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 be carried in the plasma or serum via different mechanisms, such as within RNA-protein complexes or extracellular vesicles (4,9–12).

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 ≥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 ≥80%, followed by RNAdvance (Agencourt Bioscience, Beckman Coulter, Beverly, MA), mirVana PARIS (Life Technologies, Thermo Fisher Scientific, Waltham, MA), and MagMAX Viral RNA Isolation Kit (Fisher Scientific). exRNA yield per prep ranged from 1.65 ± 0.26 ng (mean ± SEM) for the MagMAX kit to 35.17 ± 22.09 ng for the RNAAdvance kit (Supplementary Table S1). exRNA yield per 1 mL plasma (Figure 1C) was 175.8 ± 110.5 ng for the RNAAdvance kit, 30–40 ng for the miRCURY-Biofluids, Quick-RNA, and mirVana kits, and 15–20 ng for the Agencourt RNAdvance Blood Kit.

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

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

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.
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 miRNAs. In contrast, exRNA prepared with the Quick-Zol kit had good amplification for miRNAs but not miRNAs. exRNA from the MagMAX kit failed to amplify miRNAs and miRNAs. 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 miRNasy, 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 (%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.

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<table>
<thead>
<tr>
<th>Kit</th>
<th>Ladder recovery</th>
<th>Size bias</th>
<th>exRNA yield</th>
<th>cfDNA Amount</th>
<th>miRNA</th>
<th>mRNA</th>
<th>Spike-in RNA</th>
</tr>
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<tbody>
<tr>
<td>RNAdvance Blood</td>
<td>+++</td>
<td>-</td>
<td>+++*</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MagMAX Viral</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miRCURY-Biofluids</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Quick-RNA</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
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<tr>
<td>DirectZol</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>mirNeasy</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>mirVana PARIS</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

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 "full range," "60%–80%," "40%–60%," and "<40%" recovery, respectively. For ladder size bias, "+++," "++," "+," and "-" denote "full range," "loss of 5 kb," "loss of 4 kb," and "loss of 2 kb," respectively. For RNA yield per 1 mL plasma, "+++," "++," "+," and "-" denote "≥10 ng," "5–10 ng," "1–5 ng," and "<1 ng," respectively. For RT-PCR, "+++," "++," "+," 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 ladder size bias, "+++," "++," "+," and "-" denote "full range," "loss of 6 kb," "loss of 5 kb," and "loss of 4 kb," respectively. For ladder size bias, "+++," "++," "+," and "-" denote "full range," "60%–80%," "40%–60%," and "<40%" recovery, respectively. For ladder size bias, "+++," "++," "+," and "-" denote "full range," "60%–80%," "40%–60%," and "<40%" recovery, respectively.

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fication bias for a particular RNA species (e.g., mRNA-dominant amplification from the Quick-RNA exRNA sample and mRNA-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.

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Competing interests
The authors declare no competing interests.

References


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