Published in final edited form as:

Methods Mol Biol. 2010 ; 666: 43–51. doi:10.1007/978-1-60761-820-1_4.

Transcriptomic Analyses of Saliva

Viswanathan Palanisamy and David T. Wong

Abstract

Salivary biomarkers for diagnostic and prognostic assessments have become increasingly well established in recent years. Salivary mRNA transcriptomic analyses create a new paradigm in the emerging field for noninvasive molecular diagnosis. In this chapter, we will overview the development of sensitive and robust microarray and multiplex quantitative reverse transcriptase-PCR assays for the discovery and validation of mRNA biomarkers in human saliva. Total RNA isolated from human saliva is used for microarray profiling through Human Genome U133 Plus 2.0 and Exon 1.0 ST array platforms. A universal RNA linear amplification strategy was used to amplify RNA from nanogram scale followed by reverse transcription-PCR reaction, cleaned up enzymatically, and validated by quantitative PCR. Further, the integrity of RNA can be analyzed by the Agilent Bioanalyzer and quantified using a Nanodrop microvolume spectrophotometer. Using these invaluable technical tools, one can identify thousands of mRNA species in saliva. These methods indicate that salivary mRNA provides an efficient medium for biomarker discovery in oral and systemic diseases detection.

Keywords

Saliva; mRNA profiling; biomarkers; oral cancer; microarray profile; mRNA stability

1. Introduction

Messenger RNA in human saliva is an emerging field for noninvasive diagnostic applications. The discoveries of saliva-derived mRNA in normal and oral cancer patients (1-3) and other forensic applications (4, 5) have opened up a new field for studying gene expression noninvasively. Our laboratory has extensively studied microarray-based gene profiling followed by real-time quantitative reverse transcription-PCR (RT-PCR) for saliva RNA detection. The existence of saliva RNA is a remarkable finding because RNA is more labile than DNA and particularly because ribonucleases are known to be present in saliva (6) to degrade RNA molecules. We have identified certain macromolecules associated with saliva RNA which may protect it against ribonucleases (6). The other possible scenarios for protection of salivary RNA may be when it is complexed to lipids, proteins, lipoproteins, or phospholipids as in serum (7, 8) or when protected within apoptotic bodies (9) or other vesicular structures. Therefore, RNA in the saliva may not be as fragile as it was previously assumed to be.

Although a variety of platforms are available for mRNA expression analysis, currently the Affymetrix U133 Plus 2.0 (U133 Plus 2.0) and Human Exon 1.0 ST (HuEx) platforms are noteworthy. Microarray profiling followed by quantitative PCR analysis is widely accepted for biomarker discoveries. RNA is detectable in saliva but little is known about its integrity and stability. Theoretically, to maintain RNA integrity, the time and steps between

collection and RNA extraction should be reduced to the minimum. Methods such as snap-freezing of saliva or the addition of a stabilizing reagent have also been used to reduce the chance of RNA degradation. Of importance are detailed studies reported by our group (10, 11), on the extraction and stability of saliva RNA, and these will be the primary focus of this methods entity.

The purpose of this chapter is to provide simple and reliable methods for isolating and profiling saliva RNA. Here, we describe a detailed methodology for extraction, amplification, and gene expression profiling of saliva RNA using microarray and PCR techniques. Saliva contains a multitude of proteins and DNA; therefore, before performing any interpretation about mRNA species, it is critical to ensure that the isolated RNA is human RNA and not other contaminating nucleic acids. The first part describes the most common protocols used to isolate RNA from human saliva and the second part describes different microarray platforms for profiling and assessing the quality of the isolated saliva RNA. The isolation of RNA from saliva involves few centrifugation and incubation steps. Of note is that commercial RNA extraction kits are available to extract RNA from saliva (for example, RNeasy Protect Saliva Mini Kit; QIAGEN, USA and Oragene RNA kit; DNA Genotek, Ottawa, Ontario, Canada). Here we describe a robust, highly reproducible method of RNA extraction using commercial reagents, designed for isolating RNA in laboratory settings. An extra washing and incubation step in extraction of RNA from a silica column provides extremely pure mRNA for downstream applications. The final part of the unit describes methods used for gene profiling of saliva RNA using Affymetrix U133 Plus 2.0 and HuEx array microarray platforms.

We have combined universal full-length linear amplification of mRNA, a microarray platform with U133 plus 2.0 and all exon-level resolution, and comprehensive q-PCR validation. The comprehensiveness and advantages of our approach are demonstrated by the detection of many new transcript fragments and most notably the fragmented RNA can also be detected. The demonstration of the ability to identify fragmented RNA from saliva exon profiles provides early evidence that salivary exon profiles may have clinical value in disease diagnosis. This approach has provided a comprehensive method that is also applicable for biomarker studies of other body fluids and clinical samples containing fragmented RNAs.

2. Materials

2.1. Saliva Collection and Processing

1. 50 mL of sterile tube and styrofoam cup
2. Crushed ice
3. Distilled water
4. Laboratory vortex
5. Refrigerated bench top centrifuge with 50 mL tube adapters
6. RNase inhibitor (Ambion Inc., Austin, TX, USA)

2.2. RNA Isolation

1. RNeasy Micro Kit, QIAGEN, Valencia, CA, USA
2. Absolute ethanol
3. RNase-free water

4. Instrument: Nanodrop 3300 microvolume spectrophotometer (Thermo Scientific, USA).

2.3. Target cRNA Preparation

1. RiboAmp®^{Plus} kit (Molecular devices, Sunnyvale, CA USA)
2. Affymetrix RNA labeling kit (Affymetrix, Santa Clara, USA)
3. GeneChip® Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA)
4. 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA)
5. RNase-free water
6. Absolute ethanol
7. PCR machine

2.4. U133 Plus 2.0 Microarray Analysis

1. Affymetrix Human Genome U133 plus 2.0 Arrayer
2. Microarray Suite (MAS) software (Affymetrix).
3. Computer

2.5. All Exon Array and Data Processing

1. WT cDNA Synthesis Kit (Affymetrix)
2. WT Terminal Labeling Kit (Affymetrix).
3. Gene Chip Human Exon 1.0 ST arrays (Affymetrix)
4. Computer
5. Exon Array Computational Tool (ExACT) (Affymetrix)

2.6. Quantitative Gene Expression Analysis by q-PCR

1. ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA)
2. MuLV Reverse Transcriptase (Applied Biosystems)
3. Power SYBR Green PCRMix (Applied Biosystems)
4. Gene-specific primers (e.g., synthesized by Sigma-Genosys (Woodlands, TX, USA))
5. ABI 7500 Fast system software (Applied Biosystems)

2.7. Statistical Analysis

1. Software R Codes: <http://www.r-project.org>

3. Methods

Saliva collection from human subjects has to be approved by the Institutional Review Board. The inclusion criteria we used for normal subject selection were age ≥ 30 years and no history of malignancy, immunodeficiency, autoimmune disorders, hepatitis, HIV infection, or smoking. Our oral cancer patients were recruited with documented primary T1 or T2 oral squamous cell carcinoma. All of the patients had received diagnoses of primary disease or

not received any prior treatment in the form of chemotherapy, radiotherapy, surgery, or alternative remedies.

3.1. Saliva Collection and Processing

1. Unstimulated saliva samples should be collected between 9 a.m. and 10 a.m. in accordance with published protocols (12). Ask subjects to refrain from eating, drinking, smoking, or oral hygiene procedures for at least an hour before collection.
2. The subjects should be instructed to rinse their mouth thoroughly with deionized water prior to the collection and to void the mouth of saliva. The subject should be seated comfortably with eyes open, head tilted slightly forward, and (for unstimulated saliva collection) instructed to rest for 5 min and to minimize orofacial movements. The subject should allow the saliva to accumulate in the floor of the mouth before spitting it into a preweighed or graduated test tube every 60 s. 5 min of collection time is usually enough for a sufficient amount of saliva (~5 mL) for analysis.
3. Following collection, centrifuge saliva samples at 2,600g for 15 min at 4°C. Saliva supernatant will then be separated from the cellular phase. RNase inhibitor (500 units/mL) is added to the cell-free saliva supernatant (*see* **Notes 1, 2, and 3**).

3.2. RNA Isolation from Cell-Free Saliva

1. Isolate RNA from the cell-free saliva supernatant according to the modified protocol from the manufacturer (RNeasy Micro Kit) (*see* Note 4).
2. Saliva (300 µL), mixed well with 700 µL of RLT buffer (supplied with the kit; contains guanidine thiocyanate), is incubated at room temperature for 10 min with occasional vortexing.
3. Next, absolute ethanol (500 µL) is added, and the solution passed through a silica column (RNeasy MinElute spin column) by centrifugation at ≥8,000g for 1 min.
4. Wash the column with 350 µL of buffer RW1 (supplied with the kit; contains ethanol) followed by incubation at room temperature for 15 min with 10 µL RNase-free DNase (supplied with RNeasy Micro Kit) and 70 µL of buffer RDD (supplied with the kit).
5. Wash the column with 350 µL of buffer RW1 followed by 500 µL of buffer RPE (supplied with RNeasy Micro Kit).
6. Wash the column once with 500 µL of 80% ethanol and centrifuge the contents. Discard the flow through.
7. Elute the RNA with 30 µL RNase-free water at 13,000g for 2 min.
8. Measure the quantity of RNA using the Nanodrop 3300 microvolume spectrophotometer.

¹Supernatant phase of saliva has to be used to extract enough total RNA for cDNA preparations.

²The quality of RNA could meet the demand for PCR, q-PCR, and microarray assays. It is always better to add RNase inhibitors to freshly collected oral fluids followed by ultra-low-temperature storage (−80°C).

³Importantly, the conditions for separating the pellet and saliva supernatant have to be optimized to avoid mechanical rupture of cellular elements which would contribute to the RNA detected in the fluidic cell-free phase (16).

⁴There are several commercial extraction kits available, but notably QIAGEN's RNA protect saliva and DNA Genotek's Oragene RNA are good examples for rapid isolation of RNA from saliva.

3.3. Target cRNA Preparation

1. Isolated RNA is subjected to linear amplification using RiboAmp® Plus according to the protocol from the manufacturer.
2. Round one of RNA amplification utilizes 10–11 µL of eluted RNA for first-strand cDNA synthesis. Following round one, second-strand synthesis is carried out using the supplied primers from the kit, followed by in vitro transcription (IVT).
3. To maximize the RNA yield, a second round of amplification has to be carried out using the supplied primers in reverse order. After this round of second-strand synthesis (1.5 rounds of RNA amplification), the Affymetrix RNA Labeling kit can be used for the second round of IVT to biotinylate the cRNA product.
4. The labeled cRNA is purified with the use of the GeneChip® Sample Cleanup Module.
5. Small aliquots from each of the isolation and amplification steps are used to assess the quality by RT-PCR. The quality of the fragmented cRNA prepared as described in (13) is assessed by capillary electrophoresis with the 2100 Bioanalyzer.

3.4. U133 Plus 2.0 Microarray Analysis

1. The Affymetrix Human Genome U133 plus 2.0 Array, which contains 54,000 probe sets representing ~38,500 genes (i.e., each gene may be represented by more than one probe set), is used for gene expression profiling.
2. The array data can be normalized and analyzed by means of Microarray Suite (MAS) software (Affymetrix).
3. A detection *P*-value will be obtained for each probe set. Any probe set with a *P* value <0.001 and an intensity value >200 is assigned as “present,” indicating that the matching gene transcript is reliably detected (*see* Note 5).
4. The total number of present probe sets on each array can be obtained and the percentage (%) of present genes calculated.
5. Functional classification is performed on selected genes (present on all 10 arrays, *P* < 0.01) by means of the Gene Ontology Mining Tool (<http://www.netaffx.com>).

3.5. All Exon Array and Data Processing

1. Single-stranded cDNA is generated from the amplified cRNA with the WT cDNA Synthesis Kit then fragmented and labeled with the WT Terminal Labeling Kit.
2. Samples are hybridized with GeneChip Human Exon 1.0 ST Arrays and scanned at the Microarray Facility. Raw data can be processed with the Exon Array Computational Tool (ExACT) (Affymetrix) for background correction and normalization.
3. For example, the salivary mRNA Exon array raw data has been deposited in the Gene Expression Omnibus (GEO) database under series accession no. GSE7760.

3.6. Quantitative Gene Expression Analysis by q-PCR

1. q-PCR can be performed with use of an ABI 7500 real-time PCR System.

⁵The guide to interpreting detection and intensity *P*-values is detailed in the Affymetrix Technical Note: New statistical algorithms for monitoring gene expression on GeneChip® Probe Arrays (http://www.affymetrix.com/support/technical/technotes/statistical_algorithms_technote.pdf).

2. 2 μ L aliquot of the isolated salivary RNA (without amplification) is reverse transcribed into cDNA by means of MuLV Reverse Transcriptase.
3. The resulting cDNA (3 μ L) is used for PCR amplification with Power SYBR Green PCRmix.
4. The primers were synthesized by Sigma-Genosys and Table 4.1 shows the primers used and validated for these studies.
5. All reactions have to be performed in triplicate, with conditions customized for the specific PCR products. The initial amount of cDNA of a particular template is extrapolated from a standard curve with the use of 7500 Fast system software.
6. The detailed procedure for quantification by standard curve has been previously described (14).

3.7. Statistical Analysis

1. Data analysis and statistical evaluations can be performed with customized R codes (version 2.3.1, <http://www.r-project.org/>).
2. Define a probe set as present when it has a P value <0.001 and an intensity value >200 . These criteria were assessed by the results of preliminary experiments.
3. The SECT includes all probe sets present in microarrays. In addition, you can refine the SECT to remove GC-rich (i.e., $\geq 80\%$) probe sets. The concordance between the SECT and the normal salivary core transcriptome (NSCT) was evaluated assuming hypergeometric distributions.
4. For q-PCR data, we normalized all transcript abundances to the three genes in the saliva internal reference (SIR): *ANXA2* (annexin A2), *RPL37* (ribosomal protein L37), and *S100A* (see Note 6).
5. Specifically, you can subtract the mean of the SIR genes' cycle threshold (C_T) values from all of the raw C_T values and use the resulting ΔC_T values to represent the relative abundance of the transcripts (15).

Acknowledgments

This work was supported by National Institutes of Health grants R01DE017170 (D.T.W.), U01DE16275 (D.T.W.), and R00DE018165 (V.P.). We thank Kai Kao, Leo Lee, and Hui Zhou for technical suggestions.

References

1. Li Y, St John MA, Zhou X, Kim Y, Sinha U, Jordan RC, Eisele D, Abemayor E, Elashoff D, Park NH, Wong DT. Salivary transcriptome diagnostics for oral cancer detection. Clin. Cancer Res. 2004; 10:8442–8450. [PubMed: 15623624]
2. Li Y, Zhou X, St John MA, Wong DT. RNA profiling of cell-free saliva using microarray technology. J. Dent. Res. 2004; 83:199–203. [PubMed: 14981119]

⁶The three genes representing the Salivary Internal Reference are amplified using the following primers:

- a. *ANXA2* – Forward primer: TCCCTGTACTATTATATCCAGCAA, reverse primer: TTCTGGTAGTCGCCCTTAGTGT
- b. *RPL37* – Forward primer: GAAATACCACCGGAAGTGGTC, reverse primer: GAATCCATGCCTGAATCTGC
- c. *S100A8* – Forward primer: TGCTAGAGACCGAGTGTCTCTCAG, reverse primer: CATCAGTGTGATATCCAACCTCTTGA

3. Hu Z, Zimmermann BG, Zhou H, Wang J, Henson BS, Yu W, Elashoff D, Krupp G, Wong DT. Exon-level expression profiling: a comprehensive transcriptome analysis of oral fluids. *Clin. Chem.* 2008; 54:824–832. [PubMed: 18356245]
4. Juusola J, Ballantyne J. Multiplex mRNA profiling for the identification of body fluids. *Forensic Sci. Int.* 2005; 152:1–12. [PubMed: 15939171]
5. Juusola J, Ballantyne J. mRNA profiling for body fluid identification by multiplex quantitative RT-PCR. *J. Forensic Sci.* 2007; 52:1252–1262. [PubMed: 17868268]
6. Park NJ, Li Y, Yu T, Brinkman BM, Wong DT. Characterization of RNA in saliva. *Clin. Chem.* 2006; 52:988–994. [PubMed: 16601067]
7. Rosi A, Guidoni L, Luciani AM, Mariutti G, Viti V. RNA-lipid complexes released from the plasma membrane of human colon carcinoma cells. *Cancer Lett.* 1988; 39:153–160. [PubMed: 3359412]
8. Whitelegge JP, Zabrouskov V, Halgand F, Souda P, Bassilian S, Yan W, Wolinsky L, Loo JA, Wong DT, Faull KF. 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.* 2007; 268:190–197. [PubMed: 19050733]
9. Halicka HD, Bedner E, Darzynkiewicz Z. Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. *Exp. Cell Res.* 2000; 260:248–256. [PubMed: 11035919]
10. Jiang J, Park NJ, Hu S, Wong DT. A universal pre-analytic solution for concurrent stabilization of salivary proteins, RNA and DNA at ambient temperature. *Arch. Oral Biol.* 2008; 54:268–273. [PubMed: 19047016]
11. Park NJ, Yu T, Nabili V, Brinkman BM, Henry S, Wang J, Wong DT. RNAprotect saliva: an optimal room-temperature stabilization reagent for the salivary transcriptome. *Clin. Chem.* 2006; 52:2303–2304. [PubMed: 17138851]
12. Navazesh M. Methods for collecting saliva. *Ann. NY Acad. Sci.* 1993; 694:72–77. [PubMed: 8215087]
13. Kelly JJ, Chernov BK, Tovstanovsky I, Mirzabekov AD, Bavykin SG. Radical-generating coordination complexes as tools for rapid and effective fragmentation and fluorescent labeling of nucleic acids for microchip hybridization. *Anal. Biochem.* 2002; 311:103–118. [PubMed: 12470669]
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
15. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002; 3 RESEARCH0034.
16. St John MA, Li Y, Zhou X, Denny P, Ho CM, Montemagno C, Shi W, Qi F, Wu B, Sinha U, Jordan R, Wolinsky L, Park NH, Liu H, Abemayor E, Wong DT. Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma. *Arch. Otolaryngol. Head Neck Surg.* 2004; 130:929–935. [PubMed: 15313862]

Table 4.1
Selection of primers used for quantitative PCR validation of selected nine transcripts in saliva (*n* = 64)

Gene symbol	Primer sequence (5'-3')	Validated ^a	P value	Mean fold increase
<i>DUSP1</i>	F: CCTACCACTATTATCCCGACG	Yes	0.039	2.60
	R: TTGTGAAGGCAGACACCTACAC			
<i>H3F3A</i>	F: AAAGCACCCAGGAAGCAAC	Yes	0.011	5.61
	R: GCGAATCAGAACTTCAGTGGAC			
<i>IL1B</i>	F: GTGCTGAATGTGGACTCAATCC	Yes	0.005	5.48
	R: ACCCTAAGGCAGCAGTTG			
<i>IL8</i>	F: GAGGTTGTGGAGAACTTTTGG	Yes	0.000	24.3
	R: CTGGCATCTTCACTGATCTTGG			
<i>OAZ1</i>	OAZ1 F: AGAGAGAGTCTTCGGGAGAGG	Yes	0.009	2.82
	R: AGATGAGCGAGTCTACGGTTC			
<i>S100P</i>	F: GAGTTCATCGTTGCTGGCTG	Yes	0.003	4.88
	R: CTCACGGGCATCATTTGAGTCC			
<i>SAT</i>	F: CCAGTGAAGAGGGTTGGAGAC	Yes	0.005	2.98
	R: TGGAGGTTGTCATCTACAGCAG			
<i>GADD45B</i>	F: TGATGAATGTGGACCCAGAC	No	0.116	
	R: GAGCGTGAAGTGGATTTC			
<i>RG52</i>	F: CTGCCATAAAGACTGACCTTG	No	0.149	
	R: GCTTCTGATTCACCTACCCAAC			

NOTE. q-PCR was performed to validate the microarray findings on an enlarged sample size including saliva from 32 patients with OSCC and 32 matched control subjects. Nine potential salivary mRNA biomarkers were selected from the 17 candidates shown in **Table 4.1**. Seven of them were validated by q-PCR (*P* < 0.05). Sample includes 32 saliva from OSCC patients and 32 from matched normal subjects.

^aWilcoxon's signed rank test: if *P* < 0.05, validated (Yes); if *P* 0.05, not validated (No).