Chapter 2

RNA Sequencing Analysis of Salivary Extracellular RNA

Blanca Majem, Feng Li, Jie Sun, and David T.W. Wong

Abstract

Salivary biomarkers for disease detection, diagnostic and prognostic assessments have become increasingly well established in recent years. In this chapter we explain the current leading technology that has been used to characterize salivary non-coding RNAs (ncRNAs) from the extracellular RNA (exRNA) fraction: HiSeq from Illumina® platform for RNA sequencing. Therefore, the chapter is divided into two main sections regarding the type of the library constructed (small and long ncRNA libraries), from saliva collection, RNA extraction and quantification to cDNA library generation and corresponding QCs. Using these invaluable technical tools, one can identify thousands of ncRNA species in saliva. These methods indicate that salivary exRNA provides an efficient medium for biomarker discovery of oral and systemic diseases.

Key words Saliva, exRNAs, Small and long ncRNA profiling, Biomarkers, RNA sequencing

1 Introduction

Extracellular RNA (exRNA) in human saliva is an emerging field for noninvasive diagnostic applications. The discovery of saliva-derived mRNA in normal and oral cancer patients [1–3] and other forensic applications [4, 5] opened up a new field for noninvasive molecular diagnosis. Our laboratory has extensively studied microarray-based gene profiling followed by real-time quantitative-PCR (RT-qPCR) for saliva mRNA detection. We have identified certain macromolecules associated with salivary mRNA that were protecting against ribonucleases [6]. Salivary RNA was found in complexes with lipids, proteins, lipoproteins, and phospholipids as well [7, 8]. Apoptotic bodies [9] or other vesicular structures in saliva also play a protection role. Therefore, RNA in the saliva may not be as fragile as it was previously assumed to be. Despite the numerous studies based on characterizing and finding mRNA diagnostic biomarkers in saliva, the introduction of deep sequencing technologies [10, 11] has revealed a new landscape of salivary exRNA [12]: micro-RNAs (miRNAs), piwi-interacting-RNAs (piRNAs), circular-RNAs (circRNAs), and other noncoding RNAs (ncRNAs). To date, only a few

Gregory J. Seymour et al. (eds.), *Oral Biology: Molecular Techniques and Applications,* Methods in Molecular Biology, vol. 1537, DOI 10.1007/978-1-4939-6685-1_2,

studies characterizing ncRNAs in saliva have used RNA-sequencing (RNA-Seq) technologies [13]. In this chapter, we present the detailed methodology for RNA extraction, cDNA library construction and quality controls (QCs), and data analysis of sequencing data. Although a variety of platforms are available for RNA-seq, the Illumina® platform is the most used nowadays. Increasing knowledge on salivary composition thanks to this platform will make a difference in understanding the biology of the diagnostic biomarkers found in saliva for local and systemic diseases.

The purpose of this chapter is to provide robust and reliable methods for isolating and profiling of salivary exRNA, dividing it in two main sections regarding the type of the library (small and long ncRNA libraries) constructed. We also describe a protocol for RNA extraction after saliva collection, including detailed explanations of DNase treatment, RNA precipitation for sample concentration and specific QCs for the extracted RNA. The commercial kits for RNA extraction allow high RNA yield, but the eluted RNA usually is contained in big volumes and therefore low concentrations, which is not recommended for subsequent steps, since library preparation starts with little volumes and requires a high concentration sample. Either way, the lower limit of detection of the QCs makes the RNA precipitation crucial for sample concentration, resulting in high reproducibility among samples and accurate RNA and cDNA quantification, which at the same time is translated into good quality of raw read data after sequencing. Thus, our protocols are a guide for RNA-seq of salivary exRNA, but some concepts and methodology may also be applied to other types of body fluids.

2 Materials

2.1 Saliva Collection and Processing

1. 50 mL sterile tube.
2. Laboratory vortex.
3. Refrigerated benchtop centrifuge with 50 mL tube adapters.
4. SUPERase-In RNase inhibitor, Cat# AM2694 Ambion.

2.2 RNA Sequencing of Salivary ncRNAs

1. Qiazol.
2. Chloroform.

2.2.1 RNA Isolation

3. miRNeasy micro kit.
4. Absolute ethanol.

2.2.2 DNase Treatment and RNA Precipitation

1. DNase.
2. NaOAc 3 M.
3. Glycogen.

4. Absolute ethanol.
5. Nuclease-free water.

2.2.3 RNA Quantification and QCs

1. QuanTi™ RiboGreen RNA assay kit.
2. 96-Well half area microplate (black solid plate), Cat# 3694 Corning.
3. Agilent RNA 6000 Pico kit.

2.2.4 cDNA Library Preparation

1. NEBNext® Multiplex Small RNA library Prep Set for Illumina®.
2. Exiqon Spike-in miRNA kit v2, Cat# 208041 Exiqon.

Small ncRNA Library

3. 8-Tube PCR strip.
4. Thermal Cycler PCR machine.
5. 6% Novex® TBE PAGE gel, 1.0 mM 10-well.
6. SYBR® Gold Nucleic Acid Gel Stain (Life Technologies, Inc. #S-11494).
7. Gel broker tubes, Cat#, 3388-100 SeqMatic.
8. Corning®, Costar®, Spin-X® Centrifuge Tube Filters (Cellulose Acetate Filters).
9. 3 M Sodium Acetate, pH 5.5.
10. 100 and 80% ethanol (freshly prepared).
11. QIAquick PCR purification kit.

Long ncRNA Library

1. NEBNext Ultra Directional RNA Library Prep kit for Illumina®.
2. ERCC spike-in, Cat# 4456740 Ambion.
3. NEBNext Singleplex or NEBNext Multiplex Oligos for Illumina®.
4. Actinomycin D (Sigma# A1410, dissolved in dimethylsulfoxide [DMSO] to 5 μg/μL).
5. 8-tube PCR strip.
6. 80% thanol (freshly prepared).
7. Thermal Cycler PCR machine.
8. DynaMag™-2 Magnet.
9. Agencourt® AMPure® XP Beads (Beckman Coulter, Inc. #A63881).

2.2.5 cDNA Library Quantification and QCs

1. Qubit® dsDNA BR assay kit.
2. 96-well half area microplate (black solid plate), Cat# 3694 Corning.
3. Agilent High Sensitivity DNA Kit.

2.2.6 RNA Sequencing by Illumina®

1. EB buffer.
2. Tween™ 20 Surfact-Amps™ detergent solution.
3. HiSeq2000 Illumina system.

2.2.7 De-Multiplexing and Data Processing

1. Cutadapt.
2. Bowtie mapping 16 s rRNA/Microbiome.
3. Bowtie mapping Human Genome.

3 Methods

3.1 Saliva Collection and Processing

Saliva collection from human subjects has to be approved by the Institutional Review Board.

We have used the following inclusion criteria for normal subject selection: age ≥30 years, and no history of malignancy, immunodeficiency, autoimmune disorders, hepatitis, HIV infection, or smoking.

1. Ask subjects to refrain from eating, drinking, smoking, or oral hygiene procedures for at least 1 h prior to collection.
2. Instruct subjects to rinse the mouth thoroughly with water and to void the mouth of saliva. The subject should be seated comfortably with eyes open and head tilted slightly forward. For unstimulated saliva collection subjects should rest for 5 min and minimize orofacial movements.
3. To collect un-stimulated saliva (*see* **Note 1**) allow, saliva to accumulate in the floor of the mouth and ask the subject to spit into a preweighed or graduated test tube every 60 s. Collection for 5 min usually yields sufficient saliva (~5 mL) for analysis.
4. Following collection, centrifuge saliva samples at 2600 × *g* for 15 min at 4 °C. Saliva supernatant will then be separated from the cellular phase.
5. Add SUPERase-In RNase inhibitor (at a ratio of 1 μL/mL) to 1 mL of cell-free saliva (CFS) supernatant for preserving exRNA degradation.
6. Store aliquots of 1 mL CFS at −80 °C for further analysis.

3.2 RNA Sequencing of Salivary exRNA

3.2.1 RNA Isolation

1. Thaw 4 aliquots of 1 mL of saliva, resting the tubes on ice and not for more than half an hour (*see* **Note 2**).
2. Split the sample in 500 μL of CFS and centrifuge for 5 min at 10,000 × *g* (*see* **Note 3**). Collect the supernatant to proceed with **step 2**, and discard the pellet fraction.
3. Split 0.5 mL of cell free saliva (CFS) in two tubes—250 μL in each.
4. Add 750 μL of Qiazol to 250 μL of CFS. Vortex for 30 s and incubate 5 min at RT.

5. Add 200 µL chloroform and mix by vortex for 30 s, and then incubate 5 min at RT.
6. Centrifuge the sample at 12,000 × *g* for 15 min at 4 °C.
7. Carefully collect 600 µL (at least) of upper aqueous phase and transfer to the new tubes.
8. Add 900 µL (1.5 Vol) of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay to the next step.
9. Pipette 700 µL of the sample into an RNeasy MinElute spin column. Centrifuge at 9300 × *g* for 30 s at RT. Discard the flow-through. Repeat this step using the remaining sample.
10. Pipette 700 µL buffer RWT into the RNeasy MinElute spin column and centrifuge at 9300 × *g* for 30 s to wash. Discard the tube with flow-through and place the column in a new 2 mL collection tube.
11. Pipette 700 µL Buffer RPE onto the RNeasy MinElute spin column. Close the lid gently and centrifuge at 9300 × *g* for 30 s to wash the column. Discard the flow-through.
12. Pipette 300 µL Buffer RPE onto the RNeasy MinElute spin column. Close the lid gently and centrifuge at 9300 × *g* for 30 s to wash the column. Discard the flow-through.
13. Pipette 500 µL of 80% ethanol onto the RNeasy MinElute spin column (*see* **Note 4**). Close the lid and centrifuge at ≥9300 × *g* for 2 min to wash membrane. Discard the collection tube with the flow-through.
14. Place the RNeasy MinElute spin column into a new 2 mL collection tube. Centrifuge at full speed for 5 min to dry the membrane. Discard the collection tube.
15. Place the column in a new 1.5-mL tube. Add 30 µL preheated water (~50 °C) directly to the center of the membrane. Close the lid and incubate for 1–2 min at RT, and then centrifuge for 1 min at full speed.
16. Maintain the column in the same tube. Add 30 µL more (*see* **Note 5**) of preheated water directly to the center of the membrane. Close the lid and incubate for 1–2 min at RT (*see* **Note 6**), and then centrifuge for 1 min at full speed. Proceed directly to DNase treatment and RNA precipitation step (*see* **Note 7**).

3.2.2 DNase Treatment and RNA Precipitation

1. Mix the next components to perform off-column DNase treatment to the eluted RNA of 8 samples at the same time:
   - 2 µL TURBO DNase—18 µL (for 8 samples).
   - 11 µL Buffer—99 µL (for 8 samples).
   - 27 µL H_2O (nuclease-free)—243 µL (for 8 samples).

2. Add 40 µL DNase Mix/sample (100 µL final volume = 60 µL RNA + 40 µL DNase Mix).
3. Leave it for 15 min at RT. *Continue with* step 4 *for RNA precipitation*.
4. Add 10 µL (0.1 Vol) of sodium acetate 3 M pH5.5.
5. Add 1 µL (5 µg) of glycogen (Glycogen is at 5 µg/µL concentration).
6. Vortex briefly.
7. Add 250 µL (2.5Vol) of 100% ethanol (*see* **Note 8**).
8. Vortex briefly.
9. Incubate at −80 °C overnight (O/N) or for at least 1.5 h at −80 °C.

3.2.3 RNA Quantification and QCs

QuanTi™ RiboGreen RNA Assay

1. Prepare serial dilution of rRNA standards (12.5–200 ng/mL).
2. Make 70 µL aliquots of each standard and stock at −80 °C for future use.
3. Make 5 µL aliquots of fluorescent dye (Component A in RiboGreen kit) and stock them at −80 °C (*see* **Note 9**).
4. Take one set of standards and one Fluorescent Dye aliquot from freezer and thaw them at room temperature (RT) in the dark (important for the Dye) (*see* **Note 10**).
5. Prepare RNA sample dilutions at 1/30 in 1× TE buffer: Mix 1 µL of RNA/sample and 29 µL of 1× TE buffer for each sample.
6. Prepare enough working solution (WS) for all the experiment at a ratio of 1:200 dilution of Fluorescent Dye: 1× TE into a 15 mL tube (in the darkness).
7. Plate 15 µL of the standards (multichannel micropipette is recommended for reproducibility) in triplicate, and 15 µL of diluted samples in duplicate.
8. Add 15 µL of the WS into each well (standard and samples) and incubate the plate for 15 min at RT in the dark (with lid).
9. Read the plate at 480–520 nm in a spectrophotometer (*see* **Note 11**).

Agilent Bioanalyzer, Eukaryotic RNA Pico Chip

1. Take out the reagents 30 min prior to running the Chip and allow them to reach RT in the dark.
2. Follow the manufacturer instructions for preparing the gel-dye-matrix properly, and running the chip (45 min in total). Criteria of QCs for extracted RNA:
   - Quant-iT Ribogreen RNA assay: salivary RNA concentration normally ranges from 50 to 80 ng/mL saliva. If total RNA amount is <5 ng it is not recommended to proceed with the library construction.

– RNA 6000 Pico Chip, Bioanalyzer: detection of intact ribosomal RNA peak indicates residual cell contamination (eukaryotic: 18S (1869 nt), 28S rRNA (5070 nt); prokaryotic: 16S (1542 nt), 23S rRNA (2906 nt)) and excludes the sample for further analysis.

3.2.4 cDNA Library Preparation

Small ncRNA Library

Prepare the Exiqon Spike-in miRNA kit v2: Dissolve the miRCURY LNA™ Array Spike-in microRNA Kit v2 in 30 µL/vial of nuclease-free water (supplied) upon receipt. Vortex to thoroughly dissolve the lyophilized RNA, pulse briefly in a microfuge, and leave the suspension on ice for 30 min to dissolve. Aliquot the dissolved spike-in miRNAs and store at –80 °C until use and avoid repeated cycles of freeze/thawing.

Ligate the 3′ SR Adaptor

1. Mix the following components in a sterile nuclease-free PCR tube:

Saliva RNA	5.5 µL
Exiqon Spike-in	0.5 µL
(Green) 3′ SR adaptor for Illumina	1 µL
Total volume	7 µL

2. Incubate in a preheated thermal cycler for 2 min at 70 °C. Transfer tube to ice.
3. Add the following components:

RNA+ 3′ SR adaptor mix	7 µL
(Green) 3′ ligation reaction buffer (2×)	10 µL
(Green) 3′ ligation enzyme mix	3 µL
Total volume	20 µL

4. Incubate for 1 h at 25 °C in a thermal cycler.

Hybridize the Reverse Transcription Primer

1. Add the following components to the ligation mixture from **step 4** and mix well:

3′ Ligation reaction mix from **step 1**	20 µL
Nuclease-free water	4.5 µL
(Pink) SR RT primer for Illumina	1 µL
Total volume now should be	25.5 µL

2. Heat samples for 5 min at 75 °C. Transfer to 37 °C for 15 min, followed by 15 min at 25 °C.

Ligate the 5′ SR Adaptor

1. With 5 min remaining, resuspend the (yellow) 5′ SR adaptor in 120 µL of nuclease-free water and store at −80 °C (This step is only necessary when the kit is first opened).
2. Aliquot 1.1 µL×**N** of the (yellow) 5′ SR Adaptor into a separate, 200 µL nuclease-free PCR tube, with **N** equal to the number of samples being processed for the current experiment.
3. Incubate the adaptor in the thermal cycler at 70 °C for 2 min and then immediately place the tube on ice. Keep the tube on ice and use the denatured adaptor within 30 min of denaturation.
4. Add the following components to the ligation mixture from **step 6** and mix well:

Reaction *mix* from **step 2**	25.5 µL
(Yellow) 5′ SR Adaptor for Illumina (denatured)	1 µL
(Yellow) 5′ ligation reaction buffer (10×)	1 µL
(Yellow) 5′ ligation enzyme mix	2.5 µL
Total volume	30 µL

5. Incubate for 1 h at 25 °C in a thermal cycler.

Perform Reverse Transcription

Mix the following components in a sterile, nuclease-free tube:

Adaptor ligated RNA from **step 3**	30 µL
(Red) first strand synthesis reaction buffer	8 µL
(Red) murine RNase inhibitor	1 µL
(Red) ProtoScript II reverse transcriptase	1 µL
Total volume	40 µL

Incubate for 60 min at 50 °C.

Immediately proceed to PCR amplification.

Safe Stopping Point: If you do not plan to proceed immediately to PCR amplification, then heat inactivate the RT reaction at 70 °C for 15 min. Samples can be safely stored at −15 to −25 °C.

Perform PCR Amplification

Add the following components to the RT reaction mix from **step 4** and mix well:

RT reaction mix from **step 4**	40 µL
(Blue) LongAmp Taq 2× Master Mix	50 µL
(Blue) SR Primer for Illumina	2.5 µL
(Blue) Index Primer	2.5 µL
Nuclease-free water	5 µL
Total volume now should be	100 µL

PCR Cycling Conditions

Initial denaturation	94 °C	30 s	1 Cycle
Denaturation	94 °C	15 s	
Annealing	62 °C	30 s	15 Cycles
Extension	70 °C	15 s	
Final extension	70 °C	5 min	1 Cycle
Hold	4 °C		

QIAQuick PCR Purification

Purify the PCR amplified cDNA construct (100 µL) using a QIAQuick PCR Purification Kit.

1. Add 500 µL Buffer PB to the PCR reaction and mix.
2. Apply the sample to the QIAquick column and centrifuge at 15,600 × *g* for 30–60 s.
3. Add 750 µL Buffer PE to the QIAquick column and centrifuge at 15,600 × *g* for 30–60 s.
4. Centrifuge the column with the lid of the spin column open for 5 min at 15,600 × *g* (*see* **Note 12**).
5. Place each QIAquick column in a clean 1.5 mL microcentrifuge tube.
6. To elute amplified DNA add 26 µL Nuclease-free Water. Let the column stand for 1 min, and then centrifuge at 15,600 × *g* for 1 min.

Size Selection Using 6% Polyacrylamide Gel and Purification of Size Selected Library

Prepare 500 mL Running Buffer (100 mL 5× Running Buffer + 400 mL Water), leave 100 mL Running Buffer with 10 µL SYBR Gold stain for later use.

1. Mix the purified PCR product (25 µL) with 10 µL of Gel Loading Dye, Blue (6×).
2. Load 5 µL of Quick-Load pBR322 DNA-MspI Digest in a well on the 6% PAGE 10-well gel.
3. Load two wells with 17 µL each of mixed amplified cDNA and loading dye on the 6% PAGE 10-well gel.

4. Run the gel for ~1.5 h at 90 V. Do not let the blue dye exit the gel.
5. Remove the gel from the apparatus and stain the gel with SYBR Gold nucleic acid gel stain in a clean container for 2–3 min and view the gel on a UV transilluminator. The 140 and 150 nt bands correspond to adapter-ligated constructs derived from the 21 and 30 nt RNA fragments, respectively. For miRNAs, isolate the bands corresponding to ~140 bp. For piRNAs, isolate the band corresponding to ~150 bp (*see* Fig. 1).
6. Place the two gel slices from the same sample in a Gel Broker tube (SeqMatic) with a 2 mL tube, then centrifuge at 14,000×*g* for 1 min, and then soak in 400 μL DNA Gel Elution buffer (1×).
7. Rotate in eppendorf shaker for at least 2 h at RT.
8. Transfer the eluate and the gel debris to SpinX column with 1 cm diameter Whatman filter.
9. Centrifuge the filter for 2 min at >15,600×*g*.
10. Recover eluate and add 1 μL linear acrylamide, 40 μL 3 M sodium acetate pH 5.5, 500 μL of 100% ethanol, and 500 μL of isopropanol. Vortex well.
11. Precipitate at −20 °C for at least 4 h or −80 °C at least 1.5 h.
12. Spin >15,600×*g* for 30 min at 4 °C.
13. Remove the supernatant, taking care not to disturb the pellet.
14. Wash the pellet with 500 μL 80% ethanol.

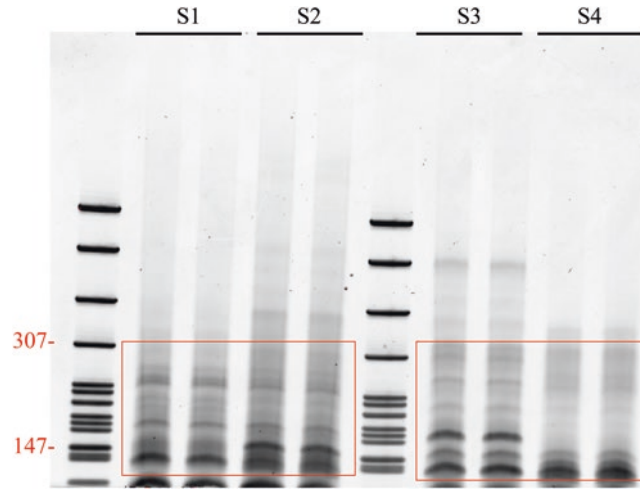

Fig. 1 Transilluminator view of miRNA and piRNA bands. The lanes S1 to S4 correspond to 4 different small ncRNA libraries. Each library has been run per duplicate and bands were cut below 140 bp and above 300 bp. miRNAs isolated bands correspond to ~140 bp. piRNAs isolated bands correspond to ~150 bp

15. Spin >15,600 × *g* for 10 min at 4 °C.
16. Air-dry pellet for up to 10 min at RT to remove residual ethanol.
17. Resuspend pellet in 12 μL EB Buffer (2 of 12 μL will be used for cDNA library quantification in Subheading 3.2.5).

Long ncRNA Library

Prepare the ERCC spike-in: Dissolve in nuclease-free water the lyophilized product making 1:100 dilution stocks. Vortex to thoroughly dissolve the lyophilized RNA, pulse briefly in a microfuge, and leave the suspension on ice for 30 min to dissolve. Aliquot (1–2 μL) the dissolved spike-in RNAs and store at –80 °C until use and avoid repeated cycles of freeze/thawing.

Preparation of First Strand Reaction Buffer and Random Primer Mix

Total saliva RNA + ERCC spike-in (4.5 μL of RNA + 0.5 μL Spike-in)	5 μL
(Pink) NEBNext first strand synthesis reaction buffer (5×)	4 μL
(Pink) NEBNext random primers	1 μL
Total volume	10 μL

RNA Fragmentation

1. Incubate the samples at 94 °C for 2 min.
2. Transfer the tube on ice.
3. Proceed to First Strand cDNA Synthesis.

First Strand cDNA Synthesis

Dilute Actinomycin D stock solution (5 μg/μL) to 0.1 μg/μL in nuclease-free water for immediate use.

The fragmented and primed mRNA	10 μL
(Pink) murine RNase inhibitor	0.5 μL
Actinomycin D (0.1 μg/μL)	5 μL
(Pink) ProtoScript II reverse transcriptase	1 μL
Nuclease-free water	3.5 μL
Final volume	20 μL

Incubate the sample in a preheated thermal cycler as follows:

10 min at 25 °C.

15 min at 42 °C.

15 min at 70 °C.

Hold at 4 °C.

Second Strand cDNA Synthesis

The First Strand Synthesis reaction mixes	20 μL
Nuclease-free water	48 μL
(Orange) second strand synthesis reaction buffer (10×)	8 μL
(Orange) second strand synthesis enzyme mix	4 μL
Total volume	80 μL

1. Mix thoroughly by gentle pipetting.
2. Incubate in thermal cycler for 1 h at 16 °C, with heated lid set at ≥40 °C.

Purify the Double-Stranded cDNA Using 2.5× Agencourt AMPure XP Beads

1. Vortex AMPure XP beads to resuspend.
2. Add 200 μL (2.5×) of resuspended AMPure XP beads to the second strand synthesis reaction (≈80 μL). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 min at RT.
4. Quickly spin the tube in a microcentrifuge to collect any sample on the sides of a tube. Place the tube on an appropriate magnetic rack (DynaMag™-2 Magnet) to separate beads from supernatant. After the solution is clear (about 5 min), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
5. Add 200 μL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at RT for 30 s, and then carefully remove and discard the supernatant.
6. Repeat **step 5** once for a total of 2 washing steps.
7. Air-dry the beads for 10 min while the tube is on the magnetic rack with lid open (recommend hood).
8. Elute the DNA target from the beads into 60 μL nuclease-free water. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and then place it in the magnetic rack until the solution is clear.
9. Remove 55.5 μL of the supernatant and transfer to a clean nuclease-free PCR tube.

Perform End Repair/dA-Tail of cDNA Library

The purified double-stranded cDNA	55.5 μL
(Green) NEBNext end repair reaction buffer (10×)	6.5 μL
(Green) NEBNext end prep enzyme mix	3 μL
Total volume	65 μL

Incubate the sample in a thermal cycler as follows:

30 min at 20 °C.

30 min at 65 °C.

Hold at 4 °C.

Proceed immediately to Adaptor Ligation.

Perform Adaptor Ligation

Dilute the NEBNext Adaptor for Illumina (15 μM) to 1.5 μM with a 10-fold dilution (1:9) with nuclease-free water for immediate use.

The dA-Tailed cDNA	65 μL
(Red) Blunt/TA Ligase Master Mix	15 μL
(Red) Diluted NEBNext adaptor	1 μL
Nuclease-free water	2.5 μL
Total volume	83.5 μL

Incubate 15 min at 20 °C in a thermal cycler.

The adaptor is provided in NEBNext Singleplex or NEBNext Multiplex Oligos for Illumina

Purify the Ligation Reaction Using AMPure XP Beads

1. To the ligation reaction (83.5 μL), add 16.5 μL nuclease-free water to bring the reaction volume to 100 μL.
2. Add 100 μL (1.0×) resuspended AMPure XP beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 min at RT.
4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from supernatant. After the solution is clear (about 5 min), discard the supernatant that contains unwanted fragments (Caution: do not disturb the beads).
5. Add 200 μL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at RT for 30 s, and then carefully remove and discard the supernatant.
6. Repeat **step 5** once for a total of two washing steps.
7. Briefly spin the tube, and put the tube back in the magnetic rack.
8. Completely remove the residual ethanol, and air-dry beads for 10 min while the tube is on the magnetic rack with the lid open (recommend hood).
9. Elute DNA target from the beads with 50 μL nuclease-free water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic rack until the solution is clear.
10. Transfer the 50 μL supernatant to a clean PCR tube. Discard the beads.

11. To the 50 μL supernatant, add 50 μL (1.0×) of the resuspended AMPure XP beads and mix well on a vortex or by pipetting up and down at least 10 times.
12. Incubate for 5 min at RT.
13. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 min), discard the supernatant that contains unwanted fragments (Caution: do not discard the beads).
14. Add 200 μL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at RT for 30 s, and then carefully remove and discard the supernatant.
15. Repeat **step 14** once for a total of two washing steps.
16. Briefly spin the tube, and put the tube back in the magnetic rack.
17. Completely remove the residual ethanol, and air-dry beads for 10 min while the tube is on the magnetic rack with the lid open (recommend hood).
18. Elute DNA target from the bead with 25 μL nuclease-free water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic rack until the solution is clear.
19. Without disturbing the bead pellet, transfer 20 μL of the supernatant to a clean PCR tube and proceed to PCR enrichment (*see* **Note 13**).

Optional stopping point: at this point cDNA library can be stored at −20 °C.

Perform USER Excision and PCR Library Enrichment

The Universal PCR primer and Index (X) Primer are contained in the NEBNext SinglePlex or NEBNext Multiplex Oligos for Illumina.

The size selected cDNA	20 μL
(Blue) NEBNext USER enzyme	3 μL
(Blue) NEBNext High-Fidelity PCR Master Mix, 2×	25 μL
(Blue) Universal PCR Primer (25 μM)	1 μL
(Blue) Index (X) Primer (25 μM)	1 μL
Total volume	50 μL

PCR cycling conditions

User digestion	37 °C	15 min	1 Cycle
Initial denaturation	98 °C	30 s	1 Cycle

Denaturation	98 °C	10 s	
Annealing	65 °C	30 s	15 Cycle
Extension	72 °C	30 s	
Final extension	72 °C	5 min	1 Cycle
Hold	4 °C		

Purify the PCR Reaction Using AMPure XP Beads

1. Vortex AMPure XP beads to resuspend.
2. Add 50 μL (1.0×) of resuspended AMPure XP beads to the PCR reaction (≈50 μL). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 min at RT.
4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from supernatant. After the solution is clear (about 5 min), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
5. Add 200 μL of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at RT for 30 s, and then carefully remove and discard the supernatant.
6. Repeat **step 5** once for a total of two washing steps.
7. Air-dry the beads for 5 min while the tube is on the magnetic rack with the lid open.
8. Elute the DNA target from the beads into 23 μL nuclease-free water. Mix well on a vortex mixer or by pipetting up and down, quickly spin the tube in a microcentrifuge and place it in the magnetic rack until the solution is clear.
9. Transfer 20 μL of the supernatant to a clean PCR tube, proceed with the QCs and quantification step and/or store at −20 °C.

3.2.5 cDNA Library Quantification and QCs

Qubit® dsDNA BR Assay

1. Prepare serial dilution of DNA standards (1–20 ng/μL).
2. Make 500 μL aliquots of each standard and stock at −4 °C for future use (use within a month).
3. Make 10 μL aliquots of fluorescent dye in Qubit DNA kit and stock at −80 °C (*see* **Note 14**).
4. Remove the high concentrated STD curve from 4 °C and one Dye aliquot from −80 °C, and bring them to RT in the dark (important for the Dye) (*see* **Note 10**).
5. Prepare enough WS for all the experiment at a ratio of 1:200 (means of 1 μL of Dye/Reagent per 200 μL of Buffer (*see* **Note 15**).
6. Dilute the DNA library samples with 1:60 against WS.

7. Prepare the low concentrated STD curve (1/10 ratio of high concentrated STD, *see* Table 1).
8. Plate 30 μL of the low concentration standard curve (multichannel micropipette is recommended for reproducibility) in triplicate, and 30 μL of sample in duplicate.
9. Incubate the plate for 15 min at RT in the dark (cover the plate well to avoid DMSO evaporation and therefore, variation in the lecture).
10. Read the plate at 485–530 nm in a spectrophotometer.

Agilent Bioanalyzer, High Sensitivity DNA Chip

1. Take out the reagents 30 min prior to running the Chip and allow them to reach RT in the dark.
2. Dilute the DNA samples 1/10 with nuclease-free water (Only for the Long RNA libraries).
3. Follow the manufacturer instructions for preparing the gel-dye-matrix properly, and running the chip (45 min in total) (*see* Fig. 2).
   Criteria of QCs of constructed library:
   - Qubit dsDNA BR assay: concentration >10 nM.
   - High Sensitivity DNA Chip, Bioanalyzer: Small RNA library should have a major peak of 140–200 bp; Long RNA library should have a major peak of 300–400 bp.

3.2.6 RNA Sequencing by Illumina®

1. Sample pooling: 8 libraries of small ncRNA are pooled at 10 nM in total, per lane; 4 libraries of long ncRNA are pooled at 10 nM in total, per lane (*see* **Note 16**). Samples are pooled with QIAGEN EB Buffer 0.1% Tween 20 in a total volume of at least 20 μL (preferable).
2. Sequencing: samples are sequenced by HiSeq Illumina system, and stranded and Single-End 50 base paired (SE50) used for the procedure (5 days long).

Table 1
Qubit DNA standards

Initial high concentration (ng/μL)	Volume of diluted DNA standard	Volume of WS (μL)	Final low concentration (ng/μL)
20	10 μL of 20 ng/μL	90	2
10	10 μL of 10 ng/μL	90	1
5	10 μL of 5 ng/μL	90	0.5
2	10 μL of 2 ng/μL	90	0.2
1	10 μL of 1 ng/μL	90	0.1
0	10 μL of 0 ng/μL	90	0

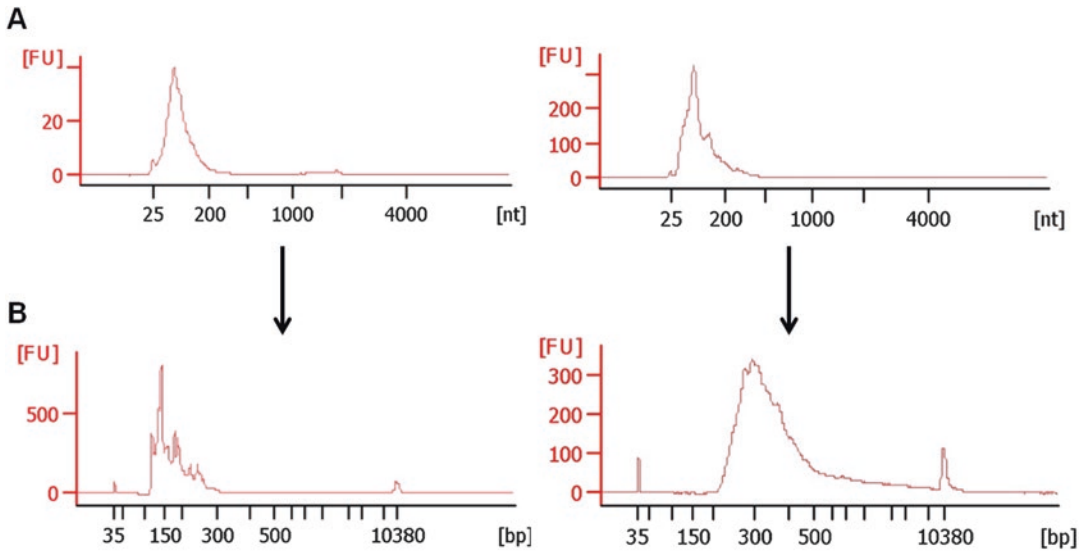

Fig. 2 Agilent bioanalyzer: eukaryotic RNA pico chip and high sensitivity DNA chip. (**a**) RNA profile of salivary exRNA after RNA precipitation. The average length of salivary exRNA is 25–200 nucleotides. (**b**) cDNA library of small ncRNAs (*left*) and long ncRNAs (*right*) after size selection and cDNA library purification

3.2.7 Barcode De-Multiplexing and Data Processing

1. Raw data: one FastQ file is obtained per lane (8 lanes/flow cell).
2. De-multiplexing: Each lane needs to be de-multiplexed following the indexing codes of the samples (1–48 index in one set).
3. Adaptor Trimmed reads: raw data of each individual sample is submitted to Cutadapt software to remove the adaptor sequences from RNA-Seq raw data.
4. Quality control: QC on adaptor-trimmed reads follows the next aspects: (a) Per Base Sequence quality, (b) Per Read quality, (c) per Base N content, and (d) Adaptor content.
5. Mapping:
   - Small RNA libraries: Bowtie mapping and Human Genome are used to align the reads to the human genome. Then, RNA read counts are measured using mapping results and RNA annotation (*see* Table 2).
   - Long RNA libraries: Bowtie mapping to 16S rRNA/microbial genome is used before mapping to the Human Genome, and RNA read counts are measured using mapping results and RNA annotation (*see* Table 2).

4 Notes

1. Unstimulated saliva is collected between 9 a.m. and 10 a.m. following published protocols [14].

Table 2
Output data of small and long ncRNA libraries

Small ncRNA libraries	Long ncRNA libraries		
Average number of RNA detected	Average number of genes detected		
miRNA	386	RefSeq genes	3050
piRNA	99	lncRNA genes	1419
Other ncRNAs[a]	145		

[a]Includes snoRNAs, tRNAs, snRNA, and others

2. Each aliquot allows two RNA extractions—starting volume of 500 μL CFS.
3. This step gets rid of residual bacteria and cell debris.
4. Adding a washing step with ethanol 80% step to the commercial protocol improves washing salts and concentrates the RNA in the silica-gel membrane.
5. Two times of elution may reduce the concentration of RNA but will translate into higher yield after RNA precipitation since more volume for eluting the RNA allows better recovery of the RNA trapped in the silica-gel membrane.
6. Waiting time (1–2 min) for the membrane to get well soaked as well as using preheated water facilitates the elution of all RNA content.
7. RNA precipitation after DNase treatment will clean the protein content in the sample regarding the DNase enzyme and will result in a high RNA concentration, suitable for starting cDNA library construction.
8. The use of ethanol instead of isopropanol is because the precipitated pellet is firmer and adheres more strongly to the tube wall with ethanol than isopropanol. Ethanol is more volatile which facilitates removal and less salt will co-precipitate with ethanol than with isopropanol.
9. Avoiding freezing and thawing of the rRNA standard and fluorescent dye improves notably the reproducibility of the Ribogreen quantification assay.
10. Little changes in temperature will affect the fluorescence lecture, so it is important to avoid heating the tubes containing Dye before plate lecture.
11. Both standard curve and samples will end up in ½ dilutions when mixed with the Dye→STD curve points: 100, 50, 25, 12.5, 6.25, and 0 ng/mL, RNA samples: 1/60 dilution.
12. Centrifugation with the lid open ensures that no ethanol remains during DNA elution. Residual ethanol may interfere with the correct loading of the sample on the PAGE gel.

13. Be sure not to transfer any beads. Trace amounts of bead carry over may affect the optimal performance of the polymerase used in the NEBNext High-Fidelity 2× PCR Master Mix in the subsequent PCR step.
14. Avoiding freezing and thawing of the fluorescent dye notably improves the reproducibility of the Qubit DNA quantification assay.
15. WS already contains the dye so, needs to be prepared freshly and protected from light.
16. Each pooled library contributes to 1.25 and 2.5 nM of the total lane, for small and long ncRNA libraries respectively.

Acknowledgments

This work was supported by National Institutes of Health grant (UH2/UH3 TR000923). We thank Kai Kao, Leo lee, and Hui Zhou for technical suggestions.

Conflict of Interests David Wong is cofounder of RNAmeTRIX Inc., a molecular diagnostic company. He holds equity in RNAmeTRIX, and serves as Scientific Advisor. The University of California also holds equity in RNAmeTRIX. Intellectual property that David Wong invented and which was patented by the University of California has been licensed to RNAmeTRIX.

References

1. Hu Z, Zimmermann BG, Zhou H, Wang J, Henson BS, Yu W, Elashoff D, Krupp G, Wong DTW (2008) Exon-level expression profiling: a comprehensive transcriptome analysis of oral fluids. Clin Chem 54:824–832
2. Li Y, Zhou X, St John MAR, Wong DTW (2004) RNA profiling of cell-free saliva using microarray technology. J Dent Res 83:199–203
3. Li Y, St John MA, Zhou X, Kim Y, Sinha U, Jordan RC, Eisele D, Abemayor E, Elashoff D, Park NH, Wong DTW (2004) Salivary transcriptome diagnostics for oral cancer detection. Clin Cancer Res 10:8442–8450
4. Juusola J, Ballantyne J (2005) Multiplex mRNA profiling for the identification of body fluids. Forensic Sci Int 152:1–12
5. Juusola J, Ballantyne J (2007) mRNA profiling for body fluid identification by multiplex quantitative RT-PCR. J Forensic Sci 52:1252–1262
6. Park NJ, Li Y, Yu T, Brinkman BMN, Wong DTW (2006) Characterization of RNA in saliva. Clin Chem 52:988–994
7. Rosi A, Guidoni L, Luciani AM, Mariutti G, Viti V (1988) RNA-lipid complexes released from the plasma membrane of human colon carcinoma cells. Cancer Lett 39:153–160
8. Whitelegge JP, Zabrouskov V, Halgand F, Souda P, Bassilian S, Yan W, Wolinsky L, Loo JA, Wong DTW, Faull KF (2007) Protein-sequence polymorphisms and post-translational modifications in proteins from human saliva using top-down Fourier-transform ion cyclotron resonance mass spectrometry. Int J Mass Spectrom 268:190–197
9. Halicka HD, Bedner E, Darzynkiewicz Z (2000) Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. Exp Cell Res 260:248–256
10. Park NJ, Zhou X, Yu T, Brinkman BM, Zimmermann BG, Palanisamy V, Wong DTW (2007) Characterization of salivary RNA by cDNA library analysis. Arch Oral Biol 52:30–35
11. Spielmann N, Ilsley D, Gu J, Lea K, Brockman J, Heater S, Setterquist R, Wong DTW (2012) The human salivary RNA transcriptome

revealed by massively parallel sequencing. Clin Chem 58:1314–1321

12. Bahn JH, Zhang Q, Li F, Chan TM, Lin X, Kim Y, Wong DTW, Xiao X (2014) The landscape of microRNA, Piwi-interacting RNA, and circular RNA in human saliva. Clin Chem 61:221–230
13. Majem B, Rigau M, Reventós J, Wong DTW (2015) Non-coding RNAs in saliva: emerging biomarkers for molecular diagnostics. Int J Mol Sci 16:8676–8698
14. Navazesh M (1993) Methods for collecting saliva. Ann NY Acad Sci 694:72–77

http://www.springer.com/978-1-4939-6683-7

Oral Biology
Molecular Techniques and Applications
Seymour, G.J.; Cullinan, M.P.; Heng, N.C.K. (Eds.)
2017, XIV, 503 p. 92 illus., 54 illus. in color., Hardcover
ISBN: 978-1-4939-6683-7
A product of Humana Press