Tutorial: Design and fabrication of nanoparticle-based lateral flow immunoassays

Claudio Parolo,[1†] Amadeo Sena-Torralba,[1†] José Francisco Bergua,[1] Enric Calucho,[1] Celia Fuentes-Chust,[1] Liming Hu,[1] Lourdes Rivas,[1] Ruslan Álvarez-Diduk,[1] Emily P. Nguyen,[1] Stefano Cinti,[2] Daniel Quesada-González,[3] Arben Merkoçi *[1,4]

1 Nanobioelectronics & Biosensors Group, Institut Català de Nanociència i Nanotecnologia (ICN2), Campus UAB, 08193 Bellaterra, Barcelona, Spain.
2 University of Naples "Federico II", Naples, Italy.
3 Paperdrop Diagnostics, Eureka building, Campus UAB, 08193 Bellaterra, Barcelona, Spain.
4 ICREA, Institució Catalana de Recerca i Estudis Avançats, Barcelona 08010, Spain.
† C.P. and A.S. equally contributed to this manuscript.
* arben.merkoci@icn2.cat

Abstract

Lateral flow assays (LFA) are quick, simple and cheap assays to analyse a variety of samples at the point of care or in the field, making them one of the most widespread biosensors currently available. They have been successfully employed for the detection of a myriad of different targets (ranging from atoms up to whole cells) in all type of samples (including water, blood, foodstuff and environmental samples). Their operation relies on the capillary flow of the sample within a series of sequential pads with different functionalities aiming to generate a signal indicating the absence/presence (and, in some cases, the concentration) of the analyte of interest. In order to have a user-friendly operation, their development requires the optimization of multiple, interconnected parameters that may overwhelm new developers. In this Tutorial we provide the readers with: 1) the basic knowledge to understand the principles governing an LFA and to take informed decisions during lateral flow strip design and fabrication, 2) a roadmap for optimal LFA development independent of the specific application, 3) a step by step example protocol for the assembly and operation of an LF strip for the detection of Human Immunoglobulin G and 4) an extensive troubleshooting section addressing the most frequent issues in designing, assembling and using LFAs.

Introduction

The simplicity and low cost of lateral flow assays (LFAs) have made them one of the most used point-of-care (PoC) sensors[1,2] in a variety of disciplines ranging from diagnostics[3–6] to environmental[7–9] and safety[10–14] analysis. Their success lies in their general design that has remained almost unchanged since their first use as pregnancy tests in 1970s.[15] Although all LFAs simply rely on capillary forces to move the sample along a test strip to generate a measurable signal, their fabrication is far from being straightforward.[16] In fact, in order to provide the end user with an easy-to-use device, developers must consider and evaluate multiple parameters, some even simultaneously, for optimal performance or market-readiness.[17] For a new researcher or developer, this task may prove to be quite daunting. Fortunately, as it is a relatively ´old´ and well-known technology, there are several resources online that discuss the general principles of

LFAs and even several guides are provided by both public and private entities.[16,18–26] In addition, searching for "lateral flow assay" in Scopus generates over 7700 documents, with many of them describing the development of a new LFA for specific applications. Although the design and optimisation of tailored LFAs remains challenging, the target-specific nature of the assay makes it impossible to write a generalizable LFA protocol that works for all applications. Therefore, instead, in this Tutorial we have collected the knowledge to provide a general roadmap that can be used by developers to design, optimize, and fabricate an LFA for their specific application(s).

General principles and components of lateral flow assays

From a user perspective, operation of a well-designed LFA is strikingly simple: the assay is executed by adding the sample onto a single paper lateral flow strip (LF strip) and after a short incubation time, the positive or negative outcome of the test is revealed by the appearance of a line. Having a more detailed look (Fig. 1) at what happens to the sample after its application (assuming for a standard immune sandwich assay, although other formats are also possible see Box 2), we can see that it first encounters the sample pad, which assures that the characteristics of the sample match those required for optimal detection (pH, ionic strength, viscosity, purity and concentration of blocking agents). Next, the sample reaches the conjugate pad, which releases the labelled detection bioreceptor (e.g. an antibody, often – conjugated to a nanoparticle for detection) upon wetting and allows for a first analyte-bioreceptor interaction. The sample then travels through the membrane (also called detection pad). In most LFAs there are at least two lines on the membrane: 1) the test line, where a capture bioreceptor binds the labelled analyte, generating a line that indicates the presence or absence of the analyte (most commonly visualised either by naked eye or by an optical reader); and 2) the control line, which assures the correct operation of the LFA (by selectively capture of the labelled bioreceptor). Finally, the sample reaches the absorbent pad, which provides enough bed volume for the complete flow of the sample.

Overview of the Tutorial

The design of the LF strip strongly depends on the type of sample and the target molecule to be detected. Therefore, we start this Tutorial by a discussion of the types of samples that can be analysed and the different challenges this raises. Next, we discuss how to select and characterise appropriate bioreceptors and how to choose the optimal type of nanoparticles for detection. Following this, we have a detailed look at the four main physical components of the LF strip: sample pad, conjugate pad, membrane and absorbent pad. We will examine in detail the effects that each component has on the LFA, and discuss the key characteristics developers should consider when choosing the most suitable materials and reagents for a specific target of interest. In addition, we provide a detailed step-by-step example procedure for the assembly and operation of an LF strip for detecting Human Immunoglobulin G (Human IgG).

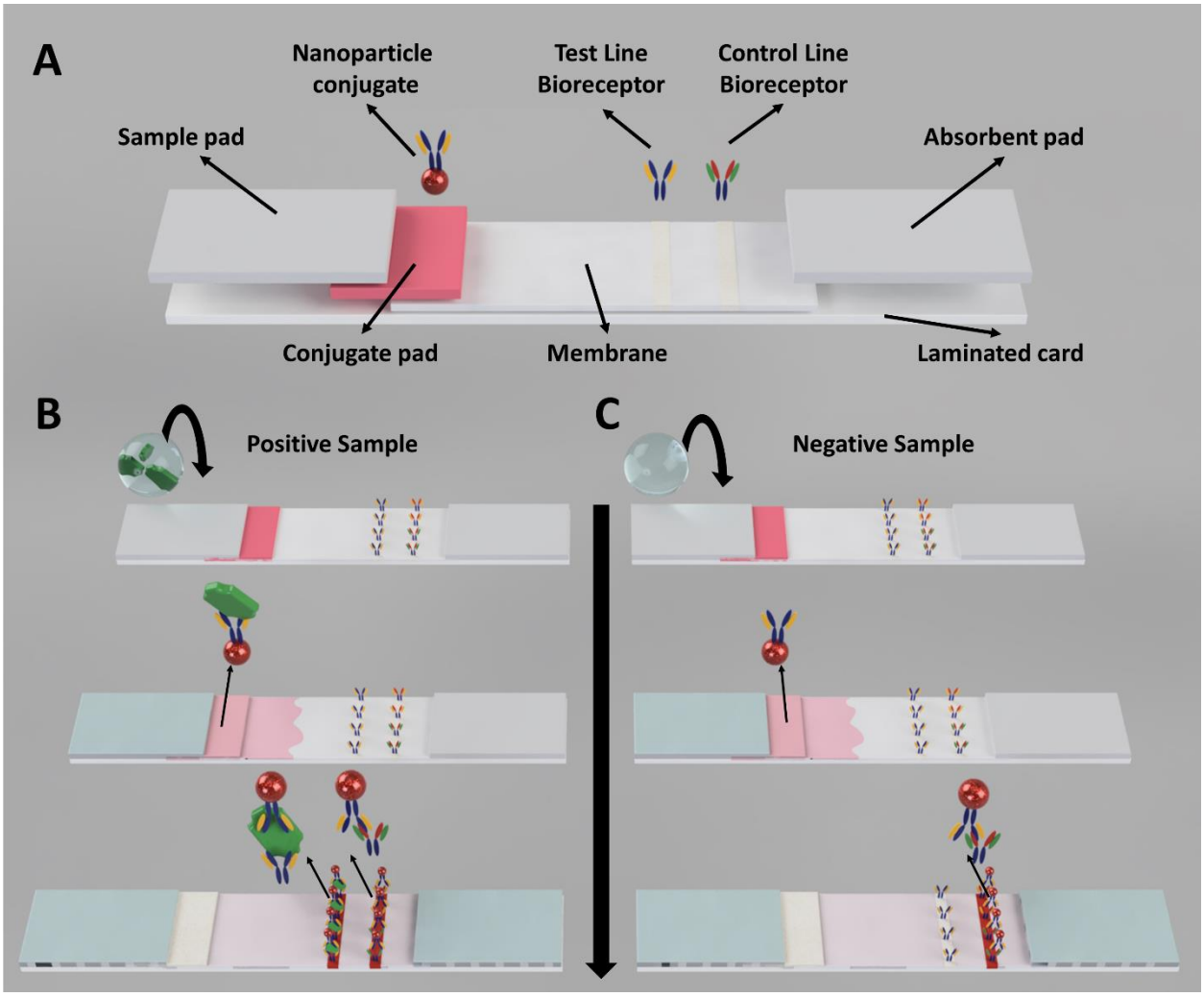

Figure 1: *schematic of the main components and operation of a typical LFA. A) A LFA is made of four main parts: the sample pad, the conjugate pad, the membrane and the absorbent pad. They are mounted on a laminated card. The membrane contains the bioreceptors that form the test and control lines. The bottom part shows the operation of a LFA based on an immunosandwich recognition. B) The presence of the target in the sample produces the accumulation of nanoparticles on the test and control lines making the classical two red lines to appear. C) Instead the absence of the target makes the nanoparticles to accumulate just on the control line, giving a single coloured line output.*

Types of samples and target analytes

As aforementioned, LFAs are used in a variety of applications with samples that present extremely different composition and characteristics (Table 1).[27] For example, clinical samples can be whole blood,[28,29] plasma,[30,31] serum,[32–34] sweat,[35,36] urine,[37,38] stool,[39,40] saliva,[41,42] cerebrospinal fluid[43,44] and nasal swabs,[45,46] while food matrices could be juices,[47,48] cereals,[49,50] meat,[51,52] vegetables,[53] and environmental (mostly water and soil) samples.[54–57] Although the sample pad provides a means to control the properties of the sample solution (see the following section), some complex matrixes may require pre-treatment

before an aliquot can be added into the LFA strip.[58] For instance, the most obvious is the requirement for homogeneity of ground solid samples in specific buffers in order to allow for an even and steady flow through the LFA. For example when analysing solid food matrices the uneven distribution in their volume of toxic/pathogenic contaminants requires the use of large volume of samples that need to be homogenised.[59] In the case of whole blood, its dark red colour and viscosity present challenges and various limitations for LFAs. Ideally, the separation of blood cells using external filtration units[60] or by using an integrated special filtration membrane[61,62] on the sample pad is recommended. However, the additional use of external filters may complicate the system for the end user and, moreover, sufficient purification is not always achieved. It is also possible to lyse the blood cells before adding the blood sample to the LF strip. However, this should be avoided (unless the target is within the cell) to prevent the release of other proteins and nucleic acids that can interfere with the specificity and sensitivity of the LFA.

Besides making the sample suitable for running in an LFA, developers must also develop a simple yet standardized specimen collection protocol. To achieve reliable analytical results from different users, it is paramount that the sample collection procedures are consistent, in particular for LFAs requiring a quantitative reading. For example, LFAs for urinary test often include a sterile cup to minimize contamination during specimen collection or porous plastic wicks to control the volume of sample and the flow speed. For stool samples, a small spatula is generally included for the uptake of a precise amount of sample and for its immersion in a provided homogenization solution. Nasal, saliva, skin and vaginal samples often provide surface contaminant controls and typically require the use of a cotton swab to capture molecules and particles from inner tissues and surfaces. The swab is then immersed into a provided solution to suspend and pre-treat the samples before its application to the lateral flow strip. In some special cases, like LFAs for whole blood samples, running and/or washing buffers are used. Their function is to guarantee the flow of the analyte along the LF strip even when the viscosity or the insufficient amount of sample would prevent it.

TABLES

Table 1: Considerations on pH, collection and treatment of different types of samples for their use in LFAs.

Sample	pH	Collection	Sample treatment	Ref.
Urine	4.5-7.2	• Can be directly applied in some LFA. • Otherwise use of sterile containers. • Some patients might not be able to urinate due to pre-existing condition, requiring catheter extraction.	• Sample pad buffer should aim at equalize the pH of different samples. • Possible dilution or centrifugation.	37,38
Blood	7.35-7.45	• Fingerprick. • Venous puncture.	• If directly applied, a second running buffer is required. • Use of extra stacking pad for sample filtration.	28,29

Plasma	pH 7.35-7.45	• Requires the separation of cells from non-clotted blood, generally by centrifugation. • Special filters can separate plasma from blood without centrifugation, making the ideal approach for LFAs.[224]	• A running/washing buffer is generally recommended upon sample addition. • It may require additional filtration prior application to the LFA.	30,31
Serum	pH 7.35-7.45	• Requires separation of the liquid portion from clotted blood, generally by centrifugation.	• A running/washing buffer is generally recommended upon sample addition. • It may require additional filtration prior application to the LFA.	32,33
Sweat	3.5-8.5	• The absorbent pad of an LFA is wiped across the skin. • An absorbent patch is sticked to the skin for several days (for example PharmCheck patches).	• Use of a running buffer to either extract the sample from the patch or make it run through the sample pad. • Sample pad buffer should aim at equalize the pH of different samples.	35,36
Mucus	5.5-8.3	• Nasal swabs (Including Universal Transport Medium).	• Use of a running buffer to extract the sample from the swab. • The buffer may contain: mucins, surfactants and high salt concentrations for viscosity reduction.	45,46
Saliva	6.75-7.25	• Collection in swabs or tubes. • Unstimulated: pooling saliva in the mouth and deposit into a specimen tube. It may involve food or drink contamination, higher viscosities, and air bubbles. • Stimulated: by chewing gum, by sucking on a lozenge or by swab-like collection devices. It may involve changes in pH,	• Running/extraction buffer may contain: mucins, surfactants and high salt concentrations for viscosity reduction. • Centrifugation and application of the supernatant. • Special salt pad made of glass fibre with highly concentrated salt, placed in the middle of sample pad.	41,42,225

		contamination, and sample dilution. • Time of collection should be registered for correction of saliva flow rates.		
Cerebrospinal fluid	7.2-7.4	• Lumbar puncture performed by a professional. • Collection in a sterile container.	• Directly onto the strip and addition of running buffer. • Or addition of diluent in the container and then apply onto the strip.	[43,44]
Stool	6-7.2	• Collection in a sterile container, followed by sampling with a dedicated spatula.	• Homogenization of the sample contained in the spatula in running/extraction buffer. • Usually with tween20 for sample disruption.	[39,40]
Water	6.5-8.5	• Can be directly applied. • Some low-concentrated analytes might require the concentration of the sample. • Dirty water may require special filtration.	• Vacuum pumps and syringes might be used for sample pre-concentration.	[54–57]
Solid Food	N/A	• Uptake of small amount in sterile bags. • Very different matrices may require different treatments	• Generally, homogenization, filtration and dilution. • Might require extra filtration capabilities. • Use of a running/extraction buffers.	[49–52,226]
Liquid Food	N/A	• Can be directly applied. • Uptake of an aliquot in sterile containers	• Might require extra filtration capabilities.	[47,48,227,228]

Bioreceptor selection

In order for a bioreceptor (typically proteins, antibodies, DNA) to be effective in an LFA, it has to comply with three main characteristics: (1) it has to be stable, (2) it needs fast association kinetics and (3) should have a strong binding affinity for the target molecule. The stability of the bioreceptor means that it has to keep its structure and functionality in a variety of environments (different temperatures, humidity %, pressures)[63] and, above all, it has to maintain its function after a cycle of drying and rewetting. In regards to this, fast target analyte binding kinetics are essential as there are practically no incubation steps on the LFA and the bioreceptors have to bind to the target within seconds. These two are important characteristics to be considered when selecting suitable antibodies as bioreceptors. In fact, most

commercial antibodies are characterized with techniques such as Enzyme Linked Immunosorbent Assay (ELISA) and western blot, which include long incubation steps, typically in the order of hours, and therefore, antibodies that work for ELISA and Western blot may not work for LFA. Finally, the binding between the bioreceptor and the target must be strong to obtain a stable signal. In practice, most of the labelled target will pass through the LFA strip and travel to the detection zone in matter of seconds. Meanwhile, the flow still continues for minutes and acts as an internal washing step. If the target-to-bioreceptor binding is weak, the signal will decrease over time once the concentration of the labelled-analyte in solution decreases.

The choice of the proper bioreceptor is probably the most important step to achieving the required analytical sensitivity and specificity in an LFA. This is particularly challenging for the detection of protein targets given the large variety of possible bioreceptors (Table 2). Ideally, during the initial phases of an LFA development, several bioreceptors should be screened using a combinatorial approach where each bioreceptor is tested both in the test line and conjugated to the label nanoparticle. Depending on the resources and facilities available, the use of standard techniques (such as ELISA,[64] Surface Plasmon Resonance (SPR),[65] Biolayer interferometry (BLI)[66] and Isothermal Titration Calorimetry (ITC)[67])[68] could speed up the screening of multiple bioreceptors. For example, ELISA can be used to evaluate dozens of antibody/antigen combinations to find those producing the most sensitive result in a relatively short amount of time. In this respect, the developer could minimize the duration of the incubation steps in order to eliminate the bioreceptors with slow binding kinetics. Moreover, SPR, BLI and ITC can provide useful information about the association and dissociation binding kinetics, although they are more expensive techniques than ELISAs. Indeed, these techniques can help to identify and shortlist the most promising bioreceptors, however, they must be finally tested in a real LFA, in order to choose those that will be used in the final assay since the kinetics can be different for binding in porous media.[69]

Capture bioreceptors vs. detection bioreceptors.In order to avoid confusion, in this manuscript we will refer to the bioreceptors conjugated to the nanoparticles as “detection bioreceptors”, to the bioreceptors on the membrane as “capture bioreceptors”, which are further divided into “test line capture bioreceptors” and “control line capture bioreceptors”.

Although it is important to test different combinations of detection and capture bioreceptors (ideally previously short-listed using standard techniques), from a theoretical perspective the latter should have a faster binding kinetic. The reason for this is that the interaction of capture bioreceptors with the analyte is limited to the instants during which the flow passes on the test line area, while the detection bioreceptors have slightly more time to bind to the analyte during the flow from the conjugate pad to the test line. Another aspect to consider for the development of a sandwich assay is that the detection bioreceptor should not interfere with the binding of the test line capture bioreceptor to the target. For example, during the testing of a sandwich assay using a monoclonal antibody and a polyclonal antibody, the monoclonal antibody should be the detection bioreceptor (it binds to just one epitope of the analyte) while the polyclonal antibody should be the test line capture bioreceptor (the binding to multiple epitopes maximizes the chances of the sandwich formation). Finally the conjugation/immobilization chemistries, stability and cost should also be considered for each assay development. For example a bioreceptor may not be compatible with the chosen nanoparticle (i.e. the absence of functional groups for its conjugation), thus forcing the developer to use it as capture bioreceptor. Or the immobilization of the bioreceptor on

the membrane leads to the unfolding of its structure and the loss of its functionality, while it may be compatible with the nanoparticle surface. Or the amount of bioreceptor needed for conjugation to the nanoparticle is considerably higher than the amount needed for its immobilization on the test line, so it behoves using the cheaper bioreceptor as detection bioreceptor.

The characteristics for the control line capture bioreceptors are less stringent, since they have to bind either the detection bioreceptor or other molecules on the nanoparticle surface. In the case of using as detection bioreceptor an antibody, the control line capture bioreceptor could be a secondary polyclonal antibody. Instead if the detection bioreceptor is a nucleic acid the control line capture bioreceptor could be its complementary sequence. Other options such as biotin/streptavidin, BSA/anti-BSA antibodies and similar can also be employed for the generation of the control line.

Table 2: Common bioreceptors used in LFA for the detection of protein targets.

BIORECEPTOR	ADVANTAGES	DISADVANTAGES	CONSIDERATIONS
Polyclonal antibodies	• Cost-effective • Fast production • Multiple binding sites	• Low specificity • Cross-reactivity • Variability between different batches	Affinity purification of the serum required to minimize cross-reactivity.
Monoclonal antibodies	• High specificity • Low batch-to-batch variability.	• Expensive • Long development process.	For immune-sandwich assays antibodies binding to different epitopes must be chosen, unless the target presents multiple-repeated antigens/epitopes. For example a membrane protein on the surface of a cell.
Fragments	• Less nonspecific binding • Once the sequence is known they are generally cheaper and easier to produce than full size antibodies • Low batch-to-batch variability • Ability to increase the number of bioreceptors per probe	• Less stable than full length antibodies since they lack of Fc region	Antibody against IgG Fc region cannot be used in control line. BSA should not be used as blocking agent, given its higher molecular weight (66 kDa) compared to the fragments (<50 kDa). The possibility to produce some of them as recombinant protein in E. Coli makes their synthesis easier and cheaper than

			the immunization of full-length antibodies or using human cell lines.
Nanobodies	• Less nonspecific binding • Once the sequence is known they are generally cheaper and easier to produce than full size antibodies • Low batch-to-batch variability • Ability to increase the number of bioreceptors per probe	• Production limited to *Camelidae* and *Sharks* species.	BSA should not be used as blocking agent, given its higher molecular weight (66 kDa) compared to nanobodies (15 kDa).
Aptamers[229]	• Cheaper than antibodies. • Ability to recognize any type of target analyte. • High stability • High batch to batch reproducibility	• Their binding activity is highly dependent on the ionic strength of the buffer and the presence of interfering molecules in the buffer (i.e. certain cations)	It is recommended to perform a denaturation and refold step before fabricating the LFA.

Selecting nanoparticles for detection

In recent years the development of new nanomaterials has broadened the type of labels available for LFA although mostly gold nanoparticles and latex beads are used in mass-manufactured tests (Figure 2).[70–75] Here, we describe what we think are the most relevant nanomaterials in regards to their type of readout (also see Table 3 for a summary of the advantages and disadvantages of the different nanoparticles).

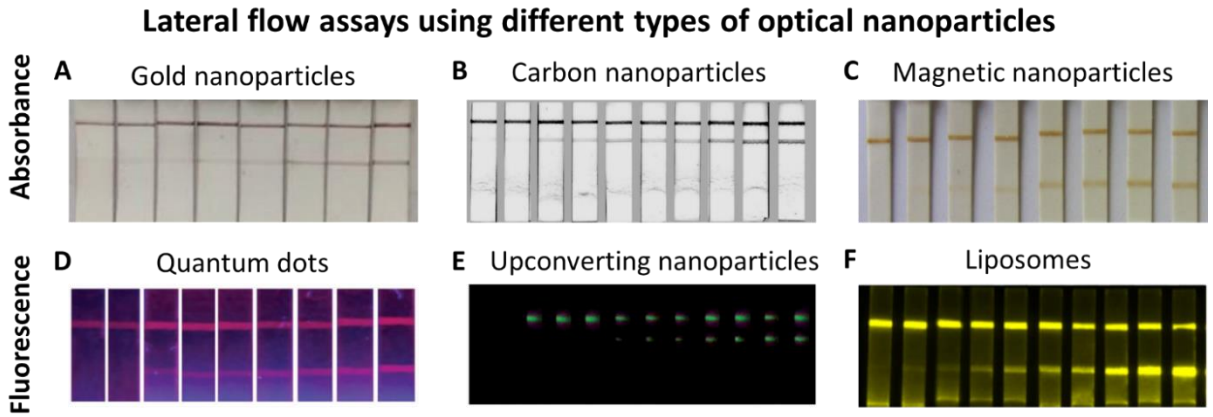

Figure 2 *– Examples of optical read outs of LFAs using different types of nanoparticles. In particular the figure shows calibration curves obtained for different targets (so non-comparable to each other in term of sensitivity) using nanoparticles providing an absorbance signal - A) gold nanoparticles,*[54] *B) carbon nanoparticles,*[55] *C) magnetic nanoparticles*[56] *- and others generating a fluorescence signal - D) quantum dots,*[57] *E) upconverting nanoparticles,*[58] *F) fluorescence-loaded liposomes.*[59]

Naked eye detection. The use of particles that allow for naked eye detection in LFAs are particularly beneficial for qualitative applications or those that aim for cost effectiveness, i.e. not requiring the use of external readers. Nonetheless, coupling these particles with an external reader may increase the reproducibility and provide the quantitative analysis required for more challenging applications.

- Gold nanoparticles: Since the 1980's,[76–78] gold nanoparticles (AuNPs) have become the most widely used detection labels in LFAs. The reasons behind their popularity are that (1) their surface plasmon resonance produces a strong red colour ideal for naked eye detection;[79] (2) there are different synthetic methods that are able to produce AuNPs with different sizes and shapes; [80,81] (3) they have low toxicity; (4) they can be easily functionalized either by nonspecific adsorption[19](see Box 1 Steps xx-yy) or *via* covalent bonds, [82–85] and (5) they are relatively stable. Given that AuNPs are the most common labels in LFAs, in this manuscript (see Box 1 Steps XX-yy) we propose an easy-to-optimize functionalization protocol. In addition, we also provide a step-by-step procedure for qualitative (by naked eye) and quantitative (camara-based) detection of an AuNPs-based LFA (see Box 1 Steps xx-yy).
- Carbon-based materials: Recently carbon nanoparticles (CNP)[86–92] and carbon nanotubes (CNT)[93,94] have provided an alternative to AuNPs. Although they do not have a plasmon resonance that provides such strong signal as AuNPs, being black offers a stronger contrast to the white background of the nitrocellulose. In addition, they are cheaper to produce, less prone to aggregation and are easy to functionalise.
- Dye loaded Latex beads: Another alternative to AuNPs are dye-loaded latex beads, as they are cheap and can be purchased in a variety of different colours. [2,95] Additionally, they are less sensitive to chemical and physical damage. [96] However, they are more suitable for qualitative assays and clinical screening, since their limit of detection tends to be higher in comparison to AuNPs. [97]

Fluorescent reading. The use of fluorescent labels is generally recommended for the detection of low target concentrations and/or application requiring quantitative results. However, this comes with a higher cost and the need for an external reader. Examples of suitable fluorescent nanoparticles are:

- Quantum Dots: In fluorescence based LFAs, quantum dots (QDs) are typically the most used labels.[98] When excited with UV light, they provide strong photoluminescence, whose emission peak can be tuned by changing the elemental composition and size of the QD. This also makes them ideal for multiplexed detection.[99–102] In addition, they can be easily conjugated to bioreceptors[103–106] and provide higher stability and resistance to photo-bleaching than organic dyes. [107] Nonetheless, their elemental composition may be toxic, their cost is generally higher than AuNPs and they require an UV-lamp for excitation. [98,108–110]

- Upconverting nanoparticles: Since the early 2000s, upconverting nanoparticles (UCNPs) have been applied as labels in LFAs.[111–113] Their excitation wavelengths, which is in the near infrared range, do not produce auto-fluorescence of the membrane (which occurs when illuminated with UV light) and their strong emission in the visible region produces more sensitive LFAs than those obtained using QDs.[114] However, the need for an expensive and bulky near infrared laser is not appropriate for many PoC applications, thus limiting the integration of UCNPs in LFAs. [114–117]
- Liposomes: In an effort to increase the sensitivity of LFAs, the possibility to encapsulate fluorescent dyes into liposomes has also been explored. Besides carrying a high number of fluorescent dyes with the capability of producing a strong fluorescence signal, the possibility to tailor the lipid bilayer composition allows for the ease of functionalization of the liposome surface with bioreceptors. [118–121] However, the two main limitations faced with liposomes as labels in LFA are the complex synthesis techniques and poor stability.

Non-optical readings. LFAs with non-optical readout are also possible and their use is comparable to that of fluorescence LFAs.[122,123] In fact, lower detection limits and more quantitative results can be achieved in comparison with fluorescence-based LFAs, but they require specific readers. Examples of nanoparticles suitable magnetic and thermal readings are:

- Magnetic nanoparticles: Magnetic nanoparticles (MNPs) are versatile labels for LFAs, since they provide both an optical and a magnetic signal. Their dark colour allows for their use as classical optical labels and their magnetic field can be harnessed for easier functionalization (e.g. removing unbound bioreceptors without the need of centrifugation), for sample pretreatment (MNPs could be incubated with the sample and separated prior their incorporation in LFA to remove any contaminant)[124] and to provide a sensitive read out. In fact, while optical readouts rely mostly on the labels close to the surface of the membrane, measuring the magnetic field allows for the use of all the labels accumulated on the test line. [71,125–132]
- AuNPs for thermal reading: There are few reports about the implementation of thermal methods on LFAs, which are based on the thermal radiation emitted by AuNPs after being excited by a laser. This strategy can be exploited in order to achieve lower limits of detection where colorimetric measurements cannot reach. Moreover, thermal contrast readings are highly reproducible over time with the same LFA strip. The main limitations of thermal contrast techniques are the need for both a laser and an infrared camera. Nonetheless, recent efforts have been dedicated to the improvement of the portability of such setups.[133,134]
- Nanoparticles for electrochemical readings: The use of an electrochemical output in an LFA can provide a variety of amplification strategies and a quantitative output.[135–137] Although there are just few examples of this type of readout (especially those employing nanoparticles as electrochemical labels) in LFAs we believe they will be more frequent during the next years.[135,138] In our opinion, the reasons behind their limited spread so far is the increased complexity in the fabrication of LFAs. In fact, although there is a variety of techniques that can be used (screen-printing, inkjet-printing, writing, pressing)[139–141] for the modification of membranes with electroactive materials, they still imply extra steps and reagents during the fabrication process. In addition to the required dedicated reader.

Table 3: Common labels used in LFA and their advantages and disadvantages.

LABEL	ADVANATGAGES	DISADVANTAGES
AuNPs	• Qualitative naked-eye detection • Well-known conjugations • Strong signal	• Quantitative detection requires extra hardware • Relative expensive if commercially bought
CNPs/CNTs	• Qualitative naked-eye detection • More stable • Cheaper than AuNPs • High signal-to-noise ratio	• Quantitative detection requires extra hardware • Unspecific adsorption • Weaker signal than AuNPs
Latex beads	• Qualitative naked-eye detection • Resistant to chemical and physical damage • Cheaper than AuNPs • Multiple colours available	• Quantitative detection requires extra hardware • Less sensitive • Weaker signal than AuNPs
QDs	• Strong fluorescent signal • Multiple colours available	• Requires extra hardware (UV-light and reader) • Higher toxicity
UCNPs	• Strong fluorescent signal • No UV source necessary • Multiple colours available	• Requires extra hardware (NIR laser) • More expensive than QDs (they are made of rare materials)
Liposomes	• Loading of multiple labels • Easy conjugation	• Require extra hardware depending on the loaded label • Delicate to pH and ionic strength
MNPs	• Dual magnetic/colorimetric signal • High signal to noise ratio • Very sensitive • Possibility to incorporate sample pre-treatment and analyte pre-concentration	• Require non-optical reader for magnetic measurements
AuNPs for thermal reading	• Dual thermic/colorimetric signal • High signal to noise ratio • Very sensitive	• Require expensive non-optical reader and a laser

Sample Pad

The sample pad represents the portion of an LFA where the sample is loaded at the beginning of the test. This component has two key functions: (1) assuring an even flow and (2) the standardization of the buffer

conditions of the sample. The material and design chosen for the sample pad can have a major influence on the overall system.[18–20,142]

Characteristics of sample pads. The geometry and characteristics of the sample pad are important for the control of the sample flow and for the design of the final product (it will affect the shape of the housing cassette). In particular, the key parameters are:

- Bed volume: The bed volume, or void/dead volume, refers to the volume of air contained within the sample pad. This parameter can be used to calculate the total amount of liquid that is required to wet the pad. It controls the volume of sample that flows to the rest of the LFA and is directly proportional to the thickness, porosity, and overall dimensions of the pad:

  Bed Volume = Total Volume of the pad * Porosity (%)

  For example, for a pad measuring 5 cm long, 0.5 cm wide, and 0.015 cm thick, with a porosity of 70%, the bed volume would be: 5 cm * 0.5 cm * 0.015 cm * 0.7 = 0.02625 cm^3 = 26.25 µL. We recommend for micron-size analytes (i.e. cells, bacteria), sample pads with high bed volumes (for example woven meshes) must be used to ensure the analyte is not trapped and retained within the sample pad. Instead for analytes at the nanoscale (i.e. proteins, toxins, nucleic acid), a commitment must be set between speed and sensitivity.

- Thickness: The thickness of the pad not only influences the bed volume but also affects the consistency of the flow. For example, a thicker sample pad provides higher buffering capabilities and therefore a slower and more stable flow. A slower flow generally produces a higher sensitivity of the LFA by increasing the probability for the successful biorecognition of the target by the labelled bioreceptor. A thick pad may also be compressed by the housing cassette, leading to a decrease in the amount of sample absorbed and a reduced flow speed. On the other hand, thin sample pads require less sample volume, but have lower buffering and faster flow speeds, which may negatively affect the wetting of the following pads and decrease the sensitivity of the test. We recommend ….
- Absence of extractable material: During the fabrication of the sample pad, manufacturers may include chemicals (referred as extractable material) to confer particular properties to the material. For the design of an LFA it is important to verify that there is no release of unwanted chemicals that may affect the flow or the biorecognition of the target.
- Particle Retention Rating: The particle retention rating refers to the particles size that the sample pad is able to remove, allowing a proper filtration of the sample. If the chosen sample pad does not provide the desired particle retention rating, it is possible to incorporate an extra pad, for example blood filtration membranes.

Types of sample pads. There are two main types materials used in commercially available sample pads: cellulose fibres and woven meshes. Sample pads made of cellulose fibres tend to be thicker (≥ 250 µm) and cheaper, but are also weaker for handling, especially when they are wet (making the fabrication more delicate). They typically have bigger bed volumes (≥ 25 µL/cm^3) and have higher tolerance towards the chemicals present in the sample pad buffer. Sample pads made of woven meshes, such as glass fibres, have good tensile strength and enable even distribution of the sample over the conjugate pad. In addition,

woven meshes can also act like a filter for removing particulates from the sample (for example Whatman LF1 and GF/DVA made of glass fibre,[143] Whatman Fusion 5[144] made of a PVA-coated glass fibre, and the Pall Vivid Plasma Separation membrane[145] made of asymmetric polysulfone), avoiding the blockage of further pads in the remaining LFA, and are able to retain minimal amounts of sample, thanks to their low bed volumes (≤ 2 μL/cm^3). On the other hand, they tend to be more expensive, are sometimes more difficult to cut than cellulose fibres and can be affected by chemicals used during the fabrication.

Sample pad buffer and preparation. In addition to filtering out impurities, the sample pad corrects the sample composition by adding regents that confer the appropriate pH, ionic strength, viscosity and blocking capabilities. In order to achieve this, the sample pad is generally soaked in a dedicated buffer and further dried, prior its application in the LFA strip. Looking at the sample pad buffer composition, we can find four main components:

- Buffering agents: The type and concentration of buffering agents will determine the pH and ionic strength of the solution for the whole duration of the assay. This affects not only the reproducibility of the assay (by creating the same conditions for different samples), but also the sensitivity and specificity of the test itself (pH and ionic strength can affect the interaction between the receptor and target as well as nonspecific binding, this should also be considered for other buffers used in the LFA, such as the running or washing buffers). The most used buffers are: phosphate (pH range between 5.8 and 8),[146,147] TRIS (pH range between 7.5 and 9),[148,149] HEPES (pH range between 6.8 and 8.2)[150] and borate (pH range between 8 and 10).[151] Their concentration depends on the specific application, but it generally varies between 10 and 100 mM, where higher concentrations are used for more complex and variable samples.
- Detergents: The use of detergents in the sample pad buffer has two main functions: (1) minimizing the nonspecific binding (disrupting weak ionic and hydrophobic bonds) and (2) facilitating the flow of the detection labels along the different pads. Common detergents used in the sample pad are SDS (concentration ranging between 0.05 - 0.5% (wt/vol)), Tween® 20 (concentration ranging between 0.01 and 0.1% (vol/vol)) and Triton (concentration ranging between 0.05%-1% (vol/vol)).
- Blocking agents: Together with detergents, the use of blocking agents in the sample pad buffer can minimize the formation of nonspecific bonds, making the LFA more specific. Furthermore, by adding the blocking agent onto the sample pad, blocking of the membrane may not be required, making the overall fabrication easier and faster. The most common used blocking agents are BSA (concentration ranging between 0.01 and 0.1% (wt/vol)), milk (concentration ranging between 0.01 and 0.1% (vol/vol)) and casein (concentration ranging between 0.1 and 2% (wt/vol)). Chaotropic agents, such as polyvinyl alcohol (concentration ranging between 0.1 and 1% (vol/vol)), polyvinylpyrrolidone (concentration ranging between 0.3 and 1% (vol/vol)) and polyethylene glycol (concentration < 0.5% (vol/vol)), are sometimes also added into the sample pad buffer to prevent non-specific interactions, however this strategy is less commonly used.[152]
- Preservatives: Finally, preservatives such as sodium azide (concentration ranging between 0.01-0.05% (wt/vol)) could be used in commercial tests to avoid microbial contamination on the LFA strip.

Generally, the drying of the sample pad follows a less delicate procedure than that for the drying of the conjugate pad or the membrane. This is mainly due to the absence of delicate reagents, such as antibodies and nanoparticles, in the sample pad. The most common drying method is placing the sample pads in an

oven at 37 °C, but this can be increased to 45-60 °C. Alternatively, the sample pads can be dried by vacuum drying.

Conjugate pad

The conjugate pad is the second pad to be encountered by the sample encounters. It has three main functions: (1) preserving the dried conjugated nanoparticles for detection, (2) releasing them upon wetting by the sample and (3) providing the first interaction between the labelled bioreceptor and the target. Given its key role in the LFA, preparation of the conjugate pad is one of the most critical stages during fabrication of the device. Overall the conjugate pad should provide low nonspecific binding (to avoid the retention of the nanoparticles or the target into the conjugate pad), a consistent flow and bed volume (achieving an homogenous and reproducible flow along the width of the membrane is essential to obtain reproducible results), and mechanical strength without extractable material (the conjugate pad must resist the fabrication process and the housing without releasing any material that can block the membrane or interfere with the signal generation). [18–20,142]

Types and characteristics of conjugate pads. The most used material for the conjugate pad is glass fibre; although cellulose and polyester can also be used. The choice of the conjugate pad material should take into account several factors such as thickness, bed volume and resistance to nonspecific binding. In particular cellulose pads provide the higher thickness (300-1000 µm), followed by glass fibre (100-500 µm) and polyesters (100-300 µm). As mentioned for the sample pad, a thicker material generally means a higher bed volume (assuming similar pore size). This in turn allows for the storage of a higher amount of nanoparticles for detection, a slower flow, and a higher sensitivity. At the same time, it is also related to a weaker mechanical strength of the material when it is wet.

Conjugate pad buffer and preparation. The conjugate pad buffer functions to maximize the stability of the particles and to completely release them upon re-wetting by the sample. In the conjugate pad buffer composition, we find two main components (besides the particles):

- The buffering agents: Given that most nanoparticles used for detection in LFAs are colloidal suspensions, their stability is generally affected by the ionic strength of the solution. Considering that the nanoparticles will undergo a drying process during the fabrication of the LFA (which produces a temporary increase in the salt concentration), it is recommended to start with a low ionic strength. For example, one of the most common buffers used for this purpose is borate buffer at 2-5 mM. It is important to note that, during the assay, it is the sample pad buffer that has the major role on the buffering of the overall test.
- Stabilizing and re-solubilisation reagents: The key components of the conjugate pad buffer are sugars, in particular sucrose and trehalose. They have two main functions: preserving the native conformation of dehydrated proteins (the hydroxyl groups of sugar molecules replace the water around the protein upon drying) and their quick re-solubilisation upon wetting.[153] Typically, they are used at concentrations ranging from 1 to 10% in volume.[154]

Once a suitable buffer has been chosen, the bioreceptor-nanoparticle conjugate can be loaded into the membrane by either air jet dispensing or by immersion process. Air jet dispensing is the most reliable non-

contact method to deliver the particles, as it provides quantitative coverage all over the membrane. The immersion method, on the other hand, is used when the air jet method is not possible, however, the major drawback is that the coverage is not uniform and may lead to high sensor to sensor variability.[155]

The drying process is crucial to maintain the stability of the dried bioreceptor-nanoparticle conjugate and determines the release efficiency from the membrane. For example, failing to completely dry the conjugate pad may generate a syrup like solution unable to run through the membrane. There are two ways to dry the conjugate pad upon loading: hot air and vacuum drying.[19] For mass production of LFAs, hot air is the most convenient method (given its ability to process a high quantity of conjugate pad and lower cost) and is typically fixed at 37 °C to not affect the stability of the bioreceptors.[34,156] In smaller laboratories or when mass production is not required, vacuum drying may be the preferred method as it is not a heat-induced drying process and therefore does not affect the stability of the bioreceptors (although it requires more expensive and delicate equipment).

Membrane

The membrane (also called detection pad) is the part of the LFA strip where the signal is generated. The key characteristics of the membrane are that it should (1) facilitates a homogenous flow, (2) provide a solid functionalization for capturing the bioreceptor and (3) show low nonspecific binding.

Types and characteristics of the membrane: Commercial membranes are generally defined by their capillary flow time, which is the time required for the sample front to cover the membrane length (generally 4 cm), and is generally expressed as sec/4cm. A few manufacturers classify their membranes based on their pore sizes, and, in that case, the conversion of pore size to capillary flow time is roughly: 8 µm = 135 s and 6 µm = 180 s. In general, the higher the capillary flow time, the slower the flow speed. This parameter is crucial not only for the overall duration of the assay, but it has a fundamental role in defining the sensitivity and specificity of the LFA. High capillary flow times allow for a longer interaction time between the target molecule and the bioreceptor, thus increasing the sensitivity of the test.[157] At the same time, high capillary flow times also increase the chance for nonspecific binding to happen. Therefore, it is crucial to evaluate the membranes, with particular attention to the different capillary flow times, during the development of an LFA.

The membrane material defines the type of interactions that govern the functionalization of the membrane with the capture bioreceptors (both for the test and control lines). Nitrocellulose is the most commonly used membrane due to its low price, its strong binding to proteins (including antibodies, the most used type of bioreceptors) and its tuneable wicking properties (obtaining different capillary flow times and the possibility to change surfactant content).[158] Regarding the other types of membranes polyvinylidene fluoride (PVDF) is mainly used for water filtration and western blot thanks to its broad solvent compatibility, low background and superior staining capabilities.[159] Similarly, nylon is also mainly used for filtering aqueous and organic solutions thanks to being strong, flexible, hydrophilic and solvent-resistant.[160,161] Finally, polyethersulfone (PES) thanks to its hydrophilicity and extremely low protein binding, is generally used to remove micro/nanoparticles, bacteria, and fungi from the sample.[162]

Immobilization buffer. Overall, membrane functionalization with the capture bioreceptor occurs *via* non-covalent binding (for example, for nitrocellulose, there are mostly electrostatic and hydrophobic

interactions),[163] and as such, it is important that the immobilization buffer is optimized accordingly. In this respect the immobilization buffer has to maximize the capture bioreceptor adsorption on the membrane, maintain the capture bioreceptor reactivity and not alter the flow properties of the membrane. Looking at its composition in more details, we find four main components:

- The buffering agents: The ideal buffer should maintain the stability of the capture bioreceptor and promote its binding to the membrane. For this reason, buffers with low ionic strength (10 mM) and a pH between +1 and -1 unit of the isoelectric point of the capture bioreceptor are preferred. Buffer compositions with high salt concentrations may screen the electrostatic interactions between the membrane and the capture bioreceptor. Additionally, slightly varying the pH from the isoelectric point of the bioreceptor assures for proper solubility without affecting its tertiary structure. The two most common buffers used for this purpose are ammonium acetate and phosphate buffers.
- Stabilizers: As mentioned for the conjugate pad buffer, the presence of sugars can help stabilizing the capture bioreceptor once the membrane is dried. For this reason, low concentrations of lactose (0.1% w/v) or trehalose (1% w/v) can be included and their concentration should be carefully optimized in order to avoid losing bioreceptor activity upon re-solubilization.
- Alcohols: Alcohols are also included in the immobilization buffer due to their ability to reduce the solution's surface tension, viscosity and static repulsion, enable faster drying times and improve the binding of the capture bioreceptor to the membrane. The most commonly used are methanol, ethanol, isopropanol at concentrations between 1% to 10% (v/v).

Membrane Striping. The immobilization buffer composition and the membrane material define the type and efficiency of membrane functionalization with the bioreceptor. The striping strategy itself (i.e. the deposition of the bioreceptor into the membrane) is crucial to define the width of the test and control lines. As this is a fundamental aspect with respect to the reproducibility of the LFA, the striping should be performed using automated dispensers, allowing for precise control over the flow rate and the speed (so the deposition amount of bioreceptor), as well as the positioning of the lines onto the membrane. There are two types of automated dispensers: contact and non-contact. Although both types provide similar results, different precautions must be taken for each instrument. For contact dispensers, it is important to verify that the dragging of the nozzle does not produce indentations, or grooves, on the membrane; whilst for the non-contact method, the dispensing height should be optimized for membranes of different thicknesses.

Looking in more detail at the striping process we find four main parameters:

- Bioreceptor concentration: The capture bioreceptor concentration will determine the maximum signal achievable by the LFA. Intuitively, the more concentrated the bioreceptors on the test line, the higher the density of labelled nanoparticles is that can be achieved. In the case of immune sandwich assays (see Box 2), it is generally recommended to use an antibody concentration of at least 1 mg/mL in the dispensing solution (see Box 1 Step xx). For competitive assays (see Box 2), where the presence of target (often, but not limited to, small molecules presenting a single binding site – atoms, toxins, pesticides, peptides) decreases the signal, the capture bioreceptor concentration should be around 0.1 mg/mL up to 1 mg/mL. In this case (or if the capture bioreceptor is particularly expensive, or the use of a highly concentrated solutions is not feasible) it is often necessary to compensate the low bioreceptor concentration with a complementary

protein, such as BSA, in order to raise the overall protein concentration in solution. This precaution is necessary to maintain the structure and functionality of the bioreceptor over time, as a low concentrations can induce protein denaturing and loss of binding activity.

- Flow rate and speed: The dispensing flow rate defines how much liquid is dispensed over time (i.e. how much bioreceptors-containing solution is dispensed), and is generally defined in µL/cm. The higher the flow rate, the large the dispensed volume (for a fixed area), and the wider the striped line will be. The speed defines the distance travelled by the nozzle over time, and is generally expressed in mm/sec. So, for a fixed flow rate, the slower the speed the higher the volume of bioreceptor-containing solution that is dispensed. During the fabrication of an LFA, the developer must optimize and balance these two values to obtain reproducible and well defined lines. Generally, it is preferred to obtain narrower lines since this allows the use higher bioreceptor concentrations and to be cost effective. For antibodies and assuming the use of a nitrocellulose membrane with average capillary flow time (120 sec/4cm), the starting parameters are typically 1 µL/cm for the flow rate and 20 mm/sec for the speed (see Box 1 Step xx).
- Position and size: Besides affecting the geometry of the housing cassette (in particular the position of the reading window), the position of the test line also contributes to the sensitivity of the LFA. The further it is placed from the conjugate pad, the more time is allowed for the labelled detection bioreceptors and the target to interact, thus increasing the number of possible immune-complexes to be captured onto the test line.[164] The only requirement for the control line is that it must be positioned after the test line, since it should confirm successful flow of the sample front until the end of the strip. Usually, the test line is dispensed 12-13 mm from the origin of a 60 mm strip, while the control line is 4 mm behind the test line. In case of a multiplexed sensor, several test lines can be dispensed onto the same membrane, but there should always be at least a 2 mm spacing between them. For a deeper understanding on the best test line position we refer the reader to the work from Ragavendar et al.[165]
- Drying: Similar to the conjugate pad, the presence of proteins (including antibodies) onto the membrane requires delicate drying, as high temperatures may denature the bioreceptors. The general conditions adopted are for 2 hr at 37 °C (see Box 1 Step xx).

Blocking conditions. Although commercial membranes can generally directly be striped with the capture bioreceptors, in some cases it is necessary to further treat them with a blocking agent. However, generally speaking, blocking of the membrane should not be performed unless it is strictly necessary, i.e. when the blocking agents added to the sample or conjugate pad are not enough. Although blocking of the membrane decreases nonspecific signals, from a fabrication point of view, this step is quite cumbersome, requiring additional reagents, incubation and drying steps. and potentially affecting the flow of the sample. Consequently, this may affect the reproducibility and the overall analytical performance of the LFA. However, if blocking the membrane is required, it should always be performed after the striping and drying of the capture bioreceptors, otherwise the blocking agent hinders the functionalisation of the membrane. Common blocking agents are: 1-2% w/v BSA, 1-2% w/v IgG, 0.1-0.5% w/v gelatine, 1-2% w/v casein, 0.5-1% w/v PVP (8-10 kDa), and 0.1-1% w/v PVA (8 – 10 kDa). The choice of the blocking agent depends on the type of membrane, the sample and the capture bioreceptor. Generally, the blocking agent should be smaller than the capture bioreceptor, so not to induce steric hindrance that may affect its binding to the target analyte. PBS (0.01 M, pH 7.2-7.4) is the most commonly used blocking buffer. For blocking, the membrane is completely immersed in the blocking solution for 5-10 min and immediately

washed (at least twice) with a weak buffer (e.g. 5-10 mM PBS, pH 7.2-7.4 with 0.005%-0.01 w/v SDS) in order to remove excess blocking reagents. Finally, the membrane is dried using the same conditions used after striping.

Absorbent pad

The last pad in an LFA is the absorbent pad and its role is to control the volume of sample that a strip can take. In the absence of the absorbent pad, once the liquid reaches the end of the membrane, the flow stops and the liquid evaporates homogenously along the strip. This means that all the labels that did not reach the last part of the detection pad can accumulate on the strip and can increase the background noise.. On contrary, the presence of an absorbent pad assures that all the labels reach the end of the strip. A typical absorbent pad is … The absorbent pad does not require any special handling during LFA fabrication, and although its use is generally recommended, considerations such as its cost and its implementation in the LFA geometry (its incorporation requiring a housing cassette that is able to accommodate it) should be evaluated by the developer.[18–20,142]

BOX 2: Types of lateral flow assays

LFAs generally use one of the following two main sensing strategies: competitive or sandwich (non-competitive) assays. Their successful use in LFAs requires the developer to consider important factors during the sensor design, fabrication and optimization.

Competitive assays. There are two main types of competitive formats: In the first type, the target in the sample and a labelled-target (alternatively a molecule with less affinity for the bioreceptor than the target itself) in the strip compete for the capture bioreceptor on the test line,[166,9]. In the second type the target in the sample and the target on the test line compete for the labelled bioreceptor.[150,167,168] A key characteristic of competitive assays is that a higher target concentration results in a lower signal. Competitive assays are generally used for small target molecules that cannot be efficiently recognized by more than one bioreceptor (such as drugs and toxins), but they could also be adapted for big analytes. Since competitive assays are not affected by the hook effect[169] (the detection and capture bioreceptors have all their binding sites occupied by single targets, preventing the sandwich formation. This would show a low signal which may be mis-interpreted as absence or low target concentration), they are particularly useful for the detection of targets with extremely high concentrations. It is important for the developer of a competitive assay to verify that the bioreceptor can recognize the competitor molecule even after it is labelled or adsorbed on the membrane (the use of a spacer to anchor the competitor to the membrane or label may expose it and favour its recognition by the bioreceptor).

Sandwich (non-competitive) assays. A sandwich (non-competitive) assay is probably the most used strategy for the detection of mid- and big-size analytes (such as proteins, antibodies, bacteria, cells) in LFAs. It functions by capturing the target molecule between a capture bioreceptor and a (nanoparticle-)labelled detection bioreceptor, producing a signal that increases proportionally with the amount of target in the sample.[34,170,171] One key aspect for the development of this type of assays is the requirement of two bioreceptors that bind different portions of the target. For example, two different monoclonal antibodies or a labelled monoclonal antibody and a capture polyclonal antibody are the most secure ways to ensure

the sandwich formation. In the case of particularly big targets, such as bacteria or cells, where the same antigen is repeated many times in the same target, the use of the same antibody (better if polyclonal) would also be a feasible option. As mentioned in the previous paragraph, sandwich assays are subjected to possible hook effects in the case of extremely high target concentrations[169] and they are more prone to false positive results than competitive assays.

- **End of BOX 2 -**

Assembly of the LFA

The assembly of the different pads and membrane on the strip is the final step prior to storing. Although sticking the pads and membrane on the supportive laminated card might seem trivial, it is actually an important step that will ensure the homogenous flow of the sample along the strip and the batch-to-batch reproducibility. The developer should assure contact between the sequential pads and their complete wettability to move all the reagents along the strip. Most laminated cards come with release liners that helps to position the different pads always at the same distance. This is convenient if the sizes of the different pads and the relative release liner are the same. There are also dedicated laminators commercially available, which would improve the consistency in the fabrication process. A detailed step-by-step procedure for assembling a standard sandwich LFA is described in Steps xx-yy of Box 1.

Finally the LF strip may be inserted in the housing cassette. It is possible to purchase pre-made housing cassettes from most of the pads/membrane providers, although in this case the width of the LF strip must be adapted to the width of the cassette (this assuming that the length and height are compatible given that they are produced by the same company). Alternatively LFAs with non-common geometries (for example using materials from different providers, or having dimensions non compatible with the cassettes) or requiring special functionalities (i.e. the application of the sample and a running buffer at different positions of the LF strip or requiring the docking of the cassette to a dedicated reader) may require the design of a dedicated cassette. In this case we recommend to test different cassette geometries using a 3D printer, which provides the freedom to vary several parameters at a low cost. Once the developer finds the final design, the cassette can be mass produced by specialized companies. Overall, once designing a housing cassette, the developer should consider three main aspects. First, the cassette should keep the LF strip in place (i.e. preventing it from flipping over or moving inside the cassette), without compressing or damaging the pads/membrane (this would affect the flow of the sample). Second, the cassette should have the hole to apply the sample positioned in the bottom part of the sample pad, so to assure the flow of the majority of the sample towards the membrane. Third, the reading window of the cassette must allow the user or the reader to easily visualize both the control and the test line(s).

Single membrane LFA. So far, we have described the materials and features of the different membranes in a typical LFA strip. Alternatively, there is one membrane marketed by GE Healthcare that simplifies the manufacturing process, as well as minimize the costs: FUSION 5.[144] This unique glass fibre-based membrane with proprietary technology, facilitates the production of the LF strips, because it can adopt any of the roles of the different pads and membrane needed for a single LFA in just one single device. In general, FUSION 5 overcomes several problems when manufacturing LF strips such compatibility and

contact issues between the membranes. When working with complex samples such as blood, it can act as a separator, obtaining a serum with a similar yield compared to conventional centrifugation procedures within 1 min following application of the blood drop. It also can act as conjugate pad due to its composition and permanent negative charge, which enables the release of the conjugate to the detection pad. As detection membrane, FUSION 5 does not need to be blocked in order to avoid unspecific adsorption. Two major drawbacks of the FUSION 5 are the need for carrier beads to load the bioreceptors of the test and control lines, and its low capillary flow time (38 sec/4 cm, so a relatively fast flow compared with some nitrocellulose membranes), which may reduce the sensitivity of the test in comparison with other types of membranes. As of March 2020 we did not find any scientific manuscript describing the development of a LFA using solely Fusion5, instead we found references using Fusion5 as part of LF strips or more complex structures.[172–179]

Non-conventional materials. Other interesting low cost alternatives to the conventional LFAs membranes, such as cotton threads, cellulose and glass capillaries have been described in the last years. For instance, cotton threads are attractive for LFA development thanks to their flexibility and wicking properties, which allow the control of smaller sample volumes.[180–183] Cellulose fibers (i.e. paper) have been extensively used for microfluidic paper analytical devices and some for LFA, because of their ubiquitous availability, biodegradability, and patterning capabilities.[184] Although, they are a convenient option for diagnostics performed in low-resource settings, their major drawback is the need of a chemical modification to guarantee the immobilization of bioreceptors. Finally, glass capillary tubes can also be used to take advantage of their transparency, smooth surface and chemical inertness. These characteristics make them especially useful to minimize the non-specific binding by contaminants present in the sample matrix.[185,186]

Storage

To ensure that the analytical performance of the LF strip remains constant over time and within different applications and environmental conditions, the storage conditions of LF strips play a vital role. There are three main components that can affect the stability of an LF strip: the bioreceptors, the membrane and the label.

In most cases, the bioreceptor will be the limiting factor for the stability of the strips. Regarding bioreceptor stability we need to distinguish between stable DNA molecules, which also includes aptamers, from more delicate proteins, such as antibodies. If properly dried, DNA molecules can withstand a wide range of temperatures and storage conditions. To maximize the shelf-life of proteins, dry and cool conditions are required (4 °C inside sealed bags with desiccants).[187] If other proteins are included on the strips (e.g. BSA as blocking agent in nitrocellulose) the same storage considerations should be taken into account. Particularly delicate bioreceptors may also require the addition of stabilizers (such as sugars, proteins, agars and gelatines[188]) to the relative buffers (for examples whey would be striped together with the capture bioreceptor or included in the conjugate buffer). Also, it should be considered that some surfactants used on LF strips to improve the flow across the paper or the release of the label particles may reduce the life-time of the proteins. Secondly, the membrane itself has an expiration date. Deteriorated nitrocellulose shows a yellowish colour, produces a sour smell and might generate faint lines after performing the assay. To maximize its life-time, nitrocellulose should be stored in dry conditions and protected from direct light (i.e. in a zipper bag with dessicants). Finally, the stability of the detection label

often depends on the nature of the label itself: properly modified (e.g. coated with a blocking protein during conjugation) and dried particles in glass fibre should not have any stability issues, whilst enzyme-based or dye-loaded particles labels may require refrigeration to preserve their activity. LF strips should not be frozen.

Assay evaluation

Qualitative evaluation. When thinking of an LFA, the first thing that comes to mind is the presence or absence of the line in a pregnancy test (Fig. 3A). This intuitive and qualitative analysis done by naked eye is the reason of LFA popularity and it is ideal for the discrimination between the presence or absence of a particular condition, such as pregnancy.

Semi-quantitative evaluation. A different approach for reading an LFA by naked eye relies on the use of barcode style LFAs, where the number and the intensity of multiple test lines facilitate a semi-quantitative reading (Fig. 3B).[189,190] These kind of strips can be of useful in three main scenarios: (1) when the concentrations of two or more analytes needs to be assessed (i.e. the user can observe and compare the signals obtained for the different analytes and quickly verify if any of the analyte has an higher/lower concentration than the other); (2) for the detection of a defined target concentration threshold (a measurable line appears if the concentration of analyte is at least at the threshold value); and (3) to group target concentrations into different categories based on concentration ranges (i.e. ranges of concentrations can be represented by using different lines). The latter scenario is useful for the detection of biomarkers which are of interest just above/below a defined concentration, such as *E. coli* in urine.

Quantitative evaluation. In recent years the development of dedicated readers and smartphones have facilitated the quantitative analysis of LFA (Fig. 3C-D).[191,192] In order to achieve quantification, the fabrication process must provide highly reproducible strips (i.e. the developers and test manufacturers must strictly assure that the LFA production is extremely rigorous, otherwise the test result can greatly differ from different batches of LFA) and an accurate calibration curve to be used as a standard. The use of a dedicated reader is still preferred to obtain accurate measurements, as it minimizes the effect of ambient light on the signal intensity (to obtain a proper quantitative result the measurement should be performed using the same light conditions as the original calibrate, therefore the need of a fully closed box with integrated light sources). Hybrid systems where a smartphone is coupled to a special support also provide excellent performances.[193,194]

Multiplexed evaluation. The detection for several targets at the point of care is highly desirable, for example for drug screening, diagnosis of infectious diseases, monitoring of pesticides. Although the fabrication of LFAs for analysis of multiple target is particularly challenging (i.e. higher cost, longer optimizations, possible cross reactivity between biomolecules, etc.), there are some successful examples of this type of sensors. [3,195–201] In particular for a few number of targets (approximately under 10 different analytes), the read out could look like a barcode assay (where each line represents a specific analyte), instead for a high number of targets (over 10 different analytes), the read out would look like a microarray (where each dot represents a specific analyte). Overall the higher the line/dots to analyse the more challenging would be the naked-eye reading, thus the use of a dedicated reader is recommended.

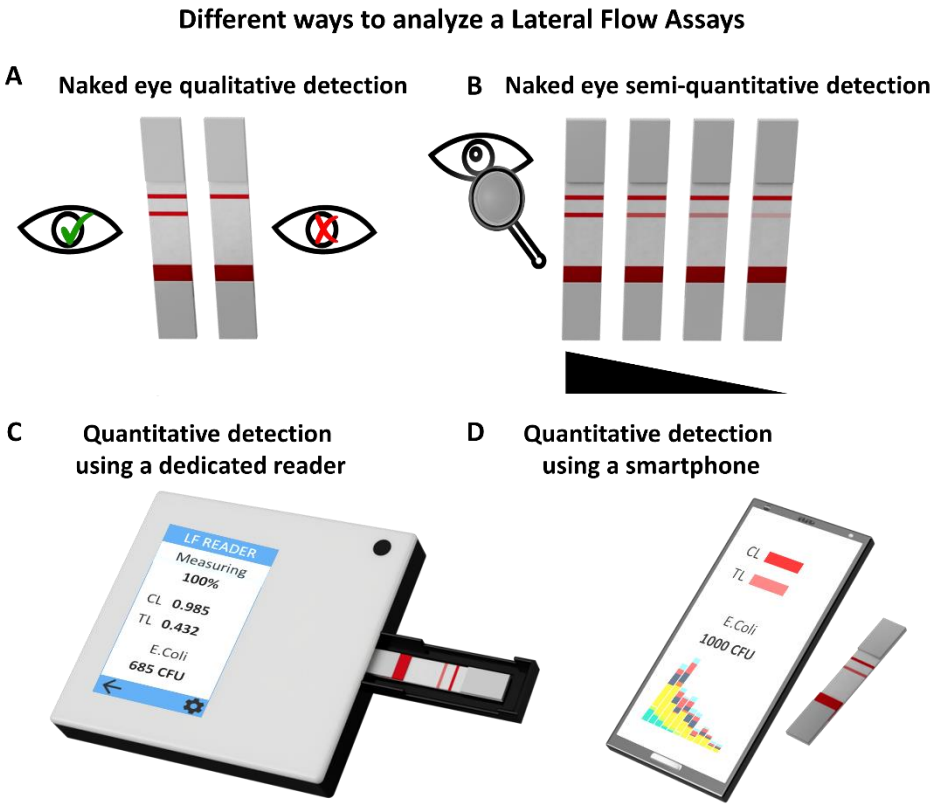

Figure 3: *Different types of read out of LFAs. A) Most LFAs are designed to give a qualitative (yes or no) response ideal for naked eye detection by non-specialized users (for example the pregnancy test). B) Similarly LFAs can also be designed to provide a semi-quantitative results. This type of reading is mostly limited to the fabrication of LFAs, when the developer needs to evaluate how different parameters affect the sensor response. C) The use of dedicated readers can make LFAs fully quantitative devices. Although most readers record an optical response, it is possible to have also magnetic and thermal readers. D) More recently smartphones are also being used as LFA readers (especially when coupled with an external platform to guarantee a constant lighting) or as user interface (receiving the LFA results via WiFi or Bluetooth).*

Iterative LFA development

As described, an LF strip is comprised of a variety of materials and reagents that are carefully interconnected to produce a sensor that aims to be easy-to-use, yet still highly sensitive and specific. The most challenging and complex task during the development stage is tuning the different parameters and components to achieve the best sensing performance. Although there are groups working on computational modelling to facilitate the optimization of LFA,[17,202,203] this approach is not yet widely implemented as it requires modelling and programming skills that are not always present in research groups or the availability of specific software.

More generally, in research and development (R+D) laboratories, the optimization of an LFA relies on an iterative approach of trial and error, as described by Hsieh et al.[16] As schematized in figure 4, the developer

first defines the analytical performance, usability and cost required by the specific application. Then, on this basis, there is the first screening (using standard techniques such as ELISA and SPR) of bioreceptors and analytes obtaining a shortlist of the most promising combinations. The resulting bioreceptors are then conjugated to the nanoparticles and the stability of the resulting conjugates is verified. At this point, developers use the half stick format (made of membrane and absorbent pad) to evaluate the combinations of membranes, blocking agents, running buffers, striping conditions and conjugates. If the original goals are met, developers use the 3/4 stick format (made of conjugate pad, membrane and absorbent pad) to evaluate the optimal preparation of the conjugate pad as well as the effect of the real sample matrix on the assay performance. Finally, there is the incorporation of the sample pad (and for commercial applications also the housing cassette) to evaluate the overall sensing performance. Although this approach for the LFA optimization heavily relies on trial and error, it is still time and cost efficient. It is important to note, as we previously mentioned, that this is an iterative process, meaning that optimization steps might have to be repeated to compensate for changes done further down the optimization procedure.

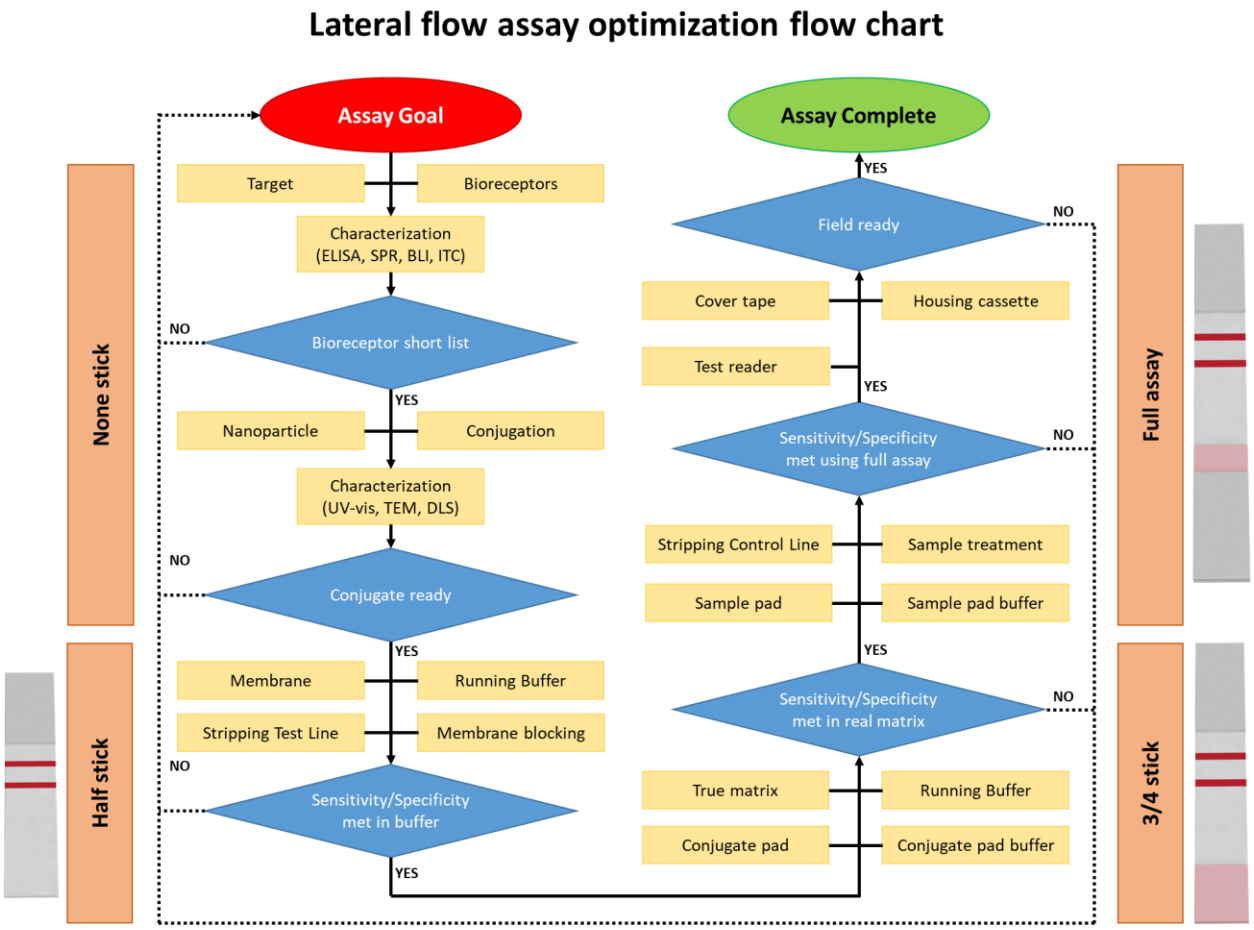

Figure 4: *Ideal optimization route for the fabrication of a LFA. After setting the assay goal the developer should short-list the most promising bioreceptors using gold standard techniques (ELISA, SPR, BLI and ITC). The next step is the optimization of the conjugation to the nanoparticle label and the characterization of the conjugate stability (using UV-vis, TEM, DLS). Subsequently using a half-stick format (just membrane and absorbent pad) the developer can test several combinations of nanoparticles and bioreceptors to find*

the most promising ones. During this stage there is also the first evaluation about the need to block the membrane. Then the developer employs a 3/4 stick format (half stick + conjugate pad) testing the conjugate pad fabrication and the effect of the real sample matrix. During this stage there is the second evaluation of membrane blocking. If the required sensitivity and specificity are met, then there is the final optimization using the full assay format (3/4 stick plus sample pad and housing cassette). During this phase the optimization of the sample pad buffer and eventually sample treatment strategies are evaluated prior using the housing cassette.

Costs, patents and production

The estimation of the cost of an LFA depends on the development phase of that particular test: R+D, large scale manufacturing or in the commercial market. During the R+D phase, most of the costs are attributed to the bioreceptors used in the LFA. Generally, we estimate the production costs of a single LF strip to be less than 1 $ (see Supplementary Table 1 for a detailed example). Despite this low cost, a R+D project for developing a novel LFA could require a lot of financing: labware, consumables, paper, chemical and biological reagents (around 30,000-40,000 $/year); new equipment and maintenance (around 60,000 $); laboratory renting, personnel and over costs (depending on the location, size, employee degree, country, etc.); subcontracting (e.g. materials characterization, regulatory control, legal advice and patentability); taxes and travels (project meetings, congresses and assay demonstrations). This cost will vary depending on the current status of the facilities, the project and the milestones. The main objective of an R+D phase should be to reach a minimum viable product (MVP), that is an LFA that has demonstrated to have enough repeatability (same response reproduced from the test over time), replicability (same response applying the LFA in different environments) and reproducibility (same response when different operators reconstruct the design), and that clearly differentiates and defines positive and negative responses. Then, with a reliable MVP, the next costs will come from proprietary and regulatory expenses.

In this regard, patentability of a relatively old technology such as LFA can be challenging, as for the patent to be accepted there should be a demonstrable and non-obvious novelty. Generally, the patentability can come from one or more of the following points:

- Biomarker – an LFA detecting a new biomarker, whose presence or absence is related to a specific disease or status.
- Bioreceptor – an LFA using a new bioreceptor for a defined application (for example an aptamer sequence).
- Label – an LFA using a new label implying a new detection method, sensing strategy or a signal enhancement. However, nanomaterial size modifications, new dyes or conjugation of enzymes on the surface of the label are often not enough to be considered as novel ideas.
- Sensing strategy – an LFA using a new sensing mechanism that leads to a demonstrated advantage over a standard assay. It may include extra steps, reactions or interactions between the different pads, labels and biomarkers.
- New materials – an LFA using new materials as pads or membrane, instead size modifications on the pads and parameters such as porosity and protein affinity are difficult to protect.
- New Design – an LFA that employs a new functionality or has an identificative trademark.

Patenting processes are long (more than one year in most cases) and the costs are generally high (evaluation and certifying costs), leading also to maintenance costs. The patent cost will also vary depending on the level of protection of the technology (country-wide, continent-wide or world-wide). In addition to the requirements of each country in which the LFA will be used, regulation and approval (see next section) expenses will also depend on the target user and environment. During this phase, the R+D laboratory will still be in charge of producing and providing the product for its evaluation.

After R+D, with the MVP protected and approved for the market, the next phase is the mass production. During the mass production in a factory, the cost per LF strip decreases to about 0.01 $, although this does not include equipment and their maintenance, the employees, insurances, taxes, quality controls and many other indirect costs. Finally, the market cost is the toughest one to estimate. For the sake of comparison, we will use the cost of "Clearblue Rapid Detection Pregnancy Test", which we can purchase for approximately 6 $ per test. Although it could seem that there is a great benefit for the company and the seller, there are also even more indirect costs such as: packaging (cassettes, bags, desiccants etc.) and storage of the product, shipping, marketing, taxes, additional reagents and tools, plus the cost of R+D and clinical validation that need to be taken into account.

Regulation and approval

An LFA for the purpose of PoC, or clinical environments, is considered a medical device and its use is regulated by governmental institutions such as the European Medicines Agency (EMA) in EU or the Food and Drug Administration (FDA) in US. In the EU, medical devices have to undergo a conformity assessment to demonstrate that the legal requirements are met to ensure they are safe. Accredited national authorities are responsible to conduct these conformity assessments that, once fulfilled, award the device the CE ("Conformité Européenne", tr. European Conformity) mark.

Furthermore, LFAs also work as *in vitro* assays, where the device does not affect the organism in which it interacts with. In this case after obtaining the MVP and, in order to obtain the CE mark (or the regional corresponding indicator), the device should be validated as indicated by the relevant institution. Depending on the origin of the samples, an ethical committee may have to evaluate the process.

Future Directions

LFAs have been some of the most popular biosensors for the past decades and we believe they still have a bright future ahead, especially if developers will design them to address the challenges that personalized/precision medicine and in-the-field environmental analysis will present. Regarding clinical purposes, we believe there are two major goals that the next generation of LFAs should tackle: (1) the sensitive and quantitative detection of multiple protein targets at under pM concentrations[3,195–199] (for example using a microarray-type approach)[204] and (2) the integration into a single LFA strip of both a nucleic acid amplification and detection.[205–211] Similarly, for environmental analysis an improvement in the sensitivity and quantitative ability of multiple targets will be required, together with the development of LFAs detecting families of compounds rather than specific molecules. Succeeding in these tasks will require a joint effort between different disciplines, ranging from the selection of bioreceptors showing desirable characteristics for LFAs (fast binding kinetics and stability), to the

development of novel materials showing superior properties (either labels or new membranes, pads, and filters[212]), to the use of programming to optimize the fabrication of LFAs and their quantitative analysis using portable readers.

BOX 1: Example protocol for a lateral flow assay to detect human IgG

This Box describes a detailed step-by-step procedure for a traditional sandwich LFA to detect human IgG. In this example, we use two different polyclonal antibodies as capture and detection bioreceptors, the latter one being coupled to gold nanoparticles for qualitative detection by the naked eye and quantitative camera-based detection. Before assembling the LF strip, we provide a detailed procedure to screen for optimal buffers to reduce aggregation of the AuNP-bioreceptor conjugates. Troubleshooting guidance is provided in Table 4.

Materials

Reagents

- Capture bioreceptor for the test line. In the example shown in this protocol we use an Anti-Human IgG (whole molecule) antibody produced in goat (Sigma-Aldrich, Cat. no. I1886) (RRID:AB_260125).
- Capture bioreceptor for the control line. In the example shown in this protocol we use a Chicken Anti-Goat IgG H&L (Abcam, Cat. No. ab86245) (RRID: AB_1951137). For general recommendations on how to select and validate control line antibodies, see … of the main text.
- Detector bioreceptor for conjugation to AuNPs . In the example shown in this protocol we use an Anti-Human IgG (γ-chain specific) antibody produced in goat (Sigma-Aldrich, Cat. no. B1140) (RRID: AB_258513).
- Target analyte. In the example shown in this protocol we use IgG from human serum (Sigma-Aldrich, Cat. no. I2511) (RRID: AB_1163604). See in the main text for a detailed description of possible target analytes and samples.
- Bovine serum albumin (BSA; Sigma-Aldrich, Cat. no. A3294)
- AuNP solution (Gold nanoparticles 20nm, BBI Solutions, Cat. No. EM.GC20)
- Hydrochloric acid, 37% (w/w) (Sigma-Aldrich, Cat. no. 320331)
  Caution: Hydrochloric acid may be corrosive to metals, causes severe skin burns and eye damage and may cause respiratory irritation. Do not breathe dust/ fume/ gas/ mist/ vapours/ spray. Wear protective gloves/ protective clothing/ eye protection/ face protection. Handle it inside a fume hood.
- Sodium hydroxide (NaOH; Sigma-Aldrich, Cat. no. S8045)
  Caution: Sodium hydroxide may be corrosive to metals and can cause severe skin burns and eye damage. Do not breathe dust/ fume/ gas/ mist/ vapours/ spray. Wear protective gloves/ protective clothing/ eye protection/ face protection. Handle it inside a fume hood.
- Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, Sigma-Aldrich, Cat. No. 71504)
- Sodium phosphate dibasic (Na_2HPO_4, Sigma-Aldrich, Cat. No. 71640)
- Sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$, Sigma-Aldrich, Cat. No. B9876)
  Caution: Sodium tetraborate decahydrate causes serious eye irritation, may damage fertility and may damage the unborn child. Obtain special instructions before use. Do not handle until all

safety precautions have been read and understood. Wear protective gloves/ protective clothing/ eye protection/ face protection.

- Boric acid (H_3BO_3, Sigma-Aldrich, Cat. No. B7901)
  Caution: Boric acid may damage fertility and may damage the unborn child. Obtain special instructions before use. If exposed or concerned get medical advice/ attention.
- Sodium citrate ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$, Sigma-Aldrich, Cat. No. 1613859)
- Sucrose ($C_{12}H_{22}O_{11}$, Sigma-Aldrich, Cat. No. 84097)
- Phosphate buffered saline tablets (Sigma-Aldrich, Cat. No. P4417)
- Tween-20 (Sigma-Aldrich, Cat. No. P1379)
- MilliQ water (produced using Milli-Q system (>18.2MΩ*cm))

Equipment

- Detection pad (mdi, Cat. No. CNPH200).
- Backing card (Kenosha, Cat. No. KN-PS1060.45).
- Sample and absorbent pad (Merck Millipore, Cat. No. CFSP001700).
- Conjugate pad (GE Healthcare, Standard 14 Cat. No. 8133-2250).
- Graduated pipettes (Gilson P2, 20, 200, 1000).
- Tips for graduated pipettes (Fisherbrand, Cat. No. 10177190 and 10677731).
- Polypropylene Graduated Microtubes (Eppendorf, 0030120248).
- Transfer Pipettes (Fisherbrand, Cat. No. 13439108)
- Lateral flow assay reader (Skannex, SkanMulti with OEM software).
- ThermoShaker for microtubes (Biosan, Cat. No. TS-100).
- Centrifuge (Beckman Coulter, Allegra 64R).
- Reagent dispenser (Imagene Technology, IsoFlow reagent dispenser).
- Programmable strip cutter (Kinbio, Cat. No. ZQ2002).
- Oven (P. Selecta, Cat. No. 2001246)
- Laboratory pump (KNF lab, Laboport Cat. No. N938.50KN.18)
- Vacuum Desiccator (Karnell, Cat. No. 550)
- pH meter (Crison, pH meter Basic 20+)
- Analytical balance (Ohaus Discovery, Readability: 0.001 g).
- Milli-Q system (>18.2MΩcm-1) (Millipore)
- Fume hood (Flores Valles)
- Fridge and Freezer (Premium NoFrost)

Software

- ImageJ - Version 1.52u - https://imagej.nih.gov/ij/
- OriginLAb – Version 8 - https://www.originlab.com/
- Excel – Version 2016 - https://products.office.com/en/excel

Reagent Setup

Phosphate Buffer Saline (PBS) (0.01 M; pH 7.4) Dissolve one PBS tablet in 200 mL of MILLIQ water. Store it at room temperature (25 °C) for up to 30 d.

Phosphate Buffer (0.01 M; pH 7.4) Dissolve 0.21 g of Sodium phosphate dibasic in 150 mL of MilliQ water. Dissolve 0.20 g of sodium phosphate monobasic monohydrate in 150 mL of MilliQ water. Add a magnetic stirrer and measure the pH of the sodium phosphate dibasic solution under agitation using a pH meter. Add the sodium phosphate monobasic monohydrate solution dropwise to the sodium phosphate dibasic

solution with a disposable Pasteur pipette until the pH value reaches 7.4. Store it at room temperature for up to 30 d.

Borate Buffer (10 mM; pH 7) Dissolve 0.58 g of sodium tetraborate decahydrate in 150 mL of MilliQ water. Dissolve 0.092 g of boric acid in 150 mL of MilliQ water. Add a magnetic stirrer and measure the pH of the sodium tetraborate decahydrate solution under agitation using a pH meter. Add the boric acid solution dropwise to the sodium tetraborate decahydrate solution with a disposable Pasteur pipette until the pH value reaches 7. Store it at room temperature for up to 30 d.

Borate Buffer (100 mM; pH 9) Dissolve 5.72 g of sodium tetraborate decahydrate in 150 mL of MilliQ water. Dissolve 0.92 g of boric acid in 150 mL of MilliQ water. Add a magnetic stirrer and measure the pH of the sodium tetraborate decahydrate solution under agitation using a pH meter. Add the boric acid solution dropwise to the sodium tetraborate decahydrate solution with a disposable Pasteur pipette until the pH value reaches 9. Store it at room temperature for up to 30 d.

Conjugate pad buffer Prepare a solution of PBS buffer containing 5% (wt/vol) sucrose, 1% (wt/vol) BSA and 0.5% (vol/vol) Tween-20. Store it at 4 °C for up to 7 days. **CRITICAL:** The sucrose and Tween-20 in the conjugate pad buffer are imperative for the release and flow of the conjugated AuNPs solution along the nitrocellulose strip.

Sample pad buffer Prepare a solution of PBS buffer containing 0.5% (wt/vol) BSA and 0.05% (vol/vol) Tween-20. Store it at 4 °C for up to 7 days.

Procedure

The following simple procedure elucidates the optimal conditions to perform the functionalization of AuNPs with the detection bioreceptor. In particular it allows the definition of the optimal pH and the minimum antibody concentration to fully cover the nanoparticles. The procedure relies on the propensity of bare (citrate-covered using the Turkevic synthesis) gold nanoparticle to aggregate in the presence of high salt concentrations.[213] Once the AuNPs are fully covered with the antibody (or with a blocking agent, such as BSA) they remain monodispersed even at high salt concentrations. The aggregation rate can be qualitatively assessed by naked eye: fully aggregated AuNPs induce a colour change of the suspension from red to transparent (due to their precipitation), partially aggregated AuNPs would change to colour of the suspension from red to blue (due to a shift and widening of their plasmonic peak), while monodispersed AuNPs should maintain the original red colour.[214] This can be quantitatively measured using UV-vis spectra, DLS, Z-potential and TEM.

Nanoparticle-bioreceptor Aggregation Test - 1 hour

1. Prepare three solutions of 20 nm diameter AuNPs at pH 7, 8 and 9 (with OD of approx. 0.450 at 520 nm). Set the correct pH with 10 mM borate buffer (for pH 7) and 100 mM borate buffer (for pH 8 and 9).
2. Prepare dilutions of the detector bioreceptor (detector anti-Human IgG in this example) in MilliQ water (200, 175, 150, 125, 100, 80, 60, 40 and 0 µg/ml). Prepare a solution of 10% NaCl (wt/vol) in MilliQ water.

CRITICAL STEP – Although we typically use a 10% NaCl (wt/vol) solution for the gold aggregation test, it is possible to substitute it with a different buffer (e.g. conjugate pad buffer or LFA running buffer) to study their effects on the stability of the conjugates.

3. In a 96 micro-wells plate put 150 µL per well of the AuNP solutions from Step 1. Prepare 2 rows of 9 wells for each AuNP solutions in order to create a duplicate test.
4. To each well, add 10 µl of one of the detector antibody dilution from Step 2 in increasing order of antibody concentration (see figure 5).
   CRITICAL STEP – For easy comparison of the effect of the pH, the wells in each column should have the same antibody concentration.
5. Place the plate on top of the Thermoshaker for 20 minutes at 600 rpm.
6. Add 20 µl of 10% NaCl (wt/vol) solution to each well.
7. Place the plate back on top of the Thermoshaker for 5 minutes at 600 rpm.
8. Measure the absorption spectra (from 400 nm to 600 nm with at least 5 nm between each measurement).
9. From each spectrum evaluate the peak intensity, peak position and spectra shape (see Fig. 5).
   CRITICAL STEP - The maximum absorbance peak of 20 nm-sized AuNPs is at 520 nm. If the AuNPs aggregate in the gold aggregation test, the absorbance peak will shift to the right and the peak becomes wider. The use of the optimum pH and antibody concentration will avoid the aggregation of the AuNPs.

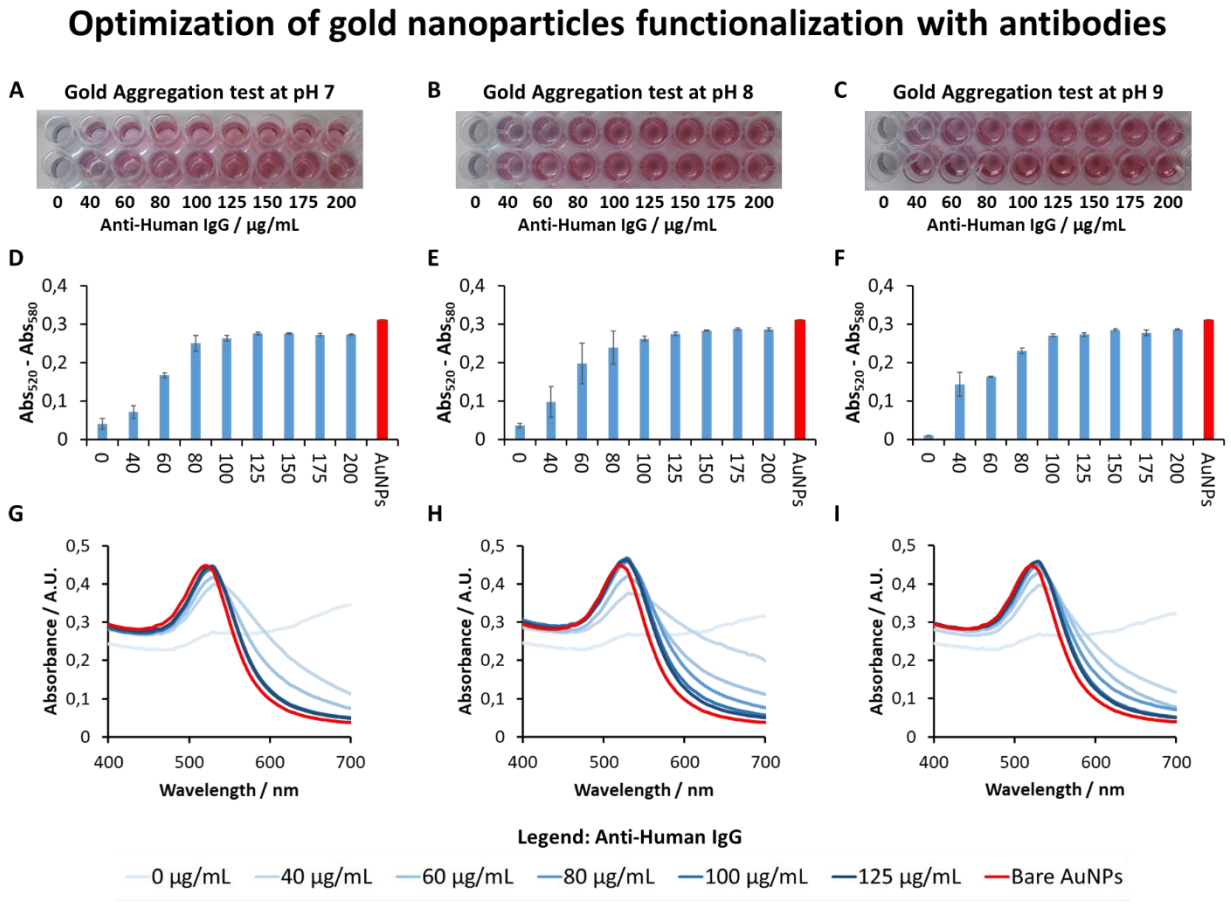

Figure 5 *- Results of the gold aggregation test for 20 nm diameter AuNPs and anti-Human IgG. A-C) Photos of the resulting GAT. Even by naked eye it is clear the colour gradient following the anti-human IgG concentrations. D-F) Bar charts obtained subtracting the OD at 520 nm from the one at 580 nm. This analysis allows to estimate the minimum antibody concentration to give stable conjugates. The higher the calculated value the narrower the peak, indicating more monodispersed AuNPs. G-I) Spectra obtained for the different tested conditions, confirmed the bar chart results. It is important to note that the peak position for stable conjugates will be slightly shifted to higher wavelengths (in this case 530 nm) due to the increased hydrodynamic diameter of conjugates (AuNPs + antibody) compared to the one of bare AuNPs (in this case 520 nm).*

Large-scale nanoparticle conjugation to the detector bioreceptor– 1 hour and 20 minutes

10. Transfer 1.5 mL of the AuNP solution with the optimal pH (as selected in Step 9) in an 2 mL Eppendorf tube.
11. Add the optimised amount of detector antibody in MilliQ water to the AuNP solution.
    CRITICAL STEP - The starting antibody concentration is defined by the gold aggregation test, but it should be further optimized, generally reducing it. For example, in this case we are using a final concentration of 2.5 µg/mL detector anti-Human IgG, which is lower than the amount defined by the gold aggregation test (6.6 µg/mL, assuming the gold aggregation test value of 100 µg/mL) because it provides a lower background signal (Fig. 6).
12. Incubate the antibody-AuNP mixture for 20 minutes at 650 rpm at room temperature using the Thermoshaker.
13. Add 100 µL of 1 mg/mL BSA in MilliQ water
    CRITICAL STEP – BSA has three functions: it covers any surface area not filled in with the antibody, it helps with stability, and it prevents nonspecific adsorption.
14. Incubate the mixture for 20 minutes at 650 rpm at room temperature using the Thermoshaker.
15. Centrifuge the mixture at 14000 rpm (30053 g) for 20 min at 4 ˚C.
    CRITICAL STEP - Centrifugation is performed to remove free antibodies that may interfere with the sensitivity of LFA and multiple centrifugation steps may be required to ensure the complete removal of unbound antibodies. The centrifuge parameters are selected depending on the size of the AuNPs and volume used. During the centrifugation the solution tends to heat and this might affect the stability of the conjugated antibodies. It is therefore recommended to adjust the temperature to 4˚C.
16. Resuspend the antibody-AuNP pellet in 500 µL of the conjugate pad buffer.
    CRITICAL STEP - The dilution factor of the conjugation solution should be carefully selected, as it will affect the dynamic range of the assay. Although a more concentrated solution of AuNPs might provide better detection limits in LFAs, it may affect the flow as well as increase the cost of the sensor.
    PAUSE POINT: The antibody-AuNP conjugate can be stored at 4 ˚C.

Lateral flow strip fabrication – 2 hours and 30 minutes

17. ***Nitrocellulose membrane striping (Steps 17-26):*** Fill the reservoirs (test and control lines pumps)

of the lateral flow dispenser with MilliQ water and dispense 2100 µL MilliQ water (dispense rate of 5 µL/cm and a speed of 50 mm/sec) in order to clean the syringe, tubing and nozzles.
CRITICAL STEP – Every lateral flow dispenser has its specific washing procedure. Consult with the equipment manual of the specific equipment for details.

18. Empty the supply lines and the reservoirs.
19. Prepare a 200 µL solution of 1 mg/ml capture antibody (capture anti-Human IgG in this example) and a 200 µL solution of 1 mg/ml of the control line antibody (anti-goat IgG in this example) in phosphate buffer**.** Add the control line antibody solution in the reservoir corresponding to pump for the control line and the capture antibody in the one corresponding to pump for the test line.
CRITICAL STEP: The volume of the dispensing solution should be enough to strip the required amount of nitrocellulose membranes. Usually 100 µL should be enough for the preparation of a 30-cm nitrocellulose membrane using a dispense rate of 0.5 µL/cm and a speed of 50 mm/sec in Step 22.
20. Prime the pumps and the tubing until a drop flows out of the nozzles.
TROUBLESHOOTING:
21. Place the CN200 nitrocellulose membrane (previously attached to the backing card) on the vacuum table.
CRITICAL STEP: The laminated card side corresponding to the absorbent pad should touch the rear of the table. The position of the nozzles must be manually adjusted in order to determine the test line and control line position in the nitrocellulose membrane.
22. Setup the pump system by adjusting the dispense rate at 0.5 µL/cm and the speed to 50 mm/sec.
23. Press Run to dispense the capture antibody solution from Step 19 onto the test line and the control line antibody solution onto the control line.
CRITICAL STEP: Be sure that the dispensed solutions are homogeneous, as this will affect the assay's results. Discard any membrane with faulty lines, see Figure 6.
24. Fix the antibodies by drying the detection pad at 37 °C for 2 hours in the oven.
TROUBLESHOOTING:
25. (Optional) Block of the membrane with BSA:
    a. Soak the membrane into the blocking buffer (2% BSA, 0.01M PBS pH7.4) for 20 min;
    b. Soak the membrane into the washing buffer (0.05% SDS, 0.005M PBS pH7.4) with a gentle shake for 15 min. Repeat this step at least 2 times;
    CRITICAL STEP: It is crucial to remove the excess of BSA from the membrane otherwise, it may cover the binding site of the capture and control bioreceptors.
    c. Dry the membrane at 37℃ for at least 2 hour.

26. Before turning off the lateral flow dispenser, perform washing procedure with MilliQ water, followed by 0.1 M HCl, 0.1 M NaOH and again MilliQ water. Remove the MilliQ from the tubing by un-priming the system.
CRITICAL STEP: Dispense at least 10 syringe volumes during each washing step (dispense rate of 5 µL/cm and a speed of 50 mm/sec). The pumps should be thoroughly cleaned monthly using a weak detergent (0.05% Sodium C14-16 olefin sulfonato), 10 % (vol/vol) bleach, HCL (0.01 M pH 2) and NaOH (0.01 M, pH 12).

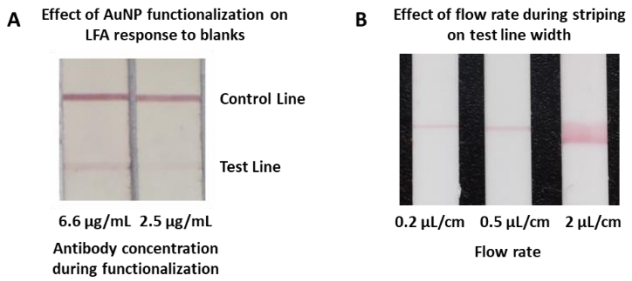

Figure 6 *– Examples of how the AuNPs conjugation and the striping of the test line can affect the LFA signal. A) Using a concentration of 6.6 µg/mL (left) vs one of 2.5 µg/mL (right) of anti-Human IgG during the AuNPs functionalization produced different signal in LFAs challenged with a blank solution (all the other parameters in the LFA fabrication were fixed). In particular, the former produced a higher non-specific signal (stronger red line) compared to the latter. Thus a careful optimization of the nanoparticle conjugation can lead to consistent improvements in the overall LFA performance. B) Fixing the dispense speed at 50 mm/sec, the use of three different flow rates during the membrane striping produced lines with different widths. As expected using a flow rate of 2 µL/cm generated a thicker line than those obtained using lower flow rates.*

27. ***Sample pad preparation (Steps 27-28):*** Pre-treat the sample pad by dipping it into sample pad buffer until fully wet.
28. Dry the sample pad at 37 °C for 2 hours in the oven.
29. ***Conjugation pad preparation (Steps 29-39):*** Set the dispense rate of the test and control line pumps to 0 µL/mm.
30. Set the air pressure at 0.3-0.6 bar.
31. Fill the spraying pump with MilliQ water and dispense at least 10 syringe volumes to thoroughly clean the pump.
32. Empty the MilliQ water of the tube and syringe.
33. Take at least 100 µL of the AuNP-antibody conjugation solution from Step 16 and prime it in the spraying syringe.
    CRITICAL STEP: the volume of AuNP-antibody conjugation solution depends on dispense distance, dispense rate and bed volume of syringe (see Step 35).
34. Prime the pumps until a drop of the conjugation solution flows out of nozzle and place the conjugate pad on the vacuum table.
    CRITICAL STEP: Place the pad on a clean and thin plastic support plate in order to avoid the conjugation solution from sticking onto the vacuum table.
35. Setup the spraying pump with a dispense rate of 5 µL/mm and a speed of 50 mm/sec
36. Press RUN to dispense the conjugation solution onto the conjugate pad.
37. Press ‘un-prime’ to recycle the conjugation solution.
38. Clean the pumps using MilliQ water, followed by 0.1 M HCl, MilliQ water, 0.1 M NaOH and again MilliQ water. Un-prime the system and keep the pumps in a liquid-free state.

CRITICAL STEP: Dispense at least 10 syringe volumes during each washing step (dispense rate of 5 µL/cm and a speed of 50 mm/sec). The pumps should be thoroughly cleaned monthly using a weak detergent (0.05% Sodium C14-16 olefin sulfonato), 10 % (vol/vol) bleach, HCL (0.01 M pH 2) and NaOH (0.01 M, pH 12)

39. Dry the conjugate pad in the vacuum chamber for at least 60 min.

    CRITICAL STEP: Check that the conjugate pad is fully dry by evaluating the stiffness of the glass fibre. Check that the AuNPs solution is homogeneously distributed throughout the conjugate pad by evaluating the colour of the test line by naked eye.

40. ***Assembly of the strips (Steps 40-41):*** Use the plastic guides of the laminated card in order to accordingly assemble the pads (see Figure 7). After the membrane, place the absorbent pad, then the conjugate pad and finally the sample pad.

    TROUBLESHOOTING:

41. Cut the strip at 3 mm width using the strip cutter.

    CRITICAL STEP: In the absence of a dedicated strip cutter, it is possible to cut the strips using a manual guillotine, although the strip to strip variability will be higher.

    PAUSE POINT: The tests can be stored in a sealed bag with desiccants at 4 °C for as long as the first reagent or material expire.

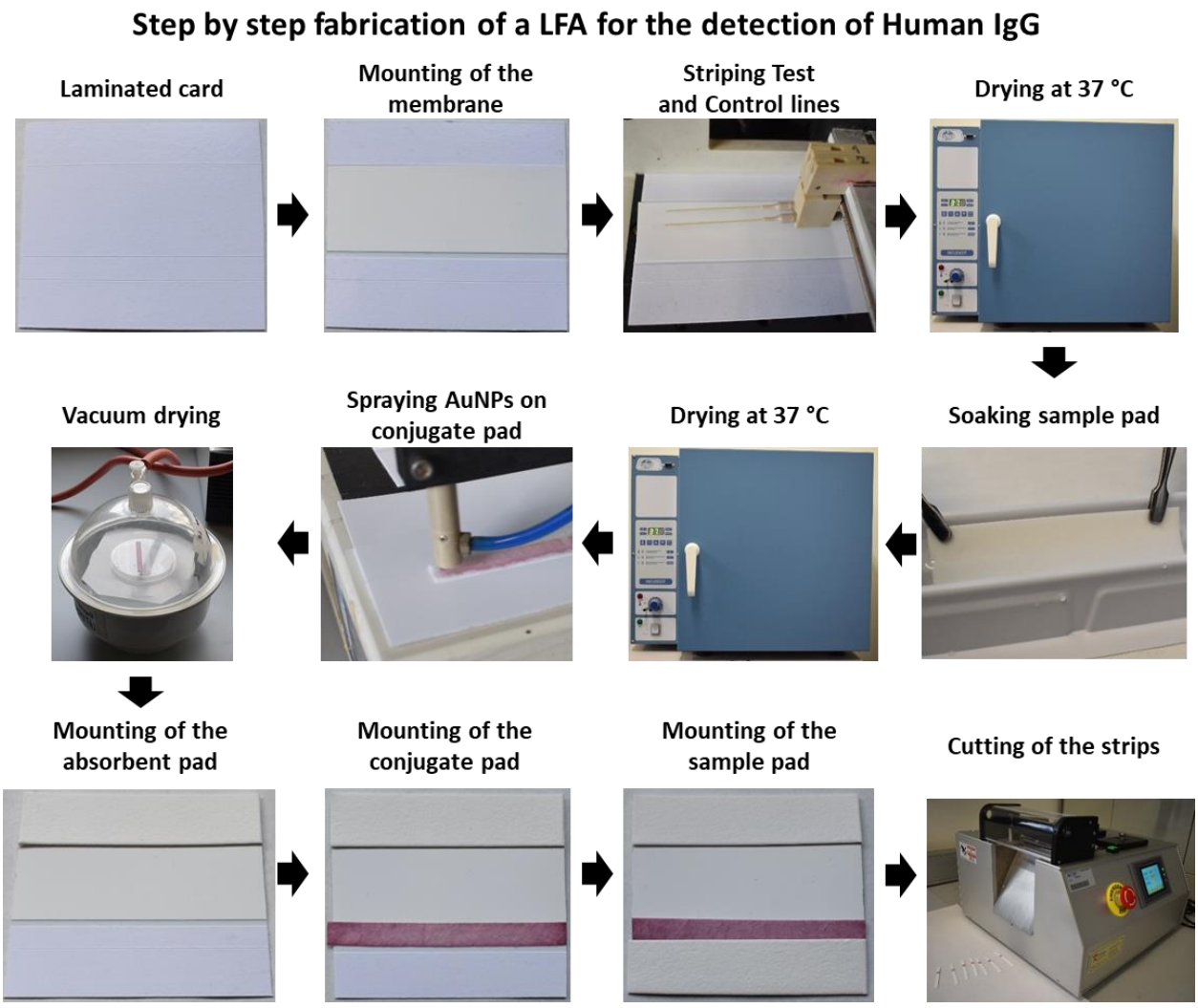

Figure 7 *– Step by step fabrication of a LFA for the detection of Human IgG.*

Assay operation and qualitative evaluation - 1 hour

42. Prepare dilutions of the sample containing target analyte. In this example we use Human IgG in PBS buffer (0, 0.01, 0.03, 0.1. 0.3, 1, 3, 10, 30, 100, 300, 1000 ng/mL). For detailed recommendations on the types of samples that can be analysed, see 'Types of Sample' in the main text.
43. Drop-cast 70 µL of the sample solution onto the sample pad.
    CRITICAL STEP: The solution should be added dropwiseon top of the sample pad to avoid overflow of the sample on top of the conjugate pad and the membrane. This will guarantee that the sample moves along the different pads of the LF strip just by capillarity. In addition commercial LF strips relying on this type of procedure are often placed in the housing cassette, which limits the possibility of overflow and guarantee the application of the sample always in the same part of the sample pad.

CRITICAL STEP: Alternatively, the strip can be dipped directly into the sample solution, paying attention that just the sample pad is in contact with the sample. This will guarantee that the sample moves along the different pads of the LF strip just by capillarity. While the dropwise method is more convenient from the user perspective (it is more stable and practical), the dipping of the LF strip may be helpful during the development phase (minimizing user variations) or when washing/running buffers are required (after adding the sample the user would just leave the sample inside a tube containing the desired buffer). In addition LFA relying on the dipping method do not require a housing cassette since the LF strip is placed vertically inside the sample.

44. Wait 15 min and evaluate the strips qualitatively by eye (Fig. 8). Proceed to the next section once the signal in the test and control lines stabilizes.

CRITICAL STEP: The appearance of signal in the test and control lines indicate the presence of the target analyte (Human IgG in this example) in the sample, while the appearance of signal in the control line only indicates the absence of the target analyte in the sample. The signal intensity in the test line correlates with the concentration of the target analyte in the sample. The absence of the control and test lines makes the LFA not trustworthy, since it indicates a malfunctioning of the strip.

CRITICAL STEP: Use a clear and constant white light source and avoid shadows while evaluating the strips by naked eye.

TROUBLESHOOTING:

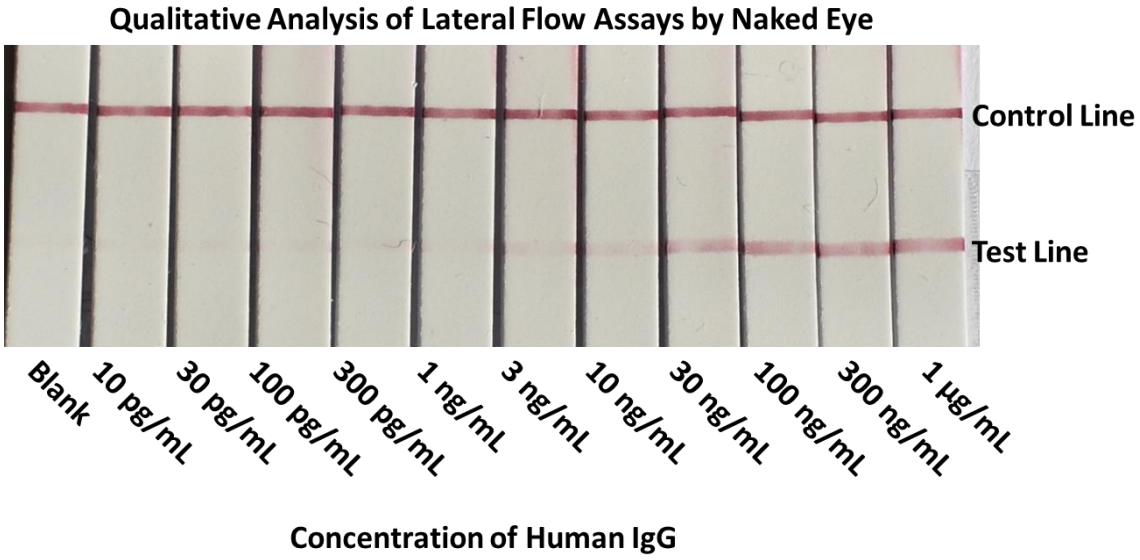

Figure 8 *– Qualitative analysis of LFAs for the detection of Human IgG. The intensity of the test line increases with the concentration of target.*

Quantitative evaluation - 30 minutes

45. Fix the strips in a flat surface under the camera holder (i.e. a tripod or similar).
46. Fix the camera on the camera holder.
47. Adjust the camera parameters and take pictures of the strips.
    CRITICAL STEP: To facilitate the comparison between different strips and different days it is essential to use always the same set-up, including the distance between the camera and the strips, the light source (ideally in a dark room to prevent contamination from ambient light), and the camera parameters (ISO, aperture, shutter speed and focus).
    CRITICAL STEP: We cannot provide specific indications on the camera parameters to employ, since they may vary from place to place and form camera to camera. Nevertheless, we recommend to fix all the set up conditions at the beginning of the development and to keep them constant. A simple "trick" to speed up the optimization of the best camera conditions is to take the first picture using the "automatic" mode of the camera. Then using such parameters as starting point to find the optimal ones.
48. Open the pictures with Image J software (see Fig. 9 A-B).
49. Select the green channel by selecting Image – Colour – Split Channel – Green channel (see Fig. 9 C).
    CRITICAL STEP: The use of the green channel is recommended for the analysis of red labels such as gold nanoparticles since it provides the highest sensitivity.
50. With the "straight" command draw a line along the membrane (see Fig. 9 D)
    CRITICAL STEP: The width of the straight line should be increased till covering most of the strip in order to obtain more reproducible results.
51. Extract the plot profile by selecting Analyze – Plot profile – List (see Fig. 9 E)
52. Export the results in a data analysis software (Excel, Origin, etc.)
53. Calculate the peak intensity of the test line and plot it vs the target analyte concentration used (Fig. 9F).
    CRITICAL STEP: the peak intensity can be obtained subtracting the peak value of the test line from the background value. Overall it is not recommended to use the peak value of the control line as reference for the test line one, since high target analyte concentrations (in a sandwich assay) may lead to a dramatic depletion in the concentration of AuNPs after the test line, which may produce a decrease in the control line intensity (if compared to strips that used low target concentrations).
54. Fit the data to a four-parameter logistic curve (sigmoidal curve).[215]
    CRITICAL STEP: In case of using a range of target concentrations that generates both non-responsive and saturated signals the best data fitting is generally obtained using a four-parameter logisitic curve (a sigmoidal curve). Instead if the calibration is done using a range of target concentration focused on the dynamic range of the fitted sigmoidal curve (between the 10% and 90% of the maximum signal) it is possible to approximate the fit to a linear curve. Overall, sigmoidal approximation is generally accepted for competitive immunoassays (since they more closely represent an ideal Langmuir model than an immune sandwich assay), whereas linear approximation of the initial part of concentration dependence is often preferred for immune sandwich assays.
55. Carry out the desired statistical analysis such as calculating the limit of detection (LoD) and the limit of quantification (LoQ).
    CRITICAL STEP: It is possible to calculate the limit of detection (LoD = value of the blank + 3 times its standard deviation) and limit of quantification (LoQ = value of the blank + 10 times its standard deviation), which in this case is 0.77 nM and 4.62 nM respectively. As shown also by this example dataset (where the LoD signal is almost undistinguishable from the non-responsive portion of the

fitted curve), although LoD is a generally accepted method to evaluate LFAs, it is sometimes not statistically robust since it does not count for the variance in the test signals. We refer the reader to the following references describing improved methods to calculate the LoD of LFAs.[203,216–218]

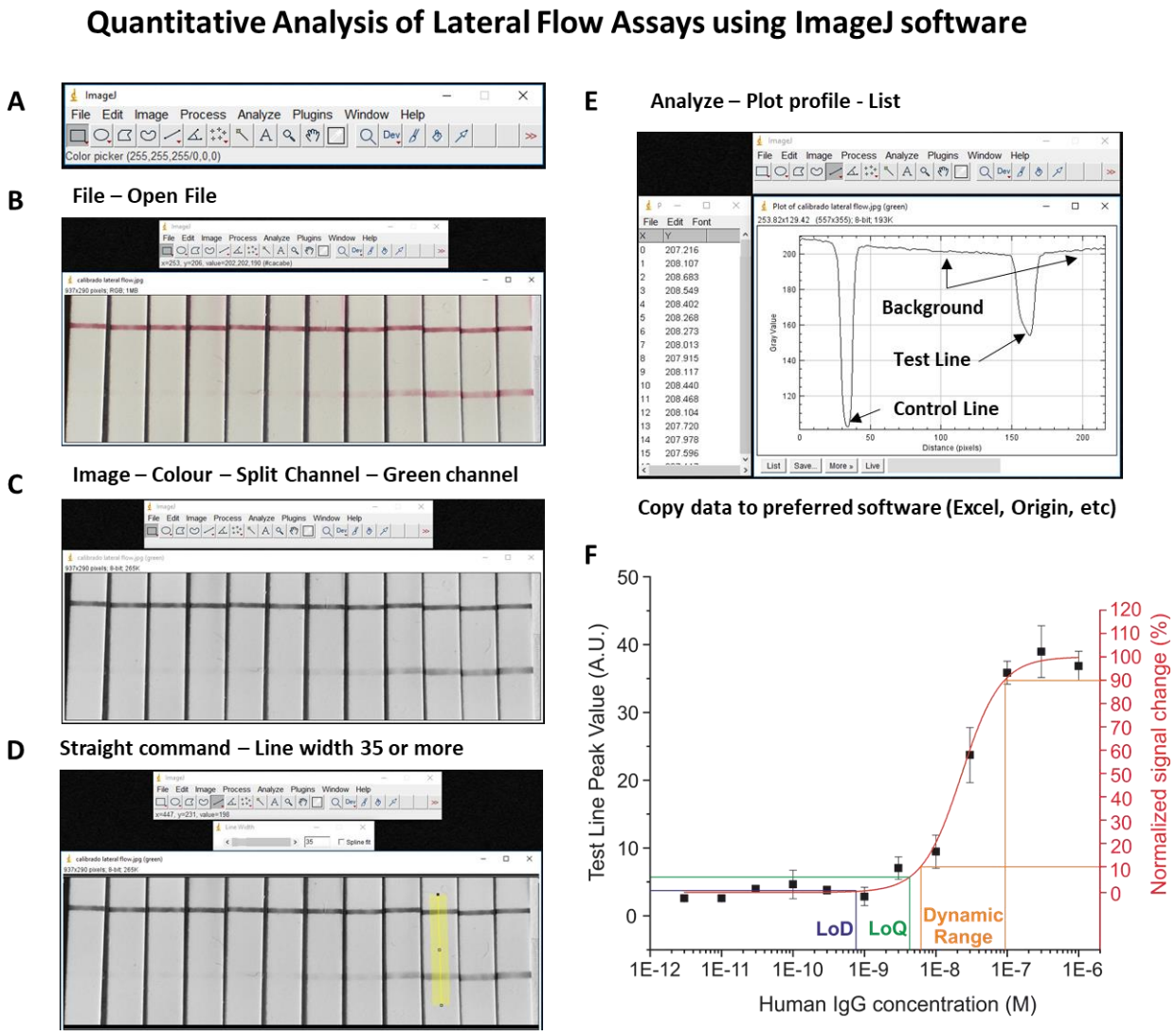

Figure 9 *– Quantitative analysis of LFA using ImageJ and fitting the results to a four-parameter logistic curve (sigmoidal curve). A) Open the ImageJ software. B) Open the file containing the photo of the strip(s). C) For LFAs employing red labels (i.e. AuNPs) the use of the green channel maximizes the sensitivity of the analysis. D) The use of the straight command followed by E) the plot profile allows the representation of the signal profile along a LFA strip. We recommend to use a line width that covers most of the strip in order to have more reliable results. From the plot profile it is possible to extract the values and use data analysis software to calculate the peak value of the test line. It is obtained by subtracting the background signal from the peak value. F) The data must then be plotted against the target concentration, in this case using a four-parameter logisitic curve (a sigmoidal curve). It is then possible to carry out statistical analysis such as calculating the limit of detection (LoD), the limit of quantification (LoQ) and the dynamic range (orange lines).*

- **END OF BOX 1 -**

Table 4: Troubleshooting Table

STEP	Problem	Reason	Solution
•	• The target analyte is not commercially available. • The target analyte is too expensive.	• The target analyte is new or not common.	• Produce your own target analyte. • Purify the target analyte from real samples.
•	• Bioreceptors are not commercially available. • Bioreceptors are too expensive.	• The target analyte is new or not common.	• Evaluate different types of bioreceptors (see table 3). • Develop new bioreceptors. • Consider changing assay format from sandwich to competitive assay.
•	• Bioreceptors are not sensitive and specific enough, as determined using standard techniques (ELISA. SPR, etc.).	• The target analyte is new or not common. • The target analyteconcentration is too low.	• Develop new bioreceptors. • Consider if sample treatment, target pre-concentration or signal amplification could solve the issue. • Re-evaluate your assay's goals.
•	• Nanoparticles are not stable in solution after conjugation with the bioreceptor.	• The ionic strength of the buffer is too high. • The conjugation was not successful.	• Decrease buffer ionic strength or increasing amount of surfactants or BSA. • Increase the amount of bioreceptor during the conjugation. • Block the nanoparticles with stabilizing agents (i.e. BSA). • Evaluate different conjugation strategies.
•	• Nanoparticles do not flow upon rewetting.	• Nanoparticles are stuck on the conjugate pad.	• Increase the sugar concentration in the conjugate pad buffer. • Verify the conjugate pad is fully dried before adding the sample. • Increase the amount of surfactant in the running buffer. • If using polyclonal antibodies control for the formation of immune-aggregates. • Change conjugate pad material. • Re-optimize conjugation.
•	• Sample does not reach the conjugate pad.	• The sample pad is clogged. • The sample pad and the conjugate pad are not in contact.	• Use a different sample pad material.

			• Introduce an extra filtration or sample treatment to remove bigger particles. • Use an extra running or washing buffer. • Assure the sample and conjugate pad are overlaid. • Assure the sample pad is not compressed by the cassette. • Change composition of the sample pad buffer increasing amount of surfactant.
•	• Test line does not show up, but the control line does.	• The target concentration is too low or too high. • The test line bioreceptor was not properly striped. • If using a sandwich assay the bioreceptors bind the same epitope.	• Check a wider range of target concentrations (sandwich assay can present also the hook effect[169]). • Decrease ionic strength of striping buffer. • Remove/decrease surfactants from striping and running buffers. • Increase concentration of bioreceptor or add other molecules (BSA) to stabilize it. • Remove membrane blocking (if any), or decrease blocking agent concentration. • Increase amount of label in conjugate pad. • Assure the bioreceptor is completely dried before using the LFA. • Change bioreceptor.
•	• Control line does not show up, but the test line does.	• The control line bioreceptor was not properly striped. • The bioreceptor-nanoparticle conjugate concentration is too low.	• Add a different nanoparticle specific for the control line. • Decrease ionic strength of striping buffer. • Remove surfactants from striping buffer. • Increase amount of nanoparticles in conjugate pad. • Change to a different capture bioreceptor.
•	• Both control and test lines do not show up.	• The bioreceptor-nanoparticle conjugation was not successful. • The striping was wrong. • Target concentration is too high.	• Change conjugation strategy. • Change striping buffer, decreasing ionic strength and removing surfactants.

			• Decrease surfactant concentration in the running buffer. • Use a new membrane (in case it is expired) or change material. • Verify the capture bioreceptors are actually striped on the membrane. • Verify the labelled-bioreceptor is not expired. • Dilute the sample.
•	• The sensor is not specific.	• Nonspecific interactions induce the accumulation of label on the test line even in the absence of target.	• Change to different bioreceptors. • Block the membrane. • Increase blocking agents in sample pad and running buffers. • Increase amount of surfactants in the striping, sample pad and running buffers. • Increase ionic strength in the sample pad and running buffers. • Change pH in the sample pad and running buffers. • Use a faster flow membrane. • Actively remove possible contaminants.
•	• The cut-off value is not adequate for the application (i.e. the sensor is too much or not enough sensitive to detect the target concentration of interest).	• The sensor cannot detect application-relevant concentrations of target.	• Increase amount of nanoparticles. • Use a slower flow membrane. • Change amount of bioreceptors on the test line. • Change ionic strength, pH and surfactants concentration in the buffers. • Change type of nanoparticles and sensing strategy. • Pre-concentrate or dilute the sample depending on what is appropriate. • Include a signal amplification step. • Change to different bioreceptors.
•	• The sensitivity is not adequate for the application	• The sensor cannot accurately differentiate two close concentration values	• Increase amount of nanoparticles.

			• Use a slower flow membrane. • Change amount of bioreceptors on the test line. • Change ionic strength, pH and surfactants concentration in the buffers. • Change type of nanoparticles and sensing strategy. • Pre-concentrate the sample. • Apply a signal amplification strategy. • Change to different bioreceptors. • Modify strip dimensions, architecture. • Consider the pre-treatment of the sample (purification, amplification, not dilution, etc.) and/or increase the sample volume. • Improve quantification device (camera, sensor, software, etc.).
•	• The sensor does not show repeatability	• Using the same target concentration, the sensor produces different signals.	• Verify the fabrication steps and the ambient conditions are constant from batch to batch. • Use a constant light source. • If using a reader, use the same parameters (i.e. exposure, focus, etc.). • Assure the proper storage of the LFA.
•	• The sensor is not reproducible	• Other developers or users do not obtain the same response.[230]	• Verify the fabrication steps and the ambient conditions are constant from batch to batch. • Use a constant light source. • Define protocols to store all the reagents involved and control parameters such as pH during all the steps. • Evaluate incubation steps at different times/temperatures. • Define expiration dates for reagents, materials and the device itself. • Provide a more precise and easier to understand protocol

			to facilitate the sensor operation by the user.

Acknowledgments: C.P. acknowledges Marie Skłodowska-Curie Actions Individual Fellowship; this project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 795635. E.C. acknowledges Ministerio de Ciencia e Innovación of Spain and Fondo Social Europeo for the Fellowship PRE2018-084856 awarded under the call "Ayudas para contratos predoctorales para la formación de doctores, Subprograma Estatal de Formación del Programa Estatal de Promoción del Talento y su Empleabilidad en I+D+i", under the framework of "Plan Estatal de Investigación Científica y Técnica y de Innovación 2017-2020". E. P. N. acknowledges funding through the EU's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 754510. We also acknowledge the Program "Centros de Excelencia Severo Ochoa", reference SEV-2017-0706, funded by Agencia Estatal de Investigación, Spanish Government.

Data Availability: The datasets generated during and/or analysed during the current study (Figures xx-yy) are available from the corresponding author on reasonable request.

References

1. Parolo, C. & Merkoçi, A. Paper-based nanobiosensors for diagnostics. *Chem. Soc. Rev.* **42**, 450–457 (2013).

2. Bahadır, E. B. & Sezgintürk, M. K. Lateral flow assays: Principles, designs and labels. *TrAC Trends Anal. Chem.* **82**, 286–306 (2016).

3. Brangel, P. *et al.* A Serological Point-of-Care Test for the Detection of IgG Antibodies against Ebola Virus in Human Survivors. *ACS Nano* **12**, 63–73 (2018).

4. Posthuma-Trumpie, G. A., Korf, J. & van Amerongen, A. Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey. *Anal. Bioanal. Chem.* **393**, 569–582 (2009).

5. Hu, J. *et al.* Advances in paper-based point-of-care diagnostics. *Biosens. Bioelectron.* **54**, 585–97 (2014).

6. Yetisen, A. K., Akram, M. S. & Lowe, C. R. Paper-based microfluidic point-of-care diagnostic devices. *Lab Chip* **13**, 2210–51 (2013).

7. Martinelli, F. *et al.* Advanced methods of plant disease detection. A review. *Agron. Sustain. Dev.* **35**, 1–25 (2015).

8. Li, P., Zhang, Q. & Zhang, W. Immunoassays for aflatoxins. *TrAC Trends Anal. Chem.* **28**, 1115–1126 (2009).

9. Anfossi, L., Baggiani, C., Giovannoli, C., D'Arco, G. & Giraudi, G. Lateral-flow immunoassays for mycotoxins and phycotoxins: a review. *Anal. Bioanal. Chem.* **405**, 467–480 (2013).

10. Luo, K., Kim, H.-Y., Oh, M.-H. & Kim, Y.-R. Paper-based lateral flow strip assay for the detection of

foodborne pathogens: principles, applications, technological challenges and opportunities. *Crit. Rev. Food Sci. Nutr.* **60**, 157–170 (2020).

11. Ahmed, F. E. Detection of genetically modified organisms in foods. *Trends Biotechnol.* **20**, 215–223 (2002).

12. Ngom, B., Guo, Y., Wang, X. & Bi, D. Development and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: a review. *Anal. Bioanal. Chem.* **397**, 1113–1135 (2010).

13. Zhao, X., Lin, C.-W., Wang, J. & Oh, D. H. Advances in Rapid Detection Methods for Foodborne Pathogens. *J. Microbiol. Biotechnol.* **24**, 297–312 (2014).

14. Ramage, J. G. *et al.* Comprehensive Laboratory Evaluation of a Specific Lateral Flow Assay for the Presumptive Identification of Abrin in Suspicious White Powders and Environmental Samples. *Biosecurity Bioterrorism Biodefense Strateg. Pract. Sci.* **12**, 49–62 (2014).

15. Grubb, A. O. & Glad, U. C. Immunoassay with test strip having antibodies bound thereto. (1976).

16. Hsieh, H. V., Dantzler, J. L. & Weigl, B. H. Analytical Tools to Improve Optimization Procedures for Lateral Flow Assays. *Diagnostics* **7**, 29 (2017).

17. Gasperino, D., Baughman, T., Hsieh, H. V., Bell, D. & Weigl, B. H. Improving Lateral Flow Assay Performance Using Computational Modeling. *Annu. Rev. Anal. Chem.* **11**, 219–244 (2018).

18. Merck Millipore. Rapid Lateral Flow Test Strips - Considerations for Product Development. Available at: http://www.merckmillipore.com/INTERSHOP/web/WFS/Merck-RU-Site/ru_RU/-/USD/ShowDocument-Pronet?id=201306.15671.

19. Nanocomposix. Lateral Flow Assay Development Guide. Available at: https://cdn.shopify.com/s/files/1/0257/8237/files/BioReady_Lateral_Flow_Handbook.pdf.

20. Wong, R. C. & Tse, H. Y. *Lateral flow immunoassay*. (Springer, 2009).

21. Bishop, J. D., Hsieh, H. V., Gasperino, D. J. & Weigl, B. H. Sensitivity enhancement in lateral flow assays: a systems perspective. *Lab Chip* **19**, 2486–2499 (2019).

22. Zhang, G., Guo, J. & Wang, X. Immunochromatographic Lateral Flow Strip Tests. in 169–183 (Humana Press, 2009). doi:10.1007/978-1-60327-569-9_12

23. Volkov, A., Mauk, M., Corstjens, P. & Niedbala, R. S. Rapid Prototyping of Lateral Flow Assays. in 217–235 (Humana Press, 2009). doi:10.1007/978-1-60327-569-9_14

24. Bailes, J., Mayoss, S., Teale, P. & Soloviev, M. Gold Nanoparticle Antibody Conjugates for Use in Competitive Lateral Flow Assays. in *Nanoparticles in Biology and Medicine* 45–55 (Humana Press, 2012). doi:10.1007/978-1-61779-953-2_4

25. Zeng, L., Lie, P., Fang, Z. & Xiao, Z. Lateral Flow Biosensors for the Detection of Nucleic Acid. in 161–167 (Humana Press, Totowa, NJ, 2013). doi:10.1007/978-1-62703-535-4_14

26. Ching, K. H. Lateral Flow Immunoassay. in 127–137 (Humana Press, New York, NY, 2015). doi:10.1007/978-1-4939-2742-5_13

27. Tang, R. H. *et al.* Advances in paper-based sample pretreatment for point-of-care testing. *Crit. Rev. Biotechnol.* **37**, 411–428 (2017).

28. Wang, Y. *et al.* A SERS-based lateral flow assay biosensor for quantitative and ultrasensitive detection of interleukin-6 in unprocessed whole blood. *Biosens. Bioelectron.* **141**, 111432 (2019).

29. Ang, S. H., Rambeli, M., Thevarajah, T. M., Alias, Y. B. & Khor, S. M. Quantitative, single-step dual measurement of hemoglobin A1c and total hemoglobin in human whole blood using a gold sandwich immunochromatographic assay for personalized medicine. *Biosens. Bioelectron.* **78**, 187–193 (2016).

30. Gao, X. *et al.* Paper-Based Surface-Enhanced Raman Scattering Lateral Flow Strip for Detection of Neuron-Specific Enolase in Blood Plasma. *Anal. Chem.* **89**, 10104–10110 (2017).

31. Dieplinger, B., Egger, M., Gegenhuber, A., Haltmayer, M. & Mueller, T. Analytical and clinical evaluation of a rapid quantitative lateral flow immunoassay for measurement of soluble ST2 in human plasma. *Clin. Chim. Acta* **451**, 310–315 (2015).

32. Ou, L. *et al.* Development of a lateral flow immunochromatographic assay for rapid detection of Mycoplasma pneumoniae-specific IgM in human serum specimens. *J. Microbiol. Methods* **124**, 35–40 (2016).

33. Huang, Y. *et al.* Development of up-converting phosphor technology-based lateral flow assay for quantitative detection of serum PIVKA-II: Inception of a near-patient PIVKA-II detection tool. *Clin. Chim. Acta* **488**, 202–208 (2019).

34. Chamorro-Garcia, A. *et al.* Detection of parathyroid hormone-like hormone in cancer cell cultures by gold nanoparticle-based lateral flow immunoassays. *Nanomedicine Nanotechnology, Biol. Med.* **12**, 53–61 (2016).

35. Dalirirad, S. & Steckl, A. J. Aptamer-based lateral flow assay for point of care cortisol detection in sweat. *Sensors Actuators B Chem.* **283**, 79–86 (2019).

36. Hudson, M. *et al.* Drug screening using the sweat of a fingerprint: lateral flow detection of Δ9-tetrahydrocannabinol, cocaine, opiates and amphetamine. *J. Anal. Toxicol.* **43**, 88–95 (2019).

37. Dalirirad, S. & Steckl, A. J. Lateral flow assay using aptamer-based sensing for on-site detection of dopamine in urine. *Anal. Biochem.* 113637 (2020). doi:10.1016/j.ab.2020.113637

38. Li, Z., Chen, H., Feng, S., Liu, K. & Wang, P. Development and Clinical Validation of a Sensitive Lateral Flow Assay for Rapid Urine Fentanyl Screening in the Emergency Department. *Clin. Chem.* **66**, 324–332 (2020).

39. Henderson, W. A. *et al.* Simple lateral flow assays for microbial detection in stool. *Anal. Methods* **10**, 5358–5363 (2018).

40. Lin, Z. *et al.* Development of an immunochromatographic lateral flow device for rapid detection of Helicobacter pylori stool antigen. *Clin. Biochem.* **48**, 1298–1303 (2015).

41. Hu, Q. *et al.* An up-converting phosphor technology-based lateral flow assay for point-of-collection detection of morphine and methamphetamine in saliva. *Analyst* **143**, 4646–4654 (2018).

42. Oh, H.-K., Kim, J.-W., Kim, J.-M. & Kim, M.-G. High sensitive and broad-range detection of cortisol in human saliva using a trap lateral flow immunoassay (trapLFI) sensor. *Analyst* **143**, 3883–3889 (2018).

43. Boulware, D. R. *et al.* Multisite validation of cryptococcal antigen lateral flow assay and quantification by laser thermal contrast. *Emerg. Infect. Dis.* **20**, 45–53 (2014).

44. Fleury, A. *et al.* A lateral flow assay (LFA) for the rapid detection of extraparenchymal neurocysticercosis using cerebrospinal fluid. *Exp. Parasitol.* **171**, 67–70 (2016).

45. Sakurai, A. *et al.* Multi-colored immunochromatography using nanobeads for rapid and sensitive typing of seasonal influenza viruses. *J. Virol. Methods* **209**, 62–68 (2014).

46. Eryılmaz, M. *et al.* SERS-based rapid assay for sensitive detection of Group A *Streptococcus* by evaluation of the swab sampling technique. *Analyst* **144**, 3573–3580 (2019).

47. Principato, M. *et al.* Detection of target staphylococcal enterotoxin B antigen in orange juice and popular carbonated beverages using antibody-dependent antigen-capture assays. *J. Food Sci.* **75**, T141-7 (2010).

48. Jiang, H. *et al.* Silver Nanoparticle-Based Fluorescence-Quenching Lateral Flow Immunoassay for Sensitive Detection of Ochratoxin A in Grape Juice and Wine. *Toxins (Basel).* **9**, (2017).

49. Kong, D. *et al.* Ultrasensitive and eco-friendly immunoassays based monoclonal antibody for detection of deoxynivalenol in cereal and feed samples. *Food Chem.* **270**, 130–137 (2019).

50. Focker, M., van der Fels-Klerx, H. J. & Oude Lansink, A. G. J. M. Cost-Effective Sampling and Analysis for Mycotoxins in a Cereal Batch. *Risk Anal.* **39**, 926–939 (2019).

51. Yang, X. *et al.* A Lateral Flow Immunochromato-graphic Strip Test for Rapid Detection of Oseltamivir Phosphate in Egg and Chicken Meat. *Sci. Rep.* **8**, 16680 (2018).

52. Magiati, M., Myridaki, V. M., Christopoulos, T. K. & Kalogianni, D. P. Lateral flow test for meat authentication with visual detection. *Food Chem.* **274**, 803–807 (2019).

53. Tan, G. *et al.* Ultrasensitive quantitation of imidacloprid in vegetables by colloidal gold and time-resolved fluorescent nanobead traced lateral flow immunoassays. *Food Chem.* **311**, 126055 (2020).

54. Hassan, A. H. A., Bergua, J. F., Morales-Narváez, E. & Mekoçi, A. Validity of a single antibody-based lateral flow immunoassay depending on graphene oxide for highly sensitive determination of E. coli O157:H7 in minced beef and river water. *Food Chem.* **297**, 124965 (2019).

55. Liu, Y. *et al.* Detection of 3-phenoxybenzoic acid in river water with a colloidal gold-based lateral flow immunoassay. *Anal. Biochem.* **483**, 7–11 (2015).

56. Wu, Z. *et al.* Aptamer-based fluorescence-quenching lateral flow strip for rapid detection of mercury (II) ion in water samples. *Anal. Bioanal. Chem.* **409**, 5209–5216 (2017).

57. Quesada-González, D., Jairo, G. A., Blake, R. C., Blake, D. A. & Merkoçi, A. Uranium (VI) detection in groundwater using a gold nanoparticle/paper-based lateral flow device. *Sci. Rep.* **8**, 16157 (2018).

58. Mosley, G. L. *et al.* Improved lateral-flow immunoassays for chlamydia and immunoglobulin M by sequential rehydration of two-phase system components within a paper-based diagnostic. *Microchim. Acta* **184**, 4055–4064 (2017).

59. Ridgway, K., Lalljie, S. P. D. & Smith, R. M. Sample preparation techniques for the determination

of trace residues and contaminants in foods. *J. Chromatogr. A* **1153**, 36–53 (2007).

60. Gong, M. M., Macdonald, B. D., Vu Nguyen, T., Van Nguyen, K. & Sinton, D. Field tested milliliter-scale blood filtration device for point-of-care applications. *Biomicrofluidics* **7**, 44111 (2013).

61. Golden, A. *et al.* Extended Result Reading Window in Lateral Flow Tests Detecting Exposure to Onchocerca volvulus: A New Technology to Improve Epidemiological Surveillance Tools. *PLoS One* **8**, e69231 (2013).

62. Sastre, P. *et al.* Development of a novel lateral flow assay for detection of African swine fever in blood. *BMC Vet. Res.* **12**, 206 (2016).

63. Choi, J. R. *et al.* Sensitive biomolecule detection in lateral flow assay with a portable temperature–humidity control device. *Biosens. Bioelectron.* **79**, 98–107 (2016).

64. Fukushi, S. *et al.* Characterization of novel monoclonal antibodies against the MERS-coronavirus spike protein and their application in species-independent antibody detection by competitive ELISA. *J. Virol. Methods* **251**, 22–29 (2018).

65. Duo, J. *et al.* Surface plasmon resonance as a tool for ligand-binding assay reagent characterization in bioanalysis of biotherapeutics. *Bioanalysis* **10**, 559–576 (2018).

66. Miller, B. S. *et al.* Quantifying Biomolecular Binding Constants using Video Paper Analytical Devices. *Chem. - A Eur. J.* **24**, (2018).

67. Dam, T. K., Torres, M., Brewer, C. F. & Casadevall, A. Isothermal Titration Calorimetry Reveals Differential Binding Thermodynamics of Variable Region-identical Antibodies Differing in Constant Region for a Univalent Ligand. *J. Biol. Chem.* **283**, 31366–31370 (2008).

68. Yang, D., Singh, A., Wu, H. & Kroe-Barrett, R. Determination of High-affinity Antibody-antigen Binding Kinetics Using Four Biosensor Platforms. *J. Vis. Exp.* (2017). doi:10.3791/55659

69. Mosley, G. L., Nguyen, P., Wu, B. M. & Kamei, D. T. Development of quantitative radioactive methodologies on paper to determine important lateral-flow immunoassay parameters. *Lab Chip* **16**, 2871–81 (2016).

70. Nguyen, V.-T., Song, S., Park, S. & Joo, C. Recent advances in high-sensitivity detection methods for paper-based lateral-flow assay. *Biosens. Bioelectron.* **152**, 112015 (2020).

71. Quesada-González, D. & Merkoçi, A. Nanoparticle-based lateral flow biosensors. *Biosens. Bioelectron.* **73**, 47–63 (2015).

72. Soh, J. H., Chan, H.-M. & Ying, J. Y. Strategies for developing sensitive and specific nanoparticle-based lateral flow assays as point-of-care diagnostic device. *Nano Today* **30**, 100831 (2020).

73. Ge, X. *et al.* Nanomaterial-enhanced paper-based biosensors. *TrAC Trends Anal. Chem.* **58**, 31–39 (2014).

74. Zhan, L. *et al.* The Role of Nanoparticle Design in Determining Analytical Performance of Lateral Flow Immunoassays. *Nano Lett.* **17**, 7207–7212 (2017).

75. Quesada-González, D. & Merkoçi, A. Nanomaterial-based devices for point-of-care diagnostic applications. *Chem. Soc. Rev.* **47**, 4697–4709 (2018).

76. Verheijen, R., Osswald, I. K., Dietrich, R. & Haasnoot, W. Development of a one step strip test for

the detection of (dihydro)streptomycin residues in raw milk. *Food Agric. Immunol.* **12**, 31–40 (2000).

77. Fong, W. K. *et al.* Rapid solid-phase immunoassay for detection of methicillin-resistant Staphylococcus aureus using cycling probe technology. *J. Clin. Microbiol.* **38**, 2525–2529 (2000).

78. Shyu, R. H., Shyu, H. F., Liu, H. W. & Tang, S. S. Colloidal gold-based immunochromatographic assay for detection of ricin. *Toxicon* **40**, 255–258 (2002).

79. Ren, W., Ballou, D. R., FitzGerald, R. & Irudayaraj, J. Plasmonic enhancement in lateral flow sensors for improved sensing of E. coli O157:H7. *Biosens. Bioelectron.* **126**, 324–331 (2019).

80. Guide, I. B. +44 (0)1223 661000 info@innovabiosciences Guide to Lateral Flow Immunoassays Innova Biosciences Guide. **44**,

81. Bishop, J. D., Hsieh, H. V., Gasperino, D. J. & Weigl, B. H. Sensitivity enhancement in lateral flow assays: A systems perspective. *Lab Chip* **19**, 2486–2499 (2019).

82. Di Nardo, F., Cavalera, S., Baggiani, C., Giovannoli, C. & Anfossi, L. Direct vs Mediated Coupling of Antibodies to Gold Nanoparticles: The Case of Salivary Cortisol Detection by Lateral Flow Immunoassay. *ACS Appl. Mater. Interfaces* **11**, 32758–32768 (2019).

83. Parolo, C. *et al.* Design, preparation, and evaluation of a fixed-orientation antibody/gold-nanoparticle conjugate as an immunosensing label. *ACS Appl. Mater. Interfaces* **5**, 10753–9 (2013).

84. Liu, J., Mazumdar, D. & Lu, Y. A simple and sensitive ‘dipstick’ test in serum based on lateral flow separation of aptamer-linked nanostructures. *Angew. Chemie - Int. Ed.* **45**, 7955–7959 (2006).

85. Mao, X. *et al.* Disposable nucleic acid biosensors based on gold nanoparticle probes and lateral flow strip. *Anal. Chem.* **81**, 1660–8 (2009).

86. Posthuma-Trumpie, G. A., Wichers, J. H., Koets, M., Berendsen, L. B. J. M. & van Amerongen, A. Amorphous carbon nanoparticles: a versatile label for rapid diagnostic (immuno)assays. *Anal. Bioanal. Chem.* **402**, 593–600 (2012).

87. Van Dam, G. J. *et al.* Diagnosis of schistosomiasis by reagent strip test for detection of circulating cathodic antigen. *J. Clin. Microbiol.* **42**, 5458–5461 (2004).

88. Blazková, M., Micková-Holubová, B., Rauch, P. & Fukal, L. Immunochromatographic colloidal carbon-based assay for detection of methiocarb in surface water. *Biosens. Bioelectron.* **25**, 753–8 (2009).

89. Blažková, M., Rauch, P. & Fukal, L. Strip-based immunoassay for rapid detection of thiabendazole. *Biosens. Bioelectron.* **25**, 2122–2128 (2010).

90. Kalogianni, D. P., Boutsika, L. M., Kouremenou, P. G., Christopoulos, T. K. & Ioannou, P. C. Carbon nano-strings as reporters in lateral flow devices for DNA sensing by hybridization. *Anal. Bioanal. Chem.* **400**, 1145–52 (2011).

91. Noguera, P. *et al.* Carbon nanoparticles in lateral flow methods to detect genes encoding virulence factors of Shiga toxin-producing Escherichia coli. *Anal. Bioanal. Chem.* **399**, 831–8 (2011).

92. Oliveira-Rodríguez, M. *et al.* Point-of-care detection of extracellular vesicles: Sensitivity optimization and multiple-target detection. *Biosens. Bioelectron.* **87**, 38–45 (2017).

93. Qiu, W. *et al.* Carbon nanotube-based lateral flow biosensor for sensitive and rapid detection of DNA sequence. *Biosens. Bioelectron.* **64**, 367–372 (2015).

94. Yao, L. *et al.* MWCNTs based high sensitive lateral flow strip biosensor for rapid determination of aqueous mercury ions. *Biosens. Bioelectron.* **85**, 331–336 (2016).

95. Greenwald, R. *et al.* Improved serodetection of Mycobacterium bovis infection in badgers (Meles meles) using multiantigen test formats. *Diagn. Microbiol. Infect. Dis.* **46**, 197–203 (2003).

96. EMD Millipore. *Rapid Lateral Flow Test Strips. Considerations for Product Development*. (2013).

97. Quesada-González, D. & Merkoçi, A. Biosensors and Bioelectronics Nanoparticle-based lateral fl ow biosensors. **73**, 47–63 (2015).

98. Morales-Narváez, E., Naghdi, T., Zor, E. & Merkoçi, A. Photoluminescent Lateral-Flow Immunoassay Revealed by Graphene Oxide: Highly Sensitive Paper-Based Pathogen Detection. *Anal. Chem.* **87**, 8573–8577 (2015).

99. Goldman, E. R. & Mattoussi, H. Quantum dot bioconjugates for imaging , labelling and sensing. 435–446 (2005).

100. Taranova, N. A., Berlina, A. N., Zherdev, A. V. & Dzantiev, B. B. 'Traffic light' immunochromatographic test based on multicolor quantum dots for the simultaneous detection of several antibiotics in milk. *Biosens. Bioelectron.* **63**, 255–261 (2015).

101. Foubert, A. *et al.* Development of a Rainbow Lateral Flow Immunoassay for the Simultaneous Detection of Four Mycotoxins. *J. Agric. Food Chem.* **65**, 7121–7130 (2017).

102. Wang, C. *et al.* Layer-by-layer assembly of magnetic-core dual quantum dot-shell nanocomposites for fluorescence lateral flow detection of bacteria. *Nanoscale* **12**, 795–807 (2020).

103. Yan, X. *et al.* CdSe/ZnS Quantum Dot-Labeled Lateral Flow Strips for Rapid and Quantitative Detection of Gastric Cancer Carbohydrate Antigen 72-4. *Nanoscale Res. Lett.* **11**, (2016).

104. Berlina, A. N., Taranova, N. A., Zherdev, A. V., Vengerov, Y. Y. & Dzantiev, B. B. Quantum dot-based lateral flow immunoassay for detection of chloramphenicol in milk. *Anal. Bioanal. Chem.* **405**, 4997–5000 (2013).

105. Bruno, J. G. Application of DNA Aptamers and Quantum Dots to Lateral Flow Test Strips for Detection of Foodborne Pathogens with Improved Sensitivity versus Colloidal Gold. *Pathog. (Basel, Switzerland)* **3**, 341–55 (2014).

106. Bruno, J. G. Evaluation of pathogenic big 7 E. coli aptamer-quantum dot lateral flow test strips. *J. Bionanoscience* **11**, 148–152 (2017).

107. Yang, H. *et al.* A novel quantum dots-based point of care test for syphilis. *Nanoscale Res. Lett.* **5**, 875–81 (2010).

108. Zamora-Gálvez, A., Morales-Narváez, E., Romero, J. & Merkoçi, A. Photoluminescent lateral flow based on non-radiative energy transfer for protein detection in human serum. *Biosens.*

Bioelectron. **100**, 208–213 (2018).

109. Chen, J. *et al.* A facile fluorescence lateral flow biosensor for glutathione detection based on quantum dots-MnO2 nanocomposites. *Sensors Actuators, B Chem.* **260**, 770–777 (2018).

110. Rong, Z. *et al.* Dual-color magnetic-quantum dot nanobeads as versatile fluorescent probes in test strip for simultaneous point-of-care detection of free and complexed prostate-specific antigen. *Biosens. Bioelectron.* **145**, 111719 (2019).

111. Niedbala, R. S. *et al.* Detection of analytes by immunoassay using Up-Converting Phosphor Technology. *Anal. Biochem.* **293**, 22–30 (2001).

112. Corstjens, P. *et al.* Use of up-converting phosphor reporters in lateral-flow assays to detect specific nucleic acid sequences: A rapid, sensitive DNA test to identify human papillomavirus type 16 infection. *Clin. Chem.* **47**, 1885–1893 (2001).

113. Hampl, J. *et al.* Upconverting phosphor reporters in immunochromatographic assays. *Anal. Biochem.* **288**, 176–187 (2001).

114. Kim, J. *et al.* Rapid and background-free detection of avian influenza virus in opaque sample using NIR-to-NIR upconversion nanoparticle-based lateral flow immunoassay platform. *Biosens. Bioelectron.* **112**, 209–215 (2018).

115. He, H. *et al.* Quantitative Lateral Flow Strip Sensor Using Highly Doped Upconversion Nanoparticles. *Anal. Chem.* **90**, 12356–12360 (2018).

116. You, M. *et al.* Household Fluorescent Lateral Flow Strip Platform for Sensitive and Quantitative Prognosis of Heart Failure Using Dual-Color Upconversion Nanoparticles. *ACS Nano* **11**, 6261–6270 (2017).

117. Gong, Y. *et al.* A portable and universal upconversion nanoparticle-based lateral flow assay platform for point-of-care testing. *Talanta* **201**, 126–133 (2019).

118. Khreich, N. *et al.* Detection of Staphylococcus enterotoxin B using fluorescent immunoliposomes as label for immunochromatographic testing. *Anal. Biochem.* **377**, 182–188 (2008).

119. Baeumner, A. J., Jones, C. & Yee, C. A generic sandwich-type biosensor with nanomolar detection limits. 1587–1593 (2004). doi:10.1007/s00216-003-2466-0

120. Edwards, K. a & Baeumner, A. J. Optimization of DNA-tagged dye-encapsulating liposomes for lateral-flow assays based on sandwich hybridization. *Anal. Bioanal. Chem.* **386**, 1335–43 (2006).

121. Edwards, K. A., Korff, R. & Baeumner, A. J. Liposome-Enhanced Lateral-Flow Assays for Clinical Analyses. in *Methods in molecular biology (Clifton, N.J.)* **1571**, 407–434 (2017).

122. Urusov, A. E., Zherdev, A. V. & Dzantiev, B. B. Towards Lateral Flow Quantitative Assays: Detection Approaches. *Biosensors* **9**, 89 (2019).

123. Yang, J. *et al.* Detection platforms for point-of-care testing based on colorimetric, luminescent and magnetic assays: A review. *Talanta* **202**, 96–110 (2019).

124. Nash, M. a, Waitumbi, J. N., Hoffman, A. S., Yager, P. & Stayton, P. S. Multiplexed enrichment and detection of malarial biomarkers using a stimuli-responsive iron oxide and gold nanoparticle reagent system. *ACS Nano* **6**, 6776–85 (2012).

125. Marquina, C. *et al.* GMR sensors and magnetic nanoparticles for immuno-chromatographic assays. *J. Magn. Magn. Mater.* **324**, 3495–3498 (2012).

126. Ryu, Y., Jin, Z., Kang, M. S. & Kim, H. S. Increase in the detection sensitivity of a lateral flow assay for a cardiac marker by oriented immobilization of antibody. *Biochip J.* **5**, 193–198 (2011).

127. Wang, D. B. *et al.* Detection of Bacillus anthracis spores by super-paramagnetic lateral-flow immunoassays based on 'Road Closure'. *Biosens. Bioelectron.* **67**, 608–614 (2015).

128. Liu, D. *et al.* A modified lateral flow immunoassay for the detection of trace aflatoxin M1 based on immunomagnetic nanobeads with different antibody concentrations. *Food Control* **51**, 218–224 (2015).

129. Zheng, C., Wang, X., Lu, Y. & Liu, Y. Rapid detection of fish major allergen parvalbumin using superparamagnetic nanoparticle-based lateral flow immunoassay. *Food Control* **26**, 446–452 (2012).

130. Panferov, V. G., Safenkova, I. V., Zherdev, A. V. & Dzantiev, B. B. Setting up the cut-off level of a sensitive barcode lateral flow assay with magnetic nanoparticles. *Talanta* **164**, 69–76 (2017).

131. Lago-Cachon, D. *et al.* Scanning Magneto-Inductive Sensor for Quantitative Assay of Prostate-Specific Antigen. *IEEE Magn. Lett.* **8**, 1–5 (2017).

132. Moyano, A. *et al.* Magnetic immunochromatographic test for histamine detection in wine. *Anal. Bioanal. Chem.* **411**, 6615–6624 (2019).

133. Qin, Z. *et al.* Significantly Improved Analytical Sensitivity of Lateral Flow Immunoassays by Using Thermal Contrast. *Angew. Chemie Int. Ed.* **51**, 4358–4361 (2012).

134. Wang, Y. *et al.* Thermal Contrast Amplification Reader Yielding 8-Fold Analytical Improvement for Disease Detection with Lateral Flow Assays. *Anal. Chem.* **88**, 11774–11782 (2016).

135. Sinawang, P. D., Rai, V., Ionescu, R. E. & Marks, R. S. Electrochemical lateral flow immunosensor for detection and quantification of dengue NS1 protein. *Biosens. Bioelectron.* **77**, 400–408 (2016).

136. Aller Pellitero, M., Kitsara, M., Eibensteiner, F. & del Campo, F. J. Rapid prototyping of electrochemical lateral flow devices: stencilled electrodes. *Analyst* **141**, 2515–2522 (2016).

137. Ruiz-Vega, G., Kitsara, M., Pellitero, M. A., Baldrich, E. & del Campo, F. J. Electrochemical Lateral Flow Devices: Towards Rapid Immunomagnetic Assays. *ChemElectroChem* **4**, 880–889 (2017).

138. Zhu, X., Shah, P., Stoff, S., Liu, H. & Li, C. A paper electrode integrated lateral flow immunosensor for quantitative analysis of oxidative stress induced DNA damage. *Analyst* **139**, 2850–7 (2014).

139. Cinti, S., Moscone, D. & Arduini, F. Preparation of paper-based devices for reagentless electrochemical (bio)sensor strips. *Nat. Protoc.* **14**, 2437–2451 (2019).

140. Gonzalez-Macia, L., Morrin, A., Smyth, M. R. & Killard, A. J. Advanced printing and deposition methodologies for the fabrication of biosensors and biodevices. *Analyst* **135**, 845 (2010).

141. Li, Z. *et al.* Pen-on-paper strategy for point-of-care testing: Rapid prototyping of fully written microfluidic biosensor. *Biosens. Bioelectron.* **98**, 478–485 (2017).

142. Merck Millipore. IVD Lateral Flow – Sample, Conjugate and Absorbent Pad Basics. (2020). Available at: https://www.sigmaaldrich.com/technical-documents/articles/ivd-

immunoassay/lateral-flow/pads-chemistries-selections-specifications-and-conjugates.html.

143. GF/DVA - GE Healthcare Life Sciences. Available at: https://www.gelifesciences.com/en/us/shop/whatman-laboratory-filtration/whatman-dx-components/lateral-flow-pads/gf-dva-p-00759.

144. Fusion 5 - GE Healthcare Life Sciences. Available at: https://www.gelifesciences.com/en/us/shop/whatman-laboratory-filtration/whatman-dx-components/lateral-flow-pads/fusion-5-p-00787.

145. Vivid™ Plasma Separation Membrane - Pall Shop. Available at: https://shop.pall.com/us/en/medical/advanced-materials/diagnostics/vivid-plasma-separation-membrane-zidgri78lls.

146. Quesada-González, D. *et al.* Iridium oxide (IV) nanoparticle-based electrocatalytic detection of PBDE. *Biosens. Bioelectron.* **127**, 150–154 (2019).

147. Parolo, C., Medina-Sánchez, M., de la Escosura-Muñiz, A. & Merkoçi, A. Simple paper architecture modifications lead to enhanced sensitivity in nanoparticle based lateral flow immunoassays. *Lab Chip* **13**, 386–390 (2013).

148. Tsai, T.-T. *et al.* Development a stacking pad design for enhancing the sensitivity of lateral flow immunoassay. *Sci. Rep.* **8**, 17319 (2018).

149. Shao, X.-Y. *et al.* Rapid and Sensitive Lateral Flow Immunoassay Method for Procalcitonin (PCT) Based on Time-Resolved Immunochromatography. *Sensors* **17**, 480 (2017).

150. Quesada-González, D., Jairo, G. A., Blake, R. C., Blake, D. A. & Merkoçi, A. Uranium (VI) detection in groundwater using a gold nanoparticle/paper-based lateral flow device. *Sci. Rep.* **8**, 16157 (2018).

151. Li, J., McMillan, D. & Macdonal, J. Enhancing the signal of lateral flow immunoassays by using different developing methods. *Sensors Mater.* **27**, 549–561 (2015).

152. West, M., Walters, F., Phillips, S. & Rowles, D. Enhanced Performance of a Lateral Flow Assay. Available at: https://www.bbisolutions.com/pub/media/wysiwyg/technical_support/BBI_WHITEPAPER_A4_MORFFI_DIGITAL-linked.pdf. (Accessed: 27th February 2020)

153. Reagent Chemistries and Labels of Choice for Lateral flow. Available at: https://www.emdmillipore.com/INTERSHOP/static/WFS/Merck-Site/-/Merck/en_US/Freestyle/DIV-Divisional/Events/pdfs/lateral-flow-presentations/reagent-chemistries-and-labels-of-choice-for-lateral-flow.pdf.

154. Shim, W.-B., Kim, J.-S., Kim, M.-G. & Chung, D.-H. Rapid and sensitive immunochromatographic strip for on-site detection of sulfamethazine in meats and eggs. *J. Food Sci.* **78**, M1575–M1581 (2013).

155. Tsai, T.-T. *et al.* Development a stacking pad design for enhancing the sensitivity of lateral flow immunoassay. *Sci. Rep.* **8**, 17319 (2018).

156. Shao, X.-Y. *et al.* Rapid and Sensitive Lateral Flow Immunoassay Method for Procalcitonin (PCT) Based on Time-Resolved Immunochromatography. *Sensors (Basel).* **17**, (2017).

157. Yahaya, M. L., Zakaria, N. D., Noordin, R. & Razak, K. A. The Effect of Nitrocellulose Membrane Pore Size of Lateral Flow Immunoassay on Sensitivity for Detection of Shigella sp. in Milk Sample. *Mater. Today Proc.* **17**, 878–883 (2019).

158. UniSart® Nitrocellulose Membranes - The Substrate of Choice for Protein Assays. Available at: https://www.sartorius.com/resource/blob/89574/dc103586e857d533c5901961867f5ed9/broch-unisart-nitro-sl-1536-e-1--data.pdf.

159. Membrana Immobilon-P, PVDF; 0,45 µm, rollo de 26,5 cm x 3,75 m | IPVH00010. Available at: https://www.merckmillipore.com/ES/es/product/Immobilon-P-PVDF-Membrane,MM_NF-IPVH00010.

160. Nylon Membranes - Membrane Disc Filters | Sterlitech. Available at: https://www.sterlitech.com/nylon-membranes.html.

161. Li, H., Han, D., Hegener, M. A., Pauletti, G. M. & Steckl, A. J. Flow reproducibility of whole blood and other bodily fluids in simplified no reaction lateral flow assay devices. *Biomicrofluidics* **11**, 024116 (2017).

162. PES (Polyethersulfone) Membranes - Membrane Disc Filters | Sterli. Available at: https://www.sterlitech.com/polyethersulfone-pes-membrane-filters.html.

163. Tovey, E. R. & Baldo, B. A. Protein binding to nitrocellulose, nylon and PVDF membranes in immunoassays and electroblotting. *J. Biochem. Biophys. Methods* **19**, 169–183 (1989).

164. Asiaei, S., Bidgoli, M. R., ZadehKafi, A., Saderi, N. & Siavashi, M. Sensitivity and colour intensity enhancement in lateral flow immunoassay tests by adjustment of test line position. *Clin. Chim. Acta* **487**, 210–215 (2018).

165. Ragavendar, M. S. & Anmol, C. M. A mathematical model to predict the optimal test line location and sample volume for lateral flow immunoassays. *Conf. Proc. ... Annu. Int. Conf. IEEE Eng. Med. Biol. Soc. IEEE Eng. Med. Biol. Soc. Annu. Conf.* **2012**, 2408–11 (2012).

166. Wen, H. W., Borejsza-Wysocki, W., Decory, T. R. & Durst, R. A. Development of a competitive liposome-based lateral flow assay for the rapid detection of the allergenic peanut protein Ara h1. *Anal. Bioanal. Chem.* **382**, 1217–1226 (2005).

167. Apilux, A., Rengpipat, S., Suwanjang, W. & Chailapakul, O. Development of competitive lateral flow immunoassay coupled with silver enhancement for simple and sensitive salivary cortisol detection. *EXCLI J.* **17**, 1198–1209 (2018).

168. Posthuma-Trumpie, G. a, Korf, J. & van Amerongen, A. Development of a competitive lateral flow immunoassay for progesterone: influence of coating conjugates and buffer components. *Anal. Bioanal. Chem.* **392**, 1215–23 (2008).

169. Rey, E. G., O'Dell, D., Mehta, S. & Erickson, D. Mitigating the Hook Effect in Lateral Flow Sandwich Immunoassays Using Real-Time Reaction Kinetics. *Anal. Chem.* **89**, 5095–5100 (2017).

170. Corstjens, P. L. A. M. *et al.* A user-friendly, highly sensitive assay to detect the IFN-γ secretion by T cells. *Clin. Biochem.* **41**, 440–444 (2008).

171. Rivas, L., Medina-Sánchez, M., de la Escosura-Muñiz, A. & Merkoçi, A. Improving sensitivity of gold nanoparticle-based lateral flow assays by using wax-printed pillars as delay barriers of

microfluidics. *Lab Chip* **14**, 4406–4414 (2014).

172. Carrell, C. S. *et al.* Rotary manifold for automating a paper-based *Salmonella* immunoassay. *RSC Adv.* **9**, 29078–29086 (2019).

173. Yang, J. J., Oh, H.-B. & Hwang, S.-H. Paper-based speedy separation of amplified DNA (PASS-DNA): Potential for molecular point-of-care testing. *Sensors Actuators B Chem.* **286**, 101–103 (2019).

174. Tang, R. *et al.* A fully disposable and integrated paper-based device for nucleic acid extraction, amplification and detection. *Lab Chip* **17**, 1270–1279 (2017).

175. Nurul Najian, A. B., Engku Nur Syafirah, E. A. R., Ismail, N., Mohamed, M. & Yean, C. Y. Development of multiplex loop mediated isothermal amplification (m-LAMP) label-based gold nanoparticles lateral flow dipstick biosensor for detection of pathogenic Leptospira. *Anal. Chim. Acta* **903**, 142–148 (2016).

176. Chua, A., Yean, C. Y., Ravichandran, M., Lim, B. & Lalitha, P. A rapid DNA biosensor for the molecular diagnosis of infectious disease. *Biosens. Bioelectron.* **26**, 3825–3831 (2011).

177. Choi, D. H. *et al.* A dual gold nanoparticle conjugate-based lateral flow assay (LFA) method for the analysis of troponin I. *Biosens. Bioelectron.* **25**, 1999–2002 (2010).

178. Hagström, A. E. V *et al.* Sensitive detection of norovirus using phage nanoparticle reporters in lateral-flow assay. *PLoS One* **10**, e0126571 (2015).

179. Kim, J. *et al.* Orientational binding modes of reporters in a viral-nanoparticle lateral flow assay. *Analyst* **142**, 55–64 (2017).

180. Zhou, G., Mao, X. & Juncker, D. Immunochromatographic Assay on Thread. *Anal. Chem.* **84**, 7736–7743 (2012).

181. Meng, L.-L., Song, T.-T. & Mao, X. Novel immunochromatographic assay on cotton thread based on carbon nanotubes reporter probe. *Talanta* **167**, 379–384 (2017).

182. Jia, X., Song, T., Liu, Y., Meng, L. & Mao, X. An immunochromatographic assay for carcinoembryonic antigen on cotton thread using a composite of carbon nanotubes and gold nanoparticles as reporters. *Anal. Chim. Acta* **969**, 57–62 (2017).

183. Seth, M., Mdetele, D. & Buza, J. Immunochromatographic thread-based test platform for diagnosis of infectious diseases. *Microfluid. Nanofluidics* **22**, 45 (2018).

184. Lappalainen, T., Teerinen, T., Vento, P., Hakalahti, L. & Erho, T. Cellulose as a novel substrate for lateral flow assay. *Nord. Pulp Pap. Res. J.* **25**, 536–550 (2010).

185. Du, S., Lin, H., Sui, J., Wang, X. & Cao, L. Nano-gold capillary immunochromatographic assay for parvalbumin. *Anal. Bioanal. Chem.* **406**, 6637–6646 (2014).

186. Qu, X. *et al.* Development of a Nano-Gold Capillary Immunochromatographic Assay for Rapid and Semi-Quantitative Detection of Clenbuterol Residues. *Food Anal. Methods* **9**, 2531–2540 (2016).

187. Cao, E., Chen, Y., Cui, Z. & Foster, P. R. Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. *Biotechnol. Bioeng.* **82**, 684–690 (2003).

188. O'Farrell, B. Lateral Flow Technology for Field-Based Applications—Basics and Advanced

Developments. *Top. Companion Anim. Med.* **30**, 139–147 (2015).

189. Tian, T. *et al.* Distance-based microfluidic quantitative detection methods for point-of-care testing. *Lab Chip* **16**, 1139–1151 (2016).

190. Leung, W. *et al.* InfectCheck CRP barcode-style lateral flow assay for semi-quantitative detection of C-reactive protein in distinguishing between bacterial and viral infections. *J. Immunol. Methods* **336**, 30–36 (2008).

191. Mak, W. C., Beni, V. & Turner, A. P. F. Lateral-flow technology: From visual to instrumental. *TrAC Trends Anal. Chem.* **79**, 297–305 (2016).

192. Shah, K. G., Singh, V., Kauffman, P. C., Abe, K. & Yager, P. Mobile Phone Ratiometric Imaging Enables Highly Sensitive Fluorescence Lateral Flow Immunoassays without External Optical Filters. *Anal. Chem.* **90**, 6967–6974 (2018).

193. Zangheri, M. *et al.* A simple and compact smartphone accessory for quantitative chemiluminescence-based lateral flow immunoassay for salivary cortisol detection. *Biosens. Bioelectron.* **64**, 63–68 (2015).

194. Roda, A. *et al.* Smartphone-based biosensors: A critical review and perspectives. *TrAC Trends Anal. Chem.* **79**, 317–325 (2016).

195. Jiang, N. *et al.* Lateral and Vertical Flow Assays for Point-of-Care Diagnostics. *Adv. Healthc. Mater.* **8**, e1900244 (2019).

196. de Puig, H., Bosch, I., Gehrke, L. & Hamad-Schifferli, K. Challenges of the Nano-Bio Interface in Lateral Flow and Dipstick Immunoassays. *Trends Biotechnol.* **35**, 1169–1180 (2017).

197. Oh, Y. K., Joung, H.-A., Han, H. S., Suk, H.-J. & Kim, M.-G. A three-line lateral flow assay strip for the measurement of C-reactive protein covering a broad physiological concentration range in human sera. *Biosens. Bioelectron.* **61**, 285–289 (2014).

198. Li, J. & Macdonald, J. Multiplexed lateral flow biosensors: Technological advances for radically improving point-of-care diagnoses. *Biosens. Bioelectron.* **83**, 177–192 (2016).

199. Gao, Z. *et al.* Platinum-Decorated Gold Nanoparticles with Dual Functionalities for Ultrasensitive Colorimetric in Vitro Diagnostics. *Nano Lett.* **17**, 5572–5579 (2017).

200. Kim, H., Chung, D.-R. & Kang, M. A new point-of-care test for the diagnosis of infectious diseases based on multiplex lateral flow immunoassays. *Analyst* **144**, 2460–2466 (2019).

201. Mohd Hanafiah, K. *et al.* Development of Multiplexed Infectious Disease Lateral Flow Assays: Challenges and Opportunities. *Diagnostics* **7**, 51 (2017).

202. Qin, Q. *et al.* Algorithms for immunochromatographic assay: review and impact on future application. *Analyst* **144**, 5659–5676 (2019).

203. Liu, Z. *et al.* An improved detection limit and working range of lateral flow assays based on a mathematical model. *Analyst* **143**, 2775–2783 (2018).

204. Gantelius, J., Bass, T., Sjöberg, R., Nilsson, P. & Andersson-Svahn, H. A Lateral Flow Protein Microarray for Rapid and Sensitive Antibody Assays. *Int. J. Mol. Sci.* **12**, 7748–7759 (2011).

205. Choi, J. R. *et al.* An integrated lateral flow assay for effective DNA amplification and detection at

the point of care. *Analyst* **141**, 2930–2939 (2016).

206. Jauset-Rubio, M. *et al.* Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay. *Sci. Rep.* **6**, 37732 (2016).

207. Deng, X. *et al.* Applying strand displacement amplification to quantum dots-based fluorescent lateral flow assay strips for HIV-DNA detection. *Biosens. Bioelectron.* **105**, 211–217 (2018).

208. Ivanov, A. V., Safenkova, I. V., Zherdev, A. V. & Dzantiev, B. B. Nucleic acid lateral flow assay with recombinase polymerase amplification: Solutions for highly sensitive detection of RNA virus. *Talanta* **210**, 120616 (2020).

209. Lafleur, L. K. *et al.* A rapid, instrument-free, sample-to-result nucleic acid amplification test. *Lab Chip* **16**, 3777–3787 (2016).

210. Xu, Y. *et al.* Nucleic Acid Biosensor Synthesis of an All-in-One Universal Blocking Linker Recombinase Polymerase Amplification with a Peptide Nucleic Acid-Based Lateral Flow Device for Ultrasensitive Detection of Food Pathogens. *Anal. Chem.* **90**, 708–715 (2018).

211. Cheng, N. *et al.* Specific and relative detection of urinary microRNA signatures in bladder cancer for point-of-care diagnostics. *Chem. Commun.* **53**, 4222–4225 (2017).

212. Tang, R. *et al.* Improved sensitivity of lateral flow assay using paper-based sample concentration technique. *Talanta* **152**, 269–276 (2016).

213. Christau, S., Moeller, T., Genzer, J., Koehler, R. & von Klitzing, R. Salt-Induced Aggregation of Negatively Charged Gold Nanoparticles Confined in a Polymer Brush Matrix. *Macromolecules* **50**, 7333–7343 (2017).

214. Ruiz-Sanchez, A. J. *et al.* Tuneable plasmonic gold dendrimer nanochains for sensitive disease detection. *J. Mater. Chem. B* **5**, 7262–7266 (2017).

215. Findlay, J. W. A. & Dillard, R. F. Appropriate calibration curve fitting in ligand binding assays. *AAPS J.* **9**, E260–E267 (2007).

216. Holstein, C. A., Griffin, M., Hong, J. & Sampson, P. D. Statistical Method for Determining and Comparing Limits of Detection of Bioassays. *Anal. Chem.* **87**, 9795–9801 (2015).

217. Faber, N. (Klaas) M. The limit of detection is not the analyte level for deciding between "detected" and "not detected". *Accredit. Qual. Assur.* **13**, 277–278 (2008).

218. Armbruster, D. A. & Pry, T. Limit of blank, limit of detection and limit of quantitation. *Clin. Biochem. Rev.* **29 Suppl 1**, S49-52 (2008).

219. Quesada-González, D. *et al.* Signal enhancement on gold nanoparticle-based lateral flow tests using cellulose nanofibers. *Biosens. Bioelectron.* **141**, 111407 (2019).

220. Zheng, C., Wang, X., Lu, Y. & Liu, Y. Rapid detection of fish major allergen parvalbumin using superparamagnetic nanoparticle-based lateral flow immunoassay. *Food Control* **26**, 446–452 (2012).

221. Bai, Y. *et al.* A sensitive lateral flow test strip based on silica nanoparticle/CdTe quantum dot composite reporter probes. *RSC Adv.* **2**, 1778 (2012).

222. Zhao, P. *et al.* Upconversion fluorescent strip sensor for rapid determination of Vibrio

anguillarum. *Nanoscale* **6**, 3804–3809 (2014).

223. Ambrosi, A., Airò, F. & Merkoçi, A. Enhanced gold nanoparticle based ELISA for a breast cancer biomarker. *Anal. Chem.* **82**, 1151–6 (2010).

224. PLASMA SEPARATION: Why do you need it and how do you achieve it |. Available at: https://dcndx.com/plasma-separation-why-you-need-it/.

225. Nawattanapaiboon, K. *et al.* Hemoculture and Direct Sputum Detection of mecA-Mediated Methicillin-Resistant *Staphylococcus aureus* by Loop-Mediated Isothermal Amplification in Combination With a Lateral-Flow Dipstick. *J. Clin. Lab. Anal.* **30**, 760–767 (2016).

226. Xu, S. *et al.* Lateral Flow Immunoassay Based on Polydopamine-Coated Gold Nanoparticles for the Sensitive Detection of Zearalenone in Maize. *ACS Appl. Mater. Interfaces* **11**, 31283–31290 (2019).

227. Anfossi, L. *et al.* A Lateral Flow Immunoassay for the Rapid Detection of Ochratoxin A in Wine and Grape Must. *J. Agric. Food Chem.* **60**, 11491–11497 (2012).

228. Berlina, A. N., Taranova, N. A., Zherdev, A. V., Vengerov, Y. Y. & Dzantiev, B. B. Quantum dot-based lateral flow immunoassay for detection of chloramphenicol in milk. *Anal. Bioanal. Chem.* **405**, 4997–5000 (2013).

229. Chen, A. & Yang, S. Replacing antibodies with aptamers in lateral flow immunoassay. *Biosens. Bioelectron.* **71**, 230–242 (2015).

230. Wiederhold, N. P. *et al.* Interlaboratory and Interstudy Reproducibility of a Novel Lateral-Flow Device and Influence of Antifungal Therapy on Detection of Invasive Pulmonary Aspergillosis. *J. Clin. Microbiol.* **51**, 459–465 (2013).

Supplementary Table 1 – Costs associated with a single LF strip

Material/Reagent	**Cost in USD**
Laminated card	0.0080
Nitrocellulose membrane	0.0204

Conjugate pad	0.0044
Sample and Absorbent pad	0.0046
Antibodies for test and control lines	0.0450
Gold nanoparticles	0.1700
Conjugate pad buffer	0.0031
Antibodies for conjugate	0.1416
Sample pad buffer	0.0047
Total	**0.4018**

This estimation is based on the product prices provided by suppliers in 2019 and considering a 6 mm wide strip. To the final cost of a single LFA of 0.4018 $ must be added the cost for the dispenser (which can be around 20,000.00 $, but can be even lower for lab scale applications) and the salary of personnel/facility (not estimable since it may vary significantly between countries).