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REVIEW ARTICLE

# Advances in paper-based sample pretreatment for point-of-care testing

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**ABSTRACT**

In recent years, paper-based point-of-care testing (POCT) has been widely used in medical diagnostics, food safety and environmental monitoring. However, a high-cost, time-consuming and equipment-dependent sample pretreatment technique is generally required for raw sample processing, which are impractical for low-resource and disease-endemic areas. Therefore, there is an escalating demand for a cost-effective, simple and portable pretreatment technique, to be coupled with the commonly used paper-based assay (e.g. lateral flow assay) in POCT. In this review, we focus on the importance of using paper as a platform for sample pretreatment. We firstly discuss the beneficial use of paper for sample pretreatment, including sample collection and storage, separation, extraction, and concentration. We highlight the working principle and fabrication of each sample pretreatment device, the existing challenges and the future perspectives for developing paper-based sample pretreatment technique.

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Concentration; extraction; paper-based device; point-of-care testing; sample collection; separation



## Introduction

Point-of-care testing (POCT) has found widespread applications in detection and monitoring of various diseases,[1–4] food safety [5] and environmental pollution,[6] in line with the ASSURED criteria laid out by World Health Organization (WHO), namely affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end users.[7] To date, microfluidic chip-based devices have been broadly used in POCT, e.g. a 3D-printed microfluidic device for detection of pathogenic *Escherichia coli* in milk [5] and a glucometer-based sensor for detecting $Cu^{2+}$ in water samples.[8] However, chip-based devices are associated with high-cost, equipment-dependent and complicated fabrication process, which has significantly limited their applications in low-resource and disease-endemic settings. Recently, paper has attracted increasing attention as a POC diagnostic tool since it is low-cost, portable and easy-to-use. The porous structure of paper allows fluid flow by capillary action and thus enables, biological sample storage, mixing and flow control, which play a fundamental role in POCT.[9,10] This readily obtained and inexpensive material has thus became an important substrate in various diagnostic assays, such as lateral flow assay (LFA),[2,11–13] dipstick assay,[14] and enzyme linked immunosorbent assay (ELISA).[15]

In clinical diagnosis, biological samples (e.g. blood, urine, saliva) are generally complex, making them difficult to be analyzed directly. For instance, blood contains the mixture of red blood cells, white blood cells and plasma, among which only a small part will be used as targets for diagnosing specific diseases. Therefore, sample pretreatment is vital to remove the large matrix and retrieve only the target analyte (e.g. DNA, RNA or protein) from the sample for downstream analysis, which can be divided into sample collection and storage, separation, extraction and concentration. Traditionally, sample pretreatment is performed by either using a vacutainer system,[16,17] a commercial kit [18] or ultra-filtration technique.[19] These methods are generally expensive, time-consuming and equipment-dependent, which are not suitable for POCT in disease-endemic settings. Therefore, there is an urgent need to develop simple, rapid and inexpensive paper-based platforms for sample pretreatment, which is compatible with paper-based diagnostic devices (e.g. LFA).

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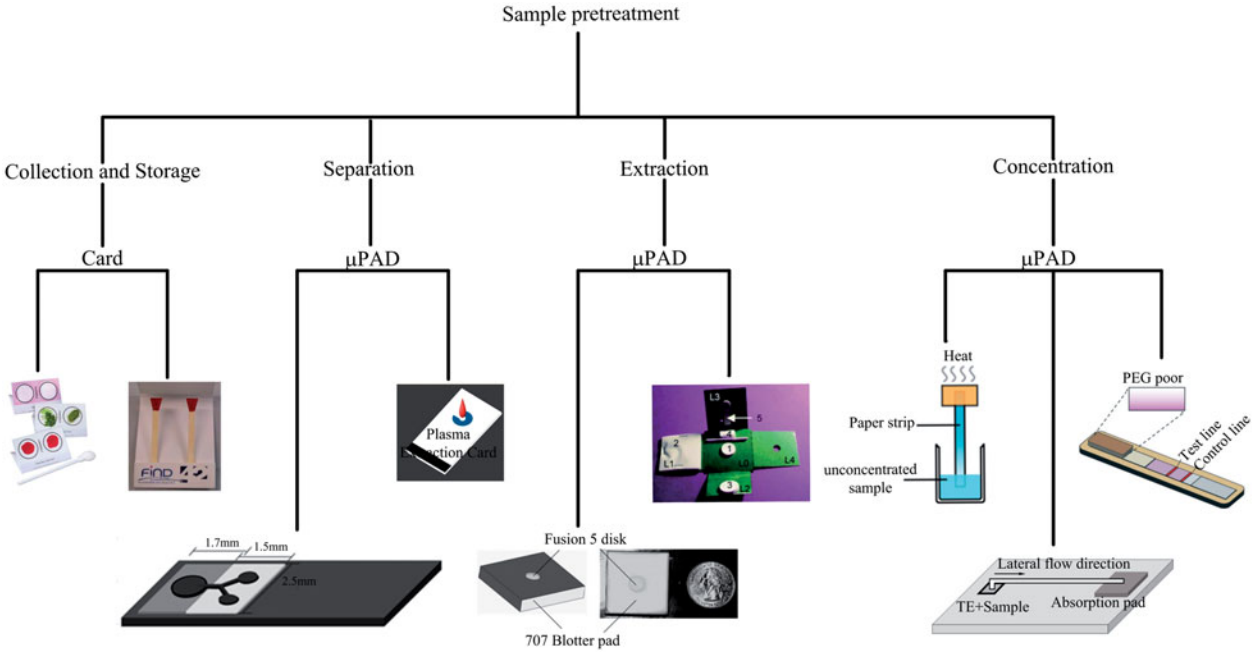


**Figure 1.** Paper-based sample pretreatment methods. Sample pretreatment are divided into collection and storage, separation, extraction and concentration. Collection and store contain commercialization, the image was reproduced from the manufacturer's instructions and the shaped-based devices,[45] different μPADs for sample separation,[47,58] extraction [27,67] and concentration.[77,80,81]

With recent advances in modification and fabrication technologies of paper, it is now possible to perform complex sample pretreatment in paper-based platforms (Figure 1). For example, the commercial chemically-modified paper (e.g. Fast Technology Analysis (FTA) card) can be used for collection and storage of blood sample,[20] cells,[21] and viruses [22,23] prior to extraction and analysis. An FTA card is able to extract the target nucleic acid by lysing the cells, denaturing the proteins, and protecting nucleic acid from nucleases, oxidative and UV damage.[24,25] In addition, paper can also be modified by using chemicals (e.g. ovalbumin (OVA) and ethylenediaminetetraacetic acid (EDTA) to name just two) to combine with a target for metal ion separation,[26] and by using paper folding techniques to develop an origami card for protein separation and DNA extraction.[27,28] These advanced technologies make paper a potential platform for sample pretreatment.

Although there exists some good reviews on the working principles,[29] analytical device,[30] inkjet-printed fabrication technology,[31] chemical measurements [32] and industrial applications and commercial opportunities [33] of paper-based POCT, the review of paper-based sample pretreatment has not yet been reported. Here, we present the start-of-the-art advances in the paper-based sample pretreatment for POCT, including sample collection and storage, separation, extraction, and concentration. We discuss the advantages of using paper as a platform for sample pretreatment. We highlight the working principles and fabrication techniques used in paper-based sample pretreatment. In addition, we discuss the existing challenges and future perspectives for developing paper-based sample pretreatment.

## Paper-based sample collection and storage

In most cases, clinical sample cannot be processed and analyzed immediately due to inadequate medical facilities and limited medical staff in disease-endemic settings. Therefore, samples may need to be stored after collection, before being transported from remote areas to the laboratories. Conventionally, various methods (e.g. Streck® Blood Collection Tubes (Streck® BCTs) [34] and equipment (e.g. refrigerator and liquid nitrogen) have been used for collection and storage of clinical samples (e.g. whole blood, mixed body fluid). However, these conventional techniques are expensive and require a large container, limiting their accessibility in resource-limited settings. Paper is composed of cellulose, which has a degree of hydroxyl groups after nitration in the surface and then the amount of surfactant is absorbed onto the paper surface by electrostatic and dispersive interactions. The chemical molecules are connected with hydroxyl groups onto the paper's surface. Chemical molecules could prevent samples from being

harmed or damaged.[33,35] Therefore, paper as a good biocompatible substrate, has been widely used in biological collection and storage, such as various commercial cards and shaped filter. A number of factors, such as the sample type, sample volume, storage condition and preservation time need to be considered in paper-based sample collection and storage.

### Commercial technologies

Nowadays, a wide range of commercial cards has been used for sample collection and storage (Table 1). Paper is mainly chemically modified for this purpose. For example, the commonly used Fast Technology Analysis (FTA) classic cards contain chemicals that are used to lyse cell, denature proteins, prevent bacterial growth, and protect nucleic acids from nucleases, oxidative and UV damage according to the manufacturer's instructions [36] (Figure 2A). On the contrary, FTA elute card elutes nucleic acid from filter paper, and retains protein and bacteria in the card.[21] In addition, the chromatography paper can be pretreated with butylated hydroxytoluene (BHT) in methanol for blood collection and storage.[37] Each card has its own working conditions (e.g. sample volume preservation duration and temperature). In terms of sample volume, for example, an FTA card requires a volume up to 125 μL according to the manufacturer's instructions, 3MM chromatography paper requires up to 50 μL,[38,39] whereas Nobuto Filter Paper strips requires the volume up to 100 μL [40–42] based on the size of sample area. In whole blood sample storage, for example an FTA card, could store whole blood sample up to 22 years, nucleic acids up to 17.5 years and buccal sample up to 12 years at room temperature (18–25 °C). The Whatman 903 protein saver card and 3MM filter paper could store whole blood sample under frozen (−80 °C and −20 °C), cold chain (4 °C) or tropical condition (35 °C) for up to 13 weeks.[25] Most samples are prepared by pipetting or spotting onto the cards,[37,38,43] in dried form at room temperature before storage, and these cards need to be cut by scissors or punched with a defined size to estimate the sample volume for downstream analysis, but this involves the potential issue of cross contamination due to the direct contact of scissors/puncher and samples. Most commercial cards (e.g. FTA cards) allow collection and storage of various samples without the need to maintain a cool condition or safety regulations with respect to the transport of hazardous reagents. Besides, these cards are compatible with many extraction kits, such as the MagMAX-96 viral RNA isolation kit (Ambion, Inc, Austin, Tx, USA),[44] for downstream analysis (e.g. Real Time-Polymerase Chain Reaction (RT-PCR)[38,44].

### Emerging technologies

#### Technologies to be improved for POC use

To address the limitation of commercial paper (e.g. cross contamination), a new shaped filter device has been developed for a quick storage of a defined volume of dried blood spots (Table 1; Figure 2B).[45] The Whatman GB003 paper is cut using a laser to a defined volume shape to absorb 20 μL of whole blood. Compared to commercial cards, blood sample with a fixed volume is dispensed onto the shaped device by touching the inverted triangle against the blood spot, and then is dried for 4 h at room temperature before being stored. The device is able to collect and store different volume of blood sample based on the shape and wicking capacity of paper. Similar to the FTA card, the device is also compatible with Qiagen DNA blood mini or Chelex extraction method for real-time PCR analysis and PURE extraction for target detection. Unlike the FTA card, this filter device is cut without contacting the sample area with scissors, reducing the potential issue of cross contamination. The storage stability of the device should be evaluated over a long period of time (6 months) for potential future applications.

## Paper-based sample separation

In practice, raw samples are often not suitable for direct analysis at POC, as they contain complex elements which might interfere with the analyte detection signal and thus lower the accuracy of clinical diagnosis. For instance, a raw blood sample contains red blood cells, plasma and amino acid, which may interfere with the optical signal of gold nanoparticle in colorimetric LFA in the absence of proper sample separation.[46–48] Conventional laboratory separation methods include discontinuous centrifugation [49,50] and filtration,[51] which involve complex operations and expensive equipment. Several novel separation techniques, such as pathogen enrichment device [52] and magnetic beads separation techniques,[53,54] have also been introduced. With the development of paper-based modification and fabrication technologies, several commercial technologies and emerging technologies have demonstrated the development of paper-based separation devices, which offers a great potential for POCT. The sample separation technique of POCT should meet the criteria of requiring low sample volume, short separation

**Table 1.** Paper-based sample collection and storage.

| Types | Types of paper | Type of sample | Time of preservation | Volume of sample | Temperature | Modification | Advantages | Disadvantages | Application | References |
|---|---|---|---|---|---|---|---|---|---|---|
| Commercial technologies | Chromatography paper | Whole blood, urine | 7-12 days | 2 mL (urine), 10-50μL (whole blood) | 4 °C or 37 °C | Impregnated with butylated hydroxytoluene (BHT) | Extends the target (e.g. fatty acid) stability during long-term storage | Requires to elute target (e.g. urine metabolites) from filter paper<br>Nucleic acids are not stable at room temperature | Detection of Metabolic disorders, gametocyte-specific mRNA, Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) level | [37–39,87] |
| | Nobuto filter paper | Whole blood, serum | 3 month | 40-100 μL | 18∼25 °C | Impregnated with chemical reagent (proprietary) | Allows one to store and collect sample at room temperature | Requires chemical reagents (e.g. phosphate buffer solution (PBS), PBS with 0.2% Tween-20 or RNAse-free water containing 0.2% Tween-20) to elute the target | Detection of Puumala and Rift Valley Fever virus, Avian influenza virus antibody and protein concentration of Canis lupus | [40–42,88] |
| | 3MM filter paper | Whole blood, Dried blood spots, anti-coagulant-coated blood, fresh blood | 9 month | 10 μL | 20∼25 °C | Impregnated with chemical reagent (proprietary) | No effect by 30-50% of the hematocrit concentration<br>Can be applied to the wide range of blood samples<br>Allows one to store and collect sample at room temperature | Needs reagent buffer (e.g. phosphate buffer solution with 1% Tween, 0.9% sodium chloride and 5% trichloroacetic acid) to extract target | Detection of urea, African swine fever virus and antibody | [39,88,89] |
| | Guthrie card | Whole blood | 26-28 years | 50 μL | 18∼25 °C | Impregnated with chemical reagent (proprietary) | Allows one to store and collect sample at room temperature<br>Can store a longer time than other cards | Needs additional DNA purification method | Detection of DNA methylome | [88,90] |
| | Whatman™ 903 filter paper | Whole blood, virus, urine, cell, serum | 13 weeks | 50-100 μL (whole blood), 15 μL (serum), 10 mL (urine) | −80° C, −20 °C, 4 °C, 35 °C | Impregnated with chemical reagent (proprietary) | Can apply to a wide range of samples | Needs additional DNA purification method<br>Nucleic acids are not stable at room temperature | Detection of DNA methylome, mucopolysacchariduria, glycosaminoglycans, protein expression profiling, HPV, Gametocyte mRNA and anti-Brucella smooth LPS antibodies | [25,82, 87, 88,90–95] |
| | Whatman No.1 Qualitative grade filter paper | feces | 37 days | 10 μL | 37 °C | No impregnation with chemical reagent because samples diluted in chemical buffer | Allows one to store, collect and transport a low concentration sample | Requires elution step for RNA amplification<br>Contains the PCR inhibitors<br>Nucleic acids are not stable at room temperature | Detection of Levivirus and Allolevivirus | [43] |

(*continued*)

**Table 1.** Continued

| Types | Types of paper | Type of sample | Time of preservation | Volume of sample | Temperature | Modification | Advantages | Disadvantages | Application | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | Whatman FTA card | Whole blood, virus, Tumor, tissue, bacterial, buccal swabs | 12-22 years | 5–125μL | 18∼25 °C | Impregnated with chemical reagent (proprietary) | Allows one to store and collect sample at room temperature<br>Can apply to a variety of sample types | Requires specific chemicals (e.g. FTA purification reagent (FTA) or water (FTA) Elute) for DNA purification<br>Requires a long time for extraction and purification of DNA from samples | Avian influenza surveillance, HLA genotyping, Bovine respiratory diseases diagnostic, molecular diagnostic of Avian metapneumo virus, tumor genotyping, diagnosis and identification of FMDV | [22,23,36,44,60,63,65,88,96–99] |
| | Whatman FTA elute card | Whole blood, cells, tissue, bacterial, buccal swabs | 8 years | 12-40μL | 18∼25 °C | Impregnated with chemical reagent (proprietary) | Allows one to store and collect sample at room temperature<br>Can apply to the variety of sample types | Requires specific chemicals (e.g. FTA purification reagent (FTA) or water (FTA Elute)) for DNA purification<br>Requires elution step for DNA amplification<br>Requires long time for extraction and purification of DNA from samples | Cervical cancer screening | [21,66,88] |
| Emerging technologies | A shaped Card | Whole blood | No | 20 μL | −20 °C | | Stores a defined volume of dried blood spots<br>Reduces the potential for cross contamination during the manipulation samples as compared to commercial technologies (e.g. this filter device is cut without contacting the sample area by scissors)<br>Good compatibility (The device is compatible with Chelex, Qigen and PURE DNA extraction methodologies) | Cannot store sample at room temperature<br>Does not evaluate the storage time | Detection of parasite chromosomal DNA of whole blood | [45] |

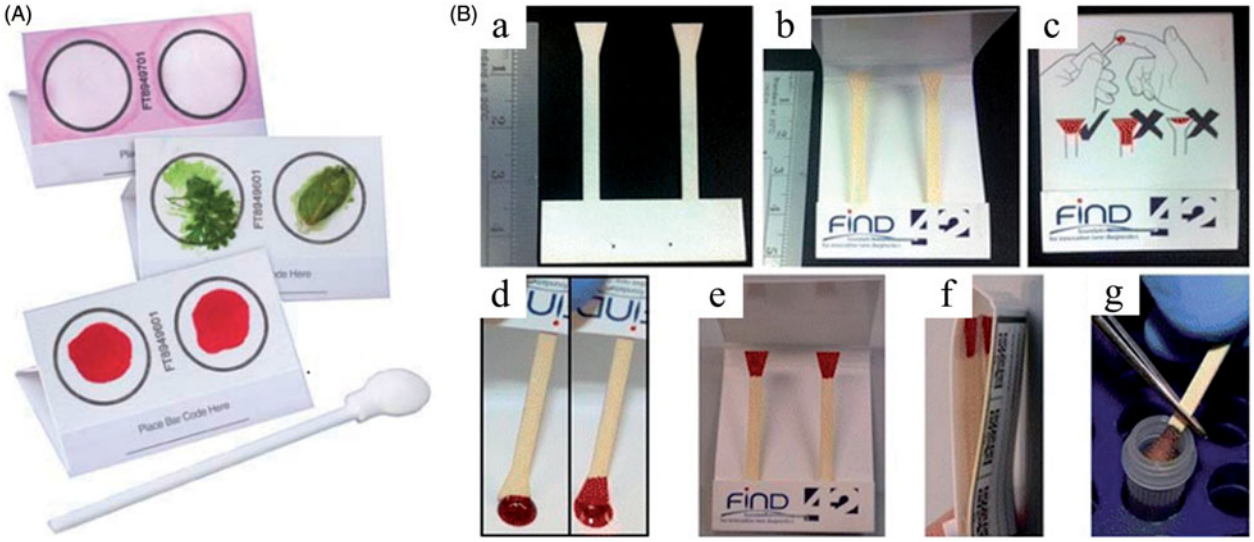


**Figure 2.** Paper-based sample collection and storage. (A) Commercial FTA card is used sample collection and storage, the image was reproduced from FTA_CardsDataSheet_FINAL_3.25.10_LR, whereas (B) shaped filter device is used for sample collection, storage and extraction.[45]

time and being flexible to be integrated into the diagnostic devices.

### Commercial technologies

Additionally, commercial blood separation membranes, such as Fusion 5, LF1, MF1, VF2, have been widely used in a POCT platform (Table 2).[47,55] In a study, a variety of blood separation membranes (LF1, MF1, VF1 and VF2) have been used for sample separation combined with a piece of Whatman No.1 paper for target analyte detection (e.g. human serum protein). LF1 is most suitable for fabrication of μPAD for whole blood separation because it requires less blood sample volume and achieves faster plasma isolation. This paper-based device is allocated between the iron mold and a permanent magnet, and the μPAD was fabricated by the wax dipping method (Fig. 3A).[47] These membranes use the principle of filtration aided by capillary force to separate plasma from whole blood. The device can be applied to separate plasma from a single drop of whole blood within 2 min. The blood volume to be separated was estimated according to the equation ($y = 3.12x + 13.38$, $y$ - size of separation membrane, $x$ - volume of sample) for a different size of separation membrane (e.g. For 28 $mm^2$, 38 $mm^2$ and 50 $mm^2$ LF1-μPAD, the best range volume of blood were 8–11 μL, 11–17 μL and 15–22 μL). In this technology, a commercial membrane has been successfully integrated into the paper-based biosensor. However, this device could use other fixation methods (e.g. chemical cross-linking [56] or physical adhesion [57]) to replace the iron mold to a permanent magnet and make it more simple and at low-cost.

### Emerging technologies

#### Technologies suitable for POC use

A microfluidic paper-based analytical device (μPAD) has been developed for separating plasma from whole blood using a red blood cell agglutination technique (Figure 3B).[48] The μPAD is fabricated by printing the hydrophobic barrier pattern onto a chromatography paper with a wax printer and melting the wax to create the hydrophobic barrier spanning the entire thickness of the paper substrate. The red blood cell agglutination technique is integrated into the μPAD, which consists of the plasma separation zone in the center and the test area in the periphery. The device is able to separate plasma from the whole blood based on the principle of agglutination, where the surface antigen of red blood cell is specially combined with agglutinating antibodies (anti-A,B), leading to red blood cells (RBCs) aggregation, that are too large to pass through the pores within the chromatography paper. To perform the assay, a small volume of whole blood (~7 μL) is spotted onto the plasma separation zone, which separates the plasma, to allow the plasma to diffuse directly to the detection zone by capillary force for target detection (e.g. glucose) in 5 min. This device has successfully realized the integration of paper-based sample separation and detection of POCT platform. However, this device needs agglutination antibodies for separating red blood cells, which increases the whole cost of the sample pretreatment method.

Similar to aforementioned μPAD, a paper-based card has also been used for plasma separation from whole blood sample.[58] The card consists of multilayer

**Table 2.** Paper-based sample separation.

| Types of technologies | Types of paper or device | Materials | Volume of sample | Time of separation | Working principle | Types of sample | Advantages | Disadvantages | Application | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| Commercial technologies | Fusion 5 | Glass fiber | 30 μL < V < 90 μL | 40 s for a 4 cm strip | Lateral flow | Whole blood | Fast flowing as compared to LF1,MF1,VF1 and VF2<br>No blocking required<br>Low background and high sensitivity<br>No surfactant needed | Sample does not wick through the test<br>Red cells passing through the Fusion 5 | Separate plasma from whole blood | [55] |
| | LF1 | Polyvinyl alcohol-bound glass fiber | V < 30 μL | 30–120 s | Lateral flow | Whole blood | No appreciable red cell hemolysis | Needs less blood volume as compared other papers | Separate plasma from whole blood | [47] |
| | MF1 | Polyvinyl alcohol-bound glass fiber | 90 μL < V < 100 μL | 30–120 s | Lateral flow or filtration | Whole blood | No appreciable red cell hemolysis | Needs a certain volume of sample (90μL < V < 100μL) | Separate plasma from whole blood | [47] |
| | VF1 | Binder-free glass fiber | V > 100 μL | 30–120 s | Filtration | Whole blood | No appreciable red cell hemolysis | Thickness (632μm)<br>Needs only large volume blood as compared to other papers | Separate plasma from whole blood | [47] |
| | VF2 | Polyvinyl alcohol-bound glass fiber | V > 100 μL | 30–120 s | Filtration | Whole blood | No appreciable red cell hemolysis | Thickness (785μm)<br>Needs only large volume blood as compared other papers | Separate plasma from whole blood | [47] |
| | μPAD | LF1, MF1, VF1, VF2, Whatman No.1 paper | 15-22 μL | 2min | Filtration | Whole blood | The integrated device could achieve separation and detection of POCT | Needs a permanent magnet on the back of the glass slide | Separate plasma from whole blood | [47] |
| Emerging technologies | μPAD | Chromatography paper | 7 μL | 2min | RBC agglutination | Whole blood | The integrated device separates plasma via red blood cell (RBC) agglutination technique | Needs antibody for agglutination red blood cell | Separate plasma from whole blood | [48] |
| | The plasma extraction card | Filtration membrane | 25 μL | 3 min | Filtration | Whole blood | This card includes test area and control spot, the control spot was indicates if lateral spreading has occurred and if there is sufficient application volume | -No integration with detection of POCT | Separate plasma from whole blood | [58] |
| | Paper-based with sample treatment | Conjugation of glass fiber | 150 μL | 10 min | Cd-EDTA complex formed | Drinking and tape water | This technology adds an extra conjugation pad into the lateral flow assay for separate $Cd^{2+}$ | No | Separate and detect metal ions (e.g., Cadmium (Cd2+)) from drinking and tape water | [26] |
| | oPAD-Ep | Whatman grade 1 chromatography paper | | 5 min | Electrophoretic | Fluorescent molecules, serum | This origami design reduces driving voltage (Just only 10V), which is more than 10 times lower than that used conventional electrophoresis | Needs electrical power for separation targets<br>Not integrated with POCT | Separate fluorescent, separates bovine albumin (BSA) from calf serum | [28] |

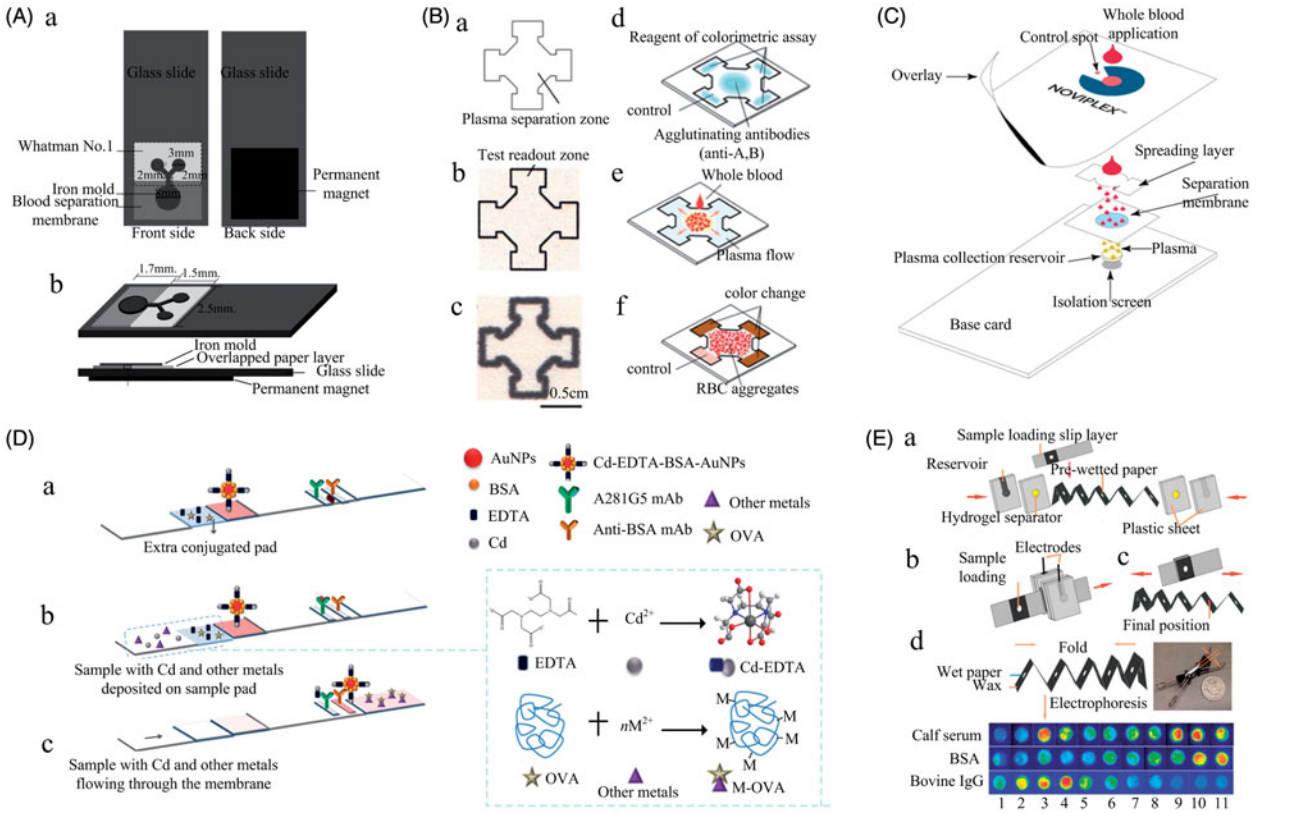


**Figure 3.** Paper-based sample separation. (A) Blood separation membrane,[47] (B) plasma was separated by μPAD via red blood cell (RBC) agglutination technique,[48] (C) paper-based separation cards,[63] (D) paper-based microfluidic device for $Cd^{2+}$ separation,[26] and (E) an origami paper-based electrophoretic device was used to separate fluorescent molecules and proteins.[28]

membranes, including overlay, spreading layer, separation membrane, plasma collection reservoir, isolation screen and base card (Figure 3C). Following the addition of whole blood onto the membrane, the plasma is drawn by capillary action, and is migrated to a collection disc at the bottom of the membrane. The card is able to separate 2.5 μL of plasma from 25 μL of whole blood within 3 min. Unlike μPAD,[48] this card is able to store the dried plasma for subsequent analysis (e.g. proteomic analysis and vitamin D quantification) after sample separation. The card still needs to be combined with paper-based biosensor for achieving the integration of separation and detection of sample, which will expand its application scope.

On the other hand, a new integrated paper based immunosensing system has been introduced, which combines both sample treatment and cadmium ($Cd^{2+}$) detection (Figure 3D).[26] An extra dual function conjugation pad is added between the sample pad and the conjugation pad of AuNPs in lateral flow format. This dual function pad was impregnated with 10% ovalbumin (OVA) and 0.05 mM ethylenediaminetetraacetic acid (EDTA) to mask interferences with albumin using OVA and to detect $Cd^{2+}$ using EDTA. The working principle is based on the competitive reaction between the Cd-EDTA complex (formed in the first conjugation pad in presence of target $Cd^{2+}$) with the cadmium – ethylenediaminetetraacetic acid – bovine serum albumin – gold nanoparticles (Cd – EDTA – BSA – AuNP) conjugate (deposited onto the second conjugation pad), to bind to the binding sites of the 2A81G5 monoclonal antibody in the test line. This cost-effective device enables rapid and highly sensitive detection of $Cd^{2+}$ for application of environmental monitoring at the POC.

### *Technologies to be improved for POC use*

Besides separating the whole blood and metal ions, an origami paper-based electrophoretic device (oPAD-Ep) (Figure 3E) has been developed to rapidly separate fluorescent molecules (e.g. $Ru(bpy)_3Cl_6$ ($Ru(bpy)_3^{2-}$, Fluka), 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene ($BODIPY^{2-}$, Invitrogen), 8-methoxypyrene-1,3,6-trisulfonic acid trisodium salt ($MPTS^{3-}$, Anaspec), 1,3,6,8-pyrenetetrasulfonic acid tetrasodium salt ($PTS^{4-}$, Fisher Scientific)) and proteins (e.g. bovine serum albumin (BSA)).[28] The molecules can be made to move through the substrate (e.g. gel, polyacrylamide and paper) by electrophoretic transport in the

presence of an external electric field (e.g. EP). The electric field consists of a negative charge at one end which pushes the molecules through the substrate, and a positive charge at the other end that pulls the molecules through the substrate, to achieve the target separation at the end of the assay.[59] oPAD-Ep contains three steps: the slip layer and origami paper were patterned by wax printing, the plastic buffer reservoirs are made using a laser cutter and the components are assembled as shown in Figure 3E. The device uses multiple thin folded paper layers to support the medium for electrophoresis. The substrate is used to suppress the thermal convection caused by Joule heating and to sieve target biomolecules (e.g. nucleic acid and protein) based on their size. The oPAD-Ep device can be used for separating a single ($BODIPY^{2-}$) or multiple ($MPTS^{3-}$ and $Ru(bpy)_3^{2-}$) fluorescent -tagged samples within 5 min to detect one or more target analytes based on their different electrophoretic mobilities. Unlike conventional electrophoresis system, the oPAD-Ep device can reach high electric field intensity at low applied voltage (~10 V), making it suitable for POC application.

## Paper-based sample extraction

After collecting the sample, an extraction step is important in order to isolate the target analyte (DNA or RNA) from a complicated biological sample for downstream analysis. Conventional extraction methods include microwave irradiation,[60] salt-precipitation and spin-column method,[61] alkaline based lysis extraction method,[62] guanidinium thiocyanate based extraction method,[63] magnetic particle-based method,[64] commercial kits.[62] However, these processes always require multistep operations, which are time-consuming. Therefore, a simple, prompt technique needs to be developed. In POCT, the sample extraction technique should meet the requirement of easy manipulation, a short extraction time and equipment-independent. At present, several commercial technologies and emerging technologies had been used for target extraction.

### *Commercial Technologies*

At present, several paper-based commercial cards, such as FTA card[44,60,65] and FTA elute card,[21,66] not only store and collect sample, but also extract nucleic acid from biological samples. These technologies have to simply the operations of extraction, but there are some challenges. The detail information can be found in Table 1.

### *Emerging technologies*

#### *Technologies suitable for POC use*

Several studies have reported a variety of sample extraction techniques using paper (Table 3). For instance, a low-cost paper microfluidic origami device has been developed for DNA extraction [27] (Figure 4A). This device is fabricated by stacking flat polymer sheets and paper. This device uses guanidinium thiocyanate method to extract DNA, and the complex chemical and physical operation steps for DNA extraction can be simplified by sequential folding. Then, DNA is absorbed on Fusion 5 membrane. The origami device could extract 150 μL of bacterial DNA from raw viscous sample (pig mucin spiked with *E. coli*) without the use of external power and high-cost equipment within 1.5 h. Besides, this device could reduce the biohazard concerns during transportation due to the use of non-hazardous reagents, such as water, ethanol and elute buffer. The operation steps should be reduced to make the assay faster and simpler.

In addition, based on filtration isolation of nucleic acids (FINA) technology, a μPAD (known as paper-based separation device) has been developed to extract DNA from whole blood. FINA modules are fabricated by sandwiching separation membrane disk between blotter pad and a thin sheet of Parafilm with a hole in the center (Figure 4B). This technology uses alkaline (e.g. NaOH) extraction method to extract DNA, where DNA is captured by Fusion 5 membrane and the lysis debris is removed into absorption pad through the vertical flow of chemical buffer. After DNA extraction, the filter paper is completely soaked in the test tube for further analysis.[67,68] This method has been proven to successfully extract human immunodeficiency virus-1 (HIV-1) DNA from 100 μL of whole blood in less than 2 min without need for any additional tools, such as laboratory equipment. The FINA test has been reported to be highly sensitive and specific, with shorter extraction time as compared to the conventional extraction methods (e.g. commercial kits).[69] Additionally, this cost-effective technique is also compatible with the commonly used blood collection tube containing anticoagulants, thus holding a great potential to be used in POC settings. This technology could rapidly extract DNA from whole blood. However, the issue of reagent storage should be achieved for future applications.

#### *Technologies to be improved for POC use*

A paper-based microfluidic device consisting of a piece of Fusion 5 filter paper has been developed for DNA extraction. The filter paper is attached to a PDMS

**Table 3.** Emerging technologies of paper-based sample extraction and concentration.

| Types of technologies | Names of devices | Types of papers | Volume of sample | Time | Types of sample | Working principle | Advantages | Disadvantages | Application | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| Paper-based sample extraction | A paper microfluidic origami device | Millipore cellulose paper, Fusion 5 | 150 μL | 1.5 h | Raw viscous sample (e.g. Pig mucin) | Guanidinium thiocyanate extraction method | This device does not need external power and high cost equipment<br>Disposable | Long time<br>Needs to use ethanol and elute buffer for washing and extracting DNA<br>Not integrated with detection of POCT | Bacterial DNA from raw viscous sample | [27] |
| | Filtration isolation of nucleic acids (FINA) technology | Fusion 5, absorption pad | 100 μL | 2 min | Whole blood | NaOH extraction method | This technology develops a vertical filtration method to extract DNA from whole blood in less time as compared to other extraction technologies | Requires extra buffer for extracting DNA<br>Requires multiplex operations, including lysis step and washing step<br>Not integrated with detection of POCT | Human immunodeficiency virus type 1 (HIV-1) DNA extraction from whole blood | [67,68] |
| | A filter paper-based microdevice | Fusion 5 | 0.25-1μL | 7 min | Whole blood, dried blood sample, buccal swabs, saliva, cigaret butts | NaOH extraction method | This device realizes the automated extraction DNA from small volume of various samples | Requires external syringe pump, valve and electrical power for automated extraction DNA<br>Not integrated with detection of POCT platform | DNA purification from diverse biological samples | [70] |
| | surface acoustic wave (SAW) | Polyester-cellulose | 40 μL | 10–15 s | Liquid of cell, protein, and bacteria | SAW atomization method | This device could extract a fluid from the paper as compared to standard microfluidics devices | Requires external electrical power for extraction target<br>Not integrated with detection of POCT | Extraction of protein molecules and yeast cells | [71] |
| Paper-based sample concentration | Aqueous two-phase system (ATPS) concentration | Glass fiber | 40 μL | 25 min | A variety of disease biomarker solution | | This technology integrates ATPS into lateral flow assay and forms portable all-in-one diagnostic<br>Achieves 10-fold concentration | Needs extra running buffer for concentration of sample flow through lateral flow assay test strip | Detection of transferrin and malaria biomarker (e.g., Plasmodium lactate dehydrogenase (pLDH)) | [77,78] |
| | Chitosan based concentration | Nitrocellulose membrane, glass fiber, Cellulose wicking pad | 100-2000 μL | 25 min | High protein, Blood, non-target DNA | Impregnated with chitosan to concentration target | This technology realizes one-step purification and concentration of DNA in porous membrane<br>Can apply to a wide range of sample types | The integration of sample-to-answer device for POCT has not been demonstrated | Purification and concentration of DNA from complex samples | [79] |
| | Evaporative concentration | Chromatography paper | 600 μL | 20 min | Urine | Use of heat to accelerate evaporation | This study reports the first demonstration | Requires external electrical power | Tuberculosis-specific glycolipid | [80] |

(*continued*)

**Table 3.** Continued

| Types of technologies | Names of devices | Types of papers | Volume of sample | Time | Types of sample | Working principle | Advantages | Disadvantages | Application | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | and concentration target | of using heat on a paper device to rapidly concentrate target from biological fluid Achieves 20-fold concentration | Needs sonly heat stable biomarker No integration with detection of POCT Needs high temperature (up to 220 °C) | lipoarbinomannan (LAM), urinary biomarker for the detection and diagnosis of tuberculosis | |
| | Isotachophoresis concentration | Cellulose filter paper | 30μL | 6min | Fluorescent molecule, Alexa Fluor 488 (AF488) succinimidyl ester | Based on effective electrophoretic mobility of target to concentration sample ions | This technology develops wax printing fabrication method and novel cross-shaped device structures to overcome Joule heating and evaporation of ITP and then concentrate the target This technology has been integrated into lateral flow assay of POCT Achieves 1000-fold concentration, Requires less volume of sample | Requires external electrical power | Concentration of molecules (e.g., fluorescent molecule or Alexa Fluor 488 (AF488) succinimidyl ester) | [73,81] |

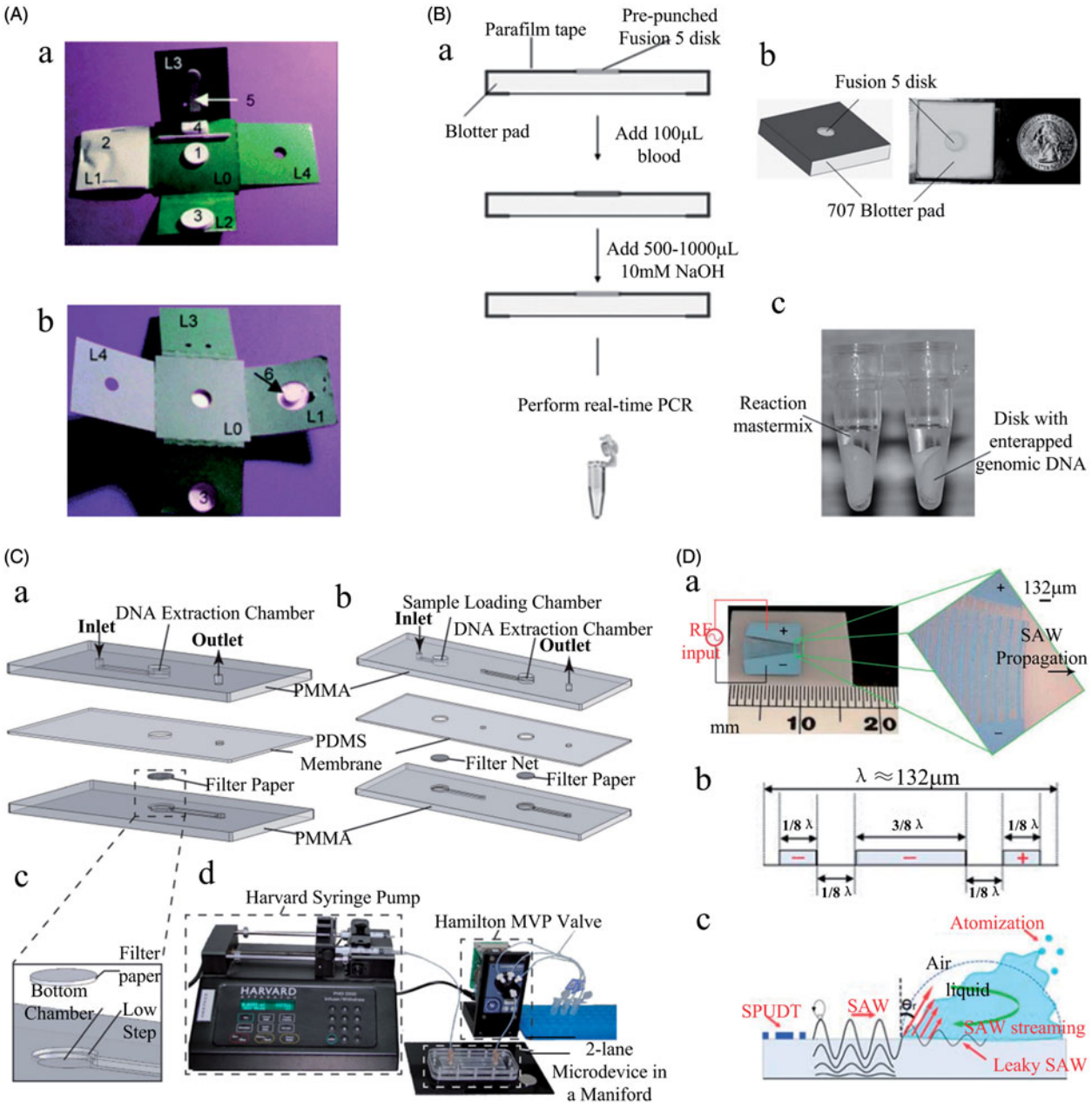


**Figure 4.** Paper-based sample extraction. DNA was extracted by (A) Microfluidic origami device.[27] (B) μPAD via FINA method [67] and (C) filter paper-based microdevice.[70] (D) Fluid, protein and yeast cell were extracted by SAW extraction device.[71]

membrane, which is sandwiched by two PMMA layers (Figure 4C).[70] Similar to the principle of the study discussed earlier, this device use alkaline lysis extraction method to isolate DNA from diverse biological samples and to capture DNA using Fusion 5 membrane. For 7 min, 0.25–1 μL of blood can be extracted using Fusion 5 filter paper and the DNA yield obtained (5.6–21.8 ng) are higher than that obtained by conventional technique of QIAamp DNA Micro kits (3.6–13.0 ng) (QIAGEN, Germantown. MD, USA). This device achieves an automated extraction of DNA from a small volume of sample, which is required to combine external syringe pump and valve. However, this device is required to be combined with paper-based pump and valve as developed in literature [16] to increase its portability for future POC use.

Surface acoustic wave (SAW) atomization has been used for extracting the target analytes (e.g. protein (ovalbumin and BSA) and yeast cells) from the sample stored in a paper.[71] The atomized target is then collected onto a clean glass slide for the next analysis. The SAW device is composed of piezoelectric substrate, single-crystal lithium niobate, with chromium-aluminum single-phase unidirectional transducer (SPUDT) electrodes patterned by UV lithography (Figure 4D). The target is extracted from the sample stored at the paper strips

using SAW atomization method. The SAW atomization extraction is performed through a high-frequency electrical power applied to inter-digital transducer to produce mechanical vibration, which enables target atomization. The device is used to rapidly extract the target from the sample. A portable battery power should be integrated into this technology to make it more portable for use in remote settings.

## Paper-based sample concentration

In POCT, the ability of a diagnostic device to detect a low concentration of target analyte remains a challenge. For instance, the currently available LFA has relatively poor detection limits ($\sim 10^{-11}$ M),[72] which are unable to detect the clinical detection limit ranging from $10^{-12}$ to $10^{-16}$ M of most diseases (e.g. cancer, neurological disorders) without a concentration step.[73] Therefore, concentration step is vital for the sample with extremely low concentration for the ease of detection to improve detection limit of target. Various traditional methods have been applied for concentrating the clinical samples, such as ultrafiltration,[19] isotachophoresis (ITP),[74,75] and temperature gradient focusing (TGF).[59,76] However, these traditional methods are always complex and expensive. Thus, a simple and easy-to-use concentration technique should be developed to substitute these complex, time-consuming and laborious traditional techniques. At present, some emerging technologies have reported the use of paper-based concentration methods to improve the sensitivity of the assay (Table 3).

### *Emerging technologies*

#### *Technologies suitable for POC use*

An aqueous two-phase system (ATPS) has been used for concentrating the target biomarkers. At present, paper-based ATPS has been applied to concentrate the sample for improving the sensitivity of LFA (Figure 5A).[77] The 3D paper architecture is composed of multilayer defined size ($8 \times 10$ mm) laser-cut strips made of glass fiber. The mixture of polyethylene glycolpotassium (PEG) and sample-phosphate in 3D paper at appropriate concentrations (PEG: sample = 9:1), produces PEG-rich phase (a more hydrophobic phase) on top layer of 3D paper and PEG-poor phase (a more dense and hydrophilic phase) at the bottom layer. As a result, this phenomenon enables sample concentration for sensitivity enhancement in LFA. This technology could achieve the detection limit as low as 0.1 ng/μL for detecting transferrin (enhanced up to 10-fold sensitivity) within 25 min. Based on the similar principle of a micellar ATPs of the Triton X-114 system, a 3D paper design is also integrated with LFA to simultaneously concentrate and detect a malaria biomarker (e.g. Plasmodium lactate dehydrogenase (pLDH)) within 20 min at room temperature. This single-step successfully detect target (pLDH) at 1 ng/μL and increase up to 10-fold.[78] Compared to conventional evaporation and ITP techniques, ATPS technology is more suitable to be integrated into paper-based device without need for additional power supply.

Additionally, a novel sample preparation system has been used for in-membrane sample purification and concentration on paper (Figure 5B).[79] Chitosan is immobilized in paper to capture DNA via anion exchange chromatography. In solution buffered with a pH below 6.3–6.5, the primary amine is protonated, chitosan binds nucleic acid (DNA and RNA) via electrostatic interactions. When adding a solution with the pH above the amine pKa (it means acidity coefficient), the primary amines are deprotonated which results in the loss of electrostatic attraction and the release of nucleic acid. Nitrocellulose membrane has been reported to have greater efficiency in sample concentration, as shown by a higher concentration factor (13.3×) as compared to glass fiber (12.3×). This system has been used to successfully purify and concentrate the complex sample containing high protein content, excess non-target DNA and blood. However, this device could be further integrated into a sample-to-answer POC platform.

#### *Technologies to be improved for POC use*

Evaporation concentration technique based on heating has been used to concentrate the sample for downstream analysis.[59,76] Similarly, a paper-based evaporative concentration device has been developed to concentrate the biological sample.[80] The device consists of a defined size of chromatography paper sandwiched between two custom-made aluminum plates, which can be heated up to 220 °C by a commercial resistive heater. The concentration is achieved through heating induced evaporation of the liquid carrier, resulting in the enrichment of the target molecule (Figure 5C). With this method, the concentration of tuberculosis biomarker is increased nearly 20-fold within 20 min. Compared to other concentration techniques (e.g. electrodynamic and nanofiltration techniques), this technology has the potential to be easily integrated into lateral flow assays, which is able to concentrate hundreds of micro liters of sample down to tens of micro liters in order to meet the requirement of a small final sample volume for downstream analysis (e.g. LFA). However, this technology can only be used for heating thermally-

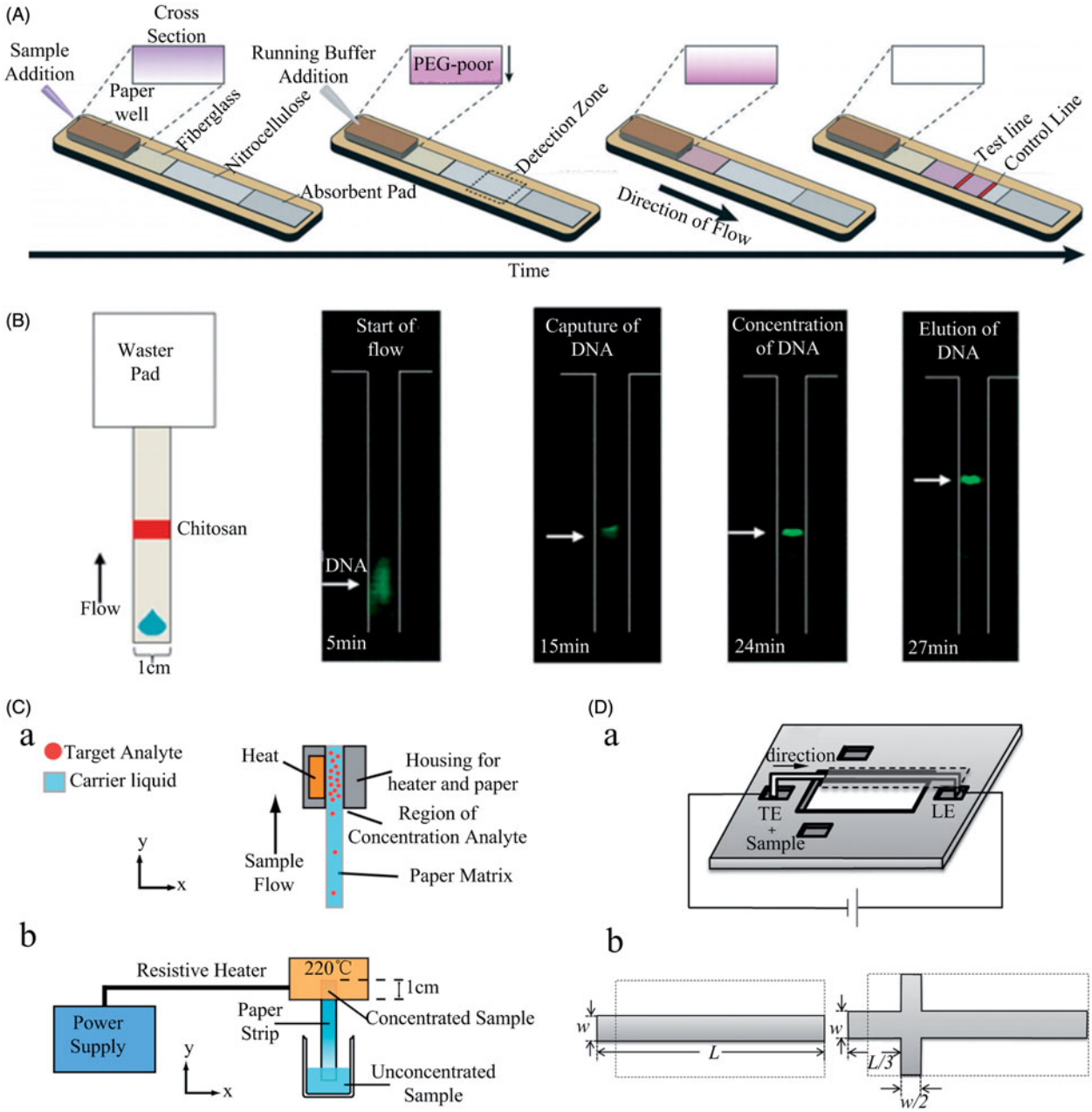


**Figure 5** Paper-based sample concentration. The target analyte was concentrated using (A) the 3D paper-based device using ATPS,[77] (B) in-membrane sample purification and concentration using chitosan,[79] (C) the evaporation concentration device,[80] and (D) paper-based ITP concentration device.[73]

stable biomarkers since the high temperature (220 °C) involves the issue of thermally-induced damage to the biomarkers. The technology should overcome the high temperature to expand its wide application.

Additionally, isotachophoretic (ITP), an electrophoresis technique, has been applied for paper-based sample concentration. An ITP technique is applied in the paper-based device based on different effective electrophoretic mobility of target to concentrate sample ions. Sample ions are focused between high electrophoretic mobility leading ions (LE) and low electrophoretic mobility trailing ions (TE). When a constant voltage or current is applied across the channel, sample ions accumulate and concentrate by electrophoresis into a number of contiguous zones between LE and TE zones according to their mobility. Each zone has concentrated molecules with similar molecular weight, as governed by electrophoresis conversation laws.[81] However, there exist the challenges of Joule heating and evaporation when using this technique on paper-based device. To overcome these challenges, several approaches have been developed. A study has created shallow channels on μPAD by wax printing to rapidly dissipate Joule heating. This device is able to concentrate 30 μL of target analyte

(fluorescent molecule) and increased 1000-fold concentration in 6 min.[81] With similar principle of ITP, another study has designed the cross-shaped membrane on ITP device to decrease the evaporation of sample from the membrane free surface and Joule heating (Figure 5D). The concentration of sample, for example, Alexa Fluor 488 (AF488) succinimidyl ester (Molecular Probes, Eugene, OR) can be increased by nearly 900-fold.[73] In addition, the cross-shaped device has been integrated into LFA to improve the sensitivity of the assay. The detection limit of sample was enhanced 400-fold in 90 s and by 160-fold in 5 min. Successfully, the technology is integrated into LFA for detection of protein. However, its efficiency is low (~10%), the improvement of which needs the optimization of all parameters. Meanwhile, this device should also be combined with the portable battery to expand the scope of its application.

## Conclusion and future perspectives

In POCT, sample pretreatment, including collection and storage, separation, extraction and concentration, are important to obtain the desired analytes from the sample for downstream analyzes. To replace the complicated conventional techniques, a low-cost, rapid, and easy-to-use technique should be developed. Paper, with low-cost, easy-to-use, eco-friendly, and portable features, holds great potential for sample pretreatment. At present, with the development of modification and fabrication techniques of paper, paper-based sample pretreatment technologies have been developed to reduce the time and step of operation, offering great potential for POCT in low resource settings.

However, most existing paper-based sample pretreatment technologies have some limitations and cannot be used for resource-poor setting. For example, most paper-based devices are portable without external power, but these require manual intervention and multistep processes (e.g. multiple reagent addition steps and extra heating step) by manual operation.[44,45,82] To address this limitation, a flow control technique and valve (e.g. two-dimensional paper networks,[83] paper-based non-mechanical valve [84]) could be integrated into paper-based POCT devices to replace multistep chemical processing sequences for sample pretreatment. Besides, some paper-based devices (e.g. paper-based ITP device,[81] paper-based SAW device [71] and paper-based evaporation concentration device [80]) are not portable and require external power supply, which are not suitable for low-resource endemic settings.[81] To overcome this challenge, a portable paper-based electronic device (e.g. paper-based flexible piezoelectric nano-generators [85]) - could be coupled with the paper-based sample pretreatment technique in the future. Additionally, in sample preparation, nucleic acid extraction always needs electrical-powered syringe pump for adding buffer [70] and heating for cell lysis. Therefore, the paper-based sample pretreatment device for nucleic acid extraction should be coupled with a simple paper-based pump,[16] and a portable miniature heater to be well-suited for POCT in resource-limited settings.

In the future, these paper-based sample pretreatment technologies will need to overcome some challenges to improve the drawback of the technology, such as, external equipment, multiplex operations, long time, cross contamination. In addition, the integration of paper-based sample pretreatment into a paper-based diagnostic device (e.g. the most commonly used LFA) is highly demanded to produce an integrated sample-to-answer diagnostic device for POCT. For example, paper-based microfluidic valve technology [9,84] can be integrated into this paper-based platform to direct the buffer or sample to flow sequentially from the area of sample pretreatment to the detection area. In particular, to accurately detect target nucleic acid at low-resource settings, a fully automated paper-based platform starting from nucleic acid extraction, amplification and the use of Smartphone's sensor [86] for quantitative detection is essential. Furthermore, these technologies should be validated by using clinical sample testing and expand its application. Finally, we envision that paper-based sample pretreatment can make a great contribution to the development of cost-effective POCT in the near future.

## Disclosure statement

The authors declare that they have no conflict of interest.

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