Chapter 1

Salivary Diagnostics Using Purified Nucleic Acids

Paul D. Slowey

Abstract

Saliva is an easily accessible fluid that has led to increasing interest in the development of salivary diagnostics. This chapter describes some of the newer tools and procedures for collection, stabilization, and storage of oral fluid matrices that aid in the successful use of saliva as a test specimen. This chapter focuses particularly on nucleic acid components for downstream molecular diagnostic (MDx) testing, since this is probably the area where saliva is likely to have the greatest impact in improving healthcare for the general population.

Key words Saliva, RNA, DNA, Nucleic acids, Stabilization, Exosomes

1 Introduction

Over the last few years, the use of saliva as a noninvasive bodily fluid for research, forensic, and clinical testing has grown tremendously and is now in use in many areas of the global in vitro *diagnostic* (IVD) market.

The number of applications for saliva is growing exponentially as evidenced by the increasing number of available tools for saliva acquisition and subsequent testing either immediately at the point of care or under controlled laboratory conditions.

Saliva is now used in tests for adverse responses to multiple therapeutics, genomics for cystic fibrosis, fragile X syndrome in autism, disorders of the salivary glands, cancers (including breast, head and neck, and oral cancers), abused drug testing in the workplace and other environments, as well as certain systemic diseases including HIV, hepatitis C, and Sjögren's syndrome.

The success of any test, whether for research or diagnostic purposes, relies on the successful harvesting of the specimen from a subject in a standardized, repeatable fashion and careful handling of the sample throughout the collection and downstream testing process. This rule applies to all specimen types, but care should

Gregory J. Seymour et al. (eds.), *Oral Biology: Molecular Techniques and Applications,* Methods in Molecular Biology, vol. 1537,
DOI 10.1007/978-1-4939-6685-1_1,

especially be taken with respect to processing and stabilizing saliva samples to ensure optimum results.

The following text describes some of the newer tools and procedures for collection, stabilization, and storage of oral fluid matrices that aid in the successful use of saliva as a test specimen. This chapter focuses particularly on nucleic acid components for downstream molecular diagnostic (MDx) testing, since this is probably the area where saliva is likely to have the greatest impact in improving healthcare for the general population. For more detailed information on current salivary diagnostics and available tools, the reader is referred to several review articles on the subject [1–5].

Dr Lawrence Tabak (Deputy Director of the NIH and former head of the National Institute for Dental and Craniofacial Research, NIDCR) characterized saliva as a "mirror of the body" and is therefore reflective of disease and disease processes going on in the human body. This precious biofluid contains many of the biomarkers that are indicative of disease and maladies affecting human beings, so saliva is the ideal sample matrix for large-scale epidemiological studies, population screening, and diagnosis of multiple diseases and conditions.

Saliva is cost-effective, noninvasive, easy to transport, amenable to simple disposal, and highly attractive in certain cultures (and religions), which find the use of blood an unacceptable option. More importantly, saliva contains many of the indicators of disease found in blood, urine, and tissue samples.

Typically, levels of biomarkers in saliva are 10–1500 times lower than in blood, but with the advent of newer, more sensitive detection technologies, the analysis of salivary biomarkers has become a much more attractive option. When patient preference to eliminate the use of needles is considered as an additive factor, the "compelling story" for saliva grows significantly stronger. These are some of the major reasons that there has been an "explosion" in research and development in salivary diagnostics, in the last few years, resulting in the development of a plethora of tools and tests using this unique bodily fluid.

A series of technological developments, which have also contributed to the growing importance of saliva as a diagnostic medium, include several high-throughput technologies such as next-generation sequencing, proteomics, mass spectrometry, genome wide association studies (GWAS), and genotyping, which allow large numbers of samples to be tested in a short time. Saliva has already been shown to be a readily adaptable specimen for use in these high-impact technologies.

Saliva is now in routine use for the diagnosis of HIV in the privacy of one's home [6, 7] and for the detection of multiple hormones as part of a "general wellness" program, sold direct to the consumer [8–10]. Saliva has also been used to detect drugs of abuse [11] and in certain situations has been shown to be a

preferable biofluid to urine, which is currently the method of choice. This is particularly true in the case of marijuana, when testing for "impairment" and whether a particular individual is fit to drive a vehicle or perform dangerous tasks.

Multiple diseases have also been detected using saliva, including caries risk [12–14]; periodontitis [15]; oral [16], breast [17–22], and head and neck cancers [23]; and salivary gland disorders [24]. Point of care tests are now also in development looking at viruses, bacteria [25], and difficult to measure hormones using saliva [26].

Perhaps the area where saliva has gained the most traction is for the collection of nucleic acids (DNA and RNA). The noninvasive nature of saliva means that samples of DNA or RNA can be collected at a remote site, sometimes without professional input, and transported to a laboratory where on-site testing is performed and the results reported back to the physician, who in turn can provide rapid feedback to the subject or patient. The elimination of the phlebotomist to collect a sample is the key driver in this instance.

1.1 Salivary DNA Collection

There are a number of tools available for genomic DNA collection from saliva and more are currently in development. These are based upon the collection of whole saliva, or in some cases buccal epithelial cells, harvested by a rinse solution or mouthwash system.

1.2 Salivary RNA Collection

Since the discovery of RNA in saliva [16], there has been a rapid uptake in transcriptomic analysis using saliva specimens. A group of RNAs termed "core" RNAs have been found to be present in both whole saliva and saliva supernatant and verified through experimental work [16].

The "gold standard" for salivary RNA collection termed "direct saliva transcriptome analysis" (DSTA) [35] has been well used routinely for collection and isolation of RNA (miRNA and mRNA) from patients with multiple diseases. The DSTA method involves processing "salivary supernatant" obtained by centrifuging saliva collected by the passive drool technique at $2600 \times g$ for 15 min at 4 °C followed by aspiration from the pellet. The salivary supernatant so obtained is stored ready for use at cool temperatures, without stabilizing agents, until use. mRNAs can be isolated by one of a number of commercial kits, but in the study by Lee et al. [35], mRNAs were isolated using the MagMAX Viral RNA Isolation kit (Applied Biosystems). The integrity of the mRNAs harvested was confirmed using a series of reference genes. This method remains the gold standard for comparative purposes.

1.3 Exosomes

The discovery [27] that small microvesicles, exosomes found in saliva, contain highly important salivary micro-RNAs (miRNAs) and messenger RNAs (mRNAs) has spawned the development of a series of tools to capture and interrogate microvesicles, exosomes, and cell-free DNA (and RNA) and miRNAs for transcriptomic analysis.

A report by Gallo et al. in 2012 [27] confirming that miRNAs in serum and saliva exist primarily inside exosomes, and that using the exosomal fractions of these bodily fluids increases the sensitivity of miRNA detection, has focused a lot of attention on various microvesicles, including exosomes.

Only recently tools for the analysis and quantification of exosomes in blood have become available, and work has begun on the evaluation of saliva as a readily available source of exosomes, and early work in this area is highly promising.

The established standard for exosome isolation involves ultracentrifugation [41]; however, exosomes have also been isolated by precipitation, microfiltration, and antibody-coated magnetic beads. Saliva exosome studies have traditionally utilized ultracentrifugation for isolation [42–44]; however, when exosomes were isolated by ultracentrifugation from glandular saliva and whole saliva by Michael et al. [42], the authors concluded that viscosity and cellular contamination in whole saliva make it a less than ideal medium for exosomal isolation, so a purified saliva specimen may be a more advantageous specimen to use.

1.4 Cell-Free DNA

Cell-free DNA (cfDNA) is an important component for evaluation of oncological markers in various malignancies [49], for noninvasive prenatal testing (NIPT, [50]), and for other diseases including rheumatoid disease, trauma, myocardial infarction, and fever and inflammatory disease [49, 51–54]. Methods for the isolation of cfDNA again typically include blood, amniotic fluid, and other invasive bodily fluids. While isolation of cfDNA has been carried out using saliva, the process involves centrifugation of a whole saliva specimen collected by the passive drool technique.

Importantly, at the heart of any successfully developed saliva diagnostic test or procedure is the need to successfully collect, stabilize, and recover the sample, so particular emphasis will be placed on these aspects in the text to follow.

2 Materials

2.1 Salivary DNA Collection Procedures

A number of commercial tools are now available for the collection of genomic DNA from saliva specimens (*see* **Note 1**).

1. The Oragene device from DNA Genotek (Ottawa, Canada) is the market-leading technology [28]. To collect a sample, subjects expectorate ("spit") into the Oragene device until a volume of 2 mL of saliva has been collected. A cap on the Oragene device containing proprietary stabilizing buffers is closed, and this causes a stabilizing buffer to flow into the saliva sample, resulting in a laboratory ready sample with long-term shelf life (1 year) (*see* **Note 2**).

2. The DNA·SAL™ device (Oasis Diagnostics®, Vancouver, USA) is a raking/scraping tool that collects cells from the inside of the oral cavity (buccal mucosa) [23, 29]. The collection head of the DNA·SAL™ tool is rubbed gently on the inside of the cheeks for 30 s, resulting in the accumulation of cells on the body of the DNA·SAL™ device. In addition, cells are abraded by the mild raking action and remain "free-flowing" in the saliva in the pool formed in the mouth. In order to harvest these cells and saliva, a small amount (2.5 mL) of a safe, stabilizing rinse solution is taken in the mouth, "swished around," and then expectorated (spat) back into a collection tube provided. The detachable head of the DNA·SAL™ device is then removed into the collection tube, to increase the yield of DNA. The sample obtained is stable for up to 30 days at room temperature.
3. Norgen Biotek (Ontario, Canada) has a device called the Saliva DNA Collection and Preservation Device [30]. The principles of this device are similar to the Oragene system. In this case, the subject expectorates into a Collection Funnel connected to a Collection Tube until a 2-mL sample of saliva has been collected (marked by a line on the Collection Funnel). The Collection Funnel is removed and may be recycled. A preservation agent is added to the saliva sample by means of an ampoule, and then the contents of the tube are mixed by shaking and are now ready for analysis or transportation to a laboratory for downstream testing. The Norgen sample is stable for up to 2 years.
4. The DNAgard® Saliva device from Biomatrica is a relatively new entrant into the field [31]. Once again, the Biomatrica device is modeled on similar principles to the Oragene and Norgen DNA devices. Subjects expectorate into a tube through a removable funnel until a "fill mark" is reached. The contents of a dropper bottle are then added to the saliva sample and the mixture inverted 5–7 times to stabilize the sample for up to 30 months at room temperature.
5. In addition to methods using passive drool and buccal cell harvesting, two well-known technologies use simple swabs. Where small to medium quantities of DNA are required, these devices may be suitable.
   (a) The Mawi Technologies iSWAB-DNA Isolation Kit [32, 33] uses a series of routine swabs (iSWABs) for sample collection. One of the "iSWABs" is placed in the mouth and rubbed against the inside of the cheek covering the whole cheek while rotating the iSWAB. The iSWAB is then placed into a Collection Vial with a narrow neck and screwed down in a corkscrew-like motion until the iSWAB reaches

the bottom of the Collection Vial containing a proprietary buffer solution. In order to mix the sample with the liquid in the Collection Vial, the iSWAB is moved up and down inside the Collection Vial 10–15 times. The iSWAB is then removed from the Collection Vial, and the entire procedure is repeated with an additional three iSWABs, by alternating between the left and right cheek. In each case, the iSWAB samples are introduced into the same Collection Vial in order to enrich the sample with DNA. Upon completion, a cap is placed on the Collection Vial and the sample stored or analyzed. Sample stability is several months at ambient temperature.

(b) The Isohelix DNA Buccal Swab kit [34] is described by the manufacturer as "using a unique swab matrix design to efficiently collect buccal cell samples." Two different swab types are available, and in each case, samples are collected by rubbing one of the swab types (designated SK-1 and SK-2) firmly against the inside of the cheek or underneath the lower or upper lip for 1 min. The head of the swab is then placed into a small Collection Tube, then the swab head removed from the shaft of the device, either by snapping the shaft at a notch etched into the side of the shaft (SK-1) or by sliding a plastic cover over the swab head and detaching the swab head by exerting pressure to dislodge the swab head (SK-2). Details of sample stability are not provided.

2.2 Salivary RNA Collection Procedures

The number of salivary RNA collection methods is fewer than for its counterpart, DNA; however, three or four technologies are worthy of mention:

1. For the Oragene RNA device from DNA Genotek (Ottawa, Ontario, Canada) [36, 37], subjects are asked to place a small amount of table sugar in the palm of their hands then touch the top of their tongue to the sugar, in order to stimulate greater saliva flow. The sugar and pooled saliva in the mouth are left there for 10–15 s without swallowing. The saliva that pools in the oral cavity is then expectorated into the Oragene container, a plastic Collection Tube. Expectoration is continued until a line on the Oragene device is reached (2.0 mL). The sample is then capped and tightened causing a buffer in the cap of the Oragene device to be released into the saliva sample causing immediate stabilization of the sample. The mixture of sample and buffer reagent is then shaken vigorously to mix the sample, which is reported to have a stability of 60 days at ambient temperature. The crude Oragene RNA mixture may be purified using a number of kits including Qiagen RNeasy Micro or Qiagen RNeasy Mini Kits using a centrifuga-

tion followed by pelleting step to obtain purified RNA for downstream analysis (*see* **Note 3**).

2. Norgen Biotek (Canada) offers "Saliva RNA Collection and Purification Devices" [38] based upon identical principles to the Saliva DNA Collection Devices branded by the company (*see* Subheading 2.1, **item 3**). The only significant difference in the collection procedure is the addition of an RNA stabilizing reagent instead of a DNA stabilizing agent. Norgen offers specific kits for isolation of RNA from saliva samples based upon a spin column technique.
3. Two devices are available from Oasis Diagnostics® (Vancouver, WA) for transcriptomic workup:
   (a) The RNAPro·SAL™ device [39] is a system for the simultaneous harvesting of two "cell-free" samples of saliva that may be used for both RNA and proteins or combined to provide a higher yield of saliva for transcriptomics or proteomics. In this device, saliva is collected from the pool of saliva in the oral cavity by means of an absorbent pad connected to a stem. After 1–3 min, saliva collection is complete, signified by a color change in a Sample Volume Adequacy Indicator (SVAI), within the device, from yellow to bright blue. The saturated absorbent pad is squeezed through a compression tube and then through a narrow bore filter containing a proprietary filtration medium. The sample is subsequently bifurcated (split into two) and collected into two equivalent 2-mL Eppendorf tubes where it may be stabilized. In the case of proteins, immediate stabilization is necessary, and this is facilitated using a protein stabilizing agent provided with the device. In the case of RNA, the purified saliva is stable for up to 14 days but may be stabilized as required by means of "off the shelf" RNA stabilizing reagents. The total yield of purified saliva is 1.0 mL.
   (b) The Pure·SAL™ device [40] may be a better option if protein is required. In this RNA is required. In this case, saliva is collected in identical fashion to the RNAPro·SAL™ device, but a *single* sample of saliva is collected by squeezing the saliva sample obtained through a compression tube into which has been inserted a proprietary separation medium.

   A minimum of 1.0 mL of cell-free saliva is collected into a single 2-mL Eppendorf tube and stabilized as above.

   Two important applications have been reported for the Pure·SAL™ device particularly, which equally apply to the "sister" RNAPro·SAL™ technology—these applications are for exosomes and cell-free DNA, each of which can provide increasingly important information on disease and disease processes of relevance to diagnosis.

2.3 Exosomes

1. Pure·SAL™ Oral Specimen Collection Device (Catalog Number PRSAL-401).
2. Precipitating reagent (ExoQuick-TC, System Biosciences, Mountain View, CA).
3. EXOCET lysis buffer (System Biosciences).

2.4 Cell-Free DNA (cfDNA)

1. Pure·SAL™ Oral Specimen Collection Device (Catalog Number PRSAL-401).
2. Falcon tubes.
3. Roche High Pure PCR Template Preparation Kit.
4. Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies).

3 Methods

Recently, the Pure·SAL™ device has been compared to whole saliva and validated for the collection of exosomes [45], quantified using precipitating reagents (ExoQuick-TC Kits) from System Biosciences [46]. Isolated exosomes were quantitated by a cholesteryl ester transfer protein (CETP) assay (EXOCET, System Biosciences) validated for the purification and quantification of exosomes [47, 48]. It was found that using the Pure·SAL™ device simplified collection significantly eliminated non-exosomal contaminating materials without loss of exosomes. A detailed description of the method comprising saliva collection, isolation of exosomes, and quantification is detailed below.

3.1 Sample Collection and Stabilization

Collect a saliva specimen by one of the methods described above in Subheading 2.2.

3.2 Isolation of Exosomes

1. Combine 1.7 g of collected sample with 340 µL of ExoQuick-TC and mix by inversion (*see* **Note 6**).
2. Incubate overnight at 4 °C.
3. Centrifuge sample at 16,000 × *g* for 5 min.
4. Resuspend resultant pellet in EXOCET lysis buffer (85 µL per tube) and incubate at 37 °C for 5 min.
5. Centrifuge at 2000 × *g* for 5 min.
6. Use resultant supernatant for analysis.

Results from the experiments are shown in Table 1. The experiment was repeated with a second saliva pool, and similar results were obtained. It was noted that if whole saliva is not processed at

Table 1
Comparison of the quantity of salivary exosomes collected by the Pure·SAL™ device and whole saliva followed by centrifugation

Process for sample isolation	Number of exosomes per mL	DNA (μg/mL)	Protein (mg/mL)
Whole saliva—centrifuged 16,000 × *g*	3.10×10^9	1.47	4.75
Pure·SAL™ device	3.25×10^9	1.19	4.58

sufficient centrifuge speeds, non-exosomal materials remaining in the exosome pellet will interfere with quantitation of exosomes by the cholesteryl ester transfer protein (CETP) assay.

3.3 Cell-Free DNA

1. *Sample collection.*
   I. Pure·SAL™: collect a saliva specimen as described above in Subheading 2.2.
   II. Whole Saliva:
      (a) Collect saliva by the passive drool technique into a 50-mL Falcon tube.
      (b) Centrifuge at 3000 × *g* for 20 min.
      (c) Take the supernatant and transfer to another centrifuge tube and centrifuge at 16,000 × *g* for 5 min.
2. Store all samples (I) and (II) at –80 °C prior to DNA isolation.
3. DNA isolation.
   (a) Isolate DNA with the Roche High Pure PCR Template Preparation Kit by using 700 μL saliva aliquots per isolation.
4. DNA quantification using PicoGreen.
   (a) Measure DNA quantity using the Quant-iT™ PicoGreen® dsDNA Assay Kit (*see* **Note** 7).
      - Prepare a standard curve using ten different concentrations of lambda DNA provided in the kit. Perform triplicate readings for increased precision.
      - Construct a standard curve using the values from the ten different concentrations of lambda DNA.
      - Measure the samples relative to the standard curve and present in a table format.
      - In the experimental work performed, it was shown that the Pure·SAL™ device removed 98.1–98.2% of all DNA, providing a total of 1.8–1.9% of cfDNA in comparison to the gold standard passive drool/centrifugation method which was effective in removing 98.9–99.1% of all DNA and providing 0.9–1.1% of cfDNA.

4 Notes

1. DNA from samples collected using one of the above commercial tools may be isolated using one of a significant number of DNA isolation kits provided by a number of manufacturers. The number of possibilities available is too numerous to cover in this manuscript; however, a number of manufacturers have developed specific saliva kits or validated certain kits to work well for saliva specimens. The list includes Qiagen Corporation (www.Qiagen.com), DNA Genotek (www.DNAGenotek.com), Norgen Biotek (www.NorgenBiotek.com), Biomatrica (www.Biomatrica.com), Oasis Diagnostics® (www.4saliva.com), Life Technologies (www.ThermoFisher.com), and others.
2. DNA Genotek received FDA 510(k) clearance for the use of Oragene in conjunction with a test for warfarin sensitivity developed by the company GenMark Diagnostics, so the device may be used *clinically* for this single application.
3. For RNA isolation, there are fewer kits available that have been specifically optimized for saliva specimens. The Qiagen miRNeasy kit has been used successfully for the isolation of purified RNA for transcriptome work, RNA sequencing, and other applications, as has the QIAzol lysis reagent from the same company. Other methods that have been used include organic extraction methods (TRIzol LS), spin filter-based methods (QIAamp Viral (Qiagen)), NucleoSpin (Clontech), and miRVana (Life Technologies) and combined method of organic extraction and spin filter clean up (miRNeasy micro (Qiagen)) and Quick-RNA MicroPrep (Zymo Research).
4. In reference to Subheading 1.4, the performance of one particular device (the Pure·SAL™ device) has been evaluated side-by-side with the "gold standard" method (passive drool/centrifugation) for cell-free DNA according to protocols outlined in the manuscript [55]. In the experiments performed, the Pure·SAL™ device was found to be a superior tool for harvesting cfDNA.
5. In Subheading 2.1, care should be taken to investigate options for DNA purification based upon the specific application required. These may include simple ethanol precipitation techniques, spin column methods, 96-well microplates, or automated methods, such as the Promega Maxwell 16 instrument or the Qiagen QIAsymphony equipment. Whole saliva contains a significant quantity of mucinous material that can have an impact on the quality of DNA obtained. It is recommended that investigators contact the individual manufacturers for details of any methods and how they may be applied to DNA isolation from saliva, prior to the commencement of any validation studies.

6. The method used in this chapter for isolation of exosomes is only one of a number of exosomal isolation kits now available. These include the Exo-spin kit from Cell Guidance Systems, Total Exosome Isolation Reagent from Thermo Fisher, miRCURY from Exiqon, PureExo Exosome Isolation kit from PureExo, and ExoCap Capture Kit from JSR Biosciences. Investigators are encouraged to validate the best method for exosome isolation in their own laboratory.
7. The authors also carried out DNA quantification by quantitative PCR (qPCR) as an alternate method of DNA assessment.

Acknowledgments

The author would like to acknowledge the support of Dr David T Wong (UCLA) for his support and encouragement in preparing this manuscript.

References

1. Punyadeera C, Slowey PD (2013) Saliva as an emerging biofluid for clinical diagnosis and applications of MEMS/NEMS in salivary diagnostics. Chapter 22 in "Nanobiomaterials in Clinical Dentistry." Elsevier, pp 453–475, ISBN: 978-1-4557-3127-5
2. Streckfus C, Bigler L (2002) Saliva as a diagnostic fluid. Oral Dis 8:69–76
3. Slowey PD (2013) Commercial saliva collections tools. J Calif Dent 41(2):97–105
4. Malamud D, Tabak LA (eds) (1992) Saliva as a diagnostic fluid. Vol 694, Annals NY Academy of Science
5. Samaranayake LP (2006) Saliva tells the body's health. In: Stephen Moss (ed) The benefits of chewing. pp 24–35
6. Zachary D, Mwenge L, Muyoyeta M, Shanaube K, Schaap A, Bond V, Kosloff B, de Haas P, Ayles HBMC (2012) Infect Dis 8(12):183
7. Testing Oral Fluid for the Presence of HIV Antibodies February 2013. Report by the Association of Public Health Laboratories (APHL) http://www.aphl.org/AboutAPHL/publications/Documents/ID_Feb2013_Testing-of-Oral-Fluid-for-the-Presence-of-HIV-Antibodies-Brief.pdf
8. See http://www.zrtlab.com/patients-standard-tests/saliva
9. See http://www.diagnostechs.com/Pages/Home.aspx
10. See https://www.labrix.com/SalivaryHormoneTesting
11. Cone EJ, Huestis M (2007) Interpretation of oral fluid tests for drugs of abuse. Ann NY Acad Sci 1098:51–103
12. Bradshaw DJ, Marsh PD (1998) Analysis of pH-driven disruption of oral microbial communities in vitro. Caries Res 32:456–462
13. Bratthall D, Hansel Petersson G (2005) Cariogram-a multifactorial risk assessment model for a multifactorial disease. Community Dent Oral Epidemiol 33:256–264
14. Larmas M (1992) Saliva and dental caries: diagnostic tests for normal dental practice. Int Dent J 42:199–208
15. Christodoulides N, Floriano PN, Miller CS, Ebersole JL, Mohanty S, Dharshan P, Griffin M, Lennart A, Ballard KL, King CP Jr, Langub MC, Kryscio RJ, Thomas MV, McDevitt JT (2007) Lab-on-a-chip methods for point-of-care measurements of salivary biomarkers of periodontitis. Ann NY Acad Sci 1098:411–428
16. Li Y, St John MA, Zhou X, Kim Y, Sinha U, Jordan RC, Eisele D, Abemayor E, Elashoff D, Park NH, Wong DT (2004) Salivary transcriptome diagnostics for oral cancer detection. Clin Cancer Res 10:8442–8450
17. Streckfus C, Bigler L (2005) The use of soluble, salivary c-erbB-2 for the detection and post-operative follow-up of breast cancer in women: the results of a five-year translational research study. Adv Dent Res 18:17–24
18. Streckfus C, Bigler L, Dellinger T, Dai X, Cox WJ, McArthur A, Kingman A, Thigpen JT

(2001) Reliability assessment of soluble c-erbB-2 concentrations in the saliva of healthy women and men. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 91:174–179

19. Streckfus C, Bigler L, Dellinger T, Dai X, Kingman A, Thigpen JT (2000) The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. Clin Cancer Res 6:2363–2370
20. Streckfus C, Bigler L, Dellinger T, Pfeifer M, Rose A, Thigpen JT (1999) CA 15-3 and c-erbB-2 presence in the saliva of women. Clin Oral Investig 3:138–143
21. Streckfus C, Bigler L, Tucci M, Thigpen JT (2000) A preliminary study of CA15-3, c-erbB-2, epidermal growth factor receptor, cathepsin-D, and p53 in saliva among women with breast carcinoma. Cancer Invest 18:101–109
22. Streckfus C, Bigler L, Dellinger T, Kuhn M, Chouinard N, Dai X (2004) The expression of the c-erbB-2 receptor protein in glandular salivary secretions. J Oral Pathol Med 33:595–600
23. Ovchinnikov DA, Cooper MA, Pandit P, Coman WB, Cooper-White JJ, Keith P, Wolvetang EJ, Slowey PD, Punyadeera C (2012) Tumor-suppressor gene promoter hypermethylation in saliva of head and neck cancer patients. Translat Oncol 5:321–326
24. Hu S, Zhou M, Jiang J, Wang J, Elashoff D, Gorr S, Michie SA, Spijkervet FK, Bootsma H, Kallenberg CG, Vissink A, Horvath S, Wong DT (2009) Systems biology analysis of Sjogren's syndrome and mucosa-associated lymphoid tissue lymphoma in parotid glands. Arthritis Rheum 60:81–92
25. Corstjens LAM, Abrams WR, Malamud D (2012) Detecting viruses by using salivary diagnostics. J Am Dent Assoc 143:12S–18S
26. Shirtcliff EA, Buck RL, Laughlin MJ, Hart T, Cole CR, Slowey PD (2015) Salivary cortisol results obtainable within minutes of sample collection correspond with traditional immunoassays. Clin Ther 37:505–514
27. Gallo A, Tandon M, Alevizos I, Illie GG (2012) The majority of MicroRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One 7, e30679, http://www.Plosone.org
28. See http://www.dnagenotek.com/US/pdf/MK-006.pdf
29. Thomas GA, Oberkanins C, Berndt A, Slowey PD (2014) Validation of a series of genomic StripAssays® to salivary DNA collection using the DNA·SAL™ Device. Paper presented at the American Society of Human Genetics Meeting, San Diego, October 2014
30. See https://norgenbiotek.com/product/saliva-dna-collection-preservation-and--isolation-kit
31. See http://biomatrica.com/dnagardsaliva.php
32. See http://www.mawidna.com/products/iswab-dna-collection-kit
33. El-Hahmawi B (2014) An efficient non-invasive sample collection technology for various population segments. Paper presented at the Qatar Foundation Annual Research Conference, At Doha, Qatar, Accessed 18 Nov 2014
34. See http://www.isohelix.com/products/isohelix-dna-buccal-swabs
35. Lee YH, Zhou H, Yan X, Zhang L, Chia D, Wong DTW (2011) Direct saliva transcriptome analysis. Clin Chem 57:1295–1302
36. See http://www.dnagenotek.com/US/products/RE100.html
37. Patel RS, Jakymiw A, Yao B, Pauley BA, Carcamo WC, Katz J, Cheng JQ, Chan EK (2011) High resolution of microRNA signatures in human whole saliva. Arch Oral Biol 56:1506–1513
38. See https://norgenbiotek.com/product/saliva-rna-collection-and-preservation-devices
39. Chiang SH, Thomas GA, Liao W, Grogan T, Buck RL, Fuentes L, Yakob M, Laughlin MJ, Schafer C, Nazmul-Hossain A, Wei F, Elashoff D, Slowey PD, Wong DT (2015) RNAPro*SAL: a device for rapid and standardized collection of saliva RNA and proteins. Biotechniques 58:69–76
40. See http://4saliva.com/products/pure%E2%80%A2sal
41. Théry C, Zitvogel L, Amigorena S (2002) Exosomes: composition, biogenesis and function. Nat Rev Immunol 2:569–579
42. Michael A, Bajracharya SD, Yuen PS, Zhou H, Star RA, Illei GG, Alevizos I (2010) Exosomes from human saliva as a source of microRNA biomarkers. Oral Dis 16:34–38
43. Lau C, Kim Y, Chia D, Spielmann N, Eibl G, Elashoff D, Wei F, Lin YL, Moro A, Grogan T, Chiang S, Feinstein E, Schafer C, Farrell J, Wong DT (2013) Role of pancreatic cancer-derived exosomes in salivary biomarker development. J Biol Chem 288:26888–26897
44. Lässer C, Alikhani VS, Ekström K, Eldh M, Paredes PT, Bossios A, Sjöstran M, Gabrielsson S, Lötvall J, Valadi H (2011) Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. J Translat Med 9:9
45. Laughlin MJ, Buck RL, Slowey PD (2014) A simplified method for the isolation of exosomes from saliva using a prototype saliva collection device (RPSAL-702). Paper presented at the

North American Saliva Symposium Boston, October 2014

46. See https://www.systembio.com/microrna-research/exoquick-exosomes/overview?gclid=CPTmjv2foMoCFQWUfgodu_gJUw
47. Savina A, Vidal M, Colombo MI (2002) The exosome pathway in K562 cells is regulated by Rab11. J Cell Sci 115:2505–2515
48. Gupta S, Knowlton AA (2007) HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway. Am J Physiol Heart Circ Physiol 292:H3052–H3056
49. Fleischhacker M, Schmidt B (2007) Circulating nucleic acids (CNAs) and cancer—a survey. Biochim Biophys Acta 1775:181–232
50. The American College of Obstetricians and Gynecologists Committee Opinion Number 640 September 2015. Cell-free DNA screening for fetal aneuploidy, http://www.acog.org/Resources-And-Publications/Committee-Opinions/Committee-on-Genetics/Cell-free-DNA-Screening-for-Fetal-Aneuploidy
51. Zhong XY, von Mühlenen I, Li Y (2007) Increased concentrations of antibody-bound circulatory cell-free DNA in rheumatoid arthritis. Clin Chem 53:1609–1614
52. Lam NY, Rainer TH, Chan LY, Joynt GM, Lo YM (2003) Time course of early and late changes in plasma DNA in trauma patients. Clin Chem 49:1286–1291
53. Chang CP, Chia RH, Wu TL, Tsao KC, Sun CF, Wu JT (2003) Elevated cell-free serum DNA detected in patients with myocardial infarction. Clin Chim Acta 327:95–101
54. Moreira VG, Prieto B, Rodriguez JS, Alvarez FV (2010) Usefulness of cell free plasma DNA, procalcitonin and C-reactive protein as markers of infection in febrile patients. Ann Clin Biochem 47:253–258
55. Slowey PD, Giese U, Hofner M, Kegler U, Weber M, Buck RL, Laughlin MJ (2014) Comparison of RNAPro·SAL™ saliva collection versus centrifugation for cell-free DNA isolation from saliva specimens. Paper presented at the Molecular Medicine Tri-Conference, San Francisco CA, February 2014