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Characterization of Human Salivary Extracellular RNA by Next-generation Sequencing

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Abstract

BACKGROUND: It was recently discovered that abundant and stable extracellular RNA (exRNA) species exist in bodily fluids. Saliva is an emerging biofluid for biomarker development for noninvasive detection and screening of local and systemic diseases. Use of RNA-Sequencing (RNA-Seq) to profile exRNA is rapidly growing; however, no single preparation and analysis protocol can be used for all biofluids. Specifically, RNA-Seq of saliva is particularly challenging owing to high abundance of bacterial contents and low abundance of salivary exRNA. Given the laborious procedures needed for RNA-Seq library construction, sequencing, data storage, and data analysis, saliva-specific and optimized protocols are essential.

METHODS: We compared different RNA isolation methods and library construction kits for long and small RNA sequencing. The role of ribosomal RNA (rRNA) depletion also was evaluated.

RESULTS: The miRNeasy Micro Kit (Qiagen) showed the highest total RNA yield (70.8 ng/mL cell-free saliva) and best small RNA recovery, and the NEBNext library preparation kits resulted in the highest number of detected human genes [5649 – 6813 at 1 reads per kilobase RNA per million mapped (RPKM)] and small RNAs [482–696 microRNAs (miRNAs) and 190 –214 other small RNAs]. The proportion of human RNA-Seq reads was much higher in rRNA-depleted saliva samples (41%) than in samples without rRNA depletion (14%). In addition, the transfer RNA (tRNA)-derived RNA fragments (tRFs), a novel class of small RNAs, were highly abundant in human saliva, specifically tRF-4 (4%) and tRF-5 (15.25%).

CONCLUSIONS: Our results may help in selection of the best adapted methods of RNA isolation and small and long RNA library constructions for salivary exRNA studies.

The landscape, biogenesis, and function of extracellular RNA (exRNA)[14] in intercellular signaling has sparked a new paradigm of research, specifically in saliva, as it is a unique biofluid. Because saliva has several advantages, including noninvasive, easy, and repeatable

[14]Nonstandard abbreviations: exRNA, extracellular RNA; RNA-Seq, RNA-Sequencing; GITC, guanidine isothiocyanate; cDNA, complementary DNA; dUTP, 2′-Deoxyuridine 5′-Triphosphate; rRNA, ribosomal RNA; CFS, cell-free saliva; RPKM, reads per kilobase RNA per million mapped; miRNA, microRNA; tRNA, transfer RNA; tRF, tRNA-derived RNA fragment; IRB, Institutional Review Board; UCLA, University of California at Los Angeles; SOP, standard operating procedure; RT-qPCR, reverse transcription–quantitative PCR; miRNA, micro RNA; piR-NAs, piwi-interacting RNA; ddPCR, droplet digital PCR; lncRNAs, long noncoding RNAs; SPIA, single primer isothermal amplification; HOMD, Human Oral Microbiome Database; UCSC, University of California, Santa Cruz; hg19, human genome; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA.

collection, it has consistently evoked interest as an attractive source for disease detection and screening. Salivary exRNAs have become a useful biomarker source for clinical detection of local and systemic diseases such as oral cancer (1, 2), Sjögren syndrome (3), pancreatic cancer (4), and breast cancer (5).

RNA-Sequencing (RNA-Seq) is a rapidly developing new high-throughput approach to transcriptome profiling that uses deep-sequencing technologies (6, 7). Having a very low background signal, it allows unique mapping to the genome of interest with an accurate and large dynamic range of expression levels with high reproducibility (8, 9). It has a wide variety of applications, but no single analysis pipeline can be used for all biofluids (10). Specifically, RNA-Seq of saliva is challenging and differs substantially from other physiological fluids (i.e., plasma, urine, etc.) owing to its specific characteristics such as low RNA abundance, low integrity of exRNA, and high abundance of bacterial content (11–14). These features of saliva pose challenges for RNA extraction, construction of RNA libraries, RNA-Seq, and bioinformatic analysis of salivary RNA-Seq data. Therefore, all the conventional RNA-Seq methods that can be applied to other biofluids need to be tested and optimized specifically to human saliva.

A wide variety of commercial kits for RNA isolation offer reliable and reproducible means of obtaining RNA for detection by RNA-Seq. Most of these kits are based on organic extraction, silica membrane–based spin column technology or paramagnetic particle technology (15). One of the most commonly used RNA isolation methods is the phenol-guanidine isothiocyanate (GITC)–based organic extraction. However, RNA samples isolated by this method are frequently contaminated with proteins, cellular materials, organic solvents, salts, ethanol, and DNA contamination. Other methods such as silica column and paramagnetic particle-based RNA isolation systems seem to be a better solution because they are relatively simple, efficient, inexpensive, and yield total RNA with low levels of proteins and cellular contamination (16). However, they also pose increased risk for DNA contamination. Therefore, DNase digestion after RNA isolation seems to be a compulsory step (15).

Similarly, a range of complementary DNA (cDNA) library construction kits have been used with RNA-Seq (17, 18) including nonstranded protocols (in which RNA sense and antisense strand information is lost) and stranded protocols (with preserved strand information). Nonstranded protocols generally cost less and have fewer steps than stranded ones but lose critical information with regard to antisense transcription (19). Strategies to preserve transcript strand information include adaptor ligation at the RNA level (20) or single-strand cDNA (21), reverse transcription with primers containing 1 adaptor (22), or 2′-Deoxyuridine 5′-Triphosphate (dUTP) incorporation during the second strand synthesis of cDNA (17). Owing to the high abundance of ribosomal RNA (rRNA) in the total RNA preparation (over 90%), most of the RNA sequencing protocols selectively sequence poly-A-tailed mRNA transcripts or deplete rRNA (10, 18).

Several publications have evaluated and compared various kits' abilities for RNA isolation and cDNA library construction from plasma, tissue samples, etc. (15–18), but none has evaluated the ability to recover RNA from saliva. Because use of saliva is increasing in

translational and clinical applications, there is a compelling need to characterize salivary exRNA by developing optimal technical capabilities to process saliva, so it can be used properly for next-generation sequencing applications and for biomarker development.

This paper aims to compare RNA isolation efficiency from saliva with commercial kits and various commercially available cDNA library preparation kits. We also discuss the analysis of small RNAs in cell-free saliva (CFS) including novel salivary transfer RNA (tRNA)-derived RNA fragments (tRFs) and study the effects of rRNA depletion on the detection of human genes.

Materials and Methods

SALIVA COLLECTION

Unstimulated human saliva was collected after obtaining informed consent from all the participants and approval by the Institutional Review Board (IRB) at the University of California, Los Angeles (UCLA), with reference number IRB#13–001455-AM-00002. The whole saliva samples were centrifuged at 2600*g* for 15 min at 4 °C, and cell-free supernatant were treated for the concurrent stabilization of proteins and RNA by the inclusion of a protease inhibitor cocktail (aprotinin, phenylmethylsulfonyl fluoride, and sodium orthovanadate) and RNase inhibitor (SUPERase·In; Ambion) based on our saliva standard operating procedure (SOP) (23). All CFS samples were stored at −80 °C until further analysis.

RNA EXTRACTION

Six commercially available kits for total RNA isolation from CFS were compared including 3 phenol-based [miRNeasy Micro Kit (Qiagen), TRIzol® Plus RNA Purification Kit (Invitrogen), and mirVana miRNA Isolation Kit (Ambion)] and 3 nonphenol-based kits utilizing nonaggressive solvents [Quick-RNA™ MicroPrep kit (Zymo Research), QIAamp Viral RNA Mini Kit (Qiagen), and NucleoSpin miRNA kit (Macherey-Nagel)] (15) (Table 1).

For all extractions, we followed the instruction protocols of the vendors. When appropriate, the isolated RNA was treated with Turbo™ DNase I (Ambion). All kits were assessed for RNA extractions from CFS samples of 5 healthy adult individuals. The obtained RNA extracts were immediately stored at −80 °C until further use.

RNA QUANTIFICATION AND QCS

For assessing RNA yield, RNA samples extracted with different kits were analyzed with a highly sensitive Ribogreen reagent (the Quant-iT RiboGreen RNA Assay Kit; Life Technologies). In addition, RNA integrity was assessed with a 2100 Bioanalyzer (Agilent) system using the Eukaryotic RNA Pico Chip. If total RNA amount was <5 ng measured by Ribogreen library construction was not performed (RNA yield should be about 20 – 80 ng/mL CFS). Recovery of mRNAs was evaluated using reverse transcriptase quantitative PCR (RT-qPCR) as described previously (24), whereas yield of micro RNAs (miRNAs) and piwi-interacting RNAs (piRNAs) after each isolation kit was measured using droplet digital

PCR (ddPCR) with a Taqman small RNA assay (ThermoFisher Scientific) as described previously (25). Detection of an intact rRNA peak excluded the sample from further analysis, because it indicated residual cell contamination (26).

cDNA LIBRARY PREPARATIONS (SMALL AND LONG RNAs)

To evaluate the performance of available methods for cDNA library generation for RNA-Seq, we used multiple commercially available kits owing to variability in their library yield when very low amounts of RNA are available such as in CFS. We prepared libraries using 5 different cDNA library preparation kits including 3 for long RNA library generation [NEBNext Ultra Directional RNA Library Prep kit for Illumina® (NEBNext), SMARTer Stranded RNA-Seq Kit (Clontech), and Ovation® RNA-Seq System V2 (Nugen)] and 2 for small RNA library preparation [NEBNext® Multiplex Small RNA library Prep Set for Illumina (NEBNext) and Ovation Ultralow Library System V2 (Nugen)]. Whereas the SMARTer Stranded RNA-Seq Kit (Clontech) cDNA protocol is based on synthesis by random priming, the Ovation RNA-Seq System V2 (Nugen) allows cDNA synthesis by oligo(dT) and random priming. In addition, NEBNext Ultra Directional RNA Library Prep Kit for Illumina and SMARTer Stranded RNA-Seq Kit (Clontech) are based on directional RNA sequencing, crucial for analysis of long noncoding RNAs (lncRNAs). The concept is to perform the cDNA reaction and to remove 1 of the 2 strands selectively, by incorporating dUTP into the second strand cDNA synthesis reaction (18). In contrast, the NuGEN Ovation RNA-seq V2 does not provide information about strand orientation, but is based on a single primer isothermal amplification (SPIA) technology (27).

Saliva samples from 5 healthy donors (approximately 30 years old) were obtained with 2 biological replicates each. The samples were processed to produce 50 ng of total RNA as an input for each library. Importantly, predefined amount of synthetic spike-in RNAs [Exiqon Spike-in miRNA kit v2 (Exiqon) for small RNA-Seq data set and ERCC spike-in (Ambion) for long RNA-Seq data set] were added into each RNA sample equivalently, which served as internal standards to evaluate library efficiency and reproducibility, to normalize data across different samples, and to calculate absolute RNA abundance, gene detection sensitivity, and evenness of transcript coverage. For all RNA-Seq library preparation kits, we followed kit protocols.

RNA LIBRARY QC AND RNA SEQUENCING

Libraries were quantified by qPCR using the Qubit® dsDNA BR Assay Kit (Invitrogen) and assessed by use of the DNA High Sensitivity LabChip kit on an Agilent Bioanalyzer. Small RNA libraries should have a major peak of 140 –200 bp, whereas long RNA libraries should have a major peak of 300 – 400 bp. Libraries were sequenced on HiSeq2000 Illumina System using 150 base-length read chemistry in a single-end mode. In addition, the quality of the RNA-seq libraries were evaluated using fastQC and quantitated with samtools idxstats. Library complexity was examined using read uniqueness.

RNA SEQUENCING AND BIOINFORMATIC ANALYSIS OF RNA-Seq DATA

The alignment was performed using Bowtie 2 (Bowtie 2.0.2) (28) with 1 mismatch permitted in the entire length. A total of 30 –50 million single-end (50 nt) reads were

obtained for each library. RNA read counts were measured using mapping results and RNA annotation. For long RNA-Seq libraries, mapping to 16S rRNA and to the Human Oral Microbiome Database (HOMD) genome was used before mapping to the University of California, Santa Cruz (UCSC), human genome (hg19), followed by the RNA annotation to Noncode database to identify lncRNAs (29). For detection of small RNAs, alignment was done for hg19 followed by the annotation to the databases such as the following: the Rfam database for tRNA/rRNA/small nucleolar RNA (snoRNA), etc. (30), mirBase for miRNAs (31), and piRNABank for piRNAs. The RPKM values were calculated for each sample using the normalized read counts for each annotated gene [(1000 × read count) ÷ (number of gene covered bases × number of mapped fragments in million)]. The Pearson correlation coefficient of the gene expressions between the different CFS samples were calculated using the R package version 3.0.2 to assess the reproducibility of the cDNA synthesis method.

rRNA DEPLETION

RNA quantification was performed by the use of the Quant-iT RiboGreen RNA Assay Kit (Invitrogen) for 5 CFS samples of healthy adult individuals. For each sample, rRNA depletion was performed and their replicates (without rRNA depletion) served as controls. rRNA depletion was done by the use of the Ribo-Zero™ rRNA Removal Magnetic Kit for bacteria (Epicenter) followed by the RNA-Seq libraries' construction using the NEBNext kits. The goal of the Ribo-Zero rRNA Removal Kit protocol is to wash and resuspend magnetic beads, which then can bind to removal probes hybridized to rRNA, producing an RNA sample ready for library preparation. We chose this kit for use in our study because other methods, such as poly(A) depletion, do not retain noncoding, regulatory information. After obtaining the RNA-Seq data, gene coverage was analyzed with different thresholds (cutoff RPKM). Human RNA-Seq reads (hg19) in rRNA depleted and nondepleted controls were compared to determine the rRNA removal efficiency, while the yield of bacterial 16S rRNA was evaluated using RT-qPCR.

SALIVARY tRFs FROM SMALL RNA LIBRARY

RNA-Seq was performed for 4 CFS samples of healthy adults. The RNA-Seq raw reads were mapped to hg19 by means of the Bowtie 1 software version (v.1.1.2) (32) and then annotated to known small RNA databases by comparing the mapping coordinates of the read with the annotation coordinates. For tRFs, 2 databases were used: tRFdb and the Thomas Jefferson University MINTbase.

Results

EVALUATION OF exRNA ISOLATION EFFICIENCY FROM CFS WITH COMMERCIAL KITS

The quality of the extractions from CFS was assessed in terms of total RNA yield, as well as yield of long RNA (mRNA) and small RNA (miRNA, piRNA; Fig. 1). Salivary exRNA samples isolated from different methods (kits) showed similar profiles of size distribution. The majority of RNA molecules were shorter than 200 nt (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue7). The miRNeasy Micro Kit (and the NucleoSpin miRNA kit (Macherey-Nagel) showed the highest total RNA yields (70.8 and 66.3 ng/mL CFS, respectively), whereas the

Quick-RNA MicroPrep kit (Zymo Research) and the QIAamp Viral RNA Mini Kit (Qiagen) provided the lowest recovery data (23.5 and 26.1 ng/mL CFS, respectively; Fig. 1A). In terms of long RNAs [mRNAs such as glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*),[15] actin beta (*ACTB*), and ribosomal protein S9 (*RPS9*)], the highest RNA yield was achieved with the mirVana miRNA Isolation Kit (Ambion), which showed mean Ct value of 22.1 over all 3 genes. The second high yield was from Quick-RNA MicroPrep kit with mean Ct of 23.8. The other 3 kits showed similar mean Ct from 24.5–25.6, except Trizol method with mean Ct of 26.4 (Fig. 1B). However, the recovery of small RNAs was the best with the miRNeasy Micro Kit (Qiagen) and NucleoSpin miRNA kit (Macherey-Nagel). The poorest performance for miRNA and piRNA was obtained with the Quick-RNA MicroPrep kit (Fig. 1, C and D). Comparing all results, the miRNeasy Micro Kit was chosen for total RNA extraction from CFS.

EVALUATION OF PERFORMANCE OF cDNA LIBRARY PREPARATION KITS FOR RNA-Seq OF HUMAN CFS

The sensitivity of gene detection was assessed by examining the read occurrence of the known set of hg19 genes in the human CFS sample-specific small and long RNA-Seq data sets (Fig. 2, A and B). In general, all libraries yielded high-quality reads. A total of >300 miRNAs, >100 piRNAs, >300 known lncRNAs, and thousands of coding genes were identified in each library with at least 1 RPKM. The RNA abundance was highly correlated across biological replicates ($r > 0.97$), across the donors ($r > 0.90$), and across libraries ($r = 0.9535$), thus proving high reproducibility among the human CFS samples. Comparison of all library generation kits revealed that the NEBNext cDNA short and long library preparation methods performed the best, yielded the highest reproducibility, and showed the best transcriptional coverage of detected miRNAs, piRNAs, and coding genes (Fig. 2, A and B). The small RNA library contained approximately 59% bacterial exRNAs. The proportion of non-coding RNA (ncRNA) included 6% miRNAs and 7% piRNAs (Fig. 2C). Fig. 2D presents the list of top 10 miRNAs and top 10 piRNAs. The majority of the top-listed salivary piRNAs are derived from 5′ fragment of tRNA, with the cleavage site localized on the tRNA anticodon-loop with tRNA-GluCTC and tRNA-GlyGCC (Fig. 3E).

ASSESSMENT OF rRNA DEPLETION

In general, the vast majority of total RNA abundance consisted of rRNA. The RNA yield with the rRNA depletion procedure decreased to 29.2%– 47.3% (mean 38.3%) of that from the protocol without rRNA depletion (Fig. 3A). The size profile of salivary exRNA before rRNA depletion and after rRNA depletion, assessed with Bioanalyzer, is shown in Fig. 2 in the online Data Supplement. No significant change in the profile of size distribution was observed after rRNA depletion. The RT-qPCR measurements showed the relative abundance of 16S rRNA was decreased to 0.8%–7.7% (mean 4.4%) of nondepleted samples (Fig. 3B). The recovery of transcripts of 3 reference human genes (*ACTB*, *RPS9*, and *GAPDH*) after rRNA depletion was also determined with RT-qPCR. The mean yield with rRNA depletion was 79.4%–95.0% (Fig. 3C). In addition, rRNA depletion resulted in almost doubly increased gene coverage (1.26 – 1.74 times) compared with controls with a 1 RPKM cutoff

[15]Human genes: *ACTB*, actin beta; *RPS9*, ribosomal protein S9; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

value. With a threshold of 10 RPKM, the proportion of detected genes was 64.28% for rRNA-depleted CFS samples, whereas controls constituted 35.72% (Fig. 3D). Finally, substantial variations, especially in gene expression patterns, were observed among the CFS samples with and without rRNA depletion (controls). The proportion of human RNA-Seq reads was much higher in rRNA-depleted CFS samples: 41% for hg19 and trancriptome compared with 14% for controls (without rRNA depletion). In case of human lncRNAs, 13% lncRNAs were detected in rRNA-depleted CFS samples, whereas only 5% were detected in controls; for small RNAs, the differences were as follows: 14.06% of piRNAs and 1.2% of miRNAs detected for rRNA-depletion vs 10.37% and 0.47%, respectively, for controls (Fig. 3E). In addition, the microbial rRNA analysis was performed (16S rRNA). The rRNA depletion resulted in substantially increased detection of new bacterial transcripts, mean 27.75% of all raw RNA-Seq reads mapped to 16S rRNA database after rRNA depletion compared with 19% of all raw reads without rRNA depletion (control), (Fig. 4).

SALIVARY tRFs FROM SMALL RNA LIBRARY

Fig. 5 presents the breakdown of the small RNAs in human CFS samples. The percentage of tRFs is shown with respect to the total number of annotated reads including snoRNAs, piRNAs, tRNAs, small nuclear RNA (snRNAs), miRNAs, 5S rRNA, and y RNAs. Among these, tRFs constituted a mean of 15.47% of all total annotated human small RNAs, whereas for other small RNAs, the following percentages were seen: miRNAs (28.22%), piRNAs (23.6%), snoRNAs (0.07%), snRNAs (15.27%), 5S rRNAs (2.5%), and y RNAs (14.8%). The analysis of the small RNA library revealed 2 novel tRFs in human CFS: tRF-4 (4%) and tRF-5 (15.25%). The proportions of tRF-1, tRF-2, and tR-3 were negligible (Fig. 5).

Discussion

COMPARISON OF RNA ISOLATION EFFICIENCY FROM CFS WITH DIFFERENT COMMERCIAL KITS

Comparison of the 6 most commonly used commercial kits for RNA isolation from human CFS revealed that for RNA-Seq of human CFS, the use of the miRNeasy Micro Kit (Qiagen), and the NucleoSpin miRNA kit (Macherey-Nagel) seemed to be the most suitable choices, producing sufficient RNA after DNase I digestion with RNA yield at least 3-fold higher than the other methods, specifically for small RNAs. The Quick-RNA MicroPrep kit (Zymo Research) and the QIAamp Viral RNA Mini Kit (Qiagen) revealed rather poor efficiencies of RNA extraction from human CFS.

PERFORMANCE EVALUATION OF DIFFERENT COMMERCIAL KITS FOR cDNA LIBRARY PREPARATION

Comparing all library generation kits, the NEBNext library generation methods (both long and small) yielded the highest reproducibility and the highest numbers of detected small and long exRNAs. They were the simplest and the most reliable in terms of library preparation workflow and final total RNA yield. By contrast, the Ovation RNA-Seq V2 systems and specifically SMARTer Stranded library preparation methods appeared to produce less satisfactory results. These are more suitable methods when RNA quantity is not limited, but not for CFS (18, 33).

In our work, the small RNA library included 6% miRNAs and 7% piRNAs. In addition, the majority of the top-listed salivary piRNAs were derived from 5′ fragment of tRNA with the cleavage site localized on the tRNA anticodon-loop with tRNA-GluCTC and tRNA-GlyGCC. tRFs can arise through 2 main processes. The first is stress-induced tRNA cleavage (34), which is angiogenin-dependent and leads to cleavage of the tRNAs at the anticodon loop. The second is the inability of the reverse transcriptase to process cDNA of the entire tRNA owing to the presence of posttranscriptional modifications (such as methylation), that hinder efficient sequencing. The 2 most highly abundant tRF species, Glu^{CTC} and Gly^{GCC}, which made up close to 70% of the tRFs seen in the exRNA, had no methylation modification at the anticodon loop. This suggests that the majority of salivary piRNAs identified in our study and derived from 5′ tRFs were due to cleavage and most probably have important regulatory functions (35, 36).

OPTIMIZED rRNA DEPLETION INCREASES THE SENSITIVITY OF HUMAN GENE DETECTION

As expected, the vast majority of total RNA yield was made up of rRNA, which is consistent with the current literature (80%–90% of total RNA) (6, 37). Because rRNA provides little information about the transcriptome, it is reasonable to remove it to increase the amount of information obtained from RNA-Seq data (gene coverage, i.e., protein-coding genes, noncoding RNAs, snRNAs, snoRNAs, and repeat elements) (38).

There are various ways of performing rRNA depletion from total RNA including enrichment of a nonribosomal fraction, digestion of highly abundant transcripts, amplification of a non-rRNA fraction or rRNA depletion (6, 37). Those methods are based on selective hybridization of oligonucleotides to rRNA, recognition with a hybrid-specific antibody or removal of the antibody-hybrid complex on magnetic beads (6, 37).

In our study, the use of the protocol that selectively removes bacterial rRNAs [Ribo-Zero Magnetic Kit for Bacteria (Epicentre)] allowed an increase in the sensitivity of detection of human transcripts and genes (almost 50%), that points to a deeper sequencing process. This is a proof of concept that the rRNA removal step improves the comprehensiveness of human exRNA profile in saliva (39). Therefore, we strongly suggest applying this treatment before library preparation to reduce sequencing costs, particularly in salivary transcriptomics studies, for which deep coverage is needed.

Several new methods are currently being developed for separation of bacterial from human RNA. Innocenti et al. used a modified RNA-seq approach, enabling discrimination of primary from processed 5′ RNA ends to provide initial information on transcripts in *Enterococcus faecalis* (40). In turn, Ettwiller et al. developed a method, Cappable-seq, that depletes rRNA and directly enriches the 5′ end of primary transcripts. This approach enables determination of transcription start sites at single base resolution by enzymatically modifying the 5′ triphosphorylated end of RNA with a selectable tag (41). Additionally, the MICROBEnrich kit (Ambion, Thermo-Fisher Scientific) allows bacterial RNA enrichment from mixed host-bacterial RNA populations. However, at this time, the most effective way of distinguishing bacterial RNA from human RNA is at the stage of bioinformatics analysis of salivary RNA-Seq data by performing the alignment to a specific genome of interest (42).

SALIVARY tRFs

tRFs are a novel, heterogeneous class of small noncoding RNAs that in our study, constituted the third most abundant class of short RNAs in human CFS (apart from miRNAs and piRNAs). This finding may hold profound significance as tRFs are believed not to be random by-products of tRNA degradation or biogenesis, but RNAs with precise sequence structure that have specific expression patterns and specific biological roles (43). The percentage of tRFs accounted for a mean 15.47% of all total annotated human small RNAs. In addition, the analysis of the small RNA library revealed 2 novel tRFs in human CFS including tRF-4 and tRF-5 (4% and 15.25% of all annotated small RNAs, respectively). The proportions of tRF-1, tRF-2, and tR-3 were negligible (Fig. 5).

In conclusion, RNA isolation and cDNA library preparation methods may affect the outcome, analysis, and interpretation of transcriptomic data. Our results may help provide guidance in choosing the methods that are best adapted for salivary exRNA studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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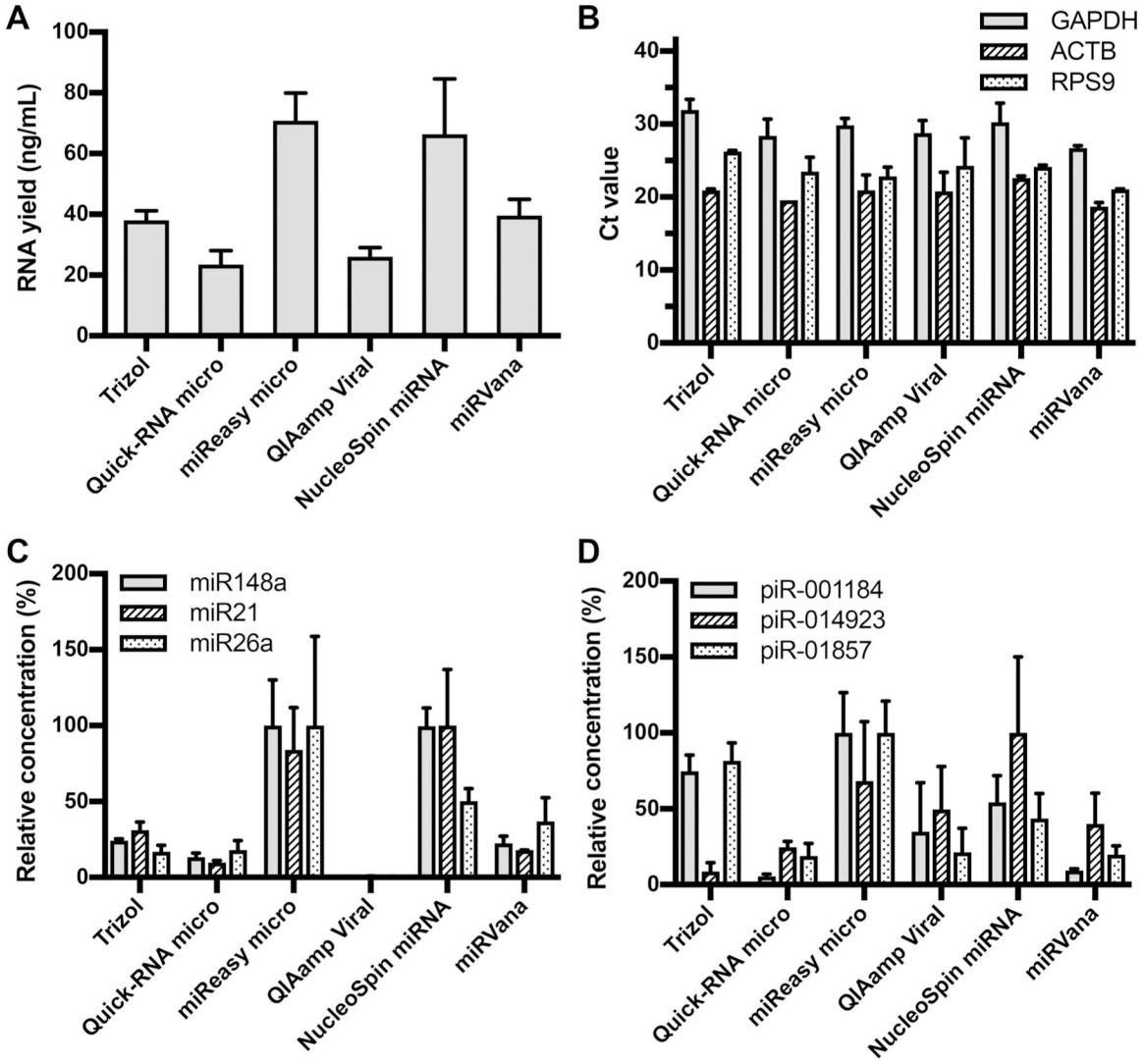


Fig. 1. Comparison of RNA isolation efficiency with commercial kits and their performance on human CFS samples.

Total RNA yield from different kits was measured with Ribogreen RNA assay (A).The recovery of mRNAs of 3 reference genes (*GAPDH, ACTB*, and *RPS9*) from different kits was determined with RT-qPCR (B). The Ct values of each gene from different kits were plotted. Recovery of miRNAs (miR148a, miR21, and miR26a) (C) and piRNAs (piR-001184, piR-014923, and piR-01857) (D) of each isolation kit was measured with droplet digital PCR(ddPCR) method. The relative yield of each small RNA was normalized by setting the highest mean copy number from 1 kit as 100%.

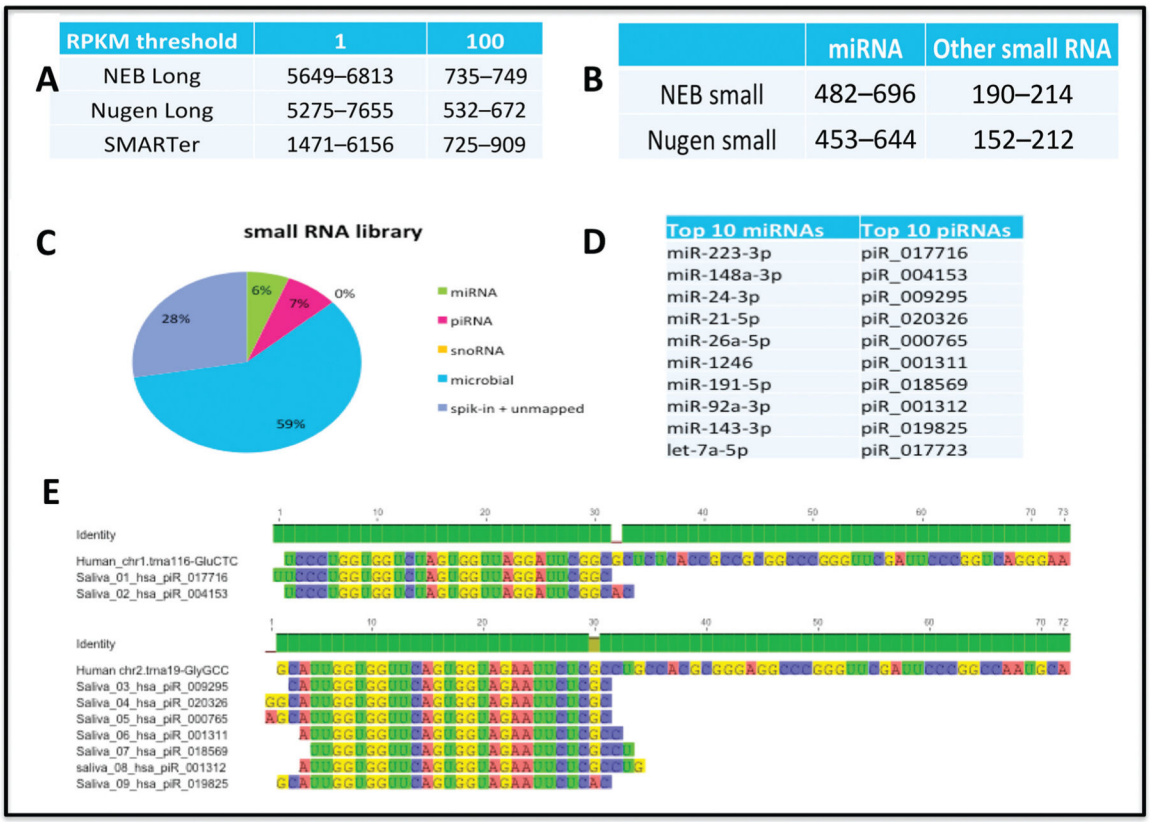


Fig. 2. Comparison of library preparation kits and their performance on human CFS samples.

Long RNA library (A) and small RNA library (B). Proportion of non-coding RNA from small RNA library (C) and lists of top 10 miRNAs and piRNAs (D). The majority of top listed salivary piRNAs are derived from 5′ fragment of tRNA (E). The cleavage site is localized on the tRNA anticodon-loop. Sequence alignment of most abundant salivary piRNAs with tRNA-GluCTC and tRNA-GlyGCC.

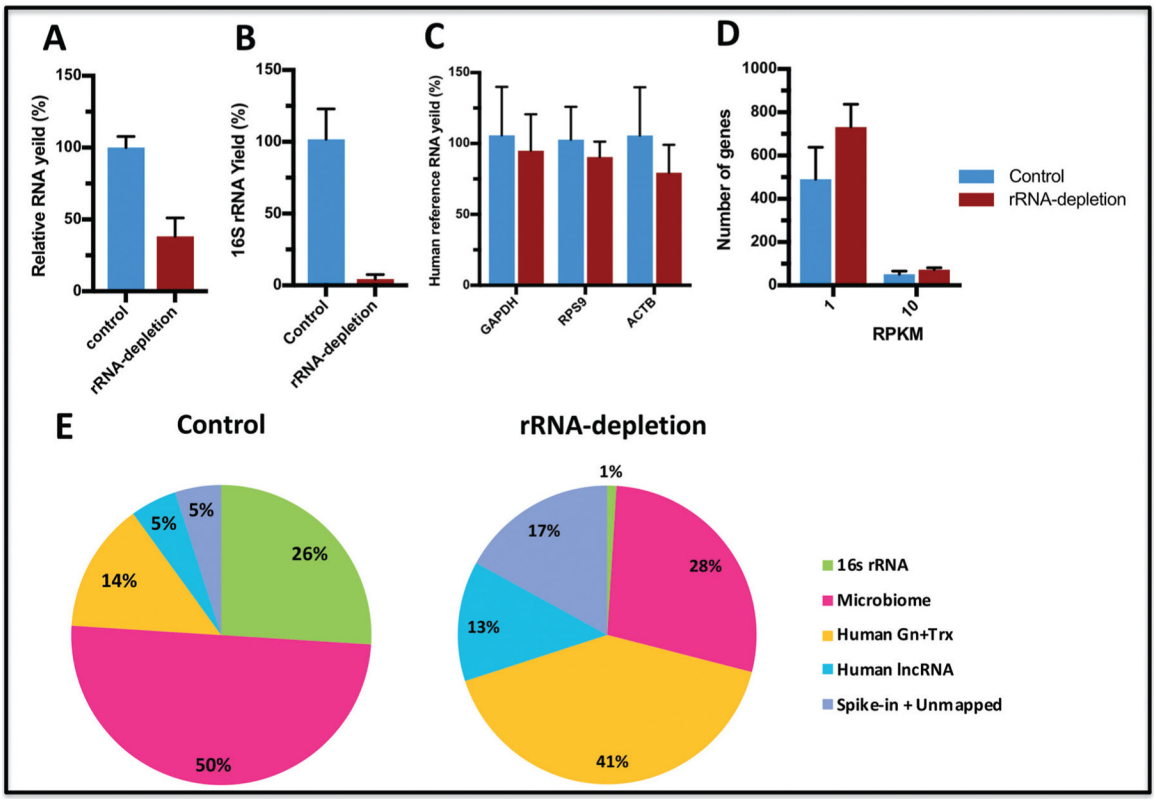
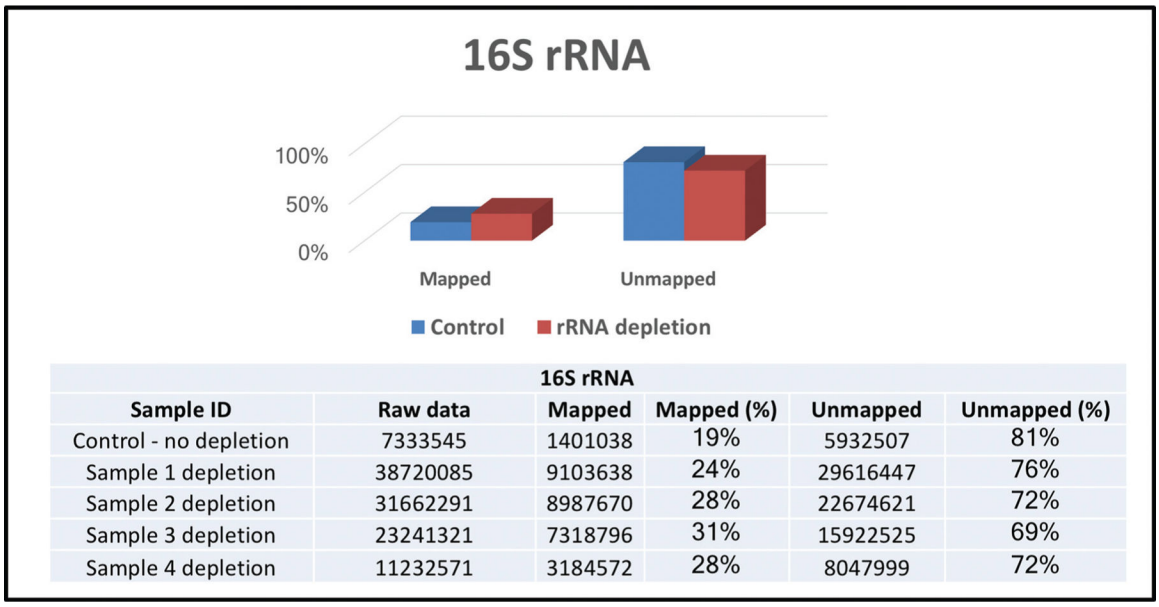


Fig. 3. Optimized rRNA depletion can increase the sensitivity of human gene detection.

Relative RNA yield after rRNA depletion measured with the Quant-iT RiboGreen RNA Assay Kit (Invitrogen) (A). The relative yield of 16S rRNA was examined with RT-qPCR (B). The relative yield of human reference RNA was measured with RT-qPCR (C). Comparison of gene coverage with different cutoff RPKM thresholds (D). Comparison of detected genes in rRNA depleted and non-depleted CFS samples for long and for small libraries (E).



16S rRNA					
Sample ID	Raw data	Mapped	Mapped (%)	Unmapped	Unmapped (%)
Control - no depletion	7333545	1401038	19%	5932507	81%
Sample 1 depletion	38720085	9103638	24%	29616447	76%
Sample 2 depletion	31662291	8987670	28%	22674621	72%
Sample 3 depletion	23241321	7318796	31%	15922525	69%
Sample 4 depletion	11232571	3184572	28%	8047999	72%

Fig. 4.
Microbial rRNA analysis (16S rRNA): rRNA depletion vs without rRNA depletion.

Proportion of small RNAs in human CFS samples

			small_RNA_species/raw read counts						
Sample No.	total reads	all_annotations	miRNA	piRNA	trf_annotated	snoRNA	snRNA	5S rRNA	y_rna
1	16636666	971369.3516	274313.1702	147130.6614	119815.8243	790.964574	151842.7137	46199.29697	231276.72
2	18494879	3441940.086	605885.3605	1455032.636	821279.1179	1173.93098	275267.9582	49412.72188	233888.361
3	27430744	282715.4454	104111.3301	37126.8	32355.5	165.459386	46344.36958	4105.310261	58506.7141
4	13916451	4474118.757	1355581.689	1071121.2	640766.7	2412.97994	941904.619	100937.1047	361394.427

Sample 1	total reads	mapped reads	all annotated	Sample 2	total reads	mapped reads	all annotated	Sample 3	total reads	mapped reads	all annotated	Sample 4	total reads	mapped reads	all annotated
tRf-4_target	19909.57	0.12%	2%	tRf-4_target	279233.1	1.51%	8%	tRf-4_target	5347.9	0.02%	2%	tRf-4_target	196881.7	1.41%	4%
U-RNA	151842.7	0.91%	16%	U-RNA	275268	1.49%	8%	U-RNA	46344.4	0.17%	16%	U-RNA	941904.6	6.77%	21%
piRNA	126355.2	0.76%	13%	piRNA	1174680	6.35%	34%	piRNA	31342.9	0.11%	11%	piRNA	871390.4	6.26%	19%
tRf-3_target	683.7952	0.00%	0%	tRf-3_target	533.8997	0.00%	0%	tRf-3_target	274.6	0.00%	0%	tRf-3_target	2342.8	0.02%	0%
piR-1	81.246	0.00%	0%	piR-1	158.066	0.00%	0%	piR-1	20.1	0.00%	0%	piR-1	261.3	0.00%	0%
Y_RNA	231276.7	1.39%	24%	Y_RNA	233888.4	1.26%	7%	Y_RNA	58506.7	0.21%	21%	Y_RNA	361394.4	2.60%	8%
tRf-2_target	31.3142	0.00%	0%	tRf-2_target	300.983	0.00%	0%	tRf-2_target	116.1	0.00%	0%	tRf-2_target	80.6	0.00%	0%
trf-5	118307.1	0.71%	12%	trf-5	815852.1	4.41%	24%	trf-5	32119.6	0.12%	11%	trf-5	633878.4	4.55%	14%
trf-3	827.9957	0.00%	0%	trf-3	5283.833	0.03%	0%	trf-3	187.2	0.00%	0%	trf-3	6518.2	0.05%	0%
tRf-1_target	49.68484	0.00%	0%	tRf-1_target	94.62452	0.00%	0%	tRf-1_target	22.7	0.00%	0%	tRf-1_target	107.6	0.00%	0%
trf-1	680.6926	0.00%	0%	trf-1	143.2164	0.00%	0%	trf-1	48.6	0.00%	0%	trf-1	370.2	0.00%	0%
mirna	274313.2	1.65%	28%	mirna	605885.4	3.28%	18%	mirna	104111.3	0.38%	37%	mirna	1355581.7	9.74%	30%
snoRNA	790.9646	0.00%	0%	snoRNA	1173.931	0.01%	0%	snoRNA	165.5	0.00%	0%	snoRNA	2413.0	0.02%	0%
5S_rRNA	46199.3	0.28%	5%	5S_rRNA	49412.72	0.27%	1%	5S_rRNA	4105.3	0.01%	1%	5S_rRNA	100937.1	0.73%	2%
piR-2	19.855	0.00%	0%	piR-2	31.9653	0.00%	0%	piR-2	2.4	0.00%	0%	piR-2	56.8	0.00%	0%
all tRFs	140490.2	1%	14%	all tRFs	1101442	5.96%	32%	all trf	38116.9	0.14%	13%	all trf	840179.4	6.04%	19%

tRf-1-4_targets are piRNAs that are predicted to be tRFs (they are not officially in the database for tRFs), while **trf1-4** were initially piRNA sequences, so their read counts are pooled in the piRNA count

Fig. 5.

Proportion of small RNAs in human CFS samples including tRFs.

Table 1.

Comparison of different RNA extraction kits.

RNA isolation kit	miRNeasy Micro Kit	TRIzol Plus RNA Purification Kit	Quick-RNA MicroPrep kit	QIAamp Viral RNA Mini Kit	NucleoSpin miRNA kit	mirVana miRNA Isolation Kit
Principle	Phenol/guanidine-based and silica membrane-based method	GITC-based method	Nonphenol-based, Zymo-Spin column technology	Nonphenol-based, silica membrane-based spin column or vacuum technology	Nonphenol-based, silica membrane-based mini spin column technology	Phenol-based, organic extraction with spin column technology (Glass Fiber Filter)
	Combined method of organic extraction and spin filter clean up	Organic extraction method	Combined method of organic extraction and spin filter clean up	Spin filter-based method	Spin filter-based method	Combined method of organic extraction and spin filter-based method
Size of RNAs	Total RNA, including miRNA and other small RNAs (smaller than ~200 nucleotides)	Total RNA	Total RNA (including small RNAs 17–200 nucleotides)	Total viral RNA greater than 200 nucleotides	Total RNA including miRNA, siRNA, shRNA[a] (small RNA: <200 nucleotides, large RNA: >200 nucleotides)	Total RNA including miRNA, siRNA, shRNA, and snRNA
Sample source	Cultured cells, various animal and human tissues	Animal and plant cells, tissue, bacteria, or yeast	Any cells (animal, blood cells, etc.), all tissues, yeast, plant, or bacteria	Plasma (treated not with heparin), serum, and other cell-free body fluids	Cultured cells, human/animal tissue, plant tissue, <150 μL reaction mixture	All cell and tissue types, bacteria, plants, viral samples, and yeast

[a] shRNA, short (or small) hairpin RNA.