Application of aptamers as molecular recognition elements in lateral flow assays for analytical applications

Application of aptamers as molecular recognition elements in lateral flow assays for analytical applications

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Abstract

Owing to their ease in operation and fast turnaround time, lateral flow assays (LFAs) are increasingly being used as point-of-care diagnostic tests for a variety of analytes. In a majority of these LFAs, antibodies are used as a molecular recognition element. Antibodies have a number of limitations such as high batch-to-batch variation, poor stability, long development time, difficulty in functionalization and need for ethical approval and cold chain. All these factors pose a great challenge to scale up the antibody-based tests. In recent years, the advent of aptamer technology has made a paradigm shift in the point-of-care diagnostics owing to the various advantages of aptamers over antibodies that favour their adaptability on a variety of sensing platforms including the lateral flow. In this review, we have highlighted the advantages of aptamers over antibodies, suitability of aptamers for lateral flow platforms, different types of aptamer-based LFAs and various labels for aptamer-based LFAs. We have also provided a summary of the applications of aptamer technology in LFAs for analytical applications.

Keywords: aptamers; lateral flow assays; analytical applications; point-of-care; diagnostics; biosensing

1. Introduction

The last few decades have witnessed an enormous surge in chemical contaminants and microbial pathogens, primarily due to unregulated and unsustainable anthropogenic activities [1,2]. These contaminants and microbes are negatively impacting human and animal health by causing various disorders and diseases and are responsible for a high morbidity and mortality. Although the majority of these disorders or diseases are treatable today, timely diagnosis is the key. The contemporary approaches to detect various analytes, such as small molecules, antigens, toxins and whole bacterial cells, require centralized laboratory equipped with many sophisticated instruments including but not limited to spectrophotometer, High-Performance Liquid Chromatography (HPLC) system, Gas Chromatography (GC) system, and Liquid Chromatography System coupled to Mass Spectrometer (LCMS), Enzyme-Linked Immunosorbent Assay (ELISA) reader[3–5] and some modern instruments like automated blood culture system (e.g., BACTEC®), thermal cycler, Cartridge-Based Nucleic Acid Amplification Test (CB-NAAT) set-up and high throughput molecular diagnostic system[6–9]. Although all these instruments/tests, as mentioned above, provide a precise detection of analytes or disease-causing organisms when operated by skilled human resources, they are not suitable for decentralized operation or on-site application. Further, high costs associated with these tests and dependence on proprietary reagents limit their applications in low and middle-income countries, where resources are limited and disease burden is high[3,10].

To address these limitations, in the last three and a half-decade, the focus of the *in vitro* diagnostic (IVD) industry has shifted towards point-of-care diagnostics (POC), as POC tests can potentially allow a short sample-to-answer time with minimum human intervention[10–12]. The World Health Organization (WHO) issued the 'ASSURED' (affordable, sensitive, specific, user-friendly, robust, equipment-free and deliverable) criteria as a good framework for evaluating POC diagnostic tests for resource-limited settings [13]. Further, in the last

decade, the POC test has also evolved with a defined target product profile (TPP) that is useful at different levels such as homes (TPP1), communities (TPP2), clinics (TPP3), external laboratories (TPP4) and hospitals (TPP5)[14,15]. To match these criteria although, a variety of miniaturized and portable POC diagnostic/biosensing platforms have been introduced in the IVD market ranging from optical and fluorescence to electrochemical, none of these platforms, except immunochromatography or lateral flow, have fulfilled the 'ASSURED' criteria completely.

Additionally, the lateral flow format is the only diagnostic platform that has an unmatched ability to be used at various TPP levels ranging from TPP1 to TPP5. Due to these attributes, the first LFA (urine-based pregnancy detection kit) has gained significant momentum since its introduction by Unipath, and subsequently a range of lateral flow assays (LFAs) have been developed and reported for infectious diseases (e.g. malaria, human immunodeficiency virus infection), environmental pollutants (e.g. pesticides and antibiotics), toxins (e.g. snake venom and endotoxins), drugs (e.g. cocaine), metabolites (e.g. ATP) and heavy metals (e.g. mercury)[16–30]. Due to its ease in operation, fast turnaround time, minimum human intervention and wide acceptability, currently LFAs constitute a major share of IVD industry with ~6.0 billion US dollar (USD) in 2018 and expected to reach ~8.7 billion USD by 2023 with a compound annual growth rate (CAGR) of 7.7%[31]. Despite such popularity and huge market share, one of the major problems that LFAs face is their inconsistent performance from batch-to-batch. This is primarily because LFA manufacturers are heavily dependent on analyte-specific antibodies, primarily polyclonal ones. These antibodies are raised in animals and thus, due to the physiological variation among animals, the antibodies evince huge inter-batch variability[32]. Further, it is difficult to obtain a high-affinity antibody for non-immunogenic analytes like small molecules (pesticides and antibiotics). To address these inherited limitations of antibodies in the last decade, a large number of the research group have used structured

nucleic acids called ‘aptamers’ for LFA[33]. Aptamers, also known as chemical antibodies, are ssDNA or RNA molecules that acquire a complex 2D or 3D structure and behave like a synthetic receptor for their cognate analyte and bind to it with high affinity and selectivity like an antibody[12,34]. They have several distinct advantages over antibodies, including the *in vitro* generation, ease in functionalization, amenability to synthesize at large scale without compromising their quality, no requirement of the cold chain for storage, and ability to undergo target-induced structural switching[12,32,35]. A brief comparison of these attributes of aptamers with antibodies are presented in **Table 1**. Due to these added advantages, aptamers are intensely investigated to replace antibodies in all possible diagnostic formats[36–39], including lateral flow devices[40]. Indeed, advent of aptamer technology has given a new dimension to the IVD industry, which is evident from the its rapid growth. The global IVD market value is forecasted to reach ~92.54 billion USD in 2023, with a CAGR of ~6.6%. The aptamer industry itself is one of the fastest-growing industries that is expected to reach ~245 million USD by 2020 with a compound annual growth rate of ~17.9 % and there is an enormous potential for application of aptamers in IVD. In the current review, we have attempted to highlight the process of aptamer selection in general, analytical application of aptamers, design of aptamer-based lateral flow assay (ABLFA), use of various signalling molecules in ABLFA, various formats of LFA and analytical application of various ABLFA, and finally discussed the future of ABLFA.

2. Aptamer development through SELEX

Aptamers are developed with an iterative *in vitro* evolution process called Systematic Evolution of Ligands by EXponential enrichment (SELEX)[41]. This process starts by subjecting a random oligonucleotide library to nonspecific analytes or matrix being used for aptamer selection to rule out the possibility of getting non-specific binders. These sequences that are bound with these non-target analytes are discarded. After this, the remaining

oligonucleotide pool (sequences that do not bind with the non-target analytes) population is incubated with a target of interest against which we intend to develop aptamers. Here, only the sequences that bind to the target are selected and the unbound sequences are discarded. The bound sequences are then eluted from the target and amplified using polymerase chain reaction (PCR). Next, the PCR-obtained dsDNA product is converted into ssDNA and subjected to another round of SELEX (**Figure 1**). Each successive round of SELEX involves a gradual increase in the selection pressure, i.e., the concentration of non-target analytes is increased in the counter selection, and the concentration of the specific target analyte is decreased. In addition, the stringency of selection is increased as it progresses by increasing the number of washes and the strength of detergent in wash buffer. This, along with the increasing iterative cycle numbers, ensures the high affinity and selectivity of aptamers to its cognate analyte by the end of SELEX. The products of different cycles are checked for their affinity and selectivity for the target analyte, and finally, the products from the best performing cycle number are cloned and sequenced and can be sequenced without cloning using next generation sequencing (NGS) approach. This concludes the process of SELEX, and the obtained sequence/s is/are then chemically synthesized and used for further characterization and downstream applications.

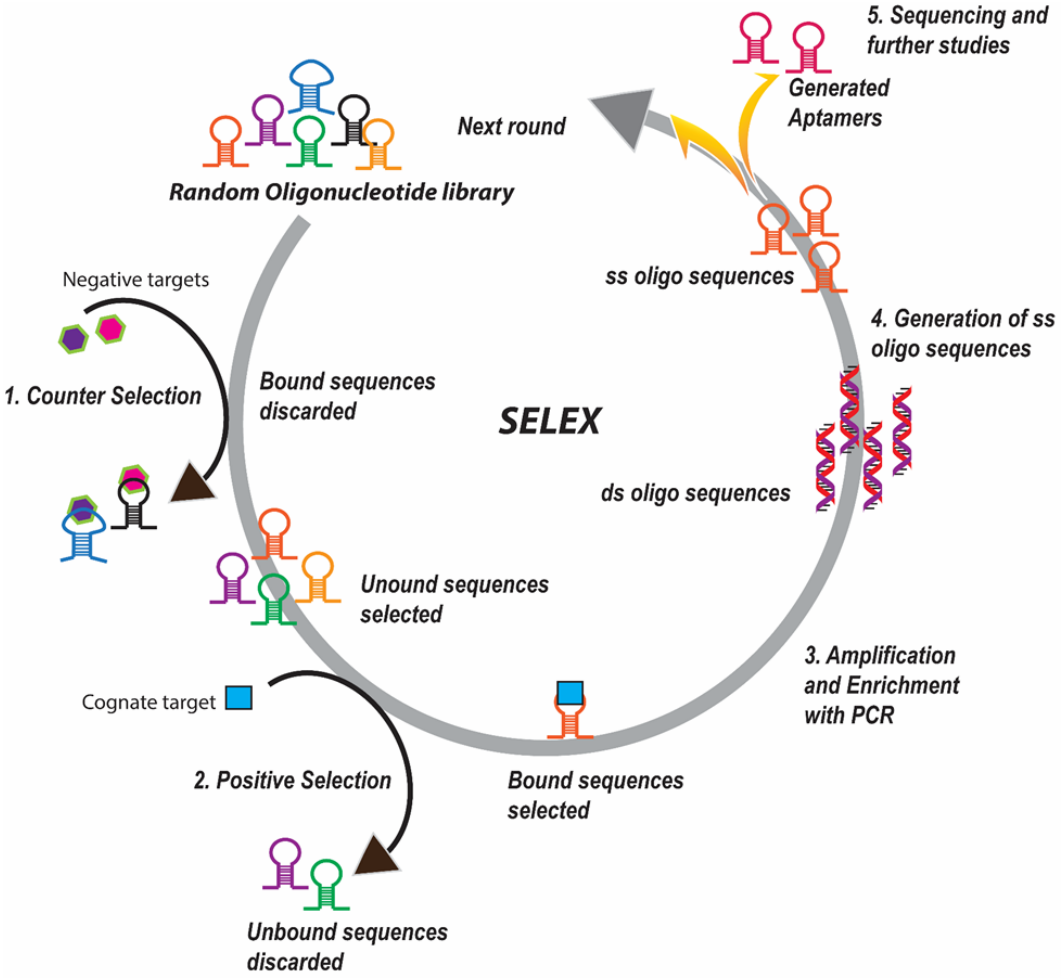


Figure 1. Schematic presentation of the SELEX process. A typical SELEX cycle involves four major steps 1. Reacting the random oligonucleotide library with nonspecific targets, called counter-selection to discard sequences having an affinity for nonspecific analytes; 2. positive selection, selecting the sequences binding with the target analyte; 3. Enrichment of the sequences from positive selection with PCR and generating dsDNA sequences; 4. These dsDNA sequences are then converted into ssDNA sequences and subjected to the next round of SELEX. After the desired number of cycles, the products are cloned and sequenced, i.e., step 5.

Traditional SELEX provides an overall approach for aptamer selection whose target range initially remained very restricted in nature. However, with the realization of the potential of the aptamers technology [42,43], tremendous amount of interest was shown by scientific

community in developing aptamer against variety of targets [40–44, 21,35,45–51]. This also resulted in various modification in SELEX strategy to suite the requirements better. For instance, many targets were difficult to immobilize particularly the small targets[56,57], with some protein molecules immobilization results in denaturation thus the developed aptamers could not recognize proteins in their native state[58]. Further, the traditional SELEX is very tedious and both labour and resource intensive. Over the years many modified SELEX approaches have been developed to make the SELEX process more efficient by addressing the shortcomings of traditional SELEX approach [48,59–62]. In the below section we would discuss some of the modified SELEX strategies.

2.1. Capillary electrophoresis SELEX (CE- SELEX)- Partitioning of the binder sequences from non-binders is one of the most crucial steps in SELEX. CE-SELEX segregates the binders from the non-binders based on the differential mobility of the non-binders and binder-target complex. Typically, first the oligonucleotide pool and the target are mixed in solution and allow the binding of the sequences with the target to form binder-target complex. After this, the solution is loaded onto a CE- capillary, and is then subjected to high voltage to resolve the components in the mixture based on their electrophoretic mobility in the capillary. The electrophoretic mobility is dependent on charge per unit area on the analytes. The free non-binders, because of their similar charge travels as a band and at a faster rate. As the CE-SELEX is performed at a pH at which the target does not carry any charge so the charge per unit area in binder-target complex is reduced, thus the mobility of the binder-target complex is considerably slowed when compared to free non-binders. This results in two discrete and distant bands which can be separately eluted to minimize cross contamination[63], (**Figure 2)**. Mendonsa *et al* used the strategy to develop high affinity aptamers against Ig E, in just 2 rounds with a pool population which depicts almost 100 % binding[64]. CE-SELEX

is also advantageous in terms of freedom provided to the sequences to bind the target as in this process target is not immobilized and it is in free state, so a greater amount of area is available for binding site selection and also offers less steric hindrance. Though CE-SELEX is highly efficient in segregating the binders from non-binders but small amount of non-binders contamination remains as some of the non-binders tends to tail along the binders[65]. To reduces this contamination, recently Le *et al* introduced a new concept of ideal-filter capillary electrophoresis (IFCE), where the binding and non-binding sequences travel in opposite direction. They used this strategy to develop aptamers against MutS protein in a single step[65]. CE SELEX is a form of microfluidics SELEX, and later many modification has been method notably Bead–based microfluidic SELEX (magnetic and non-magnetic), Sol-gel–based microfluidic SELEX and Integrated microfluidic SELEX systems, which are reviewed in detailed somewhere else[66].

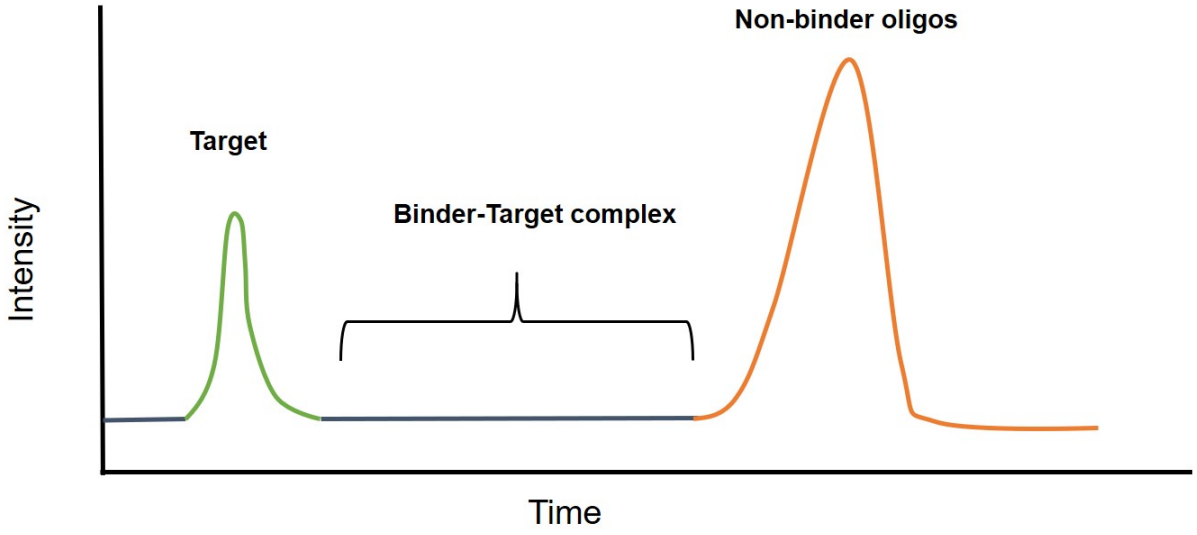


Figure 2. A typical electropherogram of CE-SELEX separation, here due to different electrophoretic mobilities, binder-target complex has different elution window than the

unbound sequences. The collection window for the binder sequences (from the complex) starts from the end of the free protein peaks to the starting of the non-binder sequence[67].

2.2. Capture-SELEX

Conventional SELEX strategies involves immobilization of the target on some matrix, this approach works comfortably with large protein targets which even after immobilization on matrix are still left with large exposed area which could be scanned for potential binding sites. However, with small targets it becomes a bit tricky, first of all it is difficult to immobilize a small target on a matrix, even if it is done it further reduces the already scanty area available for binding site (aptatope) scanning. Furthermore, immobilization of the small targets also induces steric hindrance that limit the interaction of aptamer libraries with target [56–58] . To overcome these limitations Stoltenburg *et al* banked upon the structural switching ability of the aptamers first described by Nutiu *et al* according to which a aptamer which is in duplex structure (bound to a partially complementary sequence), leaves this partially complementary sequence and forms a different complex structure upon binding with its target[44]. Stoltenburg *et al* improvised this method by immobilizing the oligonucleotide pool that was tethered with a complementary sequence which in turn is bound with the matrix. Upon binding with the target the aptamer sequences breaks the duplex structure with the capture oligo and detaches from it (**Figure 3**)[60]. This sort of SELEX strategy is very useful for small targets and targets which are difficult to immobilize likes the organic targets or targets with few or no suitable functional groups.

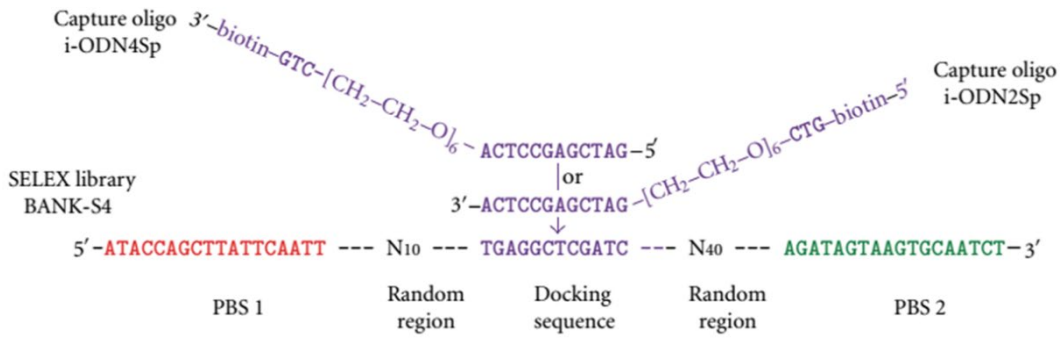


Figure 3. The oligonucleotide pool (BANK-S4 library) is hooked to capture oligo by forming a duplex between the complementary sequences. The capture oligo is immobilized on a matrix. Upon binding with a target, the oligo sequence leaves the capture oligo, which then can be harvested and amplified. (Reproduced from [60], Copyright Hindawi Publishing Corporation, 2012)

The strategy later on become the template for development of more SELEX strategies, where the targets were not immobilized but oligonucleotides were immobilized on some matrix and their structural switching abilities were banked upon for their detachment from the matrix. For instance, the ssDNA oligonucleotides are known to adsorb on the surface of Graphene oxide (GO) via π-π stacking[68], but upon binding with their target they leave they surface of GO, this property has been used to develop GO-SELEX, where first the oligonucleotides are bound on GO matrix and then oligonucleotide laden GO matrix is subjected to targets, and the binders then leave the GO matrix upon binding with the target[18,69,70]. Similarly ssDNA oligonucleotides also adsorbs onto the surface of gold nanoparticle[71] and recently this property has been employed to use gold nanoparticles based SELEX to develop aptamers against Porphyrin[72].

2.3. Cell SELEX

Cell SELEX uses live cells for aptamer selection. First described by Homann *et al* in 1999, where they used live *Trypanosoma brucei*, a parasitic protozoa causing African trypanosomiasis or sleeping sickness method for aptamer selection[73]. Here the oligonucleotide pool is subjected to the live cells directly and then the binding sequences are amplified and then enriched in an iterative manner. The strategy was particularly useful in cases where no prior knowledge of the target is available, and the targets have to be in their native conformations for successful aptamer development. As the cell membranes have numerous exposed components that all can serve as potential targets, cell SELEX can develop aptamers against many targets simultaneously[74,75]. The method has been successfully used to develop aptamers against many membrane proteins both known and unknown[62], interestingly development of the aptamers against the unknown proteins also leads to discovery of the target proteins which at times serves as biomarkers. For instance, Berezovski *et al* used cell SELEX to develop a technology, they named "Aptamer-Facilitated Biomarker Discovery (AptaBiD)", where the first aptamers are developed against whole cells and then the aptamer binding component is identified with mass spectroscopy. Using the technology, they identified previously unknown markers on immature dendritic cells (CXorf17 protein, galectin-3, glycoprotein NMB, lipoprotein lipase) and mature dendritic cells (copine-2)[51]. Such biomarkers discovery can potentially revolutionize the diagnostic research.

255 **Table 1. Comparison of aptamers with antibodies**

Properties	Antibody	Aptamer
Generation and synthesis	*In Vivo* selection, raised in animals	*In Vitro* Selection
	Selection process cannot be tailored on demand.	Selection process can be tailored as per need, selection pressure can be controlled as per requirement to obtain highly specific and affine binders.
	Produced in animal or by recombinant technology	Chemically synthesised in *in vitro* conditions
	Difficult to raise antibodies against non-immunogenic entities	Can be developed against non-immunogenic entities also
	High cost of production	Economical cost of production
	Batch-to-batch variation	Negligible batch-to-batch variation
Stability	Requires stringent storage conditions (cold storage)	Can withstand a range of storage conditions
	Low shelf life	Higher shelf life

	High susceptibility to change in pH, temperature and ionic concentrations	Relatively immune to change in pH, temperature and ionic concentrations
	Stability cannot be increased	Stability can be increased by chemical modifications
Modification, specificity and affinity	Comparable specificity and affinity with aptamers	Comparable specificity and affinity with antibodies
	Affinity and specificity can be tailored	Affinity and specificity can be tailored on demand
	Modification is challenging and sometime on modification antibody loss their binding	Amenable to modifications
	Difficulties in immobilization	Immobilization is comparatively easier
Structural Switching	On binding to its target antibody does not undergoes target-induced structural change.	Aptamers can easily undergo target-induced structural change that makes them an ideal molecular recognition element for electrochemical sensing platform.

3.Analytical applications of aptamers

Due to ease in adapting aptamers on to various diagnostic/sensing platforms in recent years, aptamers have been successfully utilized to detect a variety of analytes for clinical, biomedical,

plant pathology, food safety, and environmental applications. For the aforementioned applications, aptamers have been generated for heavy metals, drugs, hormones, proteins, glycoproteins, antigen-based biomarkers, enzymes, toxins, cancer cells, fungal, viral, and bacterial pathogens. Further, the last decade has witnessed a surge for a range of aptamer-based homogenous assays (bind and detect or no-wash assays) for a variety of analytes ranging from small molecules to whole-cell using fluorescent aptamers of a combination of aptamer with gold nanoparticles (GNPs). Easy and well-controlled synthesis of GNPs spurred great attention in the IVD community as a signalling or reporter element for antibody/aptamer-based rapid diagnostic tests (RDTs) with visual readout. Due to the unique property of aptamers to being adsorbed on to the surface of GNPs and release of such GNPs adsorbed aptamer molecules in the presence of its cognate analytes has opened a new avenue in the field of RDTs as these RDTs are immobilization free and can detect an analyte in solution within few minutes. However, signal generation (colour change) in many of these tests depends on the salt-induced aggregation of GNPs. The amount of salt required to aggregate GNPs is never uniform and highly user-dependent and also depends on the number of steps you are adding salt to GNPs. To overcome these limitations more recently, the intrinsic peroxidase-like activity of GNPs (NanoZyme) was explored in combination with the high affinity and specificity of ssDNA aptamers. In such a system similar to salt-induced aggregation-based GNPs-aptamer sensors, the aptamer is adsorbed on to the GNPs surface. This adsorption, in turn, 'turn-off' the NanoZyme activity of GNPs. However, the presence of cognate target induces a structural change in aptamers leading to the release of aptamers from GNPs surface with subsequent 'turn-on' activity of NanoZyme. This approach yields a visual readout that allows the user to measure the analytes, both qualitative and quantitatively (using UV-vis spectrophotometer). Being a generic approach, it was successfully utilized for the detection of small molecules (antibiotics and pesticide), protein (thrombin) and whole bacterial cell (*Pseudomonas*

285 *aeruginosa*). Although this approach allows qualitative and quantitatively detection, it is a
286 multistep process. As each step introduces some probability of error in a diagnostic test; thus,
287 it is imperative to have a single step diagnostic assay for POC application for the detection of
288 various analytes. Aptamer-based lateral flow format is an incredible diagnostic assay that
289 allows the detection of an analyte in a single step. Majority of LFAs requires only the addition
290 of sample followed by a room temperature incubation for 5-10 minutes. Most importantly, it is
291 suitable for TPP1 and TPP2 as even a layperson can perform this assay. In the current review,
292 we have attempted to highlight the importance and utility of aptamer technology in lateral flow
293 assays in analytical applications.

294 **4. Lateral flow assay principle and design**

295 Lateral flow assays (LFAs) have gained significant recognition, especially in resource-
296 constrained settings, primarily due to their ease of use, low cost, test rapidity, disposable
297 format, small sample volume required, and ability to use at the point-of-care [12,22,55,76].

298 LFAs are generally composed of a strip fabricated onto the surface of a porous membrane such
299 as nitrocellulose, which consists of a sample pad, conjugate pad, test, and control line on a
300 nitrocellulose membrane and an absorbent pad, all mounted on a backing card as illustrated in
301 (**Figure 4**) [12,22,55,76,77]. Nitrocellulose membranes are inexpensive and offer a high
302 affinity for proteins and other molecules. Membranes are available in different grades. The
303 grade used will affect the sensitivity due to the wicking rate and the specificity due to
304 nonspecific adsorption over test and control strips. [22].

305 The liquid sample is added to the sample pad, usually made of cellulose or glass fiber, which
306 can contain a filter and reagents that adjust the assay conditions such as the pH. The sample
307 then migrates laterally towards the conjugation pad via capillary action. The liquid in the
308 sample hydrates a detection label in the pad consisting of a molecular recognition element

(MRE, aptamer) coupled to a reporter molecule, which is always present in excess amount. If the target is present, it will form a complex [55]. The sensitivity of the assay can be adversely affected by poor preparation and release of the conjugate [22]. The sample migrates further to the test line, which contains capturing molecules such as aptamers or antibodies. These capturing molecules bind to the target, which is still coupled to the MRE and the reporter and immobilizes the complex. A signal is produced through the enrichment of reporter molecules, leading to a positive signal or colour change [55]. The colour intensity corresponds to the quantity of target with can be measured with the naked eye or quantified using an optical strip reader [12,55]

The unbound MRE-reported conjugate is then moved towards the control line, which also contains a capturing agent. This may be an oligonucleotide complementary to the aptamer, and a visible colour change or other signal production in the control line validates the performance of the LFA [12,55,77].

The purpose of the absorbent pad is to prevent backflow, maintaining the flow rate of the sample through capillary action [55,77]. To coordinate fluid flow, adjacent membranes must be overlapped [55]. The absorbent capacity can significantly affect the results of the assay [22].

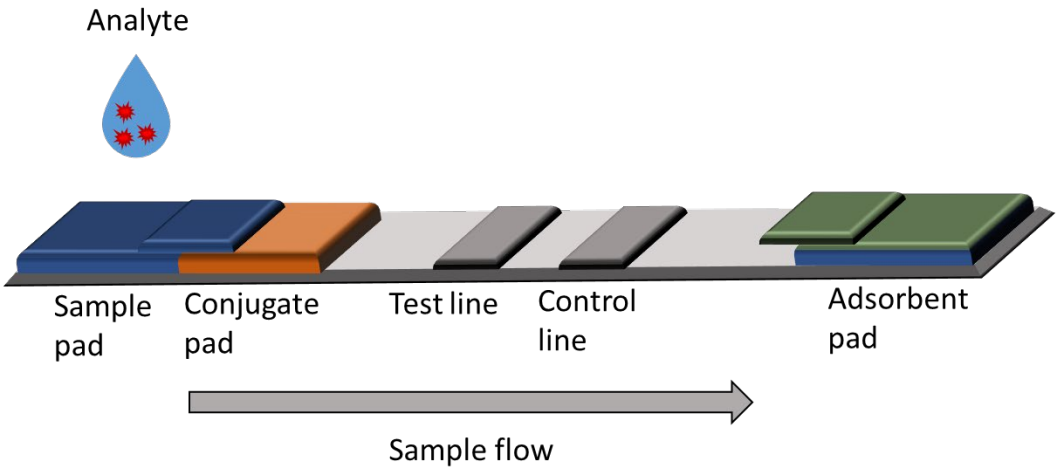


Figure 4. An example of a lateral flow assay. This consists of a sample pad, conjugate pad, test, and control line on a nitrocellulose membrane and an absorbent pad, all mounted on a backing card.

Difficulty in developing LFAs includes the possibility of false negatives due to the hook effect. The hook effect is caused by a high target concentration, which can prevent signal generation. The other difficulty is false-positive results due to non-specific binding. Additional advantages and limitations are stated in **Table 2**. Despite these difficulties, due to ease in operation and ability to deliver rapid sample-to-answer, LFAs remain potent tools for routine diagnostics. To date, these formats have been widely used to detect a large number of targets, including proteins, toxins, hormones, ions, whole cells, and viruses [55]. Multiple formats of LFAs have been published such as sandwiches, competitive, and multiplex [12].

Table 2. The advantages and limitations of lateral flow assays.

Advantages	**Limitations**
Low cost	Speed of capillary action cannot be controlled
Fast analysis of results	Generally qualitative or semi-quantitative

User-friendly	Batch reproducibility can vary
Microfluidic	Cross-reactivity can occur
Generally, it does not require sample pre-treatment	Hook effect
No or little requirement for electricity	Can be difficult to construct a successful conjugate
Wide range of applications	Optimization is difficult
Can be multiplexed	

340

341 **4.1 Sandwich format**

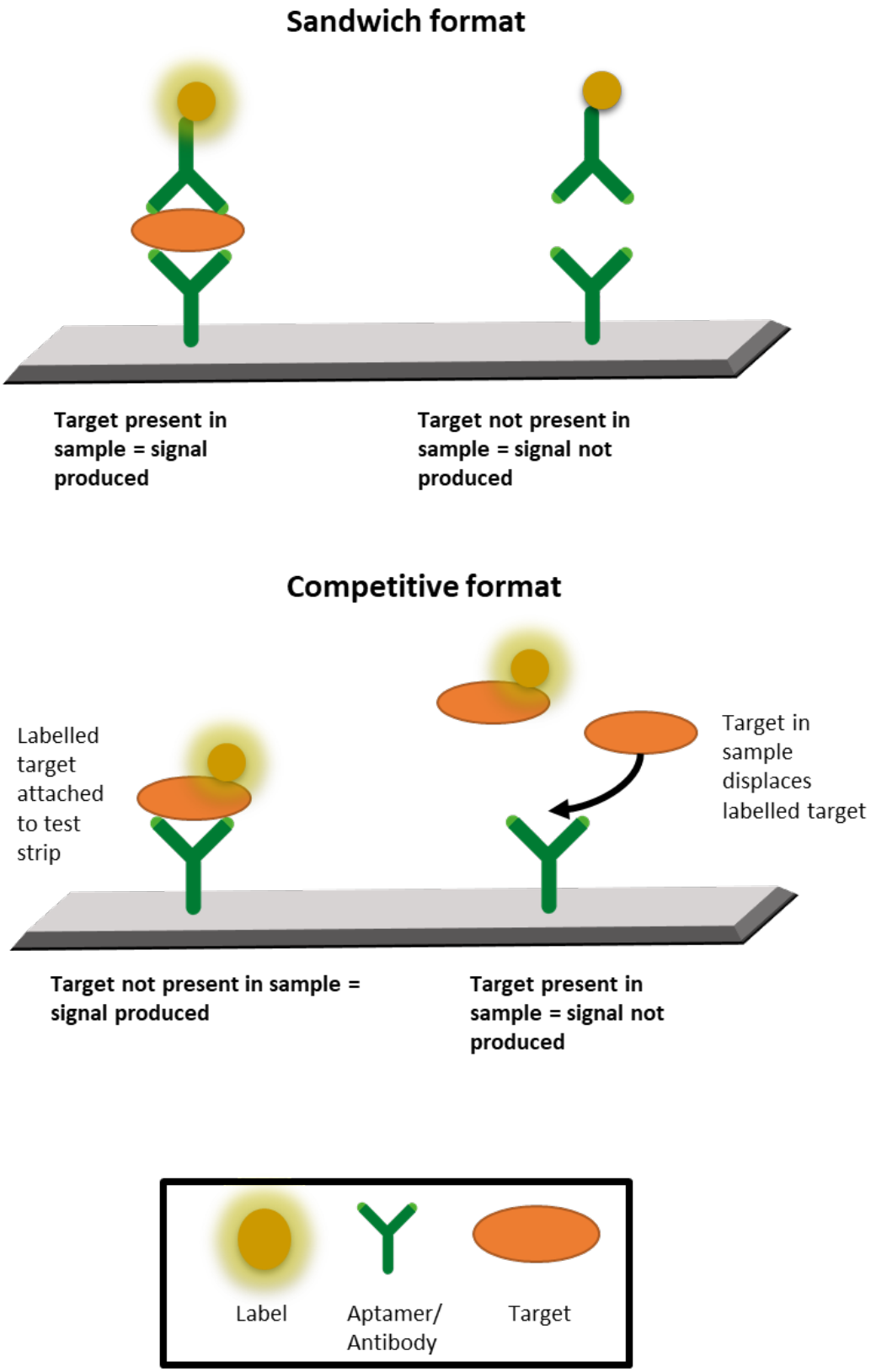
342 A sandwich LFA consists of a molecular recognition element (MRE, aptamer) coated with a
343 reporter label, which may be an enzyme/ gold nanoparticles (GNPs)/ fluorescent dye that reacts
344 with the target of interest to form a complex on the conjugate pad [29]. Labels will be discussed
345 in more detail later in this review. Capillary action then drives the target-MRE complex to the
346 test line containing a secondary MRE in the form of an aptamer or antibody, which binds to
347 form a sandwich with the target in the middle. This results in a visible change in colour on the
348 test line, as illustrated in (**Figure 5**). [12,55] . Excess MRE conjugate will be captured in the
349 control line by a secondary MRE, as described above. Generally, the sandwich format of LFA
350 is preferred, due to its high specificity and sensitivity because of the use of two MREs targeting
351 different epitopes on the same analyte. Other advantages over other formats include a simpler
352 readout mechanism and the ability to modify probes [55]. This format is more suitable for large
353 molecular weight analytes like proteins or antigens.

354 **4.2 Competitive format**

355 For some LFA designs, obtaining an adequate matching pair of MREs during selection is
356 difficult. Therefore, some assays must use a competitive format instead. This is especially true

for the detection of low molecular weight compounds [22,55]. This format consists of a sample pad pre-loaded with the coated MRE conjugate. Pre-immobilised within the test line is the target of interest that can bind with the labelled conjugate. The control line contains a pre-immobilised secondary aptamer/complementary sequence of aptamer or antibody which can bind to the MRE conjugate [12].

When the liquid reaches the test line, it contains both labelled targets and unlabelled targets. Both the targets (the one in the sample to be tested and the one pre-loaded into the test zone) compete to bind with the MRE conjugate [29]. If the sample contains the target of interest, a visible colour change can be seen in the control line, but not the test line. A negative result is indicated with the appearance of a visible colour change in both the control line and the test line, as illustrated in **Figure 5** [12,55].



368

369 **Figure 5. The sandwich and competitive format of LFA.** The sandwich format involves two

370 MREs, in this example, aptamers, binding to the target in the middle, forming a "sandwich."

371 The competitive format consists of an MRE with the target to be detected already found. If the
372 target is present within the sample, it will compete for binding.

373 **4.3 Absorption-Desorption Format**

374 It has previously been observed that target binding can induce desorption of aptamers from the
375 surface of reporter molecules because a large proportion of bases are committed to a target
376 binding secondary structure rather than binding to the reporter molecule. The desorption format
377 of LFA is whereby nonspecific adsorption to a reporter molecule is utilized to determine
378 whether an aptamer is bound to a target [25]. The aptamer and reporter molecule are mixed,
379 and the aptamer is adsorbed to the surface of the reporter molecule. When the target is
380 introduced, the aptamers bind preferentially to the target and are desorbed from the reporter
381 molecule [78].

382 Alsager et al developed a desorption based LFA to detect 17β-estradiol. In the absence of the
383 target, the negatively charged aptamers coating a GNP surface, prevent the particles from being
384 captured on the test line. In the presence of the target, target binding causes the aptamer coating
385 to be removed from the GNP surface. The GNP surface is then exposed, which can then be
386 captured by a negatively charged protein which has a high affinity for GNPs. The control line
387 in this example consisted of lysozyme, a positively charged protein which will bind to all
388 GNPs, regardless of whether they are coated with aptamers [25].

389 **4.4. Multiplex LFAs**

390 To achieve efficient and high-throughput detection of targets, the simultaneous measurement
391 of multiple analytes must be accomplished. Multiplex LFAs generally fit into three categories:
392 they detect multiple targets on one strip, they detect multiple targets on multiple strips, or, more
393 recently, the integration of LFA and microarray in one device, as illustrated in F 4) [22].
394 Multiplexing has several benefits over uniplex, including a more rapid analysis as multiple

targets can be detected simultaneously, detection of co-infections in the case of infectious diseases, increased sensitivity, and the reduced overall cost of the assay [76,77].

4.5 Detecting multiple targets on one strip

Detection of multiple targets on one strip is typically achieved through additional MREs with the same label, usually probes that are spatially separated from each other [76]. This is the most popular format of multiplexing LFAs, as it requires the least amount of design changes to the architecture of the device. The maximum number of lines that have been included in a single strip is six, including the control strip [79]. It is more common to have 2-3 targets per strip.

One way to overcome the issue with the number of targets per strip is to change the shape of the detection site from a line to a dot, leading to a microarray design. The number of targets can then be significantly increased; up to 32 targets have been reported [77,80].

However, the interpretation of the results from multiple targets on one strip platform is more complicated than that of a simplex assay or multiple strip platform. Increasing the number of targets of interest when test strips are closely spaced together can interfere with identification [81]. To overcome this issue, test strips can be spaced further apart. However, this then means that the assay requires a higher sample volume, more materials, and an increase in time to result [77]. Another alternative is to use different coloured labels as illustrated in. **Figure 6b**. Each target can be identified based on colour, thus simplifying analysis [81].

4.6 Detecting multiple targets on multiple strips

Detection of multiple targets on one strip is, however, limited by space, the number of MREs, and the effect of flow by passing through multiple MREs [76]. Various multiplex LFA platforms have been developed through modification of the architecture of the platform, including devices that use forks, peace symbols, and discs with pathogen-specific strips [76,77]. This method collects one sample and distributes it in parallel between multiple strips

[77]. Each of these strips can also contain multiple test strips. One distinct advantage of multiple strips is that each strip can be developed separately and then put in the same device, without any further optimisation.

This format of multiplexing generally overcomes the issue with interference that is seen with single strip multiplexing. However, there will be a higher fabrication cost, and a larger volume of sample required in proportion to the number of targets detected [81]. Generally, each strip requires 50-100 µl of the sample, and that amount increases as the number of strips increases. Therefore, this format is most suitable for samples that have a high volume, such as urine, saliva, food safety, and environmental samples such as water.

It may be feasible to combine several multiplexing strategies in one device to increase the high-throughput ability of the device. However, this does increase the complexity of the device for the end-user. Therefore, there should be a balance between simplicity and high-throughput ability [77].

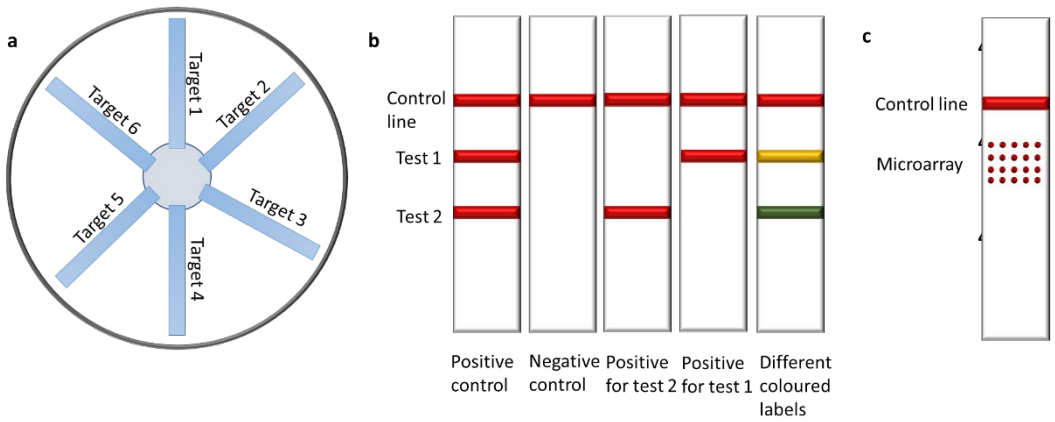


Figure 6. a. An example of a multiplex LFA system utilizing multiple strips. Each strip can target one or more analytes. The sample is added in the middle of this disk and flows along each of the strips via capillary action. Adapted from [77]. **b. An example of a multiplex LFA system utilizing multiple test lines on one strip.** Labels can be of the same colour or different

colours. Adapted from [23]. **c. An example of a multiplex LFA implementing microarray technology.** Adapted from [81].

5. Signalling/reporter molecules

The recent improvements in nanotechnology have led to the development of LFAs based on the conjugation of aptamers to a variety of nanomaterials [82]. Nanomaterials used as signalling/reporter molecules in LFAs contribute significantly to the sensitivity of the assay [22,83]. There are a variety of nanomaterials that can be used as reporter molecules in an LFA platform. These include quantum dots (QDs), coloured latex beads, liposome-encapsulated dyes, GNPs, magnetic particles, carbon, selenium and silver nanoparticles, upconverting phosphors, organic fluorophores, and textile dyes [22,77,82]. Some labels generate an immediately visible signal (colour change) while others produce a signal after additional steps. Naturally, those labels that give an immediate signal are preferred as this takes less time, less user interaction, and fewer materials.

For a label to be suitable as a reporter, it should be detectable at very low concentrations, and upon conjugation with biorecognition molecules, the properties of the label and the biorecognition probe should not change. Desirable features for a reporter molecule include stability and ease of conjugation with biomolecules [22]. The most common labels will be discussed in this review.

5.1 Gold Nanoparticles (GNPs)

For LFAs, the most common label used is GNPs due to their strong red colour upon a positive reaction, as illustrated in (**Figure 7**). GNPs are spherical particles that can be easily functionalized and have a high affinity towards biomolecules. The size and shape of the GNPs are dependent on the amount of sodium citrate utilized, and this generally determines their optical properties [82,83]. Compared to other labels, GNPs have enhanced stability, much

higher charge transfer values, and good quality of optical signalling [83]. Signal intensity is dependent on the ratio of GNPs to sample volume.

It is important to optimize the pH of the GNPs solution and the aptamer concentration to ensure the best binding efficiency. GNPs stability is greatly dependent on particle electrostatic repulsion. Therefore, the occurrence of ionic materials can cause great attractive forces, leading to aggregation of GNPs and a colour change from red to blue [83]. Stabilization of the GNP-aptamer complex is also important for eliminating the matrix effect, whereby other substances present in the sample have an unintended effect on the LFA [83,84].

Sensitivity is a function of the molar absorption coefficient, and therefore the optical properties of GNPs can improve sensitivity in LFA assays. This optical signalling sensitivity can be further improved by the mixture of silver and GNPs and also the addition of enzymes [22]. For example, horseradish peroxidase (HRP) can be immobilised onto the surface of GNPs, enabling its enzyme activity to catalyse the conversion of chromogenic substrates such as 3,3′,5,5′-tetramethylbenzidine (TMB), causing a stronger colour change than that of GNPs alone [85]. However, the addition of enzymes requires storage at a low temperature, limiting its use in low-resource areas.

Another way to improve optical sensitivity is through the addition of palladium (Pd). This composition changes the optical properties of the GNPs, resulting in a significant red-shift into the near infrared region which can be read by an infrared camera [86].

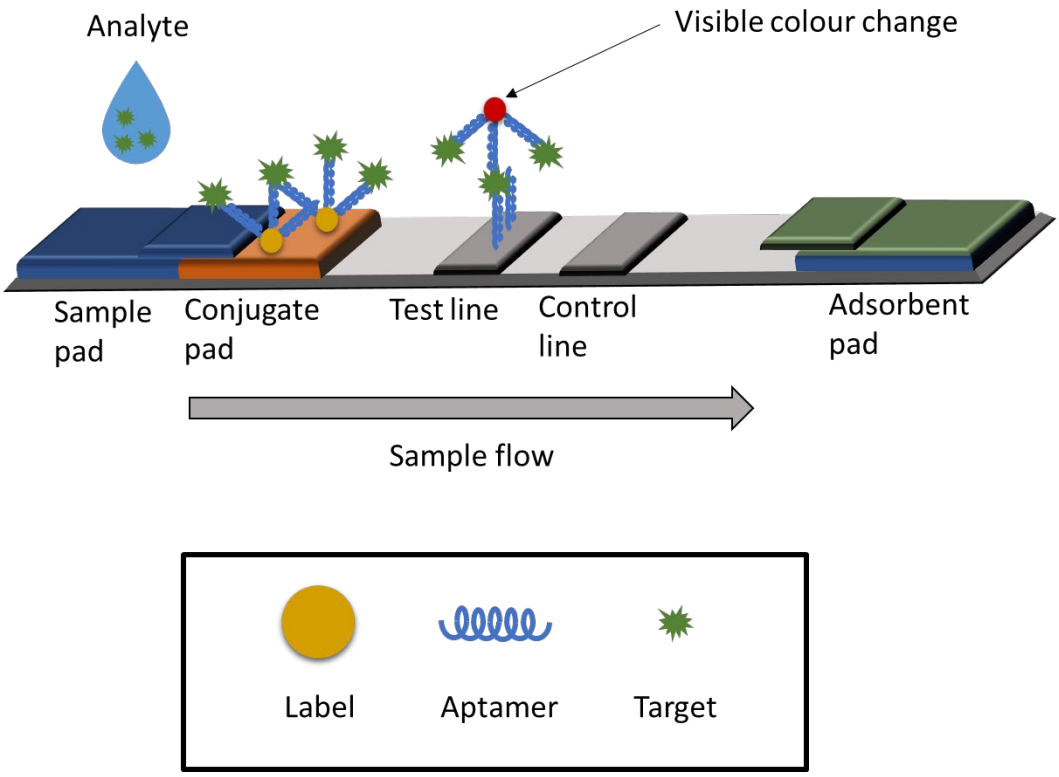


Figure 7. An example of a sandwich LFA utilizing GNPs. In this example, GNPs functionalized with aptamers are immobilized on the conjugate pad. If the target is present within the sample, it will bind and form a complex. This complex will flow to the test line, where another aptamer is immobilized. Binding to this secondary aptamer leads to a visible colour change from gold to red.

Kim et al developed a sandwich LFA for the detection of avian influenza whole virus utilizing GNPs. To our knowledge, this is the first time a sandwich format LFA has been developed for detection of avian influenza whole virus. Using this format, the authors were able to achieve a LOD of 2.09 × 105 EID_{50}/ml (egg infective dose) in duck's faeces [87].

A competitive sandwich LFA for the detection of ochratoxin A (OTA) in *Astragalus membranaceus* utilizing GNPs was developed by Zhou et al. in 2016. The authors looked at the effect of the size and shape of GNPs, as well as the amount of GNP-aptamer conjugate,

498 migration rate, and the addition of methanol on the sensitivity and general performance of the
499 LFA. This optimization leads to a visual LOD of 1 ng mL^{-1} achieved within 15 min [28]. These
500 results show why GNPs are one of the most popular labels for LFAs.

501 **5.2 Fluorescent and luminescent materials**

502 Fluorescent and luminescent molecules are another common label used in LFA due to its
503 simplicity and ability to multiplex [88]. The advantage of fluorescent molecules is that the
504 amount of fluorescence is directly related to the amount of target, making the assay
505 quantitative. However, fluorescent molecules can have a lower sensitivity due to
506 photobleaching and chemical and metabolic degradation; therefore, high stability is required
507 when using fluorescent molecules [22].

508 Research into labelling has led to the development of quantum dots (QDs) and are ideal
509 fluorescent labels [83]. QDs are semiconducting particles that have unique electrical and
510 optical properties, are water-soluble, and their surface structure allows for a wide variety of
511 molecules to be attached to their surface. Therefore, QDs are a potential substitute for organic
512 fluorescent molecules [88]. Similar to GNPs, QD size and shape also determines the optical
513 properties. A broad spectrum of wavelengths can be monitored, and a single light source is
514 required to excite QDs [81]. Like GNPs, QDs have high stability and molar absorption
515 coefficient. Compared to organic fluorescent molecules, QDs are less susceptible to metabolic
516 degradation. Bruno et al. reported a ten-fold improvement in the LOD of an LFA for the
517 detection of foodborne pathogens by changing from colloidal gold to QDs [89]. A limitation
518 compared to GNPs is that QD-biomolecule complexes are more difficult to achieve [22].
519 Combining PCR and a QD-LFA, Sapountzi et al. were able to detect SNPs in human DNA
520 with a visual LOD of 1.5 fmol. They utilized a UV lamp to enable visualization with the naked
521 eye [88].

Upconverting nanoparticles (UCNPs) are another option when choosing a label for LFA and are generally used in the sandwich format. UCNPs are excited in the infra-red region and emit in the high energy visible region, hence the term upconverting. One advantage of UCNPs over other fluorescent molecules is that they do not emit autofluorescence, and they do not photodegrade due to their excitation in the infra-red region. They can be produced from easily available bulk materials; however, preparation and labelling are laborious, requiring multiple steps, and batch to batch variability can affect the sensitivity of the LFA. When prepared correctly, 10 – 100 fold increases in sensitivity when compared to GNPs have been reported [22]. Jin et al. reported a multiplex LFA for the simultaneous detection of bacteria, small molecules, and ions utilizing UCNPs with red, green, and blue emission peaks. They also designed a portable reader in the form of a smartphone to improve the usability of this LFA [26].

In another LFA for the detection of vaspin, Ali et al. utilized UCNPs in a sandwich assay. The capture probe was immobilized on the test line, and the detection probe was functionalized with 100 nm diameter UCNPs. When vaspin is present, UCNPs are captured on the test strip, leading to fluorescence, which could be measured using a smartphone app [90]. The LOD was 39 pg mL^{-1}, far lower than that of Raston et al. [30].

5.3 Magnetic particles and aggregates

Magnetic particles are a relatively new addition to the labelling selection. An optical strip reader can measure the colour produced from these particles, or the magnetic particle signals themselves can be measured using a magnetic assay reader which is a device that measures the magnetic field change induced by magnetic particle binding. In comparison to optical signalling, magnetic signalling is more stable, has lower background noise, and sensitivity 10 to 1000-fold higher has been reported [22,83,91]. Magnetic particles have also been utilized to

reduce the matrix effect. Aptamer-magnetic particle conjugates bind with the target forming a complex. This complex can then be separated from the rest of the sample using a magnet [83].

For the detection of the Ebola virus glycoprotein, Duan et al. developed an LFA utilizing Fe_30_4 magnetic nanoparticles as a Nanozyme probe. Within 30 minutes, a LOD of 1 ng/mL could be achieved [92].

5.4 Enzymes

Enzymes can also be utilized as labels for LFAs; however, the addition of enzymes increases the number of steps required for the assay. The enzymatic reaction will produce a colour upon a positive result. The sensitivity of the LFA is dependent upon the enzyme-substrate combination; therefore, this must be considered when choosing to use enzymes with LFAs [22].

Tasbasi et al. developed an LFA for the detection of the bacteria *Listeria monocytogenes* utilizing silica nanoparticles. Silica nanoparticles were loaded with TMB, functionalized with aptamer, and then immobilized on the conjugation pad. The presence of the target releases the TMB, which causes a colour change due to horseradish peroxidase immobilized on the test line. This assay achieved a LOD of 53 cells/mL within 5 minutes [93].

5.5 Colloidal carbon

In comparison to other labels for LFA, colloidal carbon is relatively inexpensive, with a straightforward scale-up. The advantages of carbon nanoparticles are their black colour leading to easy detection and high sensitivity, simple functionalization with a wide variety of biomolecules, and they can be used to detect both low and high molecular weight targets[22]. Compared to GNPs, carbon nanoparticles show a higher colour density [83]. The limitation of carbon nanoparticles is non-specific absorption of proteins and biomolecules and the occurrence of irregularly shaped large particles [22]

5.6 Coloured latex beads

In comparison with GNPs, coloured latex beads are far more complex to synthesise. Coloured latex beads are generally prepared using a two-step method, whereby synthesis and dyeing are separate processes [94]. The advantage of using coloured latex beads is that there are multiple colours available, making them very useful for multiplexing, and the ability to conjugate them using a variety of techniques. Additionally, coloured latex beads are highly, uniform, reproducible and can be combined with a variety of detection chemicals [95].

A limitation of conventional latex beads is that they often leak, both in long term storage and in buffer solution, reducing the colour intensity. However the methods developed by Zhu et al and Liu et al can overcome this limitation [94,96].

Liu et al utilised their newly developed method for synthesising coloured latex beads to develop a LFA for the detection of brucellosis which resulted in an increased signal intensity in comparison to GNPs in the same assay [94].

6. Analytical application of aptamer-based lateral flow assays

In this section, we have discussed the utility of aptamer technology for the detection of various analytes on a lateral flow format. Broadly we have divided the application of aptamer as detection of protein and detection of small molecules.

6.1 Detection of proteins

Proteins/antigens can be key biomarkers in the detection of disease. The advantage of detecting proteins by LFA is that there are generally a diverse variety of molecules expressed on the cell surface which provide several aptamer binding sites, otherwise known as aptatopes [40]. This means that the sandwich format can easily be implemented. As an example, Frohnmeyer et al. demonstrated a semi-quantitative sandwich LFA utilizing dye-encapsulating liposomes for the

596 detection of cholera with a LOD of 2 ng ml^{-1} , achieved within 20 minutes [29]. Liposomes are
597 sphere-shaped artificial vesicles that have a large internal space for which dyes can be stored
598 [83]. In another example, Lee et al. developed a sandwich format LFA for the detection of
599 human odontogenic ameloblast-associated protein with a LOD of 1.63 nM in saliva samples
600 [18].

601

602 The detection of proteins is limited by low target concentrations and usually a considerable
603 amount of other proteins contaminating the sample solution. This leads to an issue with
604 sensitivity without the use of pre-concentration steps. Low target concentration can potentially
605 be overcome using amplification methods. This can result in higher sensitivity and a lower
606 limit of detection. Two main amplification approaches have been implemented in aptamer-
607 based biosensors, platforms using nucleic acid amplification, known as nucleic acid-lateral
608 flow assays (NALFA) and platforms using signal amplification. In contrast to aptamer-based
609 sensors, NALFAs use nucleic acid sequences as recognition elements instead of aptamers.
610 However, aptamers are single-stranded DNA molecules and can be detected by NALFAs
611 displaying the target DNA sample. LFAs for the detection of proteins, whole cells, bacteria,
612 and viruses commonly use one for these amplification approaches [55].

613 For the detection of human epidermal growth factor receptor 2 (HER2), Ranganathana et al.
614 developed two colorimetric LFAs were developed. The first included aptamers that were
615 functionalized onto the surface of GNPs. When the target, in this case, HER2 was present, it
616 binds with the aptamer, causing the GNPs to be released. Colour change and, therefore,
617 detection is induced by the addition of NaCl, causing aggregation of the GNPs. This method
618 achieved a LOD of 10 nM. Method two involved biotinylated aptamers functionalized onto the
619 gold nanoparticle surface. When HER_2 is present, it binds specifically to the aptamer, leading
620 to the release of the GNPs. This method achieved a LOD of 20 nM [27].

Similarly, Daliriradab et al. developed a cortisol LFA using GNPs. As with the LFA developed by Ranganathan et al., the presence of the target causes the desorption of the aptamer from the gold nanoparticle surface. What Daliriradab et al. did differently was including the presence of cysteamine, immobilized on the test zone of the LFA. Cysteamine captures the free GNPs, which allows visual detection without the addition of extra reagents by the user. The LOD of the LFA was found to be one ng/ml, well under the LOD required for clinical detection (8-140 ng/ml) [16].

In 2017, a sandwich LFA was developed for the detection of vaspin. This assay consisted of a capturing probe, a biotin-labelled aptamer immobilized on a streptavidin-coated membrane, and a signalling probe; a gold nanoparticle functionalized aptamer. The LOD in spiked human serum was 0.105 nM [30].

Similarly, Alnajrani et al. used the biotin-streptavidin relationship to develop a competitive LFA for the detection of progesterone in environmental samples. In this report, aptamers were functionalized onto GNPs and further hybridized with biotin modified complementary sequence. In the absence of progesterone, the GNPs aptamer conjugates will be captured by streptavidin in the test line. The presence of progesterone causes a conformational change, leading to the release of the biotin modified complementary sequence. In this case, the colour disappears. A LOD of 5 nM was achieved with spiked tap water samples [97].

For the detection of exosomes as biomarkers for non-invasive early cancer diagnosis, Cheng et al combined GNPs with Pd termed “nanopopcorns” and aptamer nanoflowers. Aptamer nanoflowers are a recent addition to the classes of biorecognition elements, characterised by their functional nucleic acid superstructures coordinated between long-chain DNA and divalent metal ions, resulting a flower-like structure. These can then be utilised as high density self-assemblies on surfaces such as nitrocellulose membranes. Results were read with a smartphone

based thermal reader, allowing quantification and achieved a 71-fold improvement in analytical performance. [86].

As mentioned previously, the hook effect can be a serious limitation of LFAs. To circumvent this drawback, Gao et al. recently developed a novel three-line LFA for the detection of thrombin. The thrombin line was introduced between the test and control lines, which minimizes the hook effect. The authors determined that the concentration of thrombin correlated quantitatively to the signal produced in the presence of thrombin. The LOD achieved in a 10 min reaction time was 0.85 nM [98]. This was an important step in the development of LFAs. However, including an extra line for every target limits the number of targets that are feasible for multiplex LFAs.

6.2 Detection of small molecules

Small molecules often lack a second binding site for a sandwich assay to be possible [25,40]. The most common mechanism for the detection of small molecules in an LFA platform is competitive fluorescence quenching. GNPs, QDs, and organic fluorescent dyes and proteins have been used for this method [83]. As aptamers are single-stranded DNA, they can bind to a DNA probe and form a duplex. The target for the aptamer then replaces the DNA probe via competitive binding [55,83]. The detection label and the DNA probe, therefore, cannot bind, and there is either no or weak visible colour change [55]. If a sample does not contain the target of interest, the detection label binds to the complementary DNA probe, leading to a visible colour change. LFAs have been successfully developed for the small molecules cocaine, ATP, antibiotics, pesticides, heavy metals and others [17,19,20,99–101]

A limitation of this method is that the design of the DNA probe is difficult; the dissociation constant must fit within a specific range. If the sequence of the DNA probe is too short, the binding affinity to the aptamer can be weak. This can lead to problems with specificity.

669 Conversely, if the DNA probe is too long, the binding affinity can be too strong, resulting in
670 reduced competition and false-negative results.

671 For the detection of kanamycin, Ou et al. developed a competitive LFA combining magnetic
672 microspheres with target-mediated chain displacement of single-stranded DNA and the capture
673 of the visible DNA functionalized GNP probe. The presence of kanamycin leads to the release
674 of cDNA into the supernatant, the concentration of which is in correlation to the concentration
675 of kanamycin. Colour change is mediated via cDNA induced hybridization onto DNA
676 functionalized GNPs, captured on the test zone. This LFA achieved a LOD of 4.96 nM in a 20
677 min reaction time [19].

678 Cheng et al. developed an LFA for the multiplex detection the pesticides chlorpyrifos, diazinon,
679 and malathion. The authors integrated quantum dot nanobeads and gold nanostars to produce
680 a quencher nano-pair. The LOD achieved was 0.73 ng/mL, 6.7 ng/mL and 0.74 ng/mL
681 respectively [100].

682 **6.3 Detection of DNA**

683 As with proteins, detection of DNA in samples is generally limited by low concentrations of
684 target. Similarly, sensitivity of LFAs for detection of DNA is increased through the use of
685 NALFAs as aptamers can target single stranded-DNA by complementary binding [40].

686 As an example of this, Fang et al developed a NALFA for the detection of *Salmonella*
687 *enteritidis* without the need for DNA extraction. In the presence of the target, an aptamer binds
688 to the outer membrane of *Salmonella enteritidis* and the aptamer itself serves as a template for
689 isothermal strand displacement amplification. The product of amplification is then detected by
690 a GNP-based LFA which targets the sequence of the aptamer previously amplified by nucleic
691 acid amplification [102].

692 Hui et al. combined amplification refractory mutation system (ARMS)-PCR with gold
693 magnetic nanoparticles and LFA for SNP detection. This was applied to the genotyping of
694 methylenetetrahydrofolate reductase. The LOD reported was five ng, and visual detection can
695 be achieved within 5 minutes after loading PCR products into the device [103]. The limitation
696 here is the addition of a PCR step before the LFA. This increases the chance of contamination,
697 the skill required for the user, the cost, and the time necessary to obtain results. Analytical
698 application of various aptamers-based LFAs are summarized in **Table 3.**

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712 **Table 3:** Analytical application and performance of aptamers-based lateral flow assays
713 described in literature.

Analyte/s detected	Label	The sequence of Aptamer/aptamer pair used	Format of LFA	The material used for control line	Turnaround time (min)	LOD	Specificity established	Reference
Cholera	dye-encapsulating liposomes	CATCCGTCACACCTGCTCGGCAAAAAGGATTGCCCAGGTCTGCTGTCTAGCCGGATTCGGTGTTCGGTCCCGTATC BiotinCATCCGTCACACCTGCTCGGCAAAAAGGATTGCCCAGGTCTGCTGTCTAGCCGGATTCGGTGTTCGGTCCCGTATC Biotin-GATACGGGACCGAACACC	Sandwich	CT-B at a concentration of 0.1 mg ml^{-1}	Around 15 min	2 ng ml^{-1}	Yes	[29]
Vaspin	GNPs	Primary aptamer V1: 5′-Biotin-CGTACGGAATTCGCTAGCTGATGGTGTGGCG GGGGCGGCCTGGGGCGGGCCGCCGATG	Sandwich	Streptavidin	5 min after sample loading	0.137 nM in the buffer and 0.105 nm in spiked	Yes	[30]

		GGATCCGAGCTCCACGTG-3′. Secondary aptamer V49: 5′- Thiolated – CGTACGGAATTCGCTAGCGGTGGCTCTAGGGCCTATCGTTGCGCCGACGGATCCGAGCTCCACGTG-3′. The complementary sequence of V49 (cV49): 5′- Biotin - CAC GTG GAG CTC GGA TCC GTC GGC GCA ACG ATA GGC CCT AGA GCC ACC GCT AGC GAA TTC CGT ACG −3′.				human serum		
Vaspin	UCNPs	Primary aptamer 5′-Biotin-CGTACGGAATTCGCTAGCTGATGGTGTGGCGGGGGCGGCCTGGGGCGGGCCGCC	Sandwich	Streptavidin	2-3 min	39 pg ml^{-1}	Yes	[90]

		GATGGGATCCGAGC TCCACGTG-3′ Secondary aptamer 5′- Thiolated – CGTACGGAATTCGC TAGCGGTGGC TCTAGGGCCTATCG TTGCGCCGACGGAT CCGAGCTCCACGTG -3′ The complementary sequence of secondary aptamer 5′- Biotin - CAC GTG GAG CTC GGA TCC GTC GGC GCA ACG ATA GGC CCT AGA GCC ACC GCT AGC GAA TTC CGT ACG −3′.						
Progestero ne	GNPs	60-mer P4 aptamer [1] 5′SHGCATCACACAC CGATACTCACCCGC CTGATTAACATTAG CCCACCGCCCACCC CCGCTGC-3′	Competit ive	Streptavidin	20 min	5 nM	Yes	[97]

		8-mer C1 complementary sequence-biotin ATGAGTGG(T5)-biotin -3′ 8-mer C2 complementary sequence-biotin 5′ TGTAATCG(T5)-biotin-3′ 8-mer C3 complementary sequence-biotin 5′ GTGTGTGA(T5)-biotin -3′ 70-mer random ssDNA 5′ SH-AGGCCTAAGGGCATAATTAGCTCGAGCTCGAAAGGGGTTATATGATGATTTGAATTCATGGGGCCCGACT-3′ 8-mer complementary sequence-biotin for						

		random ssDNA 5′ CGGGCTGA(T5)-biotin -3′						
17β-estradiol	GNPs	35-meraptamer AAGGGATGCCGTTTGGGCCCAAGTTCGGCATAGTG 35-mer random DNA ACGGGTGGCCGCCAGGTCTTGAAGTGGCAGTATTA 75-mer BPA aptamer ATACGAGCTTGTTCAATAGGAAATCACGATTAGGTCCTCCGTCTGTGTGCGGTTGTGGTGATAGTAAGAGCAATC 75-mer random DNA AGGCCTAAGGGCATAATTAGCTCGAGCTCGAAAGGGGTTATATGATGATTTGAATT	Desorption	lysozyme	15 min	50 nM	Yes	[25]

		CATGGGGCCCGACTCGGAT						
Escherichia coli, Listeria monocytogenes, and Salmonella enterica	QDs	EcO 3R (truncated to 60 bases for 3′-digoxigenin modification): CACACCTGCTCTGTCTGCGAGCGGGGCGCGGGCCCGGCGGGGGATGCGTGGTGTTGGCTC EcO 4F (73 base length): ATACGGGAGCCAACACCATAATATGCCGTAAGGAGAGGCCTGTTGGGAGCGCCGTAGAG CAGGTGTGACGGAT	Sandwich	streptavidin-colloidal gold or streptavidin-Qdots and an anti-digoxigenin antibody control	20 min	~300–600 bacterial cells	Yes	[89]
chlorpyrifos, diazinon, and malathion	QDs and gold nanostars	CBA Thio/MC6-D/-CGAATTTCTTCCATTTCTTGCTTCTTGCATGGATTCG DBA Thio/MC6-D/-ATCCGTCACACCTGCTCTAATATAGAGG	Sandwich	No control line	5 min	Chlorpyrifos – 0.73 ng/mL, diazin	Yes	[100]

		TATTGCTCTTGGAC AAGGTACAGGGAT GGTGTTGGCTCCCG TAT MBA Thio/MC6-D/- ATCCGTCACACCTG CTCTTATACACAAT TGTTTTTCTCTTAAC TTCTTGACTGCTGG TGTTGGCTCCCGTA T BCS-C Biotin/- CGAATCCATGCAAG AAGCAAGAAATGG AAGAAATTCG BCS-DBiotin/- ATACGGGAGCCAA CACCATCCCTGTAC CTTGTCCAAGAGCA ATACCTCTATATTA GAGCA GGTGTGACGGAT BCS-M Biotin/- ATACGGGAGCCAA CACCAGCAGTCAAG				on – 6.7 ng/mL, and malathion – 0.74 ng/mL		

		AAGTTAAGAGAAA AACAATTGTGTATA AGAGCA GGTGTGACGGAT						
Cortisol	GNPs	ATGGGCAATGCGG GGTGGAGAATGGTT GCCGCACTTCGGC	Competitive	No control line	5 min	2.7 nM	Yes	[16]
Thrombin	GNPs	Apt1 T-DNA C-DNA 5'-GGT TGG TGT GGT TGG AAA AAA AAA-(CH2)6-SH-3' 5'-Biotin-AAA AAA AAA GGT TGG TGT GGT TGG-3' 5'-TTT TTT TTT TTT TTT TTT-Biotin-3'	Sandwich	Streptavidin	10 min	0.85 nm	No	[98]
mercury ions, ochratoxin A and Salmonella	UCNPs	Aptamer 1 (OTA) GCTGAGTCT GAGTCG ATCGGGTGTGGGTG GCGTAAAGGGAGC ATCGGACA	Sandwich	Streptavidin	30 min	Mercury – 5 ppb, ochratoxin A – 3 ng/m	Yes	[26]

		complementary DNA 1 CGCCACCCACACCC GAT Aptamer 2 (SE) GCTGAGTCT GAGTCG TATGGCGGCGTCAC CCGACGGGGACTTG ACATTATGACAG complementary DNA 2 CTGTCATAATGTCA AG Aptamer 3 (Hg2+) GCTGAGTCT GAGTCG TCATGTTTGTTTGTT GGCCCCCCTTCTTT CTTA complementary DNA 3 AAACAAACATGA complementary DNA of control part CGACTCAGA CTCAGC				L, *Salmonella* – 85 CFU/ mL		

Ampicillin	GNPs	CRP-FAM: 5′-FAM ACA CGA TGGG GGG GTA TGA TTT GAT GTG GTT GTT GCA TGA TCG TGG-3′ Amp(short)-FAM: 5′-FAM CAC GGC ATG GTG GGC GTC GTG-3′ CRP w/o TGG-FAM: 5′-FAM ACA CGA ACC GGG CCA ATG ATT TGA TGA CCT TGT TGC ATG ATC GTA G-3′ Amp(short) w/o TGG-FAM: 5′-FAM CAC GGC ATA GAC CGC GTC GTG-3′ CRP scrambled-FAM: 5′-FAM CTT ACT AAT TAA AAC TCA TAA ATA CTC TAA TTA CCC GCC TAA TCA A-3′	Competitive	α-mouse antibodies	10 min	10.6 nmole	Yes	[17]

		Amp(short) scrambled-FAM: 5′-FAM TCT AAT ATA ATC TAT ACT CCT-3′						
odontogenic ameloblast-associated protein	Magnetic particles	OD 5R-17 CCATTCGTAC GCAACAGGGCGTG CGATATCGGACCCC CGTGGATGGCTCTG AATGC OD 5R-29 CCATTCGTAC GCAACAGGGAGCA ATCAATTCGACAAC CGTGGATGGCTCTG AATGC OD 5R-32 CCATTCGTAC GCAACAGGCTAGA GCGAAGACACCGA AAGTGGATGGCTCT GAATGC OD 5R-35 CCATTCGTAC GCAACAGGCGACCT	Sandwich	Not stated	Not stated	0.24 nM in buffer and 1.63 nM in saliva	Yes	[18]

		AAACAACGCATCA GGTGGATGGCTCTG AATGC OD 5R-39 CCATTCGTAC GCAACAGGGACGT ATGAATCACTCGCG TGTGGATGGCTCTG AATGC OD 5R-64 CCATTCGTAC GCAACAGGGATGC ATCGACTGTAAACA CGTGGATGGCTCTG AATGC						
Kanamycin	GNPs	Aptamer: 5′-Biotin-TGGGGGTTGAGGCT AAGCCGA-3’ cDNA: 5′-TTGTGCTATCGGCT TAC-3’	Sandwich	Streptavidin	10 min	4.96 nM	Yes	[19]

		DNA1: 5′-ATAGCACAACCGTC–(CH2)6–HS-3’ capDNA1: 5′- Biotin-TGACTGTAAGCCG-3’ capDNA2: 5′-GGTTGTGCTATTATGA-Biotin-3’						
Human epidermal growth factor	GNPs	HeA2_3 (HER2) 5’-TCT AAA AGG ATT CTT CCC AAG GGG ATC CAA TTC AAA CAG C-3’ HeA2_3 (Scramble) 5’-AGC CGT TAA TAA TGC CTA CGA CAC TAA TAA CGA CCT GGT A-3’ 2-2t (HER2) 5’-GCA GCG GTG TGG GGG CAG CGG TGT GGG	Competitive	Polycation polymer	Less than 1 min	20 nM	Yes	[27]

		GGC AGC GGT GTG GGG-3’ A08 minimer (control aptamer, OTA) 5’-GGC AGT G TG GGC GAA TCT ATG CGT ACC GTT CGA TAT CGT G-3’						
Listeria monocytogenes	Silica particles	A15-Biotin 5′-Biotin-TACTATCGCGGAGACAGCGCGGGAGGCACCGGGGA -3′ A15-SH 5′-Thiol-TACTATCGCGGAGACAGCGCGGGAGGCACCGGGGA -3′ A15M-SH 5′-Thiol-TACTATCGCGGAGACAGCGCGGGAGGCACCGGGGATT TTT -3′ A15-HP-Biotin 5′-Biotin-TACTATCGCGGAGA	Sandwich	No control line	Within 5 min	53 cells/mL	Yes	[93]

		CAGCGCGGGAGGC ACCGGGGATAGTA -3′ A15-HP-SH 5′-Thiol- TACTATCGCGGAGA CAGCGCGGGAGGC ACCGGGGATAGTA -3′ Scrambled A15-HP 5′-Thiol- GCGGGAGGCAAAT AGCGTCCCGCGTGG AAAGGGCC CCCGC-3′						

714

715 **7. Conclusion**

716 LFAs have made a paradigm shift in the IVD industry due to its ease in operation, rapid
717 turnaround time, and affordability with an ability to be used at home (TPP1) and community
718 (TPP2) as well. One major limitation that LFAs faces is high inter-batch variability due to the
719 use of antibodies. This limitation of LFAs can easily be addressed using synthetic receptor
720 molecules like aptamers. Similar to antibodies, the aptamer is a platform technology; thus, it
721 can replace the former in all possible diagnostic formats, including LFA. With the advent of
722 aptamer technology, LFA has assumed a new dimension and witnessed a great success in IVD
723 and has shown its utility for clinical, biomedical, food safety, agriculture, and environmental
724 application. In the last decade, several variants of lateral flow have been reported without

deviating from the basic principle of LFA design to improve the sensitivity of LFAs. With time evolution of LFAs are continue, and we are expecting to see next-generation LFAs with ultrahigh sensitivity and reproducibility to bridge the existing diagnostic gap in health care.

As the current focus of health care is on personalized medicine and companion diagnostics thus in such context, LFA appears to play an important role in the near future in managing health owing to its extraordinary ability to be used at point-of-care with patient itself. Further, it can also be used for environmental monitoring and detection of chemical and biological hazards to ensure food safety. Moreover, as it is really difficult to generate high-affinity antibodies for the detection of small molecules and toxins thus aptamer technology can bridge this gap easily by designing and developing high-affinity aptamers against these analytes and adapting them on to a lateral flow format. Taking together, aptamer-based LFA has a bright future for analytical applications due to their enormous potential and ability to tailor it on demand.

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