Enabling Accurate miRNA Biomarker Discovery from Liquid Biopsies

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Abstract

Due the lack of symptoms common to the initial stages of diseases, early detection and diagnosis is critical for successful treatment. Even in rare cases for precautionary health check-ups, diseases seldom get correctly diagnosed by inaccurate and limited clinical biopsies. Clinical biopsies are often invasive and cumbersome in nature, which make patients not only shun away but also highly impractical to do regularly. Recent advances in the field of liquid biopsies through the analysis of the cell-free nucleic acids (cfNA) hold much promise to achieve the goal of early detection. The cfNA that are released from cells throughout the body circulate in blood and may serve as biomarkers that can be used to monitor health status [1]. However, the minute amount of cfNA coupled to a lack of efficient isolation methods for cfNA serves as big hurdles for liquid biopsy research. Here we demonstrate the cfNA isolation efficiencies of two new products from Zymo Research, *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit and *Quick-cf*RNA™ Serum & Plasma Kit. We show that the kits enable up to 100-fold increased yield and up to 4-fold increased number of uniquely identified microRNA species over alternative technologies. Quantification comparison is demonstrated consistently by both RT-qPCR and fluorescence-based method (Qubit™ microRNA). In conjunction with RealSeq®-Biofluids Library Prep Kit (SomaGenics) designed for cfRNA as input, we show robust small RNA sequencing is possible from cfRNA isolated from just 200 µl of plasma. Read qualities are validated by high read alignments to both human genome (hg38) and annotated microRNA database (miRBase). Compared to alternative commercially-available technologies, both kits from Zymo Research enable greater sample input volume, provide flexibility in co-purifying or separating cfDNA and cfRNA, and are compatible with various biological fluids. In brief, coupling these isolation and library preparation technologies enable liquid biopsy research to reach a level far beyond what was possible previously.

Cell-free RNA Yield Comparison

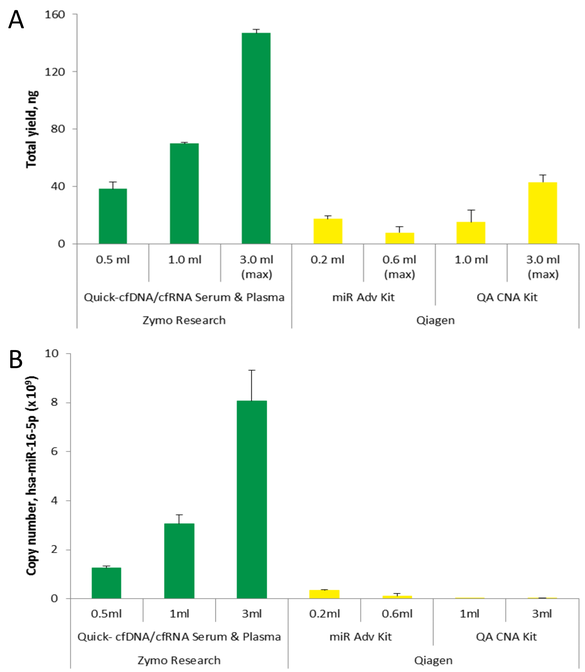

Figure 1. Efficient and linear isolation of cell-free RNA. Total cell-free RNA was purified from plasma (single donor, 61y-F) using three different commercial kits, The Quick-cfDNA/cfRNA™ Kit (Zymo Research), miRNeasy Serum/Plasa Advanced Kit (miR Adv Kit, Qiagen) and QIAamp Circulating Nucleic Acids 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[2]. Bars represent average copy numbers (x 10^9). For both graphs, error bars indicate sample standard deviations from two independent extractions.

Cell-free DNA/RNA Separation Efficiency

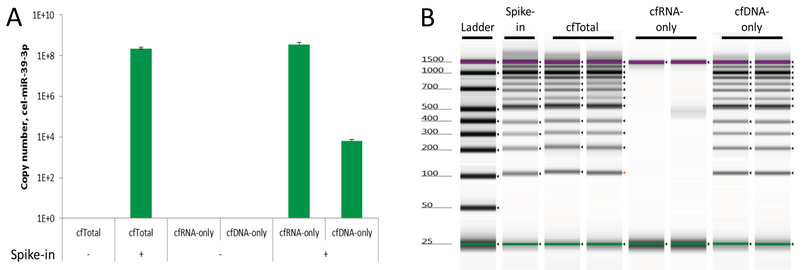

Figure 2. Efficient separation and recovery of spike-in microRNA and DNA by the *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit. Total cell-free nucleic acids were purified from plasma (identical donor, 39y-F) using co-elution and parallel protocols from Quick-cfDNA/cfRNA™ Serum & Plasma Kit. **(A)** Separation 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.

NGS Compatibility: *Quick-cf*RNA™ Serum & Plasma Kit

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	Average	1,041,574	76.6	88.8	43.6	623	323	254
Qiagen miRNeasy Serum/Plasma	Average	905,153	58.5	63.9	0.4	160	51	29

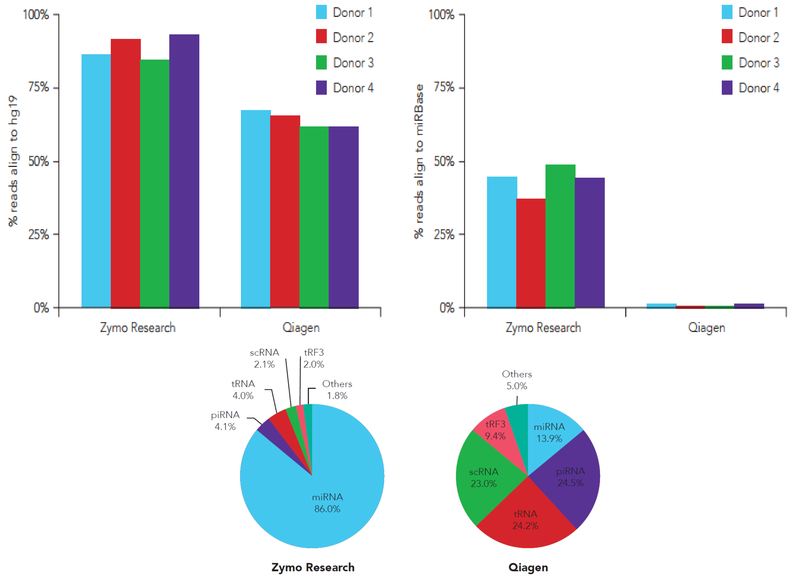

Figure 3. RNA sequencing result comparison of RNA isolated from two commercial kits. Total cell-free RNA from a set of four different donors were obtained using two cell-free RNA isolation products: *Quick-cf*RNA™ Serum & Plasma Kit (Zymo Research) and miRNeasy Serum/Plasma (Qiagen). RealSeq-Biofluids Library Prep Kit (SomaGenics) was used to generate RNA sequencing library and analyzed on the MiniSeq System (Illumina) and summarized in the table. Number of reads and read quality assessments are summarized. Values from read alignment % to human genome (hg19) and microRNA (miRBase) databases are visualized in the bar graphs. Cell-free RNA types and proportion values are visualized in the pie charts.

NGS Compatibility: *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit

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

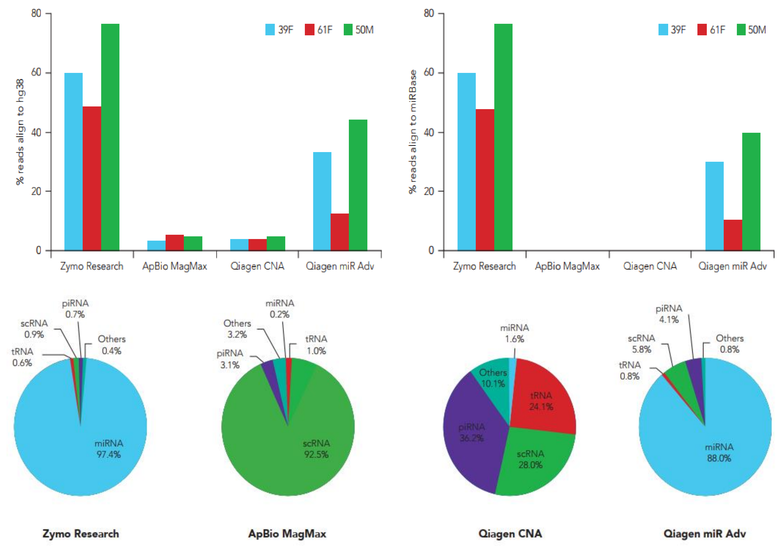

Figure 4. RNA sequencing result comparison of RNA isolated from four commercial kits. Total cell-free RNA from a set of three different donors were obtained using four cell-free RNA isolation products: *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit (Zymo Research), MagMAX Cell-Free Total Nucleic Acids (ApBio MagMax; Applied Biosystems), QIAamp Circulating Nucleic Acids (Qiagen CAN), 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) and summarized in the table. Values from % read alignment to human genome (hg38) and microRNA (miRBase) databases are visualized in the bar graphs. Proportions of microRNA and other RNA types in total cell-free RNA are visualized in the pie charts.

Conclusion

Cell-free RNA isolation from liquid biopsy samples is difficult due to inefficient nucleic acid extraction and library preparation techniques for low-input samples. By pairing the Zymo Research *Quick-cf*DNA/*cf*RNA™ Serum & Plasma Kit or *Quick-cf*RNA™ Serum & Plasma Kit and the SomaGenics RealSeq-Biofluids Library Prep Kit, a robust workflow is established for isolation and NGS analysis of cell-free nucleic acids. Assessment of yield and NGS analyses confirms these observations when compared to other commercially-available isolation technologies. Moreover, isolation of cell-free small RNAs using the Zymo Research method demonstrated higher quality reads and diverse microRNA species.

Reference:

1. Schwarzenbach H, Hoon DS, Pantel K: Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer. 2001;11:426.37.
2. Balcells I, Cirera S, Busk PK: Specific and sensitive quantitative RT-qPCR of miRNAs with DNA primers. BMC Technology. 2001;11:70.