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Methods Article

METHODS ARTICLE

A comparison of the efficiency of RNA extraction from extracellular vesicles using the Qiagen RNeasy MinElute versus Enzymax LLC RNA Tini Spin columns and qPCR of miRNA

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Abstract

One consequence of the current severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic is an interruption to the supply of laboratory consumables, particularly those used for RNA extraction. This category includes column-based RNA extraction kits designed to retain short RNA species (defined as having fewer than 200 nucleotides), from small sample volumes, e.g. exosomes or extracellular vesicles (EVs). Qiagen manufactures several kits for the extraction and enrichment of short RNA species, such as microRNA (miRNA), which contain silica-membrane columns called "RNeasy MinElute Spin Columns." These kits, which also contain buffers and collection tubes, range in price from USD380 to greater than USD1000 and have been subject to fulfillment delays. Scientists seeking to reduce single-use plastics and costs may wish to order the columns separately; however, Qiagen does not sell the RNeasy MinElute Spin Columns (in reasonable quantities) as an individual item. Thus, we sought an alternative product and found RNA Tini Spin columns from Enzymax LLC. We conducted a systematic comparison of the efficiency of RNA extraction for miRNA quantitative real-time PCR (qPCR) using the Qiagen RNeasy MinElute Spin Columns and Enzymax LLC RNA Tini Spin columns and the Qiagen total RNA extraction protocol that enriches for short RNA species. We compared the efficiency of extraction of five spike-in control miRNAs, six sample signal miRNAs, and nine low- to medium-abundance miRNAs by qPCR. The RNA was extracted from EV preparations purified from human plasma using CD81 immunoprecipitation. We report no statistically significant differences in extraction efficiencies between the two columns for any of the miRNAs examined. Therefore, we conclude that the Enzymax RNA Tini Spin columns are adequate substitutes for the Qiagen RNeasy MinElute Spin Columns for short RNA species enrichment and downstream qPCR from EVs in the miRNAs we examined.

Keywords: qPCR; exosomes; extracellular vesicles; column-based RNA extraction; miRNA

Introduction

Exosomes are small (30–150 nm) extracellular vesicles (EVs) that contain lipoproteins, DNA, messenger RNA (mRNA), and small, noncoding RNAs, including microRNA (miRNA) [1]. Many different cell types excrete exosomes, and exosomes possibly facilitate cell-to-cell communication during normal develop-

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ment (for a review, see Bakhshandeh et al. [2]) and evidence continues to build for exosomes contributing to the transmission of pathogenic proteins. For example, exosomes are known to carry genetic material involved in the spread of specific types of tumors [3], neurodegenerative diseases such as Alzheimer's disease [4], Parkinson's disease [5], and other protein-misfolding disorders [6, 7]. In recent years, researchers have recognized the therapeutic potential of exosomes and are beginning to explore them as mechanisms for the delivery of pharmaceuticals and also in the design of personalized medicine [8].

Another aspect of exosome research involves the analysis and quantitation of EV-sequestered cargo for the characterization of biomarkers in the diagnosis and prognosis of disease. This is particularly relevant for pathologies that are currently primarily diagnosed using clinical criteria, such as Parkinson's [9], Alzheimer's [10], and amyotrophic lateral sclerosis (ALS) [11]. Exosomes can provide a "window into the cell" and, in the case of neurological disorders, a "window into the brain." Their presence in blood, urine, saliva, breast milk, and other bodily fluids means access to exosomes is minimally invasive compared to procedures such as lumbar punctures required to obtain cerebral spinal fluid or tissue biopsies.

Purification of EVs from blood plasma can be accomplished by ultracentrifugation, volume-excluding polymers, or immunoprecipitation. We further enrich this preparation for exosomes of neural origin by purification using antibodies for the transmembrane protein, L1CAM/CD171 [11]. Preparations of L1CAM/CD171 purified exosomes are sometimes referred to as "neural-derived exosomes" since L1CAM/CD171 is enriched in the brain (see https://gtexportal.org/home/gene/L1CAM). However, since L1CAM/CD171 is also highly expressed in neurons in the gut, colon, and tibial nerve and "exosome" preparations can be heterogeneous in size and composition, we prefer to use the term neural-enriched EVs (NEEs). It naturally follows that we refer to the preparation left behind once the NEEs are extracted as total-minus-NEE (T−N) fraction.

As stated above, exosomes contain a wide range of proteomic and genetic material. miRNAs are small [between 18 and 22 nucleotides (nt)], noncoding RNAs that regulate gene expression and protein translation and are enriched in EVs, including exosomes and NEEs [12].

A large body of evidence reporting on disparate pathologies demonstrates that the differential expression of miRNAs can be diagnostic, prognostic, or both for various diseases, including some cancers [13] and neurodegenerative diseases [6]. For example, we recently reported an eight miRNA fingerprint in NEEs that distinguished ALS patients from healthy controls [11].

Conventional RNA extraction and quantitation techniques, such as those that preferentially capture long (>200 nt) RNA species including mRNA, are unsuitable for retaining short RNA (<200 nt) species. Furthermore, when using column-based RNA purification methods, the protocol needs to be designed specifically to retain short RNA species and this requires specialized wash buffers and optimization of the ethanol volume and concentration added to the column during the washing steps.

Qiagen is a leader in RNA extraction and makes kits designed to isolate short RNAs in a total RNA fraction from serum or plasma-derived EVs using silica-based spin columns (ExoRNeasy Midi/Maxi Kit #77144, 77164, 77023; RNeasy Micro Kit #74004; RNeasy MinElute Clean-up Kit #74204; and miRNeasy Micro Kit #217084). In the case of Qiagen's RNA ExoRNeasy Midi Kit #77144, the kit comes in two parts—Part 1: Vesicle isolation and Part 2: RNA isolation. This is appropriate for researchers who want to isolate EVs and RNA using the one kit, but for researchers who isolate EVs using a different method, such as CD81 immunoprecipitation, choosing a kit without Part I is appropriate to save costs and plastics. While the buffers for total RNA extraction enriching for short RNAs can be bought separately, the RNeasy MinElute spin columns cannot.

Enzymax LLC also makes spin columns to extract total RNA including short RNA species (RNA Tini Spin column with collection tube, #EZC1070N) and these can be purchased separately in batches of 50.

A significant hurdle in using miRNA to design diagnostic or prognostic tests for human disease is access to patient samples. Clinical trials can be a source for plasma, but samples from such trials are frequently only available in small volumes for discovery research. Thus, the researcher is working with minimal amounts of material to begin with, meaning preferred methods to extract RNA must be efficient in preserving as much RNA as possible.

When searching for miRNA biomarkers, the researcher generally carries out a global measure of all short RNA species using next-generation sequencing to identify species of interest and then quantitates these findings using real-time quantitative PCR (qPCR). Although qPCR amplifies targeted RNA many times over, it can still be a problem to obtain a robust signal after 40 cycles, especially when working with NEE, which is ∼5–10% of the fraction of total EVs [14]. For this reason, it is critical to preserve all RNA throughout the purification process, which begins with the column-based purification process.

We have previously described an eight miRNA fingerprint that distinguishes blood plasma samples from ALS patients and healthy controls [11]. Since Qiagen does not sell the RNeasy MinElute Spin Columns separately, we sought an alternative, and decided to test RNA Tini Spin columns manufactured by Enzymax LLC #EZC107N. We compared RNA yield from duplicate T−N aliquots taken from the same EV preparations, and from three individual blood plasma samples ($n = 3$), using the Qiagen RNeasy MinElute Spin Columns and the RNA Tini Spin columns manufactured by Enzymax LLC.

We report no significant differences in the mean raw quantitation cycles (Cqs) for any of the target miRNAs when comparing RNA extracted from the Qiagen columns versus the Enzymax columns. Thus, we conclude that the Enzymax RNA Tini Spin columns are a suitable substitute for the Qiagen RNeasy MinElute Spin Columns for medium to high-abundance miRNA. For low-abundant miRNA, researchers should check their extraction on a case-by-case basis.

Materials and methods

Materials

Normal single donor human plasma, frozen once, was obtained from Innovative Research (10 mL, #IPLA-N-S, Novi, MI, USA), and EVs extracted as described previously (Section 2.3 EV Extraction in Banack et al. [11]). We used the T−N fraction of the EV preparations for these experiments. T−N represents the total heterogeneous EV population minus the EVs positive for L1CAM/CD171 neural surface proteins, designated NEE [11].

The RNA extraction kit containing the RNeasy MinElute Spin Columns was from Qiagen (ExoRNeasy Midi Kit #77144, Hilden, Germany). The RNA Tini Spin columns were from Enzymax LLC (Lexington, KY, USA, #EZC107N). Although the RNeasy MinElute Spin Columns from Qiagen cannot be purchased as a separate item, the lysis and wash buffers used in the total RNA extraction process that retains short RNA (<200 nt) are available. QIAzol lysis reagent 50 mL #79306, Buffer RPE (concentrate 55 mL)

1018013, Buffer RWT (80 mL) #1067933, miRCURY RNA spike-in kit, for RT (containing UniSp2, 4, 5 and cel-miR-39-3p) #339390, and the miRCURY LNA RT Kit containing UniSp6 #339340 were from Qiagen. Chloroform ≥99%, stabilized, molecular biology grade #0219400225 was from MP Biomedicals. Ethyl alcohol, pure 200 proof for molecular biology #E7023 (Lot #SHBJ8384), was from Sigma-Aldrich (St Louis, MO, USA).

Preparation of spike-in controls for RNA extraction and cDNA synthesis efficiency

Spike-in control templates for RNA extraction (available as a premixed, lyophilized vial of UniSp2, UniSp4, and UniSp5 from the Qiagen miRCURY RNA spike-in kit, for RT #339390) and cDNA synthesis controls UniSp6 (available as a lyophilized vial as part of the miRCURY LNA RT Kit #339340), and cel-miR-39-3p (available as a lyophilized vial as part of the Qiagen miRCURY RNA spike-in kit, for RT #339390) were removed from the freezer (−20°C) and briefly centrifuged. 80 µL nuclease-free water was added to each of the vials, the vials were incubated for 20 min on ice, then vortexed, and briefly centrifuged. To avoid repeat freeze–thaw cycles, 15 µL aliquots were stored at −20°C.

RNA extraction from EVs

Extraction of total RNA retaining short RNA species from T−N was conducted following the manufacturer's instructions as described in Qiagen RNeasy Midi Kit Part 2: RNA isolation (a detailed protocol is available at www.qiagen.com/HB-2630 "exoRNeasy Midi/Maxi Handbook" beginning page 21, step 6) with the following modifications: for each T−N sample to be lysed, 1 µL of a mix of UniSp2, UniSp4, and UniSp5 (see above for preparation instructions) was added to 700 µL of QIAzol lysis reagent (#79306) and mixed by pipetting up and down.

Briefly, a total of 50 µL T−N pellets were removed from −80°C freezer and thawed on ice, the sample transferred to a fresh RNAse-free 2 mL tube, then 700 µL of the QIAzol/spike-ins mix was added, and each sample mixed by pipetting and briefly vortexed. Tubes were incubated at RT for 5 min, 90 µL chloroform (MP Biomedicals #0219400225) added, then the tube capped securely and shaken vigorously for 15 s. Tubes were incubated for 5 min at RT then centrifuged at 12 000*g* for 15 min, 4°C. The upper aqueous phase (~400 µL), containing total RNA, was carefully transferred to a new 2 mL collection tube being careful not to touch the interphase layer (this contains DNA). Two × volumes of 100% ethanol were added (~800 µL), and the sample mixed thoroughly by pipetting. The use of two volumes of 100% ethanol at this stage selects for retaining short RNA species on the spin column. Up to 700 µL sample was pipetted into either a Qiagen RNeasy RNA MinElute Spin Column or an Enzymax RNA Tini Spin column placed inside a 2 mL RNAse-free collection tube. The tube lid was closed tightly and spun at 8000*g* for 15 s at RT. The flow-through was discarded. This step was repeated until all the sample was passed through the spin column. 700 µL Buffer RWT (Qiagen #1067933) was added to each column, the lid closed, then the tube centrifuged for 15 s at 8000*g*, RT. The flow-through was discarded. 500 µL Buffer RPE (Qiagen #1018013) was added to each column, the lid closed, and the tubes centrifuged for 15 s at 8000*g*, RT, then the flow-through discarded. This step was repeated, but the second time, the tubes were spun for 2 min and the flow-through discarded. The spin column was placed inside a new 2 ml RNAse-free collection tube and the column and tube centrifuged at full speed for 5 min at RT with the lid of the spin column open to dry the membrane. This step is important because any carry-over of ethanol can interfere with downstream processes. The collection tube containing the flow-through was discarded. Finally, the spin column was placed into a 1.5 mL RNAse-free collection tube, and 15 µL nuclease-free water was added directly to the center of the column, the column was let to stand for 1 min, RT, then spun at full speed at RT for 1 min to elute the RNA. The RNA was immediately placed on ice and used for cDNA synthesis or stored at −80°C.

cDNA synthesis

cDNA was synthesized using the miRCURY LNA RT Kit (Qiagen #339340) according to the manufacturer's instructions. 0.5 µL of each spike-in control UniSp6 and *C. elegans* cel-miR-39-3p (see above for preparation instructions) was added to each reaction to monitor cDNA synthesis efficiency. To optimize the amount of

Table 1: miRCURY LNA miRNA PCR assays used for the analysis of QC and low- to medium-abundance miRNA

miRNA category	Target miRNA	Qiagen GeneGlobe ID	Catalog number
Extraction control	UniSp2	YP00203950	339306
	UniSp4	YP00203953	339306
	UniSp5	YP00203955	339306
RT synthesis control	UniSp6	YP00203954	339306
	cel-miR-39-3p	YP00203952	339306
Sample signal miRNA	hsa-miR-142-3p	YP00204291	339306
	hsa-miR-451a	YP02119305	339306
	hsa-miR-191-5p	YP00204306	339306
	hsa-miR-23a-3p	YP00204772	339306
	hsa-miR-30c-5p	YP00204783	339306
	hsa-miR-103a-3p	YP00204063	339306
Low- to medium-abundance miRNA	hsa-miR-146a-5p	YP00204688	339306
	hsa-miR-199a-5p	YP00204494	339306
	hsa-miR-4454	YP02114119	339306
	hsa-miR-10b-5p	YP00205637	339306
	hsa-miR-29b-3p	YP00204679	339306
	hsa-miR-151a-3p	YP00204576	339306
	hsa-miR-199a-3p	YP00204536	339306
	hsa-miR-151a-5p	YP00204007	339306
	hsa-miR-126-5p	YP00206010	339306

RNA used in the reaction, we conducted cDNA synthesis with 2 and 4 μL RNA, then ran qPCR of quality control (QC) and sample signal miRNAs. We subsequently used 4 μL RNA for all downstream cDNA reactions.

qPCR

qPCR QC was conducted using miRCURY LNA miRNA SYBR PCR Assays for the targets listed in Table 1, according to the manufacturer's instructions. cDNA was diluted 1/30 and 3 μL was used in each qPCR reaction. The reaction conditions are described in Table 2. A melt curve and a no-template control (NTC) were included in each assay to check for primer specificity and any nonspecific amplification. qPCR was conducted on the BioRad CFX96™ Real-Time System in 96-well plates and data acquired in Bio-Rad CFX Manager version 3.1 after 40 cycles.

Table 2: qPCR reaction conditions for miRCURY LNA miRNA PCR assays column comparison

Step	Time	Temperature (°C)
PCR initial activation	2 min	95
2-step cycling		
Denaturation	10 s	95
Combined annealing/extension	60 s	56
Number of cycles	40	
Melt curve analysis		60–95

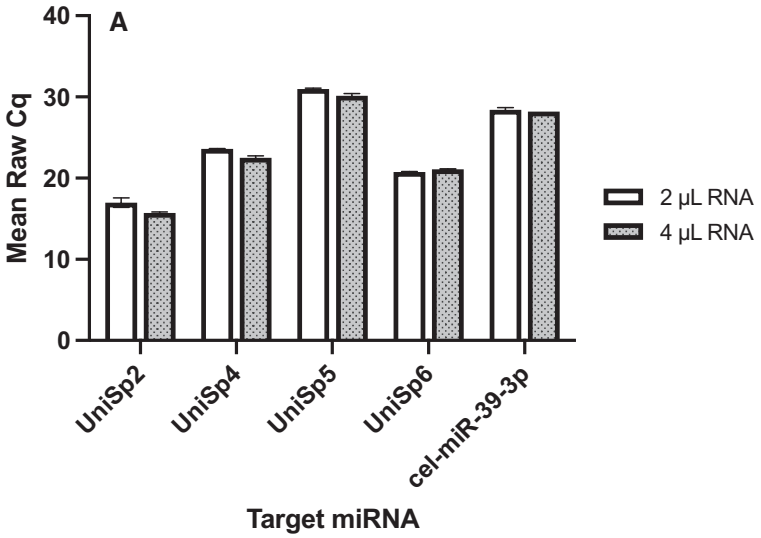
Statistics

Cqs ($n = 3$) returned for qPCR from RNA extracted using the Qiagen RNeasy MinElute Spin Column or the Enzymax RNA Tini Spin column were compared using the Mann–Whitney test for unpaired nonparametric samples, conducted in GraphPad Prism version 9.1.1. for iOS, where $P < 0.05$ was set as significant.

Results

Optimization of RNA volume in cDNA reaction

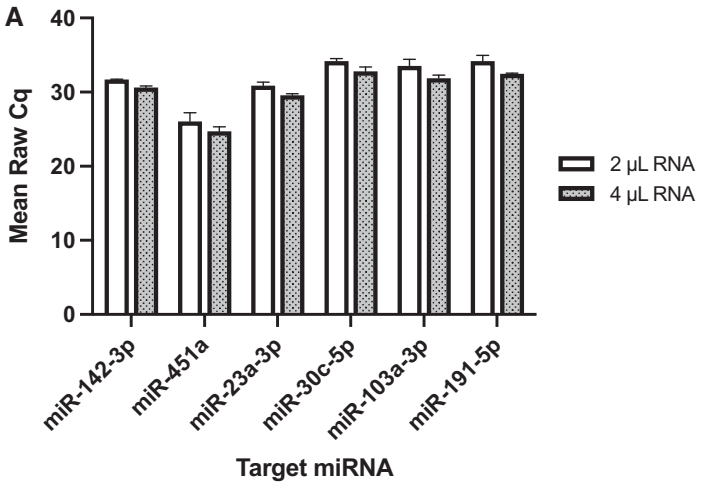
Given that we were planning to assay some reportedly low-abundance miRNAs, we increased the volume of RNA that we would typically use in a cDNA reaction to maximize the likelihood of observing a signal in the qPCR. As standard QCs, we used RNA spike-ins to check for consistency and efficiency in the RNA extraction (UniSp2, 4, and 5) and cDNA synthesis (UniSp6 and cel-miR-39-3p). We synthesized cDNA using either 2 μL RNA (cDNA1 and cDNA2) or 4 μL RNA (cDNA3 and cDNA4) extracted from T−N EVs using the Qiagen RNeasy MinElute Spin



B

	Raw Cq 2 μL RNA				Raw Cq 4 μL RNA				
	cDNA1 (2)	cDNA2 (2)	2 μL RNA mean	SD	cDNA3 (4)	cDNA4 (4)	4 μL RNA mean	SD	ΔCq
UniSp2	16.59	17.40	**17.00**	0.58	15.62	15.82	**15.72**	0.14	**1.28**
UniSp4	23.58	23.64	**23.61**	0.04	22.37	22.69	**22.53**	0.23	**1.09**
UniSp5	30.91	31.04	**30.97**	0.09	29.95	30.33	**30.14**	0.27	**0.83**
UniSp6	20.70	20.80	**20.75**	0.07	21.04	21.12	**21.08**	0.05	**-0.33**
cel-miR-39-3p	28.60	28.25	**28.42**	0.25	28.22	28.21	**28.21**	0.01	**0.21**

Figure 1: Quality control assays were run to optimize the volume of RNA for use in the cDNA reaction. UniSp2, 4, and 5 are RNA extraction spike-in controls (added to the lysis buffer before RNA extraction and to check for RNA extraction efficiency), and UniSp6 and cel-miR-39-3p are cDNA synthesis controls (added to the reaction mixture before cDNA synthesis and used to check the efficiency and reproducibility of the cDNA synthesis reaction). As expected, we report no difference in the mean raw Cqs for the cDNA synthesis spike-ins (UniSp6 and cel-miR-39-3p), but a Cq difference of ~1 was observed for the RNA extraction Cqs. The reason for this difference is because twice as much UniSp2, 4, and 5 templates were added to the cDNA reaction (contained in 2 or 4 μL RNA), whereas the same amount of template was added to the cDNA reaction for either 2 or 4 μL RNA. cDNA was synthesized using the miRCURY LNA RT Kit (Qiagen #339340), and qPCR of the QC miRNAs was run using miRCURY LNA miRNA SYBR individual PCR assays according to the conditions described in materials and methods. cDNA1 and cDNA2 were synthesized using 2 μL RNA, and cDNA3 and cDNA4 were synthesized using 4 μL RNA. Each raw Cq data point represents the miRNA signal from two separate T−N EV extractions ($n = 2$) from human plasma as described in materials and methods. Samples cDNA1 (2) and cDNA2 (2), and cDNA3 (4), and cDNA4 (4) represent 2 and 4 μL of RNA used in the cDNA reaction, respectively. A no-template control was run for each target miRNA and in each case, returned a mean raw Cq of 0 (data not shown)



B

	Raw Cq 2 µL RNA A				Raw Cq 4 µL RNA				
	cDNA1 (2)	cDNA2 (2)	**2 µL RNA raw Cq mean**	SD	cDNA3 (4)	cDNA4 (4)	**4 µL RNA raw Cq mean**	SD	**ΔCq**
miR-142-3p	31.74	31.72	**31.73**	0.01	30.77	30.49	**30.63**	0.20	**1.10**
miR-451a	26.88	25.21	**26.05**	1.18	25.15	24.27	**24.71**	0.62	**1.34**
miR-23a-3p	31.21	30.54	**30.88**	0.47	29.41	29.73	**29.57**	0.23	**1.31**
miR-30c-5p	33.96	34.42	**34.19**	0.33	33.22	32.43	**32.83**	0.56	**1.36**
miR-103a-3p	34.16	32.92	**33.54**	0.88	32.16	31.63	**31.90**	0.37	**1.65**
miR-191-5p	34.74	33.65	**34.20**	0.77	32.45	32.53	**32.49**	0.06	**1.71**

Figure 2: Quality control assays were run to optimize the volume of RNA to use in the cDNA reaction. hsa-miR-142-3p, hsa-miR-451a, hsa-miR-23a-3p, hsa-miR-30c-5p, hsa-miR-103a-3p, and hsa-miR-191-5p are used to measure each sample miRNA signal. We report raw Cqs >1 (which corresponds to a doubling of template in a 100% efficient reaction) between cDNA synthesized with 2 versus 4 µL RNA. Since we planned to assay some reported low-abundance miRNA, we selected 4 µL RNA for all downstream cDNA reactions. cDNA was synthesized using the miRCURY LNA RT Kit (Qiagen #339340), and qPCR of the sample signal miRNAs was run using miRCURY LNA miRNA SYBR individual PCR Assays according to the conditions described in materials and methods. These miRNAs were chosen to allow for comparison to previous experiments [11]. cDNA1 and cDNA2 were synthesized using 2 µL RNA, and cDNA3 and cDNA4 were synthesized using 4 µL RNA. Each raw Cq data point represents the miRNA signal from two separate T−N EV extractions ($n = 2$) from human plasma as described in materials and methods. A NTC was run for each target miRNA and in each case, returned a mean raw Cq of 0 (data not shown)

Column. QC spike-ins and six sample signal miRNAs were quantitated using qPCR, as described in materials and methods. As expected, we report raw Cq differences in the signal for the RNA extraction spike-ins since we added twice as much to the cDNA reaction (Fig. 1A and B). Conversely, but also as expected, we report no difference in the cDNA synthesis spike-ins since these were added directly to the reaction mixture in equal quantities (Fig. 1A and B).

To further check the optimal volume of RNA to use in the cDNA reaction, we conducted additional QC targeting six sample signal miRNAs, hsa-miR-142-3p, hsa-miR-451a, hsa-miR-23a-3p, hsa-miR-30c-3p, hsa-miR-103a-3p, and hsa-miR-191-5p. The smallest difference in Cq between 2 and 4 µL RNA was 1.10 for hsa-miR-142-3p, and the highest was 1.71 for hsa-miR-191-5p (Fig. 2A and B).

To maximize the likelihood of observing a signal for predicted low-abundant miRNAs, we used 4 µL RNA for all downstream cDNA synthesis reactions.

Column comparison

Spike-in controls

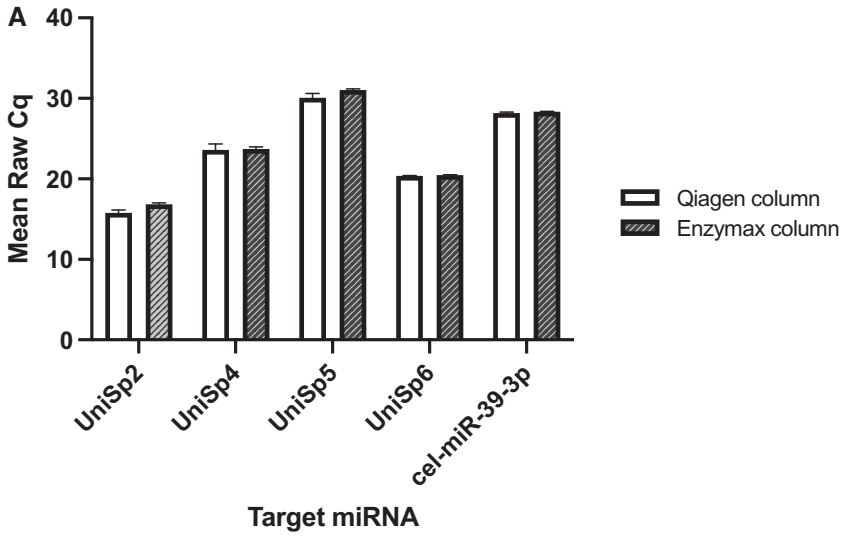
Spike-in controls were added at two stages: (i) RNA extraction (UniSp2, 4, and 5) and (ii) cDNA synthesis (UniSp6 and cel-miR-39-3p).

For the RNA extraction spike-ins, we report a difference in Cqs of 1.05 for UniSp2 and 0.97 for UniSp5 between column types (Fig. 3A and B), where the Qiagen RNeasy MinElute Spin Columns returned a lower Cq (indicating there was more miRNA in the qPCR reaction). In theory, each PCR cycle doubles the amount of amplicon in a reaction. Hence, a Cq difference of 1 equates to a doubling of material, assuming the qPCR reaction is 100% efficient. However, these differences were not significantly different (Fig. 3B), and we report no other significant differences for the RNA extraction spike-in controls (Fig. 3).

We also reported no significant difference in Cqs between column types for UniSp6 and cel-miR-39-3p (Fig. 3) for the cDNA synthesis controls.

Sample miRNA signal

Although exosomes are reported to contain mRNA, we have had difficulty using standard RNA quality/quantity methods (such as a bioanalyzer or spectrophotometer) to accurately quantitate RNA from our EV samples. Thus, to quantitate the amount of RNA extracted by the different columns, we selected five miRNAs representative of the sample miRNA signal [11]. Some of these targets are predicted to be found in low abundance and others in high abundance (Fig. 4). We then conducted qPCR on the cDNA synthesized from RNA extracted using the Qiagen RNeasy MinElute Spin columns or the Enzymax RNA Tini Spin columns. We report no difference in the mean raw Cqs for the five-sample signal miRNAs between different column types (Fig. 4A and B), suggesting the columns were equally efficient at extracting these miRNAs.



B

	Mean raw Cqs						
	Qiagen column	SD	Enzymax column	SD	ΔCq	p-value	Significance
UniSp2	15.79	0.37	16.85	0.18	-1.05	0.10	NS
UniSp4	23.62	0.72	23.70	0.27	-0.08	0.40	NS
UniSp5	30.09	0.54	31.06	0.11	-0.97	0.10	NS
UniSp6	20.38	0.04	20.49	0.03	-0.12	0.10	NS
cel-miR-39-3p	28.18	0.12	28.35	0.02	-0.17	0.10	NS

Figure 3: EVs were extracted as described previously (see materials and methods) and RNA was extracted from 50 μL of the T−N fraction and used for column comparison. 1 μL of a mix of synthetic UniSp2, 4, and 5 was added to each 700 μL lysis buffer during RNA extraction to control for extraction efficiency. 0.5 μL each of UniSp6 and cel-miR-39-3p was added to each reaction prior to cDNA synthesis to control for reverse-transcription efficiency. Following cDNA synthesis, spike-in Cqs were measured using miRCURY LNA SYBR green assays and a single qPCR analysis. Mean raw Cqs are reported. NTCs were run concurrently for each target miRNA and returned a mean raw Cq of 0 (data not shown). Each Cq represents three separate exosome preparations from the same sample ($n = 3$). Unpaired nonparametric Mann–Whitney tests were conducted using GraphPad Prism 9.11, where $P < 0.05$ was set as statistically significant

Medium and low-abundance miRNA

We have previously reported medium- to low-abundance miRNA in NEE [11]; thus, we are particularly interested in determining any differences in the extraction efficiency of these miRNAs between the two column types. We selected nine miRNA, eight of which we have previously examined [11] and measured raw Cqs in the cDNA synthesized from RNA extracted using the Enzymax RNA Tini Spin or Qiagen RNeasy MinElute Spin columns. We report no significant differences in the levels of the medium- to low-abundance miRNAs (Fig. 5A and B), suggesting that the two columns are equally efficient at extracting low-to-medium abundance miRNA.

Discussion

The 50 μL T−N samples used in these comparison experiments represent technical replicates aliquoted from the same EV preparation. From three separate human plasma sample EV preparations, we processed two technical replicates from each, for downstream analysis. The six 50 μL aliquots (2 × 3 separate sample preps) were processed in exactly the same manner, *except* three were processed using the Qiagen RNeasy MinElute Spin columns and the other three, the Enzymax RNA Tini Spin column. For this reason, we consider raw mean Cqs a suitable method for comparison of miRNA levels across the different columns and we did not conduct further normalization of these samples.

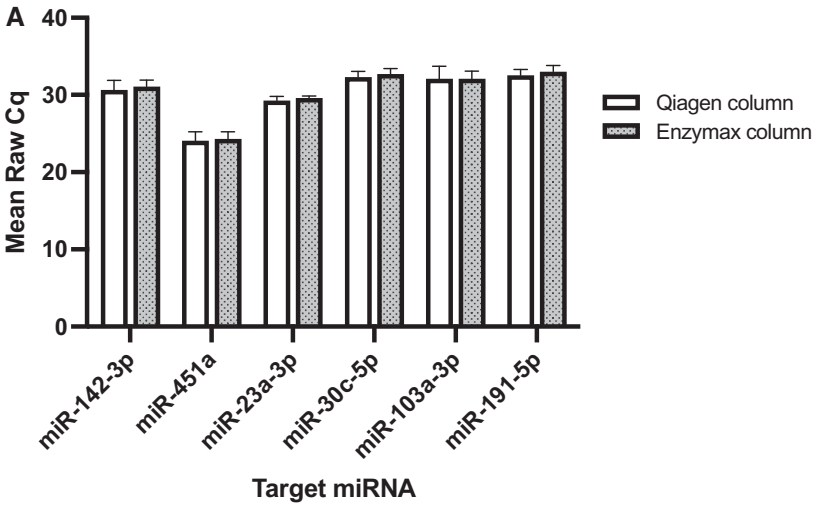
Cqs of QC miRNAs in T−N were not different in cDNA isolated using Qiagen versus Enzymax columns

Owing to the lack of affordability and availability of Qiagen RNeasy MinElute Spin columns independent of a kit, we compared the efficiency of RNA extraction with a purported equivalent, the Enzymax RNA Tini Spin columns. Qiagen RNeasy MinElute Spin columns come in various kits ranging in cost from USD380 to greater than USD1000, whereas Enzymax RNA Tini Spin columns can be bought separately in packs of 50 for USD56. Unlike the Qiagen RNeasy MinElute Spin columns, which are not sold separately, the lysis and wash buffers required to conduct the total RNA extraction protocol that retains short RNA and described herein can be purchased separately from Qiagen.

We report no significant difference in mean raw Cqs for any of the synthetic extraction spike-ins between the two brands of RNA extraction columns (Fig. 3A and B). Similarly, there was no significant difference in the mean raw Cqs for six sample miRNA signal genes, hsa-miR-142-3p, hsa-miR-451a, hsa-miR-23a-3p, hsa-miR-30c-5p, hsa-miR-103a-3p, and hsa-miR-191-5p (Fig. 4A and B).

Cqs of low- to medium-abundance miRNA in T−N were not different in cDNA isolated using Qiagen versus Enzymax columns

We compared mean raw Cqs in cDNA samples synthesized using 4 μL RNA extracted from T−N using either the Qiagen



B

	Mean raw CQs						
	Qiagen column	SD	Enzymax column	SD	ΔCq	p value	Significance
miR-142-3p	30.65	1.24	31.09	0.83	-0.44	0.700	NS
miR-451a	24.08	1.14	24.33	0.90	-0.25	0.700	NS
miR-23a-3p	29.29	0.53	29.60	0.26	-0.31	0.700	NS
miR-30c-5p	32.33	0.71	32.70	0.71	-0.37	0.800	NS
miR-103a-3p	32.09	1.61	32.07	1.03	0.02	>0.999	NS
miR-191-5p	32.55	0.75	33.02	0.80	-0.47	0.400	NS

Figure 4: EVs were purified as described previously (see materials and methods), and RNA was extracted from 50 μL of the T−N fraction, cDNA synthesized, and used for column comparison. Six miRNAs with variable predicted abundance were selected for determination of sample miRNA signal (as a measure of how efficient the RNA extraction reaction was). Following cDNA synthesis, sample signal miRNA Cqs were measured using individual miRCURY LNA SYBR green assays and a single qPCR analysis. Mean raw Cqs are reported. NTCs were run concurrently for each target miRNA and returned a mean raw Cq of 0 (data not shown). Each mean Cq represents three separate exosome and RNA preparations from three different plasma samples ($n = 3$). We report no significant difference for mean Cqs of target miRNAs, suggesting the Qiagen and Enzymax columns are equally efficient at extracting these miRNAs. Unpaired nonparametric Mann–Whitney tests were conducted using GraphPad Prism 9.11, where $P < 0.05$ was set as statistically significant

RNeasy MinElute Spin columns or Enzymax RNA Tini Spin columns. We report no difference in any of the miRNAs we targeted (Fig. 5A and B).

In the experiments described here, we used Qiagen RNeasy MinElute Spin Columns from the more expensive Qiagen exoRNeasy Midi kit (#77144). However, we note that the chemistry of the RNeasy MinElute Spin Columns found in other Qiagen kits is not different. For example, the Qiagen RNeasy MinElute clean-up kit (#74204) also comes with 50 RNeasy MinElute Spin Columns (but with different buffers than those used here) and has a list price of USD380. In addition, the miRNeasy micro kit (#217084) also comes with 50 RNeasy MinElute Spin Columns, plus buffers and Qiazol lysis reagent and has a list price of USD424.

We learned that a combination of wash buffers and the ethanol volume and concentration used in the extraction protocol determines whether short RNA species are retained on the column. Specifically, diluting the RNA aqueous phase collected after phase separation using Qiazol (Step 11 in the exoRNeasy Midi/Maxi Handbook available at www.qiagen.com/HB-2630) with two volumes of 100% ethanol adjusts the binding conditions of the column to retain short RNA species.

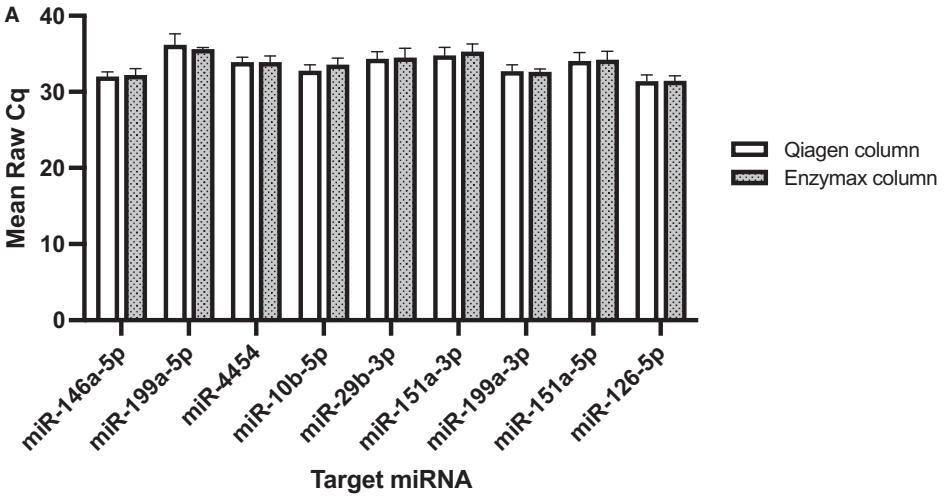
Considering this, it is entirely possible that other manufacturer's RNA extraction spin columns (e.g. syd labs, Kopkinton, MA, USA, Spin Column for DNA/RNA Tiniprep, #MB0110S pack of 50 or #MB0110L pack of 200) would also work with the protocol described herein. However, researchers should be careful to choose spin columns manufactured specifically for RNA use, (e.g. Qiagen also sells DNA clean-up kits containing "MinElute Spin Columns" but these are not designated "RNeasy," thus are not suitable for RNA applications). We recommend researchers conduct a similar comparison, using the RNA extraction protocol described here, with their current spin columns prior to putting precious samples on an unknown entity.

Conclusion

We compared the efficiency of RNA extraction by Enzymax RNA Tini Spin columns with Qiagen RNeasy MinElute Spin Columns by measuring RNA extraction QC spike-ins (UniSp2, 4, and 5), cDNA synthesis spike-in controls (UniSp6 and cel-miR-39-3p), six sample signal miRNAs, and nine low- to medium-abundance miRNA using the same extraction protocol.

We report no significant difference between columns for any of the synthetic spike-ins. We observed differences close to one Cq for UniSp2 and Unisp5, and this equates (in theory) to doubling of the amplicon in the reaction, but this was not statistically significant.

We report no difference in the Cqs for any sample signal miRNAs or the low- to medium-abundance group (Figs 4 and 5).



B

	Mean raw CQs						
	Qiagen column	SD	Enzymax column	SD	ΔCq	p-value	Significance
miR-146a-5p	32.02	0.60	32.22	0.84	-0.20	>0.99	NS
miR-199a-5p	36.18	1.44	35.61	0.22	0.57	0.70	NS
miR-4454	33.92	0.64	33.90	0.80	0.02	>0.99	NS
miR-10b-5p	32.79	0.77	33.59	0.84	-0.80	0.20	NS
miR-29b-3p	34.36	0.92	34.52	1.18	-0.16	>0.99	NS
miR-151a-3p	34.77	1.09	35.29	0.99	-0.52	0.70	NS
miR-199a-3p	32.71	0.85	32.64	0.39	0.07	0.70	NS
miR-151a-5p	34.05	1.09	34.20	1.11	-0.15	>0.99	NS
miR-126-5p	31.40	0.83	31.44	0.69	-0.04	>0.99	NS

Figure 5: EVs were purified as described previously (see materials and methods), and RNA was extracted from 50 μL of the T−N fraction and used for column comparison. Nine miRNAs with medium to low abundance were analyzed. Following cDNA synthesis, target miRNA Cqs were measured using individual miRCURY LNA SYBR green assays and a single qPCR analysis. Mean raw Cqs are reported. NTCs were run concurrently for each target miRNA and returned a mean raw Cq of 0 (data not shown). Each mean Cq represents three separate extracellular vesicle preparations and RNA extractions from three different plasma samples ($n = 3$). We report no significant differences between the mean Cqs for any miRNA, suggesting the Qiagen and Enzymax columns were equally efficient at extracting low- to medium-abundant miRNA. Unpaired nonparametric Mann–Whitney tests were conducted using GraphPad Prism 9.11 where $P < 0.05$ was set as statistically significant

In summary, the Enzymax RNA Tini Spin columns showed similar performance to the Qiagen RNeasy MinElute Spin Columns for the miRNA we examined. Therefore, we conclude that the RNA Tini Spin columns represent a suitable substitute for the Qiagen RNeasy MinElute Spin Columns using the protocol described herein, when there is a shortage, or the researcher does not want to purchase a kit. However, we recommend that researchers conduct their own QC to assess the suitability of these columns for their specific target short RNA species on a case-by-case basis.

Data availability

Data that support the findings of this study will be made available upon reasonable request from the corresponding author, R.A.D.

Author contributions

S.A.B. designed and conducted the extraction of the EVs. R.A.D. designed and performed the RNA extractions, the qPCR, and was the primary author of the manuscript and conducted the statistics. P.A.C. devised the exosome project. All authors contributed to the writing of the manuscript.

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Conflicts of interest

The Brain Chemistry Labs have submitted a patent application for the miRNA fingerprint described in Banack *et al.* [11].

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