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Sample Preparation for Lab-on-a-Chip Systems in Molecular Diagnosis: A Review

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ABSTRACT: Rapid and low-cost molecular analysis is especially required for early and specific diagnostics, quick decision-making, and sparing patients from unnecessary tests and hospitals from extra costs. One way to achieve this objective is through automated molecular diagnostic devices. Thus, sample-to-answer microfluidic devices are emerging with the promise of delivering a complete molecular diagnosis system that includes nucleic acid extraction, amplification, and detection steps in a single device. The biggest issue in such equipment is the extraction process, which is normally laborious and time-consuming but extremely important for sensitive and specific detection. Therefore, this Review focuses on automated or semiautomated extraction methodologies used in lab-on-a-chip devices. More than 15 different extraction methods developed over the past 10 years have been analyzed in terms of their advantages and disadvantages to improve extraction procedures in future studies. Herein, we are able to explain the high applicability of the extraction methodologies due to the large variety of samples in which different techniques were employed, showing that their applications are not limited to medical diagnosis. Moreover, we are able to conclude that further research in the field would be beneficial because the methodologies presented can be affordable, portable, time efficient, and easily manipulated, all of which are strong qualities for point-of-care technologies.

Microfluidics technology has been gaining attention, because of its convenience, compared to conventional benchtop testing. It combines all of the basic essential steps of a typical molecular study (extraction, amplification, and detection) in a simple automated lab-on-a-chip (LOC)-based molecular analysis device. This technology enables the reduction of processing time and the cost of the total operation, reduced contamination, and loss of sample/reagent, because of its closed structure. Moreover, this system allows device portability to resource-limited environments; the reduced reaction size allows for lower reagent consumption, the results are faster, and there is no need for highly trained personnel.[1] All the advantages listed above culminate in great potential in point-of-care testing (POCT). In this context, nucleic acid extraction plays a pivotal role in molecular analysis, since the purity of the sample directly affects downstream processes.

Nucleic acid extraction is one of the most important steps in molecular diagnosis protocols, as is frequently the outset of many downstream assays. This crucial step is available in quality-controlled commercial kits, adapted based on the nucleic acid of interest and sample source.[2] However, in most cases, nucleic acid extraction from biological samples is incompatible with POCT, because it requires column-based techniques or centrifugation, complex operations to eliminate inhibitory substances, demanding trained personnel, and expensive equipment.[3,4]

Although sample preparation is a laborious process, and quite often difficult to implement on a chip format, because of its demand for multiple steps, it has been shown numerous times in the literature.[5] POCT is paramount in resource-limited settings or situations when a fast and efficient result is needed; thus, quick, simple, and accessible nucleic acid extraction is very attractive. It is critical that these implemented techniques are understood and well-studied.

Another important component of molecular testing is sample amplification. Although it is not the focus of this Review, it is crucial to mention that the devices discussed here had all the molecular analysis steps integrated. Furthermore, the amplification step was either a polymerase chain reaction (PCR) or a loop-mediated isothermal amplification (LAMP), or a variation of these methods (reverse transcriptase-PCR, for example). PCR was chosen because of its use as a "gold standard" procedure in molecular studies. The LAMP assay was chosen for its technology; it was the first published isothermal reaction, utilizing four to six primers that ensure a strong specificity and high sensitivity with higher tolerance to PCR inhibitors.[6]

This Review aims to look at the automated nucleic acid extraction protocols integrated with amplification and detection in microfluidic systems that have been described in the past 10 years and discuss the latest nucleic acid extraction procedures found in automated LOC devices for molecular

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testing. Here, we describe the procedures that have been reported and what they were used for, limiting the amplification assays to PCR and LAMP variations. Discussions about which methodologies would be more useful in low-resource situations are also included.

NUCLEIC ACID EXTRACTION BY SOLID PHASE

Solid-phase extraction, as the name suggests, utilizes a solid medium to extract nucleic acids, usually taking advantage of the difference in chemical or physical affinities between the genetic material and the unwanted substances that can be present in a sample. Several different techniques can fit into this category, such as the use of micropillars, membranes, and beads.

Nucleic Acids Extraction by Micropillars. This very simple method relies on the micropillar affinity for nucleic acid binding. The extraction was conducted at room temperature, followed by nucleic acid binding to the micropillars and sample purification by washing and elution steps. This technique has shown promising results in a semiautomated device developed by Petralia in 2016, which was used to extract genetic material to identify the hepatitis B virus (HBV) from analytical samples with concentrations comparable to those of clinical samples (105 copies/μL).[7] This report shows the fabrication of micropillars made of silicon, used to increase the device's surface area, which enable the capture of a greater volume of nucleic acid while also sparing the need for chaotropic agents and ethanol, which are well-known PCR inhibitors. Instead, the nucleic acids are bound through hydrogen bonds to silanol groups on the pillar surface, formed during the chemical treatment of the micropillar array with a solution of ammonium hydroxide, hydrogen peroxide, and water, the authors reported an extraction efficiency of ~40%, superior to commercial kits tested (Promega, Qiagen).[7] Furthermore, the method has been used to extract genetic material for pharmacogenetic typing.[8] In a 2016 report by Zhuang, it used to extract nucleic acid to successfully detect warfarin-related single-nucleotide polymorphisms (SNPs) in the VKORC1 gene and the '3 SNP in the CYP2C9 gene from both dried bloodstains and oral swabs, the authors used multiplex allele-specific PCR assay for detection of the polymorphisms.[8] In addition, with a fully automated chip (Figure 1a), in 2011, Wu was able to extract DNA using pyramidal glass pillars obtained by glass etching, to which nucleic acids would attach in the presence of the chaotropic salt guanidine hydrochloride.[9] The method efficiency was equivalent to that of a commercial kit for whole blood DNA extraction (QIAamp Minelute Column (Qiagen)).[9]

Moreover, UV-activated polycarbonate micropillars have been employed for solid-phase extraction. In 2012, Chen used the enrichment module made from PMMA and contained channels coated with polyclonal antibodies to select target cells.[12] In the device, lysis was performed at a given temperature, and it was employed to extract *E. coli* O157:H7 strain genetic material from spiked water samples, followed by genotyping in a fully automated modular-based microfluidic system. The UV irradiation generated carboxylate groups, plus an immobilization buffer containing polyethylene glycol (PEG), NaCl, and ethanol, which were responsible for selectively immobilizing nucleic acids onto the polycarbonate (PC) surface. Finally, the concentrated genetic material was eluted with water or PCR buffer.

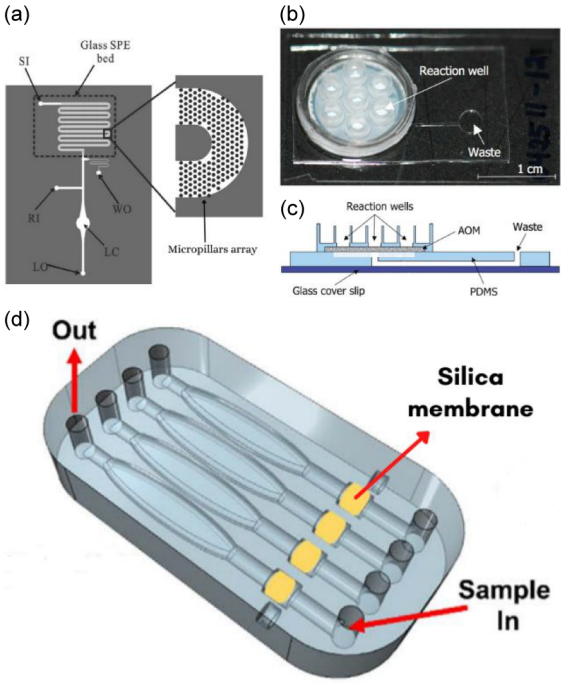

Figure 1. (a) Nucleic acid extraction and amplification device made of glass. The sample is added through the sample inlet with a loading buffer. The nucleic acid binds to the silica micropillars in the presence of a high concentration of chaotropic salt agent and is eluted with an aqueous salt buffer. The other components seen in the image are the waste outlet (WO), reagent inlet (RI), LAMP chamber (LC) and LAMP outlet (LO). [Reprinted, with permission, with modifications, from ref 9. Copyright 2011, American Chemical Society, Washington, DC.] (b) Image of a sample-to-answer chip fabricated with glass for nucleic acid extraction by an aluminum oxide membrane. (c) Schematic cross-section of the chip. The samples are added to the reaction wells, which are made of PDMS. The process can be multiplexed since different sets of primers can be used in each well. Vacuum is applied after the sample solution is added, so that the entire sample is pulled through the AOM, and the waste is directed into the waste reservoir. Then, PCR mix is added, and the chip is thermocycled. The results are obtained by fluorescence imaging. [Reprinted, with permission, with modifications, from ref 10. Copyright 2013, The Royal Society of Chemistry, London.] (d) 3D-printed microfluidic device fabricated with acrylate-based resin. The device was fabricated in two different pieces that were bonded together by photopolymerization after the silica membrane was added to the extraction chamber. The sample is pulled through the membrane by a suction pump placed at the outlet port. LAMP is then performed with a heating block. [Reprinted, with permission, with modifications from ref 11. Copyright 2018, Elsevier.]

Extraction by Silica Membrane. A silica monolith works similarly to the silica micropillars mentioned previously. Nucleic acids are prone to bind to silica monoliths in the presence of a chaotropic agent. One advantage of this membrane over others is that it can be cut to fit into specific devices. The potential of this method has already been reported by Shaw in 2011 and employed to extract DNA from human buccal samples for amplification of the Amelogenin locus in a microfluidic device.[13] The authors applied electro-osmotic pumping (EOP) for fluidic control, minimizing user intervention, so the voltage (100 V cm^{-1}) was responsible for the sample migrating through the silica monolith, as well as the washing and elution buffers. In addition, all the reagents necessary for extraction and amplification were encapsulated in

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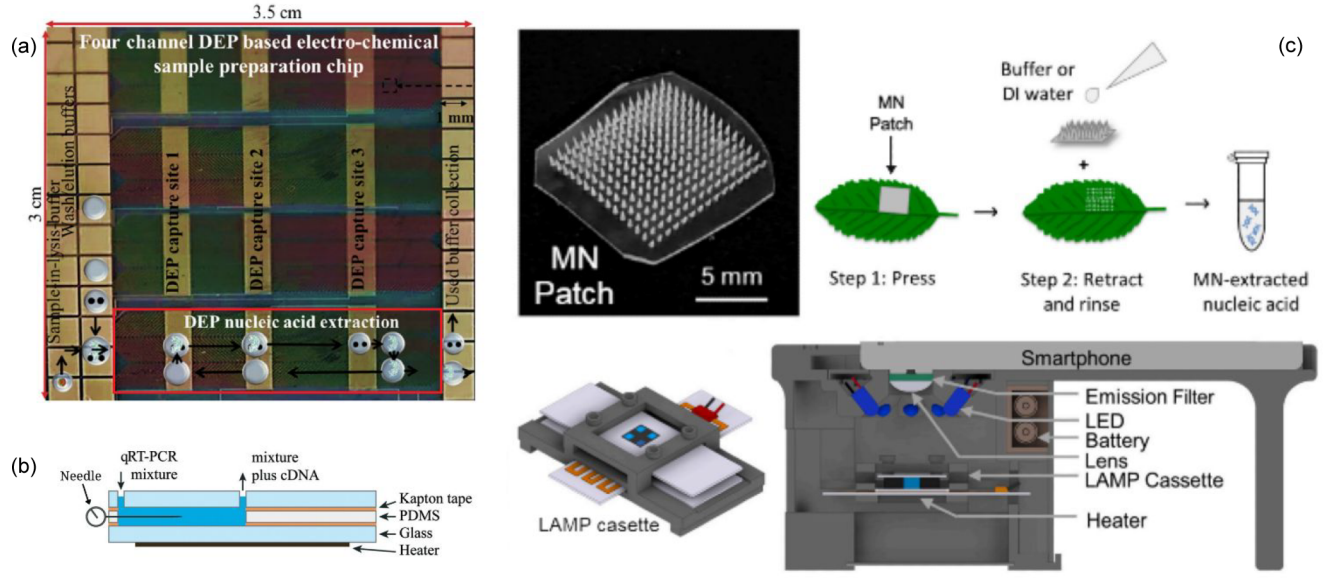

Figure 2. (a) Sample preparation chip module fabricated in glass with additional layers of metal and insulating material, designed by Prakash in 2016. It consists of four independent sample preparation channels (a single channel is identified by a red square), each with three different DEP capture sites. The sample is mixed with lysis buffer and microbeads and then added to the module in the left corner over the EW electrode array. The microbeads are carried through the channel by an electric current (100 Vpp; 60 Hz) onto the DEP capture zone, where the high frequency negative DEP capture field applied (~100 Vpp; 750 kHz), so the microbeads are immobilized on the chip surface while the residues are performed of the capture zone by the electric current. This is repeated three times, each followed by a washing step, to ensure that all the beads have been captured. Then, the nucleic acids were eluted and subjected to the amplification assay. [Reprinted, with permission, with modifications from ref 17. Copyright 2016, Springer.] (b) Cross-section of the microfluidic device used for nucleic acid extraction and amplification developed by Nestorova in 2017. The needle captures mRNA molecules from the sample by affinity between the $d(T)$ oligonucleotides immobilized on the stainless-steel surface and the poly(A) tail present in the RNA molecules. Then, the needle is inserted into the device through the PDMS wall and heated to 60 °C by a thin-film heater attached to the outer surface of the device to release the hybridized RNA. The needle is then removed, and the RNA is reverse transcribed and amplified by PCR. [Reprinted, with permission, with modifications from ref 18. Copyright 2017, The Royal Society of Chemistry, London.] (c) Disposable poly(vinyl alcohol) (PVA) microneedle (MN) patch developed by Paul in 2019. The nucleic acids, either RNA or DNA, are extracted by pressing the MN patch on top of a leaf and then rinsing with DI water or TE buffer or simply dipping the patch in the LAMP mixture. The authors attributed the extraction mechanism to the poly(vinyl alcohol) (PVA) material, capable of producing a capillary flow effect. It is hypothesized that the MN patch extracts capsid-free viral mRNA from the plant tissue. After the extracted materials are transferred to the LAMP cassette, amplification is performed without further purification steps. The bottom-right illustration shows the smartphone-based platform where amplification and detection are performed. The extraction process can be performed in less than a minute and the pathogens can be detected in the field within 30 min. [Reprinted, with permission, with modifications from ref 19. Copyright 2021, Elsevier.]

low-melting temperature agarose and preloaded onto the device. They reported a DNA extraction efficiency of ~52% before treating the chip channels with hexadimethrine bromide (Polybrene), whuch is a cationic polymer. This treatment allowed inverting the usual direction of the electro-osmotic flow from cathode to anode, therefore enhancing DNA mobility, as this is its natural electrophoretic migration, increasing silica-monolith DNA extraction efficiency to 74.5% ± 20.3%. Moreover, in 2018, Kadimisetty also used a silica membrane for the extraction of nucleic acids in a three-dimensional (3D)-printed microfluidic device,[11] as shown in Figure 1d. They successfully detected *Plasmodium falciparum* DNA in plasma samples and *Neisseria meningitides* DNA in cerebrospinal fluid (CSF) samples. The samples were first lysed and then loaded into the inlet port and flowed through the membrane, because of a suction pump placed at the outlet port. Then, they were washed twice with ethanol buffer to remove the remaining contaminants. The nucleic acid yield of the isolation membrane was 62% ± 24% (n = 3).

Similarly, a silica membrane for genetic material extraction was integrated into a fully automated microfluidic device developed by Zhao in 2014 to detect the GAPDH gene in whole blood samples.[14] The authors lysed the sample off-chip, and after loading onto the device, the flow was controlled through electromagnetic valves. Two washing steps and one elution step were performed to achieve successful extraction and purification. Real-time PCR cycle threshold values were reported as 25.3 and 26.9 for the silica-based extraction method and 22.5 and 23 for manual extraction using a centrifuge, which was performed for comparison.[14]

In a 2018 report by Song, a silica membrane was used to extract both RNA and DNA.[15] The authors used spiked saliva with HPV-16 DNA to test the feasibility of the method, and also in another assay, spiked plasma with HIV was used. Interestingly, Song used paraffin-encapsulated reagents pre-stored within the reactor; these solutions melt when chaotropic agents filter through the membrane, and the assay continues to downstream reactions. In this case, a LAMP assay was performed for amplification.[15] A very similar device as the aforementioned was manufactured, although it also aimed at HPV and HIV detection, the membrane used was porous.[16] The porous membrane has its advantages, depending on pour diameter and salt concentrations. For the fabrication of the device, the authors used an "smart connected pathogen tracer" (SCPT), which has its principles in a smartphone-based molecular analysis. This shows the variety in which the method can be used for biomedical research. Also, a computer numerical control (CNC) machine was used for the making

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of the PMMA device.[16] Furthermore, a silica membrane is used for immobilization in a POC device for the detection of ZIKA virus from a spiked saliva sample; nucleic acids from the sample bound to the silica membrane, and anything that did not bind, was discharged to waste. After extraction, RT-LAMP was performed in an insulated portable cup, in which the heat source came from a single-use Mg−Fe alloy pack.

Extraction by Aluminum Oxide Membrane (AOM). This technique consists of a monolithic aluminum oxide membrane, which is used for sample filtration. The extraction is dependent on the sample affinity to the membrane, and since it is composed of anodized aluminum, nucleic acids can easily bind. Hence, the amount of genetic material extracted is dependent on the pore size, salt concentration, and membrane pH. This simple but efficient extraction technique has been used in an automated sample-to-answer device for the identification of *Streptococcus mutans* in saliva samples and the detection of both methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) genomic DNA.[10] In a 2013 report, Oblath used genomic DNA from the targets (purchased from TCC—Global Bioresource Center) and spiked into saliva samples from healthy patients.[10] Thermal lysis was performed off-chip, the extraction and PCR were performed directly in the aluminum oxide membrane, as shown in Figure 1b. No washing or elution steps were required, and DNA elution from the membrane was expected, because of the basic pH of the PCR master mix added after extraction.

Extraction by Dielectrophoresis. Dielectrophoresis (DEP) utilizes a spatially nonuniform electric field that affects the behavior of dielectric microbeads bound to nucleic acids, helping to separate nucleic acids captured by microbeads from unwanted components in the sample. The electric field is usually generated by electrically energized microelectrodes built directly into the chip platform. The efficiency of the method is dependent on the conductivity of the aqueous fluidic medium, the size of the microbeads, and its surface conductance, among other factors. In 2016, Prakash applied this system to extract RNA from clinical samples acquired from nasopharyngeal swabs to detect influenza A, influenza B, respiratory syncytial virus (RSV), coxsackievirus, and echovirus.[17] The authors integrated DEP and electrowetting (EW) in a single surface multilayered design to achieve successful extraction, as shown in Figure 2a. They reported extraction efficiency comparable to commercial extraction kits and high reproducibility.

Use of a Probe-Coated Needle for Extraction of Nucleic Acids. As an alternative to the methods mentioned previously, there is a method of genetic material extraction through a stainless-steel needle, to which amino-modified sequence-specific oligonucleotides are linked; the needle serves as the column that will retain the desired genetic material. To prevent the need for off-chip washing and elution steps and prevent exposure of the reagents to the environment, in 2017, Nestorova designed a microfluidic device with sidewalls made of elastomer polydimethylsiloxane (PDMS).[18] As shown in Figure 2b, the simple motion of inserting the needle through this material, without washing, into the reaction chamber finalizes the extraction and purification steps of the nucleic acids. The piercing force through the microfluidic device is sufficient to remove all but the immobilized molecules. In addition, this material has self-sealing properties, averting any leakage after the needle is inserted or removed. The nucleic acids are released by thermal elution, and heat is generated by a thin-film heater coupled at the bottom of the device. The authors used this method for selective extraction of mRNA from rat glioblastoma cell spheroids, followed by on-chip RT-PCR amplification. The extraction efficiency is not mentioned, but an amplification efficiency of 87% is reported.

Use of Chitosan-Coated Beads for Extraction of Nucleic Acids. Another example is the use of chitosan, a cationic polysaccharide, as a solid phase for extraction of genetic material. Chitosan can be bound to a needle or bead to facilitate the extraction procedure, but it can also be used alone in SPE. Its deacetylated form bonds strongly with nucleic acids, which enables the purification of the sample in a very straightforward manner. In 2011, Hagan used chitosan-coated silica beads in a glass microfluidic device to extract and detect influenza A virus RNA from nasal swabs.[20] The authors mentioned that this technique is advantageous over silica-based extraction procedures, since it is a completely aqueous method and does not require the use of RT-PCR inhibitory compounds, such as guanidine and isopropanol, which are commonly used in silica-based extraction protocols.

Use of PDDA-Coated Capillary for Extraction of Nucleic Acids. Furthermore, capillary-based solid-phase extraction is also a possibility. Poly(diallyl dimethylammonium chloride) (PDDA) is a water-soluble cationic polyelectrolyte that has an abundance of highly hydrophilic and positively charged quaternary ammonium groups, which enables robust binding with negatively charged nucleic acids. This allows the purification of a high concentration of the genetic material in the capillary within <20 s. Therefore, in 2017, Fu employed silica capillaries coated with PDDA to extract DNA from milk for the identification of *Escherichia coli* in an automated sample-to-answer device.[21] The cells were lysed off-chip and added to the PDDA-modified capillary. Flow control is achieved through capillary force. The nucleic acids are captured on the surface within <20 s, and then the device is inclined so that the lysate flows out under the force of gravity, avoiding the need for washing and elution steps. The authors loaded the PCR mix, and the capillary was sealed for post-amplification and detection.

Use of Poly(vinyl alcohol) (PVA) Microneedles for Extraction of Nucleic Acids. An interesting option for automated extraction was recently proposed by Paul. As shown in Figure 2c, it consists of a portable system containing a polymeric microneedle patch, where nucleic acid extraction occurs.[19] The microneedles are made of poly(vinyl alcohol) (PVA) materials; thus, it allows for fast and complete separation, because of characteristics such as low nonspecific protein binding properties. The device consists of a PDMS stand, and a smartphone attachment for imaging. In this report, the authors selected *Phytophthora infestans* and tomato spotted wilt virus (TSWV) to test their devices, the pathogens' genetic material are DNA and RNA, respectively. Assays with the pathogens isolated and multiplexed were performed via LAMP reactions. Table 1 shows a summary of the microchips characterized in the articles mentioned here.

EXTRACTION USING MAGNETIC BEADS

This extraction procedure uses magnetic beads and an external magnetic field to separate the bound genetic material from waste and undesired compounds. In principle, nucleic acids bind to magnetic beads in the presence of a high salt solution. Binding occurs because of the affinity between ligands present

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Table 1. Summary of the Solid-Phase Extraction Methods Mentioned in This Section

Article	Device's Material	Extraction Method	Amplification Method	Buffer Information
Shaw[13] (2011)	glass	silica membrane	PCR	lysis/binding solution (5 M guanidine hydrochloride (Gu-HCl), 10 mM TE buffer), wash solution (50% (v/v) ethanol in 100 mM sodium chloride), elution buffer (10 mM TE buffer)
Kadimisetty[11] (2018)	acrylate-based resin	silica membrane	LAMP	loading buffer (Qiagen AL lysis buffer, 20 μL ethanol (contains chaotropic salt)), wash buffers (ethanol based 75 μL of AW1 and 150 μL of AW2 from Qiagen kits), no elution
Zhao[14] (2014)	PDMS	silica membrane	real-time PCR	lysis buffer (QIAamp DNA Micro Kit), wash buffers (Aw1 and Aw2 - QIAamp DNA Micro Kit), elution buffer (AE - QIAamp DNA Micro Kit)
Song[15] (2018)	PMMA	silica membrane	LAMP	Qiagen lysis buffer, Qiagen wash buffer 1, Qiagen wash buffer 2, and 25 μL of the nonbuffered LAMP reaction solution
Yin[16] (2020)	PMMA	silica membrane	LAMP	lysis buffer, 1.25 M sodium chloride and 10% polyethylene glycol, other buffers are not specified and exact measures are not cited.
Song[22] (2016)	Thermos cup body with 3D-printed cup lid	silica membrane	RT-LAMP	binding/lysis buffer (AVL buffer) (QIAamp Viral RNA Mini Kit), 100 μL of Qiagen ethanol-based wash buffer 1 (AW1) and 200 μL of Qiagen ethanol-based wash buffer 2 (AW2)
Petralia[7] (2016)	PDMS glass	silicon micropillars	real-time PCR	loading buffer (5 μL TE buffer), washing buffer (10 μL TE buffer), elution solution (water)
Wu[9] (2011)	glass	glass micropillars	LAMP	loading buffer (6 M guanidine hydrochloride, 10 mM Tris, 1 mM EDTA, pH 6.0), wash solution (2-propanol/water, 80/20 (v/v)), elution buffer (10 mM TE at pH 8.0)
Zhuang[8] (2016)	PMMA layers and PDMS membrane disks	glass capillary electrophoresis	allele-specific PCR	100-μL NaOH (20 mM), diluted HCl (1.8 mM) and 1 × TE buffer.
Chen[12] (2012)	polycarbonate	polycarbonate micropillars	continuous-flow PCR	immobilization buffer (3% PEG, 0.5 M NaCl and 63% ethanol), wash buffer (85% ethanol), elution buffer (30 μL of the PCR buffer or water)
Oblath[10] (2013)	PDMS	aluminum oxide membrane	real-time PCR	washing and elution steps not specified
Prakash[17] (2016)	glass	dielectrophoresis (DEP)	real-time RT-PCR	TNA lysis buffer (pH 5.95), extraction buffer (VHB wash buffer; pH 4.89), wash buffer (SPR wash buffer; pH 7.98); elution solution (RNase free water; pH 6.38); all supplied by Omega Bio-Tek
Nestorova[18] (2017)	PDMS glass	probe-coated stainless-steel needle	real-time RT-PCR	washing and elution steps not specified
Hagan[20] (2011)	glass	chitosan-coated beads	RT-PCR	loading buffer (75 μL of purified influenza A viral RNA and 10 mM MES buffer), wash buffer (25 μL 10 mM MES), elution buffer (10 mM Tris/50 mM KCl, pH 9.0).
Fu[21] (2017)	silica	capillary-based	real-time PCR	washing and elution steps not specified

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Table 1. continued

Article	Device's Material	Extraction Method	Amplification Method	Buffer Information
Paul[19] (2021)	PDMS stand	microneedles of polyvinyl alcohol (PVA)	LAMP/RT-LAMP	50 μL of TE buffer (for *P. infestans* detection) or DI water (for TSWV detection)

on the bead surface and the nucleic acid, assisted by an electromagnet that creates an external magnetic field when turned on. As the electromagnet is turned off and the beads are washed, genetic material and beads are separated, resulting in a highly concentrated and pure nucleic acid template for the amplification process. The beads' large surface area allows easy nucleic acid binding, making this methodology an extremely efficient approach.

Use of Magnetic Beads To Extract Bacterial Genetic Material. A very interesting report of this methodology was reported in 2016 by Chan; they used a modified 3D printer as the device for the entire molecular diagnosis of *Bacillus subtilis*.[23] In 2015, Sun used magnetic beads to extract genetic material from important pathogenic bacteria, such as *Salmonella spp*.[24] The nucleic acids were extracted from buffered peptone water-enriched pork meat and used in a LAMP fluorescent intercalating dye assay. The bead could also be covered with a layer of a substance that strongly binds to nucleic acids to optimize the extraction.[25] In a 2018 report by Fu, silica-covered beads were employed to extract genetic material from *Listeria monocytogenes* from cultured broth, PCR assays were performed, and detection was performed using paper strips.[25] As shown in Figure 3a, Wang also used coated beads; theirs included anti-*Salmonella* antibodies in propidium monoazide (PMA) solution (PMA is a photoreactive DNA-binding dye), resulting in the extraction of *Salmonella* DNA from spiked chicken meat supernatants for a subsequent LAMP assay.[26]

Another coating of beads was performed in 2017 by Shu, in which vancomycin magnetic beads were utilized to extract the genetic material of *Staphylococcus aureus*; using this method, a capture efficiency of 86.3% was achieved.[28] The authors combined magnetic field with a laser diode, which resulted in bacterial capture, concentration, and photothermolysis; furthermore, the nucleic acids were extracted from bacterial samples and utilized for a PCR assay.[28] In addition, the bead could be coated with a specific nucleotide, as reported by Wang in 2011, wherein genetic material from methicillin-resistant *Staphylococcus aureus* (MRSA) was extracted from spiked milk, sputum, and serum for its detection via LAMP assay.[29]

The magnetic beads can be coupled with capillaries and droplet formation techniques, forming a water-in-oil droplet that prevents evaporation of reagents during amplification.[30] In 2013, Liu used this particular method for the extraction of genetic material of *Mycobacterium tuberculosis* from the Bacillus Calmette Guerin (BCG) vaccine and sputum saliva samples and later identified using LAMP reaction.

In a recent report, Lee used magnetic beads with a rapid shaking of the fixed magnet for the extraction of salmonella DNA for downstream PCR detection. The assay was performed in an airtight cassette, where the pipetting of the reagents was done in each well via pump, which is located on the outer case. The use of magnetic beads held an efficiency similar to a commercial kit, according to the authors, and a multiplex-PCR was used to achieve genotyping.[31] Furthermore, magnetic beads have been used as particles alongside a neodymium magnet for extraction of *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* from oropharyngeal swabs and bronchoalveolar lavage fluid specimens.[32] Lysis was performed on-chip, and resulted in similar DNA yields, compared to the phenol-chloroform method, according to the authors. In 2019, Wang manufactured a bilayer device, in which one layer was

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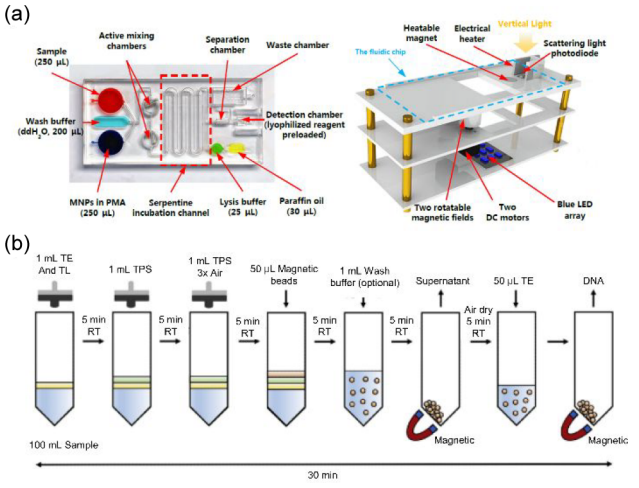

Figure 3. (a) Images illustrating the design of the polydimethylsiloxane (PDMS) fluidic chip (left) and the structure of the periphery where the chip is mounted (right). Here, the images show an example of how magnetic bead extraction could occur. First, the sample, wash buffer and MNPs (magnetic nanoparticles) are mixed, and then magnetic capture and bead treatment occur in the serpentine incubation channel. Magnetic separation and washing follows the nucleic acid extraction. Finally, the LAMP assay is performed. [Reprinted, with permission, with modifications, from ref 26. Copyright 2020, Royal Society of Chemistry, London.] (b) Iimage showing step-by-step of the syringe-filter based DNA extraction adapted by Lee.[27] First, the sample is passed through a porous syringe filter. The filter then is washed with TE buffer in the direction of filtration into a sterile tube. Then, TL buffer, TPS buffer, and TPS buffer and air flow through the filter in the opposite direction of filtration. Last, magnetic beads are added to the tube to bind the DNA and manually shaken. The magnetic beads are then separated from the supernatant by a magnet. Washing buffer is added, then the beads were permitted to air-dry. Finally, TE buffer is added to elute the extracted and purified bacterial DNA. The results obtained are comparable to a commercial kit method. The entire process, from DNA extraction to detection, can be performed within 1 h. [Reprinted, with permission, with modifications, from ref 27. Copyright 2019, Elsevier.]

manufactured from PDMS and, underneath, another layer was made of glass.[32] In addition, silica-coated magnetic beads have been alongside sterile syringe filters (Figure 3b), assisting in filtration, cell lysis, cell debris removal, and DNA purification, as shown in a 2019 report by Lee. This was done to extract genetic material from water samples and identify fecal indicator bacteria (FIB), in this case, *Escherichia coli* and *Escherichia faecalis*.[27]

Use of Magnetic Beads To Extract Virus and Protozoa Genetic Material. The methodology of using beads coated with specific nucleotides was efficient for extracting DNA from an HIV-infected Jurkat T cell line to identify HIV-1 virus;[33] after extraction, a PCR assay was performed to identify the virus. Also, the 2019 work of Ma succeeded in rapidly extracting the Influenza A (H1N1) virus using a single-stranded H1N1-specific aptamer to coat the beads for the binding with the virus, and antibiotic vancomycin-coated beads were used to extract genetic material from MRSA.[34] The use of both viral and bacterial samples suggests that the method is very versatile, and the whole molecular analysis was controlled by a smartphone. Another use of probe-conjugated beads was reported by Wang in 2011, who extracted RNA from whole tissue lysates to identify nervous necrosis virus via RT-LAMP assay.[35] In 2015, Lin described an automatic diagnosis of viral pathogens in Phalaenopsis spp. (orchids) was performed, where RNA was extracted from ground leaves and flowers mixed with a lysate buffer, and the bead was coated with sequence-specific nucleotides.[36] Furthermore, magnetic silica beads were used to extract λ-phage DNA, where guanidinium chloride was used to promote nucleic acid binding to the beads, the method is called dynamic solid-phase extraction (dSPE), and the extraction efficiency was reported to be 69.7% $\pm$ 5.7%.[37] A palm-sized microfluidic cartridge (CARD) made of polystyrene was recently manufactured by Wang; the device used silicon-based magnetic beads to extract human papilloma virus (HPV), and chemical lysis was done before the extraction.[38] Protozoan genetic material has also been extracted using this procedure, most notably by Choi in 2018, who used a DNA-binding bead to extract DNA from infected blood samples to identify two of the most life-threatening malaria species, *Plasmodium falciparum* and *Plasmodium vivax*, via LAMP assay.[39] The emergence of the SARS-CoV-2 in the past year has rushed many scientists to come up with a rapid and easy detection platform for the virus. Rodriguez-Mateos recently reported a device that successfully detects the virus within an hour, the efficient detection occurs due to the use of oligo (dT)-coated magnetic beads extraction of viral RNA for a LAMP assay, all in a single apparatus.[40] This was performed in a PMMA device where the extraction method was based on immiscible filtration assisted by surface tension (IFAST).

Use of Magnetic Beads To Extract Genetic Material for Multiplex Assays. Magnetic bead extraction has also been used in multiplex analysis, where silica-coated beads have been used to extract DNA from different microorganisms simultaneously from urine samples to identify *E. coli*, *Proteus mirabilis*, *Salmonella typhimurium*, and *S. aureus* via LAMP assays.[41] Moreover, in 2018, Wang et al. were able to concurrently extract genetic material using monodisperse magnetic beads from clinical respiratory viral samples to identify influenza A virus subtypes (H1N1, H3N2, H5N1, and H7N9), Influenza B virus, and the hexon gene sequence of human adenovirus (HAdV) in a LAMP assay.[42]

Use of Magnetic Beads To Extract Genetic Material for Methylation Analysis of DNA. In a 2018 report by Stark, the authors used a dropper containing magnetic particles to extract DNA from cells to identify methylation patterns.[43] They used bisulfite conversion, and, because of the high salt content of the bisulfite solution, this method can also be used for human cell lysis, reducing the assay time. The quantification of methylation markers was performed directly on the chip via quantitative PCR. An example of the method's efficiency in purifying samples for detection through LAMP or PCR was demonstrated in 2017 by Sandetskaya, who built a fully automated table-top device that could perform both amplification techniques depending on its configuration.[44] The authors used *E. coli* for the PCR assay and *Salmonella* for the LAMP assay to showcase the versatility of the device. The main difference between each extraction was the preparation of the beads for LAMP and PCR; for LAMP, the beads were washed twice with buffer, and for PCR, they were washed, resuspended in water, and heated. Another versatile example is from the 2019 work of Yin; their device can extract DNA or RNA using superparamagnetic particles, although the study focused on extracting DNA from cells and blood for detection by digital PCR.[45]

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Table 2. Summary of Solid-Phase Extraction Methods by Magnetic Beads Previously Mentioned

Article	Device's Material	Extraction Method	Amplification Method	Buffer Information
Chan[23] (2016)	modified 3D printer	paramagnetic silica beads	PCR	200 μL of NucliSENS lysis buffer, 3 washing buffer at 200 μL each and 50−100 μL of elution buffer (BioExpress, Kaysville, UT)
Sun[24] (2015)	disposable cyclic olefin co-polymer	magnetic beads	LAMP	lysis buffer (Dynabeads DNA DIRECT Universal kit, ThermoFisher Scientific, MA, USA), 200 μL washing buffer
Fu[25] (2018)	copper block	silica-coated mag-netic beads	continuous-flow PCR	400 L MBSL (lysis) buffer, wash with 500 L of 70% ethanol solution, TE buffer to release DNA
Wang[26] (2020)	polydimethylsiloxane (PDMS)	immunomagnetic separation	LAMP	lysis buffer (25 μL) at 70 °C from Huafeng 138 Biotech (Guangzhou, China), wash buffer (doubly distilled (dd) H_2O, 200 μL)
Shu[28] (2017)	polytetrafluoroethylene (PTFE)	vancomycin-coated beads	real-time PCR	photothermal lysis, elution by distilled water
Wang[29] (2011)	PDMS	probe-conjugated magnetic beads	LAMP	thermal lysis (95 °C), phosphate buffered saline (PBS) for washing step
Liu[30] (2013)	PTFE	magnetic beads cou-ples with droplet formation in capil-laries	LAMP	washing step by 7.5× ThermoPol buffer and alkaline buffer with a low ionic strength (~0.2× ThermoPol buffer, pH 8.9) for elution step
Lee[31] (2021)	inexpensive 3D-print parts, off-the-shelf electronics and motors	magnetic beads	PCR	5 mL PBS buffer mixed with 200 μL of lysis buffer, then 20 μL of proteinase K (20 mg/mL) was added. To capture DNA, 20 μL of a suspension of magnetic beads (20 mg/mL) and 200 μL of isopropanol. After separation, 200 μL of washing buffer I and II. Lastly, 200 μL of elution buffer was used
Wang[32] (2019)	PDMS and glass	magnetic beads	LAMP	buffer concentration not mentioned
Lee[27] (2019)	syringe	magnetic beads	LAMP	1 M Tris/HCl pH 8.0, 0.5 M EDTA), 1 mL of TE/lysozyme buffer (TL) (1 M Tris/HCl pH 8.0, 0.5 M EDTA, 7.5 mg/mL lysozyme), 300 mL of TL, 1 mL TE/proteinase K/SDS buffer (TPS) (1 M Tris/HCl pH 8.0, 0.5 M EDTA, 300 mg/mL proteinase K, 1% SDS [w/v]),1 mL of TPS, washing buffer (1 mL) (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 70% ethanol) and 1 mL of TE buffer as an elution buffer
Wang[33] (2013)	PMMA plate with a PDMS cast	probe-conjugated magnetic beads	PCR	thermal lysis, washing buffer (dd H_2O)
Ma[34] (2019)	PDMS	immunomagnetic separation	LAMP	thermal lysis and 20 μL of lysis buffer (H1N1: Favnk 001 (0.5×), 25 μL of dd H_2O for washing, elution buffer not mentioned
Wang[35] (2011)	PDMS	probe-conjugated magnetic beads	RT-LAMP	thermal lysis (95 °C), washed then resuspended into dd H_2O (50 L)
Lin[36] (2015)	buried optical fibers	probe-conjugated magnetic beads	RT-LAMP	500 μL of lysis buffer (100 mM tris(hydroxymethyl)aminomethane hydrochloride, 1.0 M potassium chloride, and 10 mM ethylene diamine tetraacetic acid, pH 8.4) and n, 25 μL of dd H_2O was used to purify and unbind DNA
Duarte[37] (2011)	polyester film coated with toner (PeT)	magnetic silica beads	PCR	loading buffer (4 μL of 8 M GuHCl pH 7.6), wash buffer 1 (μL of 80% isopropyl alcohol (IPA)), wash buffer 2 (8 μL of 0.1× Tris-EDTA (TE)), elution buffer (0.1× TE buffer)

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Table 2. continued

Article	Device's Material	Extraction Method	Amplification Method	Buffer Information
Choi[39] (2018)	poly(methyl methacrylate) (PMMA)	magnetic bead	real-time fluorescence LAMP	amphiphilic lysis buffer, washing and elution buffers not specified
Rodriguez-Mateos[40] (2021)	poly(methyl methacrylate) (PMMA)	oligo (dT)-coated magnetic bead	RT-LAMP	lysis buffer (concentration not specified) containing strong surfactant and 748 μL of 5 M GuHCl
Wang[38] (2021)	polystyrene	magnetic bead	PCR	lysis buffer, washing buffer, elution buffer was mentioned; however, the concentrations were not specified
Li[41] (2020)	polycarbonate	silica-coated magnetic bead	LAMP	lysis buffer, binding buffer, washing buffer IR, washing buffer WB, and elution buffer from bacterial DNA extraction kit (AU2001, BioTeke Corporation, Beijing, China)
Wang[42] (2018)	low-cost polymer material	monodisperse magnetic beads	LAMP	buffers were not specified
Stark[43] (2018)	PDMS	magnetic beads	methylation-specific PCR	bisulfite conversion buffer for lysis (23 μL of Lightning Conversion reagent (Zymo) and 1 μL of Proteinase K (NEB P8107S)), 105 μL of M-Binding buffer, 20 μL of M-Wash Buffer, 20 μL of M-elution buffer
Sandetskaya[44] (2017)	polycarbonate	immunomagnetic separation	PCR/LAMP	thermal lysis, 20 μL 0.5 M binding buffer (CH_3COOK/HCl, pH 4.4), 10 μL PCR buffer + 1 μL 0.25 M binding buffer were used for both washing step, PCR mix used as elution buffer (pH 8.5)
Yin[45] (2019)	PTFE	superparamagnetic particles	digital PCR	5% Triton-X 100 lysis buffer was used and 5 M GuHCl was used as the washing buffer and 20 μL elution buffer
Dong et al.[46] (2021)	polypropylene and some parts are made of 3-aminopropyltriethoxysilane	magnetic beads	multiplex-PCR	buffers used from a whole blood DNA extraction kit (TianLong Science and Technology, Xi'an, China); all the items were used

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Use of Magnetic Beads To Extract Genetic Material for Genotyping. Magnetic beads have also been used to extract DNA for genotyping, as described in the recent work of Dong et al.; in that work, the authors analyzed five single nucleotide polymorphisms (SNPs) using blood samples. The assay was performed in an airtight cassette, where the pipetting of the reagents was done in each well via pump, which is located on the outer case. The use of magnetic beads held an efficiency similar to a commercial kit, according to the authors, and a multiplex-PCR was used to achieve genotyping.[46] A summary of these methodologies is given in Table 2.

EXTRACTION USING CENTRIFUGAL MICROFLUIDICS

This method has been widely used and reported in the literature. It also relies on a bead (magnetic or not) extraction system but with the addition of centrifugal support in charge of rotation so that the movement is responsible for the nucleic acid extraction. Sample preparation can be easily controlled due to centrifugal forces, and the contamination risk is very low, because of minimal sample manipulation.

Use of Magnetic Beads for Extraction in Centrifugal Microfluidics. In this approach, centrifugal forces help the beads flow through the device, functioning with the help of two stationary magnets in the bottom of the turning table and controlling the motion of the magnet bead during the extraction of the nucleic acids. The binding of the beads with the genetic material occurs because of the rapidly alternating rotation frequency. After extraction, the beads were washed and eluted, and the outcome was a purified template for the amplification assay. Centrifugal microfluidics has been used for the detection of genetic material from bacteria, viruses, and protozoa.

The technique has been used for the extraction of nucleic acids to identify Influenza A H3N2 virus strains, where the authors prestore beads and reagents necessary for all the diagnostic steps.[47] The device is fixed to a rotational axis that allows for alternating frequencies from 0 to 90 Hz and fluctuating temperatures, depending on the step being performed. The buffers for the extraction are stored in miniature aluminum pouches, which are open at 55 Hz, because of the liquid pressure, and buffers are released onto the device to initiate lysis of the sample. As the frequencies fluctuate, the reagents are released up into the last frequency change to 20 Hz when the samples are aliquoted into the reaction chamber for the RT-PCR assay. Furthermore, the method has been employed by Choi to extract DNA from the human whole blood to detect *Plasmodium falciparum* via LAMP assays.[48] In 2019, Li also employed extraction through the rotation of the disk against a stationary magnetic field.[49] The reagents were also preloaded, and the method was used to extract DNA from a spiked sample solution to detect the hepatitis B virus via PCR assay. Also, in 2020, Sciuto developed a portable system that used magnetic beads through centrifugation to extract RNA from spiked samples containing the hepatitis B virus, synthetic clones were used as well, and, with both samples, the device performed according to standards.[50] In 2020, Rombach designed the RespiDisk, which enables for multiplex detection of respiratory tract infection (RTI) pathogens from a single sample.[51] It is completely automated and uses RT-PCR as its downstream assay for detection. Here, extraction was done using magnetic beads via centrifugal force as well; some of the pathogens detected were influenza viruses, coronaviruses, parainfluenza viruses, RSV/hMPV, and Adeno/Boca/Rhino/Entero viruses.[51] Furthermore, in 2015, Czilwik used this method to extract genetic material from serum samples for the multiplex PCR detection of *Staphylococcus warneri*, *Streptococcus agalactiae*, *E. coli*, and *Haemophilus influenzae*.[52]

Use of Acid-Treated Beads for Extraction in Centrifugal Microfluidics. The acid treatment of the beads increases the binding of the genetic material. In these cases, the device is equipped with a zigzag-like chamber where extraction occurs. The centrifugal force helps the sample flow through this chamber binding to the acid-treated beads. The samples are washed and eluted, and an amplification assay is performed. In 2017, Park et al. were able to extract genetic material from spiked water and milk samples with 80% efficiency to detect the presence of *Salmonella typhimurium* and *Vibrio parahemolyticus*.[53] In another report, it was used to extract DNA from a spiked sample solution for the identification of *E. coli*, *S. typhimurium*, and *Vibrio parahemolyticus*.[54] In the same year, Oh used a similar system (Figure 4) for DNA extraction to determine the presence of *E. coli*, *S. typhimurium*, *Vibrio parahemolyticus*, and *L. monocytogenes* in real milk samples.[55] In addition, it was utilized on a device that successfully extracted RNA from clinical samples to identify three Influenza A strains: H1N1, H3N2, and H5N1.[56] All of the extractions mentioned previously here were used in subsequent LAMP reactions or a variation of the assay (RT-LAMP) to identify the pathogens.

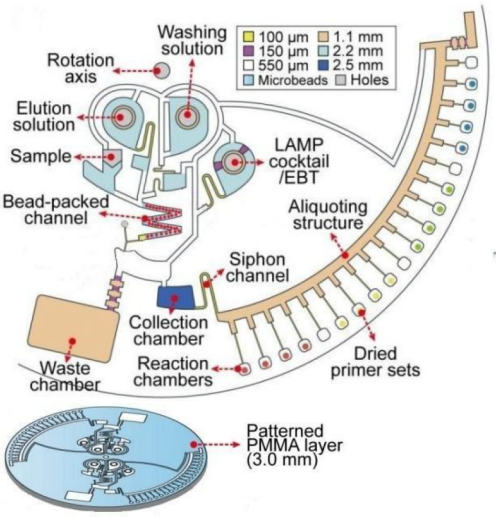

Figure 4. Illustration of the design and components of a centrifugal microfluidic device with a PMMA layer disk. The gray circles represent the places where the sample, washing solution, elution solution, and LAMP cocktail are added. The "bead-packed channel" is where DNA extraction occurs via silica-coated microbeads. In this particular disk, there are 20 reaction chambers where multiplex LAMP is performed. [Reprinted, with permission, with modifications, from ref 55. Copyright 2019, Royal Society of Chemistry, London.]

Use of Miscellaneous Beads for Extraction in Centrifugal Microfluidics. Zeolite and zirconia have also been used for extraction in these devices.[57−59] The porous structure and ion exchange capability of these stones enables them to absorb impurities from the samples. Zeolite coupled with a hand-generated centrifugal force was used to extract genetic material from pathogens responsible for urinary tract infections, and the nucleic acid templates were used in a

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Table 3. Summary of the Solid-Phase Centrifugal Microfluidcs Methodologies Discussed in the Section, with the Specified Bead Used in Centrifugation and the Buffers Where They Were Directly and Clearly Stated

Article	Device's Material	Extraction Method	Amplification Method	Buffer Information
Stumpf[47] (2016)	PMMA casted with PDMS	silica-coated magnetic beads	RT-PCR	200 μL highly wetting binding buffer, (180 μL lysis buffer, 200 μL washing buffer 1, 200 μL washing buffer 2, and 120 μL elution buffer
Choi[48] (2016)	PMMA	magnetic beads	LAMP	30 μL of binding buffer (pH 5.0), 150 μL of washing buffer (pH 7.0), 25 μL of LAMP master mix used for elution
Li[49] (2019)	PMMA	magnetic beads	real-time PCR	800 μL of lysis buffer, 800 μL of washing buffer 1, 800 μL of washing buffer 2, 150 μL of elution buffer all from the same kit (kit not mentioned)
Sciuto[50] (2020)	polycarbonate disk and silicon chip	magnetic beads	qRT-PCR	lysis buffer, 1.25 M sodium chloride and 10% polyethylene glycol, other buffers are not specified and exact measures are not cited
Rombach[51] (2020)	polycarbonate	magnetic beads	RT-PCR	150 μL lysis buffer, 440 μL binding buffer, 200 μL washing buffer I, 200 μL washing buffer II, and 180 μL elution buffer
Czilwik[52] (2015)	cyclic olefin polymer foils	silica-coated magnetic beads	geometrically multiplexed species-specific real-time PCR	binding buffer, washing buffer HS, washing buffer LS, and elution buffer from a commercial kit (Instant MP Basic kit A, Analytic Jena, Germany). The lysis buffer was obtained from the EasyMag kit (Biomérieux, France).
Park[53] (2017)	PMMA/ polycarbonate	guanidine hydrochloride (Gu-HCl) washed beads	LAMP	5 μL lysis buffer by a QIAamp DNA mini kit (Qiagen, Germany), 5 μL of a washing buffer (35% ethanol), 3.5 μL of an elution solution (DEPC-DW)
Nguyen[54] (2019)	PMMA	guanidine hydrochloride (Gu-HCl) washed beads	LAMP	lysis buffer (Qiagen, The Netherlands), washing solution (70% ethanol), elution solution (DNase/RNase water)
Oh[55] (2019)	PMMA	guanidine hydrochloride (Gu-HCl) washed beads	LAMP	lysis buffer (75 μL: 25 μL of lysis buffer, 25 μL of pure ethanol and 25 μL of 6 M guanidine hydrochloride), a washing solution (50 μL), an elution solution (33 μL)
Jung[56] (2015)	polycarbonate	guanidine hydrochloride (Gu-HCl) washed beads	RT-LAMP	lysis by a QIAamp Viral RNA Mini kit (Qiagen), washing solution (70% ethanol), elution solution (RNase-free water)
Zhang[57] (2018)	PMMA	zeolite beads	LAMP	alkaline lysis solution (0.2 M NaOH, 1% SDS, a volume ratio of sample to lysis solution of 1:9)
Yan[59] (2017)	PMMA	zirconia beads	LAMP	lysis, washing and elution buffers not specified
Roy[58] (2015)	thermoplastic elastomer	zirconium/silica beads	PCR	10 μL lysate buffer, washing buffer

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multiplex LAMP assay.[57] The pathogens in which nucleic acids were extracted were *Pseudomonas aeruginosa*, *S. typhimurium*, *Vibrio parahemolyticus*, *Vibrio vulnificus*, *Streptococcus iniae*, and *Vibrio alginolyticus*, all from urine samples. Zirconia was utilized to extract DNA from *E. coli*, *Bacillus subtilis*, *S. typhimurium*, *S. aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* from spiked serum for a multiplex LAMP assay.[59] The authors' experiments resulted in a lysis efficiency of 90.2% for *E. coli*, a *B. subtilis* efficiency of 83.3%, a *S. aureus* efficiency of 64.6%, and a *S. uberis* efficiency of 76.8%. In addition, zirconia has been coupled with silica in a zirconium/silica bead to extract genetic material from distilled water spiked with bacterial spores for the detection of *Bacillus atrophaeus* subsp. *globigii*, through a PCR assay.[58] The aim of the study was to employ a method for the detection of most respiratory DNA or RNA viruses, bacteria, and spores. A summary of these methodologies is given in Table 3.

EXTRACTION USING PAPER

The basis of this procedure is the flow of samples through paper membranes and nucleic acid binding, because of its affinity for the membrane. After the washing and elution steps, the purified nucleic acid template is ready for the amplification assay. This type of extraction has been widely used for extracting genetic material for LAMP assays. The major difference between each device is the type of paper used in the extraction process, such as Whatman FTA cards, glass filter paper, Fusion 5 paper, and poly(ether sulfone) (PES) filter paper, used alone or in combination. Moreover, some devices are manufactured using only paper.

Use of Whatman FTA Cards for Extraction of Genetic Material. Whatman FTA cards have been used in devices for the extraction of genetic material from infected human plasma to amplify a portion of the *malB* gene from *E. coli*.[60] This was achieved through a sliding mechanism to enable serial operation of sample preparation from cell lysis to purification, LAMP amplification and detection, the device was manufactured using magnetic sheets and stacked laminated layers.[60] Another application is inserting the paper in a PMMA cartridge to extract RNA from spiked oral samples for the detection of HIV-1 via LAMP assay.[61] Similarly, Whatman FTA cards were used in a PMMA cage to extract bacterial DNA for a LAMP multiplex assay to identify *Salmonella spp.*, *Staphylococcus aureus*, and *E. coli* in spiked juice and milk.[62] Furthermore, it has been combinations, in which glass fibers were utilized to perform the LAMP amplification assay and the FTA for extracting *E. coli* DNA from phosphate buffered saline (PBS), drinking water, milk, blood, and spinach.[63] Also, to extract *Streptococcus pneumoniae* DNA from clinical blood samples, interestingly, the entire molecular procedure occurs in only 1 h, regardless of the type of sample.[63] The method was demonstrated being used inside a PMMA cartridge to extract DNA from the legs of the mosquitoes *Anopheles gambiae* and *Anopheles arabiensis* for molecular identification through a LAMP assay and posterior genotyping.[64] An epidermal growth factor receptor (EGFR L858R) in lung adenocarcinoma cells was successfully detected with high specificity and sensitivity; after this method of extraction was performed, the device was manufactured from the FTA card and had PDMS reservoirs.[65] Furthermore, FTA paper has been used in a PDMS device for extraction of genetic material from samples containing *Proteus hauseri*, *Salmonella* subsp. *enterica*, and *E. coli*.[66] Whatman CloneSaver was utilized for sample preparation, including DNA extraction and purification; the device was coupled with a finger-actuated microfluidic chip (μFAchip), which contributed to a rapid and easily operable platform for amplification assays, in this case, gLAMP.[66] Moreover, in some assays, authors will coat the paper with a chitosan layer, such as in a 2019 report by Trieu. The authors manufactured a paper origami device composed of chitosan-coated paper disks and MB-infused paper disks, as shown in Figure 5a, for the extraction of *E. coli* O157:H7 and *Salmonella* spp.[67]

Use of Fusion 5 Paper for the Extraction of Genetic Material. In 2020, Hu and Lu conducted an interesting approach to paper microfluidics, where initially the authors used Fusion 5 paper to extract DNA from fruit juice.[68] In this assay, filter paper was coated with a silica membrane to

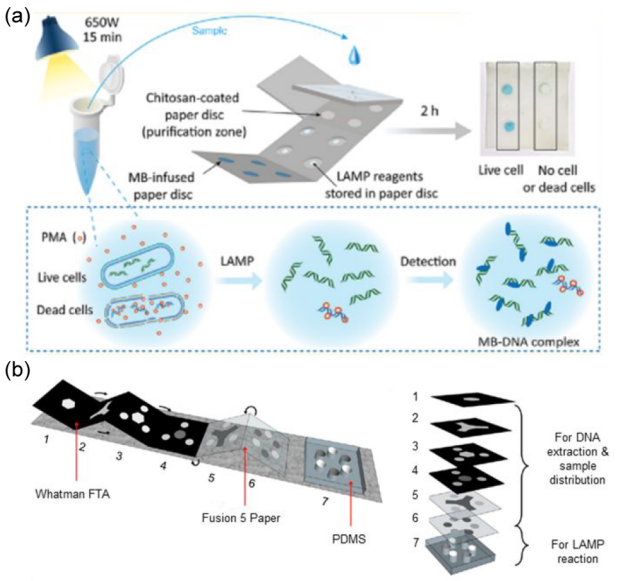

Figure 5. (a) Image illustrating the operation of the origami device developed by Trieu for DNA extraction, followed by LAMP and colorimetric detection to detect *Escherichia coli* O157:H7 (*E. coli* O157:H7) and *Salmonella* spp., foodborne pathogens. First, the sample, containing viable and dead pathogens, is treated with propidium monoazide (PMA), which reacts with DNA from nonviable cells under photoactivation, inhibiting its amplification. The treated sample then is added to the origami paper microdevice, where DNA is purified by a chitosan-coated paper disk. LAMP reagents are then added and the purification zone is flipped onto the reaction pad. LAMP reaction is performed. Lastly, after bleaching solution being added, the dye pad, containing Methylene Blue (MB) is folded onto the amplification pad for colorimetric detection. The blue color indicates a positive result and its intensity is proportional to the target concentration. The overall operating time reported for this method was 2 h. [Reprinted, with permission, with modifications, from ref 67. Copyright 2019, American Chemical Society, Washington, DC.] (b) Image illustrating the hybrid paper/polymer-based microfluidic device. Whatman FTA was used in layers 1−4, and the central gray area on those layers is composed of Fusion 5 paper. The arrows, shown in the left picture, represent the direction in which the device is to be folded during each step of the molecular analysis. Layer 7 consists of the LAMP reaction wells, indicated by the white circles. Layers 1−3 are folded so that the sample is added to the gray circle in layer 4. For washing and elution, layers 5 and 6 are folded over layer 4, and the folded device is placed over the PDMS layer, where the LAMP assay occurs. [Reprinted, with permission, with modifications, from ref 68. Copyright 2020, American Chemical Society, Washington, DC.]

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Table 4. Summary of the Solid Phase Methodologies Discussed in the Previous Section, with the Specified Paper Used and Buffer Information Where They Were Directly and Clearly Stated

Article	Device's Material	Extraction Method	Amplification Method	Buffer Information
Connelly[60] (2015)	magnetic sheets and laminated layers	Whatman FTA	LAMP	40 μL of FTA purification buffer, 80 μL of nuclease-free water and a washing buffer
Liu[61] (2012)	PMMA	Whatman FTA	LAMP	lysis and binding buffer (6 M solution of chaotropic salt guanidinium HCl) from DNeasy Blood and Tissue kit and AW1 and AW2 ethanol-based wash buffers, was purchased from Qiagen, Inc. (Valencia, CA, USA) and no elution step
Trinh[62] (2019)	PMMA	Whatman FTA	LAMP	strong buffers and chemicals (cell lysis, protein denaturants, free radical trap) for extraction and purification, washing step with 25 μL of FTA purification reagent and TE buffer (10 nM Tris-HCl, 0.1 nM EDTA, pH 8.0) from Thermo Fisher Scientific (CA, USA)
Choi[63] (2016)	glass fiber paper	Whatman FTA	LAMP	FTA purification reagent (Whatman, U.K.) and TE buffer (Sigma−Aldrich)
Liu[64] (2011)	PMMA	Whatman FTA	LAMP	binding/lysis buffer (Roche Diagnostic, IN, USA), wash buffer by Roche Diagnostic (IN, USA) without a separate elution step
Chen[65] (2018)	PDMS	Whatman FTA	LAMP	40 μL FTA purification buffer and 80 μL TE buffer as washing step and 50 μL of water for elution
Chen et al.[66] (2021)	PDMS	Whatman FTA	gel-LAMP	extraction buffer information was not specified
Trieu[67] (2019)	PDMS	Whatman FTA	LAMP	buffer information was not specified.
Hu and Lu[68] (2020)	Whatman FTA/Fusion 5	Whatman FTA/Fusion 5	LAMP	50 μL of lysis buffer (8 M guanidine hydrochloride, 1% polyvinylpyrrolidone, 50 mM Tris-HCl, 20 mM EDTA, and 21.3 mM Triton-X-100, pH 6.4), 50 μL of washing buffer I (8 M guanidine hydrochloride, 50 mM Tris-HCl, pH 6.4) once and 50 μL of washing buffer II (10 mM TrisHCl, 100 mM NaCl, pH 8) twice and 60 μL of sterile water for elution
Han[69] (2017)	PMMA and PDMS	Fusion 5	PCR	10 μL TE buffer to release cells. 200-μL distilled water, NaOH (20 mM), diluted HCl (1.8 mM), and 1 × TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for DNA extraction. No elution step
Gan et al.[70] (2017)	PMMA and PDMS	Fusion 5	in situ PCR	50-μL lysis buffer (0.1% CTAB, 1.5 M NaCl, MES, pH 5.0). Samples prepared in a 1 × TE buffer (TrisEDTA, pH 9.0). In addition, 1 mL DI water, 100-μL PCR mix (pH 8.5), and 100-μL TE buffer (pH 9.0) to dissociate DNA
Hui[71] (2018)	PMMA	glass fiber paper	LAMP	lysis buffer (0.5% cetyltrimethylammonium bromide, Sigma−Aldrich (CTAB), 1.5 M NaCl, and MES at pH 5.0), and 48 μL of proteinase K (20 μg μL^{-1}, Generay Biotech, Shanghai, China). Washing step with deionized water. Elution with distilled water
Yang[72] (2018)	glass fiber paper	glass fiber paper	LAMP	40 μL of lysis buffer (1.20 g mL^{-1} of GuSCN, 0.1 M Tris hydrochloride, 0.04 M EDTA, adjusted with NaOH to pH 8.0, 26 mg mL^{-1} Triton X-10029), 100 μL of washing buffer (30 mM NaOAc in 70% v/v ethanol), elution buffer (30 μL, 10 mM TE buffer, pH 8.0)
Ye[73] (2018)	glass fiber paper	glass fiber paper	LAMP	50 μL sample lysis buffer, 200 μL washing buffer washed and eluted the samples
Batule et al.[74] (2020)	PES filter paper and different types of glass fiber paper	SS DNA-modified glass fiber	RT-LAMP	lysis buffer (Triton X-100; 20 μL), hybridization buffer (4X SSPE) and elution solution (10 μL of NaOH, 30 mM)
Seok et al.[76] (2020)		polysulfone filter paper	RT-LAMP	not clearly stated
Rodriguez et al.[75] (2016)	PES filter paper and laminated sheets	PES filter paper	LAMP	100 μL Lysis buffer (3 M guanidinium thiocyanate, 300 mM sodium chloride, 35% v/v 1-butanol (Sigma−Aldrich, St. Louis, MO), and 3 μL of 15 mg mL^{-1} Glycoblue coprecipitant (Life Technologies, Grand Island, NY)), ethanol washes (200 μL of 70% ethanol, followed by 100 μL of 100% ethanol, 50 μL of water for elution
Trinh[77] (2019)	polycarbonate film	polydopamine-coated paper	LAMP	buffer information not specified

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facilitate DNA capture, and the DNA was examined using PCR and LAMP. However, there is also a sample-to-answer hybrid paper/polymer-based device that is fully integrated and automated and uses Whatman FTA paper for the extraction processes and Fusion 5 paper to retain DNA. This device has a microfluidic pattern design, which was wax printed onto the filter paper, and for the LAMP assay chamber, PDMS was used.[68] Thus, a hybrid paper filter was used to extract DNA from fruit juice to identify apple and grape juice in adulterated pomegranate juice, as shown in Figure 5b.

Fusion 5 paper was the sole paper used by Han in their 2017 work, in a microchip system in which the extraction portion consisted of PMMA and PDMS layers and the Fusion 5 paper was situated in the middle.[69] Furthermore, chitosan-modified Fusion 5 filter paper that is embedded in a thermoplastic (PMMA and PDMS) microfluidic device has been developed.[70] This extraction method was based on the fact that long-strand DNA molecules become entangled within the fiber matrix of the paper and DNA is electrostatically absorbed to the chitosan-modified fibers. As the elution was impeded by entangling between DNA and paper, PCR was performed directly on the extraction chamber. This was tested using standard K562 human genomic DNA and bacteriophage λ-DNA, long and short DNA fragments, respectively, from either whole blood or blood stain samples, and it was further tested for genetic analysis of the forensic STR-(short tandem repeat) assay. Lysis, extraction, and amplification were performed on-chip; this device possessed an extraction efficiency of 98% for K562 DNA and 95% for λ-DNA.[70]

Use of Glass Filter Paper for the Extraction of Genetic Material. Glass filter paper can also be used for extraction, as shown by Hui in their 2018 work, where chitosan-modified glass filter paper disks were used for the extraction of genomic DNA from *Escherichia coli, Klebsiella pneumoniae*, and *Staphylococcus aureus* for multiplex detection.[71] The chip was composed of an array of capillary actuated pipettes, the paper disks were placed onto a PMMA base, the capillaries were inserted on top, and the paper fiber bulged into the capillary, enabling DNA extraction and LAMP amplification inside the capillary. This has been applied in a paper origami device using glass filter paper, which has a five-channel hydrophobic wax printed onto a microfluidic platform.[72] The device was folded during the cell lysis, extraction, elution, and amplification steps, enabling the isolation of DNA from cattle semen to subsequently detect a viral pathogen, such as bovine herpes virus-1 (BoHV-1), and two bacterial pathogens (*Brucella* and *Leptospira*).[72] Furthermore, in 2018, Ye et al. used a very simple glass fiber paper disk to extract nucleic acids from stool samples to detect rotavirus A via LAMP assay.[73] PES filter paper has been used combined with different types of glass fiber paper to extract viral RNA, the mix of paper was utilized to extract and later identify the Zika virus (ZIKV), Dengue virus (DENV), Chikungunya virus (CHIKV), Yellow Fever virus (YFV), Norovirus I, and Norovirus II via RT-LAMP assays. The authors used probes to aid the binding of the DNA to the paper strip, and they also reported that the proposed method performed better when compared to spin-column-based methods.[74]

Use of Poly(ether sulfone) (PES) Filter Paper for Extraction of Genetic Material. In 2016, Rodriguez et al. manufactured a device consisting of self-adhesive laminating sheets to provide a hydrophobic barrier around the filter paper.[75] The design enables easy ripping and folding whenever necessary during the molecular process. In addition, the authors optimized the cell lysis and DNA extraction in a single step using alcohol precipitation and chaotropic lysis. In the report, DNA was extracted from clinical cervical specimens for the detection of human papillomavirus (HPV) via LAMP assay.[75]

Use of Polysulfone Filter Paper for Extraction of Genetic Material. This methodology has been used in a device combined with different glass and PES fiber papers.[76] The papers coupled with serum and lateral flow assay, successfully extracted viral RNA, which was later used in a LAMP assay to identify the ZIKV, DENV, and CHIKV viruses via RT-LAMP.[76]

Use of Polydopamine-Coated Paper for Extraction of Genetic Material. A foldable polycarbonate film device consisting of sample, reaction, and detection zones was manufactured.[77] The extraction procedure was performed in the device in polydopamine-coated paper, where its nanofibrous nature facilitated DNA adhesion. The authors executed both single-plex and multiplex LAMP assays, both fully on-chip. For the single-plex assay, DNA was extracted from spiked milk to identify *Salmonella spp*. For the multiplex assay, the same procedure was performed and used to determine *Salmonella spp.*, alongside *E. coli*.[77] A summary of these methodologies is given in Table 4.

NON-SOLID-PHASE EXTRACTION

Extraction by Liquid Phase. Usually, liquid phase extraction occurs by separation of a compound based on its relative solubility, which is seen in a device developed by Le Roux in 2014 that uses mainly a cyclo olefin polymer (COP).[78] The microfluidic device is fully automated and includes DNA extraction, PCR amplification and laser-induced fluorescent detection, all enclosed in a disposable chip. It also has electrodes and a pneumatic interface, which allows better fluid movement, as shown in Figure 6a. Because of the pneumatic module, the extraction reagents can flow from one port to the sample collector, where the liquid extraction reagents are in contact with the sample. When the reagents make contact with the extraction reagents, heaters clamp the sample collector, and the temperature increases; this is required for efficient extraction. In this paper, DNA was extracted from FTA paper and brush buccal swabs and subjected to 18-plex STR amplification via on-chip PCR. These data are very similar to those of conventional methods, such as benchtop extraction and amplification systems.[78]

Extraction by Isotachophoresis. Isotachophoresis (ITP) is a separation procedure that applies an electric field and two buffers to extract and purify nucleic acids in a single step. The methodology uses two buffers: one that serves as a leading electrolyte (LE), and another that acts as a tailing electrolyte (TE). These buffers have different mobilities: one buffer has a higher mobility than the fastest component in the sample, and the other has a lower mobility than the slowest component. As electric fields are applied, the fastest electrolyte moves rapidly toward the sample ions, creating zones according to their mobility. The aforementioned method of extraction was used in 2015 by Borysiak for the purification of *Escherichia coli* from whole milk samples.[79] Their device, called NAIL (Nucleic Acid Isotachophoresis LAMP), shown in Figure 6b, uses capillaries and heated air chambers as passive pumps and valves to automate fluid actuation and minimize user intervention. It operates with LAMP amplification assays, as well as a mobile

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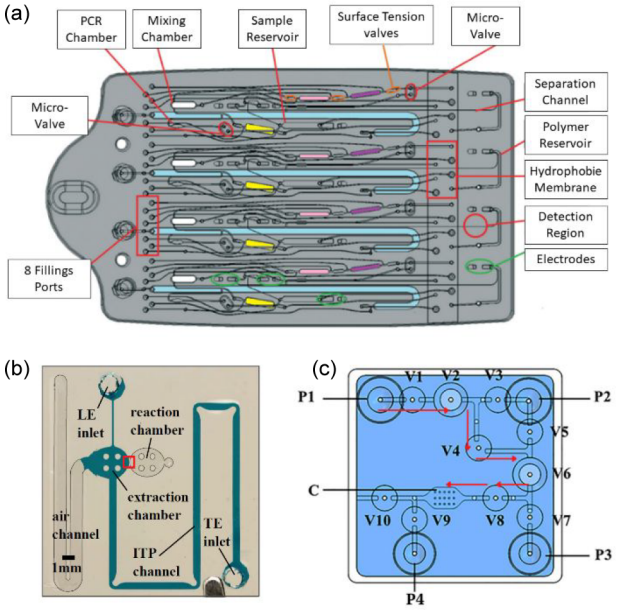

Figure 6. (a) Illustration of the cyclo olefin polymer (COP) microfluidic chip. The structures identified in pink and purple represent the PCR reagent reservoirs, blue is the extraction reagent reservoir, and yellow is the separation reagent reservoir. The device has the extraction reagents preloaded. The polymer pneumatically loaded into the microchannel allows the liquid to come into contact with the electrodes almost immediately upon entering the buffer and sample waste reservoirs, which push the extraction reagents from one port into the sample collector. The extraction reagents then come into contact with the sample within the sample collector, and temperature-based DNA liberation occurs. The chip comes into contact with a platform that is software-controlled, which allows for clamping of the sample collector to heat the extraction solution to the required temperatures. Afterward, the solution is taken back to the extraction reservoir, and the downstream procedures begin. [Reprinted, with permission, with modifications, from ref 78. Copyright 2014, Royal Society of Chemistry, London.] (b) Nucleic acid isotachophoresis LAMP (NAIL) device fabricated with SEBS (styrene−ethylene/butylene−styrene) polymer. The device is filled with colored LE via capillary flow. TE buffer mixed with the sample is pipetted into the TE inlet. The electrodes that create the ITP field are placed into the LE inlet and TE inlet chambers to apply a 450 μA constant current. The ITP is monitored using DNA-intercalating SYBR Green Dye. Once the DNA plug enters the extraction chamber, the current is stopped, and the electrodes are removed. The red square identifies a capillary valve that prevents the LE buffer from wetting the reaction chamber. Reprinted, with permission, with modifications, from ref 79. Copyright 2016, Royal Society of Chemistry, London.] (c) Microfluidic device for nucleic acid extraction, amplification and detection fabricated with PDMS and a glass substrate developed by Zhu[80] in 2019. The cell suspension from the cervical swabs is added to storage pouch 1 (P1) and mixed with the extraction buffer stored in P2 by peristaltic pumps (V1−V3) controlled with solenoid valves (not pictured in the illustration). The mixture is kept in V2 for pyrolysis extraction at 95 °C for 15 min. After cooling, the extracted DNA is mixed in V6 with the PCR master mix previously stored in P3 by switching the V5 and V7 microvalves. Then, it is transferred to the solid PCR chamber, identified in the figure by the symbol "C". Wash buffer, which is employed after the PCR amplification reaction, is stored in P4. The results are obtained using fluorescence detection. [Reprinted, with permission, with modifications, from ref 80. Copyright 2019, MDPI.]

device to image those results, eliminating the need for fluorescence detection.[79]

Extraction by Pyrolysis. This methodology relies on the use of high temperatures to lyse cells by disassembling the cellular membrane and hence isolating the DNA. It was employed by the Zhu group in 2019 for the extraction step to use in solid-phase PCR for the identification of five HPV genotypes (HPV16/HPV18/HPV31/HPV33/HPV58) from cervical swabs.[80] The sample is loaded into the microfluidic device, mixed with extraction buffer (Herogen Biotechnology), and incorporated into the device with the help of pumps and valves. Then, the mixture is kept at 95 °C for 15 min to release the DNA. Finally, the mixture is cooled to room temperature, and the extracted DNA is merged with the PCR master mix; the process is depicted in Figure 6c. The authors reported that (i) the extracted DNA satisfied the requirements of real-time PCR and (ii) the results were reproducible.[80]

In 2016, Gumus et al. also employed this method to purify fecal samples and isolate *Vibrio cholerae* nucleic acids.[81] The group developed a solar-thermal sample processing system to achieve a 95 °C pyrolysis temperature in less than 5 min through a black polycarbonate solar incubator. The samples were kept at that temperature for 10 min. Bacterial DNA was then isolated using magnetic microbeads and a binding solution (pH < 6.0) with the help of a small magnet. The magnetic beads were washed with a buffer, and then elution buffer was added, mixed and incubated at 60 °C for 5 min using a solar incubator. The beads were removed with a magnet, and the DNA was transferred to a clean tube for the PCR amplification reactions using a solar thermal system.[81] A summary of these methodologies is given in Table 5.

CONCLUDING THOUGHTS

Nucleic acid extraction is a crucial step in any molecular analysis. It is often the starting point and involves the separation of the desired genetic material from unwanted nucleic acids, soluble proteins, and cell debris. The purity of the template determines the quality of the results that are obtained in downstream experiments. Traditionally, the extraction step is performed using a commercially available kit; although it is very practical, it demands highly trained personnel and expensive equipment, and it is time-consuming and very laborious.

The potential of microfluidic devices over conventional benchtop testing has been increasingly reported. However, embedding an automated nucleic acid extraction method into these devices has been a challenge, because it involves multiple steps (lysis, extraction, washing, recovery), it demands some sort of manipulation, and there is a need for the equipment to eliminate impurities from samples. As shown in this Review, many automated or semiautomated nucleic acid extraction methods have been studied for integration into microfluidic devices to achieve successful point-of-care testing. These devices eliminate the need for extremely skillful professionals, because of their simple workflow. They also reduce user intervention, so contamination is minimal, compared to traditional methodologies, and, importantly, they are very time efficient. In fact, we highlight that most of the extraction methods mentioned in this Review could be performed in 15 min or less, restating the feasibility of these microfluidic approaches for POCT.

Solid-phase extractions such as silica membranes are easily implemented, since they can be cut to size for specific applications. However, the need for chaotropic salts and alcohol as a wash buffer may reduce amplification efficiency,

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Table 5. Summary of the Extraction Methods Mentioned Previously in This Section

Article	Device's Material	Extraction Method	Amplification Method	Buffer Information
Le Roux[78] (2014)	cyclo olefin polymer (COP)	liquid phase	PCR	846 μL of extraction buffer and 54 μL of EA1 enzyme based on the prepGEM Saliva kit (ZyGEM Corp., Hamilton, New Zealand). Twenty-five μL of a separation reagent mix of 18 μL of Internal Lane Standard (ILS) 500 (Promega) and 90 μL of dilution buffer (Hi-DI Formamide, Life Technologies, Carlsbad, CA, USA)
Borysiak[79] (2015)	SEBS polymer	isotachophoresis (ITP)	LAMP	LE buffer (600 mM Tris, 200 mM HCl buffer with 0.001% (w/v) Tween-20, 0.075% (w/v) polyvinyl pyridine (PVP), 1x SYBR green (Life Technologies)), TE buffer (90 mM Tris buffer, 37.5 mM HEPES buffer, 0.5 mg/mL proteinase K (Thermo Scientific), 0.1% (w/v) Tween-20, 0.1% (w/v) PVP, 1x SYBR green, 5000 U Ready-Lyse Lysozyme (lysis buffer; Epibio)).
Zhu[80] (2019)	PDMS	pyrolysis	solid-phase PCR	100 μL extraction buffer (Herogen Biotechnology); no washing or elution steps
Gumus[81] (2016)	polycarbonate thermoplastic	pyrolysis	PCR	binding solution (pH <6.0), wash buffer (200 μL, pH 7.0), elution buffer (80 μL, pH 8.5)

since these reagents are PCR inhibitors. Magnetic beads or silica beads, regardless of whether they are coated with other reagents or not, can also be considered for use in solid-phase extraction methods. Their major advantage is their large surface area, which enables easy nucleic acid binding, making this methodology an extremely efficient approach. Similarly, centrifugal microfluidics seems to be a great alternative, since it requires minimal sample manipulation and avoids possible contamination and user errors by pre-storing reagents in the devices in pouches that open due to pressure at certain frequencies or by simply drying the reagents onto the microchip. In addition, the use of beads guarantees highly efficient purification of nucleic acids. However, some of these require electromagnetic fields and/or centrifugal support for rotation to manipulate the beads, which can be disadvantageous in low-resource scenarios.

In addition, there is a completely automated electrically induced nucleic acid extraction method, such as the dielectrophoretic method, for which user intervention is not needed but can be complicated to install in a device since a complex electrode array is necessary. Moreover, the need for a battery or other sources of energy is a disadvantage in low resource circumstances. To overcome this problem, extraction and amplification methods that rely on other power sources, such as sunlight, are very convenient; devices that make use of such methods are presented in this Review. To overcome such difficulties, pyrolysis is effective and easily implemented, it has been tested in solar energy-powered devices, making it very attractive for point-of-care applications in low-resource communities. The use of thermal lysis eliminates the need for lysing reagents that are known to interfere with downstream applications. Other methods, such as isotachophoresis and liquid extraction, have the benefits of reduced manual steps, as others mentioned before, and because of their peculiarities, such as the pressure that builds in their channels, these methods have automated fluid actuation.

The material chosen as the substrate of the device can also present advantages or disadvantages. For instance, glass-based devices, such as those fabricated by glass etching or lithography, can be expensive and difficult to produce on a large scale. 3D-printed devices are less expensive and are easier to manufacture but are also less thermoresistant. Currently, the most frequently employed materials are poly(methyl methacrylate) (PMMA) and polydimethylsiloxane (PDMS). Thermoplastics, such as polycarbonate and PMMA, are great for microfluidic devices since these materials can be produced on a larger scale and have a relatively low cost. However, as stated by Weerakoon-Ratnayake et al. in 2017, polymers have a highly disorganized distribution of surface functional groups, which can lead to flow recirculation.[82] This could disrupt the performance of the material, impairing the overall molecular analysis procedure. To avoid such problems, there are surface modification protocols that can be employed to improve flow dynamics, such as O_2 plasma treatment.[82]

Paper seems to be the most cost-effective extraction method presented thus far. This method is also considered solid-phase extraction, and its mechanism relies on affinity between the material and the nucleic acids and the fact that long-stranded DNA can become entangled in the paper matrix.[83] The channels in paper devices could be fabricated using wax printing, which does not require trained personnel and is very cost-effective. Although waxing only allows for simple devices, the paper can be folded to form complex 3D shapes, allowing for a very compact and portable device. There are other types of printing that could be used, such as laser printing, but these are much more expensive; however, they are more accurate and allow for complicated channels. Furthermore, the high availability, low cost and simple assembly production of paper makes it a very promising material for fully automated microfluidic devices.

It is evident that the field of fully automated microfluidic devices has evolved, with many possibilities of functional, portable, and low-cost devices. The multiple successful extractions used in a variety of samples show the high applicability of the methodologies discussed, highlighting their utility not only in medical diagnostics but also in food safety analysis and many other applications.

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M.L.C. was responsible for the conceptualization, writing of the original draft, review, and editing. S.S.da S. was responsible for writing of the original draft, revisions, and editing. M.C.S. was responsible for revisions and editing. D.L.Z. was responsible for revisions and editing. M.N.A. was responsible for revisions, and L.B. eas responsible for revisions and supervision.

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Notes

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