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Assessment of Cell-Free Nucleic Acid Isolation Efficiencies for Liquid Biopsy Research

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ABSTRACT

Cell-free nucleic acids (cfNA) originate from two primary sources – via cell lysis, or secretory pathways.[1,2] Previous efforts have revealed that stages of disease progression are associated with changes in the profile and abundance of cfNA in various biofluid samples. Consequently, the use of cfNA as biomarkers could facilitate the early detection of diseases such as cancer and enable simple, specific monitoring of disease progression.[1,3] The emergence of relatively non-invasive liquid biopsy procedures as an alternative to surgical biopsies has fueled an intensive research effort and investment into the detection of cell-free disease biomarkers. Here, we discuss the development of this field and the challenges associated with extracting and identifying nucleic acids from cell-free biofluids. Using state-of-the-art cfNA extraction and library preparation methods, we have established a workflow for small RNA biomarker identification, which couples the *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit (Zymo Research) to the RealSeq-Biofluids Library Prep Kit (SomaGenics). Combined, these protocols improve total cell-free nucleic acid yield, microRNA capture, small RNA alignment to the established microRNA reference set (miRBase.org) by up to 100-fold, and increases the diversity of detectable species by up to 4-fold over alternative workflows.

INTRODUCTION

There is significant potential for liquid biopsy analysis to revolutionize clinical disease diagnoses and prognoses. Commonly, patient blood plasma is collected and analyzed for cell-free nucleic acids (cfNA) which consists of cell-free DNA (cfDNA) and cell-free RNA (cfRNA) molecules. These nucleic acids originate from necrotic and apoptotic cells from various tissues, or are secreted by live cells contained in microvesicles, such as exosomes, to communicate with cells at distant sites.[1,4] Cell-free nucleic acid abundance can be influenced by an individual's health, the level of nucleases in the blood, and the physiological clearance rate of cfNA by the liver and kidney.[5] Certain populations of cfNA are protected from degradation in the circulatory system by binding to proteins such as histones and RNA-processing proteins like AGO2.[2,6] Exosomes actively secreted from living cells also protect cfNA in blood and are found to have physiological functions, such as increasing cell growth or reprogramming target cells at distant sites during cancer metastasis.[7,8]

The presence of certain cell-free nucleic acids correlate with various health conditions and have the potential to enhance the efficacy of clinical diagnosis by identifying specific diseases based on cfNA profiles. The relatively non-invasive nature of simple blood draw or biological fluid collection allows for straightforward disease monitoring, which may otherwise require complicated, site-restrictive, and invasive surgical biopsies. Repeated sampling over time by liquid biopsy is practical and near painless for both patients and physicians and will likely facilitate more effective and widespread screening during routine health exams. All-in-all, these analyses would provide a more comprehensive view of patient health status for physicians to evaluate during diagnosis and treatment.

Plasma cfDNA has already had a profound impact on prenatal genetic testing. Currently, maternal plasma cfDNA allows for prenatal genetic testing of fetal genetic material that is shed into the maternal blood stream.[9] This test is significantly safer and easier compared to amniocentesis, which poses a higher health risk for both the mother and the fetus. The success of using fetal cfDNA profiles to diagnose genetic diseases prompted scientists to survey the potential of plasma cfDNA biomarkers for the diagnosis and monitoring of other diseases such as cancer and cancer staging.[3] Active ongoing academic efforts, and overall investment of more than $1.3 billion USD into biotech companies such as Grail, Guardant Health and Foundation Medicine[10-12], are bringing routine liquid biopsy screening closer to reality.

However, there still exist challenges to face in the field of liquid biopsy. Although the amount of cfNA can significantly increase during certain diseases,[5] typical cfDNA and cfRNA levels in healthy individuals are low. Values range from 1-100 ng per 1 ml of plasma, with an average of 30 ng, which poses several technical challenges to analysis. First, the low amount of bulk cfNA currently necessitates large sample volumes to obtain sufficient levels of cfNA for assay sensitivity. Secondly, high plasma protein content and the low molecular mass of most cfNA[2,6] complicates the removal of impurities and efficient cfNA recovery. Finally, current protocols for cfNA biomarker analysis by techniques including Next-Generation Sequencing and qPCR have much room to improve.[1,13-16] To tackle these issues, Zymo Research has partnered with SomaGenics, Inc. to develop and optimize technologies to efficiently isolate both cfDNA and cfRNA from plasma and other challenging biological samples. The Zymo Research *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit is capable of much higher yields from even lower sample volumes, and when combined with the improved efficiency of small RNA library preparation in the SomaGenics RealSeq-Biofluids Kit, read alignment of small cfRNA improves by up to 100-fold, and increases the diversity of detectable species by up to 4-fold over alternative workflows.

RESULTS

Assessment of Cell-Free RNA Yields

To compare cell-free RNA recovery efficiencies between currently available solutions, the following kits were tested: The Zymo Research *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit, the Qiagen QIAamp Circulating Nucleic Acid Kit (CNA; microRNA protocol), and the Qiagen miRNeasy Serum/Plasma Advanced Kit (miR Adv). These three kits were used to isolate cfRNA from the same plasma sample. A range of input volumes were isolated, including the maximum input volume for each kit. Quantification of RNA yield was assessed by the Qubit™ microRNA Assay Kit (Figure 1A).

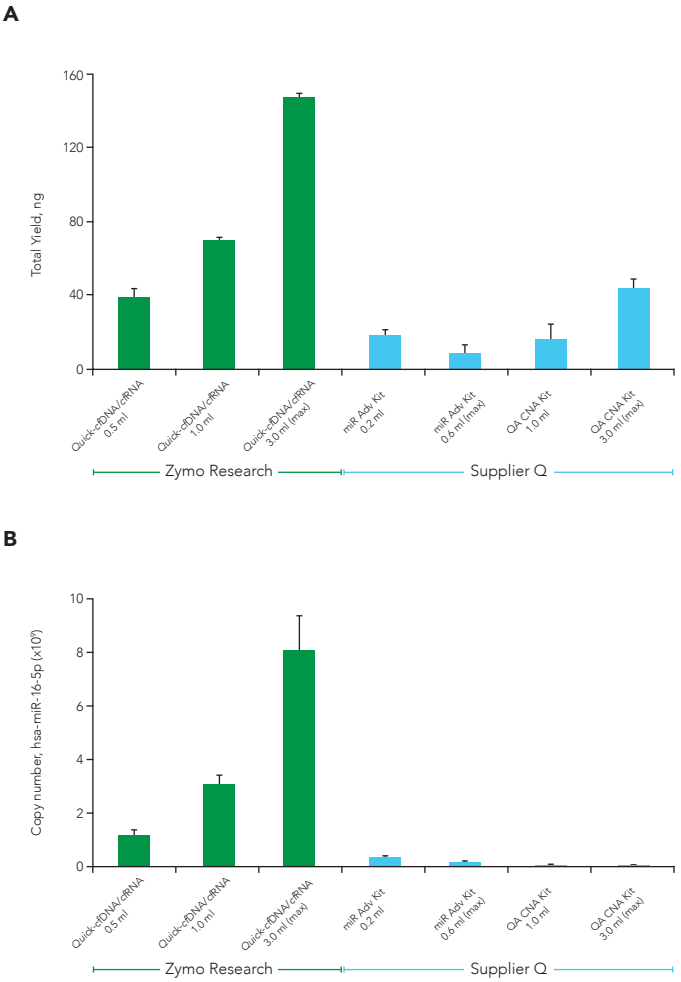


Figure 1 – Efficient and linear isolation of cell-free RNA. Total cell-free RNA was purified from plasma (single donor, 61y-F) using three different commercial kits, The *Quick-cf*DNA/*cf*RNA™ Kit (Zymo Research), miRNeasy Serum/Plasa Advanced Kit (miR Adv Kit, Qiagen) and QIAamp Circulating Nucleic Acids Kit (QA CNA Kit, Qiagen). **(A)** Total cell-free small RNA yield from each extraction kit was assessed using Qubit quantification method specific for small RNAs. Bars represent average total yield recovered in nanogram (ng). **(B)** Quantification of microRNA hsa-miR-16-5p isolated by the different extraction kits was assessed using RT-qPCR. Bars represent average copy numbers (x 10^9). For both graphs, error bars indicate sample standard deviations from two independent extractions.

The Zymo Research *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit consistently recovered higher total small RNA over all ranges of input volumes when compared to the CNA and miR Adv protocols. Roughly linear recovery of total cfNA yield (~2-fold for 0.5 ml to 1 ml and ~3-fold for 1 ml to 3 ml) was observed using the *Quick-cf*DNA/*cf*RNA™ technology. Both the miR Adv and CNA kits yielded low total cfNA levels and did not demonstrate linear recovery with sample volume input. Of note, a decrease in total yield was observed for the miR Adv kit when higher volume of sample was extracted. These results demonstrate higher total recovery of small RNA using the Zymo Research *Quick-cf*DNA/*cf*RNA™ Kit than the CNA and miR Adv kits.

To further test the observed increases in small RNA yield, we measured the abundance level of a specific microRNA, hsa-miR-16-5p, which is present in blood plasma. After cfRNA extraction by the three kits, the total copy number of hsa-miR-16-5p was measured by RT-qPCR (Figure 1B). The copy number increased linearly with input volumes using *Quick-cf*DNA/*cf*RNA™ Kit, which was not observed when using the miR Adv kit – hsa-miR-16-5p levels surprisingly decreased when the input volume was increased 3-fold to the manufacturer's recommendations. Similarly, both low hsa-miR-16-5p copy number and an absence of linear recovery were observed for the CNA kit. This may have been caused by the inability of the CNA kit to purify very small RNAs like microRNAs, which resulted in unreliable/low RT-qPCR data. Of note, we observed that Zymo Research samples quantified ~10.7-fold more hsa-miR-16-5p than miR Adv kit at similar input volumes (0.5 ml vs. 0.6 ml). Additionally, cfRNA isolated using *Quick-cf*DNA/*cf*RNA™ Kit recovered ~515-fold more hsa-miR-16-5p than the CNA kit at the 3 mL input volume.

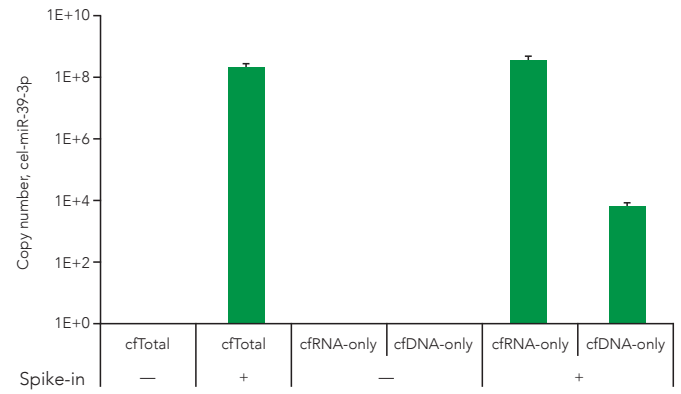
Separation of cfDNA and cfRNA from a Single Sample

Current methodologies in library preparation for Next-Generation Sequencing requires the separate isolation of cfDNA and cfRNA. Having the option to co-purify, or to purify cfDNA and cfRNA from the same sample in parallel, provides investigators the ability to control cfNA input into downstream applications. The *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit is optimized for separation of cfDNA and cfRNA from the same sample input. The 'parallel' protocol separates cfDNA and cfRNA into separate eluates, while the 'co-purification' protocol collects both cfDNA and cfRNA into the same eluate. To assess separation efficiency of cfRNA from cfDNA using the 'parallel' protocol, an exogenous microRNA spike-in and a DNA ladder were added to a plasma sample then subjected to the purification protocols. Quantitation of copy number was then assessed using RT-qPCR for the exogenous microRNA and capillary gel electrophoresis for the DNA ladder.

We first sought to test the capability of *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit to efficiently isolate cfRNA into cfRNA-only eluates without spilling into cfDNA-only eluates. A spike-in of 2.5 x 10^8 copies of an exogenous microRNA not found in mammals (cel-miR-39-3p from *C. elegans*) was added to plasma samples, mixed

with digestion buffer, purified, and then recovery was assessed by RT-qPCR. This spike-in technique is typically used to normalize sample inputs and assess processing errors.[17,18] Eluates from both the 'co-elution' and the 'parallel' protocols were quantified for cel-miR-39-3p copy number by RT-qPCR (Figure 2A). The input cel-miR-39-3p was efficiently recovered using both protocols. Eluates from both protocols showed greater than 80% recovery of cel-miR-39-3p. Moreover, less than 0.003% of the microRNA was retained in the cfDNA-only eluate from 'parallel' protocol. All negative controls lacking the added microRNA had no detectable levels of the exogenous microRNA.

A



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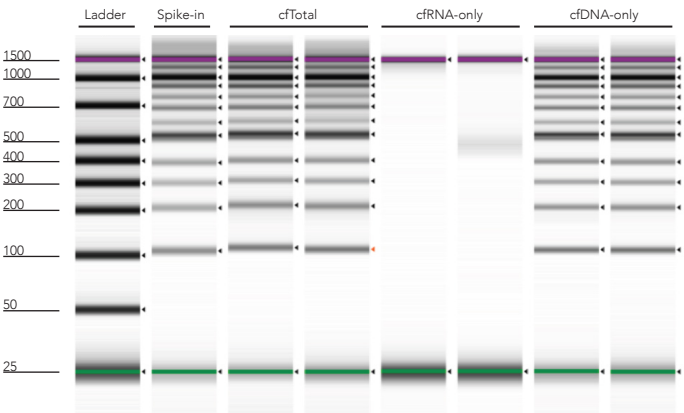

Figure 2 – Efficient separation and recovery of spike-in microRNA and DNA by the *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit. Total cell-free nucleic acids were purified from plasma (identical donor, 39y-F) using co-elution and parallel protocols from *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit. (A) Separation and recovery of spiked-in microRNA, cel-miR-39-3p, were quantitatively assessed using RT-qPCR. Values indicate the total average copy number +/- sample standard deviations from two independent extractions. (B) Separation of spike-in DNA (100 bp DNA ladder) was qualitatively assessed using capillary gel electrophoresis (TapeStation). cfTotal = eluate from 'co-purification' protocol which elutes cfDNA and cfRNA into same eluate. cfDNA-only, cfRNA-only = eluates from 'parallel purification' protocol which separates cfDNA and cfRNA into separate eluates. Spike-in = 100 bp DNA ladder spike-in.

To assess the capability of *Quick-cf*DNA/*cf*RNA™ to efficiently isolate cfDNA without contaminating cfRNA-only eluates, a DNA ladder was spiked into a plasma sample and tracked using a capillary gel electrophoresis system (Figure 2B). Approximately 50 ng of 100 bp DNA ladder was added to samples during the initial digestion step. Eluates from all samples were analyzed using DNA High-Sensitivity Tape Assay on TapeStation 2200 (Agilent). Qualitative assessment of all DNA bands showed robust recovery of added DNA in eluates from the 'co-elution' protocol and the 'cfDNA-only' eluates from the 'parallel' protocol. The cfRNA-only eluates from the 'parallel' protocol showed no detectable level of the added DNA ladder. This result confirms efficient isolation of the cfDNA into the intended cfDNA-only eluates without contaminating the cfRNA-only eluates. Efficient separation of the smallest DNA band at 100 bp size is noteworthy, since majority of the cfDNA are in the size range of 100-240 bp, with the median length of 167 bp.[6] This allows for excluding post-fractionation DNase treatments on cfRNA eluates and at the same time have the option to separately and efficiently isolate cfDNA.

Capacity to Produce Robust Cell-Free RNA Sequencing Runs

RNA Sequencing (RNA-Seq) has become one of the most important downstream applications for RNA-based investigations. The ability to profile RNA expression as well as detecting novel targets supports RNA-Seq as a crucial research tool. With cell-free RNA as input, producing robust RNA-Seq results become difficult due to multiple challenges. First, cell-free biological fluids have very low amounts of circulating cfDNA and cfRNA, making it difficult to obtain enough material above the minimum input requirements necessary for many library preparation kits. Secondly, due to their short length, microRNAs require highly optimized isolation conditions to prevent losses during purification.

MicroRNAs, which range from 20-25 nucleotides in length, have high potential to serve as disease biomarkers from biofluids.[1,13-18] Therefore, reliable and robust isolation of microRNA from cell-free samples is of great interest. To evaluate cfRNA profiles, both Zymo Research and SomaGenics independently extracted cfRNA, generated libraries, and performed RNA-Seq data. Zymo Research extracted cfRNA from 200 µl plasma derived from three different healthy donors using four different commercially available kits. SomaGenics extracted cfRNA from 200 µl plasma derived from four different healthy donors using two commercially available kits. Both prepared RNA libraries using the SomaGenics RealSeq-Biofluids Library Prep Kit. Library preparations were sequenced on the Illumina HiSeq 1500 v4 System (for Zymo Research) and Illumina MiniSeq System (for SomaGenics).

Small RNA sequencing data generated by Zymo Research, as shown in Table 1, indicates higher quality reads for *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit compared to other isolation kits. The *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit achieved an average of 4% to 44% higher usable reads than other cfRNA isolation technologies. Samples isolated by *Quick-cf*DNA/*cf*RNA™ Serum &

Plasma Kit also achieved significantly higher aligned reads to human genome (hg38) and annotated microRNA (miRBase) databases. Zymo Research averaged 32% to 57% higher hg38 alignment and 35% to 61% higher miRBase alignment (Figure 3). Moreover, the identifications of different types of small RNAs demonstrate that samples isolated using *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit contain higher proportions of microRNA than other commercial kits (Figure 4). This indicates efficient isolation all cell-free RNAs, including microRNAs, by cell-free isolation technology from Zymo Research. Observation of total number of microRNA species (defined as individual microRNA sequences with 5 or more reads) showed Zymo Research identifying a range of 61 to 393 more microRNA species compared to other isolation kits.

Kit Name	Donor Detail	Total Reads	Reads Passing Filter	% Reads Passing Filter	Reads Align to hg38	% Reads Align to h38	Reads Align to miRNA Base	% Reads Align to miRBase	microRNA Species (≥5 Reads)
Zymo Research	39F	7,916,894	7,058,146	89.15	4,730,494	59.75	4,727,725	59.72	470
	61F	6,076,424	5,224,768	85.98	2,965,305	48.80	2,903,820	47.79	377
	50M	7,507802	7,224,051	96.22	5,765,842	76.80	5,719,721	76.18	367
ApBio MagMax	39F	6,200,169	2,366,814	38.17	234,735	3.79	599	0.01	10
	61F	6,277,729	2,585,422	41.51	356,982	5.73	840	0.01	16
	50M	5,764,620	3,365,366	58.38	296,453	5.14	698	0.01	11
Qiagen CNA	39F	6,803,973	6,039,433	88.76	265,060	3.90	875	0.01	13
	61F	10,000,174	8,255,089	82.55	403,953	4.04	1,295	0.01	16
	50M	7,425,887	6,491,404	87.42	347,609	4.68	2,969	0.04	20
Qiagen miR Adv	39F	6,332,879	4,020,305	63.48	2,109,098	33.30	1,881,828	29.72	400
	61F	7,348,572	2,998,441	40.80	937,726	12.76	769,498	10.47	287
	50M	7,574,555	5,316,767	70.19	3,358,267	44.34	3,015,690	39.81	344

Table 1 – RNA sequencing result comparison of RNA isolated from four commercial kits. Total cell-free RNA from a set of three different donors were obtained using four cell-free RNA isolation products: *Quick-cf*DNA/*cf*RNA™ Serum & Plasma (Zymo Research), MagMAX Cell-Free Total Nucleic Acids (ApBio MagMax; Applied Biosystems), QIAamp Circulating Nucleic Acids (Qiagen CNA), and miRNeasy Serum/Plasma Advanced (Qiagen miR Adv). RealSeq-Biofluids Library Prep Kit (SomaGenics) was used to generate RNA sequencing library and analyzed on the HiSeq 1500 v4 System (Illumina). Number of reads and read quality assessments are summarized.

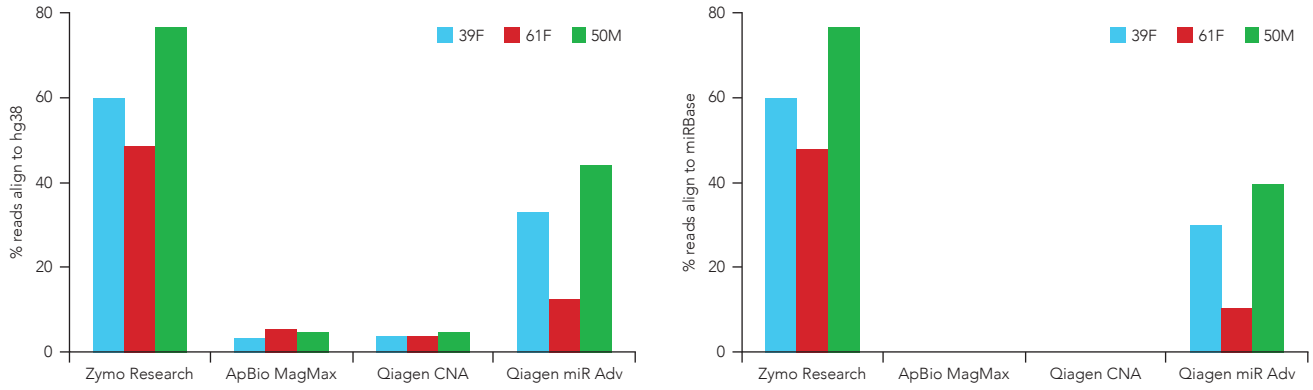

Figure 3 – Read alignment comparison of RNA isolated from four commercial kits. Total cell-free RNA from a set of three different donors were obtained using four cell-free RNA isolation products: *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit (Zymo Research), MagMAX Cell-Free Total Nucleic Acids (ApBio MagMax; Applied Biosystems), QIAamp Circulating Nucleic Acids (Qiagen CNA), and miRNeasy Serum/Plasma Advanced (Qiagen miR Adv). RealSeq-Biofluids Library Prep Kit (SomaGenics) was used to generate RNA sequencing library and ran on HiSeq 1500 v4 System (Illumina). Values (from Table 1) indicate the percentage of reads aligning to the human genome (hg38, left) and microRNA (miRBase, right).

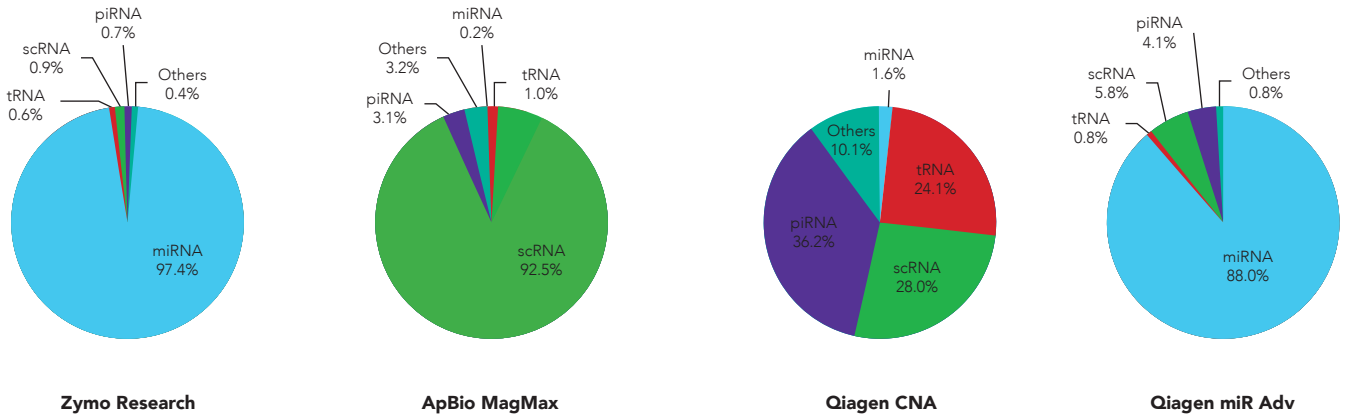

Figure 4 – Proportions of microRNA in total cell-free RNA. Total cell-free RNAs from 50 year old male donor was obtained using four different cfRNA isolation kits: *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit (Zymo Research), MagMAX Cell-Free Total Nucleic Acids (ApBio MagMax; Applied Biosystems), QIAamp Circulating Nucleic Acids (Qiagen CNA), and miRNeasy Serum/Plasma Advanced (Qiagen miR Adv). RNA types and proportion values are represented.

Independently generated small RNA isolations, library preparation and sequencing results by SomaGenics shown in Table 2 agree with data generated by Zymo Research. Higher average quality reads were observed for Zymo Research (76.6%) compared to Qiagen (58.5%). Additionally, RNAs isolated using the *Quick-cf*RNA™ Serum & Plasma Kit achieved higher average percent reads (88.8%) aligning to human reference genome (hg19) compared to Qiagen (63.9%), as well as to annotated microRNAs in miRBase (43.6% and 0.4%, respectively) (Table 2, Figure 5). This confirms efficient isolation of microRNA species by cell-free RNA isolation technology from Zymo Research.

As represented in Figure 6, Zymo Research obtained higher proportions of microRNA from total cfRNA (86.0%) compared to Qiagen (13.9%). Congruent to the small RNA-seq data, the *Quick-cf*RNA™ Serum & Plasma Kit samples achieved more diverse species of microRNA (with Zymo Research identifying 623 and Qiagen identifying 160 microRNA species that resulted in 5 or more reads). This observation is consistent with observation of the microRNA isolated using *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit. Both independent extractions and small RNA-sequencing results from Zymo Research and SomaGenics indicate overall significantly better results obtained by using cfRNA isolation technologies from Zymo Research.

Kit Name	Donor Detail	Total Reads	% Reads Passing Filter	% Reads Align to hg19	% Reads Align to miRBase	miRNAs Detected	≥ 5 Reads	≥ 10 Reads
Zymo Research *Quick-cf*RNA Serum & Plasma Kit	Donor 1	1,195,837	67.2	86.5	44.2	601	307	236
	Donor 2	1,252,927	83.7	91.6	37.3	775	420	330
	Donor 3	562,876	71.7	84.4	48.7	399	182	138
	Donor 4	1,154,655	83.9	92.8	44.2	717	382	310
	Average	**1,041,574**	**76.6**	**88.8**	**43.6**	**623**	**323**	**254**
Qiagen miRNeasy Serum/Plasma	Donor 1	1,092,135	70.5	67.3	0.9	232	86	49
	Donor 2	951,591	64.1	67.5	0.3	144	51	25
	Donor 3	775,414	60.7	61.7	0.3	159	45	30
	Donor 4	801,471	38.6	61.5	0.3	104	23	13
	Average	**905,153**	**58.5**	**63.9**	**0.4**	**160**	**51**	**29**

Table 2 – RNA sequencing result comparison of RNA isolated from two commercial kits. Total cell-free RNA from a set of four different donors were obtained using two cell-free RNA isolation products: *Quick-cf*RNA™ Serum & Plasma Kit (Zymo Research) and miRNeasy Serum/Plasma (Qiagen). RealSeq-Biofluids Library Prep Kit (SomaGenics) was used to generate RNA sequencing library and analyzed on the MiniSeq System (Illumina). Number of reads and read quality assessments are summarized.

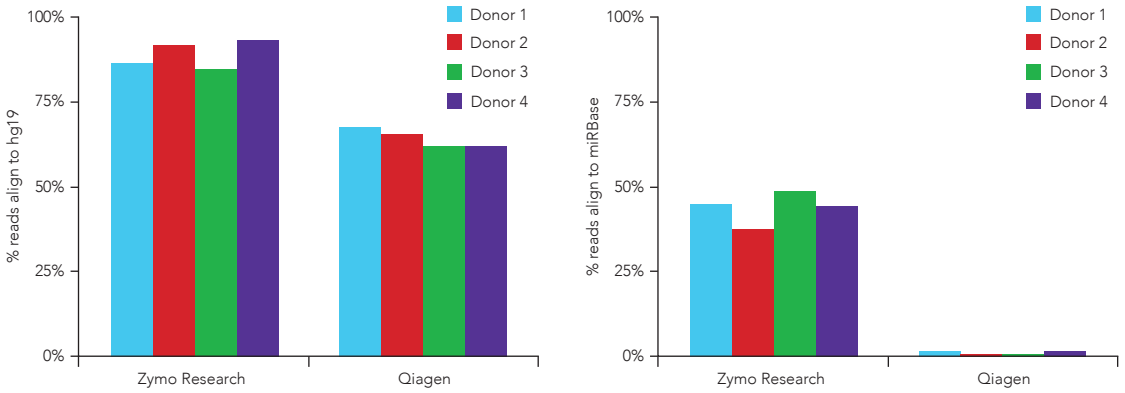

Figure 5 – Read alignment rate comparison of RNA isolated from two commercial kits. Total cell-free RNA from a set of four different donors were obtained using two cell-free RNA isolation products: *Quick-cf*RNA™ Serum & Plasma Kit (Zymo Research) and miRNeasy Serum/Plasma (Qiagen). RealSeq-Biofluids Library Prep Kit (SomaGenics) was used to generate RNA sequencing library and ran on MiniSeq System (Illumina). Values (from Table 2) indicate the percentage of reads aligning to the human genome (hg19, left) and microRNA (miRBase, right).

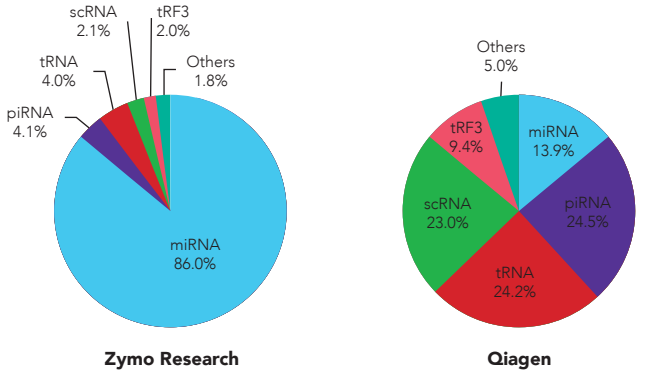

Figure 6 – Proportions of microRNA in total cell-free RNA. Total cell-free RNAs from four donors were obtained using two different cfRNA isolation kits: *Quick-cf*RNA™ Serum & Plasma Kit (Zymo Research) and miRNeasy Serum/Plasma Kit (Qiagen). RNA types and proportion values are represented.

DISCUSSION

As the liquid biopsy field grows and adopts new technologies, collection and analysis of the small amounts cell-free nucleic acids in biological fluids becomes increasingly important. Currently available isolation technologies, especially for cfRNA, lack efficient recovery. This becomes an even more significant problem when surveying for rare cell-free disease biomarkers. Inability to recover these rare species during extraction will result in missed target identification and potential misdiagnosis. Additionally, lack of biomarker detection at early stages of disease progression may be partly due to inefficient extraction technologies. The analyses shown here indicate that the *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit from Zymo Research had significant yield and quality improvements over the current most widely used product, the Qiagen QIAamp Circulating Nucleic Acid Kit (Figure 1). Moreover, *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit also outperformed the Qiagen miRNeasy Serum/Plasma Advanced Kit (Figure 1). In both cases, the *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit showed high yield recovery of cfRNA as well as abundant and diverse microRNA species. Additionally, it includes the flexibility to separate cfDNA and cfRNA into separate fractions taken from the same input sample (Figure 2). This provides an advantage over Qiagen's Circulating Nucleic Acid Kit, which can process cfDNA or cfRNA from a single sample input but lacks the versatility to co-purify or fractionate the cfNA.

The ability of nucleic acid purification technologies to produce pure and consistent DNA/RNA isolations is essential to sensitive applications like Next-Generation Sequencing. When working with biological fluids, highly effective extraction methods are required to collect total cell-free nucleic acids. Moreover, efficient isolation allows for reduced sample input needs, which is important when processing clinical samples with limited volume. As shown in Tables 1 and 2, cell-free RNA isolation technology from Zymo Research enables robust RNA sequencing results from plasma inputs as low as 200 µl of starting material. The importance of isolation technology is emphasized by comparison of extraction kits, where significant differences between isolation technologies lead to differences in yield and downstream read qualities and read alignments to established databases. Additionally, higher microRNA diversity (61 to 393 more microRNAs identified) was observed when combining the *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit with the RealSeq-Biofluids Library Prep Kit, as compared to other extraction kits. This emphasizes the importance of high cfRNA recovery rate and sensitive library preparation methods to maximize the opportunity to identify relevant rare biomarkers. Small RNA sequencing data presented in this article not only demonstrates superior results achieved by *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit but also denotes the classic importance of using the right isolation technology to yield meaningful data. With a recent increase in research and discovery efforts to characterize cfDNA and cfRNA biomarkers in biofluids, the innovative cell-free DNA/RNA purification technologies from Zymo Research and library preparations from SomaGenics will be crucial tools to obtain consistent and reliable results.

METHODS

Plasma Samples, Reagents and Kits

All plasma samples were purchased from Cardinal Biologicals. All plasma samples are derived from bloods collected in K2EDTA blood tubes and double centrifuged (1,900 x g for 30 minutes to separate plasma from blood, then another 1,900 x g for 30 minutes to further clear plasma from residual cells or debris) prior to shipping. Plasma samples were stored at -80°C. Prior to use, plasma samples were thawed at ambient temperature and centrifuged at 12,000 x g for 15 minutes at ambient temperature to remove any visible cryoprecipitate.

Spike-in microRNA cel-miR-39-3p mature sequence was obtained from mirbase.org, purchased from Integrated DNA Technologies, solvated in DNase/RNase-free water (Zymo Research), and stored at -80°C prior to use. Spike-in used 2.5 x 10^8 copies of cel-miR-39-3p per sample. The 100 bp DNA ladder (Zymo Research) was diluted in DNA Elution Buffer (Zymo Research) and stored in -20°C prior to use. A total of 50 ng of 100 bp DNA ladder spike-in was used per sample.

*Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit was obtained in-house (Zymo Research). All kit components were stored at the manufacturer's recommended temperature (ambient) prior to use. Nucleic acids were eluted in 30 µl, then immediately stored at -80°C. Eluates were thawed on ice prior to use.

QIAamp Circulating Nucleic Acid Kit was purchased from Qiagen. All kit parts were stored at the recommended temperatures (ambient and 4°C) prior to use. This kit contains several different protocols specific for isolation of cfDNA, cfRNA and input volumes, and in this short the 'Protocol: Purification of Circulating microRNA from 1 ml, 2 ml, or 3 ml Serum, Plasma, or Urine' was used to compare cfRNA recovery yield comparison. All steps were followed as the manufacturer recommended. Nucleic acids were eluted in 30 µl, then immediately stored at -80°C. Eluates were thawed on ice prior to use.

miRNeasy Serum/Plasma Advanced Kit was purchased from Qiagen. All kit parts were stored at the manufacturer's recommended temperatures (ambient and 4°C) prior to use. All steps were followed as the manufacturer recommended. Nucleic acids were eluted in 30 µl, then immediately stored at -80°C. Eluates were thawed on ice prior to use.

MagMax Cell-Free Total Nucleic Acid Isolation Kit was purchased from Applied Biosystems. All kit parts were stored at the manufacturer's recommended temperatures (ambient, 4°C, and -20°C) prior to use. All steps were followed as the manufacturer recommended. Nucleic acids were eluted in 30 µl, then immediately stored at -80°C. Eluates were thawed on ice prior to use.

Real-Time Quantitative PCR Assessment of Cell-Free RNA Recovery

Protocol from published open access methods were followed to generate primer sequences and RT-qPCR data.[19,20] Briefly, samples were thawed on ice and 3 µl of sample input was used for poly-A tail elongation and reverse transcription reactions. GoTaq® qPCR Master Mix (Promega) was used for 10 µl qPCR reactions in duplicate per sample, with primers generated using an open access software.[19] The primer sequences for hsa-miR-16-5p are (Forward) 5´ - CGC AGT AGC AGC ACG TA and (Reverse) 5´ - CAG TTT TTT TTT TTT TTT CGC CAA. The primer sequences for cel-miR-39-3p are (Forward) 5´ - GTC ACC GGG TGT AAA TCA G and (Reverse) 5´ - GGT CCA GTT TTT TTT TTT TTT TCA AG. The CFX96 Touch™ Real-Time PCR Detection System (BioRad) platform was used. Biological duplicates were used to calculate average threshold cycle and sample standard deviation values. Sample copy numbers were calculated using positive controls with known amount of copy numbers.

Fluorescence-Based Quantifications and Capillary Gel Electrophoresis

For quantification of total small RNA yield by fluorescence-based quantification, Qubit™ microRNA Assay Kit and Qubit® 3.0 instrument (Thermo-Fisher) were used per manufacturer's recommendations. For qualitative assessment of DNA separation efficiency and yield, the High Sensitivity D1000 Kit and 2200 TapeStation (Agilent Technology) were used per manufacturer's recommendations.

RNA Sequencing Library Generation and Sequence Analysis

Plasma samples from three donors were used to isolate cell-free RNA using *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit (Zymo Research), MagMax Cell-Free Total Nucleic Acid Isolation Kit (Thermo-Fisher Scientific), QIAamp Circulating Nucleic Acids Kit (Qiagen) and miRNeasy Serum/Plasma Advanced Kit (Qiagen). Libraries were prepared from cfRNA extracted from 200 µl of plasma per library. The RealSeq-Biofluids Library Preparation Kit (SomaGenics) was used to generate RNA library preps. A total of 17 PCR cycles were used for the amplification step. Library quantification was done using the KAPA Library Quantification Kit (Roche). Library preps were pooled for sequencing on HiSeq 1500 v4 System (Illumina) for Zymo Research samples and the MiniSeq System (Illumina) for SomaGenics samples. Reads were trimmed and filtered (retained ≥15 nt length) using Cutadapt [-a TGGAATTCTCGGGTGCCAAGG -O 3 -q 20 -m 15].[21] Resulting reads were then mapped using either STAR[22] (Small RNA-seq single-end pipeline) or Bowtie2 with vsl option.[23] Small RNA types were profiled using YM500v3 database.[24]

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