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Salivary MicroRNAs and Oral Cancer Detection

Janice M. Yoshizawa and **David T. W. Wong**

Abstract

MicroRNAs (miRNAs) in human saliva have recently become an emerging field in saliva research for diagnostics applications and its potential role in biological implications. miRNAs are short noncoding RNA molecules that play important roles in regulating a variety of cellular processes. Dysregulation of miRNAs are known to be associated with many diseases. miRNAs were found present in the saliva of OSCC patients and could serve as potential biomarkers for oral cancer detection. Understanding the biological function of miRNAs in association with diseases is important towards utilizing miRNAs as diagnostic markers. There are currently a variety of profiling methods available for detecting miRNA expression levels. In this chapter, we overview the Applied Biosystem Stem-loop RT based Taqman MicroRNA Assay for salivary miRNA profiling. Using this highly sensitive and specific assay, miRNAs in saliva are profiled with only a few nanograms of starting RNA. This method is also applicable for studying biomarkers in other body fluids or clinical samples that contain small amounts of RNA.

Keywords

Saliva; MicroRNAs; Biomarkers; Diagnostics; Oral cancer; qPCR; TaqMan Micro RNA assays

1. Introduction

Oral cancer, most commonly oral squamous cell carcinoma (OSCC) in ~90% of oral cancer patients, is the sixth most common cancer in the United States. If OSCC is detected at the early stage (T-1 stage), the 5-year survival rate is close to 80%. If OSCC is detected at the later stages (T-3 or T-4 stage), the 5-year survival rate decreases to 20–40%, indicating early detection methods are necessary for increasing long-term patient survival. Previously, protein, mRNA, and DNA extracted from saliva have been used to detect OSCC (1– 4). By using transcriptomic and proteomic technology, mRNA and protein salivary biomarkers, respectively, were discovered and validated to be highly discriminatory for oral cancer detection (2, 5–7).

The presence of microRNAs (miRNAs) in human saliva has recently become an emerging field for monitoring oral diseases using salivary diagnostics. miRNAs are short noncoding RNA molecules 19–24 nt in length that were first identified in 1993 as small RNAs in *Caenorhabditis elegans* (8). Since then, miRNAs have been categorized according to mass (9– 11) and the biogenesis of miRNAs and its mode of action have been well characterized (12, 13). miRNAs binding to complementary sequences in the 3′-untranslated region (3′-UTR) of mRNAs to regulate gene expression by inhibiting protein translation and/or causing mRNA degradation (14). miRNAs play important roles in regulating various cellular processes such as cell growth, differentiation, apoptosis, and immune response (15– 17). Due to imperfect complementary binding by miRNAs, a single miRNA can potentially bind

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Conflict of Interest: D.T.W. Wong is cofounder of RNAmeTRIX, a molecular diagnostic company.

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to >100 different mRNAs. To date, there are over 1,000 known miRNAs in the human genome and over 30% of human mRNAs are post-transcriptionally regulated by miRNAs (14, 18, 19). In saliva, miRNAs were found present in both whole saliva and saliva supernatant. At the time of study, 314 of the 708 human miRNAs registered in the miRBase version 12.0 were profiled (20, 21). Combined with transcriptomic and proteomic approaches, miRNA represents the third diagnostic alphabet in saliva.

Dysregulated expression of miRNAs is known to affect cell growth and can function as tumor suppressors or oncogenes in various cancers (22– 24). In oral cancer, miRNAs have been shown to affect cell proliferation (25), apoptosis (26), and even chemotherapy resistance in OSCC patients (27). miRNAs have also been shown in OSCC to be epigenetically regulated by DNA methylation (28, 29). miRNAs have distinct expression profiles due to being differentially expressed in cancer cells in comparison to normal cells (15). These distinct miRNA expression profiles have also appeared between OSCC and normal tissue (26, 29). Additionally, the expression level of many miRNAs in cancer cells and normal cells exhibit fold changes tens to hundreds of times higher than the expression levels of mRNAs (30). This data suggest that miRNAs can be potentially used as biomarkers to detect early-stage diagnosis of oral cancer and lead to the development of miRNA-based cancer-treatment and therapies.

In the past few years, several research papers have been published that showed the presence of miRNAs in saliva and their potential as noninvasive biomarkers in oral cancer detection. Park and colleagues compared the expression level of approximately 50 miRNAs in whole saliva and saliva supernatant between OSCC patients and normal controls. Two saliva miRNAs, miR-125a and miR-200a, were significantly decreased ($P < 0.05$) in OSCC patients than in normal controls (20). A preliminary analysis from another group showed miR-31 over-expressed in the saliva of OSCC patients (31). A group studying oral rinses from patients with squamous cell carcinoma of the head and neck (SCCHN) detected miR-137 promoter methylation associated with gender and body mass index (32).

How salivary mRNA and miRNA biomarkers are protected and shuttled from the source of the tumor to the saliva remains a current interest. Salivary mRNAs were discovered to be protected from ribonucleases present in the saliva by macromolecules called exosomes (33). These exosomes are small cell-secreted vesicles around 30–100 nm in length that are known to package and transport mRNAs and miRNAs (34). By being localized inside exosomes, salivary mRNAs were found to be remarkably stable (33). This stability has also been shown with endogenous salivary miRNAs degrading at a much slower rate than exogenous miRNAs (20). Exosomes have been shown to transfer mRNA from different cell types and activate or modulate gene expression in oral keratinocytes (33). Recently, miRNAs were extracted from exosomes in human whole saliva from both normal controls and Sjögren's syndrome patients (21). These findings of salivary exosomes and how they regulate cell-to-cell interaction and gene expression may allow us to understand the molecular basis of oral diseases.

Since 2004, there are now a variety of profiling methods that are available for detecting miRNA expression levels. The main principal methods include quantitative PCR (qPCR) (35, 36), microarray hybridization (37, 38), and next-generation sequencing (NGS) (39). For detecting salivary miRNA expression, there are currently several challenges. miRNAs present in saliva are relatively low, typically in the nanogram range upon extraction. Profiling arrays typically require several micrograms or more of input RNA, causing problems with sensitivity and specificity. miRNAs are short and similar in sequence, making it difficult to design RT-qPCR assays or hybridization probes. There are also three different forms of miRNA (pre-miRNAs, pri-miRNAs, and mature miRNAs) so the profiling method

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needs to be able to detect and distinguish between each type. While there have been challenges in detecting miRNA expression, there have also been numerous advances as sequence technology continues to improve each year as the discovery of new miRNAs and modifications to existing miRNAs expands (40).

For miRNA expression profiling in saliva, we focused particularly on the Applied Biosystem Stem-loop RT based Taqman® MicroRNA Assay. This method measures quantitation for mature miRNA expression and is believed to be the gold-standard method with a large dynamic range, high specificity, and high sensitivity (35, 41, 42). Far less input amount of RNA is required as compared to microarray and other technologies, making Taqman® MicroRNA assays extremely suitable for salivary miRNA analysis.

In this chapter, we provide a detailed method for the isolation and profiling of salivary miRNAs. The first part describes a modified protocol for isolating total RNA from human saliva and the second part describes the method for reverse transcribing and profiling the miRNAs of saliva on Taqman® miRNA arrays. A pre-amplification step between reverse transcription of the RNA to cDNA and miRNA array analysis during the second part is required due to the low amount of total RNA present in the saliva sample. This approach is also applicable for studying biomarkers in other body fluids or clinical samples that contain a few nanograms of starting total RNA.

2. Materials

2.1. Saliva Collection and Processing

1. 50 mL Sterile tube and Styrofoam cup.
2. Crushed ice.
3. Distilled water.
4. Laboratory vortex mixer.
5. Refrigerated bench top centrifuge that can accommodate 50 mL tubes.
6. SUPERase Inhibitor (Ambion).

2.2. Total RNA Isolation

1. mirVana PARIS Kit (Ambion).
2. Crushed ice and container.
3. 100% Ethanol, ACS quality or better.
4. DNase I stock solution (QIAGEN).
5. RDD buffer (QIAGEN).
6. Elution solution.
7. Nuclease-free water.
8. Heat block.
9. Microcentrifuge.

2.3. Taqman® MicroRNA Array

1. TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems).

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2. Megaplex RT Primers for array platform: Human Pool A and B (Applied Biosystems).
3. TaqMan® PreAmp Master Mix (Applied Biosystems).
4. Megaplex PreAmp Primers for array platform: Human Pool A and B (Applied Biosystems).
5. Nuclease-free water.
6. GeneAmp PCR System 9700 Thermal Cycler.
7. Taqman® 2× Universal Master Mix II, No UNG (Applied Biosystems).
8. Taqman® Human MicroRNA Arrays Card Set v3 (Applied Biosystems).
9. Applied Biosystems 7900HT Fast Real-Time PCR Instrument with special card holder.
10. PCR tubes or 96-well PCR plates.

2.4. MicroRNA Array Data Processing

1. RQ Manager 1.2.1 Enterprise Software.

3. Methods

3.1. Saliva Collection and Processing

1. Un-stimulated saliva samples should be collected from patients between 9 and 11 a.m. following standard operating procedures. Patients should refrain from eating, drinking, smoking, or using oral hygiene procedures for at least 1 h prior to collection.
2. Ask patients to rinse their mouth well with distilled drinking water for 1 min. Subjects can either expectorate or swallow the water.
3. Five minutes after the oral rinse, ask subjects to spit ~5 mL of saliva into a 50 mL sterile tube placed on ice. It is encouraged that the tube remains on ice while collecting saliva.
4. Following collection, the saliva samples should be brie fl y vortexed (~20 s). Centrifuge the saliva samples at 2,600 × *g* for 15 min at 4°C. Collect the saliva supernatant that has separated from the cellular phase (see Notes 1 and 2).
5. For every milliliter of saliva supernatant collected, add 1 μL (20 U) of SUPERase Inhibitor. Unless proceeding to next step, freeze at −80°C immediately.

3.2. Total RNA Isolation from Saliva Supernatant

1. Total RNA is isolated from the saliva supernatant according to a modified protocol from the manufacturer (mirVANA PARIS kit) (see Notes 3 and 4).
2. If saliva supernatant is frozen, thaw on ice until liquid and start protocol immediately. In the meantime set heat block at 95°C and put a microcentrifuge tube with elution buffer or nuclease-free water into a well. Warm 2× Denaturing Solution at 37°C if it appears solid.
3. To 300 μL of saliva supernatant, add 1.5× amount of 2× Denaturing Solution (450 μL). Mix thoroughly and incubate solution on ice for 5 min.

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4. To the saliva supernatant/2× Denaturing Solution mix, add 1.25 volume of room temperature 100% ethanol (937.5 μL). Mix thoroughly (see Note 5).
5. Add 700 μL of the saliva supernatant/2× Denaturing/Ethanol mix onto a Filter Cartridge placed in a collection tube. Spin for 60 s at 8,000 × *g*. Discard flow-through and repeat until rest of mix has been processed.
6. Wash the Filter Cartridge with 350 μL miRNA Wash Solution 1. Spin for 60 s at 8,000 × *g*. Discard flow-through.
7. Combine 10 μL DNAse I stock solution to 70 μL RDD buffer. Mix by gently pipetting solution several times in tube. Pipet the DNase I mix (80 μL) directly onto the filter membrane. Incubate sample on the bench top at room temp for 15 min (see Note 6).
8. Add 350 μL miRNA Wash Solution 1 to the Filter Cartridge. Spin for 60 s at 8,000 × *g*. Discard fl ow-through.
9. Wash the Filter Cartridge with 500 μL Wash Solution 2/3. Spin for 60 s at 8,000 × *g*. Discard fl ow-through. Repeat process again. After flow-through has been discarded a second time, spin Filter Cartridge for 1 min at 10,000 × *g* to remove any residual fluid from the filter.
10. Transfer the Filter Cartridge to a new collection tube. Apply 100 μL of elution solution or nuclease-free water heated at 95°C to the filter membrane. Let sit for 1 min. Spin for 60 s at 10,000 × *g*. One hundred microliters of total RNA elute collected. If 100 μL is not collected, spin again for another 60 s. Store at −80°C (see Notes 7 and 8).

3.3. Taqman® MicroRNA Arrays

Isolated total RNA undergoes reverse transcription, pre-amplification, and real-time PCR using a modified protocol from the manufacturer.

3.3.1. Megaplex RT

1. Thaw the following reagents on ice: Megaplex RT primers, $MgCl_2$ (included with Megaplex RT primers), and all Taqman® MicroRNA Reverse Transcription components. Spin down components. Do not vortex the RT enzyme mix.
2. In a microcentrifuge tube, prepare the RT Enzyme mix by combining the following reagents listed below. A stock solution is recommended if several RT reactions are to be prepared.

Components	Megaplex RT reaction volume (μL)
Megaplex RT primer mix A or B (10×)	0.75
dNTPs with dTTP (100 mM total)	0.15
MultiScribe Reverse Transcriptase (50 U/(μL)	1.50
RT Buffer (10×)	0.75
$MgCl_2$ (25 mM)	0.90
RNase Inhibitor (20 U/μL)	0.09
Nuclease-free water	0.36

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Total	*4.5*

3. Mix RT Enzyme mix gently and spin briefly. Aliquot 4.5 μL of RT Enzyme mix per well in a 96-well PCR plate or PCR tubes.
4. Add 3 μL of total RNA (1–350 ng) into each well. Mix thoroughly by pipetting up and down several times. Seal the plate or cap the PCR tubes and spin down briefly.
5. Incubate on ice for 5 min.
6. Set up the RT protocol in the 9700 Thermocycler, incubating (16°C for 2 min, 42°C for 1 min, and 50°C for 1 s) for 40 cycles, inactivating the RT reaction at 85°C for 5 min and then holding at 4°C.
7. Load plate or tubes in thermocycler and start the RT run.
8. Proceed to pre-amplification step immediately or store samples at −20°C.

3.3.2. Megaplex Pre-amplification

1. Thaw the following reagents on ice: Megaplex Pre-amplification primers and 2× Taqman® PreAmp Master Mix. Spin down components. Do not vortex.
2. In a microcentrifuge tube, prepare the pre-amplification mix by combining the following reagents listed below. A stock solution is recommended if several RT reactions are to be prepared.

Components	Megaplex Pre-amplification reaction volume (μL)
Megaplex Preamp Primer Mix A or B(10×)	4.0
Taqman® Preamp Master Mix (2×)	20.0
Nuclease-free water	8.5
Total	*32.5*

3. Combine Preamp mix with RT product (40 μL total). Mix thoroughly by pipetting up and down several times. Seal the plate or cap the PCR tubes and spin down briefly.
4. Set up the Preamp protocol in the 9700 Thermocycler, incubate at (95°C for 10 min, 55°C for 2 min and 72°C for 2 min), then (95°C for 15 s and 60°C for 4 min) for 14 cycles, 99.9°C for 10 min to inactivate reaction, and then hold at 4°C.
5. Do not dilute the product. Directly proceed to real-time PCR immediately or store samples at −20°C for up to 1 week.

3.3.3. Real-Time PCR

1. Prepare RT-PCR reaction at room temperature. Let all reagents sit at room temperature for at least 30 min.
2. In a microcentrifuge tube, prepare the RT-PCR reaction mix by combining the following reagents listed below.

Components	RT-PCR reaction volume (μL)
Pre-Amplification product (no dilution)	9.0

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Taqman® Master Mix, no UNG (2×)	450.0
Nuclease-free water	441.0
Total	*900.0*

3. Mix thoroughly and spin down briefly.
4. Load 105 μL of the RT-PCR reaction per port in the MicroRNA Array Card.
5. Spin down and seal the array card.
6. Create plate document on the 7900HT Fast Real-Time PCR Instrument with the following conditions, cycling at 95°C for 10 min, and then (95°C for 15 s, and 60°C for 60 s) for 40 cycles. Use default TLDA setting and FAM as reporter.
7. Load card into instrument. Start run. More detailed information on how to run the arrays can be found at the Applied Biosystem Taqman® Array User Bulletin.

3.4. Data Analysis

For RT-PCR, there are two common methods used for data analysis: absolute quantification and relative quantification. Absolute quantification determines the copy number of a target by using a standard curve with a sample of known concentration. However, this method is usually not preferred due to variations in sample quantity, sample quality, and variable PCR efficiency. The more commonly used method is relative quantification. In relative quantification, target molecules are compared to a reference target relative to a reference group (control sample). Typically, endogenous controls are used as reference targets because the gene expression remains relatively constant across tissue and cell types. Since endogenous controls vary from each target type, the chosen endogenous control should always be validated first. In saliva, RNA polymerase III-transcribed U6 snRNA has been used as an endogenous control for RT-qPCR data analysis (20). Information on how to select endogenous controls has been mentioned elsewhere (43). Relative quantification in RT-PCR data can be calculated by using the comparative C_T method, a convenient way for determining relative changes in gene expression. The comparative C_T method is also referred to as the $2^{-\Delta\Delta C_T}$ method, the same as calculating fold change. For fold change, the standard deviation (SD) is calculated to determine if the expression fold change between groups is significant.

Normalization of expression data is necessary due to many factors in the RT-PCR reaction (e.g., RNA quality, RNA quantity, efficiency) that can contribute to the variation in the expression level. Data normalization is performed by using endogenous controls to correct for variation. We performed data normalization with RQ Manager 1.2.1 from Applied Biosystems (see Note 9). Detailed information on data analysis using Taqman® Arrays has been previously published (43).

3.4.1. Calculating Fold Change

1. First determine the most appropriate endogenous controls for your samples. Several endogenous controls are recommended for data normalization when small differences in expression levels are involved.
2. Determine which of your sample will be your reference sample (calibrator). Reference samples are usually the untreated sample (control or normal) or a sample that is to be compared.

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3. Using the endogenous controls selected for your experiment, normalize the C_T values with the average C_T of the endogenous controls: $\Delta C_T = C_T$ miRNA $- C_T$ endogenous control.
4. Calculate the standard deviation (SD) for each ΔC_T.
5. Calculate $\Delta\Delta C_T$: $\Delta\Delta C_T = \Delta C_T$ experimental sample $-\Delta C_T$ of reference sample.
6. Calculate fold change: fold change = $2^{-\Delta\Delta C_T}$.
7. Incorporated the SD into fold change as a range: 2^{-DDC_T+SD} and $2^{-\Delta\Delta C_T-SD}$.

4. Notes

1. Some saliva samples are very viscous, so vortexing helps in collecting the saliva supernatant after centrifuging. However, do not vortex too much because mechanical rupture of cellular elements that may come from the pellet would interfere with RNA collected from the cell-free supernatant (6).
2. If there is no clear separation of the saliva supernatant from the pellet after the first spin, you may spin again at 2,600 × *g* for 10 min. It is critical that only the cell-free supernatant is collected.
3. Other miRNA extraction kits are commercially available for saliva miRNA extraction. While the mirVANA miRNA isolation kit is only for extracting miRNAs, the mirVANA PARIS kit contains a lysis buffer that is used for saliva optimization.
4. It is recommended to follow the procedure for extracting total RNA from saliva than for only small RNAs so that the quality of RNA can be verified for array analysis and the quantity can be measured.
5. The acid-phenol: chloroform step listed in the original protocol is skipped. An on-column DNase treatment step is included for removing DNA.
6. Another on-column DNase treatment that can be used instead of DNase 1 is TURBO DNase (Ambion). Close to 80 U of Turbo DNase would be required per sample. The wash steps after will inactivate the DNase.
7. Elution solution or nuclease-free water heated at 95°C and directly put on the column for elution results in the highest quantity of total RNA. If samples need to be concentrated in a speed-vacuum, elute samples using only nuclease-free water.
8. There are several quality controls test that can be performed on the extracted total RNA before proceeding to the Taqman Microarray RT step:
   - **(a)** Run a 260/280 reading of your total RNA on a spectrophotometer. Clean RNA has a ratio of approximately 1.8–2.2.
   - **(b)** Run samples on a gel to make sure rRNA bands are intact.
   - **(c)** Run samples on Agilent Bioanalyzer or Biorad Experion.
   - **(d)** Run a qPCR of your samples with a known reference gene.

   Even if rRNA is somewhat degraded, this does not indicate your miRNA quality is bad. miRNAs are small so they usually will not be affected. Samples with mRNA and rRNA compromised by fragmentation have been shown to give good miRNA data. If you want an estimate on how much miRNA is in the sample, the Agilent Bioanalyzer Small RNA gel measures the quantity of small RNAs and miRNAs present.

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9. Global normalization is an alternative method for data normalization that does not use endogenous controls (44). Global normalization takes the median C_T of C_T s common among all samples. The median C_T is used for normalizing the $\Delta\Delta C_T$ calculation on a per sample basis. This method follows the assumption that even though genes are differentially expressed, the amount of transcription remains similar across all samples. Global Normalization can be performed with Data Assist™ Software from Applied Biosystems.

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