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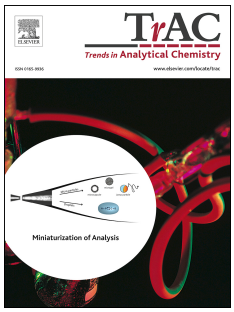

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Advances and challenges of fully integrated paper-based point-of-care nucleic acid testing

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ABSTRACT: Nucleic acid testing (NAT) has been studied extensively in paper-based diagnostics, presenting a substantially higher sensitivity and specificity than immunoassays. Paper-based NAT provides an alternative to laborious, expensive and time-consuming conventional NAT. Recent advances in paper fabrication and modification technologies have made it possible to integrate all key steps of NAT (*i.e.*, sample preparation, nucleic acid amplification and amplicon detection) into one single paper-based device, and are hence suitable for resource-poor settings. However, multiple challenges are yet to be addressed to translate the technologies into practical applications. In the present review, we discuss the current status and challenges in accomplishing each key step of NAT using low-cost paper substrates. We highlight the most recent advances, challenges and possible solutions in integrating all these steps into a compact paper-based device. We also review the latest progress towards commercialization and future perspectives on the development of an ideal sample-in-answer-out device.

Keywords: low-cost paper substrates; nucleic acid testing, sample preparation, nucleic acid amplification; amplicon detection; challenges; commercialization

1. Introduction

Paper-based devices have attracted significant interest in point-of-care (POC) diagnostic field due to their good biocompatibility, cost-efficiency, simplicity, biodegradability and ability to transport fluids through capillary action [1]. The availability of papers with different physical properties (*e.g.*, pore size, porosity, thickness, capillary flow rate *etc*) for achieving different function of the assay has further added significant advantages. These technologies offer great potential to meet ASSURED criteria recommended by World Health Organization (WHO), *i.e.*, affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end-users [2]. Recent advances in molecular diagnostics have led to the development of paper-based platform to accomplish each individual step of nucleic acid testing (NAT), including sample preparation [3-8], nucleic acid amplification [5, 9-12], and nucleic acid detection [10, 13-19]. To meet the increasing needs in developing countries where high-end infrastructures and well-trained personnel may not be available, integrating all key steps of NAT into one single paper-based platform is highly desirable [20]. This strategy would enable untrained personnel to readily perform the point-of-care (POC) test for healthcare application, food safety analysis and environmental monitoring.

Recent advances in paper fabrication and modification technologies, namely “paper patterning”, have made it possible to integrate all essential steps of NAT into one single paper-based device [21]. For instance, the Whitesides group has recently created a patterned “paper machine” to perform the nucleic acid extraction, amplification and detection by a simple sliding motion of the strip [22]. A foldable paperfluidic chip made entirely of paper and adhesive sheets has been reported by the Klapperich group to realize sample-in-answer-out NAT [23]. Our group has recently developed a four-layer integrated paper-based device separated by hydrophobic polyvinyl chloride (PVC) to achieve the three key steps of NAT [24]. Even though these studies have reported full sample-to-answer functionality of paper-based device, developing a fully integrated device in a robust, cost-effective and more importantly, user-friendly format remains challenging to date [20]. Significant challenges are yet to be addressed to bring the technologies to market (**Table 1**).

There have been multiple reviews focused on paper-based point-of-care diagnostics [20, 25-29] and point-of-care NAT [30-32]. To date, in light of the advancement of the NAT and the growing need for low cost paper-based diagnostic devices, there is a strong demand for a timely and comprehensive review on challenges and solutions in developing fully integrated paper-based NAT. In the present review, we first summarize the latest development in this field, highlighting the technical challenges of accomplishing each individual step of NAT at the POC using low-cost paper substrates. We further discuss the recent advances and challenges in integrating all key steps into a fully integrated paper-based sample-in-answer-out device and possible solutions for real world applications. Lastly, we review the significant progress made towards commercialization.

2. Recent advances and challenges in realizing each steps of paper-based NAT

Conventionally, NAT has relied heavily on well-established laboratories, high-end equipment (*e.g.,* thermocycler, quantitative real-time polymerase chain reaction (qPCR) machine, electrophoresis unit) and well-trained personnel [33]. Alternatively, researchers have sought to utilize simple, disposable and cost-effective paper substrate to conduct each key step of NAT (*i.e.*, sample preparation, nucleic acid amplification and amplicon detection), which are compatible with low-resource POC settings.

2.1. Paper-based sample preparation

Cost-effective paper-based devices have been recently introduced to extract the nucleic acids from diverse types of biological samples at the POC[34]. Commercially available extraction cards, such as Fast Technology Analysis (FTA) cards have been introduced, in combination with microcentrifuge tube, to extract DNA from various raw samples [35, 36]. Since then, several emerging paper-based extraction devices have been developed to improve the performance and robustness of existing extraction device [7, 37]. However to date, some challenges need to be addressed before bringing the product to market. In our opinion, an optimal paper-based nucleic acid extraction device should fulfill the following criteria: (a) capable of producing highly purified DNA or RNA, (b) inexpensive, (c) rapid, (d) capable of processing diverse sample types, (e) automative, (f) capable of storing reagent, (g) flexible to be integrated into sample-in-answer-out analytical device.

Rapidity and cost-efficiency. The early development of the devices was focused on achieving high efficiency of nucleic acid extraction [38]. Recently, more focus has been shifted to other criteria. To reduce the cost of the device without compromising the extraction efficiency, several studies have integrated the cost-effective extraction card into the paper-based extraction devices. For instance, commercial FTA classic cards have been utilized in several integrated paper-based devices for sample preparation (**Figure 1A**) [22, 24]. As compared to the conventional extraction kits, which costs around $25 USD per test, the FTA card only costs <$0.10 USD per test [22]. These commercial cards are impregnated with chemicals with patented formulation that readily lyse cell and denature proteins, producing highly purified nucleic acids within only 1 hr as compared to the conventional extraction which takes almost 2 hr [39, 40]. However, the FTA card-based extraction method is still considered time consuming (>15 min) for POC applications where rapid turnaround time is required. To this end, Fusion 5 disc, a single layer matrix membrane with a lower cost (<$0.05 USD per test) has been introduced to extract the nucleic acids within a few min (<10 min) using an approach termed filtration isolation of nucleic acid (FINA) (**Figure 1B**) [41]**.** In this context, detergent is used to lyse the blood cells, followed by a single wash of alkaline solution (*i.e.,* NaOH) to remove all the undesirable proteins. Similar to an FTA card, the fusion 5 disc could entrap the DNA for downstream processes. As compared to the FTA card-based extraction, the FINA allows nucleic acid extraction in a more rapid and lower-cost manner, which is more applicable in developing countries [38].

Capability of processing diverse sample types. In addition to rapidity and cost-effectiveness, it is essential to be capable of extracting nucleic acids (either RNA or DNA) from diverse sample types such as blood, urine, saliva and buccal cells with varying viscosities (*e.g.*, high viscosity as sputum and low viscosity as urine) and volumes (from µl to ml) [7]. For instance, an integrated paper-based device that implemented with FTA card has shown its capability of detecting targets from blood, milk, food and water without the need for laboratory equipment [24]. Besides, it has been reported that integrating a piece of fusion 5 disc into a microfluidic device has shown to successfully extract DNA from various biological samples, such as blood, buccal swab, saliva *etc*. [38]. In another study, a paper-based microfluidic origami composed of a stack of flat polymer sheets and paper has been developed for

extracting DNA directly from viscous sample (*i.e.*, sputum), which normally requires additional off-chip complex sample preparation step (**Figure 1C**) [7]. This device was activated by sequential folding of paper sheet to programme multiple processing steps required in nucleic acid extraction, such as cell lysis and sample washing steps.

Besides DNA, detecting RNA is challenging in many POC settings. As compared to DNA, RNA is less stable and more prone to hydrolysis by the environmental RNAses [42]. One study has demonstrated paper-based RNA extraction using one-step lysis, RNA extraction and alcohol precipitation method, involving the mixing of specimen with lysis buffer and Glycoblue coprecipitant, followed by rinsing using ethanol (**Figure 1D**) [3]. The entire RNA extraction process is performed in low-cost polyethersulfone (PES) filter paper, but an elution step is required prior to downstream processes (*i.e.*, qPCR). Overall, although the capability of processing diverse samples and the low cost of these devices are impressive, these methods require lengthy and bulky sample preparation steps, which remain a big challenge to date.

Automation. As discussed earlier, automation is one of the main challenges in paper-based assays [38]. Several known extraction methods require multiple operation steps in the process of sample preparation. For instance, a portable paper microfluidic chip has been developed for DNA extraction and direct fluorescent detection by using a smartphone (**Figure 1E**) [8]. However, multiple reagent addition and timing steps are required, which fail to reduce the complexity of the assay. To address this challenge, several studies have incorporated paper-based flow control technologies into the paper-based device to enable automated sequential fluid delivery. For example, the simplest geometry-based flow control strategies have been introduced by incorporating a shunt [43, 44] or manipulating the width or length of different channels of paper [45-47] without introducing incompatible chemicals in the flow path and thus avoiding potential influence on the biochemical reactions. Novel three-dimensional paper-based devices embedded with multiple directional valves have also been developed to autonomously handle multiple reagents [48, 49], which could potentially simplify the complex multistep fluidic operations in paper-based NAT.

On-board reagent storage. Further, storing reagent on paper would be another challenge in paper-based NAT. The reagent storage capability of the device at room temperature could eliminate the need for laboratory storage unit (*e.g.*, refrigerator). For instance, commercial FTA cards are impregnated with dry lysis reagent, which could simply be activated upon addition of sample [50]. Similarly, a study has implemented dry lysis reagent into a paper-based microfluidic origami for DNA extraction, which could be activated by addition of water [7]. The wet lysis buffer is first pipetted onto the storage pad, dried in the vacuum and stored until use. The entire sample preparation process using this device only requires a little of non-hazardous reagent (*i.e.,* elute buffer and ethanol), reducing the risk of exposure to chemical hazards during the process. Despite the advantages of dry reagent storage capability, the requirement of multiple purification and washing steps upon the addition of sample into these devices has sacrificed the simplicity of the extraction process. Taken together, the challenges of reagent storage capability and the simplicity of operations must be addressed concurrently.

Flexibility of being integrated into sample-in-answer-out device. Another challenge to be addressed would be the capability of being easily integrated with downstream steps, creating a fully integrated paper-based sample-in-answer-out device. Some extraction devices employ a bind-wash-elute process [51]. Considering the operational simplicity and the assay rapidity, following DNA extraction, *in situ* amplification should be performable within the same paper matrix. For example, FTA card has been successfully integrated into a paper-based sample-in-answer-out device for DNA extraction and *in situ* loop mediated isothermal amplification (LAMP) [24]. Besides, *in situ* helicase dependent amplification (HDA) has been performed in a 3MM chromatography paper supported by a pipette tip following the DNA extraction on the same paper substrate without DNA elution (**Figure 1F**) [5]. This strategy has not only simplified the structure of the device, but also significantly improved the performance of the assay due to the efficient use of the extracted DNA for downstream processes.

2.2. Paper-based nucleic acid amplification

Nucleic acid amplification is a mandatory step in NAT as the amount of extracted nucleic acid is usually too low to be detected by existing technologies [32]. Several

studies have demonstrated the utilization of paper as a platform for amplification [44, 52]. In particular, isothermal amplification methods (*e.g*, LAMP, HDA, nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), recombinase polymerase amplification (RPA)), which necessitate only a single reaction temperature, have been widely used in diagnostic studies to substitute conventional PCR that requires three precisely controlled temperatures [53]. Besides the requirement of having the above-mentioned automation capability and on-board reagent storage, an appropriate paper-based amplification platform should satisfy the following criteria: (a) able to produce high quantities of nucleic acids, (b) rapid, (c) capable of multiplexing amplification, and (d) cost-effective.

Rapidity and efficiency. To achieve rapid and highly efficient amplification, selecting an appropriate type of isothermal amplification technology is important. The principle and mechanism of each type of isothermal amplification technology have been recently reviewed [32, 54-56]. Several studies have demonstrated paper-based LAMP, followed by paper-based target detection. For instance, a multiple-LAMP paper device has been developed to detect Zaire ebolavirus, where the sample-inlet is connected to three paper matrices pre-coated with target specific primer, positive LAMP primers and negative control (without primer), respectively (**Figure 2A**) [9]. More recently, our group has developed a paper-based LAMP-LFA device, coupled with a small pocket created by a disposable adhesive tape, which acts as a LAMP reaction chamber (**Figure 2B**) [52]. The device could successfully detect dengue DNA by LFA in 1 hr, achieving a detection limit of as low as 3×10^3 copies of target DNA, which is comparable to the tube-based LAMP-LFA in an unintegrated format. With an amplification period of 45-60 min, the analytical sensitivity of paper-based LAMP has been shown to be comparable to that of conventional PCR, which takes more than 1 hr. Complicated primer design has been known as a main challenge in most studies, but a LAMP primer designing software has been developed to address the issue [32, 56].

Paper-based HDA has also been demonstrated in several studies. Thermostable helicase Tte-UvrD is an enzyme used in HDA, which works efficiently at 65 °C for a long reaction time (*i.e.,* 60-120 min) [32, 56]. However, this shortcoming can be addressed through optimizing reagent concentration or primer sequences. A study has

demonstrated HDA at 65 °C using 3MM chromatography paper supported by a pipette tip for 30 min (**Figure 2C**) [5]. *Another study has also demonstrated HDA for rapid amplification of Mycobacterium tuberculosis on a chromatography paper substrate using a heater [1] (**Figure 2D**). The paper substrate was coated with bovine serum albumin (BSA) to prevent enzyme and DNA absorption. This study has achieved both dried reagent storage and HDA on a single paper disc.* Notably, HDA is appropriate for use in POC settings due to its simplicity. However, the modest detection limit requires further analytical sensitivity improvement. *In addition to LAMP and HDA, paper-based rolling circle amplification (RCA) has also been reported. RCA was performed on a wax-patterned paper device by incubation at room temperature for 40 min [2]. Interestingly, this isothermal amplification method was found to be more proficient on paper than in solution due to the significantly higher localized concentration of primers and immobilized DNA capturing molecules on paper. This approach is suitable for POC testing in remote settings.*

To further improve the simplicity and rapidity, RPA has been developed. Unlike other isothermal amplification technologies, RPA does not rely on temperature-dependent primer-annealing process, making it very suitable for POC applications, where precise temperature control is often challenging [59]. The low incubation temperature (37-40°C) and rapid amplification (~10-20 min) would minimize the time and power requirement of the amplification reaction, thus reducing the cost of the assay [32, 56]. For instance, a paper-plastic device has been developed to perform RPA in 15 min, followed by lateral flow assay (LFA) in an unintegrated format to detect human immunodeficiency virus (HIV), achieving a detection limit of as low as 10 copies of target DNA (**Figure 2E**) [11]. The function of the device has been improved in another study, where a compact paper-plastic device was developed to perform RPA and LFA within one single device in < 1 hr (**Figure 2F**) [12]. The device could successfully detect *Plasmodium sp.* with a detection limit of as low as 5 copies/μL of target DNA. This rapid, sensitive and user-friendly DNA amplification-to-detection process suggests its great potential to serve as part of a POC test. *In addition to paper-based RPA, reverse transcription (RT)-RPA has also been introduced using wax-patterned paper (**Figure 2G**) [3]. RT-RPA mixture was first freeze-dried on paper and rehydration was performed with distilled water followed by heating at 40°C for amplification. Fluorescent signals were then monitored over time.*

Other isothermal amplification technologies (*e.g.*, NASBA, SAMBA *etc*) have been performed using the cartridges or tubes followed by LFA [18, 61, 62]. However, there have been no studies demonstrating the utilization of paper matrix as a platform for these amplifications, possibly due to several challenges. For example, NASBA and SAMBA require extra 95 °C denaturation step prior to amplification, which complicates the assay [32, 56]. The inability of NASBA to amplify DNA makes it less versatile. Furthermore, the reaction time of these amplifications is >1 hr, limiting their use in POC testing where assay rapidity is preferred.

Multiplex amplification. Capability of simultaneous amplification is essential for subsequent differentiation and detection of multiple targets, which enables users to test several diseases that present similar clinical features but require different treatments [16]. Even though multiplex isothermal amplification has been introduced in several studies, the reaction takes place in a single tube rather than within a paper matrix [63, 64]. Given the fact that paper has the capability of conducting isothermal amplification within their pores followed by lateral flow detection [10, 23], it shows great potential to conduct multiplex amplification and detection. Multiplex amplification is usually performed using the target specific primers with different labels. For instance, in a multiplex LAMP reaction for multiple *P.aeruginosa* gene detection, a series of target-specific labels were tagged with the FIP and BIP primers (*e.g.*, biotin-, FITC-, hex-, digoxin-modified primers), producing amplicons with different labels to be readily detected by a lateral flow test strip [17]. In another study, multiplex RPA was performed to simultaneously amplify and detect *Giardia*, *Entamoeba* and *Cryptosporidium* by using the specific probes labelled with Fluor488, fluorescein and digoxigenin, respectively [16]. Clearly, given that these amplicons are double-stranded, multiple labeled primers should be used in amplification to achieve multiplex detection. As other amplification technologies such as NASBA produces single-stranded amplicons, which could be detected through the principle of DNA-DNA hybridization, the primers involved in amplification could be unmodified [65]. The methods of multiplex nucleic acid detection will be briefly discussed in the **Section 2.3**.

Cost efficiency. Besides reagents, the cost of the assay is mainly dependent on the heater used for amplification. Electrical-dependent water baths, dry block heaters or

incubators are still utilized in several studies to perform the cost-effective paper-based amplification, which do not fully meet the need of developing countries. Since then, battery-powered approaches have been introduced to perform amplification at the POC. Recent studies have demonstrated the utilization of a handheld battery-powered heater to perform paper-based isothermal amplification (*e.g.*, LAMP) [10]. These heaters allow precise temperature control, which helps to achieve the optimum device performance. However, the function of the rechargeable heater can only be supported if electricity is available for recharging batteries, and the intermittent supply of electricity in most developing areas may reduce the productivity.

Recently, chemical heaters have been developed which show great promise for expanding NAT to developing countries [66]. For instance, a non-instrumented nucleic acid (NINA) heater with a lid filled with a temperature-regulating phase change material (PCM) has been developed to perform LAMP at the POC [67]. A constant temperature of 60 °C was achieved by the exothermic reaction of calcium oxide (CaO) and water. The NINA heater has been further improved by replacing CaO with lower-cost magnesium iron alloy (MgFe) (~$0.06 US per test) to meet the thermal requirement of LAMP [68]. Another study has also developed a toe warmer-Styrofoam cup which provides a constant 65 °C for HDA reaction [69]. These heating technologies suggest that laboratory equipment-like molecular diagnostic is possible in disposable and low cost devices.

2.3. Paper-based amplicon detection

In NAT, nucleic acid amplification is usually followed by detection and quantification of the amplicons. Researchers have sought to develop paper-based devices with a simple detection method (*i.e.*, colorimetric detection), particularly lateral flow test strips [70-72]. As these devices are to be used in the developing countries, they should meet the following criteria: (a) rapid and simple (with visual readout), (b) capable of multiplexing, (c) sensitive and (d) capable of semi-quantifying or quantifying the targets.

Rapidity and simplicity. Among various detection approaches, the simplest would be visual colorimetric detection. This could be readily achieved by the widely used LFA in < 20 min. *Generally, lateral flow detection involves the utilization of*

oligonucleotide-functionalized AuNP with unique optical properties [22]. The target DNAs would bind to the functionalized AuNP to form a complex, which would in turn bind to the capturing molecules at the test zone to produce a visible red band. The free oligonucleotide-functionalized AuNP would then bind to the control probe at the control zone to produce another red band [22].

Given that most amplicons are double-stranded (*e.g.*, PCR, LAMP and RPA amplicons) [11, 74, 75] and are normally biotinylated, they could be captured by streptavidin at the test zone of lateral flow test strip. *For instance, based on the similar working principle, one study has demonstrated the detection of biotinylated PCR amplicon [23]. Briefly, the biotinylated PCR amplicon would bind to the functionalized AuNP and subsequently bind to the streptavidin at the test zone to produce a visible signal. The free AuNP-DP would then hybridize with the control probe to produce another red signal. Similarly, in LAMP amplicon detection, the biotinylated LAMP amplicon would be first denatured and bound to the single-stranded gold-nanoparticle detector probe* ***(Figure 3A)*** *[19]. The AuNP-DP-biotinylated-amplicon would be captured by the streptavidin at the test zone to produce a visible signal. Based on the same principle, in another study, LAMP amplicon would bind to the FITC-labeled DNA probe, forming a complex with the gold-labeled anti-FITC antibodies (Ab), which would then be captured by the streptavidin at the test zone (****Figure 3B****) [20]. In addition, RPA has also been used for amplicon detection by LFA where double-stranded amplicon is labeled with both rabbit Ag and digoxigenin (****Figure 3C****) [24]. The rabbit Ag would bind with the Ab-tagged AuNP at the conjugate pad, forming a complex, which would in turn bind with the digoxigenin Ab at the test zone to produce a positive signal.*

As for the detection of single-stranded amplicons (*e.g.*, NASBA amplicons), the most common detection approach would be by nucleic acid hybridization (**Figure 3D**) [18]. The single-stranded target RNA would bind to the AuNP-labeled detector probe, forming a complex, which would then bind to the single-stranded capture probe at the test zone. Besides, detection of single-stranded amplicons via Ab-Ag or biotin-streptavidin interactions would also be possible. Apart from that, recent advances in synthetic biology have successfully created colorimetric output in paper-based microfluidics, which is detectable by the unaided eye via a novel technology called

"toehold switch" (**Figure 3E**) [15, 19]. These sensors are programmable synthetic regulators that control the gene translation through the binding of a trigger RNA. In the presence of target RNA, the b-galactosidase (LacZ) cleaves chlorophenol red-b-D-galactopyranoside (a yellow substrate), to produce chlorophenol red (a purple product). This technology allows the biosensor to specifically detect a target RNA sequence in a low-cost and simple manner based on colour changes, offering great potential to be applied in paper-based sample-in-answer-out NAT.

Multiplex detection. To date, multiple target detection remains a challenge as it induces the risk of cross-reactivity among target analytes [20]. To this end, recent studies have reported a multiple-zone test strip with different capturing molecules to detect amplicons tagged with different Ab. For instance, a lateral flow test strip coated with three different types of Ab, anti-FITC Ab, anti-hex Ab and anti-digoxin Ab has been developed to detect FITC-, hex- and digoxin-tagged LAMP amplicons, corresponding to three main toxin genes of *P.aeruginosa* (*i.e.*, ecfX, ExoS and ExoU genes) (**Figure 3F**) [17]. Based on the same principle, a multiple-zone test strip has been developed to detect diarrhea-causing protozoa *Giardia*, *Cryptosporidium* and *Entamoeba* simultaneously (**Figure 3G**) [16]. The strip is coated with anti-alexa fluor Ab, anti-fluorescein Ab and anti-digoxigenin Ab to detect three different tagged RPA amplicons. These strategies could differentiate the diseases with similar signs and symptoms for timely and appropriate medical treatment. However, no study has demonstrated multiple target amplification and detection in one single paper-based sample-in-answer-out device to date.

Sensitivity. In fact, LFAs always suffer from poor sensitivity, which fails to meet the detection limit required in clinical settings. To this end, several efforts have been made to develop novel methods for sensitivity enhancement. For example, it has been demonstrated that LFA sensitivity could be improved by using liposomes [77] or probe-based aggregation of AuNP [78]. Besides, the ability of enzymatic amplification has been reported for signal improvement in nucleic acid-based LFAs [79]. More recently, fluidic-control sensitivity enhancement strategies, which involve simple strip fabrication and operation steps have attracted significant research interest. For instance, paper architecture modification [45] and wax-printed pillars [80, 81] have been introduced for on-board fluid control. However, these approaches are less

appropriate to be integrated into a paper-based sample-in-answer-out device as former may result in consumption of more sample and reagent, which undermines the benefits of POC use, whereas the latter may cause wax melting throughout the heating process in amplification [82], hence influencing the fluidic control strategy. To this end, our group has recently demonstrated a novel strategy of incorporating a polydimethylsiloxane (PDMS) barrier and a paper-based shunt into a lateral flow strip to achieve optimum fluidic delays for detection sensitivity enhancement [44]. PDMS is heat-resistant, complementing the heating process usually needed in amplification. Therefore, it is a promising method for sensitive paper-based sample-in-answer-out NAT.

Semi-quantification or quantification. Medical diagnosis usually requires accurate measurements of biomolecules such as nucleic acids and proteins. However, visual colorimetric detection by the naked eye only enables qualification (yes or no), which is insufficient for the quantitative purpose due to the effect of different lighting conditions and the variation in colour perception among end-users [20]. Therefore, significant efforts have been made to improve the quantification capability of gold nanoparticle-based LFAs. Several studies have developed cost-effective multiple zone LFAs to provide semi-quantitative measurements of target analytes [83-85].

In order to achieve quantitative analysis in paper-based diagnostics, particularly in LFAs, which could provide more accurate quantitative result, desktop scanners and commercially available portable strip readers have been used to record the color intensity, which is corresponding to the amount of analytes, and the intensity of test zones can be analyzed by image-processing software [86]. However, this method requires additional instrumentation, which adds significant cost, hence hindering its use at the POC. Recently, smartphones have emerged as promising platforms for optical readout of assays. Development of a smartphone carrier, coupled with a cost-effective smartphone (~$ 100 US) is broadly applicable in the developing world [87, 88]. For example, it has been reported that a smartphone-based rapid diagnostic test reader performs well with LFAs for diagnosis of many diseases, including malaria, tuberculosis (TB) and HIV, thus allowing quantitative real-time detection of target analytes [89]. With advance in these mobile technologies, the data can be transferred from remote areas to the off-site laboratory, analyzed by trained personnel, and then

returned to the onsite personnel or end-users for decision-making.

3. Advances and challenges in developing fully integrated paper-based sample-in-answer-out device

As discussed earlier, recent studies have successfully orchestrated the three key steps of NAT into one single fully integrated paper-based device [20]. For example, the Whitesides' group has developed an integrated paper-based microfluidic device that enables a central patterned paper substrate to slide in and out of fluidic path, allowing sample addition, washing, nucleic acid amplification and detection with minimal operation, which is applicable in resource-poor settings (**Figure 4A**) [22]. A dynamic seal has been created to prevent evaporation of reagent especially during nucleic acid amplification at high temperature (~65 °C). However, this device relies on a UV light source and imaging equipment for endpoint detection, which may not be readily available in developing areas. To this end, another study has developed a fully integrated paperfluidic that combines three key steps to provide immediate visual readout by LFA (**Figure 4B**) [23]. However, an external heat block is required for amplification, which may hinder its applications at the POC. Our group has recently developed a four-layer integrated paper-based sample-in-answer-out device, with integrated LFA to enable rapid on-site naked eye detection **(Figure 4C**) [24]. Both Fast Technology Analysis (FTA) card and glass fiber are integrated into lateral flow strip, acting as substrates for nucleic acid extraction and amplification, respectively, and a valve is created to control the fluid flow from extraction to amplification zone and lateral flow strip, which are initially separated by PVC layers. *More recently, a fully disposable and integrated paper-based device was introduced (**Figure 4D**) [4]. It consists of a sponge-based reservoir and paper-based valve for nucleic acid extraction, integrated battery and heater for HDA and lateral flow test strip for colorimetric detection. This device allows on-chip dried reagent storage and equipment-free amplification with simple operations steps, which could be easily performed by untrained users.*

Even though all these efforts have successfully increased the simplicity, portability and usability of paper-based devices in NAT, as discussed in the previous sections, the challenges of further simplifying user operation steps and multiplex detection should be addressed for practical applications. *A recent study has demonstrated*

*multiplexed detection of Plasmodium sp. from whole blood using a paper-based origami device, which allows the sequential steps of DNA extraction, LAMP and fluorescent detection using one single device (**Figure 4E**) [5]. Multiplexed detection was achieved by controlling the flow of samples to four independent areas on the paper where LAMP reagents were deposited. Ideally, operation of the device should be simplified and easily performed even by an untrained user in remote settings.* Apart from addressing all these challenges, an ideal, powerful, well-integrated sample-in-answer-out device should be robust in all ways, including having the capability of on-board sample collection, on-board low cost, accurate readout timing, and having the ability of maintaining optimum testing environmental conditions.

Current medical diagnostics require the utilization of an extra blood collection tool (*e.g.,* lancet devices) for off-chip blood collection before applying it to an analytical device [23, 24]. Integrating a robust blood collection tool (*e.g.,* a minimally invasive hollow microneedle) into one single sample-in-answer-out device would enable one-touch activation of the device, which could further simplify the assay and enhance user performance [92]. As for the water quality analysis, coupling a sample concentration tool with the device to concentrate the target before nucleic acid extraction [4, 6] would enable highly sensitive detection of low concentration target in large volume of sample, which could also tremendously improve the functionality of device. Besides, NAT generally involves time-dependent nucleic acid extraction, amplification and detection for accurate readout [20]. Determining the endpoint of assay is critical for accurate quantitative read out, hence eliminating the need for an extra timing device. For example, a paper-based passive timer has been demonstrated in a multiplexed paper analytical device [93]. A blue dye (food colouring), which acts as a colorimetric indicator is printed and allowed to dry at the back of the paper-based detection channel. Once a sample or eluent reaches the end of detection channel, the printed dye is solubilized and observable from the top of the device, indicating the completion of an assay. In addition, testing in the field with variations in environmental conditions (*e.g.,* temperature and relative humidity) could present different performance from the test run in a typical controlled laboratory [94]. Therefore, a closed temperature-humidity control system should be integrated into the sample-in-answer-out paper-based platform to achieve a contamination-free and stable biochemical reaction for optimum assay performance.

4. The latest progress towards commercialization

Todate, several low-cost paper-based devices have been commercialized to realize the key steps of NAT (**Table 2**). As discussed, Whatman FTA card has been introduced into the market to provide a reliable approach for sample collection, storage and extraction of nucleic acids [35, 36] (**Figure 5A**). Lateral flow test strip has also been incorporated into the BioHelix Express Strip (BeSt) Cassette by BioHelix (USA) and disposable nucleic acid detection device by Ustar Biotech (China) (**Figure 5B**) to provide a simple colorimetric detection [95]. Abingdon Health (UK) and Milenia Biotec, Germany (**Figure 5C**) have also commercialized the disposable strips for biotinylated amplicon detection [95]. However, laborious off-chip extraction and amplification are still required, hence does not fully meet the demand of developing countries [96, 97].

To this end, Diagnostic for All (USA), a non-profit enterprise founded by Dr. George Whitesides in 2007, has developed low cost, disposable nucleic acid amplification-based paper microfluidic devices for diagnosis of various diseases in developing world, including infant HIV (**Figure 5D**), Ebola (**Figure 5E**) and Hepatitis C [22]. The all-in-one device basically consists of port layers for sample and buffer addition, a sliding layer, a detection layer, which consists of lateral flow test strip and an electronic housing to generate heat for amplification (**Figure 5F**). These extremely low cost diagnostics require only a drop of blood for processing in less than 1 hour, require no external electricity, additional equipment or resources, which is operable by minimally trained personnel, making it ideal for use in remote settings.

5. Conclusion and future perspective

The recent advances in paper-based diagnostics have made it possible to carry out sophisticated NAT using extremely low cost paper-based substrates. The three key steps of NAT have been successfully introduced into fully integrated paper-based devices to address the limitations of conventional NAT. However, at the current stage of their development, it is still challenging to bring the paper-based NAT technology into market. To fully meet the ASSURED criteria, there exist several challenges that require significant attention. Goals for the coming years include simplifying the user operation steps by creating an automated fluidic delivery in paper [38]. Besides that,

the capability of preserving all reagents on-board is important to eliminate the requirement for laboratory storage unit, and the ability of multiplexed detection would remarkably increase the assay productivity [7]. *For instance, the reagent could be stored within polymeric sugar film formed from pullulan and integrated into paper-based devices [2, 25]. This encapsulation enables stable and long-term storage of the entrapped reagent at room temperature.* The ability to detect multiple targets simultaneously using three-dimensional paper-based microfluidic device or paper-based origami device could enhance the functionality of a single device [60].

To further improve the performance of integrated device, a great challenge remains in incorporating simple readout timer to track the assay endpoint for accurate readout [93]. Integrating an appropriate sample collection tool, particularly a biocompatible and economical microneedle with good mechanical strength for blood collection would immensely increase the usability of sample-to-answer device [92]. Additionally, creating a robust, handheld closed system with integrated quantitative analytical module (*e.g.,* smartphone) would make the assay quantification easier [99]. Having the capability of on-board maintenance of optimum environmental conditions would achieve optimum assay performance even in the settings with extreme temperature and humidity [94]. While addressing all the above-mentioned concerns, cost-effectiveness, a key metric in the development of the fully integrated device, should be maintained to make it fully accessible in the developing world.

In fact, better understanding of the fundamentals of paper in terms of structural, chemical and physical properties would allow engineering of the cellulose fibers (*e.g.*, surface modifications), producing more biocompatible substrates [100]. *The ideal characteristics of paper substrates for each step of NAT are summarized in* ***Table 3***.
Integrating novel materials (*e.g.*, nanoparticles, electrodes, polymers) into paper to produce biofunctionalized hybrid device will also play essential roles in real world applications at a minimum cost. Collectively, an ideal paper-based sample-to-answer NAT should be low cost, robust, portable, easy to use with sensitive, multiplexed detection, and addressing all the existing challenges for future real-time POC applications and commercialization.

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Figure captions

Figure 1. Paper-based sample preparation. *(A) Commercial Fast Technology Analysis (FTA) card was used to lyse cell, denature proteins and extract nucleic acids for downstream applications [6, 7]. (B) Filtration isolation of nucleic acid (FINA) method was performed to extract DNA following the process of cell lysis by detergent and washing with alkaline solution [8]. (C) Paper-based microfluidic origami was activated by sequential folding of paper sheet to programme multiple processing steps required in the nucleic acid extraction [9]. (D) Polyethersulfone (PES) filter paper-based RNA extraction device was developed for one-step cell lysis and RNA extraction [10]. (E) A portable paper microfluidic chip was developed for DNA extraction with integrated sample lysis and DNA elution for downstream process [11]. (F) An extraction device consisting of chromatography paper supported by a pipette tip was developed for DNA extraction without an elution step [12].*

Figure 2. Paper-based nucleic acid amplification. *(A) A multiple-LAMP paper device was developed where the sample-inlet is connected to three paper matrices pre-coated with primers for LAMP [13]. (B) A paper-based LAMP-LFA device was developed for LAMP coupled with a small pocket created by a disposable adhesive tape, which acts as a LAMP reaction chamber [14]. (C) A piece of 3MM chromatography paper supported by a pipette tip was used for HDA [12]. (D) HDA was performed on a chromatography paper with dried reagent storage using a heater [1]. (E). A paper-plastic device coupled with lateral flow test strip was used for RPA followed by DNA detection in an unintegrated format [15]. (F) An integrated paper-plastic device was developed to perform RPA and LFA within one single device [16].(G) RT-RPA was performed using a wax-patterned paper with freeze-dried amplification reagents [3].*

Figure 3. Paper-based amplicon detection. LAMP amplicon (A-B) [10, 13] and RPA amplicon detection (C) [75] detection via streptavidin-biotin interactions. NASBA amplicon detection via nucleic acid hybridization (D) [18]. “Toehold switch”

technology for specific target RNA detection (E) [15]. Multiple test zone LFA for multiple amplicon detection (F-G) [16, 17].

Figure 4. Fully integrated paper-based sample-in-answer-out device for nucleic acid testing. *(A) A "paper machine" was developed for NAT by a simple sliding motion of the strip, allowing sample addition, washing, FTA card-based DNA extraction, amplification and detection with minimal operation [6]. (B) A foldable fully integrated paperfluidic chip was developed to achieve RNA extraction and amplification using polyethersulfone as a substrate, followed by lateral flow detection (Scale bar: 2.5 cm) [17]. (C) A four-layered integrated paper-based device was developed for NAT, which was activated by connecting each layer of paper to control the fluid flow from FTA card to glass fiber and lateral flow strip for sample-to-answer process (Scale bar: 1 cm) [7]. (D) A fully disposable and integrated paper-based device for NAT was introduced, which allows on-chip reagent storage and equipment-free amplification with minimal operation steps [4]. (E) A paper-based origami device was developed to achieve DNA extraction, LAMP and fluorescent detection using one single device [5].*

Figure 5. Commercial paper-based devices for nucleic acid testing. Whatman FTA cards commercialized by GE Healthcare Life Sciences, USA for nucleic acid extraction (A) [22, 24]. Lateral flow test strip commercialized by Ustar Biotech (China) (B) [95]. Generic lateral flow test strip commercialized by Milenia Biotec, Germany for amplicon detection (C) [13]. Paper-based sample-in-answer-out devices developed by Diagnostic for All, USA for infant HIV (D), Ebola (E) and other disease detection (F) (http://dfa.org/nucleic-acid-testing/).

Table 1. Challenges to be addressed for creating an ideal paper-based nucleic acid testing.

Function	Challenges	Possible solutions	Advantages	Disadvantages	Refs
Paper-based sample preparation	Rapidity and cost-efficiency	Fast technology analysis (FTA) card	More rapid and cheaper than conventional phenol-chloroform extraction method	Slower and more expensive than FINA Requires multiple washing steps	[21, 22]
		Filtration isolation of nucleic acid (FINA)	More rapid than FTA card-based extraction Low cost (<$0.05 USD per test)	Requires multiple processing steps	[32]
	Capability of processing diverse sample types	FTA card	Capable of processing diverse sample types, such as sputum, whole blood, stool, swabs, urine *etc*.	Requires multiple washing steps	[21, 22]
		FINA	Capable of processing diverse sample types, such as whole blood, buccal swab, saliva, sputum *etc*.	Requires multiple processing steps	[32]
	Automation	Incorporating paper-based fluidic control methods (*e.g*, geometry-based flow control strategies)	Simplify the assay Operable by untrained users	Special design of channels is required Optimization is needed	[39]
	On-board reagent storage	Dry reagent storage activated by water addition	Stable storage	Requires freeze drying step Requires water addition step	[42]
		Liquid reagent storage in integrated sponges	Simple activation without requiring an additional water addition step	Risk of evaporation	(unpublished)
	Flexibility of being integrated into sample-in-answer-out device	Extraction is followed by *in situ* amplification in the same paper matrix	Simple and efficient without elution step	Type of paper matrix should be optimized to be compatible for both extraction and amplification	[21]
Paper-based nucleic acid amplification	Rapidity and efficiency	Using a rapid isothermal amplification technology (*e.g.*, RPA)	Allows rapid and efficient amplification	-	[52]

		Using a proper type of paper matrix (*e.g.*, polyethersulfone)	Enables efficient amplification	-	[54]
	Multiplex amplification	Using the target specific primers with different labels (*e.g.*, for LAMP and RPA)	Producing amplicon with different labels to be readily detected by a lateral flow test strip	Need multiple types of antibodies	[16, 17]
	Cost-efficiency	Low cost chemical heater is proposed (*e.g.*, pocket warmer, toe warmer)	Cost effective and readily available	Requires optimization to produce consistent heat	[43, 60-64]
	Capability of combining with simple detection method	In situ amplification is followed by direct fluorescent detection	Enables *in situ* detection	Requires an extra UV light source or fluorescent imaging system	[22]
		Amplification is combined with lateral flow test strip	Simple colorimetric readout	Normally requires an extra step of buffer addition	[23, 48]
Paper-based amplicon detection	Rapidity and simplicity	Lateral flow assay (*e.g.*, streptavidin at the test zone to capture biotinylated amplicon)	Simple colorimetric readout	Poor quantification	[10]
		Paper-based "toehold switch" technology to detect amplicon via colour changes	Simple colorimetric readout Enables quantification	Only applicable to RNA viral detection at this stage	[15, 19]
	Multiplex detection	Using multiple-zone test strip	Easily detectable	Limited target could be detected using a test strip	[16, 17]
		Using multi-colored silver nanoparticles	Readily distinguishable	Requires preparation of multiple colored nanoparticles	[67]
	Sensitivity	Probe-based sensitivity enhancement method	Sensitive	Normally involves multiple steps	[69]
		Enzyme-based method	Sensitive	Normally involves multiple steps	[70]
		Thermal contrast	Sensitive	Requires an extra UV light source	[72]
		Fluidic control method	Simple	Longer assay time	[73, 74, 92]
	Semi-quantification and	Multiple test zone semi-quantification	Simple readout	Do not provide exact target	[76]

	quantification			concentration	
		Smartphone app quantification	Provide accurate target concentration	Requires a specific apps and smartphone accessory	[79, 80]
Fully integrated paper-based sample-in-answer-out assay	On-board sample collection	Creating an on-board blood collection tool	Simple and user-friendly	Limited to blood-based diagnostic assay	[81]
		Incorporating the function of sample concentration	Enables sensitive detection of low amount of target in large volume of sample	Multiple processing steps are required	[4, 6]
	On-board paper-based readout timer	Using printed dye on paper to track the assay completion time	Simplify the assay	Requires optimization process to accurately track the assay time	[82, 83]
	On-board maintenance of optimum testing environmental conditions	Creating a closed temperature-humidity control device	Enables optimum biomolecule reaction under optimum environmental conditions	Requires optimization for precise temperature and humidity control Requires the use of an extra closed system	[85]

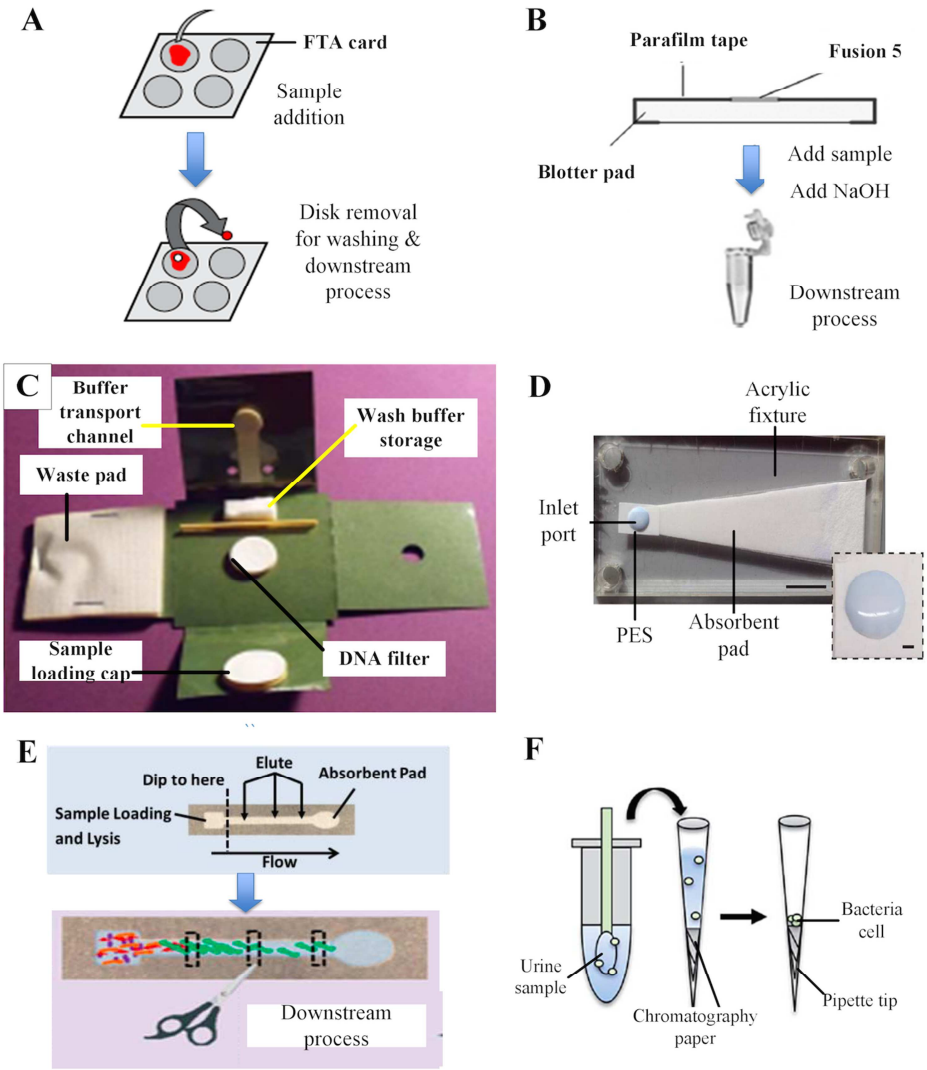
Table 2. Commercial paper-based devices for nucleic acid testing.

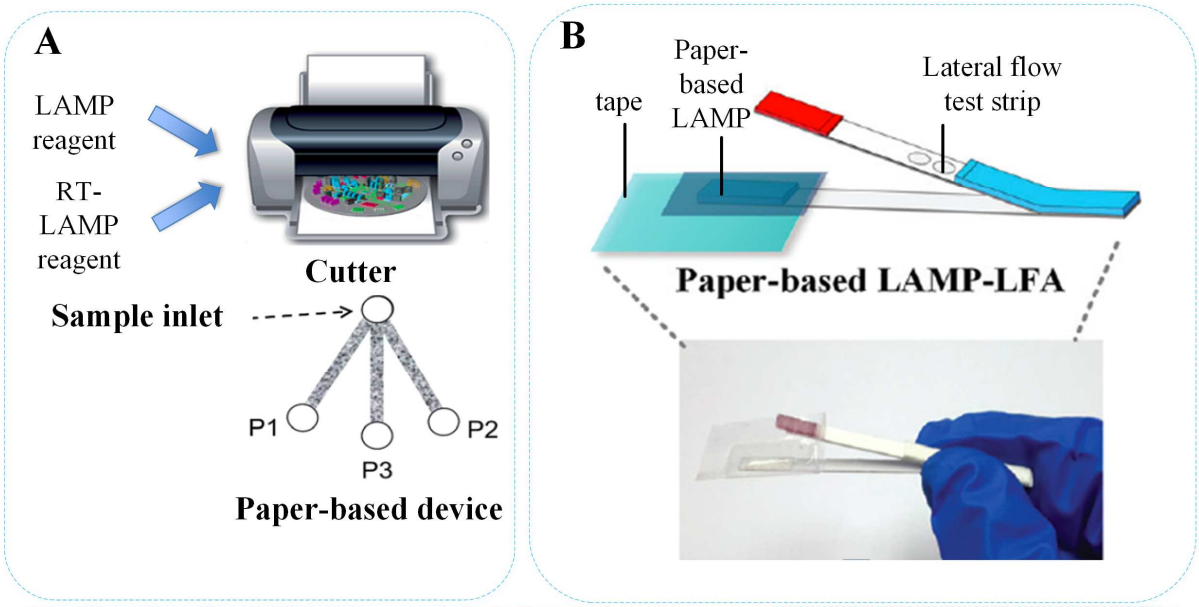
General Function	Paper-based product	Company name	Country	Applications	Stage of product	Year founded
Nucleic acid extraction	FTA card	GE Healthcare Life Sciences	USA	Extract nucleic acid from diverse samples. Widely used in drug metabolism (DM), pharmacokinetic (PK), and toxicokinetic (TK) studies.	FDA approved	2004
Nucleic acid detection	PCRD nucleic acid detector kit	Abingdon Health	UK	To detect amplicons labeled with DIG/biotin and /or FITC (or FAM)/biotin to test various diseases	FDA approved	2008
Nucleic acid detection	Disposable nucleic acid detection device	Ustar Biotechnologies	China	To detect biotinylated amplicons for various applications, such as detection of tuberculosis (TB), TB multidrug-resistance (TB-MDR), sexually-transmitted diseases (STD)	FDA approved	2005
Nucleic acid detection	Milenia ® HybriDetect (generic lateral flow dipstick)	Milenia Biotec	Germany	To detect all the biotin- and FITC-labeled analytes for various applications	FDA approved	2000
Sample-in-answer-out	HIV, Ebola and Hepatitis C diagnostic test kit	Diagnostic for All	USA	HIV, Ebola and Hepatitis C infection	Under development	2007

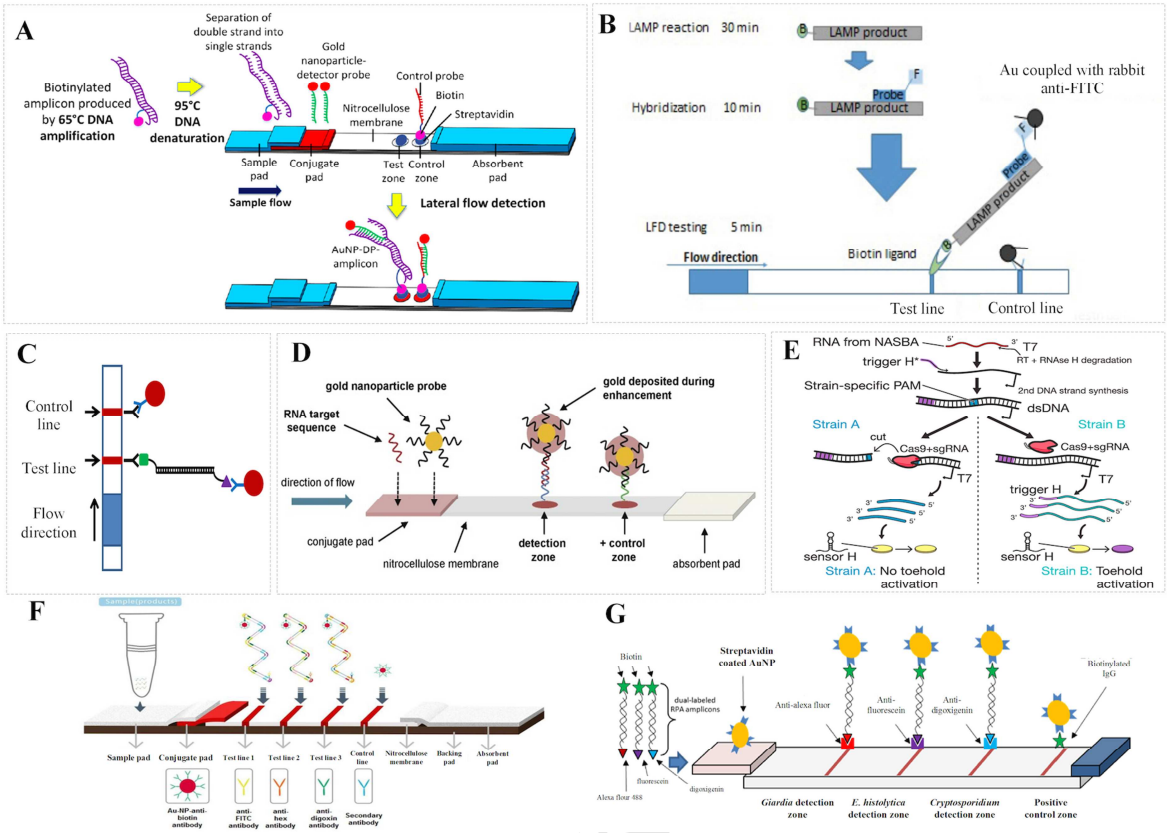
Table 3. Ideal characteristics of paper substrates for each step of nucleic acid testing.

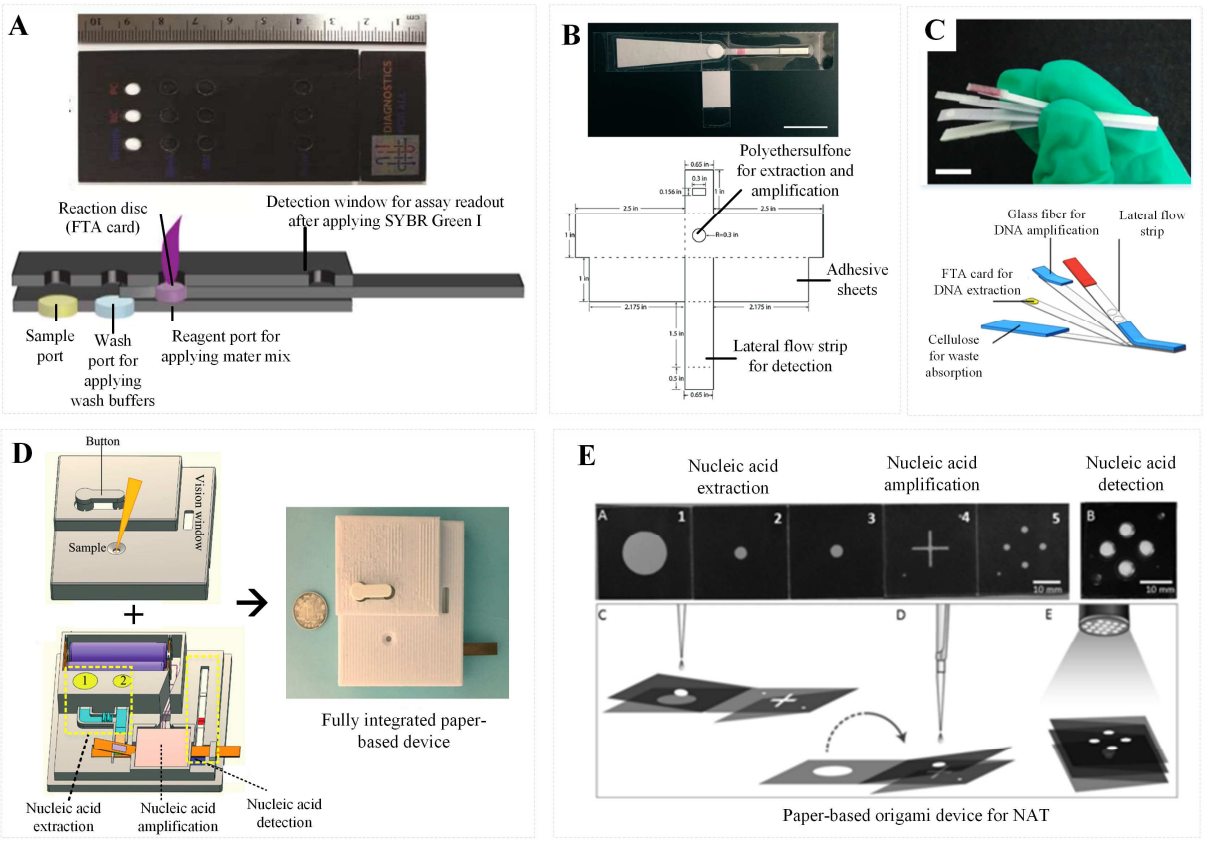
NAT step	*Ideal characteristics of paper*	*Example*	*Material*	*References*
Nucleic acid extraction	*High hydrophilicity* *Deposited with lysis reagent* *Sample preservation properties* *Non-protein binding in nature to enhance DNA purity*	*FTA card* *(Impregnated with chemicals for sample storage and lysis, including surfactant, chelating agent, buffer and free radical trap)*	*Cellulose*	*[6, 7, 18]*
Nucleic acid amplification	*High hydrophilicity* *Average pore size (0.3-0.5 μm)* *Low protein binding efficiency (e.g., non-specific polymerase enzyme binding to paper might decrease amplification efficiency)* *Low water absorbency rate to avoid significant amount of reagent absorption*	*Polyestersulfone (PES) membrane* *(Average pore size: 0.5 μm; weak zwitterionic surface treatment to enhance hydrophilicity, low protein binding efficiency, low water absorbency)*	*Cotton linter and cellulose*	*[10, 16]*
Nucleic acid detection	*High hydrophilicity* *Less swelling characteristics which allows even wetting of*	*Reaction membrane of lateral flow test strip* *(Facilitates the immobilization of*	*Nitrocellulose membrane*	*[19-21]*

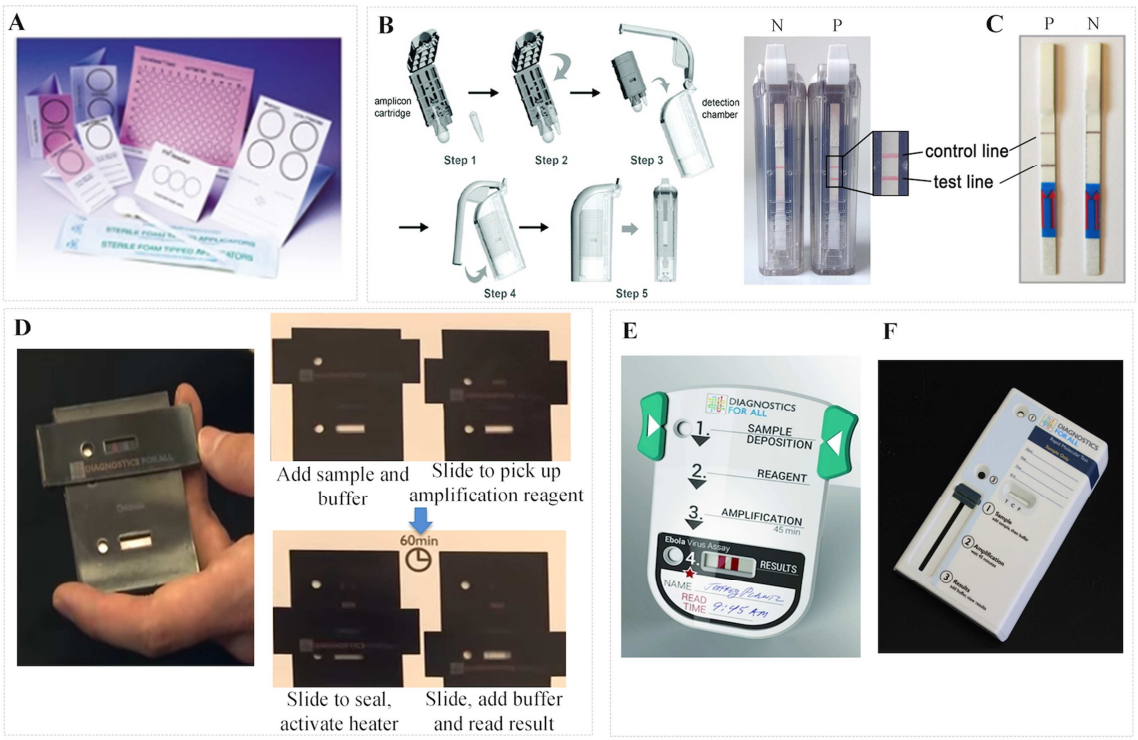
membrane and optimum flow to produce optimum colour intensity of test zone in LFA	*capturing molecules to capture the targeted nucleic acids)*
Strong protein and DNA binding properties for biomolecule immobilization	




A
LAMP reagent
RT-LAMP reagent
Cutter
Sample inlet
P1
P2
P3
Paper-based device
B
tape
Paper-based LAMP
Lateral flow test strip
Paper-based LAMP-LFA
C
Amplified DNA
Pipette tip
Chromatography paper
D
Amplification mix on paper
Plastic enclosure
Paper-based HDA
E
Base layer
Acetate layer
Sample wick strip
wax
Master mix pad
Magnesium acetate pad
1 cm
F
Base layer
Running buffer pad and slider
Sample slider
Base layer
RPA pad
Dry dilution pad
Wet dilution pad
Lateral flow strip
Mixing pad
1cm
G
RPA on wax-patterned paper
water
sample
Positive control
Negative control
Test group
Freeze-dried RPA reagent







Highlights

-The status and challenges in accomplishing each key NAT step using paper substrates are discussed.

-The advances and challenges in integrating all NAT steps into a single paper-based device are highlighted.

-The possible solutions for real world applications are discussed.

-The latest progress towards commercialization and future perspectives are reviewed.