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Paper-based sample-to-answer molecular diagnostic platform for point-of-care diagnostics

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ABSTRACT

Nucleic acid testing (NAT), as a molecular diagnostic technique, including nucleic acid extraction, amplification and detection, plays a fundamental role in medical diagnosis for timely medical treatment. However, current NAT technologies require relatively high-end instrumentation, skilled personnel, and are time-consuming. These drawbacks mean conventional NAT becomes impractical in many resource-limited disease-endemic settings, leading to an urgent need to develop a fast and portable NAT diagnostic tool. Paper-based devices are typically robust, cost-effective and user-friendly, holding a great potential for NAT at the point of care. In view of the escalating demand for the low cost diagnostic devices, we highlight the beneficial use of paper as a platform for NAT, the current state of its development, and the existing challenges preventing its widespread use. We suggest a strategy involving integrating all three steps of NAT into one single paper-based sample-to-answer diagnostic device for rapid medical diagnostics in the near future.

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1. Introduction

Molecular technology platforms are critical for the diagnosis of numerous diseases (*e.g.*, human immunodeficiency virus (HIV), dengue, cardiovascular diseases, and stroke) for timely medical treatment (Akhtar et al., 2011; Katsanis and Katsanis, 2013; Krebs et al., 2014). Nucleic acid testing (NAT), a widely used molecular diagnostic technique, promises rapid, and greater specificity and sensitivity target detection, over the other conventional assays (*e.g.*, viral isolation and immunological assays) (Lollo et al., 2014; Niemz et al., 2011; Warren, 2012). Nonetheless, NAT, which generally consists of three key steps, *i.e.*, nucleic acid extraction, amplification and detection, currently involves labor-intensive, high-cost, and time-consuming processes, significantly limiting its applications to diagnostics at the point of care (POC) (Martinez et al., 2010b; Wang et al., 2012). With increasing spread of infections with no specific medication for treatment (*e.g.,* dengue infections or HIV), rapid diagnosis is imperative to prevent a life-threatening complication (Wang et al., 2010). Therefore, there is an urgent need to develop a portable, fast and accurate NAT diagnostic tool, which can be readily implemented in disease-endemic, low resource settings to overcome the shortcomings of conventional NAT (Niemz et al., 2011).

With advances in microfluidic technologies, integrated chip-based biosensors have emerged with great potential to address the limitations of conventional equipment for point-of-care (POC) NAT. These microfluidic devices can process a small volume of liquid, significantly reducing the consumption of samples and reagents, and thus the cost. However, most chip-based devices are made of glass (Kumbhat et al., 2010; Ngo et al., 2014), silicon (Huang et al., 2013a) or polymer (*e.g.,* polydimethylsiloxane (PDMS)) (Lien et al., 2006) with integrated functional units (*e.g.,* pumps and valves) (Hawkins and Weigl, 2010) which require complicated fabrication processes, hence reducing their suitability for POC testing (Nilghaz et al., 2012).

Recently, paper has been unitized as a feasible and high potential platform for NAT, and has gained increasing interest as a platform in the diagnostic field, especially for low-resource settings due to its simplicity, cost-efficiency, biodegradability, and biocompatibility (Hu et al., 2014). It is predominantly composed of cellulose fibers with a porous structure, allowing fluids to wick through *via* capillary force (Chen et al., 2015). This special characteristic confers advantages in sample storage, mixing, filtering, transport, volume control, timing control and multiplex detection. With flow rate control, paper-based assays could eliminate multiple washing and incubation steps, and minimize the need for highly trained personnel, showing great potential to improve the existing NAT techniques (Parolo and Merkoçi, 2013). Recently, several molecular studies have demonstrated the use of paper in all three steps of NAT, such as the extraction of nucleic acid using filter paper or Fast Technology Analysis (FTA) card (Lange et al., 2014; Liang et al., 2014), nucleic acid amplification by a plastic and tape-covered glass fiber (Rohrman and Richards-Kortum, 2012), and nucleic acid detection by the most commonly used lateral flow assay (LFA), and paper-based microfluidic devices (He et al., 2010; Hu et al., 2013). Thus, the integration of the three main steps into one single rapid, simple, affordable and easy-to-use paper-based sample-to-answer diagnostic device may pave the way for rapid disease detection (Fig. 1).

There exist a number of excellent review articles on the evolution of NAT. However, they emphasize on microfluidic NAT chips (Lee et al., 2006; Mauk et al., 2015; Wu et al., 2014; Zhang and

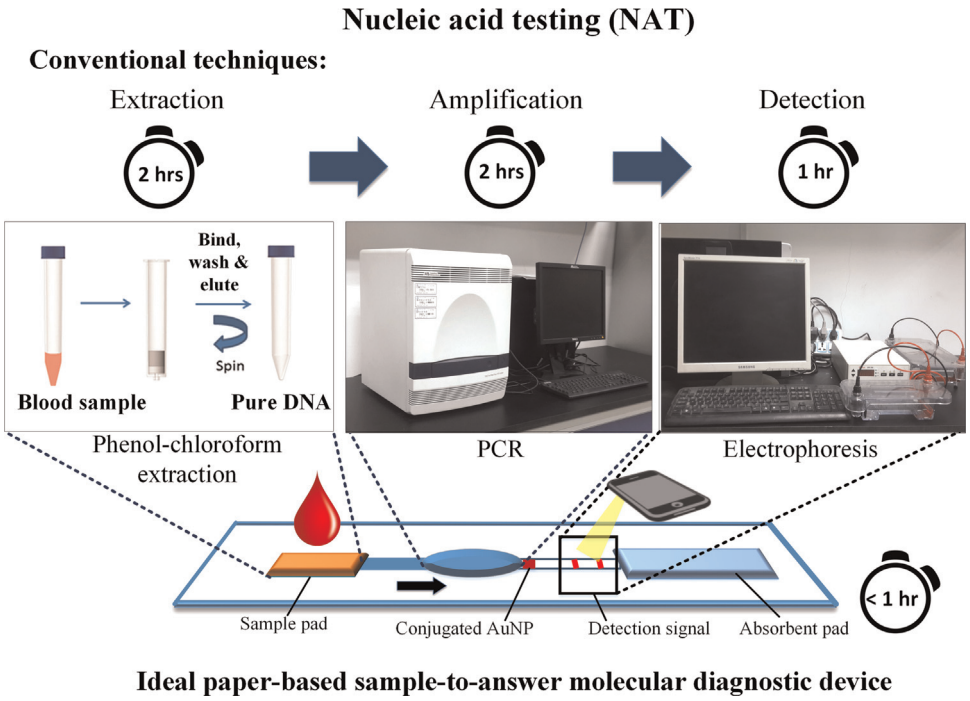

Fig. 1. An ideal paper-based sample-to-answer molecular diagnostic device for rapid disease detection. The integration of three main steps of NAT into one single paper-based device is in high demand to improve the labor-intensive, high-cost and time-consuming phenol-chloroform extraction technique, PCR and one of the amplicon detection techniques, electrophoresis.

Table 1
The comparison of different types of commercial device for NAT.

Types of paper	Purpose	Sample type	Sample volume	Sample preservation period	Temperature	Working mechanism	Advantages	Disadvantages	References
FTA card	Sample storage and nucleic acid extraction	Blood, urine, saliva, cells, buccal cells and plant materials	5–500 μl/ collection area	> 17 years	21–25 °C	Impregnated with chemicals, including surfactant, chelating agent, buffer and free radical trap that lyse cells, capture, stabilize and protect DNA	Stabilization and protection of nucleic acid for long term storage at room temperature Consisting of indicating dye for use with clear samples DNA is free of PCR inhibitors Wraparound cover is provided for protection	Washing step required Purification step required	(Abdelwhab et al., 2011; Ahmed et al., 2011; Aye et al., 2011; Gustavsson et al., 2009; Guzman and Kouri, 2004; Liang et al., 2014; Parsons and Bright, 2012; Stangegaard et al., 2013; Yan et al., 2014)
FTA Elute card	Sample storage and nucleic acid extraction	Blood, urine, saliva, cells, buccal cells and plant materials	12–40 μl/ collection area	> 8 years	21–25 °C	Impregnated with chemicals, including a chaotropic salt	Cell storage at room temperature Cell lysis and purification can be performed within the card matrix Elution can be done with sterile water Eluted DNA can be directly used for downstream analysis Wraparound cover is available for protection	Washing and elution step required	(Gray et al., 2013; Harahap et al., 2012; Namangala et al., 2013; Parsons and Bright, 2012; Santos et al., 2012; Wang et al., 2013)
903 protein saver card	Sample storage	Blood, urine	75–80 μl/ collection area	< 1 year	−80 to −20 °C	High purity cotton linters with no wet-strength additives	Wraparound cover available for protection Whatman foil barrier Ziploc bag available for storage	No stabilizing properties for sample preservation Extraction kit required	(Breier et al., 2014; Duncombe et al., 2013; Fuehrer et al., 2011; Lima et al., 2012; Rottinghaus et al., 2013)
Generic lateral flow dipstick	Amplicon detection	Amplicon	120–130 μl/ dipstick	–	–	Streptavidin is coated on the test line which would bind to the biotinylated amplicon and produce color	Low cost Easy to use, Rapid detection (5–10 min)	No sample storage and processing prior to target detection	(Arunrut et al., 2011; Khunthong et al., 2013; Prompamorn et al., 2011; Rigano et al., 2014)

Xing, 2007) or only discuss the broad range of amplification techniques (Asiello and Baeumner, 2011; Chang et al., 2012; Craw and Balachandran, 2012; de Paz et al., 2014). Although NAT chips have been widely developed, in order to overcome the financial barriers in the underdeveloped or developing countries, low-cost paper-based diagnostic devices with simple fabrication and operation process are preferred, which offer great potential to substitute the existing NAT chips. In view of the rising need for the low cost paper-based diagnostic devices, we here highlight the importance of integrating all necessary molecular diagnostic steps into one single paper-based device, and review the latest advance of paper-based diagnostic assays in this regard. We first discuss the importance of performing NAT in POC diagnostics with various existing techniques. We emphasize the beneficial use of paper as a platform to perform all necessary diagnostic steps, from sample collection to nucleic acid extraction, amplification and detection. We compare the potential integrated paper-based devices with the existing integrated chip-based devices. We also provide a glimpse of the existing challenges and the potential deployment of a paper-based sample-to-answer diagnostic platform in the near future.

2. Paper as a platform for nucleic acid extraction

In a molecular assay, nucleic acid extraction step plays a significant role in isolating DNA or RNA from the complicated biological sample (*e.g.*, blood sample) (Niemz et al., 2011). This process is important to eradicate the substances, which may interfere with the subsequent amplification process (*e.g.*, heme from red blood cells). Conventional nucleic acid extraction techniques require lengthy processes, high-end equipment, dedicated laboratory space, and skilled personnel (Dineva et al., 2007). Thereafter, on-chip nucleic acid extraction techniques (e,g., chip-based solid phase extraction (SPE) (Wolfe et al., 2002), dielectrophoresis trapping (Prinz et al., 2002) or isotachophoresis separation techniques (Wainright et al., 2003), have been introduced, which, however, may compromise the simplicity of the fabrication and operation process (*e.g.*, require multiple processing steps and external power source). Considering the simplicity and the cost entailed with the isolation of high quality and quantity of nucleic acid in low-resource and disease-endemic settings, a rapid, low-cost and uncomplicated nucleic acid extraction technique is in high demand for the subsequent nucleic acid amplification and detection.

2.1. Advantages of using a paper-based device in nucleic acid extraction

In recent years, apart from achieving high extraction efficiency, attention has been focused on the use of paper in fabricating diagnostic devices. Rapid nucleic acid detection has been attempted, especially in the LFA format (Mao et al., 2009a). However, in the absence of sample preparation, these devices are unable to detect the nucleic acid in raw biological samples. As such, an inexpensive material is an attractive platform for nucleic acid extraction, as an upstream module of a paper-based device to be combined with the existing downstream detection module (*e.g.*, LFA). Besides having a porous structure, cellulose, as a source of paper, enables proper mixing of sample and reagents, as well as immobilization of functional biomolecules (*e.g.*, DNAs or proteins), which can be utilized for nucleic acid extraction (Pelton, 2009). As cellulose is thermally stable (can be heated up to 300 °C), it fully meets the requirement for heating in the extraction process (Wong et al., 2014). Besides enabling a simple fabrication process, the biodegradability and disposability of cellulose eliminates the risk of cross-contamination by allowing only one-time use of the extraction device.

2.2. Recent advances in paper-based nucleic acid extraction

A variety of commercial extraction devices are currently used in sample collection and nucleic acid extraction. These devices are compared in terms of their general characteristics and working mechanism in Table 1. Guthrie card or dried blood spot (DBS) filter paper, consisting of 903 filter paper, made of $>90\%$ cellulose, has been used for blood collection from a pricked finger for subsequent analysis (de Vries et al., 2012). As an alternative, 903 protein saver card or WhatmanTM 903 filter paper has been introduced for sample collection and nucleic acid extraction (Kudo et al., 2004). These cards have no stabilizing properties, thus requiring lower temperature (-80 to -20 °C) for storage. To improve the extraction efficiency and facilitate high throughput, FTA cards and FTA Elute cards have been introduced. Unlike 903 protein saver card, FTA cards (Lange et al., 2014; Liang et al., 2014) are chemically-treated for DNA storage and extraction. FTA card, in particular, has been most commonly used for routine production of nucleic acid samples from a broad range of biological specimens, such as whole blood, plant cells, tissue culture cells and microorganisms (Fig. 2A). FTA card consists of filter matrices impregnated with a patented chemical formula that lyses cell membranes and denatures proteins (Aye et al., 2011). It immobilizes the DNA to produce a web-like structure around the filter matrices and protects the DNA from oxidation, nucleases, UV radiation damage or microbial contamination (Beckett et al., 2008). The cellular debris, inhibitors and stabilizing chemicals can be readily washed off with washing buffer (Goldsborough and Fox, 2006). The DNA can be stored in the form of dried whole blood spots for at least 7.5 years at room temperature, and the extracted DNA can remain stable in the card, circumventing the need for centrifugation and refrigeration equipment (Pezzoli et al., 2007).

With the simple storage technique, the FTA card can be shipped to the laboratories for subsequent analysis through regular postal service, making them a very attractive tool for field sample collection (Lange et al., 2014; Liang et al., 2014). The filter membrane can be directly added to the tube for further processing. Recently, another type of FTA cards for DNA extraction purposes, namely FTA Elute cards, has been made commercially available. Instead of keeping the DNA attached to the paper matrix for analysis, these cards release the DNA upon the addition of sterile water, hence making the extraction much easier (de Vargas Wolfgramm et al., 2009). A study has demonstrated the use of the FTA card in a microcapillary for sample-to-answer NAT (Zhang et al., 2014) (Fig. 2B). The FTA card has been proven to be successful in lysing the blood cells and extracting the DNA in 60 min. However, punching these commercial extraction devices into a tube with a single punch for downstream analysis (*e.g.*, PCR) could easily lead to cross-contamination between blood spots, and the manual process fails to meet the needs of large-scale samplings. To address this problem, a low-cost and disposable puncher could be developed for future application. Having the special features such as simple collection, room temperature storage and simple processing technique, FTA Elute cards may offer the greatest potential in nucleic acid extraction among various types of commercial extraction devices even that they require simple washing and elution steps. In the future, the use of a handheld battery-powered heater coupled with FTA Elute card to achieve the high temperature for DNA extraction could further increase its potential use in remote settings.

With advances in fabrication technologies for paper, such as printing (Rosenfeld and Bercovici, 2014; Yang et al., 2012), stacking (Liu and Crooks, 2011), and folding (Luo et al., 2014; Martinez

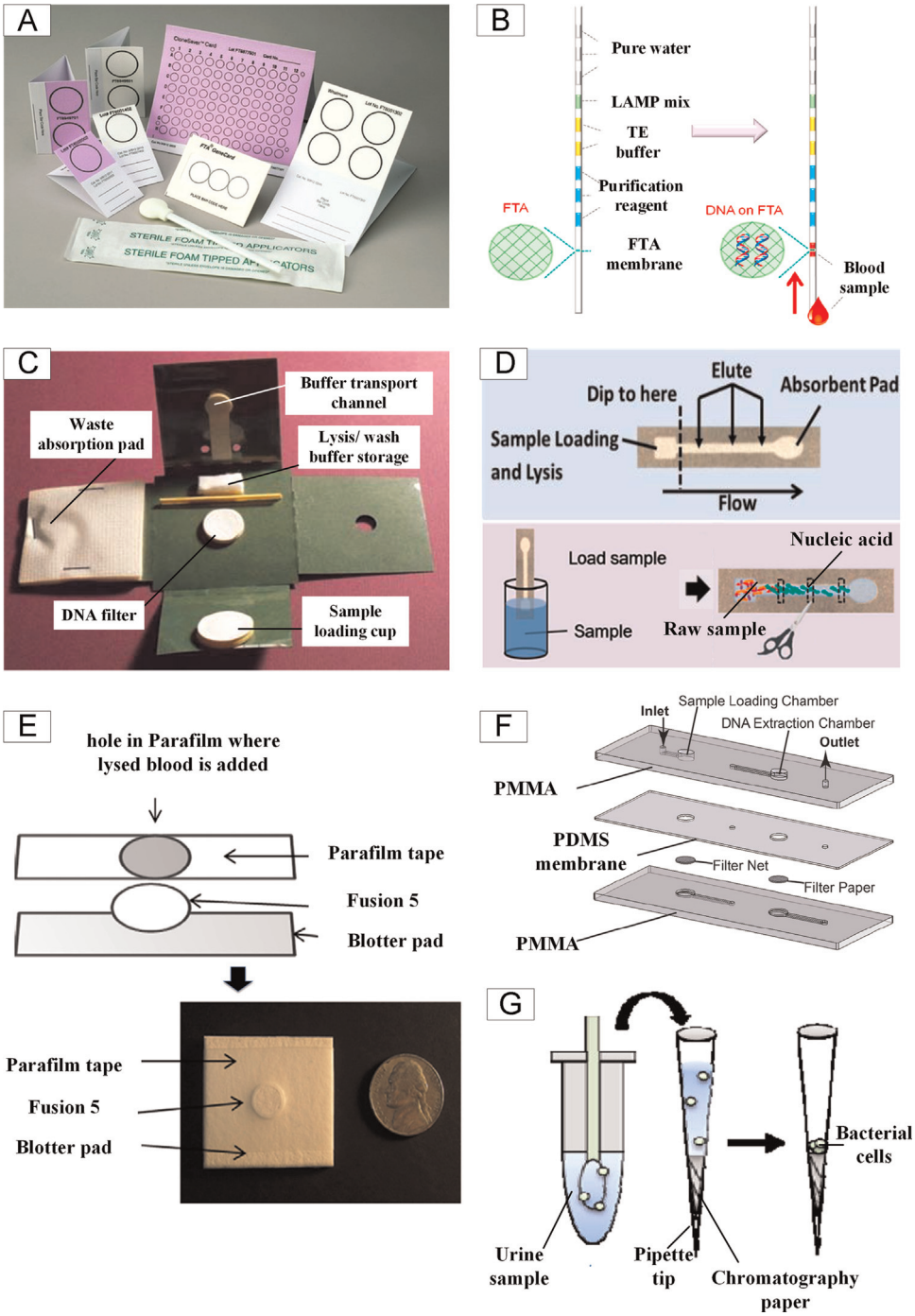

Fig. 2. Paper-based devices for nucleic acid extraction. (A) commercial FTA cards (Liang et al., 2014), (B) integration of FTA card into microcapillary (Zhang et al., 2014), (C) paper-based microfluidic origami fabricated by paper folding technique (Govindarajan et al., 2012), (D) paper-based microfluidic device for DNA extraction from the real sample (Fronczek et al., 2014), (E) DNA extraction by FINA (McFall et al., 2015), (F) paper-based extraction device coupled with PMMA and PDMS (Gan et al., 2014), and (G) an extraction model consisting of a chromatography paper and a pipette tip (Linnes et al., 2014).

et al., 2008), several studies have demonstrated the fabrication of simpler and lower-cost paper-based microfluidic devices for storage and extraction of nucleic acid (Govindarajan et al., 2012; McFall et al., 2015). For instance, a paper-based microfluidic origami consisting of a stack of polymer sheets and papers has been fabricated by a simple paper-folding technique in fewer than 30 min (Govindarajan et al., 2012) (Fig. 2C). Similar to a commercial FTA card, this origami enables dry reagent storage, and allows cell lysis and extraction of a high amount of DNA (150 ml) at room temperature. Further, the ability to store the extracted DNA at room temperature allows easy transportation of the sample to more centralized diagnostic laboratories in the absence of cold shipment. In contrast to the FTA card, which requires additional reagents during the process (*e.g.*, Tris/EDTA (TE) buffer, purification reagents, Tris HCl and EDTA), this device requires only a small amount of non-hazardous elute buffer (150 μl), which reduces the biohazard concerns during the sample preparation and transportation. Thus, this cost-effective technique offers the possibility of processing raw samples in POC settings.

In addition, an easy-to-use paper microfluidic chip has been developed to extract the DNA from a real sample in conjunction with direct detection of nucleic acid by a smartphone (Fronczek

et al., 2014) (Fig. 2D). As compared to nitrocellulose, cellulose has been reported to be more suitable for nucleic acid extraction as the large DNA genome can be easily entrapped within the cellulose fibers. Given that both DNA and nitrocellulose are negatively charged, the charge repulsion could impede their interactions, hence resulting in a lower ability to extract the DNA, as compared to cellulose (Fronczek et al., 2014).

Further, fusion 5, a single layer matrix membrane, has been used in a DNA extraction technique, termed filtration isolation of nucleic acid (FINA) (McFall et al., 2015) (Fig. 2E). This technique has been introduced for DNA extraction at POC in less than 2 min. Similar to the FTA card, the fusion 5 membrane can trap the DNA from the lysed whole blood, which can then be directly inserted into a tube for polymerase chain reaction (PCR) or stored for more than a month for future use. This extraction technique has demonstrated high sensitivity and specificity with only 100 μl whole blood specimen. Additionally, another study has also demonstrated the use of fusion 5 membrane for DNA extraction in a microfluidic device that consists of PMMA and PDMS (Gan et al., 2014) (Fig. 2F). As compared to the commercial QIAamp® DNA Micro kits, this device has been proven to yield a high volume of DNA from a variety of raw samples in 8 min, including whole blood, dried blood stain from both FTA card and Whatman 903 filter paper, buccal swabs, cigarette butts and saliva (Gan et al., 2014). In another study, a model consisting of a piece of 3MM filter paper and a pipette tip has been used for DNA extraction (Linnes et al., 2014). This model was fabricated by cutting and folding the paper, followed by the insertion of the paper into the pipette tip. Apart from enhancing the success rate of amplification, this platform has been proven to be successful in lysing the bacterial cells and extracting the target DNAs (Fig. 2G). In short, the use of paper as a platform for nucleic acid extraction can fulfill the criteria of being low-cost, automated, rapid, portable, user-friendly, having the capability to process a broad range of raw samples, and being flexible enough to be integrated into a sample-to-answer analytical system.

3. Paper as a platform for nucleic acid amplification

In the absence of an amplification step, the extracted nucleic acid is usually undetectable by existing technologies due to the low concentration of target nucleic acid in the body (Craw and Balachandran, 2012). Therefore, nucleic acid amplification is mandatory in NAT (Niemz et al., 2011). Apart from the various cost-effective paper-based sample pre-treatment techniques, a low-cost nucleic acid amplification technique is also required for downstream nucleic acid detection at POC. To determine an appropriate amplification technique for POC settings, amplification duration, reaction temperature, amplification efficiency and assay complexity (*e.g.*, requirements for handling steps or reagents) are all factors to be considered (Craw and Balachandran, 2012).

The requirement for laboratory space, electricity, expensive equipment, and trained personnel in the most-widely used PCR has limited its applicability in low resource areas. Several studies have focused on novel droplet digital PCR (DDPCR), which is capable of precisely quantifying the target sequence (Tadmor et al., 2011). However, the significantly higher reagent cost and exorbitantly expensive high-end instrumentation remains prohibitive for POC adoption (Pinheiro et al., 2012). To this end, recent effort has demonstrated the technique of amplifying nucleic acid at a single temperature (*i.e.*, isothermal nucleic acid amplification), alleviating the requirement for a time-consuming and equipment-dependent thermal cycling process (Crannell et al., 2014a). Various isothermal nucleic acid amplification techniques have been developed, such as loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), helicase-dependent amplification (HDA), recombinase polymerase amplification (RPA) and simple amplification based assay (SAMBA) (de Paz et al., 2014). Since only a fixed temperature heater is required in an amplification process, the assay cost can be significantly reduced. Additionally, some isothermal techniques (*e.g.*, RPA and LAMP) have the capability in processing raw samples directly without DNA purification step, thus increasing simplicity and reducing the total cost and time over PCR (de Paz et al., 2014). In fact, on-chip nucleic acid amplification techniques have also been introduced with implemented amplification microreactor (Liu et al., 2011; Yang et al., 2002), yet requiring complicated fabrication process.

3.1. Advantages of using a paper-based device in nucleic acid amplification

In nucleic acid amplification, reagent storage plays a key role. As a porous material, paper is capable of storing thermally stable reagent in a dry form for prolonged usage (Stevens et al., 2008). Papers with a large pore size, such as glass fiber and polyester, are capable of capturing the molecules, enabling all reagents to be stored stably in a dried formulation at ambient temperature. The dried reagents can be activated by addition of the sample or buffer. This method can therefore prolong the shelf life of paper-based diagnostic devices (Yetisen et al., 2013).

After adding the buffer or sample diluent, the papers with a larger pore size enhance the component diffusion and mixing rate within the pores, resulting in a higher amplification rate compared to the paper with smaller pore size (Yetisen et al., 2013). In comparison with other materials, such as cellulose and the blood separators, Mifare 1 (MF1), vinylidene fluoride 2 (VF2) cards, polyvinyl alcohol-bound glass fiber (GF/DVA) and Fusion 5, glass fiber with larger pore size provides more spaces for diffusion and mixing of reagent components, leading to a higher production yield (Rohrman and Richards-Kortum, 2012). Therefore, glass fiber has been reported as the most suitable matrix for amplification.

3.2. Recent advances in paper-based amplification

A study has reported the use of glass fiber as a platform for nucleic acid amplification (Rohrman and Richards-Kortum, 2012) (Fig. 3A). The study integrates the RPA technique with a paper-based device, coupled with reagent storage and mixing both in the device. RPA has been reported as a breakthrough alternative to PCR due to its simplicity and rapidness. Unlike other isothermal amplification techniques, RPA does not rely on temperature-dependent primer annealing and extension, but rather depends on enzymatically driven primer-annealing process (Lillis et al., 2014). Therefore, it does not require the precisely controlled reaction temperature. The short amplification time (~10–15 min) and low incubation temperature (37 °C) reduce the heating power required without compromising the amplification efficiency. These features further reduce the cost and complexity of the amplification process.

Several studies have investigated the potential of performing RPA at ambient temperatures in the absence of power source. A low-cost and reusable exothermic chemical heater ($0.1 US) containing a mixture of sodium acetate trihydrate (SAT) have been suggested to provide heat for RPA (Lillis et al., 2014). This heating device can be coupled with paper-based device to achieve optimum RPA performance. Surprisingly, it has been recently reported that human body heat is able to support the RPA reactions. Axilla, in particular, has been suggested as an ideal body part for incubating the reactions (Crannell et al., 2014b). Collectively, the rapid RPA with simple body heat incubation, coupled with a

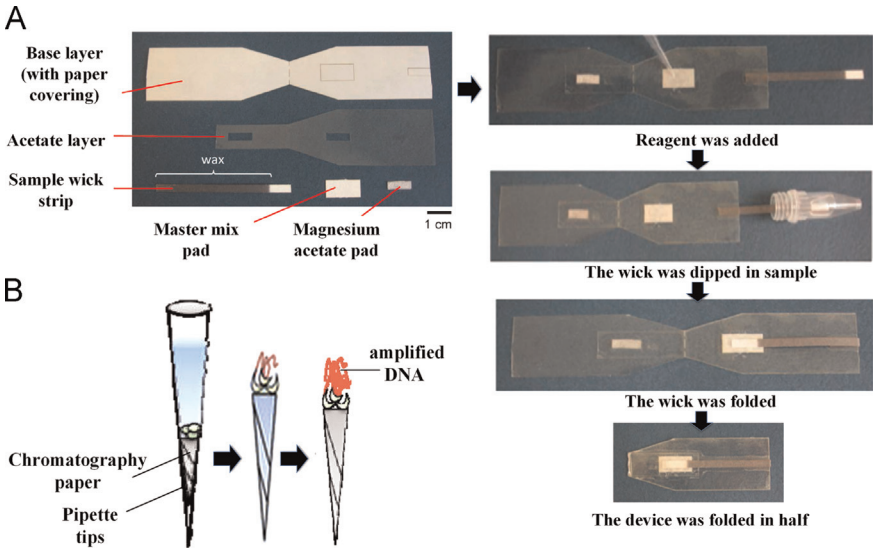

Fig. 3. Paper-based devices for nucleic acid amplification. Paper as a platform for (A) RPA (Rohrman and Richards-Kortum, 2012) and (B) HDA (Linnes et al., 2014).

paper-based amplification device, provides an extremely low-cost amplification technique in POC settings.

In addition to RPA, LAMP has also been suggested as one of the most effective techniques for POC diagnostics. Among various types of amplification techniques, LAMP has higher specificity as it requires six different primers recognizing eight distinct regions on the target sequence. Further, LAMP provides a higher production rate, which is at least 100 times higher than that of PCR. The reaction is performed at a fixed temperature (normally in the range of 60–65 °C) in a short period (30–60 min). Even though the assay time is slightly longer than that of RPA, the highly efficient amplification makes it more reliable and more suitable to be implemented in low-resource settings. However, LAMP requires high heating power to achieve the high amplification temperature needed, which normally depends on heat block or water bath. To address this, portable battery powered heaters have been developed to supply a fixed temperature for LAMP without using external electrical power (Myers et al., 2013). Recently, the development of non-instrumented nucleic acid amplification (NINA) has been reported (LaBarre et al., 2010, 2011). An engineered phase change material, calcium oxide, has been successfully used to incubate LAMP reactions based on exothermic chemical reactions. The technique has been further improved by using magnesium iron alloy (MgFe) with lower cost ($0.06 US per test) to replace calcium oxide for the exothermic reactions (Singleton et al., 2014). Therefore, paper-based LAMP can be achieved by using the suggested heating techniques.

Other isothermal amplification techniques, such as HDA and SAMBA, have also been demonstrated for nucleic acid amplification in POC settings. Recent study has demonstrated a paper-based amplification technique by HDA (Linnes et al., 2014) (Fig. 3B). The isothermal amplification was performed in the cellulose chromatography paper supported by a pipette tip, which was sealed with waterproof adhesive tape. Similar to LAMP, the amplification process requires a high temperature (65 °C). It has been suggested that a low-cost Styrofoam cup holder with commercially available toe warmers could provide an electricity-free heat sources in a POC settings, which showed comparable result to that of dry-block heater (Huang et al., 2013b). Direct DNA amplification following DNA extraction in the low-cost substrate minimizes the overall cost and processing time. However, the process needs further improvement as it requires a pressure supply (20 psi) and multiple reagent addition steps. In addition, this technique has only been used to lyse and filter bacterial cells, which have a larger size than viruses. On the other hand, SAMBA has been performed using a portable cartridge and POC machine for rapid LFA detection (Lee et al., 2010). The integration of simpler and lower-cost paper-based amplification device in these portable equipment coupled with LFA would be suitable for use in the areas with poor infrastructure (Huang et al., 2013b). Based on the aforementioned molecular isothermal amplification technologies, the introduction of a paper-based amplification device would accelerate progress towards an ideal molecular diagnostic device for low-resource settings (Pai et al., 2012).

4. Amplicon detection in paper-based diagnostics

Following nucleic acid amplification, an appropriate detection technique is required to accurately detect and quantify the amplicons. Conventional detection techniques (*e.g.,* agarose gel electrophoresis and fluorescence detection of LAMP amplicons) are time consuming and usually involve complicated and costly equipment, such as electrophoresis units and gel documentations (Ahmad and Hashsham, 2012) while as-described chip-based detection techniques involve complicated device fabrication and operation procedure. Recently, researchers have sought to develop paper-based devices (*e.g.*, LFA) for rapid detection of target nucleic acid (Hu et al., 2013; Mao et al., 2009b). These affordable devices can easily be chemically modified and conjugated with many types of biomolecules (*e.g.*, protein and DNAs), which are compatible with numerous bioassays (Martinez et al., 2010b; Yetisen et al., 2013). Additionally, the devices can produce rapid visual readouts ($<$ 20 min), which meet the requirements of POC testing.

In most LFAs, commercial DNAs have been normally used, which are usually single-stranded, allowing them to easily hybridize with the single-stranded capture probe (CP) at the test line (Hu et al., 2013). In contrast to these synthesized DNAs, most amplicons are double-stranded (*e.g.*, PCR, RPA or LAMP products) (Kersting et al., 2014; Kim et al., 2014; Rohrman and Richards-Kortum, 2012), and cannot hybridize with the CP. Streptavidin coated-generic lateral flow dipsticks (LFD) are commercially available which are able to detect biotinylated double-stranded amplicons (Rigano et al., 2014; Siah and McKenna, 2013; Surasilp et al., 2011) (Fig. 4A). As most amplicons are double-stranded, denaturation at high temperature (95 °C) is required to separate double-stranded DNAs into single strands, allowing the single-stranded DNA to bind with the complementary DNA probe. For

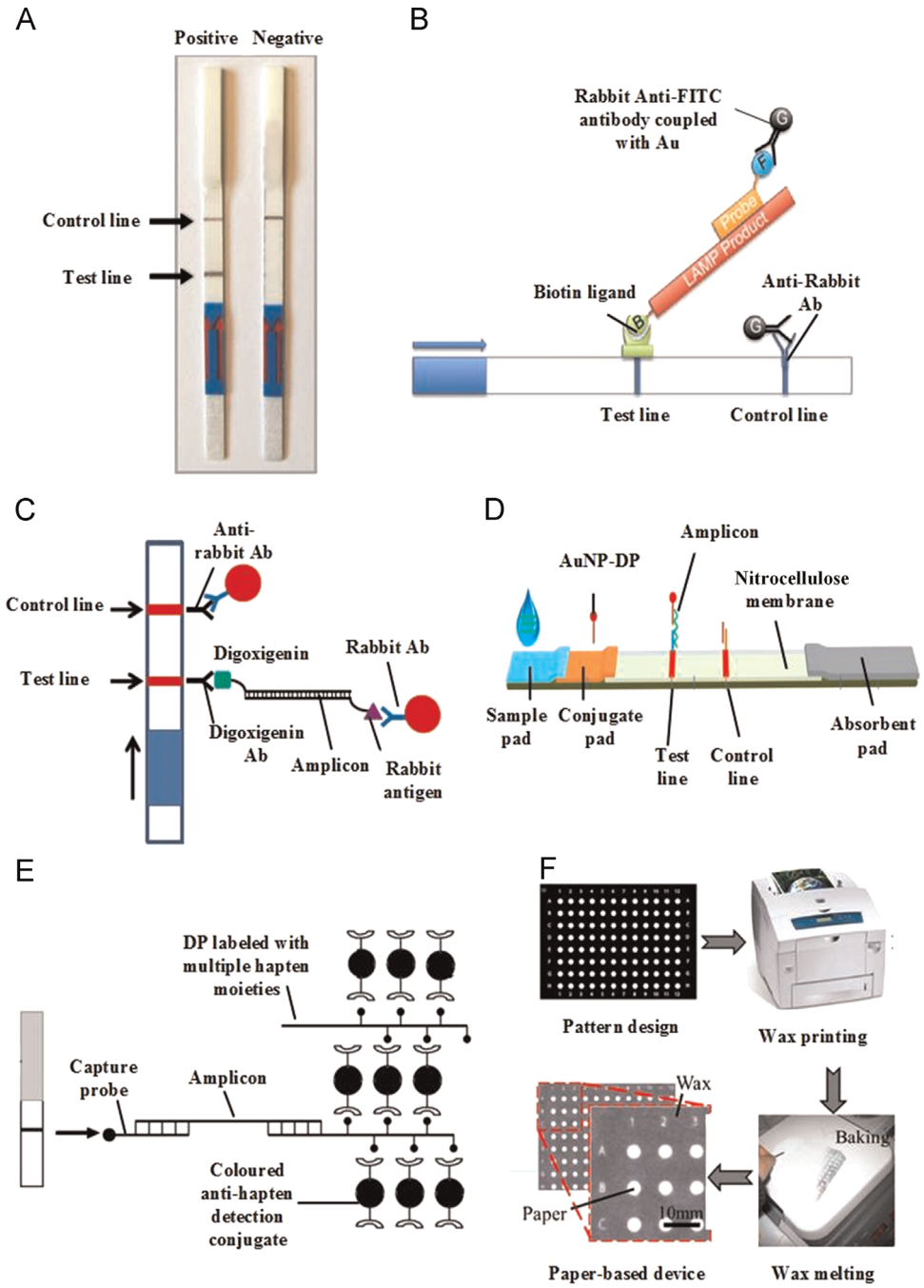

Fig. 4. Paper-based devices for amplicon detection. (A) Commercial generic lateral flow dipsticks (LFD) for detecting amplicon, paper-based devices for (B) LAMP (Khunthong et al., 2013) and (C) RPA product (Kersting et al., 2014) detection *via* streptavidin–biotin interaction, (D) NASBA (Liu et al., 2014) and (E) PCR product (Dineva et al., 2005) detection *via* nucleic acid hybridization, and (F) paper-based microfluidic devices fabricated by wax printing technique for amplicon detection (Lo et al., 2012).

example, LAMP product could be detected *via* a streptavidin–biotin interaction. At the initial stage of LAMP product detection, the biotinylated LAMP amplicons produced from biotinylated primers are denatured. The single-stranded biotinylated amplicons are then allowed to bind to the FITC-labeled DNA probe, which in turn, form complexes with the gold-labeled anti-FITC antibodies and further interact with streptavidin at the test line, producing a red signal observable by the naked eye (Kersting et al., 2014). The excess products would bind to a protein (*i.e.*, FITC) to give a red signal at the control line for assay validation (Khunthong et al., 2013) (Fig. 4B). Besides that, the double-stranded amplicon could also be detected by Ag–Ab interactions. For instance, RPA product labeled with two different antigenic tags, digoxigenin and rabbit Ag could be detected by LFA. Rabbit Ag could bind with the tag-specific Ab on the AuNP. The AuNP-amplicon conjugate in turn binds with the digoxigenin Ab on the test line and produces a positive signal, whereas the control line with immobilized anti-rabbit Ab serves as a control (Kersting et al., 2014) (Fig. 4C).

Besides detecting the amplicons by biotin–streptavidin or Ag–Ab interactions at the test line, the detection of amplicons can also be achieved by nucleic acid hybridization, provided that these amplicons are single-stranded (*e.g.*, NASBA products) (Liu et al., 2014) (Fig. 4D). As NASBA produces single-stranded RNA, the amplicons can hybridize with CP. The target RNA would bind to the AuNP labeled detector probe (AuNP-DP) to form complexes, which in turn, bind to the CP at the test line to produce an observable

signal. For example, a nucleic acid based LFA has been coupled with NASBA to quantitatively detect amplified HIV RNA concentration at a clinically relevant range of HIV viral load (500–1000 copies/mL) (Rohrman et al., 2012). In fact, double-stranded amplicons can also be detected by nucleic acid hybridization at the test line upon the denaturation process. For instance, three different PCR products, hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV 1) were simultaneously detected by LFA based on hybridization of the amplicon with target-specific CP and DP (Dineva et al., 2005) (Fig. 4E).

Most nucleic acid based LFAs involve in diverse detection approaches including colorimetry, fluorescence and chemiluminescence (Cate et al., 2014; Martinez, 2011). As these LFAs usually suffer from low sensitivity, there have been several attempts to develop multiple techniques for enhancing sensitivity. The common signal enhancement techniques include probe-based signal enhancement (Hu et al., 2013), enzyme-based signal enhancement technique (He et al., 2011), thermal contrast (Qin et al., 2012), or fluidic control technique (Parolo et al., 2013; Rivas et al., 2014). It has been reported that the sensitivity of LFA could be enhanced by using particles such as liposomes (Connelly et al., 2008; Kumanan et al., 2009) or the aggregation of AuNP aggregates to achieve almost 3-fold signal amplification (Hu et al., 2013). Besides that, the potential of enzymatic amplification has been demonstrated for signal enhancement. For instance, horseradish peroxidase (HRP) has been used to catalyze luminol in the presence of hydrogen peroxide (H_2O_2), and generate chemiluminescent signal with improved sensitivity over the gold nanoparticle (Kim et al., 2010).

In addition, a molecular beacons (MB)-based LFA has been developed for sensitive detection of nucleic acids (Mao et al., 2009b). MBs are oligonucleotide probes, which are highly selective in oligonucleotide recognition, producing fluorescent signal upon hybridization. This technique results in a higher sensitivity of the assay compared to conventional dye-labeled MBs and commonly used DNA–Au-NP-based LFA. Besides, hairpin oligonucleotide (HO)-gold nanoparticle has been proven to be successful in enhancing the sensitivity of single-nucleotide polymorphism (SNP) detection. The unique recognition characteristic of HO to specific DNA and single-base mismatched DNA lowers the detection limit (He et al., 2010). Additionally, an ultrasensitive nucleic acid-based LFA has been further developed using HRP–AuNP dual labels. In the presence of H_2O_2, the enzymatic reaction between HRP and 3-amino-9-ethyl-carbazole (AEC) produces insoluble red products, which are deposited on the AuNP. This technique enables the detection of as low as 0.01 pM without using instrumentation, showing a great promise for POC diagnosis (He et al., 2011).

Further, sensitivity of LFA can also be improved using metal particles with thermal contrast. Upon the optical stimulation by laser, the metal particles released heat, producing colorimetric signal with 36-fold signal enhancement (Qin et al., 2012). Additionally, several fluidic control techniques have also been reported to improve assay sensitivity of LFA, including paper architecture modifications to increase the amount of sample (Parolo et al., 2013) and the use of wax-printed pillars as delay hydrophobic barrier to slightly reduce flow rate and allow the sufficient mixture of sample and reagents prior to the detection (Rivas et al., 2014).

Microfluidic paper-based analytical devices have also been used for amplicon detection. For instance, a paper-based device fabricated by wax printing technique has been used to detect LAMP products based on biotin–streptavidin interaction (Lo et al., 2012) (Fig. 4F). However, the binding affinity of the biotin–DNA complex to the streptavidin–HRP complex in the paper-based system was relatively low. To this end, a more stable detection approach has been developed using fluorescent DNA probes to accurately detect DNA and produce a fluorescence signal (Lo et al., 2013).

In fact, the highly versatile paper-based device could be readily integrated into small-sized portable instruments. Therefore, more studies are required to provide further insights into the functional development of the combination of small-scale heating devices or portable detectors (*e.g.* to detect fluorescence and chemiluminescence signals) and a simple paper-based device suitable for POC molecular diagnostics.

5. Potential development of an integrated paper-based sample-to-answer molecular diagnostic device

Although each step of paper-based NAT has been briefly discussed earlier, a key challenge for the development of an ideal POC nucleic acid detection technique is integrating all these steps into a single diagnostic device. The advantages of developing a fully integrated paper-based system for NAT include cost reduction (can be as low as $0.01, considerably lower than chip-based microfluidics (Carrilho et al., 2009)), simplification of device fabrication and operation process, increased portability of the device and simplicity of data read out, suitable for POC applications. The comparison between the existing integrated chip-based devices and the potential integrated paper-based devices is listed in Table 2.

5.1. The existing integrated chip-based sample-to-answer devices

Several studies have developed an integrated chip-based sample-to-answer diagnostic device, which incorporates on-chip nucleic acid extraction, amplification and detection for rapid NAT. For instance, a glass-made microfluidic device has been developed for on-chip nucleic acid extraction, amplification and detection but this technique requires a syringe pump and an electric-powered block heater to complete the process (Wu et al., 2011). In another study, an integrated microfluidic system has been developed containing a suction-type, pneumatic microfluidic control module and a self-compensated temperature control module for sample-to-answer process (Wang et al., 2011). However, tedious step of

Table 2
The comparison between integrated chip-based and paper-based devices.

	Integrated chip-based devices	Integrated paper-based devices
Components	Polymers (PDMS or PMMA), glass, silicon	Paper and membrane
Fabrication technique	Photolithography, wet chemical etching or high precision laser cutting technique	Hydrophilic–hydrophobic patterning, paper stacking and folding
Driving force	Pump	Capillary force
Advantages	A fully enclosed microfluidic system reduces the opportunity for contamination from conventional liquid transfer steps	Low cost, simple fabrication process, allows reagent storage
Limitations	Potential adsorption of the reagents to the chip surface, risk of gas bubbles formation	Need improvement in terms of sensitivity, quantification and functionality

purification process (*e.g.*, interference removal and washing step controlled by external vacuum pump and permanent magnet) is required which is laborious for POC applications (Fang et al., 2012). To address this problem, a portable integrated microchip composed of a DNA release chamber and amplification reaction chamber has been developed to achieve on-chip sample preparation to target detection without the process of nucleic acid separation or purification. However, this device requires manual control of screw valve to enable the fluid flow from one chamber to another coupled with an electrical powered heater (*e.g.*, water bath) for cell lysis (80 °C) and nucleic acid amplification, hence making the device less suitable for use in remote settings (Fang et al., 2012).

5.2. Advantages of developing integrated paper-based sample-to-answer devices

Whilst a number of fully integrated chip-based sample-to-answer molecular diagnostic devices have been reported, it is important to emphasize critical features that distinguish the potential fully integrated paper-based devices with the existing integrated chip-based devices. Firstly, the integrated chip-based devices are normally composed of PDMS and PMMA, which require complicated fabrication techniques using standard photolithography, wet chemical etching or high precision laser cutting technique (Legendre et al., 2008). In contrast, integrated paper-based diagnostic devices, which are composed of membrane and/or paper and can be fabricated using simple and low-cost hydrophilic–hydrophobic patterning (Rosenfeld and Bercovici, 2014; Yang et al., 2012), paper stacking (Liu and Crooks, 2011) or folding techniques (Luo et al., 2014; Martinez et al., 2008), are thus more cost-effective than the integrated chip-based devices.

Secondly, integrated chip-based devices are often coupled with various functional units (*e.g.*, pumps and valves), which entail multiple operating steps. With simple capillary effect, the paper-based device allows the flowing of biological sample on paper without external power sources. Today, a variety of innovative paper-based fluidic control techniques have been introduced. The delivery of fluid in paper could be accelerated (by crafting hollow channels (Renault et al., 2013), longitudinal razor-crafted open channels (Giokas et al., 2014)), or by using film-sandwiched paper channels (Jahanshahi-Anbuhi et al., 2012)), or delayed (by the use of dissolvable sugar (Lutz et al., 2013), wax-printed pillars (Rivas et al., 2014) or tunable-delay shunts (Toley et al., 2013)). Besides that, the fluidic transport can be switched on or off by integrated paper-based valve controls (Martinez et al., 2010a, 2010b). The advanced fluidic control technologies in paper could autonomously direct multiple samples and reagents to flow sequentially to a detection zone without the need for external power supply (Chen et al., 2014; Gerbers et al., 2014), hence increasing potential use of paper in an integrated device. Additionally, paper allows reagents to be pre-stored in an integrated device in a dry form, which eliminates the need for cold chain transportation and storage.

To our best knowledge, the fully integrated molecular diagnostic equipment with sample-to-answer capability is still rare in the market (*e.g.*, GeneXpert system by Cepheid (Sunnyvale, CA) and Liat Analyzer by IQuum (Allston, MA)) (Wu et al., 2014). Although integrated chip-based sample-to-answer devices are cheaper and smaller than conventional benchtop NAT equipment, they are still sophisticated analytical tools for use in rural areas and resource-poor settings. Owing to the excellent capability of paper-based platform with advanced paper-based fabrication and modification technologies, it can be anticipated that in the near future, more paper-based sample-to-answer molecular diagnostic devices will be developed to overcome the financial barriers and improve public health in the underdeveloped and developing countries.

6. Summary and conclusion

NAT, which involves the three main steps of nucleic acid extraction, amplification and detection, plays an essential role in molecular diagnosis. Recently, paper-based devices have been developed to enable low-cost and simple nucleic acid extraction, amplification and detection. The low-cost material dramatically simplifies the fabrication process, and shortens the assay period, and hence it is very suitable for POC settings. However, thus far, there are no LFAs commercially available for specific nucleic acid detection. Without the nucleic acid extraction and amplification, LFA is unable to detect the DNA from raw blood samples, making them impractical in resource-limited settings. Therefore, we suggest the integration of the three main NAT steps into a single paper-based device, termed a “sample-to-answer” device for POC settings. The needs for no more than a single drop of blood obtained by a finger prick for analysis would bring this simple molecular diagnostic technology to the next level.

7. Future perspectives

In the future, one of the challenges in developing the paper-based sample-to-answer diagnostic device would be the lengthy and tedious nature of the multiple reagent addition steps (*e.g.*, the reagents for extraction, amplification, and detection are added at different time points). To tackle this problem, fluidic control technologies (*e.g.*, a novel microfluidic valve technology (Gerbers et al., 2014) or dissolvable sugar time delays (Lutz et al., 2013)) could be incorporated into this diagnostic device to program the multistep process by permitting timed sequential delivery of fluid. Another challenge would be the stability of the device, which is particularly related to the reagent storage. The capability of reagent storage allows the activation of the device upon the addition of buffer, making the device more user friendly. In addition, the ability of the device to store the reagent at room temperature circumvents the need for laboratory storage facilities, such as a refrigerator. Besides that, this low cost device should be fully independent of external equipment such as heater or pumps. In remote areas, rechargeable batteries are required in conjunction with paper-based devices, which aids in providing continuous electrical energy for heating processes in both nucleic acid extraction and amplification. An automated system starting from nucleic acid extraction, amplification to result analysis with minimal intermittent technical disruptions is of paramount importance for rapid, easy and contamination-free nucleic acid detection.

In an effort to realize the quantitative analysis of LFAs, the use of a low cost reader with recognition elements or smartphone’s built-in sensor to detect optical signals (colorimetric, fluorescent or chemiluminescent signals) or electrochemical signal of the assay is particularly useful in resource-limited settings (Xu et al., 2015). The result can be analyzed by the end-users for quick decision-making or transferred to the off-site laboratory to be analyzed by trained personnel. Further, we believe that highly sensitive colorimetric signal, which could be easily detected through the naked eye, coupled with a handheld solar-powered reader or a readily available smartphone-based reader would allow low cost and accurate quantification in the future. With a simple and portable sample-to-answer molecular diagnostic device coupled with a cost-effective highly sensitive signal detection technique, rapid and accurate diagnosis of diseases could be achieved in low-

resource settings for prompt medical treatment. We envision that in the future there will be more studies focusing on paper-based sample-to-answer devices dedicated to disease diagnostics.

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