B) Simulated gel image from Agilent RNA 6000 Pico chip showing RNA length distributions of the same amount of recovered RNAs by indicated kits. nt: nucleotides. (C) Bar graph of estimated plasma exRNA yields per 1 mL plasma by the indicated kits. (D) Bar graph of estimated DNA quantities per 1 mL plasma in exRNA samples purified by the indicated kits. (E) Representative agarose gel images of PCR products for *hsa-miR-16*, *hsa-miR-150*, β -actin (*ACTB*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) from cDNA inputs. Reactions lacking reverse transcriptase (-RT) and with water input served as negative controls. Error bars indicate SEM. $N = 3$ biological replicates.

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Table 2. Summary of extracellular RNA (exRNA) isolation kit comparison results.

Kit	Ladder recovery	Size bias	exRNA yield	cfDNA Amount	miRNA	mRNA	Spike-in RNA
RNAdvance Blood	++	-	+++*	+++	++	+	++
MagMAX Viral	++	+++	-	+	-	-	+
miRCURY-Biofluids	+	++	++	++	++	+	+++
Quick-RNA	+++	+++	++	+	+	++	+++
DirectZol	+++	+++	+	+	+++	-	+
miRNeasy	++	+++	+	+	+++	+++	++
mirVana PARIS	++	+++	++	++	+++	+	+++

Ladder recovery, size bias, extracellular RNA (exRNA) yield, cell-free DNA (cfDNA) co-purification, and reverse transcription PCR (RT-PCR) for microRNAs (miRNAs), messenger RNAs (mRNAs), and spike-in RNA of each kit were evaluated based on following criteria: For ladder recovery, “+++,” “++,” “+,” and “-” denote “≥80%,” “60%–80%,” “40%–60%,” and “<40%” recovery, respectively. For ladder size bias, “+++,” “++,” “+,” and “-” denote “full range,” “loss of 6 kb,” “loss of ≥4 kb,” and “loss of ≥2 kb,” respectively. For RNA yield per 1 mL plasma, “+++,” “++,” “+,” and “-” denote “≥50 ng,” “20–50 ng,” “10–20 ng,” and “<10 ng,” respectively. For cfDNA amount per 1 mL plasma, “+++,” “++,” “+,” and “-” denote “≥10 ng,” “5–10 ng,” “1–5 ng,” and “<1 ng,” respectively. For RT-PCR, “+++,” “++,” “+,” and “-” denote “good,” “moderate,” “weak,” and “not detected” amplification, respectively. * indicates possible overestimation of exRNA yields, due to the high Qubit reading of the lysis buffer used in the indicated kits.

~10 ng for the DirectZol and miRNeasy kits. The MagMAX kit yielded only 4.1 ± 0.6 ng.

The high reading seen with the RNAdvance may be partly due to contamination from the lysis buffer. RNAdvance lysis buffer alone results in a high background reading in the Qubit Assay (58.7 ± 0.9 pg/μL) (mean $\pm$ SEM; 3 technical replicates), corresponding to an RNA concentration of 4.4 ng/μL. The MagMAX lysis buffer also generated a Qubit reading of 22 pg/μL, while the same volume of lysis buffers from the other kits was not measurable by the Qubit Assay.

DNA contamination was detected in samples prepared by all seven kits using the Qubit DNA HS Assay. DNA contamination ranged from 1.2 ± 0.5 ng per 1 mL plasma for the MagMAX kit to 12.9 ± 4.7 ng per 1 mL plasma from the RNAdvance kit (Figure 1D and Supplementary Table S1). In addition, PCR amplification of β -actin (*ACTB*) was positive for samples purified by the MagMAX, miRCURY-Biofluids, Quick-RNA, DirectZol, and mirVana kits (Supplementary Figure S1).

To assess the composition and ability to amplify RNA species from purified exRNA samples, we performed reverse transcription and PCR (RT-PCR) targeting *hsa-miR-16*, *hsa-miR-150*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and *ACTB* (Figure 1E). RNA isolated using the miRNeasy kit was readily amplified for both miRNA and mRNA targets. While exRNA purified by the mirVana kit had the highest amplification for miRNAs, and the RNAdvance, miRCURY-Biofluids, and DirectZol kits also showed

robust amplification for miRNAs, they failed to readily amplify mRNAs. In contrast, exRNA prepared with the Quick-RNA kit had good amplification for mRNAs but not miRNAs. exRNA from the MagMAX kit failed to amplify miRNAs and mRNAs. To determine if an RT-PCR inhibitor was present in purified exRNA samples, we spiked synthetic *EGFP-zgpr125* RNA into purified exRNA samples and compared its amplification to water containing the same concentration of the RNA spike-in. *EGFP* amplification levels were similar to that of spike-in alone for the miRCURY-Biofluids, Quick-RNA, and mirVana exRNA samples, slightly decreased for the RNAdvance and miRNeasy samples, and greatly reduced for the MagMAX and DirectZol samples (Supplementary Figure S2).

Our study provides a comparative evaluation of seven RNA isolation kits (Table 2). Although these kits had good overall performance in recovering synthetic RNAs ($\geq 50\%$ recovery), some displayed biases in the length of RNAs that could be isolated. exRNA yields from plasma differed widely depending on the kit used, and some kits might introduce contaminants that could falsely increase Qubit RNA Assay readings. In addition, DNA contamination was present at different levels in all purified RNA samples. Differences in RT-PCR amplification among exRNA samples were apparent, partly due to the presence of RT-PCR inhibitors in some exRNA samples.

Most critically, exRNA samples purified with the majority of kits displayed ampli-

fication bias for a particular RNA species (e.g., mRNA-dominant amplification from the Quick-RNA exRNA sample and miRNA-exclusive amplification from the DirectZol exRNA sample). Therefore, different kits will reflect plasma exRNA content to different degrees, making it difficult to directly compare exRNA analyses using different RNA isolation kits. Our finding could help explain the lack of reproducibility of exRNA results between studies (13–15).

Authors contributions

X.L., M.M., and Z.W. designed the research. X.L. and M.M. performed the experiments. X.L., M.M., and Z.W. analyzed the data and wrote the paper.

Acknowledgments

We thank Miao Shi, Vivian Wei, Klaas Max, Kemal Akat, Thomas Tuschl, and Andrzej Breborowicz for their valuable input on the experimental design and preparation of the manuscript, and Jan Vijg for sharing equipment. This work was supported by NIH grants 1U19CA179564-01 and K08HD068546. This paper is subject to the NIH Public Access Policy.

Competing interests

The authors declare no competing interests.

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Received 23 March 2015; accepted 14 April 2015.

BioTechniques 59:13-17 (July 2015)
doi:10.2144/000114306

Supplementary material for this article is available at www.BioTechniques.com/article/114306.

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