Saliva Collection Devices and Diagnostic Platforms

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Abstract

Over the last few years, there has been a tremendous growth in less invasive diagnostic testing as a replacement for painful and expensive blood draws. The role of saliva in this growth trend has been enormous, and this has been driven in part by a growing awareness of the broad utility of saliva as a diagnostic medium and reinforced by a rapidly growing number of publications supporting new and varied applications for saliva. The aim of this chapter is to highlight some of the tools now available that are responsible for this growth trend and provide a glimpse into the future for salivary diagnostics in the research and clinical environments.

Salivary Diagnostics: The Future of Diagnostic Testing

Over the last few years, there has been a tremendous growth in less invasive diagnostic testing as a replacement for painful and expensive blood draws. The role of saliva in this growth trend has been enormous, and this has been driven in part by a growing awareness of the broad utility of saliva as a diagnostic medium and reinforced by a rapidly growing number of publications supporting new and varied applications for saliva. The aim of this chapter is to highlight some of the tools now available that are responsible for this growth trend and provide a glimpse into the future for salivary diagnostics in the research and clinical environments.

Over the last decade, the opportunity to use saliva as a noninvasive testing option has been reinforced by a number of high-profile organizations including the National Institutes of Health (NIH) [1], National Institute of Dental and Craniofacial Research (NIDCR) [2, 3], American Dental Association [4], American Association of Dental Research [5], and the Federation Dentale Internationale (FDI, World Dental Federation) [6], among others, who have endorsed approaches using salivary diagnostics. Some of these institutions have also provided valuable funding that has resulted in new technologies that overcome many of the barriers that slowed down the earlier development of oral fluid-based diagnostics.

Funded by the NIH, the Salivary Proteome Project [7] was a "landmark" undertaking that led to the characterization of 1,166 proteins in saliva

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and elucidation of the roles of many of these proteins in disease and disease progression. This initiative provided the impetus and foundation for a future generation of oral tools. In this groundbreaking initiative, data was collected from 23 adults from both sexes and multiple races. Using salivary diagnostics, the research team was able to detect and monitor changes in the individual proteome as a means of accurately and painlessly identifying the onset of a particular disease at the molecular level, which has obvious implications in disease identification at an earlier time point leading to the saving of lives. The legacy of the Salivary Proteome Project is that it has spurred a significant amount of activity and new research in this area.

The Streckfus group at Mississippi provided early evidence of the potential role of saliva in studies looking at a number of malignancies, particularly breast cancer using salivary c-erbB-2 (also known as Her-2/neu) and other biomarkers [8–10], and these studies provided an essential building block that formed a solid foundation for others to follow.

A "landmark meeting" in Lanier Lakes, near Atlanta Georgia in 2006, entitled "Oral Diagnostics," was attended by many of the world's leading minds in the saliva area and culminated in the publication of a successful monograph of the same name [11]. This "coming together" of many of the world's "leading lights" in a single focused event also moved the field forward by a significant margin.

Early in 2013, the State of Massachusetts provided significant funding ($4.1 million) to the Forsyth Institute for Salivary Diagnostics in Cambridge Massachusetts, an affiliate of the Harvard School of Dental Medicine, to build a center devoted entirely to research, development, and commercialization of saliva-based diagnostic tests [12], a move that clearly signals an expanding interest in providing answers through saliva.

The aforementioned examples simply illustrate some of the factors pointing to the immediate and growing interest in salivary diagnostics. Many others exist. The reader is referred to a number of important reviews on salivary diagnostics, which will serve to provide excellent background information (see [11, 13–21]). Many other good reviews on salivary diagnostics exist, so these are just a selection of what the reader may wish to view in order to understand this subject in greater detail.

Typically levels of analytes in saliva/oral fluids are significantly lower than in serum/blood, so the current growth in the development of oral fluid technologies has coincided with improvements in detection sensitivities, which now allow low-level quantification of biomarkers in lateral flow, lab-on-a-chip, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), microarrays, mass spectrometry, and multiple other technologies. As a result, the number of new oral fluid diagnostics in the development pipeline or commercially available has increased exponentially. Add to this significant technological advances in the manufacturing of oral-based devices, which are now produced with much greater precision on a lot-to-lot basis, and clear cost efficiencies and you have three of the major factors that are contributing to the salivary diagnostics "success story" and are responsible for the growth in oral fluid technologies.

Saliva is an "ultra-filtrate" of blood and as such acts as a "mirror of the body's health" [4] offering many unique advantages over other bodily fluids. Most analytes, biomarkers of disease, and drugs appear in the saliva through passive diffusion and other mechanisms. Saliva offers several advantages for downstream diagnostic work-up. These include the fact that saliva is the easiest sample to collect, offering considerable cost and disposal advantages to the user; specimens can be collected in observed fashion by minimally trained individuals, eliminating the need for costly phlebotomists and additional processing steps once the sample has arrived at the laboratory or alternate testing site. In addition, saliva samples are noninfectious and may be readily disposed with minimal cost or the need for biohazardous waste containment during transportation. When required, sample transportation is also significantly cheaper, and saliva also eliminates certain cultural "taboos" associated with blood collection prevalent in certain international cultures.

An additional contributing factor to the growth of oral-based technologies is the availability of *standardized* saliva collection methodologies offering the ability to successfully and *consistently* collect and test for a rapidly growing number of diseases and biomarkers from small quantities of saliva. Examples now abound in the literature and include infectious diseases, drugs of abuse, hormones for general wellness, oncology markers, DNA, RNA, and multiple proteins among others.

The subject topic is very broad, so this chapter will focus on four key areas, which are intended to provide a snapshot in time of current and future saliva collection tools and platform systems that are likely to positively impact salivary diagnostics over the next few years.

Following an illustrated history of salivary diagnostics and factors influencing the future impact of saliva as a bodily fluid for diagnosis, this chapter will cover the following major subtopic areas:

1. Whole versus glandular saliva secretions
2. Collection devices for whole saliva and various salivary gland secretions
3. Preservation of saliva specimens
4. Saliva diagnostic platforms

Note: Salivary diagnostics is a highly dynamic area that is in a major growth spurt, so while the author makes every effort to include all technologies of relevance, it may be that certain newer technologies may not be included. Readers are encouraged to review a number of excellent review and scientific articles cited here [11, 13–21].

History

The modern history of salivary diagnostics is fairly recent (1990s); however, saliva actually has a much longer and quite "checkered" history. In ancient Chinese times, for instance, authorities conceived the world's first "lie detector" test using the properties of saliva. At that time, those suspected of a crime were asked to chew rice, while being questioned for a suspected misdemeanor. After question time was over, if the rice was dry, the suspect was assumed to be guilty. Authorities believed then, which is now supported by recent evidence, that nervous tension created by falsifying statements slows or blocks the flow of saliva from the glands leading to a dry mouth condition and hence dryer than normal saliva. Under such circumstances, suspects are unable to moisten the rice and found guilty of the charges.

Saliva still has a very important role to play in traditional Chinese medicine. Saliva is considered a "precious" fluid, and this comes from a Chinese theory known as the "Fluids of the Five Organs." There are five critical fluids that support healthy life, according to ancient Chinese medicine, and of these, saliva is the "fluid of the spleen and the kidneys." Since these organs are particularly important for a long and active life, saliva as the bodily fluid that maintains the spleen and kidneys has become a highly precious fluid. Taoists in particular believe that chewing, producing saliva, and swallowing saliva are important contributory factors to a healthy mind and body. The manuscript Huangdineijing Lingshu [22] is considered the "Bible" of traditional Chinese medicine books, and in that text saliva is considered the "spiritual fluid" implying a relationship between the mind, brain function, and saliva. Taoists firmly believe that in order to have longevity in life, saliva should not be expectorated, but instead kept in the mouth then swallowed. According to belief, the net effect of this is a clear mind and moist skin.

In the Middle Ages, spitting was an accepted practice and was commonplace in normal life in the Western world. At that time swallowing one's spit was considered rude, but, obviously, things have changed since then. Public spitting was outlawed in the 1700s when officials introduced legislation prohibiting the practice. It was at that time that public spittoons appeared, providing a place for the public to discard unwanted "oral material."

The military has always had a use for saliva in "spit shining" boots, but this practice was eventually replaced by warm water instead of the more traditional saliva, to create the optimum boot shine.

Irwin Mandel [23], well-renowned saliva researcher and noted historian on salivary matters, recounts that many centuries ago early

physicians believed that the salivary glands were "lowly excretory organs" functioning to rid the body of toxins and evil spirits from the brain. During those times "doctors" would carry out strange acts, including administering poisonous mercury chloride to patients, causing saliva to ooze from the mouth. In Mandel's mind "saliva is a latecomer," and he commented that scientists in general only began to seriously look at saliva after they had looked at other (more) traditional bodily fluids, particularly blood. Mandel's now well-known statement "saliva doesn't have the drama of blood, it doesn't have the integrity of sweat and it doesn't have the emotional appeal of tears" captured the moment of the time, but from the 1950s onward, this statement became less and less accurate as many scientists, including Mandel, went on to provide evidence and new discoveries in the field, including the important observation that human saliva is actually bursting with hundreds of useful chemical components mixed in with millions of bacteria, viruses, yeasts, and skin cells in a concoction now known to provide excellent protection to the oral cavity. Many salivary proteins have since been characterized that are absolutely critical for the maintenance of good oral health and work in the proteomics area continues to be a burgeoning field where many new and fascinating discoveries are made on a daily basis.

Some recent historical milestones belong to OraSure Technologies (formerly Epitope, USA, http://www.orasure.com) and a former rival Company Saliva Diagnostic Systems (SDS, USA, now StatSure Diagnostics, http://www.statsurediagnostics.com), which led the race for early superiority in the commercial end of the salivary diagnostics arena in the early 1990s. In the end, history shows that it was OraSure who succeeded in producing both the first laboratory oral human immunodeficiency virus (HIV) test kit to gain US Food and Drug Administration (FDA) approval (OraSure HIV-1) and the first and only *rapid, oral point-of-care* (POC) test to gain marketing approval through the FDA Pre Market Approval (PMA) process (OraQuick HIV 1/2).

In 1994, Epitope, Inc. obtained FDA approval to collect oral fluid and reflex to an ELISA test (the Vironostika HIV MicroElisa test kit from Organon Teknika). This remained the only laboratory test that processed oral fluid specimens for HIV diagnosis until a few years ago when the company Avioq, Inc. successfully gained FDA approval for a second oral HIV ELISA test. OraSure Technologies' rapid oral POC test, OraQuick HIV 1/2 later successfully passed FDA scrutiny through an arduous Biological License Application (BLA) process and is now sold directly to consumers, who can purchase the OraQuick HIV 1/2 test in certain pharmacies to test themselves in the privacy of their own homes for $39.99.

Saliva Diagnostic Systems was later able to gain FDA clearance for its Saliva•Sampler® Oral Fluid Collection device, but the limited regulatory approval was for saliva collection only. Despite the lack of approvals for clinical applications, the device found widespread use in the research community.

Perhaps one of the most important areas where saliva can play a role is in the collection of nucleic acids. The harvesting of nucleic acids from bodily fluids and tissue is a rapidly growing area, particularly in life science research and more recently in the clinical realm. A major milestone was reached in 2011 when DNA Genotek (Canada, http://www.dnagenotek.com) successfully obtained FDA clearance for its Oragene DNA Collection Device when used in conjunction with the GenMark Diagnostics e-Sensor Warfarin Sensitivity polymerase chain reaction (PCR) Assay. Although the FDA status is confined to a single application, this clearly opens up the door for other clinical tests to be validated for saliva/orally collected specimens.

The major history of saliva diagnostics remains to be written, but all signs point to a major role to play for this underutilized fluid.

Factors Likely to Positively Impact the Role of Salivary Diagnostics in the Future

The importance of saliva as a bodily fluid for diagnosis of diseases is finally rising rapidly in significance due to a number of important contributing factors. There has never been any doubt on

the ease of use of saliva as a diagnostic specimen, or the fact that it is noninvasive, is considered a "safe" sample, or may be readily transported at lower cost than traditional blood, urine, or serum specimens. Very few dispute the fact that when offered the option of providing a blood or saliva sample, most people opt to provide a saliva specimen. It is also an easy specimen to dispose of, without the need for biohazardous waste management, and contains many of the important components of blood that are relevant to disease detection. So, why has saliva not been the ideal medium for test developers in the past and what are the reasons for the current "exploding" demand for "salivary diagnostics"? Opinions may vary, but in this author's opinion, there are a number of reasons for this change, and these may be broken down into specific categories. Some of these are described as follows:

1. *Technological Developments*: Briefly discussed above, one of the most fundamental changes over the last 20 years has been the development of newer saliva specimen collection technologies as well as downstream testing technologies with enhanced sensitivity and specificity characteristics, which now allow very small concentrations of analytes to be detected and quantified in saliva, with high precision. Typically, analytes in saliva are present in much lower quantities than in blood (1/100th to 1/2000th the concentration) so the advent of technologies such as PCR for detection of nucleic acids, tandem mass spectrometry to detect small concentrations of proteins and chemiluminescence, fluorescence, and magnetic bead technologies to detect small- and medium-sized molecules in lateral flow immunochromatographic platforms are just some of the reasons for the rapid growth in oral specimen testing. Specifically in the point of care area, improvements in manufacturing technology and knowledge of how lateral flow test strips are carefully assembled have resulted in more consistent products, which result in much less variability from strip lot to strip lot.

   Another contributory factor is a historical use of collection materials that were not totally appropriate for the collection of saliva. Certain absorbent materials that were used in a multitude of early studies as the vehicle for saliva collection have been shown to bind specific molecules tightly, leading to poor recoveries of target analyte and inferior correlation with serum or whole blood assays. Multiple publications have confirmed that various cotton-based products and, to a lesser extent, certain cellulose products can have less-than-desirable effects on recovered concentrations from oral specimens. While a number of publications [24–27] now highlight this phenomenon, it has taken many years to overcome this negative "perception" of saliva, and even now further education is necessary to inform potential users tainted by earlier negative reports. Due caution should always be observed when choosing the most appropriate tools for collection of saliva for analysis or disease diagnosis.
2. *External Forces*: A number of key organizations now put their weight behind the movement for more and better salivary diagnostics, and the reasons behind this and some important details have already been discussed. NIH, ADA, AADR, FDI, and NIDCR are just some of the organizations that have made policy statements supporting the development and implementation of salivary diagnostic tools. In addition to these contributions, several external "market forces" come into play that make oral diagnostic systems even more attractive:

   The *in vitro diagnostics* (IVD) market into which all diagnostic products fit was estimated to be worth $46 billion USD in 2012 according to Frost and Sullivan [28] and growing at an annual rate of 7 %, leading to an estimated market of $65 billion by 2017. Within the overall market, there are multiple segments; however, as a general rule, diagnostics typically fit into one of the three broad categories: laboratory-based diagnostics, molecular diagnostics (MDx), and point-of-care (POC) systems. Certain products could fit into one or more categories, but for the purposes of looking at broad diagnostic areas, these three sectors define the overall market well. If each of these sectors is looked at independently, there is significant growth in all three areas;

however, the areas of greatest growth are in the fields of MDx and POC diagnostics. Multiple factors affect the commercial markets for diagnostic tests, and some of these factors impact individual sectors more than others. As an example, cost plays an important role in the decision of laboratorians, medical directors of testing organizations, public health departments, and other healthcare decision makers involved in choosing tests for implementation. Reimbursement for those tests from Medicare, Medicaid, or private insurance companies also plays a key role.

Turnaround time (TAT) is now a much more important consideration for stakeholders as testing laboratories look to provide results "quicker, faster, and better" than the competition. Decentralization of testing over the last 10 years or so has also changed the landscape for diagnostics as customers look to options for testing in outpatient centers, clinics, and less traditional settings to move testing out of the laboratory and closer to the patient.

Since the introduction of molecular diagnostics with the advent of polymerase chain reaction, the role of traditional immunoassays has continued to grow but is now supported by an ever-expanding demand for molecular assays that can provide new and valuable information on genetic susceptibility, genotype, phenotype, familial traits, origins of our species, and many other pieces of fundamental information relevant to gaining a complete picture of disease and disease processes. It is estimated that 500 million MDx tests were performed in 2010, and this number is expected to climb to 750 million in 2015 [28]. Although the costs of MDx tests are typically higher than traditional immunoassays, the additional information on disease processes provided by such tests outweighs any cost implications. Saliva as a convenient and cost-effective bodily fluid for diagnostic use has probably found its greatest potential in the MDx field, but applications in traditional diagnostics and point of care are now appearing in greater numbers. These aspects will be covered in greater detail in the section devoted to saliva collection tools.

3. *Market Trends*: There has been a lot of focus in the medical literature on "personalized medicine" and the tailoring of therapeutics to each individual. Certain medicines have been found to be ineffective in people who possess certain genotypes/phenotypes, which has resulted in greater levels of testing to identify patients who will benefit from new therapeutics, those who will remain unaffected, and those who might even suffer adverse effects by being enrolled on such a treatment. Various drugs prove not only to be ineffective in certain patients with known genotype/phenotype, but have been shown to have adverse effects on patient outcomes, so this important finding ensures that greater levels of testing will need to be done in the future prior to enrollment in treatment regimens.

Another phrase that is used liberally in the therapeutic/diagnostic area is the term "companion diagnostics" (CDx). This terminology generally refers to the requirement to have a diagnostic test available at the same time as the market entry for any new drug entity likely to require some degree of monitoring. Specific companion diagnostic tests will typically be recommended on labeling by the FDA during the approval process for the drug and will be used to test all candidate patients to ensure that only those patients who will benefit from a given new drug are treated. Particularly, if it is known that patients with certain genetic profiles will benefit from a therapeutic and others will not, or patients with a specific genetic profile will suffer adverse effects by taking a new drug, the FDA will exert its authority to mandate that a diagnostic test be available to screen patients prior to enrollment in treatment regimens.

The development of a companion diagnostic is a cooperative effort where pharmaceutical companies and diagnostics manufacturers enter into some sort of "partnering agreement" and work together to develop a test that will be cleared or approved for marketing in the United States at the same time as the medicine. One of the first examples of this was the combination of therapeutics for colorectal

cancer from Amgen (cetuximab) and Bristol Myers Squibb (Erbitux), with the "Scorpions" KRAS PCR diagnostic test from DxS (now part of Qiagen Corporation, Germany).

There are a number of CDx tests in development, and this number is forecast to grow rapidly in the years ahead. Saliva is an ideal matrix for CDx testing.

Another market, which is experiencing significant growth, is noninvasive prenatal testing. A number of companies now offer tests for prenatal screening. Examples include the Panorama test from Natera, Inc., the Harmony test from Ariosa Diagnostics, Verifi from Verinata Health (an Illumina Company), and the MaterniT21 test from Sequenom that use cell-free DNA (cfDNA) to look for various chromosomal abnormalities in the DNA of the maternal blood that has been passed to the mother by the fetus while still in the womb. Some of the conditions that can be detected from defects in the chromosomal order are Down syndrome, Edwards syndrome, Patau syndrome, and certain sex chromosome trisomies.

While these tests use blood (which in the author's opinion is not truly "noninvasive"), the attention focused on these tests brings greater awareness for the need for less intrusive forms of testing, including urine, finger stick blood, tears, and saliva.

4. *Milestones*: There are a number of events in the history of salivary diagnostics that will be seen in the future very much as landmark events that put oral testing "on the map." Two of the most recent occurrences have been even more effective in capturing the imagination and interest of the consumer market in general and have led to a new generation of diagnostics under development.

   The approval of the OraSure HIV-1 Oral Specimen Collection Device and the Organon Teknika Vironostika HIV-1 ELISA Test Kit in 1994 was a major achievement at the time, and this paved the way for the subsequent approval of the first rapid, oral HIV test 10 years later. Again OraSure Technologies was responsible for developing the OraQuick HIV 1/2 initially for sale to healthcare professionals only. In retrospect these were both highly significant events, but even more "powerful" was the subsequent approval of the OraQuick HIV 1/2 test for consumer/over-the-counter use in 2012. This milestone signaled the first ever oral test for a potentially high-risk infectious disease to be sold directly to consumers through independent pharmacies throughout the nation.

   In the molecular area, DNA Genotek (Ottawa, Canada) was successful in obtaining an FDA clearance for its oral DNA specimen collection device (OraGene) when used with the GenMark Diagnostics e-Sensor Warfarin Sensitivity Tests. During the FDA regulatory process, DNA Genotek was able to demonstrate that saliva proved equivalent in performance to blood and as a result users of the e-Sensor Warfarin Test can now opt to use either saliva or blood. The clearance for the OraGene device has since opened up the door for many other potential clinical applications for saliva and the use of salivary samples for nucleic acid testing. At this point in time, this single application remains the only test cleared by the FDA for clinical use, but many companies now see saliva as a viable fluid and are proceeding with the development of orally based clinical tests.

5. *Funding for Salivary Diagnostics*: The topic of endorsement by key organizations and the availability of funding for research has been covered in some depth above, but some other recent funding events have brought increased attention to salivary diagnostics.

   In August 2013, the NIH, through its National Center for Advancing Translational Services, provided $17 million in funding for 24 projects under a new "Extracellular RNA Communication Program."

   A significant share ($5.5 million) of the $17 million went to UCLA Dentistry to study biological markers in saliva in order to develop functional tools for the detection of pancreatic cancer. According to a press release from UCLA, "the study will create a new paradigm in the field of salivary diagnostics, and it could

supply concrete evidence that saliva can be used in the detection of life threatening diseases, including diabetes and cancers of the pancreas, breasts, ovaries and stomach" [29].

In 2010, NIH through the NIDCR (National Institute for Dental and Craniofacial Research) funded two important studies entitled "salivary biomarkers for early oral cancer detection" and "salivary proteomic and genomic biomarkers for primary Sjögren's syndrome." Scientists have now identified the genes and proteins that are expressed in the salivary glands, so using the vast accumulated information as their guide, they will define the patterns and certain conditions under which these genes and proteins are expressed in the salivary glands and how these parts function as a fully integrated biological system.

Back in 2002, NIDCR provided funding for the development of new salivary POC testing platforms. At that time, seven research groups received funding to develop tools focused on the use of microfluidics and micromechanical systems. The projects were directed at detection and analysis of the constituents of saliva, including miRNA, mRNA, proteins, DNA, electrolytes, and others. Second round funding was provided to four of the seven, and NIDCR has recently reported that each is on the way to completing development of a platform based upon oral specimens.

Whole Versus Glandular Saliva Secretions

The word "saliva" has been used in widespread fashion in the published literature to describe secretions in the oral cavity; however, a number of different subcomponents exist in saliva, and various terms may be used to describe fluids collected from the mouth, including the broad term saliva, oral fluids, gingival crevicular fluid (GCF), and others. It is important, therefore, to provide brief definitions of the most important terms used when discussing salivary tools with potential diagnostic or investigative applications:

Saliva – this is a watery substance located in the mouth of organisms, secreted by the three main salivary glands (the submandibular, the parotid, and sublingual), as well as hundreds of other minor salivary glands and gingival crevicular fluid. Human saliva is composed of 95 % water, but also electrolytes, mucus, antibacterial compounds, and enzymes, and performs many normal functions including food digestion, lubrication, taste facilitation, and bolus formation.

Oral Fluids – this is a term often used interchangeably with "saliva" and used very often in forensic toxicology and, in particular, the drug testing world.

Gingival Crevicular Fluid – a fluid occurring in minute amounts in the gingival crevices, believed by some authorities to be an inflammatory exudate and by others to cleanse material from the crevices. GCF contains sticky plasma proteins, which improve adhesion of the epithelial attachment, has antimicrobial properties, and exerts antibody activity[1]. Definitions of other oral secretions are included below in the text.

Saliva is produced in the salivary glands and secreted from there as clusters of cells known as acini. The acini secrete fluids containing a mixture of enzymes, water, mucinous material, and various electrolytes. This concoction is collected from the acinus into specific collection ducts, where the composition of the fluid may be altered. These ducts lead into much larger ducts that eventually form a single duct that delivers the saliva mixture into the oral cavity.

Humans have three pairs of salivary glands that each delivers different secretions. The parotid gland produces a serous watery secretion, the submaxillary (mandibular) glands empty a mixed serous and mucous containing secretion, and the sublingual glands secrete a fluid that is essentially mucinous in character. The basis for the different compositions of saliva secreted by each of the various gland types has been proven histologically, and early studies by oral biologists

[1] Definition from Jablonski S. *Illustrated Dictionary of Dentistry*, W B Saunders Co. July 1982.

have shown that the composition of certain components can vary significantly from one type of salivary gland to another. For this reason it is important that the differences are understood and that the proper mouth location for appropriate sample collection is consistently used. This is also the reason that tools exist to collect whole saliva and also for various glandular types that may provide different and additional informative information on events happening in the oral cavity and in general disease processes.

Collection Devices for Whole Saliva and Various Salivary Gland Secretions

Tools for Collection of Whole Saliva

Nonmolecular Tools

The modern history of salivary collection tools with high commercialization potential can be traced back to the early 1990s when two companies based in the Pacific Northwest of the United States—Epitope Inc. (Beaverton, OR) and Saliva Diagnostics Systems (Vancouver WA)—vied for early market supremacy. The first "entrées" for each of these companies were unique, and distinct saliva collection devices that two decades later have gone on to become the most successful salivary collection tools so far produced. Epitope Inc. (which became OraSure Technologies www.orasure.com following the acquisition of Solar Technologies Corporation, STC in 2000) is the most successful saliva diagnostic company in the world with revenues of just under $88 million in 2012. Epitope originally developed the OraSure® Oral Fluid Collection Device for general purpose saliva collection, but did not realize the full potential for the device until it successfully partnered with a Dutch Company (Organon Teknika, Boxtel, Netherlands) to enable the use of the device for collection of salivary samples for HIV diagnosis. By linking the OraSure device to an HIV-1 ELISA test from Organon Teknika, the OraSure device would become a component of the first FDA-approved oral test for detection of the HIV virus. The major market applications for the OraSure HIV-1 product include public health screening, surveillance, life insurance risk assessment, and outreach programs. The OraSure device consists of a rectangular cellulose pad attached to a detachable blue plastic stem. The pad material is rubbed across the surfaces of the cheeks adjacent to the gum line for a period of time then left in the gap in the oral cavity between the teeth and gum line to absorb a salivary sample. The current manufacturer, OraSure, describes this sample as "oral mucosal transudate." The pad material is pretreated with certain proprietary salts designed to facilitate more rapid sample collection. The average specimen collection time is between 2 and 5 min, after which the OraSure device is placed into a collection tube containing a buffer and transported to a laboratory. Prior to analysis the sample must be centrifuged. Following centrifugation, the specimen is assay ready.

OraSure's expertise extends into drugs of abuse testing, so in order to capitalize on this core competency, the company developed a very similar device (using the Intercept® brand name) specifically for substance abuse testing for the main NIDA-5 accepted panel of drug entities (THC/marijuana, cocaine, opiates, methamphetamines, and PCP) and other abused drugs. The Intercept® device features an identical specimen collection device to the OraSure HIV-1 product, but differs in the proprietary buffer used to dilute the collected specimen and also the unique packaging used to brand the product. Intercept® is currently used in many areas of abused drug testing including workplace testing, drug courts, forensic toxicology, and various criminal justice settings. In the case of both OraSure HIV-1 and Intercept®, samples collected using the devices are processed in the laboratory.

Across the Columbia River from Epitope in Washington State was another emerging company in oral diagnostics, Saliva Diagnostics Systems (SDS, Vancouver, WA), now StatSure Diagnostic Systems (New York, NY, www.statsurediagnostics.com). SDS was the original developer and manufacturer of the Saliva•Sampler® Collection Device, which was also known in other countries around the world as Omni•SAL®.

The Saliva•Sampler®/Omni•SAL® device was used for general purpose standardized saliva

collection and received 510(k) marketing clearance from the FDA strictly for saliva collection only. At the time, SDS chose to market the collection tool as a collection tool only and did not "pair" the device to any specific diagnostic (e.g., HIV) or abused drug tests as its rival Epitope/OraSure had done. Later, the manufacturer transferred the rights to the product to California-based Immunalysis Corporation (Pomona, www.Immunalysis.com), a company with an existing presence in the drugs of abuse testing market through strong sales of urine-based ELISA tests. Immunalysis subsequently rebranded the product as Quantisal™ and validated saliva collection to a series of their ELISA-based drug test assays, which have received FDA clearance and are now routinely sold for workplace testing, forensics, criminal justice, and other applications.

The Quantisal™ Saliva Collection Device also uses a cellulosic (paper-based) material attached to a stem to harvest saliva from the mouth. An absorbent pad is placed in the mouth and saliva collected until a sample volume indicator built into the device changes color from white to blue (usually approximately 2 min) indicating sufficient saliva (1.0 mL + or –10 %) has been collected to perform any subsequent analysis. The absorbent pad has a series for perforations near the top of the cellulose pad, which allows easy detachment of the pad into a transportation tube containing a stabilizing buffer to ensure safe delivery of the sample to the laboratory for testing.

A third early saliva innovator in the saliva collection area was Sarstedt (Germany, www.sarstedt.com), which introduced "Salivette" to the commercial market in 1987. This device has been used extensively by the research community for a wide assortment of applications ranging from detection of steroid hormones from saliva, HIV antibody detection, markers of oxidative stress, and others. Salivette does not have any regulatory clearances from the FDA but is CE marked in the EU.

The Salivette device is available as either cotton or polyester rolls or sponges, and each configuration includes a sample transport tube. To collect a sample, the Salivette is placed in the mouth and chewed for approximately 2 min then placed into the transport tube for dispatch to a testing laboratory. The device does not incorporate any means of sample sufficiency, and the specimen must be centrifuged prior to analysis.

Note: As mentioned earlier, there are now a number of published articles that caution against the use of salivary collection devices that use cotton as the collection media, including Salivette, for certain applications, particularly the detection and quantification of steroid hormones, marijuana, and others. In such cases, use of cotton-based collection media can lead to an over- or underestimation of actual concentrations of target analyte in oral fluids. A few examples are cited here as references [24–27].

Neogen Corporation (Lexington, USA, www.neogen.com) purchased International Diagnostic Systems (IDS, St Joseph, MI) in 2009 and at that time gained the rights to the UltraSal-2™ Saliva Collection Device, manufactured by IDS. UltraSal-2™ is a large-volume saliva collection device that provides a capability to "split" the collected sample into two mutually distinct samples collected simultaneously. The device includes two collection tubes connected to a single mouthpiece into which the user expectorates. The mouthpiece can be tilted/rotated during collection to direct saliva into one or the other of the two tubes. In this way, sufficient sample can be collected into both tubes for subsequent analysis in the laboratory. In total, this device can collect up to 24 mL of whole saliva by the drool technique. UltraSal-2™ is used mainly for drug testing purposes.

The SalivaBio Saliva Collection Aid was originally developed by SalivaBio LLC (USA, www.salivabio.com) in collaboration with researchers at the Center for Interdisciplinary Salivary Bioscience Research at the Johns Hopkins School of Nursing for Hormonal Analysis, but this device has been shown to have broader applicability and may be used for most applications where saliva is required. The device works by expectorating/spitting saliva into the Saliva Collection Aid, a plastic funnel-type device. The "plastic funnel" component is connected directly to a transport tube provided by the manufacturer,

so when sample collection is finished, the tube is capped and sent to a laboratory for processing. The manufacturer has ensured that the device is compatible with multiple cryovials so samples may be collected directly into Wheaton, Sarstedt, Nalgene, or Greiner cryovials for ready storage in a freezer. SalivaBio is now also available through Salimetrics (State College, PA, www.salimetrics.com), a leading manufacturer of salivary tests for steroid hormones, neurotransmitters, and others.

In addition to marketing the SalivaBio device, Salimetrics also produces the Salimetrics Oral Swab (SOS) Device. The SOS device uses a 10×30 mm "interference free" pad as the collection medium. This is fabricated from an inert polymer material that is used as part of a kit that includes a conical tube storage box, storage tube, and bar-coded labels. The sample is collected by placing the absorbent pad in the mouth of pediatric patients for between 1 and 5 min, after which the pad is placed into the conical tube provided, labeled, and shipped to a laboratory or frozen for storage purposes.

The British Company Malvern Medical Developments (www.malmed.co.uk) developed the ORACOL Collection Kit that uses an absorbent foam material in a swab format to collect up to 1 mL of whole saliva. The ORACOL Collection Kit consists of an absorbent foam swab, centrifuge tube, and cap. To collect saliva, the ORACOL swab is placed in the mouth and allowed to absorb saliva for a period of time. The sample is removed from the swab by centrifugation using a tube provided in the kit. The processed specimen is typically used for infectious disease testing particularly measles, HIV, hepatitis A and B, mumps, syphilis, and rubella, but has also been used for substance abuse testing.

Greiner Bio-One (Kremsmünster, Austria, www.gbo.com) is the manufacturer of the SCS Saliva Collection System, a device for the collection of whole saliva by trained professional users that incorporates a series of tubes, reagents, and a sample cup for general purpose saliva collection. The first step in a series of steps with the SCS system is to rinse the mouth with a safe and proprietary reagent provided by Greiner. The second step involves taking a sample of 4 mL of a tartrazine solution in the mouth for 2 min then spitting the entire contents into a clean tube containing preservative agents that stabilize the saliva sample for long periods of time. A separate evacuated tube is used to take the collected specimen from one container into the final transportation tube. Once filled, the sample is stable for analysis or for transportation to a laboratory. An advantage of the Greiner system is that the internal colored dye (tartrazine) is used as a means of calculating the exact saliva quantity present in the total solution using colorimetric analysis.

Oasis Diagnostics® Corporation (Vancouver WA USA, www.4saliva.com) is involved in the manufacture of a series of oral-based tools. Historically the first device to be introduced by the company was Versi•SAL®, a device for standardized whole saliva collection. Versi•SAL® uses an absorbent pad made out of noncellulosic pad material to collect saliva from under the tongue until a sample volume adequacy indicator in the device changes, signifying sample sufficiency. Typical collection time using the device is approximately 1–2 min. Saliva is separated from the absorbent collection pad by expressing the sample through a plastic compression tube provided and into a standard delivery tube (2 mL Eppendorf or 1.5 mL microfuge tube). Various configurations of the device can provide between 0.5 and 1.4 mL of whole saliva, with the option to obtain two samples from the same patient using a modified compression tube. The Versi•SAL® Oral Fluid Collection Device is currently used for general purpose saliva collection for downstream testing in the laboratory. Applications include hormone testing for general wellness, substance abuse testing, cotinine (nicotine), infectious diseases, and others.

Oasis also recently introduced a second device, Super•SAL™, for standardized saliva collection with medium to large volume saliva collection capability. Super•SAL™ collects saliva using a cylindrical absorbent pad along the side of the tongue between the tongue and gum line. This device also includes a sample sufficiency indicator, which changes once an adequate sample has been obtained for subsequent analysis.

Super•SAL™ has a shorter collection time due to the higher surface area of pad material exposed to fluid in the oral cavity. In this case, the sample is expressed through a compression tube resembling a syringe barrel and into a standard receptacle (2 mL Eppendorf tube or 1.5 mL microfuge tube). A sample of 1.0 mL is typically collected in approximately 1–3 min.

The area of drug testing from oral fluids is a significant business in the United States and other parts of the world, and a number of devices exist for rapid drug testing at the point of care. Similarly, a number of oral collection devices exist that use absorbent materials to collect salivary specimens. In both cases, many of these have suffered from poor recoveries of marijuana (THC) caused by the binding of the "sticky" THC molecule to the collection matrix. The Accu•SAL™ Oral Fluid Collection Device (Oasis Diagnostics®) was designed to overcome this particular problem. A proprietary collection strip attached to a handle is placed in the mouth until the strip is saturated (as indicated by the change in the sample volume adequacy indicator incorporated as part of the device, approximately 1 min). Upon saturation the collection strip is placed in a tube containing a predetermined quantity of a reagent buffer that stabilizes the sample during transportation to the laboratory. Upon receipt at the laboratory, the handle and collection strip are removed, and the tube containing the sample can be immediately used for ELISA, homogeneous immunoassays, or GC-MS as required without further dilution. An added feature of this device is that in cases of insufficient saliva collection, there is a procedure that can be followed to provide an accurate dilution of the saliva obtained. This device may be used for multiple applications including steroid hormones for general wellness, therapeutic drug monitoring, workplace testing, and forensic applications.

In 2011, two of the IVD industry leaders, Quest Diagnostics (USA, www.Questdiagnostics.com), the largest reference testing laboratory in the United States, and Thermo Fisher Scientific (USA, www.thermofisher.com), a major *in vitro* diagnostics manufacturer, formed a partnership and sought FDA clearance for a series of homogeneous immunoassays for abused drugs produced by ThermoFisher under the CEDIA® brand. Sample collection is performed using the Quest Diagnostics Oral-Eze® Device. According to the summary of the FDA (510 K) regulatory clearance documentation, samples collected using the Oral-Eze® device may be used in the ThermoFisher assays for the NIDA-5 drugs (amphetamines, cannabinoids [THC], opiates, cocaine, methamphetamines, and phencyclidine [PCP]). Quest similarly promotes a list of drugs including the NIDA-5 series of drugs that have been validated to the Oral-Eze® collection tool in its marketing literature and Website. Quest also performs analytical testing for various drugs in its laboratory facilities at several locations around the United States.

To collect samples using the Oral-Eze®, the donor inserts the pad of the device into the mouth between the lower cheek and gum line. Collection is complete when a white indicator in a viewing window changes to a blue color. Time of collection is suggested to be up to 10 min, but four out of five collections are complete within 5 min. The saturated collection pad is then ejected from the handle of the device using pressure on a series of ridges on the side of the plastic housing, into a collection tube containing a stabilizing buffer. A cap is placed on the tube, which is then protected with a "tamper proof" seal across the top of the specimen vial. The sealed collection tube is then shipped in a specimen bag to a laboratory for subsequent testing. Quest promotional materials suggest that the main areas of application for Oral-Eze® are in preemployment, random, reasonable decision, return to duty, and postaccident testing.

Another new drug collection system, the Wolfe Reality CHECK Premium Oral Fluid System, is available through Wolfe Workplace Protection (Ashville, NC, www.wolfeinc.com). This device collects a neat oral fluid specimen by expectoration through a plastic mouthpiece into a connected transport tube. A fill line on the transport tube provides an indication of the quantity of saliva required for downstream drug analysis. After collection the mouthpiece is removed and the sample capped and sealed for protection, then shipped to the laboratory, where ELISA, homogeneous

immunoassays, or GC-MS among others may be ran on the samples.

In addition, there are a number of other "specialized" collection tools for salivary hormone detection developed by manufacturers of microplate ELISA kits as "companion tools" for collection that are sold in conjunction with various test kits. Examples include DiaMetra (Italy), IBL (Germany), Salimetrics (USA), DRG International (USA), and others.

Molecular Tools

The growth in molecular technologies (PCR, genotyping, microarrays, genome-wide association studies, sequencing, and others) has coincided with the expanding interest in saliva as a specimen, which in turn has spawned a number of very important commercial tools for salivary collection of nucleic acids. This area of salivary diagnostics is probably growing faster than any other market sector at the present time. The reasons have a common thread with those for general saliva collection, particularly that it has been proven that salivary samples are equivalent in performance to blood sampling and more cost-effective, convenient, and simpler to use.

In the molecular diagnostics space, DNA Genotek was the early market leader introducing salivary collection technologies for DNA and RNA and today continues to hold that position. DNA Genotek (Ottawa, Canada) was acquired by OraSure Technologies in 2011 but continues to operate as a wholly owned subsidiary of OraSure. DNA Genotek now offers several tools for nucleic collection from salivary samples. Originally the company launched the OraGene® DNA device in two formats, which simplified the collection and stabilization of DNA from saliva samples, and these two configurations are sold widely in the life sciences research area as well as in the personal genomics field. In December 2011, the OraGene® DNA device became the first salivary collection tool for nucleic acids cleared by the FDA for *clinical* use when used in conjunction with the GenMark Diagnostics eSensor test for warfarin sensitivity. To date this remains the only cleared device for clinical use. FDA regulatory clearance is currently restricted to collection of oral fluids and application for clinical testing with the GenMark eSensor test only.

The OraGene® DNA device is set up for home collection and has been used by a number of the early adopters of "consumer genomics" (i.e., 23andMe, Navigenics, now part of Life Technologies, Pathway Genomics and others). An OraGene® DNA specimen is collected when a subject expectorates into the OraGene collection tube until a fill line on the OraGene® device is reached. The volume is set at 2 mL, and the collection time ranges from 2 to 30 min, depending upon the subject. Once sample collection is complete, a special cap, prefilled with a proprietary and patented buffer, is attached to the OraGene® collection tube and screwed into place, resulting in the release of the buffer into the saliva specimen. The buffer has the effect of immediately stabilizing the DNA present in the sample. OraGene® DNA collects a large quantity of DNA, for a range of downstream applications. DNA Genotek also supplies reagents for the isolation of DNA and RNA from the sample and works with other reagent suppliers to offer multiple options for nucleic acid isolation.

Following up on their success with OraGene DNA, DNA Genotek has launched a series of other devices for molecular fragments including the OraGene® RNA device, the ORAcollect™, and the OMNIgene™ DISCOVER.

OraGene® RNA is described by DNA Genotek as "an all in one system for the collection, stabilization, transportation of RNA from human saliva." OraGene® RNA is basically a plastic collection vial that donors use to spit into until a specific volume of saliva (2 mL) is collected. The cap of the collection vial conceals an RNA preservative liquid that is released when the cap is placed on the vial and sealed. The RNA-preserving liquid confers several months of stability on the sample at room temperature. In order to isolate RNA from the sample, DNA Genotek supplies reagent kits. Alternately kits from outside suppliers, such as Qiagen (Germany), Norgen Biotek (Canada), or Mo Bio Laboratories (USA), may be used.

ORAcollect™ is another innovation for the collection of DNA from oral fluids. ORAcollect™

comprises a swab-shaped sponge attached to a cap that screws into a transportation tube containing buffer. Prior to use, the sponge end of the device is on the outside of the transport tube and is used to rub the lower gums ten times back and forth in one direction then a further ten times in the opposite direction. After sample collection is complete, the screw cap containing the sponge is unscrewed and inverted and into the bacteriostatic buffer solution to stabilize and transport the sample. After shaking vigorously ten times, the sample is ready for shipment to a laboratory or immediate DNA isolation. DNA Genotek promotes this particular device as an affordable alternative to buccal swabs.

A similar device, called PERFORMAGENE™-LIVESTOCK is sold into the large animal veterinary area. PERFORMAGENE™-LIVESTOCK™ collects DNA from the nasal passages of cattle and other livestock. The device uses a similar or identical sponge to the ORAcollect™ device, but in this case, the sponge is rubbed inside the nostrils of the animal for up to 5 s in order to collect an adequate specimen. The remaining procedure is identical to that of the ORAcollect™ tool, and the resulting DNA collected may be immediately purified or taken directly to downstream testing (PCR, genotyping, etc.).

OMNIgene™ DISCOVER is a specific saliva collection kit for harvesting and stabilizing *microbial* DNA. The device is in fact a tube identical or very similar to the OraGene® DNA Device that subjects spit into (through a funnel-shaped head) until a fill line marked on the device is reached. The sample is then capped, releasing a stabilizing agent into the saliva that preserves bacterial DNA in the specimen. The funnel-shaped lid may be removed by unscrewing a complete section of the device, then the lower section of the tube is capped and the mixed sample shaken then processed using off-the-shelf kits capable of isolation of microbial DNA.

According to OraSure Technologies' public financial statements, its DNA Genotek subsidiary had revenues for OraGene® DNA and other devices of $14.3 million in fiscal year 2012.

Oasis Diagnostics® uses a different approach to collection of nucleic acids from saliva. Its first market entry in 2011 was the DNA•SAL™ Salivary DNA Collection Device. DNA•SAL™ is an ergonomically correct device that has a collection "head" connected to a detachable handle. The collection head has a series of sharp edges that are rubbed across the surfaces of the inside of the cheek area, gently, for 30 s. This action captures buccal cells on the device head but also causes a significant number of detached cells to remain free flowing in the oral cavity. The loose cells are "harvested" using a small quantity of a safe stabilizing solution that is "swished" around in the mouth for 15 s then retransferred back into the same sample tube by spitting. The stabilizing rinse solution present in the sample tube confers long-term stability on the sample. DNA can then be immediately processed or transported to the laboratory for extraction followed by downstream analysis (PCR, genotyping, etc.).

Oasis provides a method for *immediate* downstream testing *without DNA isolation* with a simple sample manipulation and also supplies DNA isolation kits specifically optimized to samples collected using the DNA•SAL™ Device (which are not strictly saliva but a more complex mixture of stabilizing solution, cells, and saliva).

A new tool, RNAPro•SAL™, was recently launched by Oasis Diagnostics® for the isolation of RNA and/or proteins from saliva. This device integrates certain elements from the Oasis Super•SAL™ Universal Saliva Collection Device (for whole saliva) with components necessary to separate and independently stabilize both RNA and proteins for downstream research or clinical studies. RNAPro•SAL™ incorporates a proprietary secondary filtration unit, which functions to provide cell-free saliva. The procedure involves placing a cylindrical pad in the inside of the mouth along the gum line next to the teeth and collecting saliva until a sample volume adequacy indicator changes appearance, confirming that sufficient sample has been collected (minimum 1.0 mL, typical collection time approximately 1–2 min). The absorbent pad used to collect the salivary specimen is then placed in a compression tube that is connected to the secondary "splitting unit" that in turn connects to two collection tubes (Eppendorf or microfuge

tubes). The saturated saliva collection pad is pushed through the compression tube and through the secondary filtration unit and the eluted sample subsequently separated into two distinct fractions. The secondary filter may contain variable media, but each acts to remove cells from the sample, allowing two samples of purified saliva to be received in the sample collection tubes. These two fractions are stabilized independently with specific reagents provided with the RNAPro•SAL™ Device to yield long shelf-life fragments of RNA and proteins that are assay ready.

The last 2–3 years has seen a number of new oral nucleic acid collection tools based on expectoration (spitting) that are beginning to find application in research studies. For instance, Norgen Biotek, a Canadian company based in Thorold, Ontario (www.norgenbiotek.com), introduced a "convenient saliva collection and preservation device" as a kit known simply as the "Saliva DNA Collection, Preservation, and Isolation Kit." The kit provides an "all-in-one" procedure for the collection, preservation, and isolation of salivary DNA at ambient temperature. The technology resembles the OraGene® DNA technology, where the sample is collected by spitting into a collection tube (with a funnel connected to the top of the tube to direct the sample) until a sample fill line is attained. The funnel piece is removed and in this case a preservative is added directly from an ampoule provided. The preservative has a dual function to lyse the cells as well as preserve DNA in the sample. One positive feature of the device is that each device is uniquely numbered for positive sample identification.

Another similar device is the SalivaGene Collector from Stratec Molecular (Berlin, Germany, www.stratec.com). As for the OraGene® DNA and Saliva DNA Collection and Preservation Device from Norgen Biotek, the SalivaGene device connects a basic funnel to a collection and transport tube. Saliva is expectorated through the funnel and into the tube until a minimum saliva volume is collected, then detached and capped. One distinct feature of SalivaGene is that the buffer is pre-dispensed into the collection tubes in a lyophilized format.

Devices for Collection of Oral Specimens from Salivary Gland Secretions

There are three major salivary glands (submandibular, sublingual, and parotid) that contribute to 90 % of total (whole) saliva. The remaining 10 % comes from a number of smaller (minor) glands, particularly the buccal, lingual, labial, and palatal glands. The composition and quantities of saliva secreted by each of the different glands differ, so in order to evaluate individual salivary gland function, it is important to use tools specifically designed for collection from each of the glands. A good account of saliva composition and quantities can be found in the work of Mese and Matsuo [30]. Saliva can be collected from these individual salivary glands using a number of available tools. These tools are not used routinely, so none of them are commercialized on a widespread basis and are typically used for research studies only. Due to the limited application for these devices, innovation in this area is limited; nevertheless, these tools find application in research protocols around the world.

Parotid Gland Collection Methods

Parotid gland collections are the easiest of the individual glandular secretions to collect and may be accomplished by modifications to a device known as the Carlson-Crittenden collector, originally reported in 1910, but still in use due to the reliability and accuracy of the device. Although the Carlson-Crittenden Collector (also known as the Lashley cup) is a robust system, it needs to be expertly fitted by a skilled person. The device is used sterile, fitted with polyvinyl chloride (PVC) tubing. The inner portion of the device is connected to a bulb or a suction pump, and the device is placed over the main parotid excretory duct (Stensen's duct) in the oral cavity. Samples are collected via suction onto ice using an induced stimulation (typically a sterile aqueous citric acid solution applied to the tongue by means of a cotton swab at periodic intervals). Samples can be collected bilaterally, allowing for simultaneous collection from both parotid glands to increase yield and shorten collection times.

Cannulation is also used to obtain parotid saliva specimens. In this case, a thin tube is placed directly at the outlet of the main parotid excretory duct (Stensen's duct). This method suffers from certain drawbacks including discomfort and requires a skilled operator. In some cases, application of a local anesthetic is required.

Submandibular/Sublingual Gland Collection Devices

The most widely used device for submandibular (SM) and sublingual (SL) saliva collection is the device invented by Wolff and Davis [31], which may be used to collect either specimen type (SM or SL) using slightly modified procedures. The "Wolff" device consists of four components: tubing for collection (cellulose acetate butyrate), a polycarbonate buffering chamber (to avoid saliva being sucked into the suction device and to also remove bubbles from the collected sample), a storage tube, and a vacuum device to produce suction. The device produces pure saliva and appears to be efficient, reportedly collecting 90 % of the fluid that enters the device into the storage chamber [32]. As for parotid saliva collection, some sort of stimulus is required (usually 2 % citric acid applied directly to the tongue) with this device.

To collect saliva from the submandibular gland, the openings to each of the two parotid glands are typically blocked using 2″×2″ cotton gauze. The floor of the mouth is then dried, and the openings to the sublingual glands on both sides of the mouth are also blocked. The subject/patient is required to raise their tongue into an elevated position allowing access to the submandibular gland. At that point, collection using the Wolff device can successfully begin. To collect saliva from the sublingual gland only, a similar procedure is used, except in this case access to the submandibular gland is blocked in preference to the sublingual gland.

A 1998 study from Chile by Morales et al. reported new devices for the collection of saliva from both the parotid and major salivary glands producing on average 1.0–1.5 mL of saliva in a 10–15 min period [33]. Although the devices were not described in detail, they were used repeatedly by the Chilean authors in subsequent studies [34, 35]. Flow rates obtained from submandibular/sublingual glands were on average 180 μl per minute and from the parotid gland 80 μl per minute. One advantage of the reported devices is that collection of both parotid and submandibular/sublingual saliva may be achieved simultaneously under the supervision of a solo healthcare professional. As for other similar devices, artificial saliva stimulation using citric acid is required and samples must be collected on ice.

More recently, researchers in New Zealand have reported a custom fabricated device for the collection of submandibular saliva that is less invasive than those previously available [36]. Although the device collects a lower quantity of saliva than that collected by expectoration (spitting), the stimulated saliva specimen has a pH close to that of unstimulated saliva. The authors suggest that the device minimizes sample contamination due to the fact that the unit is a sealed device. Validation experiments performed on the device compared to saliva collected by expectoration (spitting). In each case, samples were collected over 5 min. In the case of expectoration, samples were collected by "forcible expectoration" every 30 s, and using the device, by inserting the device in the mouth and directing a tube so that saliva flowed freely from Wharton's duct into the collection cup provided. The customization element of this device requires that an accurate impression of the mouth cavity is taken, which uses a modification of the altered cast technique for lower Kennedy Class I impressions [37].

Older research from Sweden [38], published online for the first time in 2007, describes a device for submandibular/sublingual saliva using a modified Block-Brotman device, a tool originally described in 1962 [39]. The study compared the results of submandibular/sublingual collection versus collection of parotid saliva by means of standard Carlson-Crittenden cups, and results indicate that the device successfully provided higher flow rates of saliva than parotid collection at two time points throughout the day.

A more invasive option for submandibular collection is cannulation via Wharton's duct. This may be carried out using one of a number of available metal cannulae including blunt

hypodermic syringe needles, catheters with a metal tip, and a device known as the Schaitkin Salivary Duct Cannula from Hood Laboratories (Pembroke, MA), which is designed for short-term intubation of the salivary ductal system and for holding open the ductal tissue. The Walvekar Salivary Duct Stent (also Hood Laboratories) has also been used to hold open salivary ductal tissue to allow the flow of fluids from the glands and collection into a suitable receptacle.

For more in-depth information on devices for submandibular and sublingual collection, see reference [40].

Devices for Collection from the Minor Salivary Glands

There are many minor glands that make up the remainder of salivary gland secretions, including the labial, buccal, lingual, and (glosso) palatine glands. Typically, minor gland salivary secretions are less useful in providing meaningful information for diagnostic purposes, so the number of available devices for collection from these glands is limited. Samples from the minor glands are more viscous in nature, so this has also hampered studies using minor gland secretions. A variety of qualitative methods have been tried, including filter papers, capillary tubes, sponges, and micropipettes. In addition, semiquantitative assessment has been done using weight measurements, and methods involving measurement of colored spots on chromatography paper have been used to determine flow rate.

The most widely used method in current practice is an electronic device known as the Periotron® from Pro-Flow, Inc. (Amityville, NY), which has made quantitative assessment of minor salivary gland secretion much more accurate and precise. The Periotron® method uses a piece of blotting paper to harvest moisture from the mucosa. The blotting paper is subsequently placed between two plates on the Periotron® measuring instrument across which a voltage is applied. The dielectric properties of the saliva are used to calculate the volume of moisture absorbed by the blotting paper. The moisture collected is recovered, and then the blotting paper is once again returned to the instrument. A standard curve constructed from known volumes of water added to blotting papers is finally used to back calculate the amount of moisture originally present on the paper.

Interestingly, minor salivary gland secretions are reported to be less likely to respond to stimulation than the major gland secretions, although additional data confirms that mechanical stimulation of a denture "base plate" adjacent to the palatal mucosa can induce increased salivary secretions from the minor glands.

Preservation of Saliva Specimens

Saliva is a complex mixture of electrolytes, proteins, bacteria, various glycoproteins, mucins, and aqueous material, among others. While some molecules in the saliva (certain drugs, drug metabolites, steroids, cancer markers, and others) remain relatively stable in oral fluids, others, for example, RNA and proteins, are notoriously unstable and require the addition of validated stabilization reagents in order to preserve the integrity of the sample, prior to the analysis phase.

The science of sample stabilization, and in particular salivary sample stabilization, is a growth area, and a number of commercial companies have active strategies to support saliva stabilization for genomics, proteomics, and transcriptomics. Without the activity in this area, the salivary diagnostics area would stagnate.

Stabilization of Analytes in Saliva

With the appearance of new and better tools for saliva collection and clean up, opportunities to use saliva as the ideal specimen are increasing. In order to capitalize on this growth trend, adequate methods of saliva stabilization were needed and have recently been developed. Multiple strategies now exist for sample stabilization, and the number of companies offering suitable products is growing rapidly to the benefit of the entire salivary diagnostics space, another small sign that saliva is attracting greater attention from companies supporting the *in vitro diagnostics* market.

Stabilization of Nucleic Acids

RNA

Commercial sources of RNA protection are designed to halt RNA degradation at the time of salivary specimen collection until the time of analysis, which may be one of a number of downstream applications such as PCR, RT-PCR, qRT-PCR, RNA sequencing, or others. From a user standpoint, such reagents should be capable of stabilization of saliva at ambient temperature, be relatively simple to use, and remain cost-effective in comparison to the downstream technology applied.

The stability of RNA often depends upon the method of collection, pre-analytical steps taken to clean up the specimen prior to analysis, and the absolute purity of RNA. For this reason, there are multiple literature reports claiming RNA to be highly unstable, whereas others report longer shelf life, even at ambient temperature. This is clearly an area where new approaches are leading to better solutions. Due to the conflicting reports, precautionary measures are usually taken to stabilize RNA in bodily fluids, including saliva.

A number of manufacturers have introduced reagents targeting this area, and each competes for a growing market share. Reagents for the stabilization of RNA appear to be broadly applicable to the stabilization of RNA in multiple bodily fluids, including saliva, and may not be specific to application with oral specimens. Some of the most widely used are described as follows:

Qiagen, Inc. (Germany, www.qiagen.com) now provides a number of reagents for protection of transcriptomic elements from human specimens, including the RNAlater® and RNAprotect® systems for tissue and bacteria. For saliva samples particularly, the RNAprotect™ reagent [41, 42] has been commonly used. According to the manufacturer, the RNeasy® Protect Saliva Micro Kit (which includes RNAprotect® Saliva Reagent) "stabilizes RNA in saliva samples to preserve gene expression profiles." The RNeasy® Micro Kit purifies and concentrates total RNA using a spin column technique. Stabilized saliva samples can be shipped at 37 °C for 1 day, for 14 days at 15–25 °C, or for 4 weeks at 2–8 °C, prior to RNA purification. The kit provides sufficient reagents for 50 preparations. The current cost of the RNAprotect® saliva reagent and RNeasy® Micro Kit (sold together) is approximately US $660.

Qiagen also supplies the RNAlater® RNA stabilizing reagent for the immediate stabilization of RNA from multiple bodily fluids. Originally developed for tissue samples, Qiagen supports a modified protocol for adaptation to saliva. RNAlater® is sold in 50 mL or 250 mL bottles for $73.10 and $302.00, respectively. The stability of samples reported using RNAlater® is the same as for RNAprotect®/RNeasy®.

Life Technologies (USA, www.lifetechnologies.com) also markets the same RNAlater® brand in a number of kits that have been validated with Life Technologies' own RNA isolation kits from the Ambion® company (owned by Life Technologies). Pricing and sample sizes for the RNAlater® stabilizing solution from Life Technologies is $116.00 for 100 mL and $363.00 for 500 mL. Life Technologies also offers a second option for RNA stabilization, known as SUPERase•In™ RNase Inhibitor at a concentration of 20U/μ(mu)L. SUPERase•In™ Inhibitor is a nonhuman protein-based inhibitor that binds the interfering RNases including RNase A,B,C,1, and T1. The company promotes this product as "a reagent for the removal of RNases in any application where RNase contamination can be problematic." The material itself is shipped on dry ice but is conveniently priced at $125.00 for 2,500 units. Other reagents available from Life Technologies include RNaseOUT™ Recombinant Ribonuclease Inhibitor, RNase Inhibitor, and RNAsecure™.

A 2006 publication by Wong et al. [42] reported a side-by-side evaluation of the stability conferring properties of SUPERase•In™, RNAlater®, and RNAprotect® Saliva Reagent and concluded that RNAprotect® Saliva was the "optimal room temperature stabilization reagent for the salivary transcriptome." A later publication by Andreas Kurth from the Center of Biological Safety at the Robert Koch Institute in Berlin, Germany, looked at the stability of samples stabilized with RNAlater® and cautioned that samples so treated can still harbor viral infectivity and should be treated as potentially

hazardous and capable of transmitting disease, if handled inappropriately [43]. This study was somewhat limited in that it did not investigate any other RNA protective agents, so caution should be observed when looking at similar reagents, as these may or may not harbor similar properties.

Later work on RNAprotect™ Saliva Reagent has reinforced the applicability of this product for long-term stabilization of the salivary transcriptome. Jiang et al. reported 10 week stability on salivary DNA/RNA specimens, as well as 6 days stability on salivary proteins in a saliva filtrate, all carried out at ambient temperature [41].

Biomatrica (USA, www.biomatrica.com) now offers RNAstable® for the preservation of RNA at room temperature without degradation. RNAstable® is available either in liquid form, or as a dried reagent, which can be added directly to RNA samples in tubes, multi-well plates, or other suitable containers. The Biomatrica Website provides support information describing the protection of total RNA, messenger RNA (mRNA), and microRNA (miRNA) for 12 years at ambient temperature without degradation. RNAstable® is available in various sizes and specifications depending upon customer requirements. Pricing information is not immediately available.

US Company Zymo Research (www.zymoresearch.com) is now commercializing a dual function reagent that effectively stabilizes DNA and/or RNA upon contact, allowing shipping of stabilized nucleic acids at ambient temperatures with long shelf life. DNA/RNA Shield™ preserves genetic integrity and expression profiles of samples (including cells, blood, tissue, saliva, urine, and others) at ambient temperatures. In addition, DNA and RNA can be isolated directly without precipitation or reagent removal and have been shown to be compatible with most DNA and RNA purification kits. DNA/RNA™ Shield, which also inactivates infectious agents (viruses), is sold in units of 50 mL for $62.00, or 250 units for $221.00. DNA/RNA Shield™ is also sold as part of a "Mini-Prep Kit" for RNA Isolation for $239.00 (50 mL/50 preps).

The ClonTech division (www.clontech.com) of Takara Bio (Japan, www.takara-bio.com) offers the Takara RNase Inhibitor, a ribonuclease inhibitor material, expressed in *E. coli* and purified by affinity chromatography. This inhibitor is available in two sizes of 5,000 units for $112.00 and 25,000 units for $450.00. This product can be used in most applications where protection of RNA is critical.

Creative Biogene Biotechnology (USA, www.creative-biogene.com) also offers a similar "RNase Inhibitor," which is an acidic 52 kDa protein that is a potent inhibitor of pancreatic-type ribonucleases such as RNases A, B, and C. This product is offered as a 20,000 unit size, described more fully as an enzyme, which is a fusion of the RNase Inhibitor gene with a 22.5 kDa protein tag attached. This reagent must be stored at −15 to −25 °C.

OraGene RNA (DNA Genotek, Canada, www.dnagenotek.com) reagent has also been used successfully for the stabilization and rapid isolation of RNA from saliva in the OraGene RNA Self Collection Device. RNA collected using OraGene RNA is stable "for months" according to the manufacturer and may be used for human mRNA expression profiling.

At the time of this writing, the author learned of a new RNA-stabilizing agent from Norgen Biotek (Canada, www.norgenbiotek.com), a supplier of multiple kits for DNA and RNA isolation and stabilization from multiple sources and specimen types. The new reagent from Norgen is available only in liquid format and additional details on the product were unavailable at the time of writing.

For each of the aforementioned commercial RNA stabilization reagents, the actual compositions of the stabilization reagent are not reported in detail.

Finally, early methods for stabilization of RNA included "snap-freezing" saliva samples at −80 °C until analysis. This method is still commonly used in the area of transcriptomic research.

DNA

Like RNA, similar stabilization processes are carried out on crude DNA samples in saliva, until DNA isolation can provide highly stable DNA free from impurities in an assay ready format for downstream processing. The number of manufacturers and commercially available salivary

collection devices for DNA is increasing, and each supplier typically provides reagents for oral DNA stabilization, either with the collection tool or as standalone isolation kits that can be purchased as optional components.

Salivary DNA collected using the popular Oragene Device (DNA Genotek, Canada, www.dnagenotek.com) is protected by an addition of proprietary reagents including a bacteriostatic compound to inhibit the growth of the bacteria. This is introduced into the sample immediately after collection. The protected sample is said to be stable for a minimum of 1 year.

The DNA•SAL™ Salivary DNA Collection Device (Oasis Diagnostics®, USA, www.4saliva.com) includes a stabilizing solution containing alcohol and glycerol that also acts to prevent bacterial growth. Again the stabilizing agent is introduced immediately after sample collection, minimizing any potential for DNA degradation. The sample is stable for a minimum of 30 days. Additional stabilizing agents are available for longer-term storage.

DNA/RNA Shield™ from Zymo Research (USA, www.zymoresearch.com) is a reagent that enables prolonged nucleic acid stability during sample storage/transport at ambient temperatures. The DNA/RNA Shield™ reagent works efficiently for both RNA and DNA, acting to effectively lyse cells and inactivate nucleases and viral activity. Zymo has validated DNA/RNA Shield™ to a number of collection and storage devices including oral fluid collection tools.

The SalivaGene collection device from Stratec Molecular (Germany, www.stratec.com) includes a lyophilized stabilizing reagent, which is reconstituted upon sample collection. According to the same manufacturer, the PSP SalivaGene DNA Kit [44] is intended for genomic, mitochondrial, and bacterial DNA isolation from stabilized saliva samples. SalivaGene DNA includes a proprietary buffer, which acts to immediately stabilize saliva samples on contact, by effecting inactivation of DNases. It also acts to preserve the microorganism titer and pre-lyses bacteria. The stability of the sample is 12 months at room temperature and several years at −20 °C.

Norgen Biotek provides a device known simply as the "Norgen Saliva DNA Collection and Preservative Device." In this device, whole saliva is stabilized by addition of an "aqueous storage buffer," which, according to the manufacturer, is designed for rapid cellular lysis and subsequent preservation of DNA. The buffer prevents growth of gram-negative and gram-positive bacteria and fungi and also inactivates viruses [45].

Salimetrics Corporation (USA), a leader in salivary hormone ELISA test kit production, has partnered with DNA Genotek (Canada) to offer opportunities to carry out genotyping and hormone measurement on the same saliva sample [46]. In this case, whole saliva is collected by the passive drool technique into cryovials that are held on ice. Samples may be collected using the Salimetrics Saliva Collection Aid, then are rapidly frozen at −20 °C.

Biomatrica (USA, www.biomatrica.com) has developed the DNAguard™ Saliva Reagent for preservation of the integrity of genomic DNA both at ambient and elevated temperatures. The company claims that the product is based upon "an innovative technology platform applied to the chemical design of a long term saliva preservative that protects DNA in saliva with high yield and quality in comparison to cold-stored samples, but at ambient temperatures." DNAguard™ Saliva is one of the products from Biomatrica that is said to "disrupt the cellular membranes, penetrate immediately other cellular structures and inhibit nuclease activity as well as free radical activity." Further specified attributes include "protection of nucleic acids in the biosample specimen from hydrolysis such as depurination."

There are a number of other commercial sources of DNA stabilization reagents that are too numerous to mention within this brief review, so the reader is encouraged to review this subject further when trying to identify the most efficient reagents for DNA stabilization.

Proteins

Knowing that proteomics has become a major area of study, it is perhaps difficult to understand why relatively little work has been done on the stability and stabilization of salivary proteins.

Saliva has been shown to consist of 1,166 proteins [7], so the salivary proteome is a valuable tool to investigate ongoing disease processes. Evidence exists to show that the salivary proteome is very easily degraded, so methods capable of stabilizing saliva samples to protect the integrity of the salivary proteome are necessary. A compelling overview on the subject of "whole saliva proteolysis" by Oppenheim et al. [47] provides excellent background on this subject and also describes valuable solutions to slow down proteolytic activity in saliva, allowing effective downstream testing of saliva to take place. This body of work also provides strong evidence to support the hypothesis that the addition of suitable stabilizing agents to protein moieties is critical to successful salivary testing. A number of suitable stabilization cocktails are discussed by the authors, particularly the development and implementation of protease inhibitors. In all, Oppenheimer et al. tested 19 potential inhibitor cocktails and showed that a mixture of AEBSF, aprotinin, pancreatic trypsin inhibitor, leupeptin, and antipain (serine protease inhibitors) supplemented with EDTA, prevented noticeable degradation in synthetic substitutes for the proteins histatin 5, statherin, and PRP1. The authors were also able to eliminate degradation by reducing the pH of the saliva to 3.0.

Xiao et al. published a method for stabilization of the salivary proteome using ethanol [48]. Using reference proteins (beta-actin and interleukin 1-ß[beta]), the authors were able to show that the salivary proteome was stable if held at 4 °C for up to 2 weeks and using ethanol as a stabilizing agent, proteins were stable for up to 2 weeks at ambient temperature.

Sample stability and protein composition were evaluated extensively by Dutch researchers Esser et al. who examined protein stability at room temperature in freshly collected whole saliva, with and without protease inhibitors and inhibitors of bacterial metabolism, using Surface Enhanced Laser Desorption/Ionization (SELDI) [49]. Degradation was evaluated using gels followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The results confirmed rapid protein degradation within 30 min with decomposition beginning immediately after sample collection. Improved stability was observed using a cocktail of phenylmethylsulfonyl fluoride (PMSF) and leupeptin (both serine and cysteine protease inhibitors) and EDTA, a metalloprotease inhibitor, but protein breakdown was still noticeable. Addition of sodium azide, on the other hand, did not confer any stability on protein samples, indicating that bacterial metabolism is not contributing significantly to protein breakdown. This study also postulates at least six proteases are at work to potentially degrade saliva specimens.

A standard method of protein stabilization that has been used routinely in the research laboratory involves the addition of a cocktail consisting of aprotinin (1 µ[mu]L, 10 mg/mL solution), sodium orthovanadate (Na3OV4, 3 µ[mu]L, 400 mM solution), and PMSF (10 µ[mu]L, 10 mg/mL). This cocktail is added to the supernatant fraction obtained from 1.0 mL of centrifuged whole saliva and preserves saliva samples for extended time periods (up to 2 weeks).

A similar mixture comprising of sodium orthovanadate (1 mM) and a commercially available protease inhibitor cocktail (Sigma, 1 mg/mL of whole saliva) was incorporated in pioneering work on saliva protein profiling for breast cancer detection in women by Streckfus et al. [8]. Even with the stabilizing cocktail present, the researchers kept samples on ice throughout the process, then aliquoted their samples and froze them at −80 °C for long-term storage.

A "universal" stabilizing agent capable of stabilizing nucleic acids (DNA and RNA) *and* proteins would be highly advantageous for research and clinical protocols, so the 2009 finding by Jiang et al. [41] that RNAprotect™ Saliva Reagent (Qiagen, Germany) functions not only to stabilize RNA and DNA for up to 10 weeks at ambient temperature but also stabilizes proteins in saliva filtrates for 6 days, was a valuable contribution to the field. Despite this, the use of RNAprotect™ Saliva Reagent has not been broadly adopted for the protection of the integrity of proteins, mainly due to the requirement for a high dilution relative to the saliva sample (reagent must be added in a ratio of 5:1 versus saliva),

resulting in a less-than-desirable solution from a cost standpoint.

Most other reported methods for protein stabilization require low temperatures, which only serve to minimize the attractiveness of salivary testing, so it is clear that there are opportunities for more effective protective agents for the salivary proteome. Recently the activity in this area of research has increased considerably.

Saliva Diagnostic Platforms

The early success of companies like Epitope (OraSure), SDS (StatSure), Sarstedt, and others has paved the way for a much broader array of integrated saliva diagnostic platforms that are now available targeting two main areas of the IVD and life sciences markets. In addition to nonmolecular saliva platform devices targeting proteins, infectious disease antigens, and antibodies, on which most of the current technologies are based, a new area has emerged from the combination of non- and minimally invasive specimen collection with point of care molecular testing platforms that incorporate on board nucleic acid purification, hybridization, and amplification. This newly emerging market segment, which we will refer to as point of care molecular diagnostics (POC MDx), offers up the perfect combination of rapid diagnostic results with immediate diagnosis for most, if not all, diseases or conditions, so this particular segment of the IVD business could rapidly become the fastest growth area in oral diagnostics.

In the remainder of this section, some of the tools/devices that have already made an impact in the aforementioned important areas of the *in vitro* diagnostic industry will be described:

Nonmolecular Platforms

Most of the current oral point-of-care tests combine the ability to collect saliva specimens in standardized fashion with functional lateral flow immunochromatographic (LFT) test strips to deliver real-time results in 20 min or less. The number of commercially available targets is still relatively few, but platforms now exist to significantly increase the number of disease targets over the next few years. Areas that have seen the most significant growth include substance abuse detection and HIV diagnosis, but newer targets aimed at salivary hormone detection, other infectious diseases and more recently systemic diseases are now emerging.

Of all the salivary diagnostics on the market today, there is no doubt that OraSure Technologies' OraQuick Advance HIV 1/2® rapid, oral fluid test for the HIV virus has made the greatest impact. This product was launched in the year 2000 internationally. Since then the device has received FDA approval and has changed the whole paradigm for clinical testing for HIV in the United States. OraQuick Advance® HIV 1/2 has been adopted widely by governmental public health organizations including the Centers for Disease Control (CDC), Substance Abuse Mental Health Services Administration (SAMHSA), and WHO overseas as a tool to identify HIV-infected individuals in nontraditional settings including mobile vans, bathhouses, and emergency room situations and in publicly funded screening programs.

The OraQuick Advance® HIV 1/2 consists of a fairly rigid pad connected to a lateral flow immunochromatographic (LFT) test strip. The user swabs the area under the lips and around the top of the gum line for a few seconds in order to collect an adequate specimen. The sample device is then immersed in a buffer/reagent solution in a tube provided by OraSure and the buffer allowed to migrate up and onto the LTF test strip embedded in the device. After 20 min the results of the (qualitative) test are read. If a single line is observed, the sample is negative. If two lines appear, the result is classified as a "preliminary positive" result until the result can be confirmed by a more accurate test (usually Western blot analysis). The performance of OraQuick Advance® is equivalent or better than many FDA-approved ELISA tests for the HIV virus and has become a standard for diagnosis in the industry.

In 2012, the FDA-approved OraSure Technologies' Biological License Application

(BLA) submission allowing for the first time home users to purchase the OraQuick Advance® kit to test themselves for HIV in the privacy of their own homes. The over-the-counter approval for OraQuick Advance® HIV 1/2 has catalyzed new activity in the oral diagnostics arena, which eventually will result in the development and commercialization of a new generation of saliva-based lateral flow (LTF) assays that "piggyback" on a number of available enabling technology platforms.

Chembio Diagnostics, Inc. (Long Island, NY, www.chembio.com) followed OraSure becoming only the second company to achieve FDA approval status for a rapid HIV diagnostic test using oral fluid samples. At the beginning of 2013, Chembio obtained FDA approval for its "Dual Path Platform (DPP)" point-of-care HIV antibody test. This device accepts a number of sample types including saliva, serum, fingerstick whole blood, venous whole blood, or plasma specimens. In the case of oral fluid specimens, a swab is used to capture saliva from under the lips around the gum line top and bottom. It is recommended that the swabbing action is done four times around the outside of the gums on the top and bottom of the mouth. The swab is inserted into a buffer tube called a SampleTainer containing a proprietary buffer and the handle of the swab removed by snapping off the head at a carefully positioned notch. Two drops of the saliva buffer mixture is then added to a well (Well 1) on the DPP Oral Fluid HIV 1/2 Device. After 5 min, a secondary buffer reagent is added to a second well (Well 2) and the reaction allowed to proceed to completion. The visual read on the qualitative test is 25–40 min. A positive result is considered presumptively positive for either HIV-1 or HIV-2 and should be confirmed by a secondary method.

Ahead of the anticipated emergence of new oral fluid tests, the author notes the existence of a number of other rapid oral LTF tests. Microimmune in the United Kingdom and its partner at the Public Health Laboratory Branch at Colindale in London (UK) have introduced an oral-based test for measles IgM [50] that may be used for either saliva or serum specimens. The microimmune measles IgM test is a qualitative test that requires oral specimen collection using the ORACOL Oral Swab (Malvern Medical Developments, UK) followed by analysis using LTF strips. Samples are extracted from the ORACOL device and placed on the microimmune IgM LTF strips and incubated for a period of 20 min. The signal line is evaluated relative to a control line. If two lines are present, the test is positive for measles IgM antibodies.

Researchers from the University of Queensland [51] have developed a "one-step homogeneous C-reactive protein assay from saliva." This assay is a bead-based assay using streptavidin-coated donor beads that bind to anti-CRP antibody conjugated to acceptor beads. The assay time is approximately 15 min and the sample required is unstimulated whole saliva. At the time of writing, this assay has not reached the commercialization stage.

Foresite Diagnostics (UK) is now commercializing a rapid saliva kit for the detection of cortisol levels in pigs to determine stress levels [52]. The Foresite kit, developed at the Central Science Laboratory in York (UK), requires four drops (70 µ[mu]L) of pig saliva, collected using a large cotton bud that the pig chews until the pad is saturated. The saliva-soaked pad is then separated from the bud with scissors and placed in a plastic plunging unit (resembling a syringe), which expels up to 1.0 mL of saliva ready for analysis. Results are available in 5 min and read visually as a qualitative test. The device can also be used in a semiquantitative manner using a bench top reader, for instance, a Biodot reader connected to a PC, or alternately one of a number of generic handheld readers capable of quantifying signals on LTF strips.

In August 2013, Oasis Diagnostics® [53] was awarded a Phase II Small Business Innovation and Research grant to complete the development of a test for *human* salivary cortisol levels in its VerOFy® rapid POC platform. VerOFy® is another LTF device that uses an absorbent pad to collect saliva from under the tongue until a sample volume adequacy indicator built into the device changes its appearance (pale yellow-green to dark blue). The test device is removed from the mouth and allowed to sit while the test runs for an additional 10–15 min, after which results are

available. VerOFy® may be configured for visual or instrument readout, but in the case of the VerOFy® cortisol test, levels of the hormone are quantified using fluorescently labeled particles that are read by a small, portable reading device known as LIAM™ (Light Image Analysis Module). Results may be downloaded to a Smartphone or PC. The VerOFy® platform may be configured to evaluate multiple biomarkers simultaneously in quantitative fashion.

A team at Rice University headed by Dr. John McDevitt has been pioneering the development of next-generation Lab-on-a-Chip (LOC) systems for oral-based cardiac diagnostics, under a project funded by the NIH/NIDCR through a long-term (U01) grant, among others. The unique approach of the McDevitt group has resulted in the development of the "Texas Bio Nano-chip (NBC)" sensor system [54] that is based upon the use of micro-bead arrays. Using microfabrication tools, McDevitt et al. have been able to micro-etch pits within silicon wafers that have on them a variety of chemically sensitized bead "micro-reactors." The Rice group describes these as "chemical processing units." The NBC has ultra-sensitive multi-analyte detection capabilities in a miniaturized format and has already been adapted to diagnostic tests for cardiac surveillance, electrolytes, sugars, proteins, toxins, antibodies, and others. Tools from the McDevitt initiative are available through a company formed by McDevitt called LabNow, Inc. Other applications in development include tests for periodontitis, C-reactive protein (CRP), CD4 counts in HIV-infected individuals, and oral cancer.

Washington-based Seattle Sensors is working with surface plasmon resonance (SPR) technology and has developed an alternate instrument-based system for the detection of cortisol in saliva [55]. The portable instrument from Seattle Biosensors is based upon the technology developed in the laboratory of Dr. Clement Furlong at the University of Washington using a competitive assay and cortisol-specific monoclonal antibodies, with a six channel (portable) SPR biosensor. The technology is built upon Texas Instruments' Spreeta 2,000 sensor chips and has a published detection limit of 0.36 ng/mL. A pre-purification step in the instrument separates small molecules from larger macromolecules in saliva, prior to sample presentation to the sensor resulting in enhanced sensitivity. The system is reported to be useful for a wide range of applications, particularly detection of small molecules in complex mixtures. Results are available using the technology in 10–20 min, but require a separate (whole) saliva collection process using one of a number of commercially available saliva collection tools.

Drug testing is one particular area where saliva testing has gained a strong foothold, and this may be attributed to the convenience factor of being able to collect samples noninvasively from would-be "drugged" drivers and employees under workplace conditions, for example. Over the last 10 years, many companies have emerged with new innovations, each providing unique methods for detecting the NIDA-5 series of drugs (THC/marijuana, opiates, cocaine, amphetamine/methamphetamine, and PCP), but most still rely on an approach that includes the application of (stalwart) lateral flow technology. A number of companies have had some commercial success, and this may in part be due to the large number of drug tests that are performed in the United States each year. It is estimated that between 50 and 60 million drug tests are performed annually in the United States alone, so even a small fraction of this represents strong revenues for commercial suppliers. There are still technological challenges to be overcome, most notably the ability to detect marijuana effectively in saliva samples at low levels, but despite this, many oral drug tests are processed on a daily basis.

The early leaders in the oral drug testing field were Cozart Biosciences (UK www.concateno.com now Alere, Inc.), Securetec (Germany, www.securetec.net), Branan Medical (www.brananmedical.com), and Mavand (Germany, www.mavand.com).

Cozart/Concateno's early versions of the RapiScan Drug Testing Unit combined saliva collection using the Saliva Diagnostic Systems Omni•SAL® Device with rapid lateral flow test strips that were immediately read on a handheld reading unit (RapiScan). Test results were delivered in 10–15 min for a series of six abused substances. Cozart/Concateno's parent Company (Alere, Inc.) now market an upgraded version of

the RapiScan Device, called DDS2, which uses an absorbent pad-based system to collect oral fluids. Once collected the sample is immediately transferred onto a test cartridge on board the DDS2 instrument. Oral fluids mix with buffers in the device and then flow along LTF test strips in the unit. The DDS2 analyzes for five drug classes in 5 min. The results of a recent study [56] are encouraging; however, in 24 % of cases, the DDS2 unit failed to provide a valid result. Cozart's systems have been employed successfully in Europe and Australia in particular.

Securetec AG's DrugWipe 5 S (Germany) is a 10 min test that detects the NIDA-5 panel of drugs following a very rapid collection of specimen. The DrugWipe Device cassette itself houses a unique saliva collection pad that is removed from the cassette and then used to collect saliva by wiping the insides of the moistened cheeks. An indicator dye on the pad changes color from red to yellow signifying that an adequate sample has been collected. The collection pad is placed back in the device cassette, and results are read visually on the test strip in 8 min.

Branan Medical markets the OraTect III as the "first single step" oral fluid drug test. Collection on the OraTect is by means of an absorbent pad connected to the Branan test cassette. The subject rubs the absorbent pad across each cheek 15–20 times, then rubs the tongue 15–20 times then places the absorbent pad under the tongue until a series of blue lines begin to flow and are visible in the test cassette. Results are available for up to six drugs in 5–30 min using the OraTect device.

Mavand offers a multidrug screen known as RapidStat that can detect up to eight drugs in about 6–8 min. Collection of saliva is easy (less than 30 s) using a swab. Once collected the saliva is transferred to sample wells on the Mavand RapidStat Device, which are connected directly to lateral flow test strips embedded in the device.

There are many other devices in the marketplace that successfully test saliva for drugs of abuse, so two additional resources are provided that will enable the reader to learn more about this field.

The European body known as ROSITA (ROadSIde Testing Assessment, www.ROSITA.org) is an independent body responsible for evaluation and validation of tools for drug testing at the roadside, so for further information on salivary devices with applicability in law enforcement screening, the reader is referred to the ROSITA Website.

In addition, Table 3.1 includes Websites for a number of companies marketing other handheld drug tests that are commonly used for substance abuse drug testing in forensics, employment screening, workplace testing, and criminal justice. This list is not comprehensive, but provided as a reference resource only.

Table 3.1 List of representative rapid oral drugs of abuse tests/manufacturers

Manufacturer	Website	Product name
Confirm Biosciences	www.confirmbiosciences.com	SalivaConfirm
Drug Testing America/others	www.drugtestingamerica.com	i-Screen
ASC	www.americanscreeningcorp.com	Discover
American Biomedica Corporation	www.abmc.com	OralStat
JAJ Scientific	www.jajinternational.com	QikTech
Innovacon (Alere)	www.innovaconinc.com	OrALert
Mavand	www.mavand.com	RapidSTAT
Envitec	www.envitec.com	SmartClip
Branan Medical	www.brananmedical.com	Oratect XP
Ulti-med	www.ultimed.org	SalivaScreen
Varian	www.varian.com	OraLab 6
Securetec	www.securetec.net	DrugWipe 6
Express Diagnostics	www.drugcheck.com	SalivaScan

While rapid POC saliva tests are definitely growing in significance and certain tools have made a clear impact, point-of-care diagnosis using oral samples is still in the embryonic phase.

Molecular Platforms

Since the discovery of PCR and other molecular techniques, the use of DNA as a building block for diagnostics has grown rapidly. Market sources estimate that more than 500 million molecular tests are done annually in the United States (2010 numbers) and that this number will grow to 750 million by 2015 [28, 57].

Other statistical reports estimate that the worldwide market for molecular diagnostics was $5.5 billion in 2013 and on a growth curve [58]. Already a small fraction of the estimated 500–750 million tests use saliva as a sample source, particularly in the research and life sciences environments, but trends indicate that as current studies are published confirming the efficacy of saliva as an ideal specimen, the proportion of oral-based tests will rise sharply. In addition, new high-profile research projects targeting salivary RNA (including mRNA and miRNA) and proteins (proteomics) will magnify the interest in oral testing, resulting in new diagnostic areas where saliva will be a specimen of choice. A significant example of this is the recent grant award of more than $5 million to the Wong group at UCLA to examine extracellular RNA in exosomes and other microvesicles in gastric cancer [29].

A recent publication by Gallo et al. [59] has surprisingly shown that the majority of microRNAs detectable in serum and saliva are concentrated in exosomes, so this is likely to lead to a focus in this area of salivary research.

The literature supports the widespread use of saliva as an ideal medium for SNPs, genotyping, microarrays, genome-wide association studies (GWAS), and other molecular technologies; however, most of the current applications are confined to the life sciences research area. At this time the only clinical test using saliva specimens on an automated platform is the eSensor Warfarin Sensitivity Test, which uses samples collected using the OraGene Saliva DNA Device. Saliva samples are pipetted into a cartridge that fits into the eSensor XT8 multiplex PCR system. Microfluidic chambers in the cartridge deliver diluted specimens to the PCR reaction site. The eSensor XT8 delivers results for multiple genetic mutations (in this case VCORC-1 and CYP2C19) in 30 min.

A number of other tests have been validated to saliva and are available as Lab Developed Tests (LDTs) in the United States. These tests are run in CLIA (Clinical Lab Implementation Amendments Act 1988) certified laboratories, who perform internal validations and obtain state approvals to begin running the tests. This list is not exhaustive, but some of the tests/platforms validated to saliva specimens include human papillomavirus (HPV) and periodontal disease detection at Oral DNA Laboratories (Brentwood TN, now part of Access Genetics), personal genomic profiling at 23andMe (Mountain View, CA), and whole genome sequencing at the Personalized Genome Project (PGP) headed by Dr. George Church at Harvard University. The Mayo Clinic now runs a series of genotyping tests including CYP2C19, CYP2D6, HLA B1502, HLA B5701, UGT1A1, and many others using a single saliva sample. Samples are analyzed using sequencing and single gene/gene mutation techniques.

The literature abounds with new applications for saliva, too many to provide an exhaustive list, but some of the newer molecular tests to be validated to saliva include the InPlex Cystic Fibrosis Test from Hologic (Bedford MA), which simultaneously detects 23 mutations in the cystic fibrosis transmembrane receptor (CFTR) gene and the IVS8/5T/7T/9T markers, the Asuragen AmplideX FMR1 Gene test for Fragile X Syndrome in autism and the multiparameter Affymetrix GeneChip Scanner 3,000 Targeted Genotyping System, capable of detecting close to 3,000 SNPs. In this latter example, saliva was shown to be equivalent or better than blood for genotyping 2,918 SNPs from Human Ch12 (developed during the HapMap Project). Positive results for saliva were also observed with the Affymetrix Drug Metabolism Enzymes and Transporters (DMET) Microarray system. In this example, simultaneous genotyping of a large number of

known markers (1,936 markers in 225 genes) was carried out. Earlier work validated the use of saliva on the Illumina Hap370 Microarray technology [60], as well as across two genotyping platforms (the Applied Biosystems Taqman™ and Illumina BeadChip™ genome-wide arrays [61], so it is hoped that over the course of the next few years, some of these and the many other research applications published translate into future clinically relevant tests.

The advent of point-of-care devices for nucleic acid testing (POCMDx) from companies such as TwistDx, Biohelix, Rheonix, Douglas Scientific, Alere, and others could also offer up new opportunities for oral testing in the future. Currently these devices are based upon blood sampling technologies and would clearly benefit from validated noninvasive protocols using saliva.

Conclusion

In summary, the future of saliva testing is extremely bright with a number of exciting and functional techniques offering up noninvasive and cost-effective solutions for diagnosis that will find value in disease diagnosis all over our planet. The number of companies involved in salivary diagnostics has risen sharply over the last 2–3 years, and the industry now looks favorably at opportunities to look at clinically relevant biomarkers in saliva samples. Later in 2014, the first annual North American Saliva Symposium is planned, and it is hoped that this landmark meeting will bring together the greatest minds in the saliva world to share ideas on research and clinical diagnosis. The NIH has embraced saliva as a biologically important specimen, and the FDA has already cleared tests through the 510(k) and PMA processes. The "ice has been broken," and in the eyes of this author, the time of saliva as a mature body fluid has arrived!

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