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Short Communication

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Validation of Collection Techniques and Extraction Methods of Saliva for Use in Biomarker Research

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Abstract

Regarding personalized medicine, there is increasing interest in biomarker research as these specific genes and/or proteins can be used to identify people who are at risk of a disease years before symptoms appear. Therefore, one of the most important goals is to develop and validate biomarkers that can detect and identify diseases early. Moreover, the use of saliva collection to investigate biomarkers is growing in popularity as this method is inexpensive, non-invasive, and easy to process. As this process is relatively new and still not widely utilized, we tested two methods of saliva collection using different collection vessels as not and well as multiple RNA, DNA, and protein extraction kits/techniques. Based on the samples collected, we concluded that there were no significant differences in the quality or quantity of RNA based on the kit used. When we tested differences in the methods, and kits, there were no significant differences in DNA quantity and quality. However, we did see significant differences in the quantity and quality of protein based on the collection method, tube, and technique employed. All of our results support the use of saliva collection as a means to investigate biomarkers for identifying individuals at risk of developing future diseases.

Keywords: Biomarker; DNA; Personalized medicine; Protein; RNA; Saliva collection

Introduction

Regarding personalized medicine, there is increasing interest in biomarker research as these specific genes and/or proteins can be used to identify people who are at risk of a disease years before symptoms appear. A biomarker is defined as a characteristic that is measured and evaluated as an indicator of a normal biological process, a pathogenic process, or a pharmacologic response to a therapeutic intervention [1]. These parameters are measured either by physical methods, or by using methods of biochemistry and molecular biology, providing valuable information on certain disease or metabolic processes. Therefore, one of the most important goals of research in this field is to develop and validate biomarkers that can detect and identify disorders early. In medicine, biomarkers are often compounds isolated from serum, urine, or other fluids like saliva. The use of saliva collection to investigate biomarkers is growing in popularity as this method is inexpensive, non-invasive, and easy to process. Saliva is useful for investigations into prognosis, laboratory or clinical diagnosis and monitoring and management of patients with both local and systemic disorders as most of the biological markers present in blood and urine can also be detected in saliva [2]. As this process is relatively new and still not widely utilized, we tested two methods of saliva collection using different collection vessels as well as multiple RNA, DNA, and protein extraction kits/techniques. The objective of our study is to find a robust, relatively inexpensive method of saliva collection, and to develop DNA, RNA, and protein extraction techniques for biomarker investigation using saliva.

Methods and Materials

Saliva Collection

Although simplistic in many ways, there is currently no universally accepted technique for saliva sample collection [3]. Draining, spitting, and suctioning remain the most common approaches. The first method tested in this study was the "Drool" method, where the volunteer simply drools into the collection tube

[4,5]. The second method tested was the "Stimulated" version [4-7] a modified version of the "Spitting" method. In our "Stimulated" method, the volunteer chews on a piece of para film for 1 minute, and then the saliva produced is collected into a tube. Before either method employed, subjects were instructed to clean his or her oral cavity by rinsing with water for 1 minute. Afterwards, subjects waited 10 minutes before collecting the saliva sample as collecting saliva too soon after rinsing may reduce the amount of DNA that can be extracted, and it can also affect hormone/biomarker analyses [4]. All subjects (n = 2) provided informed written consent, in accordance with the tenets of the Declaration of Helsinki, under South College IRB-approved protocol 14-012. A number of companies have introduced a variety of devices aimed at collecting saliva. For this study, we used a instead of one common one from DNA Genotek, and the others were simple 15 mL conical tubes (Fisher brand and Corning).

DNA, RNA, and Protein Extraction

For RNA isolation, we tested 3 extraction kits -RNEasy Mini kit (Qiagen), Pure Link RNA Mini kit (Thermo Fisher Scientific), and SV Total RNA Isolation system (Promega). For each kit, the manufacturer's instructions were followed to extract total RNA. Once quantified on a Nano drop (Thermo Fisher Scientific), the total RNA was stored at -20 °C for use at a later date. For DNA isolation, we tested 2 extraction kits-prep IT L2P (DNA Genotek) and ReliaPrep Blood g DNA Mini prep system (Promega). The total DNA extracted was quantified on a Nano drop (Thermo Fisher Scientific), and stored at -20°C until further use.

We tested whether or not a lysis buffer was needed for saliva samples. Therefore, the samples tested either had 1X RIPA buffer, or no buffer at all. Briefly, those lysates isolated using RIPA buffer were done so according to standard methods. Briefly, 500 uL of saliva was lysed in RIPA buffer containing a protease inhibitor cocktail at 4°C for 30 minutes. Lysates were centrifuged at 16,000 g for 30 minutes at 4°C. Those lysates where RIPA buffer was not used underwent similarly conditions, but without the buffer added. Protein estimation was performed using the BCA kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

Immunoblot analysis was carried out according to standard procedures. Equal concentrations (25 μgs) of proteins were resolved on 8% SDS-PAGE, using 2X Laemmli sample buffer. Cell lysates were denatured by heating before being applied to SDS-PAGE gel. After electrophoresis, proteins were transferred to nitrocellulose membranes, blocked for 1 hour in blocking solution (5% BSA in TBST buffer), and incubated with GAPDH primary antibody overnight at 4°C. GAPDH was detected with HRP-conjugated secondary antibodies, and antibody-protein complexes were visualized using ECL (Thermo Fisher Scientific).

Densitometry Analysis

Relative densitometry analyses of the immuno blots were determined using Image J (http://rsb.info.nih.gov/ij/index.html) analysis software. A ratio of relative density was calculated for each protein of interest.

Statistical Analysis

For all experiments, we performed statistical calculations in Microsoft Excel and Graph Pad Prism 6. One-Way Repeated-Measure Analysis of Variance (ANOVA) with Bonferroni adjustment was used to compare differences between multiple groups. Post hoc analysis was conducted if warranted; Tukey tests (95%). The probability of significant differences between two groups was determined using Student's t- test and the p-values were adjusted using Benjamini-Hochberg principles for correct for false discovery rate. The data are expressed as Mean ± Standard Error (SEM) of two independent experiments performed in duplicate. A p-value of 0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Saliva Collection

Salivary diagnostics is a dynamic field that is being incorporated as part of disease diagnosis, clinical monitoring and for making important clinical decisions for patient care [1]. Our results show that there is a significant difference (p-value = 0.01) in un stimulated vs. stimulated protein expression as shown in Figure 1.

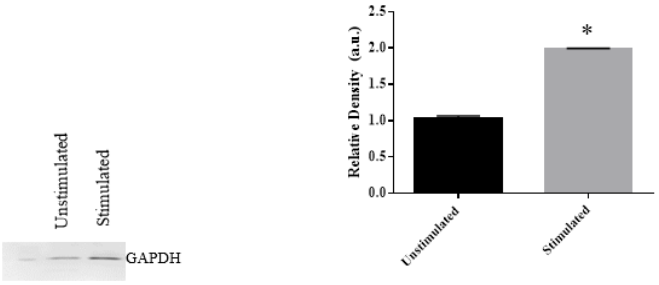

Figure 1: Significant differences in protein expression observed in unstimulated vs. stimulated saliva collection. Samples from a single subject (#4) were collected under both unstimulated and stimulated conditions using the same type of sample collection device (DNA Genotek). Protein lysates were subjected to Western blot analysis with antibody directed against GAPDH. Band intensities were quantified from the 16-bit digital image by densitometry in Image J (NCBI). All data are representative of two experiments with each sample repeated in duplicate.

For our experiments, we were able to collect about 2 mL of saliva from the stimulated method versus 0.5 mL of saliva via

the unstimulated method. Our findings lead to using our modified stimulated method for saliva collection, as the gold standard drool method as well as other stimulated methods have limitations. Patients with dry mouth or issues with salivation (especially the elderly) cannot produce enough saliva for collection, and trying to have them do so takes a greater amount of time compared to stimulated collection [8]. Moreover, men tend to produce more saliva than women without stimulation [5], whereas with stimulation, the volumes are the same (data not shown). As a result, all subsequent experiments utilized the stimulated method for collection. While collection of saliva is undemanding, non-invasive, safer to handle, easier to ship and store, and economical [3,8], the use of saliva as a diagnostic fluid has yet to become commonplace. One reason for this maybe that while most biomarkers detected in the blood are also found in saliva, their levels are substantially diminished [3,9].

DNA, RNA, and Protein Extraction

Once all the samples were collected, the DNA and RNA kits were tested. First, we found that there were no significant differences between the DNA kits tested with regards to quality [F (1, 4) = 0.3726, p = 0.5746] or quantity [F (1, 4) = 0.3332, p = 0.5947] as shown in Figure 2.

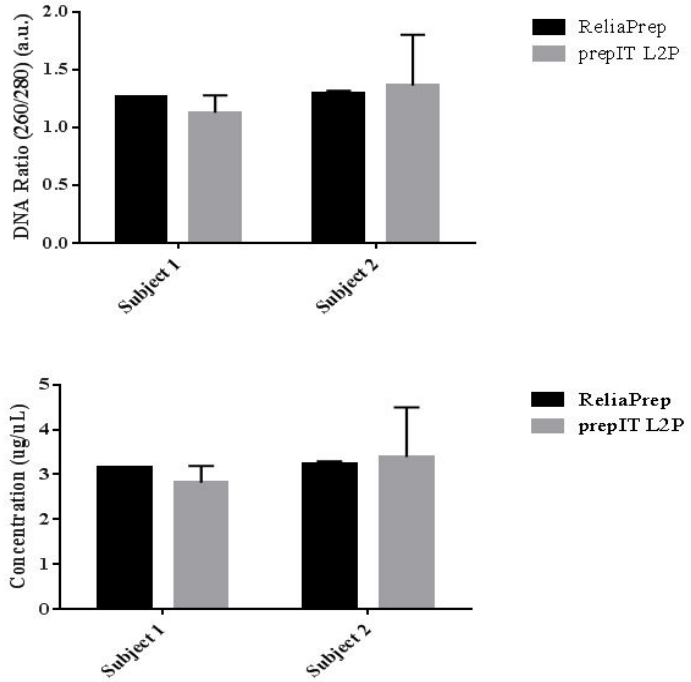

Figure 2: No significant differences between DNA kits. Samples from Subject #1 and #2 were collected under stimulated conditions. For each kit, the manufacturer's instructions were followed to extract total DNA, which was quantified on a Nano drop and stored for use at a later date. We found that there were no significant differences between the DNA kits tested with regards to quality [F (1, 4) = 0.3726, p = 0.5746] or quantity [F (1, 4) = 0.3332, p = 0.5947]. All data are representative of one experiment with each sample repeated in triplicate (n=6).

Similar results were seen in other studies [8-10]. Furthermore, we also observed no significant differences between the RNA kits tested with regards to quality [F (2, 18) = 1.225, p = 0.3171] or quantity [F (2, 18) = 1.265, p = 0.3061] (Figure 3). These results make sense as most commercially available kits and tubes are comparable to one another. Neither of the kits yielded high purity samples, but this may be due to contaminants not being removed completely during the extraction procedure, or was indicative of the lower concentration of nucleic acids.

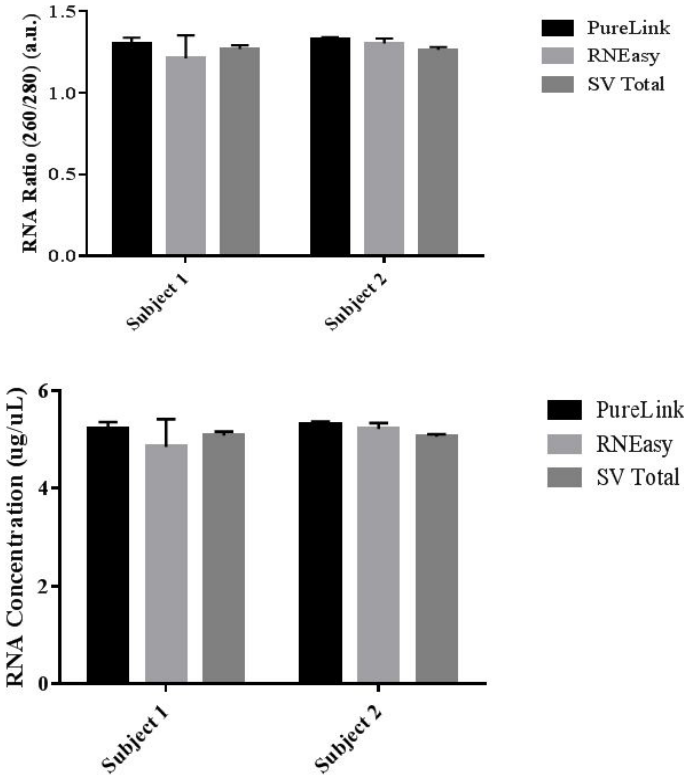

Figure 3: No significant differences between RNA kits. Samples from Subject #1 and #2 were collected under stimulated conditions. For each kit, the manufacturer's instructions were followed to extract total RNA, which was quantified on a Nano drop and stored for use at a later date. We found that there were no significant differences between the RNA kits tested with regards to quality [F (2, 18) = 1.225, p = 0.3171] or quantity [F (2, 18) = 1.265, p = 0.3061]. All data are representative of one experiment with each sample repeated in triplicate (n=6).

As for the protein, the concentration of protein ranged from 0.5 mg/mL to over 2.0 mg/mL. Protein samples collected in the Fisher brand and Corning tubes yielded the highest protein concentrations, while the commercial Oragene tubes yielded very little protein (Figure 4). We noted significant differences between

the groups [F (2, 6) = 150.1, p <0.0001]. Oragene tubes contained a preservation buffer to allow for longer storage and transportation. This buffer probably diluted the proteins in the saliva leading to the lower concentrations for those samples. The use of a lysis buffer like RIPA was also not necessary.

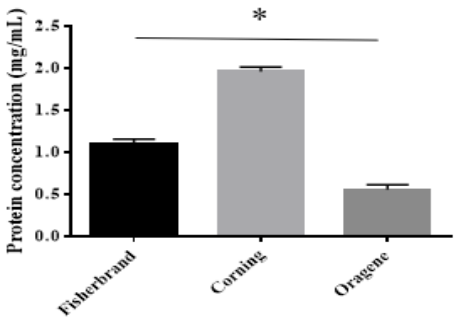

Figure 4: Significant differences in protein expression observed in collection devices. Samples were collected under stimulated conditions for each collection device. Proteins were extracted and quantified using standard methods. All data are representative of two experiments with each sample repeated in duplicate (n=6); [F (2, 6) = 150.1, p <0.0001].

Recently, scientists demonstrated a unique proteomic signature in saliva obtained from type 2 diabetics as compared with matched controls [1]. Over 60 proteins showed a greater than a 2-fold change, and many of these proteins were associated with metabolic and immune regulatory pathways. Further studies are needed, but these findings suggest that there may be unique salivary biomarkers associated with diabetes. Additionally, a panel of salivary biomarkers which can distinguish Primary Sjögren's Syndrome (PSS) patients from healthy ones was reported in the literature [1]. The future of salivary biomarkers will depend on further validation of disease specific biomarkers, and their broad implementation in diagnostic programs.

Conclusions

All of our results support the use of saliva collection as a means to investigate biomarkers for identifying individuals at risk of developing future diseases. Our results show that our modified stimulated saliva collection is better for collection means compared to the good standard non-stimulated one. Moreover, results show that commercially available kits are of similar utility with regards to RNA and DNA extraction. Lastly, differences in protein quantity were observed in the collection devices, so care must be taken when saliva samples are collected for protein analysis. Further efforts in this area should focus on the following: (1) optimizing point-of-care saliva-based testing for diagnosis, (2) ensuring that testing methods remain noninvasive and provide reliable results rapidly, and (3) improving accessibility for patients and providers while limiting health care costs. The exploration and establishment of saliva as a diagnostic tool may fulfill this objective by providing a safe and effective means by which to evaluate patients and personalize their treatment [3].

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Conflicts of Interest

S. E. Hurst is a member of the Biomarkers and Applications Editorial Board. No other authors report any conflict of interest.

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