The Pharma Innovation Journal 2022; SP-11(4): 2067-2071

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(4): 2067-2071

www.thepharmajournal.com
Received: 01-02-2022
Accepted: 04-03-2022

Deepali Sakunde
Department of Livestock Products Technology, KNP College of Veterinary Science, (MAFSU), Shirwal, Satara, Maharashtra, India

Rajiv Ranjan Kumar
Division of Livestock Products Technology, ICAR-IVRI, Izatnagar Bareilly, Uttar Pradesh, India

Sanjod Mendiratta
Division of Livestock Products Technology, ICAR-IVRI, Izatnagar Bareilly, Uttar Pradesh, India

Mukesh Gangwar
Division of Livestock Products Technology, ICAR-IVRI, Izatnagar Bareilly, Uttar Pradesh, India

Pranav Chauhan
Department of Livestock Products Technology, College of Veterinary Science and Animal Husbandry, (NDVSU), Mhow, Madhya Pradesh, India

Corresponding Author
Deepali Sakunde
Department of Livestock Products Technology, KNP College of Veterinary Science, (MAFSU), Shirwal, Satara, Maharashtra, India

Paper-based nucleic acid testing: A review

Deepali Sakunde, Rajiv Ranjan Kumar, Sanjod Mendiratta, Mukesh Gangwar and Pranav Chauhan

Abstract
Tests based on nucleic acid are vital in the field of medicine, agriculture, and forensics due to their accuracy and rapidness. Some limiting factors hinder the use of these tests, which include the cost of infrastructure, trained workforce, and the cost of reagents. The paper has recently been unitized as a viable and high potential platform for nucleic acid testing. It has gained increasing interest as a platform in the diagnostic field, especially for low-resource settings. Mainly due to its simplicity, cost-efficiency, biodegradability, and biocompatibility. It is predominantly made up of cellulose fibers, allowing fluids to wick through via capillary force. This characteristic confers advantages in sample storage, mixing, filtering, transport, volume control, timing control and multiplex detection. These paper-based assays could minimize the need for highly trained personnel, showing great potential to improve the existing Nucleic acid testing techniques.

Keywords: Nucleic acid testing, Paper based nucleic acid extraction, loop mediated isothermal amplification, lateral flow assay

Introduction
Nucleic acid test is a test for the presence of specific genetic material in a laboratory specimen, e.g., a specific segment of viral DNA or RNA in a blood sample. NATs are used, e.g., to detect contamination of blood by hepatitis viruses and HIV during the initial period of infection, before viral antigens appear in the blood of infected patients. It's a vital technique in biotech and molecular biology widely used in research, medicine, agriculture and forensics. Currently, it's a critical molecular diagnostic tool for applications in medical diagnostics (Chin *et al.*, 2011, Pardee *et al.*, 2016) [5, 36], food safety analysis (T. Denes and M. Wiedmann, 2014) [13] and environmental monitoring (Bohmann *et al.*, 2014) [4]. Nucleic acid testing is a widely used molecular diagnostic technique that promises rapid target detection over the other conventional assays with greater specificity and sensitivity, such as immunological assays and viral isolation (Lollo *et al.*, 2014; Niemz *et al.*, 2011) [30, 35]. NAT, which generally consists of three key steps, i.e., nucleic acid extraction, amplification and detection, currently involves labour-intensive, high-cost, and time-consuming processes, significantly limiting its applications at the Point of Care (POC) diagnostics (Martinez *et al.*, 2010; Wang *et al.*, 2012) [32].
Recently, the paper has been unitized as a feasible and high potential platform for NAT 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) [19]. It is predominantly composed of cellulose fibers with a porous structure, allowing fluids to wick through via capillary force (Chen *et al.*, 2015) [5]. This unique 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 skilled personnel, showing great potential to improve the existing NAT techniques (Parolo and Merkoçi, 2013) [37].

A) Paper based Nucleic Acid Isolation/ Extraction
In recent years, apart from achieving high extraction efficiency, attention has been focused on paper in fabricating diagnostic devices because paper is an inexpensive material. Furthermore, its porous structure enables proper mixing of samples and reagents and immobilizes functional biomolecules (e.g., DNAs or proteins), which can be utilized for nucleic acid extraction (Pelton, 2009) [38]. As cellulose is thermally stable (it can be heated up to 300 °C), it fully meets the requirement for heating in the extraction process (Wong *et al.*, 2014) [48].

A variety of commercial extraction devices are currently used in sample collection and nucleic acid extraction; some of them are discussed as follows:

1) Guthrie card or dried blood spot (DBS) filter paper: consisting of 903 filter paper, made of 490% cellulose, has been used for blood collection from a pricked finger for sub-sequent analysis (de Vries *et al.*, 2012) [15].

2) 903 protein saver card or Whatman™ 903 filter paper: has been introduced for sample collection and nucleic acid extraction (Kudo *et al.*, 2004) [23]. These cards have no stabilizing properties, thus requiring lower temperatures (-80 to -20 °C) for storage.

3) FTA cards and FTA Elute cards: FTA paper was developed by Lee Burgoyne at Flinders University Australia as a method for the storage of DNA. Patented cards of Whatman International Ltd from Flinders Technology Associates (FTA) have patented cards that offer a simple and fast method for the room temperature collection, transport and storage (short and long term) of DNA.

FTA cards are chemically-treated filter matrices for DNA extraction and storage (Aye *et al.*, 2011; Lange *et al.*, 2014; Liang *et al.*, 2014) [2, 25, 26]. FTA card has been used for routine extraction of nucleic acid from whole blood, plant cells, tissue culture cells and microorganisms. 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) [3]. The cellular debris, inhibitors and stabilizing chemicals can be readily washed off with washing buffer (Goldsborough and Fox, 2006) [16]. The DNA can be stored in the form of dried whole blood spots for at least 7.5 years at room temperature, thus eliminating the need for centrifugation and refrigeration equipment (Pezzoli *et al.*, 2007) [39]. Thus, 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) [25, 26]. The filter membrane can be directly added to the tube for further processing.

FTA Elute cards are another type of FTA card for DNA extraction purposes that 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) [14].

4) Paper-based microfluidic origami: consists of a stack of polymer sheets, and papers have been fabricated by a simple paper-folding technique in fewer than 30 min (Govindarajan *et al.*, 2012) [17]. 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. This device requires only a small amount of non-hazardous elute buffer (150 ml), 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.

5) 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) [33]. 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 of five discs 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 in developing countries.

6) 3MM filter paper and a pipette tip have been used for DNA extraction: 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 (Linnes *et al.*, 2014) [28]

B) Paper-Based 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) [12]. Therefore, nucleic acid amplification is mandatory in NAT (Niemz *et al.*, 2011) [35]. 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.

In nucleic acid amplification, reagent storage plays a key role. As a porous material, paper is capable of storing thermally stable reagents in a dry form for prolonged usage (Stevens *et al.*, 2008) [46]. Papers with large pore sizes, 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 the addition of the sample or buffer. This method can therefore prolong the shelf life of paper-based diagnostic devices (Yetisen *et al.*, 2013) [49]. A study has reported the use of glass fiber as a platform for nucleic acid amplification (Rohrman and Richards-Kortum, 2012) [43]. several studies have been demonstrated paper-based isothermal amplification by using a water bath or a small external heater, including paper-based loop-mediated isothermal amplification (LAMP) to address the limitations of large equipment-dependent polymerase chain reaction (PCR) (Choi *et al.*, 2016) [7], isothermal helicase-dependent amplification (HDA) (Linnes *et al.*, 2014) [28] and recombinase polymerase amplification (RPA) (Cordray *et al.*, 2015) [10].

1) Recombinase Polymerase Amplification (RPA)

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-de-pendent primer annealing and extension but rather depends on an enzymatically driven primer-annealing process (Lillis *et al.*, 2014) [27]. Therefore, it does not require a 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. 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*., 2014) [11]. Collectively, the rapid RPA with simple body heat incubation, coupled with a paper-based amplification device, provides an extremely low-cost amplification technique in POC settings.

2) Loop-Mediated Isothermal Amplification (LAMP)

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 to recognize 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 a 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) [34]. Recently, the development of non-instrumented nucleic acid amplification (NINA) has been reported (LaBarre *et al*., 2011) [24]. 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 a lower cost ($0.06 US per test) to replace calcium oxide for the exothermic reactions (Singleton *et al*., 2014) [45]. Therefore, paper-based LAMP can be achieved by using the suggested heating techniques.

3) Helicase-Dependent Amplification (HDA)

A recent study has demonstrated a paper-based amplification technique by HDA (Linnes *et al*., 2014) [28]. 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 electricity-free heat sources in POC settings which showed comparable results to that of a dry-block heater (Huang *et al*., 2013) [20]. Direct DNA amplification following DNA extraction in the low-cost substrate minimizes the overall cost and processing time.

C) Paper-based Nucleic Acid Detection

Following nucleic acid amplification, an appropriate detection technique is an essential part of accurate detection and quantification of the amplicons. While, 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 documentation (Ahmad and Hashsham, 2012) [1]. Researchers have sought this problem with developement of paper-based devices like LFA, for rapid target nucleic acid detection (Hu *et al*., 2013; Mao *et al*., 2009) [18, 31].

Lateral Flow Assay (LFA)

These affordable devices can easily be chemically modified and conjugated with several types of biomolecules e.g., protein and DNAs, those are compatible with numerous bioassays (Martinez *et al*., 2010; Yetisen *et al*., 2013) [32, 49]. In addition, the devices can produce fast visual readouts (20 min), which meet the needs 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) [18]. Streptavidin coated-generic lateral flow dipsticks (LFD) are commercially available and are able to detect biotinylated double-stranded amplicons (Rigano *et al*., 2014; Siah and McKenna, 2013; Surasilp *et al*., 2011) [40, 44, 47]. 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-labelled DNA probe, which in turn form complexes with the gold-labelled 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) [21]. 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) [22]. For. For instance, RPA product labelled 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) [21]. 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 (Liu *et al*., 2014) [29].

D) Integrated paper-based Nucleic Acid Testing device

Several studies have attempted to integrate nucleic acid extraction, amplification and detection into one single paper-based sample-to-answer device (Rodriguez *et al*., 2015; 2016 & Choi *et al*., 2016) [41, 42, 8]. For instance, a "paper machine" has been developed to perform FTA card-based nucleic acid extraction, in situ amplification and fluorescent detection by a sliding motion of the device. To achieve simple colorimetric readout, an integrated paper-based sample-to-answer device has been reported combining FTA card-based extraction, glass fiber-based amplification and lateral flow assay (LFA) (Connelley *et al*., 2015) [9]. Additionally, paper allows reagents to be pre-stored in an integrated device in a dry form, which removes the necessity for cold chain transportation and storage. A fully integrated paper fluidic device has also been developed based on polyether sulfone (PES)-based DNA/RNA extraction, in situ amplification and LFA detection (Rodriguez *et al*., 2015; 2016) [41, 42]. These paper-based devices have dramatically reduced the detection time as compared to conventional methods (from ~4 hours to ~1 hour).

Conclusion

Integrated paper-based NAT can be performed by integrating paper-based nucleic acid extraction, paper-based isothermal

amplification and LFA into one paper device. This device only needs simple operations for the entire sample-to-answer nucleic acid testing; the paper-based technique could be directly utilized in resource-limited settings without special training, external equipment (e.g., thermal cycler, refrigerator) and complex operation rapidly. In comparison with existing conventional NAT techniques, integrated paper-based sample-to-answer disposable devices would be a powerful tool for nucleic acid testing in resource-limited settings.

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