Rapid Saliva Processing Techniques for Near Real-Time Analysis of Salivary Steroids and Protein

Kelly R. Atkinson, Kim R. Lo, Steve R. Payne, John S. Mitchell, and John R. Ingram

Functional Foods and Health Programme,

The Horticultural and Food Research Institute of New Zealand Ltd., Auckland, New Zealand

Introduction: Point-of-care (POC) measurements using saliva samples have immense potential to assess systemic health and wellbeing, but sample viscosity and contaminants can affect analyses. We sought a portable clean-up method for whole saliva appropriate for use with POC measurement techniques such as biosensors.

Methods: Whole saliva from each of 13 male subjects was split into 5 fractions. Each fraction was treated with a different clean-up process: a freeze–thaw–centrifuge (FTC) step; centrifugation alone; or passage through a Mini-UniPrep polyether-sulfone filter, cotton Salivette[®], or foam Oracol device. Following clean-up, each subject's treated saliva fractions were assayed for cortisol, testosterone, dehydroepiandrosterone (DHEA), and protein

concentrations. The effects of clean-up methods on nonspecific binding (NSB) in a biosensor were also assessed.

Results: Compared with FTC, no analytes were affected by centrifugation alone. Cotton Salivettes significantly altered all analytes, with increases in cortisol (+64%), testosterone (+126%), and DHEA (off-scale) levels, and decreased protein (–21%) and biosensor NSB (–75%). Oracol foam devices decreased DHEA levels by 28%. Mini-UniPrep filtration decreased testosterone (–45%) and DHEA (–66%) concentrations while increasing cortisol (+40%).

Conclusion: No method was optimal for all analytes, highlighting the need for validation of saliva treatment methods before their adoption in rapid POC analyses. *J. Clin. Lab. Anal.* 22:395–402, 2008. © 2008 Wiley-Liss, Inc.

Key words: biosensor; cortisol; DHEA; point-of-care; testosterone

INTRODUCTION

The development of point-of-care (POC) diagnostics utilizing microfluidic and lab-on-chip formats is set to revolutionize the measurement of biomarkers of health, wellbeing, and performance (1). Recent reports have illustrated the potential for combining POC measurement with saliva as the diagnostic medium (2,3). The advantages and disadvantages of saliva as a diagnostic medium have been extensively reviewed (4–8). The ease and acceptance of repeated saliva collection by subjects of various ages, its noninvasive nature, and ability for self-collection outside of the clinical setting have led to increased interest in saliva-based monitoring in both clinical research and the wider healthcare community (9–12).

A number of circulating steroid and protein biomarkers of physiological processes are present in saliva (13). Correlations between salivary and serum concentrations

have been established for a number of small molecular weight analytes, including steroid hormones (14–16), pharmaceuticals (17), and drugs of abuse (18). In particular, measurement of steroid hormones in saliva is now commonplace as these levels correlate very well with those of unbound plasma steroids (13,19). In addition, as venipuncture itself can induce a stress response, salivary measurement is preferred over blood

Grant sponsor: New Zealand Foundation for Research, Science and Technology; Grant number: C06X0410.

*Correspondence to: Kelly R. Atkinson, Functional Foods and Health Programme, The Horticultural and Food Research Institute of New Zealand Ltd., Private Bag 92169, Auckland 1142, New Zealand. E-mail: katkinson@hortresearch.co.nz

Received 16 April 2008; Accepted 27 August 2008

DOI 10.1002/jcla.20281

Published online in Wiley InterScience (www.interscience.wiley.com).

collection for studies of stress hormones such as cortisol and dehydroepiandrosterone (DHEA) (20). The pulsatile nature and ultradian variation of steroid hormone secretion (21) make real-time POC measurement of these analytes particularly important for elucidating their relationship to biological states.

The relationship between salivary and serum peptide and protein hormones is not as straightforward as for steroid hormones, primarily owing to the differing mechanisms of serum protein transport into saliva (6) and to the presence of serum proteins in saliva only at very low levels (22). Despite this, oxytocin (22), growth hormone (23), and adiponectin (24,25) have been measured in saliva. Though these studies are in their infancy, salivary oxytocin measurements do reflect lactation-related expression changes (22). Consequently, saliva is of interest as a source of biomarkers of systemic health and wellbeing.

Whole saliva is a complex fluid comprising secretions of the major and minor salivary glands, bronchial and nasal mucosal secretions, gingival crevicular fluid, microorganisms, and food particulates (26). The composition and consistency of saliva can vary greatly between and within individuals and also with physical activity (27,28). This offers a considerable challenge for analytical pretreatment methods as these physical characteristics can influence the performance of conventional and POC measurement systems (2). Consequently, the reproducible removal of contaminants and viscous components is important for precise analyses. Ideally, a POC pretreatment method should rapidly clean up the sample by removing these components without altering the analytes of interest, and should be performed without laboratory equipment such as centrifuges and freezers.

Commercially available oral fluid collection devices have been widely utilized as combination saliva collection and pretreatment step, though use of these devices can have profound effects on the concentrations of steroids and proteins measured in saliva. Cotton collection swabs (e.g., Salivette[®]; Sarstedt, Nümbrecht, Germany) cause notable variations in androgen measurements, indicating issues with artifact contamination and steroid adsorption to the cotton matrix (29,30). Levels of secretory IgA (29,31,32) and of amylase (33) were also decreased by saliva collection with cotton swabs. Foam collectors (e.g., Oracol; Malvern Medical Developments, Worcester, UK) have previously been used for measurement of virus-specific IgG proteins (34), and may also adsorb a range of steroids (35). In addition, both foam and cotton collection methods require centrifugation (33). No studies have yet considered newer membrane-based filtration methods that

do not require centrifugation to retrieve fluid for analysis. No existing treatment methods have been optimized for measurement of both steroids and proteins from the same sample in rapid POC devices such as biosensors.

It is clear that standardization of saliva collection and pretreatment procedures is essential in obtaining precise and accurate measurements of steroid and protein analytes. In this study we evaluated the effects of a number of commercially available saliva collection and processing devices on measurement of the steroid hormones cortisol, testosterone, and DHEA. Device effects on protein quantitation were also compared to indicate likely interference with later measurement of specific protein biomarkers. We also assessed the effects of these methods on levels of nonspecific binding (NSB) within a surface plasmon resonance (SPR) biosensor device as a model of POC diagnostic systems used for the measurement of steroids and proteins (36).

MATERIALS AND METHODS

Subjects and Saliva Collection

This study was approved by the New Zealand Northern Y Regional Ethics Committee (NTY/07/02/017). Written informed consent was obtained from all the participants. Thirteen healthy male subjects aged from 23 to 47 years (37 ± 2 years, weight 86 ± 4 kg, and height 180 ± 2 cm as mean $\pm$ SEM) participated in the study. Each participant provided 10 mL of whole saliva during stimulation by chewing sugar-free gum (Extra; Wm. Wrigley Jr. Company, Peoria, IL). Saliva was provided by spitting into a 15 mL plastic tube. The participants were asked not to brush their teeth, drink hot liquids, or eat hard foods for at least an hour before providing samples. Samples were provided at 8:30 AM on the day of analysis and were immediately subjected to pretreatment and analyte measurements as described below and in Figure 1.

Saliva Pretreatment

Each 10 mL whole saliva sample was split into five 2 mL aliquots, with these aliquots treated as follows: (1) snap-frozen in liquid N₂, thawed, and centrifuged at $4,000 \times g$ for 10 min (referred to as freeze-thaw-centrifugation (FTC) treatment); (2) centrifuged as above without prior freezing; (3) passed through a 0.2 μ m polyethersulfone (PES) Mini-UniPrep membrane filter system (Whatman[®], Brentford, UK) that uses hand pressure to force the sample from an outer container through the membrane into an inner container; (4) pipetted onto a plain cotton Salivette contained in a plastic tube and centrifuged as above to recover saliva;

Fig. 1. Schematic showing experimental design, including different pretreatment methods applied to each subject's whole saliva sample.

(5) applied to an Oracol foam device and centrifuged as above to recover saliva. Samples were assayed for cortisol, testosterone, DHEA, total protein, and for NSB in an SPR biosensor.

Cortisol Radioimmunoassay

Each sample (50 μ L) was assayed for cortisol in triplicate using a commercial serum cortisol radioimmunoassay (RIA) (Diagnostic Systems Laboratories, Webster, TX) adapted for salivary cortisol measurement as follows. A secondary antibody precipitation step was included using goat anti-rabbit IgG (R2004, Sigma-Aldrich, Auckland, New Zealand) diluted in 4% (w/v) polyethylene glycol 6000 (BDH, Palmerston North, New Zealand) in assay buffer (phosphate-buffered saline (PBS; P4417, Sigma-Aldrich)+0.02% bovine serum albumin (BSA; A7030, Sigma-Aldrich)). This antibody was added after the tracer incubation step to a concentration of 133 ng/mL and samples were further incubated at room temperature for 1 hr. The cortisol standard curve ranged from 0.05 to 51.2 ng/mL in assay buffer, and exhibited parallelism with standards spiked into a whole saliva matrix. Pooled saliva controls with low, medium, and high cortisol concentrations were also assayed. All samples and controls were run in a single assay. The measured intra-assay CVs for low, medium,

and high controls were 5.2, 7.1, and 5.1%, respectively. The limit of assay detection was 0.059 ng/mL.

Testosterone RIA

Each sample (200 μ L) was assayed for testosterone in triplicate using Granger et al.'s (37) adaptation of a commercial serum testosterone RIA (Diagnostic Systems Laboratories). Pooled saliva controls with low, medium, and high testosterone concentrations were also assayed. All samples and controls were run in a single assay. The measured intra-assay CVs for low, medium, and high controls were 5.5, 8.0, and 7.1%, respectively. The limit of assay detection was 0.1 pg/mL.

DHEA Enzyme Immunoassay

DHEA was measured in saliva samples using a commercial salivary enzyme immunoassay kit (Salimetrics, State College, PA) according to the manufacturer's instructions. Samples (50 μ L) were assayed in duplicate. The intra-assay CVs for low and high controls provided in the kit were 2.0 and 1.6%, respectively. The limit of assay detection was 6.3 pg/mL.

Protein Assay

Total protein in saliva samples was measured using the CBQCA protein quantitation kit (Invitrogen,

Auckland, New Zealand). This kit provides a general-purpose fluorimetric assay for total protein content, similar to colorimetric Bradford or bicinchoninic acid assays, appropriate for any protein-containing samples (38), and assays of this type are regularly used to measure salivary protein concentrations for proteomic studies, e.g., (39). Saliva samples were diluted 1:100 with 0.1 M $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{ H}_2\text{O}$, pH 9.3, and 10 μL of each resulting diluted sample was assayed in duplicate in a 96-well plate according to the manufacturer's instructions. Fluorescence data were read on a SynergyTM HT multimode microplate reader (BioTek Instruments, Winooski, VT) using excitation at 465 nm and emission at 550 nm. The standard curve ranged from 74 ng/mL to 74 $\mu\text{g/mL}$. Protein amounts are expressed as micrograms of protein per microliter of undiluted saliva.

SPR Biosensor

A microfluidic biosensor system developed in-house and based on the Spreeta[®] SPR chip (Texas Instruments, Dallas, TX) was used to assess performance in a biosensor device. SPR is a biosensor transduction technology that measures very small changes in the refractive index of a noble metal surface, usually gold, upon binding of mass to that surface. It is used extensively in monitoring biomolecular binding interactions and in biosensor immunoassays (40). Briefly, the SPR biosensor system was composed of a six-port two-way HPLC grade injection valve (Rheodyne, Rohnert Park, CA), an M6 pump (VICI Valco Instruments, Houston, TX), and a fluidics interface to the Spreeta chip, all enclosed in a temperature-controlled box and maintained at 25°C. All interconnecting fluidics were HPLC grade 0.25 mm internal diameter Teflon or stainless steel tubing (Upchurch Scientific, Oak Harbor, WA). The output of the Spreeta chip was recorded using a custom data acquisition interface written in LabView (National Instruments, Austin, TX).

The sensing surface of the Spreeta chip was blocked with 1% BSA (ImmunoCoat; ImmSolv, Seattle, WA). Sixty microliters of each treated saliva sample was injected followed by PBS running buffer at 100 $\mu\text{L/min}$. To clean the surface between each sample, 60 μL of regeneration solution (200 mM NaOH) was injected. Measurements of sensor output as refractive index units (RIU) were taken before injection of saliva and postinjection when a stable baseline was observed. NSB was calculated as the change in RIU between preinjection and postinjection baseline values. The baseline noise of the biosensor was typically 5 response units (5×10^{-6} RIU) and the refractive index range was 1.33–1.40.

Data Analysis

All data were converted to the change in percent from standard FTC treatment by dividing each individual's results by their FTC result, allowing comparison of pretreatment effects across individuals. Statistical analysis using repeated measures one-way analysis of variance was performed to identify significant changes between the measured analyte changes from FTC for each preparation method. Dunnett's post hoc test was used to identify significant differences between FTC and each alternative pretreatment method. Analyses were performed using GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA). Raw data are presented as means and ranges (minimum–maximum) and percentage data are presented as mean $\pm$ SEM.

RESULTS

To analyze the effects of preparation techniques on analyte concentrations in whole saliva, we collected saliva samples from 13 males, split each sample into 5 portions, and treated each portion with a separate preparation method. Methods used were: (1) a standard FTC step; (2) centrifugation alone; (3) passage through a Mini-UniPrep PES membrane; (4) centrifugation through a cotton Salivette device; or (5) centrifugation through a foam Oracol device (Fig. 1). Each subject's saliva subsamples prepared in this manner were analyzed for concentrations of cortisol, testosterone, DHEA, protein, and for their NSB in a biosensor device (Table 1). For each subject's sample, we compared measurements resulting from the FTC pretreatment with the four other pretreatment methods by dividing each method's results by the FTC results (Table 2).

There were no significant differences between FTC and centrifugation alone for any of the analytes/sensor metrics measured (Figs. 2 and 3). Filtration of the saliva samples using a 0.2 μm PES membrane (Mini-UniPrep) significantly increased measured cortisol concentrations by 40% and decreased measurement of testosterone and DHEA by 45 and 66%, respectively, compared with FTC pretreatment. There was also a tendency for total protein concentrations to be reduced following Mini-UniPrep pretreatment. This trend was not significant ($P = 0.09$). This filtration step did decrease NSB in the biosensor device, but not to a significant level (Fig. 3).

Pretreatment with cotton Salivettes had a large effect on all analytes/sensor metrics measured. Measured concentrations of cortisol, testosterone, and DHEA were all significantly higher than FTC values. Cortisol levels were elevated by 64% over FTC, and testosterone was increased by 126%. DHEA values were elevated so much that they were beyond the range of the assay used and hence are not shown in Figure 2. In addition, cotton

TABLE 1. Effects of Saliva Preparation Methods on Measurement Ranges of Four Analytes and SPR Biosensor Nonspecific Binding

Preparation method	Analyte									
	Cortisol (ng/mL)		Testosterone (pg/mL)		DHEA (pg/mL)		Protein (μg/mL)		SPR nonspecific binding (RIU)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Freeze-thaw-centrifugation (FTC)	2.1	0.4–6.5	99.7	54.0–166.3	195.6	84.3–763.0	21.4	14.8–32.3	1,766	998–3,244
Centrifugation only	2.5	0.4–10.2	98.3	56.6–162.0	234.5	85.9–1,081.0	20.7	14.1–29.6	1,832	1,402–2,272
Mini-UniPrep	2.8	0.5–10.3	54.3	29.3–79.3	66.5	7.3–236.8	18.6	10.4–29.9	1,532	346–3,263
Salivette [®]	2.9	0.7–7.4	214.1	138.9–281.3	N/A	N/A	17.0	9.3–29.1	305	205–951
Oracol	2.4	0.4–9.2	91.3	61.4–141.7	126.4	43.9–296.3	20.9	11.1–31.0	1,671	785–2,251

The range shown is the minimum to maximum over all subjects. N/A—all results for salivary DHEA in the Salivette[®]-treated samples exceeded the value of the highest standard in the assay. SPR: surface plasmon resonance; DHEA: dehydroepiandrosterone; RIU: refractive index units.

TABLE 2. Effects of Saliva Preparation Methods on the Percentage Change of Four Analytes and SPR Biosensor Nonspecific Binding Vs. Freeze-Thaw-Centrifugation (FTC) Treatment

Preparation method	Analyte (mean ± SEM change from FTC)				
	Cortisol (ng/mL)	Testosterone (pg/mL)	DHEA (pg/mL)	Protein (μg/mL)	SPR nonspecific binding (RIU)
Centrifugation only	10 ± 5%	0 ± 3%	12 ± 6%	−2 ± 2%	15 ± 10%
Mini-UniPrep	40 ± 12%*	−45 ± 2%**	−66 ± 4%**	−14 ± 4%	−4 ± 15%
Salivette [®]	64 ± 16%**	126 ± 12%**	N/A	−21 ± 4%**	−75 ± 14%*
Oracol	7 ± 6%	−4 ± 6%	−27 ± 8%**	0 ± 9%	−12 ± 13% ^a

N/A—all results for salivary DHEA in the Salivette[®]-treated samples exceeded the value of the highest standard in the assay. * $P < 0.05$ and ** $P < 0.01$ vs. freeze-thaw-centrifugation (FTC). SPR: surface plasmon resonance; DHEA: dehydroepiandrosterone; RIU: refractive index units.

^aNonspecific binding measurements were only available for 4 samples treated with the Oracol device, but for all 13 samples from other methods. Statistical analyses used only the four samples for which Oracol results were present.

Fig. 2. Percentage change of steroid hormone and protein concentrations from four saliva preparation techniques compared with freeze-thaw-centrifugation (FTC) treatment. Samples were treated by centrifugation alone, a Mini-UniPrep syringeless filter, a cotton Salivette[®], or a foam Oracol device. Salivary DHEA results from Salivette-treated samples exceeded the highest assay standard and are not shown. Significant results are indicated as * $P < 0.05$ and ** $P < 0.01$. Data are shown as mean ± SEM ($n = 13$). DHEA: dehydroepiandrosterone.

Fig. 3. Percentage change of four saliva preparation techniques on nonspecific binding in an SPR-based biosensor compared with standard freeze-thaw-centrifugation (FTC) treatment. Samples were treated by centrifugation alone, a Mini-UniPrep syringeless filter (MUP), a cotton Salivette[®], or a foam Oracol device. A significant result is indicated with an asterisk as $*P < 0.05$. Data are shown as mean $\pm$ SEM ($n = 13$ for spin, MUP, and Salivette methods and $n = 4$ for Oracol). SPR: surface plasmon resonance.

Salivette pretreatment significantly decreased both salivary protein concentrations (by 21%) and biosensor NSB (by 75%). Pretreatment with foam Oracol devices had no significant effects on the analytes/sensor metrics measured apart from producing a decline of -28% in measured DHEA concentrations.

DISCUSSION

Rapid POC monitoring requires robust sample collection and treatment procedures that do not introduce artifacts into the analysis process yet minimize sources of error resulting from the biological matrix being analyzed. The results presented in this study identify artifacts produced by salivary collection/pretreatment devices in the measurement of steroids and protein. Our results support and extend previous work on the specific effects of these devices on measurement of salivary analytes, and highlight the lack of suitable treatment methods for rapid monitoring of saliva using conventional and rapid POC measurement technologies.

Saliva collection with foam or cotton devices causes well-recognized changes in fluid composition (35,41). In order to reduce these effects, we collected large volumes of saliva from each subject by a single method, and split each of these into subsamples to be treated with different methods. The Oracol and Salivette devices we used could have been employed for direct saliva collection, but would have produced a different type of saliva sample to that collected by drooling. Using our strategy of collecting a single sample and pretreating subsamples, we were able to standardize the postcollection treatment of whole saliva samples as much as possible. This allowed the objective comparison of saliva subsamples across all pretreatment methods.

Cortisol

In our study, cortisol concentrations were increased following the use of Mini-UniPrep filtration or Salivettes. Cotton Salivettes are reported to decrease or have no effect on measured cortisol concentrations in comparison with passively collected saliva (16,35,41,42). Our results may indicate immunoassay-specific artifacts derived from the cotton or filtration membrane, which cross-react with or decrease the binding affinity of our antibody, causing inflation of apparent concentrations. It should be noted that these pretreatment methods may affect the matrix components of saliva, which can influence assay results. These matrix effects could account for some of the variability observed between methods. Further studies utilizing solvent extraction of analytes or spiking with labeled analyte (e.g., (13,35)) would help to assess the contribution of matrix effects to our results. Salivary cortisol levels were affected least by foam Oracol treatment; therefore, these devices could be used for future studies of this glucocorticoid and other potential analytes. Oracol devices currently require centrifugation for sample removal, though a version without this limitation is being developed (<http://www.malvernmedical.uk.com/oracol.htm>) and would be suitable for rapid POC analyses.

Androgens

Saliva collection using cotton Salivettes has been shown to influence measurement of salivary analytes including steroids and proteins. The effects of cotton on salivary androgen levels have been well described, with increased testosterone concentrations measured using immunoassays (29,43) and decreases in recovery noted when androgens were directly measured using LC-MS (35). We identified similar increases in testosterone and DHEA concentrations measured with immunoassays following Salivette treatment, with DHEA levels so elevated that they were unable to be quantified. An androgen-like factor released from cotton, possibly a plant hormone, which interferes with antibody binding but is not measured using direct LC-MS approaches, could be responsible for this phenomenon as suggested by Shirtcliff et al. (29).

Androgen concentration decreases identified here following Mini-UniPrep filtration indicate that the PES membrane in these devices may retain androgens, preventing their measurement by immunoassay. Recently, decreased testosterone recovery was noted when PES membranes were used in on-chip membrane filters designed for steroid screening in whole blood (44). Syringe filtration of saliva with a $0.2\mu\text{m}$ filter of unknown composition before freezer storage has

also been reported to decrease measured testosterone concentrations (45). Testosterone and DHEA were the analytes most strongly affected by our saliva pretreatment methods, and we suggest that no suitable pretreatment methods currently available are compatible with rapid POC analysis of these androgens.

Protein and Biosensor NSB

Our results confirm previous reports (29,31,41,46) that cotton Salivettes significantly reduce salivary protein concentrations and should not be used for protein analyses from saliva. However, Salivette use did decrease biosensor NSB, a likely consequence of the reduced protein levels. Reducing NSB is an important factor in improving detection limits for biosensor devices given that most analytes measured in saliva are present at far lower concentrations than in blood (29). Saliva collection using Oracol devices also decreases salivary IgA concentrations (46), though we did not observe any significant decrease in total protein concentrations following this method. Specific absorption of certain protein species (including IgA) may occur with this method and could be verified through, for example, proteomic analyses. In the interim, Mini-UniPrep filtration could be a suitable rapid POC saliva pretreatment method for analyses where proteins alone are examined. Applicability to biosensor-based analyses is highly dependent upon the specific analyte being measured in such a device; therefore, a pretreatment method compatible with a biosensor should be tested for its effects on both NSB and the analyte of interest.

CONCLUSIONS

Though no method tested was comparable to FTC or centrifugation pretreatments for all analytes, certain devices (e.g., Oracol or Mini-UniPrep) may be appropriate for specific analytes or analyte combinations if they are tested before use. Further development of a robust POC-ready pretreatment method able to cope with multiple analytes would be welcomed. Until such devices become available, our results highlight the need for validation of saliva sampling conditions and procedures before their adoption in routine analyses.

REFERENCES

1. Tabak LA. Point-of-care diagnostics enter the mouth. *Ann N Y Acad Sci* 2007;1098:7–14.
2. Christodoulides N, Mohanty S, Miller CS, et al. Application of microchip assay system for the measurement of C-reactive protein in human saliva. *Lab Chip* 2005;5:261–269.
3. Gau V, Wong D. Oral fluid nanosensor test (OFNASET) with advanced electrochemical-based molecular analysis platform. *Ann N Y Acad Sci* 2007;1098:401–410.
4. Lewis JG. Steroid analysis in saliva: An overview. *Clin Biochem Rev* 2006;27:139–146.
5. Levine A, Zagoory-Sharon O, Feldman R, Lewis JG, Weller A. Measuring cortisol in human psychobiological studies. *Physiol Behav* 2007;90:43–53.
6. Chiappin S, Antonelli G, Gatti R, De Palo EF. Saliva specimen: A new laboratory tool for diagnostic and basic investigation. *Clin Chim Acta* 2007;383:30–40.
7. Vining RF, McGinley RA. The measurement of hormones in saliva: possibilities and pitfalls. *J Steroid Biochem* 1987;27:81–94.
8. Streckfus CF, Bigler LR. Saliva as a diagnostic fluid. *Oral Dis* 2002;8:69–76.
9. Lawrence HP. Salivary markers of systemic disease: Noninvasive diagnosis of disease and monitoring of general health. *J Can Dent Assoc* 2002;68:170–174.
10. Cone EJ, Huestis MA. Interpretation of oral fluid tests for drugs of abuse. *Ann N Y Acad Sci* 2007;1098:51–103.
11. Granger DA, Kivlighan KT, El-Sheikh M, Gordis EB, Stroud LR. Salivary alpha-amylase in biobehavioral research: recent developments and applications. *Ann N Y Acad Sci* 2007;1098:122–144.
12. Langman LJ. The use of oral fluid for therapeutic drug management: Clinical and forensic toxicology. *Ann N Y Acad Sci* 2007;1098:145–166.
13. Gröschl M, Köhler H, Topf HG, Rupprecht T, Rauh M. Evaluation of saliva collection devices for the analysis of steroids, peptides and therapeutic drugs. *J Pharm Biomed Anal* 2008;47:478–486.
14. Kirschbaum C, Hellhammer DH. Salivary cortisol in psychoneuroendocrine research: Recent developments and applications. *Psychoneuroendocrinology* 1994;19:313–333.
15. Gallagher P, Leitch MM, Massey AE, McAllister-Williams RH, Young AH. Assessing cortisol and dehydroepiandrosterone (DHEA) in saliva: Effects of collection method. *J Psychopharmacol* 2006;20:643–649.
16. Poll EM, Kreitschmann-Andermahr I, Langejuergen Y, et al. Saliva collection method affects predictability of serum cortisol. *Clin Chim Acta* 2007;382:15–19.
17. Yager P, Edwards T, Fu E, et al. Microfluidic diagnostic technologies for global public health. *Nature* 2006;442:412–418.
18. Lennox R, Dennis ML, Scott CK, Funk R. Combining psychometric and biometric measures of substance use. *Drug Alcohol Depend* 2006;83:95–103.
19. Gröschl M, Wagner R, Rauh M, Dorr HG. Stability of salivary steroids: The influences of storage, food and dental care. *Steroids* 2001;66:737–741.
20. Gozansky WS, Lynn JS, Laudenslager ML, Kohrt WM. Salivary cortisol determined by enzyme immunoassay is preferable to serum total cortisol for assessment of dynamic hypothalamic–pituitary–adrenal axis activity. *Clin Endocrinol (Oxf)* 2005;63:336–341.
21. Lightman SL. The neuroendocrinology of stress: A never ending story. *J Neuroendocrinol* 2005;20:880–884.
22. Carter CS, Pournajafi-Nazarloo H, Kramer KM, et al. Oxytocin: behavioral associations and potential as a salivary biomarker. *Ann N Y Acad Sci* 2007;1098:312–322.
23. Rantonen PJ, Penttilä I, Meurman JH, Savolainen K, Narvanen S, Helenius T. Growth hormone and cortisol in serum and saliva. *Acta Odontol Scand* 2000;58:299–303.
24. Toda M, Morimoto K. Comparison of saliva sampling methods for measurement of salivary adiponectin levels. *Scand J Clin Lab Invest* 2008 (in press; e-pub ahead of print at <http://dx.doi.org/10.1080/00365510802147006>).

25. Toda M, Tsukinoki R, Morimoto K. Measurement of salivary adiponectin levels. *Acta Diabetol* 2007;44:20–22.
26. Lamster IB, Ahlo JK. Analysis of gingival crevicular fluid as applied to the diagnosis of oral and systemic diseases. *Ann N Y Acad Sci* 2007;1098:216–229.
27. Gann PH, Giovanazzi S, Van Horn L, Branning A, Chatterton Jr RT. Saliva as a medium for investigating intra- and interindividual differences in sex hormone levels in premenopausal women. *Cancer Epidemiol Biomarkers Prev* 2001;10:59–64.
28. Rantonen PJ, Meurman JH. Viscosity of whole saliva. *Acta Odontol Scand* 1998;56:210–214.
29. Shirtcliff EA, Granger DA, Schwartz E, Curran MJ. Use of salivary biomarkers in biobehavioral research: cotton-based sample collection methods can interfere with salivary immunoassay results. *Psychoneuroendocrinology* 2001;26:165–173.
30. Granger DA, Shirtcliff EA, Booth A, Kivlighan KT, Schwartz EB. The “trouble” with salivary testosterone. *Psychoneuroendocrinology* 2004;29:1229–1240.
31. Aufricht C, Tenner W, Salzer HR, Khoss AE, Wurst E, Herkner K. Salivary IgA concentration is influenced by the saliva collection method. *Eur J Clin Chem Clin Biochem* 1992;30:81–83.
32. Michishige F, Kanno K, Yoshinaga S, Hinode D, Takehisa Y, Yasuoka S. Effect of saliva collection method on the concentration of protein components in saliva. *J Med Invest* 2006;53:140–146.
33. Holm-Hansen C, Tong G, Davis C, Abrams WR, Malamud D. Comparison of oral fluid collectors for use in a rapid point-of-care diagnostic device. *Clin Diagn Lab Immunol* 2004;11:909–912.
34. Vyse AJ, Cohen BJ, Ramsay ME. A comparison of oral fluid collection devices for use in the surveillance of virus diseases in children. *Public Health* 2001;115:201–207.
35. Gröschl M, Rauh M. Influence of commercial collection devices for saliva on the reliability of salivary steroids analysis. *Steroids* 2006;71:1097–1100.
36. Mitchell JS, Wu Y, Cook CJ, Main L. Sensitivity enhancement of surface plasmon resonance biosensing of small molecules. *Anal Biochem* 2005;343:125–135.
37. Granger DA, Schwartz EB, Booth A, Arentz M. Salivary testosterone determination in studies of child health and development. *Horm Behav* 1999;35:18–27.
38. You WW, Haugland RP, Ryan DK, Haugland RP. 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde, a reagent with broad dynamic range for the assay of proteins and lipoproteins in solution. *Anal Biochem* 1997;244:277–282.
39. Siqueira WL, Salih E, Wan DL, Helmerhorst EJ, Oppenheim FG. Proteome of human minor salivary gland secretion. *J Dent Res* 2008;87:445–450.
40. Lakowicz JR, Malicka J, Matveeva E, Gryczynski I, Gryczynski Z. Plasmonic technology: Novel approach to ultra-sensitive immunoassays. *Clin Chem* 2005;51:1914–1922.
41. Strazdins L, Meyerkort S, Brent V, D’Souza RM, Broom DH, Kyd JM. Impact of saliva collection methods on sIgA and cortisol assays and acceptability to participants. *J Immunol Methods* 2005;307:167–171.
42. Morelius E, Nelson N, Theodorsson E. Saliva collection using cotton buds with wooden sticks: A note of caution. *Scand J Clin Lab Invest* 2006;66:15–18.
43. Dabbs Jr JM. Salivary testosterone measurements: Collecting, storing, and mailing saliva samples. *Physiol Behav* 1991;49:815–817.
44. Thorslund S, Klett O, Nikolajeff F, Markides K, Bergquist J. A hybrid poly(dimethylsiloxane) microsystem for on-chip whole blood filtration optimized for steroid screening. *Biomed Micro-devices* 2006;8:73–79.
45. Whembolua GL, Granger DA, Singer S, Kivlighan KT, Marguin JA. Bacteria in the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. *Horm Behav* 2006;49:478–483.
46. Nurkka A, Obiero J, Kayhty H, Scott JA. Effects of sample collection and storage methods on antipneumococcal immunoglobulin A in saliva. *Clin Diagn Lab Immunol* 2003;10:357–361.