Direct Saliva Transcriptome Analysis

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BACKGROUND: Current standard operating procedures for salivary transcriptomic analysis require low temperatures and lengthy mRNA isolation, which substantially hamper its use in the clinic. We developed a streamlined, ambient-temperature processing, stabilization, and storage protocol for clinical analysis of salivary RNA.

METHODS: The direct saliva transcriptome analysis (DSTA) used cell-free saliva supernatant instead of isolated mRNA for saliva transcriptomic detection, and all procedures, including processing, stabilization, and storage of saliva samples, were performed at ambient temperature without a stabilizing reagent. We evaluated this streamlined protocol by comparing the mRNA expression levels of 3 saliva internal reference genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin, beta (ACTB), and ribosomal protein S9 (RPS9)) to levels measured with standard procedures, and detecting the variation of their expression levels under long-term ambient temperature storage. The clinical utility of DSTA was assessed by use of 7 oral cancer salivary mRNA biomarkers in a clinical study.

RESULTS: Each saliva internal reference gene mRNA showed similar expression levels when assayed by the DSTA or standard procedures, and remained stable under ambient temperature storage for at least 10 weeks without significant degradation (P = 0.918, 0.288, and 0.242 for GAPDH, ACTB, and RPS9, respectively). Compared with standard procedures, the performance characteristics of oral cancer salivary transcriptomic markers were retained as assayed by DSTA after 10 weeks of storage at ambient temperature. These results indicate that the DSTA is a suitable alternative method for saliva transcriptomic analysis and is feasible for use in clinical cancer research applications.

CONCLUSIONS: The impact of the streamlined DSTA protocol on saliva handling improves the standard operating procedures for clinical saliva transcriptomic diagnostics.

Interest in using saliva as a diagnostic biofluid for disease detection and health surveillance is increasing owing to its noninvasive accessibility, cost-effectiveness, and accumulating scientific rationale (1). The human salivary transcriptome in cell-free saliva was first discovered in 2004 by use of microarray technology (2). Investigations into the characteristics of salivary RNA followed (3, 4), leading to the development of salivary transcriptomics as an intense research subject in the last 5 years. Forensic scientists have used a small set of saliva-specific mRNAs for body fluid identification (5). Inflammatory mRNA markers can be detected in whole saliva to monitor the status of periodontal disease in type II diabetes patients (6). Expression levels of salivary amylase transcript can be correlated to sleep deprivation (7). In addition, results of studies from our laboratory have further demonstrated the utility of salivary mRNAs for detection of oral cancer (8), pancreatic cancer (9), breast cancer (10), and Sjögren syndrome (11).

A robust streamlined clinical assay for the detection of salivary biomarkers would greatly increase the translational and clinical utility of saliva. Current standard procedures for salivary transcriptomic diagnostics require mRNA isolation, which is time-consuming and labor-intensive. In addition, operator differences increase as procedural complexity increases. Although several automated devices (e.g., KingFisher®, QIAcube, MaxWell® 16) are commercially available to enhance mRNA isolation efficiency, throughput is still limited by the number of samples processed per run.

In addition to salivary mRNA isolation, particular care is required when working with RNA owing to its inherent instability and the ubiquitous presence of RNases. Current strategies rely on maintaining saliva...
samples at low temperatures, a requirement that increases logistical complexity during sample handling. Hence, one desirable clinical goal is to develop an ambient temperature protocol for salivary transcriptomic analysis. We previously tested several commercial stabilizing reagents, including SUPERase-In™ RNase inhibitor (12), RNALater® (13), and RNAProtec® saliva reagent (12, 13), and found that only RNAProtec saliva reagent with a 5-fold volume in relation to the volume of a saliva sample (saliva/reagent = 1:5; v/v) can stabilize salivary RNA at room temperature for long-term storage. However, the large volume of stabilizing reagent needed (5×) is cumbersome and will reduce the efficiency of salivary mRNA isolation. Therefore, a streamlined and robust protocol for salivary RNA analysis that can be performed at ambient temperature is needed to facilitate the translational and clinical applications of saliva diagnostics.

In this report we describe direct saliva transcriptome analysis (DSTA), a method in which saliva samples can be stored at ambient temperature without stabilizing reagent and used directly for salivary mRNA detection without the need for mRNA isolation. The clinical utility of the DSTA method was demonstrated by a case-control clinical study of oral cancer salivary biomarkers.

Materials and Methods

DSTA Protocol

The DSTA procedures, including processing, stabilization, and storage of saliva samples, were performed at ambient temperatures and used saliva supernatant (SS) instead of isolated mRNA for salivary transcriptomic detection. SS was prepared by centrifuging collected unstimulated whole saliva at 2600g for 15 min at 4°C, followed by aspiration from the pellet. The harvested cell-free SS was then sealed and stored in a cool, dry environment at ambient temperature without stabilizing reagent until use. The salivary mRNA was directly detected by a reverse transcription quantitative real-time PCR (RT-qPCR) assay with the stored SS used as the template.

Saliva Sample Collection and Processing

Saliva samples were collected, according to protocols approved by the institutional review board, from 5 healthy individuals (mean age 34 years) who gave informed consent. None of the individuals had a history of malignancy, immunodeficiency, autoimmune disorder, hepatitis, or HIV infection (See Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue9). Unstimulated whole saliva samples were collected between 9 and 10 AM as described previously (14), and processed with centrifugation to obtain cell-free SS (2). The absence of cells in the harvested SS was confirmed by microscopy. The collected SS of each study participant was then split into 3 aliquots (300 μL each) as diagrammed in Fig. 1. Aliquot #1 was directly transferred into a 1.5-mL microcentrifuge tube and stored. Aliquots #2 and #3 were immediately processed by DNase treatment and salivary mRNA isolation, respectively. The DNase-treated SS (product from aliquot #2) was employed as a contrast group to reflect DNA interference in the raw SS (product from aliquot #1), if there was any. Isolated mRNA (product from aliquot #3) was applied to standard procedures of saliva RNA detection, and was employed as a positive control to evaluate the DSTA method performance. All the SS samples (products from aliquots #1 and #2) were stored at room temperature [25 (2) °C] without stabilizing reagent, and the isolated mRNAs were frozen at −80 °C until use. At day 0 (i.e., the day that all samples were collected), and after 1, 2, and 10 weeks of storage, we used the RT-qPCR assay in all samples to detect

*Nonstandard abbreviations: DSTA, direct saliva transcriptome analysis; SS, saliva supernatant; RT-qPCR, reverse transcription quantitative real-time PCR; SIRG, saliva internal reference gene; OSCC, oral squamous cell carcinoma; RT-PCR, reverse-transcription-PCR; Cq, quantification cycle; AUC, area under the curve.
mRNA expression levels of 3 saliva internal reference genes (SIRGs): glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), and ribosomal protein S9 (RPS9). The effectiveness of the DSTA method was assessed by comparing mRNA expression levels from 3 SIRGs obtained from raw SS and isolated mRNA. The stability of salivary mRNA stored at ambient temperature without stabilizing reagent was evaluated by detecting the change of each mRNA expression level in raw SS during 10 weeks of storage.

We further initiated a case-control salivary biomarker study to examine the feasibility of clinical applications of the DSTA method associated with long-term ambient temperature storage. We collected 90 samples from 3 institutions, including 27 samples from patients with oral squamous cell carcinoma (OSCC) and 63 samples from healthy controls (see online Supplemental Table 2). All patients had a diagnosis of primary OSCC and had not undergone chemotherapy and/or radiotherapy. The controls were matched by sex, age, ethnicity, and smoking history to the OSCC group as described in Table 1. The saliva collection procedures were approved by the ethics review boards of all participating institutions. All participants provided written informed consent before sample collection. In this study, we used mRNAs from 7 OSCC salivary biomarker genes: human H3 histone family 3A (H3F3A), interleukin 1-beta (IL1B), interleukin 8 (IL8), ornithine decarboxylase antizyme 1 (OAZ1), spermidine/spermine N1-acetyltransferase 1 (SAT1), dual specificity phosphatase 1 (DUSP1), and S100 calcium binding protein P (S100P) (8). These biomarker genes were used as the proof-of-concept markers and tested in all study participants. Quantification of the 7 transcripts in 90 samples was performed concurrently by standard and DSTA methods at day 0 and after 10 weeks of ambient temperature storage without a stabilizing reagent. The feasibility of the DSTA method for the clinical applications was evaluated by the numbers of markers that could be discriminated, and their diagnostic performances were compared to the results obtained by standard procedures.

**DNAse treatment**

We eliminated genomic DNA in 40 μL SS (Fig. 1, aliquot #2 of each study participant) by rigorous DNase treatment using a TURBO DNA-free™ kit (Applied Biosystems) followed by DNase inactivation according to the manufacturer’s instructions. The effect of DNA removal was demonstrated by applying human cell genomic DNA (300 μg/mL) to the above DNase treatment procedures. (see online Supplemental Fig. 1).

**SALIVARY mRNA ISOLATION**

We isolated salivary mRNA from 300 μL SS (Fig. 1, aliquot #3 of each study participant) using a KingFisher® instrument (Thermo Electron Corporation) with a MagMAX Viral RNA Isolation Kit® (Applied Biosystems). The isolated mRNA was then treated with a TURBO DNA-free kit, followed by DNase inactivation to remove DNA contamination. The purity of the isolated mRNA was assessed by use of the A260/A280 ratio (accepted range: 1.8 –2.0) with an ND-1000 spectrophotometer (Thermo Scientific). The complete removal of DNA in the isolated mRNA was demonstrated by qPCR without RT (see online Supplemental Fig. 1). Furthermore, we evaluated the quality of isolated mRNAs by detecting GAPDH, ACTB, and RPS9 mRNA expression levels using an RT-qPCR assay. Only those samples exhibiting PCR products for all 3 genes were used for subsequent analyses (8).

**RT-qPCR ASSAY**

A 2-step RT-qPCR [reverse transcription-PCR (RT-PCR) followed by qPCR operated separately] was performed for detection of salivary transcriptomes in this study. Multiplex RT-PCR preamplification of 3 SIRG mRNAs was performed by using a SuperScript III platinum qRT-PCR System (Invitrogen) with a pool of outer primer sets (200 nmol/L for each; see online Sup-

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**Table 1. Demographic information for participants in the OSCC mRNA biomarker validation study.**

<table>
<thead>
<tr>
<th>Demographic variable</th>
<th>OSCC (n = 27)</th>
<th>Healthy control (n = 63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD), y</td>
<td>65.00 (9.56)</td>
<td>61.29 (9.82)</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>23 (85.2)</td>
<td>55 (87.3)</td>
</tr>
<tr>
<td>Female</td>
<td>4 (14.8)</td>
<td>8 (12.7)</td>
</tr>
<tr>
<td>Ethnicity, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>23 (85.2)</td>
<td>54 (85.7)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>3 (11.1)</td>
<td>7 (11.1)</td>
</tr>
<tr>
<td>Asian</td>
<td>1 (3.7)</td>
<td>2 (3.2)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12 (44.4)</td>
<td>27 (42.9)</td>
</tr>
<tr>
<td>No</td>
<td>15 (55.6)</td>
<td>36 (57.1)</td>
</tr>
</tbody>
</table>

*Detailed information on individual characteristics, such as age, sex, ethnicity, smoking history, is presented in online Supplemental Table 2.*

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*Human genes: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACTB, β-actin; RPS9, ribosomal protein S9; H3F3A, human H3 histone family 3A; IL1B, interleukin 1-beta; IL8, interleukin 8; OAZ1, ornithine decarboxylase antizyme 1; SAT1, spermidine/spermine N1-acetyltransferase 1; DUSP1, dual specificity phosphatase 1; S100P, S100 calcium binding protein P.*
plemental Table 3), and conducted by a GeneAmp PCR-System 9700 (Applied Biosystems) with a fixed thermal cycling program (see online Supplemental Table 4). Each study participant provided 6 μL raw SS, 7.08 μL DNase-treated SS, and 2 μL isolated mRNA as 3 different templates for RT-PCR, in which the samples were equalized by the mRNA volume. In addition to the 15 experimental RT-PCR samples (5 study participants × 3 templates per study participant), a negative control with nuclease-free water as the reactive template (i.e., a blank group) was prepared. The total volume of each reaction was 30 μL adjusted by nuclease-free water. The RT-PCR products were purified by ExoSAP-IT (USB) and immediately applied to qPCR or stored at −20 °C until use.

SYBR Green qPCR was performed to quantitatively detect the expression levels of salivary transcripts. The qPCR sample was prepared by combining 2× qPCR Mastermix (Applied Biological Materials), inner primers (900 nmol/L; see online Supplemental Table 3), and 2 μL cDNA template. The total volume of each reaction was 10 μL adjusted by nuclease-free water. The qPCR associated with melting-curve analysis was conducted by use of an AB-7500HT System (Applied Biosystems) with a fixed thermal-cycling program (see online Supplemental Table 5). Each gene was tested in triplicate for all samples, including the negative control in which the cDNA template was the product of negative control in RT-PCR preamplification. All primers used in RT-qPCR were designed with intron spanning by use of PRIMER3 software (http://frodo.wi.mit.edu/primer3/), and produced by Sigma after a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

STATISTICAL ANALYSIS

The expression levels of 3 SIRG mRNAs and 7 OSCC salivary transcripts detected by the streamlined and standard procedures were analyzed by raw quantification cycle (Cq) values. All qPCR experiments were performed in triplicate and presented as mean (SD) Cq. Statistical comparison by ANOVA was performed at a significance level of P < 0.05 based on the Wilcoxon signed-rank test. In the case-control salivary biomarker study, the transcript was validated when it showed a significantly different level (P < 0.05) between the OSCC patients and controls. In addition, we constructed the ROC curve and calculated the value of the area under the curve (AUC) by numerical integration of the ROC curve using MedCalc software for each transcript detected. The P values between OSCC and controls combined with AUC values represent the diagnostic performance of the biomarker.

GUIDELINES FOR MINIMUM INFORMATION FOR PUBLICATION OF QUANTITATIVE REAL-TIME PCR EXPERIMENTS

The DSTA method is an RT-qPCR–based technique for detection of salivary transcriptomes. In this study, the RT-qPCR assay was in compliance with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (15). Some procedures for sample qualification and data analysis were modified from conventional approaches because of characteristics of salivary transcriptomes and/or design of the study.

Results

To explore whether salivary transcriptomes can be directly detected without the need for RNA isolation, we used cell-free SS as the template to detect mRNA expression levels for 3 SIRGs and compared the results to results obtained with standard procedures. The results shown in Fig. 2 are for detection performed immediately after saliva samples were collected (day 0). The Cq value in the water group is the mean of triplicate qPCR experiments, and showed >33 for all 3 genes. In addition, the water group did not show any peak in the melting-curve analysis (data not shown), no matter which SIRG primers were used, indicating there was no reagent contamination during the RT-qPCR procedures. The Cq value of each gene in the experimental setups [SS (DSTA), SS + DNase, and isolated mRNA] was the mean of results for samples from 5 healthy study participants, each of which was assayed in triplicate (total 15 data points). The Cq values obtained from the raw SS (DSTA) were 22.84 (2.36), 21.57 (1.63), and 20.35 (1.39), whereas the Cq acquired from isolated mRNAs were 25.28 (1.44), 23.16 (2.2), and 21.42 (1.33) for GAPDH, ACTB, and RPS9, respectively. The P values for association, obtained by comparing the Cq values from SS (DSTA) and isolated mRNA for each SIRG were 0.092, 0.233, and 0.247 for GAPDH, ACTB, and RPS9, respectively. To ensure that the obtained Cq values resulted from the specific mRNA without genomic DNA interference, melting-curve analyses were conducted along with each qPCR run. A single peak with similar melting temperature was observed for the same gene in all samples (data not shown). Furthermore, when we compared the Cq values of the SIRGs in the DNase-treated SS group to the values for the SS (DSTA) group, the P values were 0.645, 0.13, and 0.58 for GAPDH, ACTB, and RPS9, respectively [Fig. 2; P value: SS (DSTA) vs SS + DNase]. These results indicated that the results in the SS (DSTA) group were exclusively from the mRNA without DNA interference, and the performance of the DSTA method was comparable to the standard procedures for salivary mRNA detection. It should be noted
that both the outer and inner primers of SIRGs were designed by intron spanning, which provided additional specificity to the mRNA assays.

To evaluate the stability of saliva mRNA at room temperature without stabilizing reagent and/or nuclease inhibitor, saliva samples were stored at 25 (2) °C (laboratory ambient temperature), and the 3 SIRG mRNA expression levels were assayed by using RT-qPCR at day 0 and after 1, 2, and 10 weeks of storage. As shown in Fig. 3 for the 3 SIRGs evaluated, the mean Cq values detected by SS (DSTA) increased slightly after 10 weeks of preservation and showed no significant difference throughout the time course \( P > 0.05 \); Fig. 3; \( P \) values: SS(DSTA)-Week X vs SS (DSTA)-Day 0 at X = 10. In addition, the Cq values obtained by using SS were all similar to those detected by isolated mRNA \( P > 0.05 \) at each time point [Fig. 3; \( P \) value: SS (DSTA) vs isolated mRNA at day 0 and weeks 1, 2, and 10]. DNase-treated SS samples were used to assess DNA contamination for the duration of ambient temperature storage. As shown in Fig. 3, the mean Cq values were all similar to the results detected by raw SS \( P > 0.05 \) at each time point, indicating no DNA interference was present in the DSTA procedure [Fig. 3; \( P \) values: SS (DSTA) vs SS + DNase at day 0 and weeks 1, 2, and 10]. These results demonstrated that mRNA in SS can be stable at ambient temperature in the absence of stabilizing reagent for up to 10 weeks without significant degradation, and analyzed by the DSTA method.

With the observed performance of the DSTA method, we proceeded to evaluate the feasibility of DSTA in a clinical study. Ninety saliva samples (27 from OSCC patients and 63 from matched controls) were assayed for RNA markers for 7 previously identified OSCC salivary genes: SAT1, OAZ1, H3F3A, IL1B, IL8, DUSP1, and S100P \( (8) \). To examine the effect of long-term ambient temperature storage on marker discrimination, we assayed the 7 salivary transcripts by the DSTA method at day 0 (i.e., immediately after sample collection) and after 10 weeks of room temperature storage without stabilizing reagent. Identification of the 7 salivary transcripts by using standard procedures was performed in parallel as the positive control. The quantitative distributions of Cq values for each transcript in healthy controls and patients with OSCC are shown in online Supplemental Fig. 2 and statistically described in Table 2. All 7 salivary oral cancer RNA markers exhibited upregulation in the OSCC cohort assayed by both DSTA and standard procedures. By standard procedures, 6 of the 7 gene transcripts, H3F3A, IL1B, IL8, OAZ1, SAT1, and DUSP1, showed significantly different expression levels between normal and OSCC samples \( P < 0.05 \). With the DSTA method, 6 \( (H3F3A, IL1B, IL8, OAZ1, SAT1, and S100P) \) and 5 \( (H3F3A, IL1B, IL8, DUSP1, and S100P) \) of the 7 oral cancer markers were validated \( P < 0.05 \) at day 0 and week 10, respectively. Five \( (H3F3A, IL1B, IL8, OAZ1, and SAT1) \) and 4 \( (H3F3A, IL1B, IL8, and DUSP1) \) of the salivary oral cancer markers were validated by both standard procedures and the DSTA method at day 0 and week 10, respectively. Of note, 4 markers \( (H3F3A, IL1B, IL8, and SAT1) \) at day 0, and 3 markers \( (H3F3A, IL1B, and IL8) \) at week 10 exhibited higher ROC-plot AUC values when assayed by use of the DSTA protocol (see Table 2 and online Supplemental Fig. 3). These results indicate that the DSTA method is comparable to standard procedures in discrimination of oral cancer salivary mRNA biomarkers.
Discussion

Saliva RNA detection is an emerging field in molecular diagnostics (16). We reported comprehensive characterizations of salivary RNA (3) and investigated reagent-mediated ambient temperature storage for saliva samples (12, 13). In this study we aimed to develop a robust, easy-to-use, ambient-temperature compatible, and cost-effective protocol to further advance the use of saliva transcriptomes for translational and clinical applications.

In this study we showed that 3 SIRG mRNA expression levels remained stable in ambient temperature-stored saliva supernatant for up to 10 weeks. This outcome is consistent with our previous study, in which we showed that salivary RNAs are protected by specific mechanisms against nucleases in saliva. This protective phenomenon likely occurs because salivary RNAs are associated with macromolecules such as mucines (3), AU (adenine and uridine)-rich element-binding protein (17), salivary chaperone Hsp70 (18), and apoptotic bodies (19, 20). Results of more recent studies revealed that the exosomes play an important role in protecting salivary transcriptomes (21, 22). Exosomes are vesicles for intercellular mRNA transfer that have been found in saliva (23), in which they provide a shelter to confer salivary mRNA stability in the presence of extracellular RNases. Furthermore, analyses of the RNA profiles in exosomes showed that ribosomal RNA was absent and most of the RNA mol-
ecules were <200 nucleotides in length (27), which is in alignment with the mean size of salivary mRNA (4).

Results of these studies indicated that mRNAs in human saliva are indeed protected by macromolecules and/or exosomes, and hence exhibit robust stability despite storage at ambient temperature without a stabilizing reagent.

How can protected mRNAs be detected if there is no lysis step conducted during isolation procedures? We surmise that mRNA molecules are released from protein associations or exosomes while the saliva samples are heated at ~60 °C in the beginning of RT-PCR preamplification. Heat can disrupt hydrogen bonds and nonpolar hydrophobic interactions between RNA molecules and proteins because kinetic energy generated from thermal transformation will cause the molecules to vibrate rapidly and intensively, and hence break RNA–protein associations. In addition, exosomes are derived from endosomal membrane compartments with a lipid-bilayer structure (24). Under conditions of low hydration and/or low temperatures, the lipid bilayer is in a gelatinous state ordered with straight alkyl chains, typically between 14 and 20 carbon atoms in length. When exposed to high water content and/or increased temperatures (e.g., ~60 °C), the lipid bilayer will transform to a fluid state wherein encapsulated mRNAs can be released to interact with other molecules.

A desirable outcome of this study would be to take advantage of the developed streamlined procedures for translational and clinical applications. We performed a clinical validation study of 7 oral cancer salivary mRNA biomarkers (8) to evaluate the clinical performance of the DSTA method. We benchmarked the number of validated salivary RNA markers (i.e., the transcript showing significant up-regulation in OSCC patients; \( P < 0.05 \)) and their diagnostic performances, and compared these results with the results assayed by the DSTA method. The quality of the products obtained by standard and DSTA procedures was evaluated by running melting curves along with all qPCR assays. All samples exhibited a single peak with a similar melting temperature for the same gene, indicating that no DNA contamination, mispriming, and/or primer–dimer artifacts occurred in the experiments. When the saliva was assayed immediately after samples were collected, equal validation efficiencies (6 of 7 markers were validated) were obtained by both standard and DSTA procedures, in which 5 markers overlapped. After 10-week storage at ambient temperature, expressions of all 7 transcripts were still increased in the OSCC patients, and 4 markers were validated by both procedures. Most markers validated by the DSTA method showed higher ROC-plot AUC values than those assayed by standard procedures, even after 10 weeks of ambient temperature storage, indicating that the DSTA method can confer enhanced performance for detection of oral cancer salivary biomarkers.

The DSTA method not only overcomes the issue of lengthy mRNA isolation, it also eliminates the requirement of low temperatures for processing, stabilization, delivery, and storage of saliva samples, advantages that advance the throughput of saliva transcriptomic detection and analysis.

### Table 2. Statistical analyses of 7 OSCC salivary mRNA biomarkers assayed by the streamlined and standard procedures and detected at day 0 and after 10 weeks of storage.a

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Standard procedures (Isolated mRNA)</th>
<th>DSTA day 0 (Saliva supernatant)</th>
<th>DSTA week 10 at room temperature (Saliva supernatant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( P )</td>
<td>AUC</td>
<td>( \Delta C_q )</td>
</tr>
<tr>
<td>H3F3A</td>
<td>0.004(^b)</td>
<td>0.655</td>
<td>1.77</td>
</tr>
<tr>
<td>IL1B</td>
<td>0.002(^b)</td>
<td>0.677</td>
<td>1.73</td>
</tr>
<tr>
<td>IL8</td>
<td>0.005(^b)</td>
<td>0.655</td>
<td>1.78</td>
</tr>
<tr>
<td>OAZ1</td>
<td>0.003(^b)</td>
<td>0.688</td>
<td>1.59</td>
</tr>
<tr>
<td>SAT1</td>
<td>0.044(^b)</td>
<td>0.667</td>
<td>1.40</td>
</tr>
<tr>
<td>DUSP1</td>
<td>0.008(^b)</td>
<td>0.644</td>
<td>1.56</td>
</tr>
<tr>
<td>S100P</td>
<td>0.092</td>
<td>0.611</td>
<td>0.93</td>
</tr>
</tbody>
</table>

a RT-qPCR was performed to validate the 7 previously identified OSCC biomarkers in an independent clinical saliva sample, including 27 OSCC patients and 63 healthy controls.

\( \Delta C_q \): the mean \( C_q \) value of 63 healthy controls — the mean \( C_q \) value of 27 OSCC patients.

b The marker is validated if \( P < 0.05 \) based on Wilcoxon signed-rank test.


