Considerations for Developing Saliva-based Lateral Flow Immunoassays

Lateral flow assays (LFAs) designed for diagnostic applications at home (over-the-counter, OTC) and point-of-care (PoC) are simple to use and can test for a wide variety of biomarkers in a diverse set of matrices including saliva, nasal swabs, blood, and urine. The demand for rapid SARS-CoV-2 testing drove worldwide familiarity with the use of LFAs in OTC and PoC settings, bringing increased attention to sample type and collection method. As a sample matrix in an LFA, saliva is highly attractive due to its ease of access, non-invasive sample collection process, reduced risk of disease transmission, and growing familiarity to consumers and patients. Saliva is also highly valued for diagnostic purposes due to the presence of a wide and diverse range of biomarkers that are readily accessible for rapid testing (**Table 1**).

Biocomponent Class	Examples
Hormones	Cortisol, androgens, estriol, estrogen, progesterone, aldosterone, melatonin, insulin
Cytokines	Interleukins (IL-1beta, IL-6, IL-8), tumor necrosis factor, troponin
Antibodies	IgG, IgA
Proteins/Enzymes	Amylase, pepsin, matrix metalloproteinases, C-reactive protein (CRP), mucins, lactoferrin, antimicrobial peptides (cystatin, hystatin, statherin, calprotectin)
Growth Factors	Epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) insulin-like growth factor
Nucleic Acids	Human and microbial DNA, mRNA, microRNA, tRNA-derived small RNA (sRNA)
Viruses	HIV, HSV-1, HSV-2, EBV, HPV, CMV, VZV, HCV, *Anelloviridae*
Bacteria	*P. gingivalis, S. mutans, Lactobacillus spp, T. forsythia, E. coli, H. pylori, M. tuberculosis*
Fungi	*Candida, Aspergillus*
Drugs	Anticonvulsants, chemotherapeutic agents (including antibiotics and antineoplastic agents), analgesics, drugs of abuse, ethanol
Metabolites/ Electrolytes	Phosphate, calcium, sodium, potassium, glucose, chloride, nitrate, uric acid, amino acids, lipids, carbohydrates
Tumor Markers	CA 15-3, HER2/neu, CA 19-9, p53, leptin, CA 125, alpha fetoprotein, CEA, somatic mutations in tumor suppressor genes, loss of heterozygosity, promoter hypermethylation of genes, microsatellite DNA alterations

Table 1: A diverse set of biomarkers are present in saliva making it a valuable sample matrix for LFAs.

Considerations for Saliva-Based OTC LFAs

Saliva-based LFAs need to have reduced complexity to minimize the possibility of human error and secure approval by regulatory agencies. The OraQuick (OraSure Technologies) in-home human

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immunodeficiency virus (HIV) test, approved in 2012 by the United States Food and Drug Administration (FDA), is considered a gold-standard for saliva OTC LFAs.[1] This single-use, qualitative immunoassay is easy to use for at-home detection of antibodies to HIV-1 and HIV-2.

Sample collection is performed using a filter pad attached to a test stick device; there is no need for a lancet or needle, which reduces the risk of spreading infection. The sample collection pad is inserted in the mouth to collect the sample. The test stick is then inserted into a vial containing developer solution for sample processing; the solution extracts protein contained within the sample, ensuring that it doesn't stick to the sample pad. The developer solution also enables flow of the specimen into the diagnostic device and onto the test strip. In this LFA, the saliva collection method connects to the nitrocellulose membrane and the conjugate pad, which is contained in a Mylar pouch with a desiccant. To reduce the complexity of this test, a sample dropper is not needed to move the sample from place to place; the sample swab is dropped directly into the vial.

The LFA contains HIV antigens immobilized on the nitrocellulose membrane and a conjugate pad containing colloidal gold conjugated to Protein A which captures antibodies from the sample. If the specimen contains antibodies that react with the HIV antigens on the nitrocellulose membrane, a line will appear at the test zone.

This whitepaper summarizes key considerations for the development of saliva based LFAs that are reflected in the design of the OraQuick LFA test, including:

- Sample collection, volume control, and sample normalization
- Sample processing to achieve the desired sensitivity, the dynamic range, time to results, and assay kinetics
- Results interpretation and the metric to be used, whether the test will be quantitative, semi-quantitative, or qualitative, and whether a reader will be incorporated

Sample Collection

Saliva is a complex biological fluid, and its composition can vary at the time of collection, particularly if collected following eating, drinking, or smoking. Unfortunately, there is no established uniform criteria for the collection of oral fluid for use in diagnostic applications, and as such, developers must evaluate existing options and understand the collection device landscape.[2] Customization of the sample collection process and device may be necessary if suitable solutions are not available.

The most common sample collection methods are passive drool and oral swabbing (**Table 2**).

Passive drool collection into a plastic device via a funnel is user-friendly, offers cost advantages, reduces analyte loss to material, and has a high-volume capacity. The funnels are inexpensive, and because there are no paper-based materials, there is no loss of material which can occur when using oral swabs for collection. Passive drool collection may be difficult, however, for pediatric patients, patients in critical care settings, and those with chronic dry mouth or lesions. An additional challenge is that passive drool can be highly variable in terms of viscosity.

Passive Drool	Oral Swab
Pros	**Pros**
• Inexpensive • Reduced analyte loss to material • High volume capacity • Simple	• Consistent • Filtrates/normalizes • Resistant to viscosity variability • Versatility in deployment • Variety of off-the-shelf options
Cons	**Cons**
• Participants may not be able to drool • Variability • Viscosity	• May lose analyte in swab • Limited volume extraction • Procedural importance
	

Table 2: Comparison of passive drool and oral swab sample collection processes.

An alternative to the collection of drool is the use of an oral swab, which provides a more consistent

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sample collection and is resistant to variations in viscosity. It is important to evaluate the composition of the swab itself, however, to ensure minimal analyte retention and maximal sample extraction prior to analysis.

Challenges presented by oral swabs include analyte retention and potential reactivities of the sample with different swab materials. Because of this, a spike-in recovery using these swabs must be assessed to determine whether analyte retention is an issue. This involves adding a fixed amount of viral protein into a sample, "collecting" the sample using the swab, and determining how much viral protein remains on the swab.

Other considerations include the fact that a smaller volume of saliva can be collected as compared to passive drool and the specific procedure for collection must be defined, whether it is from under the tongue for a defined amount of time, the roof of the mouth, cheeks, or the throat.

Because some collection devices can be expensive, the design of the test system, the desired cost point, and sample retention should be considered early in the development process. Fortunately, many options for customizing oral swab sample collection exist.

Sample Processing

Whatever method is used for collection, sample processing is essential, in large part, due to the presence of mucin in saliva which affects viscosity and sample flow. Other options for sample processing include filtration/separation, dilution, and additives and should be considered within the context of whether processing will occur at the same time as sample collection.

Passive drool samples can be filtered prior to being applied to the test strip. Saliva collection devices are available which are designed to filter out mucinous material for downstream assays; the filter removes large molecules and interferants as the sample is expressed into the collection tube. Other devices collect, filter, and normalize the pH of the sample to ensure the right environment for the LFA antibody to function.

Oral swabs offer a more consistent sample collection process because filtration can be directly incorporated. The level of sample filtration and normalization depends on the swab material, which can be nylon, cotton, or polyethylene, and the bed volume of the swab. Some saliva collection methods have indicators once a certain volume is reached and this helps ensure assay precision.

For samples collected via oral swabbing, the swab can be inserted into a mesh filter that is built into the cassette to extract the liquid phase before the sample reaches the cassette. This step helps to ensure the sample properly absorbs into the sample pad. If the viscosity is too high at this point, absorption will be too slow, affecting the reaction kinetics.

Another option for sample processing is dilution, in which the saliva sample or the swab itself is mixed with a buffer to normalize pH and solubilize mucins. With OTC tests, this process is completed by the user and adds complexity to the test, and as such, is more suitable for qualitative tests. For a quantitative test, a fixed volume of diluent must be added to a fixed volume of the sample to achieve the calibration that has been built into the assay.

Additives such as salts, surfactants, and blocking proteins can also be used for sample processing. Salts are highly effective for normalizing the pH to ensure the proper environment for assay antibodies to function. Surfactants help block non-specific binding through the sample pad, conjugate pad, and onto the membrane, which is critical to sample flow and specificity. Blocking antibodies can also be used in the sample pad to deal with the proteins and enzymes present in saliva that may interfere with assay results.

Results Interpretation

Reader-based and visual methods are available for the interpretation of LFA assay results (**Table 3**).

A reader is required when fluorescent or colorimetric tags are used in the LFA and can deliver both quantitative and semi-quantitative readouts and be integrated with cloud systems. The use of a reader is important if results should be read and interpreted by a clinician versus the user. There is an

Reader Based	Visual
Measurements via fluorescent or colorimetric reader Capable of doing quantitative and semi quantitative readouts	Visualization of the test line and control line with nanoparticles
• Integration with cloud • Multiple off-the-shelf options • Price sensitive • Uncertainty on regulatory route	• Inexpensive • Simple • Qualitative • Regulatory route carved out

Table 3: Comparison of reader-based and visual approaches for results interpretation.

added cost with a reader-based test, however, which can be a one-time fee for the user or embedded into the cost per test. There can also be some uncertainty in terms of the regulatory route as the reader must be part of the overall submission.

In contrast to reader-based results interpretation, visual approaches are simple, binary, and designed primarily for semi-quantitative assays. LFAs for SARS-CoV-2 use visual results interpretation, which is well-suited for untrained operators.

Different types of nanoparticles are available to enable a visual readout; among the most used, included in most if not all pregnancy tests, for example, are 40nm gold colloid. The ongoing evolution in the nanoparticles field, however, is delivering higher sensitivity options for LFAs, particularly for complex matrices such as saliva with lower concentrations of analytes than found in blood.

Higher sensitivities can be achieved using 150nm gold nanoshells, for example, which absorb more light and provide a more intense visual signal per binding event because of their larger size. These nanoshells are designed with a silica core which is significantly less dense than a conventional solid gold nanoparticle, enabling them to flow better in an LFA than a comparable-sized solid particle. Nanoshells are available with a citrate surface option for passive adsorption and a carboxyl surface option for covalent conjugation.

The following case studies demonstrate the advantages in sensitivity offered by nanoshells as compared to traditional gold nanoparticles.

Case Study: Quantitative Detection of Troponin I

Troponin I is a marker commonly used to identify patients suffering from cardiac distress. Both 40 gold nanoparticles and 150 gold nanoshells can be used for the quantitative detection of troponin I as shown in **Figure 1**. Nanoparticles offer a range of sensitivity from 0.5 to 30 ng/mL of the biomarker, while the 150 gold nanoshells extend sensitivity of the assay down to 0.05 ng/mL.

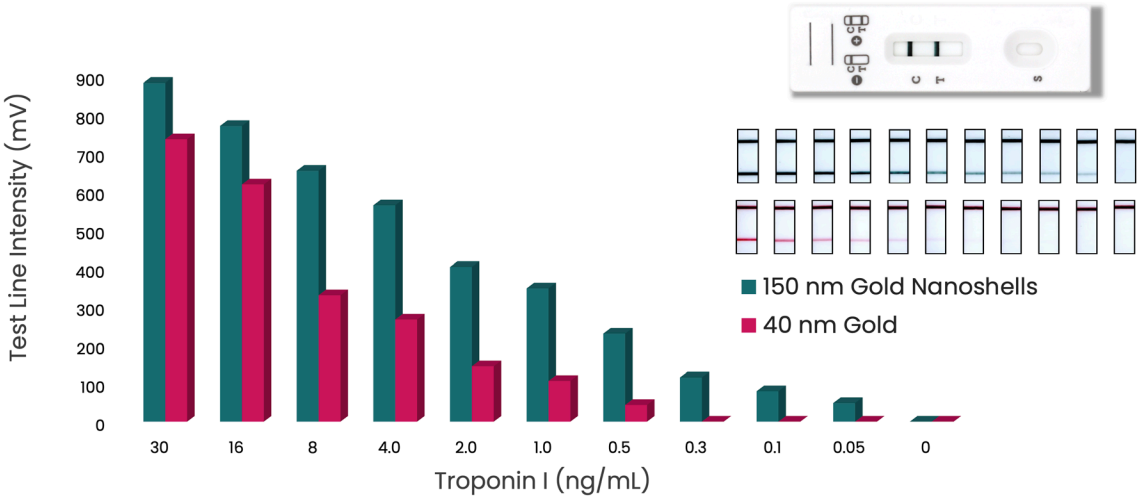

Figure 1: Comparison of 40nn gold nanoparticles and 150nm gold nanoshells for the quantitative detection of troponin I.

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Figure 2 shows a scanning electron micrograph (SEM) of gold nanoshells (white dots) bound to the nitrocellulose membrane in a troponin I lateral flow device. The SEM shows the extent of packing on the membrane and the relative size of the nanoshells versus the pore structure of the membrane. As intense as the line is on the device, a good deal of additional package space remains available.

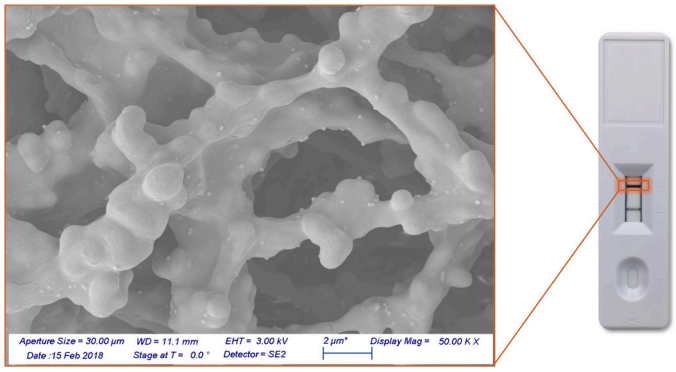

Figure 2: SEM image of 150 nm gold nanoshells bound to a nitrocellulose membrane in a lateral flow device.

Case Study: CoV-2 Serological Test

The VacCheck rapid immunoassay is a saliva-based, semi-quantitative test designed to detect human IgG antibodies with an affinity for the SARS-CoV-2 receptor binding domain (RBD) of the spike protein. Samples are collected using an oral swab and an extraction buffer. An external dropper tube is used to extract IgG and two drops are transferred to the cassette, delivering results in 10 minutes.

Figure 3 shows a direct comparison of test line intensity results from 24 serum samples requiring a blood draw and saliva samples collected on the same day. While the signal intensity from the serum is higher than saliva in most instances, the trend remains the same between these samples. This indicates that results from the non-invasive saliva based LFA are comparable to those requiring invasive sample collection.

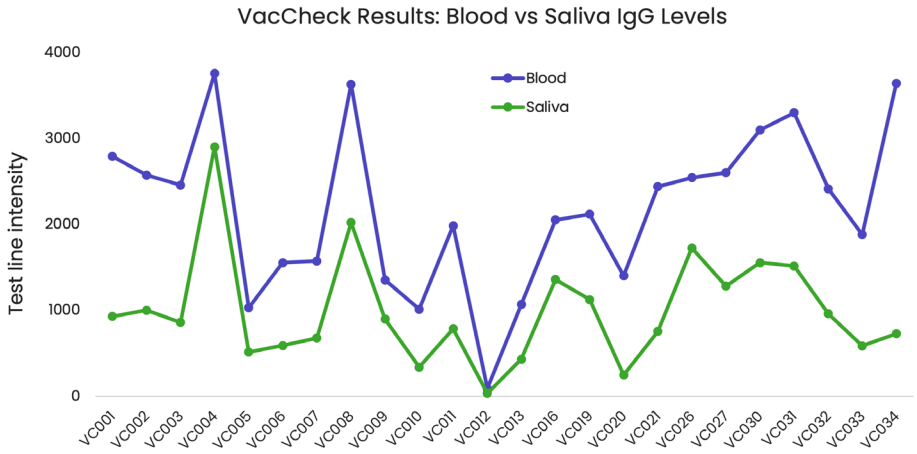

Figure 3: Comparison of test line intensity of serum and saliva samples collected on the same day.

Manufacturability

A final consideration when developing an LFA is manufacturability. To be commercially successful, the assay must be manufactured at a cost point that supports a target price amenable to customers and reimbursement programs. The ability to automate steps is critical to achieving the desired cost point and can include buffer filling using liquid handling systems, conjugate spraying and dispensing, and sample pad blocking treatments using web handling systems. Another critical consideration at this stage is the supply chain; it is essential to partner with suppliers that are strong collaborators and can meet the necessary lead times and scale.

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Conclusion

LFAs are rapid and inexpensive diagnostic devices that can be used to test for a wide range of analytes in a variety of sample types. The LFA format offers a long shelf life, does not require refrigerated storage, and delivers results in a short time without complicated processing, additional equipment, or extensive training. The simplicity of this approach is especially important for OTC and PoC applications. Saliva-based LFAs offer further advantages through the ease of sample collection and the wide range of analytes present in saliva.

A successful diagnostic lateral flow assay requires a series of optimizations including sample collection, sample processing, and interpretation of results. How, where, and by whom the assay will be used should all be considered during development to make the most effective and commercially attractive assay.

For more information, visit fortislife.com

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2. Ghosh S, Dhobley A, Avula KK, et al. (2022) Role of Saliva as a Non-Invasive Diagnostic Method for Detection of COVID-19. Cureus 14(7): e27471. doi:10.7759/cureus.27471

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