REVIEW

Microfluidics-based strategies for molecular diagnostics of infectious diseases

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Abstract

Traditional diagnostic strategies for infectious disease detection require benchtop instruments that are inappropriate for point-of-care testing (POCT). Emerging microfluidics, a highly miniaturized, automatic, and integrated technology, are a potential substitute for traditional methods in performing rapid, low-cost, accurate, and on-site diagnoses. Molecular diagnostics are widely used in microfluidic devices as the most effective approaches for pathogen detection. This review summarizes the latest advances in microfluidics-based molecular diagnostics for infectious diseases from academic perspectives and industrial outlooks. First, we introduce the typical on-chip nucleic acid processes, including sample preprocessing, amplification, and signal read-out. Then, four categories of microfluidic platforms are compared with respect to features, merits, and demerits. We further discuss application of the digital assay in absolute nucleic acid quantification. Both the classic and recent microfluidics-based commercial molecular diagnostic devices are summarized as proof of the current market status. Finally, we propose future directions for microfluidics-based infectious disease diagnosis.

Keywords: Microfluidics, Molecular diagnostics, Infectious disease, Point-of-care testing (POCT), Digital assay

Background

Infectious diseases arise from pathogens, including bacteria, viruses, and parasites, with a global distribution. Unlike other diseases, pathogens rapidly infect and are transmitted between human and animal carriers through inoculation, air, and water media [1]. It is essential to prevent infectious diseases as a public health measure. There are three fundamental strategies for managing infectious diseases: (1) controlling the source of infection; (2) blocking transmission pathways; and (3) protecting susceptible populations. Among the fundamental strategies, control of the infectious source is considered the most crucial strategy because of convenience and low cost. Prompt

diagnosis, isolation, and treatment of infected persons are essential, which require rapid, sensitive, and accurate diagnostic strategies [2]. The current diagnosis of infectious diseases usually combines clinical examinations based on signs and symptoms and laboratory tests, such as cell culture and molecular diagnostics, which require well-trained personnel, time-consuming procedures, and expensive testing equipment [3, 4]. Prevention of infectious disease outbreaks calls for rapid, low-cost, accurate, and on-site diagnosis, particularly in resource-poor areas where infectious diseases are usually prevalent and severe [5], as is treatment in the wilderness or battlefield where emergencies unpredictably occur, but medical assistance is limited [6]. In such cases, microfluidics, a technology that combines micro-electro-mechanical system technology, nanotechnology, or materials science for precise fluid manipulations [7–10], offers a new opportunity for point-of-care testing (POCT) of infectious pathogens outside of hospitals and laboratories. Microfluidic technology enables a sample- and cost-saving route for molecular diagnostics during disease outbreaks compared with

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traditional laborious diagnostics. The worldwide spread of corona virus disease 2019 (COVID-19) was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); as a result, the importance of microfluidics for timely prevention and control of the pandemic has again been emphasized [11–13]. Compared with traditional diagnostics, microfluidic POCT utilizes miniaturized and portable devices, ranging from benchtop analyzers to small lateral flow strips, that conduct tests nearby the sampling sites [14]. These tests are advanced for simplified or omitted sample preparation, rapid signal amplification, and sensitive signal readout, leading to a short duration and accurate results within minutes. The availability and massive production of microfluidics-based point-of-care tools have expanded their applications for cost-effective and straightforward diagnosis outside the hospital, near the patient, or even at home.

Among the existing strategies for diagnosing infectious diseases, molecular diagnostics are among the most sensitive methods [15, 16]. Moreover, molecular diagnostics usually serve as the gold standard method for ongoing COVID-19 detection, allowing direct detection of virus-specific RNA or DNA regions prior to onset of the immune response [17, 18]. In the current review, we present the latest advances in microfluidics-based processes for molecular diagnostics of infectious diseases, from an academic perspective to future industrial outlook (Fig. 1). We start with the three steps critical for nucleic acid testing: on-chip sample pre-processing; nucleic acid amplification; and signal read-out. We then compared various types of microfluidic platforms with their structures and functions, which showed unique features (both pros and cons). The digital nucleic acid assay is further discussed and exemplified as the third-generation technology for

the absolute quantification of infectious pathogen molecules. Additionally, several typical and latest commercial POCT devices will be introduced, which display the current state of the microfluidic POCT market for molecular diagnostics. Our outlooks towards future applications will also be discussed and explained.

On-chip nucleic acid testing

Based on the implemented functions, the modules of a microfluidic chip for nucleic acid testing can be divided into three categories (sampling, sensing, and signaling) [19]. Among these modules, the sampling module mainly realizes sample lysis and nucleic acid extraction. The sensing module primarily operates the conversion and amplification of nucleic acid signals. The signaling module achieves detection of the signal after conversion and processing by the sensing module. We will summarize different chips that can achieve the "sample in and answer out" function according to the on-chip nucleic acid testing procedure.

Sampling module: lyse the original samples and extract nucleic acids

The foremost step of nucleic acid testing is nucleic acid extraction, which refers to the isolation of targeted nucleic acid from the original samples. Nucleic acid extraction is performed to purify nucleic acids from other molecular pollutants, ensure the integrity of the primary structure of nucleic acid molecules, and to optimize outturns. Nucleic acid extraction requires essential sample lysis and nucleic acid capture, the quality and efficiency of which have a huge impact on the research and diagnosis results. Any subtle adverse effects during extraction limit downstream detections. For example, polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) approaches are inhibited by some residual organic solvents in nucleic acid extraction reagents such as ethanol and isopropanol [20]. Liquid–liquid extraction and solid-phase extraction are among the most popular modes of nucleic acid extraction [21]; however, liquid–liquid extraction on the chips is extremely limited because the reagents used in liquid–liquid extraction are corrosive to most microfluidic chips. Herein we emphasize solid-phase extraction methods based on microchips and compare the strengths and weaknesses.

Silicon-based strategies
Silicon is a compatible substrate material for nucleic acids because silicon is biocompatible, stable, and has easily modifiable properties [22]. Importantly, when modified by silica or other materials, this composite exhibits the characteristic of adsorbing negatively-charged nucleic acids in low pH and hypersaline conditions, while eluting
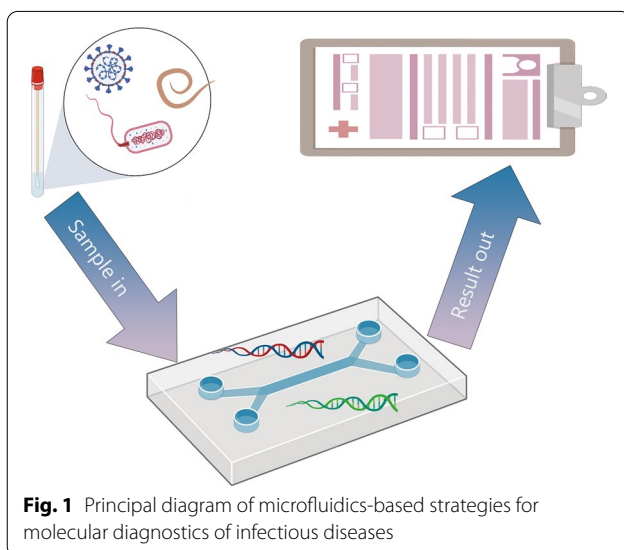

Fig. 1 Principal diagram of microfluidics-based strategies for molecular diagnostics of infectious diseases

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with high pH and low-salt solutions. Based on this phenomenon, nucleic acids can be purified.

Silicon-based materials of various forms have been exploited for nucleic acid extraction in microfluidics, such as silica beads, powder, microfiber filters, and silica gel membranes [23–26]. Depending on the material properties, silicon-based materials can be utilized in various ways on microchips. For example, silica beads, powders, and commercial nanofilters can be simply placed into the wells or microchannels of the microfluidic chip and assist the extraction of nucleic acids from samples [27–29]. Surface-modified silica gel membranes can also be used to rapidly purify DNA from pathogens at low cost. For example, Wang et al. [30] introduced a universal and portable system by combining a denaturation bubble-mediated strand exchange amplification reaction with chitooligosaccharide-coated silica gel membranes through which 10^2–10^8 colony-forming units (CFU)/ml of *Vibrio parahaemolyticus* were successfully detected, and the existence of the virus was easily visualized. Powell et al. [31] then used the silicon-based microchip to detect hepatitis C virus (HCV), human immunodeficiency virus (HIV), Zika virus, and human papilloma virus multiply and automatically, in which 1.3 μl of meandering microreactors were designed to capture RNA of viruses and perform in situ amplification. In addition to these methods, surface-modified silicon micropillars play a key role in nucleic acid extraction because the geometrical dimension and modifying material properties significantly improve extraction efficiency. Chen et al. [32] proposed a microfluidic platform to extract RNA at low concentrations based on amino-coated silicon micropillars. The microfluidic device integrates micro-pillar arrays within an area of 0.25 cm^2 on the silicon substrate to substantiate a higher extraction efficiency with high surface-to-volume ratio designs. As a benefit from this design, the microfluidic device achieves up to 95% nucleic acid extraction efficiency. These silicon-based strategies demonstrated the value of rapid isolation nucleic acids at low cost. When combined with microfluidic chips, silicon-based extraction strategies not only improve the efficiency of nucleic acid testing, but also facilitate miniaturization and integration of analytical devices [20].

Magnetic-based strategies

The magnetic-based isolation approach exploits magnetic particles to extract nucleic acids at the circumstance of external magnetic fields. The commonly utilized magnetic particles include silica-coated, amino-coated, and carboxyl-coated Fe_3O_4 or $γ$-Fe_2O_3 magnetic particles [33–36]. Compared with silicon-based, solid-phase extraction techniques, a distinct feature of the magnetic particles is ease of manipulation and control using an external magnet.

Utilizing the electrostatic interactions between nucleic acids and silica, nucleic acids are adsorbed to the surface of silica-encapsulated magnetic particles under hypersaline and low pH conditions, while the molecules can be eluted again under hyposaline and high pH conditions. The silica-coated magnetic beads allow for DNA extraction from large-volume samples (400 μl) with the help of magnet-guided movement [37]. As a demonstration, Rodriguez-Mateos et al. [38] used a tunable magnet to manipulate the transfer of magnetic beads in different chambers. Based on silica-coated magnetic particles, 470 copies/ml of genomic SARS-CoV-2 RNA can be extracted from wastewater samples for reverse-transcription LAMP (RT-LAMP) detection, and the answer can be read out within 1 h by the unaided eye (Fig. 2a).

The positively-charged magnetic particles are ideal for the nucleic acid phosphate backbone to attach. At a specific salt concentration, the negatively-charged nucleic acid phosphate groups can be absorbed to the surface of magnetic composite particles by positive charges. Thus, the magnetic nanoparticle with a rough surface and a high density of amino groups has been developed for nucleic acid extraction. After magnetic separation and blocking, the magnetic nanoparticles and DNA complexes can be used directly for PCR, omitting complex and time-consuming purification and elution operations [35]. The negative carboxyl-coated magnetic nanoparticles are also made to isolate nucleic acids, which are adsorbed to the surface in high concentrations of polyethylene glycol and sodium chloride solutions [36]. Utilizing these surface-modified magnetic beads, DNA extraction is compatible with downstream amplification. Dignan et al. [39] described an automatic and portable centrifugal microfluidic platform for nucleic acid pre-processing that allows in situ use by non-technical personnel. Moreover, the compatibility of the extracted DNA with LAMP, a technique ideal for point-of-care nucleic acid analysis, was further demonstrated for minimal hardware requirements and adaptability with a colorimetric assay (Fig. 2b).

The magnetic bead methods provide the possibility for automated extraction, of which some commercial automatic nucleic acid extractors exist [KingFisher; ThermoFisher (Waltham, MA, U.S.), QIAcube®HT; CapitalBio (Beijing, China), and Biomek®; Beckman (Miami, FL, U.S.)]. The advantages of magnetic beads in combination with microfluidics for automated nucleic acid extraction with high efficiency have the potential to facilitate the growth of molecular diagnostics; however, magnetic beads in combination with microfluidics are still largely dependent on complex control systems to

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precisely manipulate magnetic beads, which explains why prevailing commercial products are bulky and expensive, restricting the further application of magnetic beads in POCT.

Porous materials-based strategies

Several porous materials, such as modified nitrocellulose filter, Finders Technology Associates (FTA) cards, polyethersulfone-based filter paper, and glycan-coated materials, have also been utilized for nucleic acid detection [40–44]. Porous fibrous materials, such as fibrous papers, are first used for DNA extraction utilizing the physical entanglement of long-chain DNA molecules with the fiber. Small pores lead to strong physical constraints on DNA molecules, which has a positive effect on DNA extraction. The extraction efficiency does not satisfy the need for DNA amplification due to the varying sizes of pores of the fibrous paper [45, 46]. The FTA card, a commercial filter paper used in the forensic field, has been widely applied to other molecular diagnostics. By using cellulose filter paper impregnated with various chemicals to help lyse cellular membranes from samples, the released DNA can be protected from degradation for up to 2 years. More recently, impregnated cellulose paper has been developed for molecular testing of various pathogens, including SARS-CoV-2, leishmaniasis, and malaria [47–49]. The HIV in separated plasma is directly lysed, and viral nucleic acids are enriched by an integrated, flow-through FTA® membrane in the concentrator, which enables nucleic acid preparation with high efficiency [50] (Fig. 2c). The main challenge for nucleic acid testing using FTA cards is that the chemicals, such as guanidine and isopropanol, will inhibit subsequent amplification reactions. To solve the problem, chitosan-modified Fusion 5 filter paper was developed for high-efficiency nucleic acid extraction by combining the strengths of both leveraging the physical entanglement of DNA molecules with the fiber filter paper and the electrostatic adsorption of DNA to the chitosan-modified filter fibers [51] (Fig. 2d). Similarly, Zhu et al. [52] demonstrated a chitosan-modified capillary assist, a microfluidic-based in situ PCR method, to rapidly extract and detect Zika virus RNA. Based on the features of the chitosan with pH-responsive "on and off" switches, nucleic acids can be adsorbed/desorbed in a lysate/PCR mixture environment, respectively.

As described, these strategies incorporate the strengths of different solid-phase materials and increase the performance of nucleic acid extraction in microfluidics. In practical applications, extensive use of these materials is not economical, while using the materials for proper processing or surface modification of common materials can also maintain their functions. Thus, it is believed that cost can be decreased by implementing these strategies after pilot studies.

Sensing module: convert and amplify nucleic acid signals

Nucleic acid testing on microfluidic platforms often uses small sample volumes (< 100 μl), therefore requires amplification of the target nucleic acids with specific probes for conversion to a signal that is convenient for downstream detection (optical, electrical, and magnetic) [53, 54]. Nucleic acid amplification in microfluidics can also speed up the reaction, optimize the limit of detection, lower the sample demand, and increase the detection accuracy [55, 56]. Recently, with the achievement of fast and accurate detection, various nucleic acid amplification methods, including PCR and some isothermal amplification reactions, have been applied in microfluidics. This section will summarize those promising techniques based on microfluidic systems for nucleic acid testing.

PCR

PCR is a simulation of the DNA replication procedure from organisms, the theory of which is detailed elsewhere and thus will not be discussed herein. PCR can amplify very few target DNA/RNA at an exponential rate, thus making PCR a powerful tool to detect nucleic acids rapidly. In recent decades, many portable microfluidic devices equipped with thermal circulation systems to perform PCR have been developed to satisfy the needs of point-of-care diagnosis [57, 58]. According to different temperature control methods, on-chip PCR can be divided into four types (traditional, continuous-flow, spatially-switched, and convective PCR) [59]. For example, Ji et al. [60] established the direct reverse-transcription quantitative PCR (RT-qPCR) assay on a self-designed microfluidic platform to multiply detect SARS-CoV-2, and influenza A and B viruses in pharyngeal swab samples (Fig. 3a). Park et al. [61] established a simple pathogen analytic chip by integrating the film-based PCR, electrode, and polydimethylsiloxane-based finger-actuated microfluidic modules. Nevertheless, both works exemplify the common disadvantage of traditional PCR. Thermal cycling is necessary for PCR, which restricts the further miniaturization for the device and shorter testing time.

The development of microfluidics-based continuous flow and spatially-switched PCR is essential to solve this problem. Utilizing a long serpentine channel or short straight channel, continuous flow PCR can achieve rapid amplification by actively pushing reagents with a pump outside of chips to three pre-heated zones in sequence and circularly. The operation successfully avoids the transition stage between different reaction temperatures,
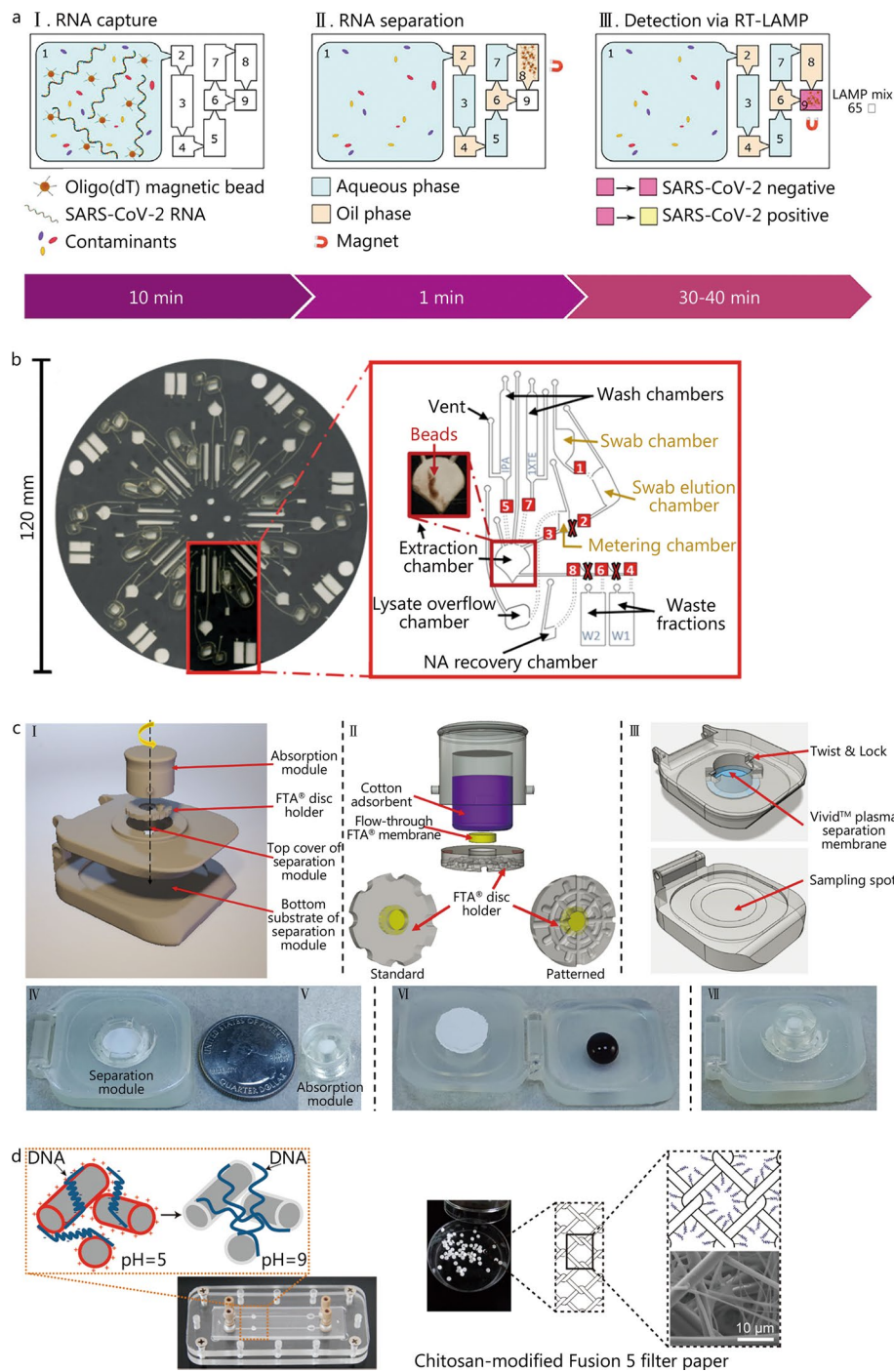
Fig. 2 Magnetic- and porous material-based devices. **a** Conceptual scheme for the microfluidic IFAST RT-LAMP device for SARS-CoV-2 RNA detection (adapted from [38]). **b** Centrifugal microdevice for dSPE of nucleic acids from buccal swabs (adapted from [39]). **c** Self-powered integrated sample concentrator using FTA® card (adapted from [50]). **d** Chitosan-modified Fusion 5 filter paper (adapted from [51]). SARS-CoV-2 severe acute respiratory syndrome coronavirus 2, RT-LAMP reverse-transcription loop-mediated isothermal amplification, FTA finders technology associates, NA nucleic acid

which significantly reduces the testing time [62] (Fig. 3b). In another study, Jung et al. [63] proposed a novel Rotary PCR Genetic Analyzer to perform the ultrafast and multiple reverse-transcription PCR in combination with the features of the stationary and flow-through PCR (Fig. 3c). The PCR microchip will rotate through three thermal blocks with different temperatures for nucleic acid amplification, as follows: I. block at 94 °C for denaturation; II. block at 58 °C for annealing; and III. block at 72 °C for the extension.

Through capillary tubes and loops, or even thin disks, convective PCR can rapidly amplify nucleic acids with naturally induced free thermal convection without an external pump. For instance, a cycle olefin polymer microfluidic platform was developed on a fabricated rotating heater stage utilizing a centrifugation-assisted thermal cycle in a ring-structured microchannel for PCR [64] (Fig. 3d). The reaction solution is driven by thermal convection and continuously exchanged high/low temperatures in the ring-structured microchannel. The whole amplification process can be finished in 10 min and the limit of detection goes to 70.5 pg/channel.

As expected, rapid PCR is a powerful tool for both fully-integrated "sample-to-answer" molecular diagnostic systems and multiplex analysis systems. With rapid PCR, the time spent on detecting SARS-CoV-2 is significantly decreased, which helps to control the COVID-19 pandemic efficiently.

Isothermal amplification

A complex thermocycler is required for PCR, which is inappropriate for POCT. Recently, isothermal amplification methods have been applied to microfluidics, including but not limited to LAMP, recombinase polymerase amplification (RPA), and nucleic acid sequence-based amplification [65–68]. With these technologies, nucleic acids are amplified at a constant temperature, thus promoting portable POCT devices for molecular diagnostics with low cost and high sensitivity.

High-throughput microfluidics-based LAMP analysis enables multiplex detection of infectious diseases [42, 69–71]. In combination with centrifugal microfluidic systems, LAMP can further promote the automation of nucleic acid detection [69, 72–75]. A rotate and react SlipChip was developed to visually detect multiple bacteria in parallel by LAMP [76] (Fig. 4a). With optimized LAMP in the assay, the fluorescent signal-to-noise ratio is approximately fivefold, and the limit of detection reached 7.2 copies/μl genomic DNA. Moreover, the existence of five common digestive bacterial pathogens, including *Bacillus cereus*, *Escherichia coli*, *Salmonella enterica*, *Vibrio fluvialis* and *Vibrio parahaemolyticus*, were visualized based on the method in < 60 min.

The advantages of LAMP in microfluidics include, but are not limited to rapid reaction and miniaturized detection. Yet, due to the reaction temperature during LAMP (approximately 70 °C), aerosols are inevitably produced, which results in a high rate of false-positive results. Detection specificity, primer design, and temperature control also need to be optimized for LAMP. Moreover, chip designs that implement multiple target detection on one chip are of significant value and should be developed. Furthermore, LAMP is suitable for multiple target detection integrated into one chip, which is of great significance, but still has a large room for growth.

RPA can partially reduce the high false-positive rates of LAMP because the relatively low reaction temperature (approximately 37 °C) causes a relatively small evaporation problem [77]. In the RPA system, two opposing primers initiate the DNA synthesis by combining with the recombinant enzymes and the amplification can be completed within 10 min [78–81]. Therefore, the entire process of RPA is much faster than PCR or LAMP. Microfluidic technology has been demonstrated to further improve the velocity and accuracy of RPA in recent years [82–84]. For example, Liu et al. [85] developed a microfluidic-integrated lateral flow recombinase polymerase amplification assay to rapidly and sensitively detect SARS-CoV-2, integrating the reverse-transcription RPA (RT-RPA) and a universal lateral flow dipstick detection system into a single microfluidic system (Fig. 4b). The assay can be finished in approximately 30 min with a 1 copy/μl or 30 copies/sample limit of detection. A wearable microfluidic device was developed by Kong et al. [86] for rapid and straightforward detection of HIV-1 DNA through RPA utilizing body temperature and a cellphone-based fluorescence detection system (Fig. 4c). The wearable RPA testing can detect target sequences at 100 copies/ml within 24 min, showing great potential for rapid diagnosis of HIV-1-infected infants in resource-limited areas.

RPA based on microfluidics has witnessed rapid advances; however, the cost from chip fabrication and reaction consumption is too high and is supposed to be lowered to increase the accessibility of the technique. In addition, the high sensitivity of RPA may influence the amplification of non-specific products, especially when contamination exists. These limitations may affect the application of RPA in microfluidic systems and deserve further optimization. Well-designed primers and probes for different targets are also required to increase the feasibility of RPA-based microfluidic strategies in POCT.

Clustered regularly interspaced short palindromic repeats (CRISPR)-based methods for nucleic acid testing

Cas13 and Cas12a have the ability to cut nucleic acids indiscriminately, and thus can be developed as detection
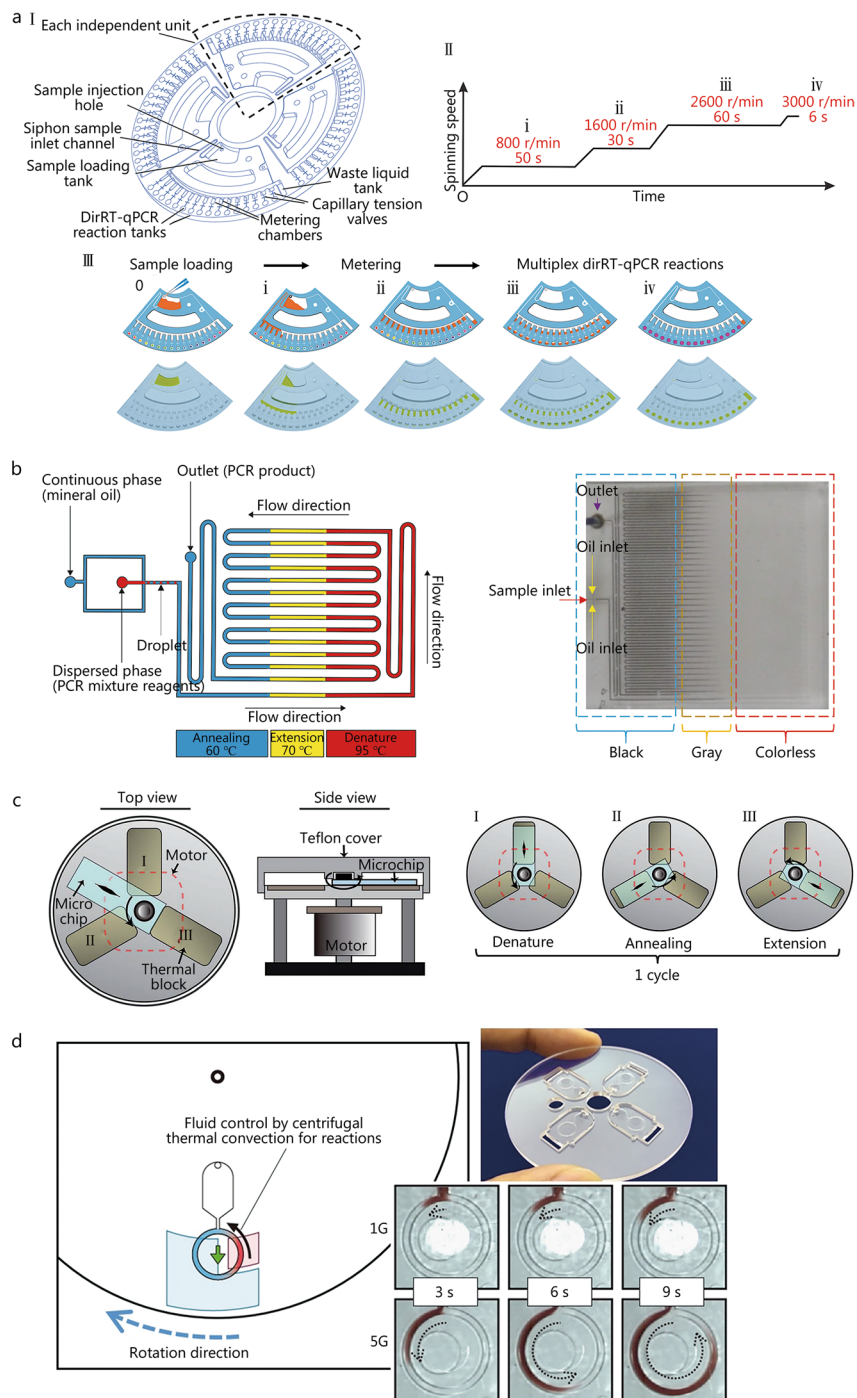
Fig. 3 Applying PCR in microfluidics. **a** Schematics of the dirRT-qPCR in the microfluidic platform (adapted from [60]). **b** Schematics of long serpentine channel based continuous flow PCR microchip (adapted from [62]). **c** Schematic illustration of a Rotary PCR Genetic Analyzer, which consists of a microchip, three heat blocks, and a stepper motor (adapted from [63]). **d** Schematic diagram of centrifugal-assisted thermal convection PCR and devices (adapted from [64]). DirRT-qPCR direct reverse-transcription quantitative polymerase chain reaction

and diagnostic tools. Cas13 and Cas12a are activated when binding the target DNA or RNA, respectively. Once activated, the proteins then start to cut other nucleic acids nearby, after which the guide RNA that targets pathogen-specific nucleic acids can cut off a quenched fluorescent probe and unleash fluorescence. Based on
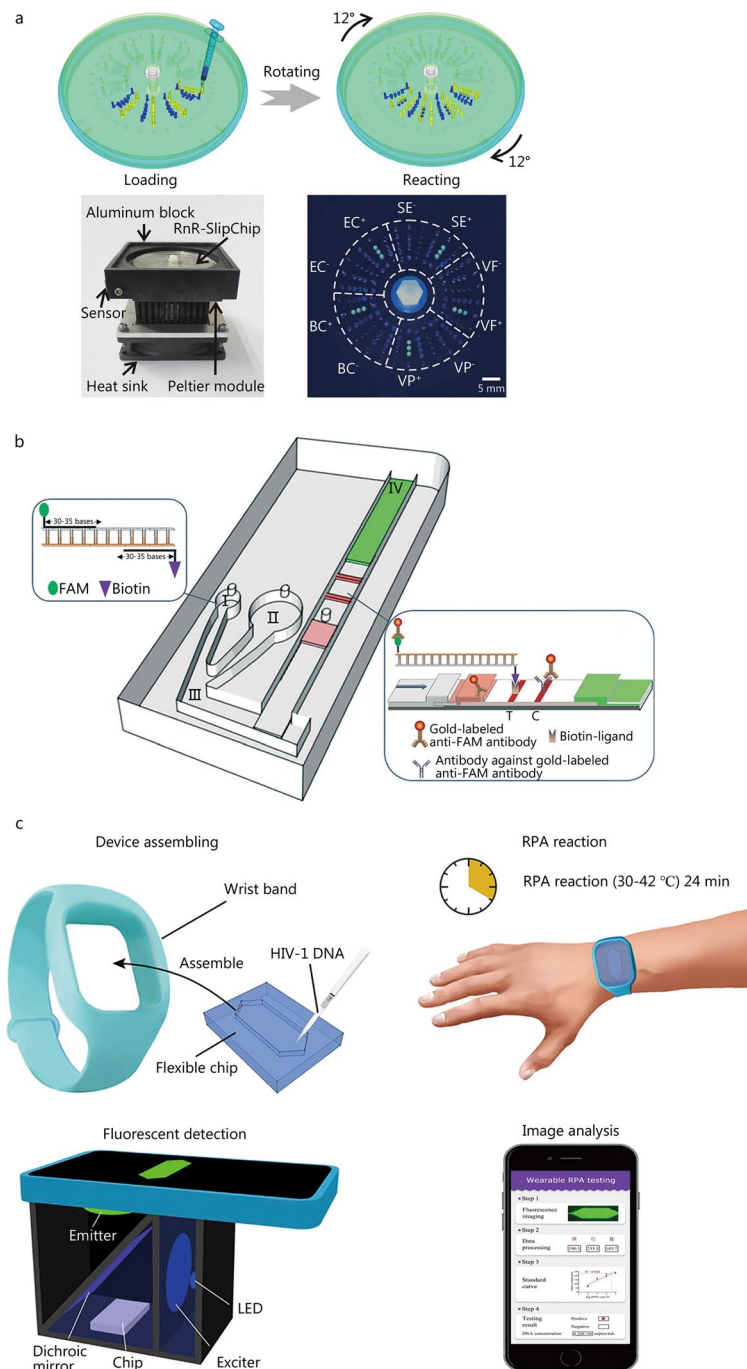
Fig. 4 Isothermal amplification in point-of-care testing (POCT). **a** Design and fabrication of the rotate and react SlipChip. After plasma bonding, a screw-nut suite was used to assemble the upper and lower chips to form the final chip (adapted from [76]). **b** Schematic illustration of the MI-IF-RPA system for COVID-19 detection (adapted from [85]). **c** Schematic of wearable RPA testing for rapid detection of HIV-1 DNA (adapted from [86]). *SE Salmonella enterica*, *VF Vibrio fluvialis*, *VP Vibrio parahaemolyticus*, *BC Bacillus cereus*, *EC Escherichia coli*, FAM carboxyfluorescein, HIV human immunodeficiency virus, RPA recombinase polymerase amplification, LED light emitting diode, MI-IF-RPA microfluidic-integrated lateral flow recombinase polymerase amplification

the theory, Kellner et al. [87] developed a Cas13-based method [Specific High-sensitivity Enzymatic Reporter UnLOCKING (SHERLOCK)], while Broughton et al. [88] developed another Cas12a-based method [DNA Endonuclease Targeted CRISPR Trans Reporter (DETECR)].

In recent years, various CRISPR-based nucleic acid assays have emerged [89, 90]. Traditional CRISPR-based methods are usually time-consuming and labor-intensive because of multiple procedures encompassing nucleic acid extraction, amplification, and CRISPR detection. The likelihood of false-positive results may be increased for exposing liquid to air. Given the above, the CRISPR-based systems are in urgent need of optimization.

A pneumatically-controlled microfluidic platform that can run 24 assays in parallel was designed for CRISPR-Cas12a and CRISPR-Cas13a detection applications [91]. The system is equipped with a fluorescence detection device, thus can automatically detect femtomolar DNA and RNA samples bypassing nucleic acid amplification. Chen et al. [92] integrated recombinase-aided amplification with CRISPR-Cas12a system in centrifugal microfluidics (Fig. 5a). This work overcomes the difficulty in integrating these two processes because Cas12a can digest the template DNA and inhibit the amplification process. In addition, Chen et al. [92] further pre-stored reaction reagents into centrifugal microfluidics to complete the whole process automatically. In another work, Silva et al. [93] developed an amplification-free CRISPR/Cas12a- and smartphone-based diagnostic method to detect SARS-CoV-2 (Fig. 5b). This assay is referred to as a cellphone-based amplification-free system with CRISPR/Cas-dependent enzyme, relying on smartphone imaging of a catalase-generated gas bubble signal in a microfluidic channel. Less than 50 copies/μl nucleic acids can be sensitively detected without pre-amplification and the full process from sample inlet to signal readout takes only 71 min.

Signaling module: detect signals processed by sensing module

As the final step of the nucleic acid testing, signal detection directly reflects the diagnostic result and is determinative in developing efficient, sensitive, and accurate POCT. Signals can be read out through various methods, such as fluorescence-based, electrochemical, colorimetric, and magnetic-based strategies. In this section, we will introduce the principle of each approach and make a comparison for infectious disease molecular diagnostics in microfluidics.

Fluorescence-based strategies are extensively applied to POCT to diagnose infectious diseases owing to their significant benefits of superior sensitivity, low cost,

easy operation, and instant analysis [94, 95]. These strategies make use of labeling fluorophores, such as fluorescent dyes and nanomaterials, to produce detectable signals (fluorescence enhancement or quenching). This finding suggests that fluorescence-based strategies can be categorized into direct fluorescence labeling, "signal-on" and "signal-off" fluorescence detection [96]. Direct fluorescence labeling detection makes use of special fluorescent tags labeling specific ligands to generate a certain amount of fluorescence when selectively binding to the targets. For "signal-on" fluorescence detection, the quality of the fluorescence signal is positively correlated with the target quantity. The fluorescence intensity is insignificant in the absence of the target and detectable as the target is sufficient. Conversely, the fluorescence intensity of the "signal-off" fluorescence detection is negatively correlated with the target quantity, which is initially at a maximum and decreasing, while the target is enhancing. For example, by utilizing the target-dependent trans-cleavage mechanism of CRISPR-Cas13a, Tian et al. [97] developed a novel sensing strategy to detect RNA directly bypassing reverse transcription (Fig. 6a). Binding to a complementary target RNA, the CRISPR–Cas13–RNA complex can be activated, triggering collateral cleavage of a non-specific RNA reporter in trans. The fluorescently-labeled reporter [fluorophore (F)] is quenched by a quencher (Q) when intact and generates fluorescence when cleaved by the activated complex.

Electrochemical detection has advantages, such as rapid detection, easy fabrication, low cost, portability, and self-control, making it a powerful analytical method for POCT applications. Based on a graphene field-effect transistor, Gao et al. [98] developed a nano-biosensor to multiply detect antigens of Lyme disease from *Borrelia burgdorferi* bacteria, exhibiting a 2 pg/ml limit of detection (Fig. 6b).

Colorimetric assays have been applied for POCT applications, benefitting from the dominance of portability, low cost, ease of preparation, and naked eye readout. Colorimetric detection can convert the information of target nucleic acid existence to visible color change utilizing oxidation of peroxidase or peroxidase-like nanomaterials, aggregation of nanomaterials, and addition of dye indicators [99–101]. Notably, gold nanoparticles are broadly applied in colorimetric strategy establishment and have attached increasing interest to develop colorimetric POCT platforms for on-site infectious disease diagnostics because of the ability to cause fast and significant color changes [102]. Utilizing an integrated centrifugal microfluidic device [103], foodborne pathogens within a contaminated milk sample can be automatically
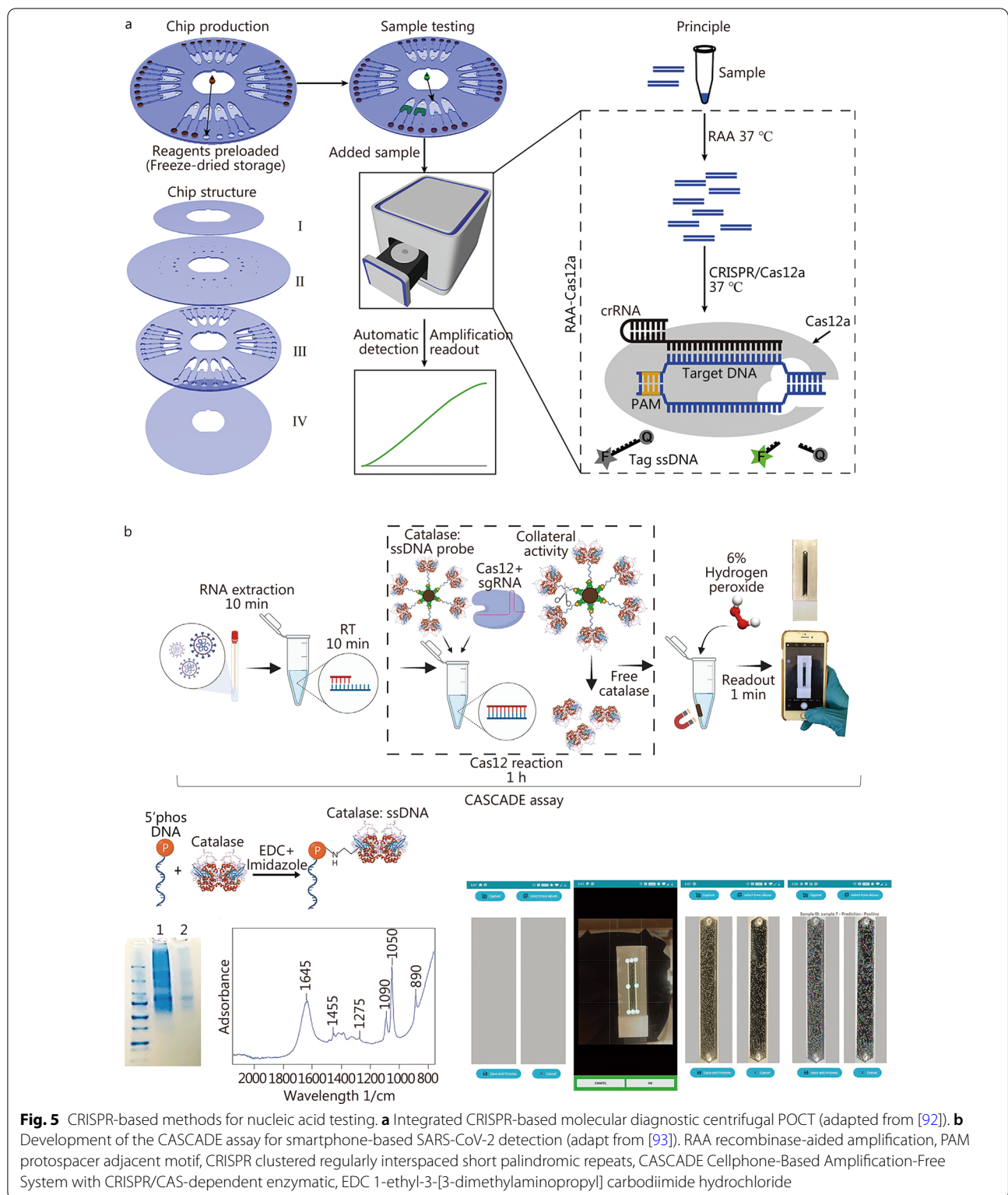
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Fig. 5 CRISPR-based methods for nucleic acid testing. **a** Integrated CRISPR-based molecular diagnostic centrifugal POCT (adapted from [92]). **b** Development of the CASCADE assay for smartphone-based SARS-CoV-2 detection (adapt from [93]). RAA recombinase-aided amplification, PAM protospacer adjacent motif, CRISPR clustered regularly interspaced short palindromic repeats, CASCADE Cellphone-Based Amplification-Free System with CRISPR/CAS-dependent enzymatic, EDC 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride

detected down to 10 bacterial cell levels, the outcome of which can be read out by the unaided eye in 65 min (Fig. 6c).

Magnetic-based sensing methods can sensitively detect analytes by employing the magnetic materials, and have obtained a surging interest for POCT applications in

recent decades. Magnetic-based sensing methods have some unique advantages, such as low-cost magnetic materials rather than expensive optics components. Even so, the detection efficiency is improved and the sample preparation time is decreased utilizing magnetic fields [104]. Moreover, magnetic-based sensing results exhibit great specificity, sensitivity, and high signal-to-noise ratio because of the insignificant magnetic background signal of biological samples [105]. A magnetic tunneling junction-based biosensor was integrated onto a portable microchip platform by Sharma et al. [106] for the multiplex detection of pathogens (Fig. 6d). The biosensor sensitively detects extracted nucleic acids below the nanomole range from pathogens.
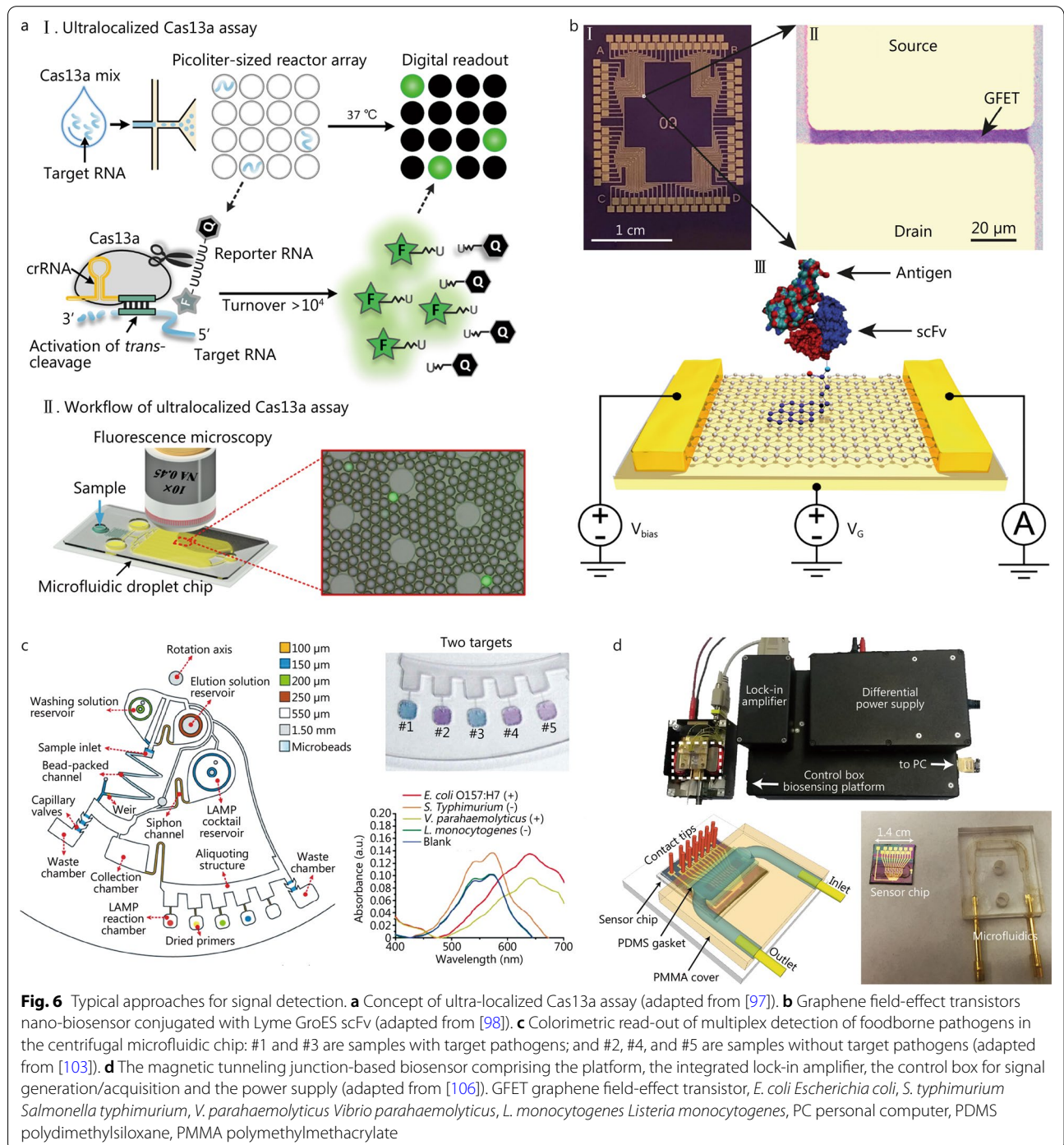

Fig. 6 Typical approaches for signal detection. **a** Concept of ultra-localized Cas13a assay (adapted from [97]). **b** Graphene field-effect transistors nano-biosensor conjugated with Lyme GroES scFv (adapted from [98]). **c** Colorimetric read-out of multiplex detection of foodborne pathogens in the centrifugal microfluidic chip: #1 and #3 are samples with target pathogens; and #2, #4, and #5 are samples without target pathogens (adapted from [103]). **d** The magnetic tunneling junction-based biosensor comprising the platform, the integrated lock-in amplifier, the control box for signal generation/acquisition and the power supply (adapted from [106]). GFET graphene field-effect transistor, *E. coli Escherichia coli*, *S. typhimurium Salmonella typhimurium*, *V. parahaemolyticus Vibrio parahaemolyticus*, *L. monocytogenes Listeria monocytogenes*, PC personal computer, PDMS polydimethylsiloxane, PMMA polymethylmethacrylate

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Despite the outstanding performance of the detection methods mentioned above, drawbacks still exist. These methods were compared (Table 1), including some applications with detailed information (both advantages and disadvantages).

Integrated microfluidic platforms for infectious disease diagnosis

With the development of microfluidics, micro-electromechanical system, nanotechnology, and materials science, the application of microfluidic chips for infectious disease detection has been promoted continuously [55, 96, 107, 108]. The miniaturized devices and precise manipulations of fluids facilitate the accuracy and economy of diagnosis. Therefore, great efforts have been made to optimize and innovate chips for further development, which leads to different microfluidic chips of various structures and functions. Herein, we briefly introduced a few common types of microfluidic platforms and compared their features (advantages and disadvantages). Furthermore, most of the examples listed below mainly focus on targeting SARS-CoV-2.

Lab on a cartridge chip (LOCC)

LOCC is the most common micro total analysis system, in which manipulation is highly miniaturized, integrated, automated, and parallelized from sample input and preparation, flow control, and liquid detection [109, 110]. Manipulation of fluids are performed by well-designed geometries and the interplay of multiple physical effects, such as pressure gradients, capillarity, electro-kinetics, magnetic fields, and sound waves [111]. LOCC shows excellent advantages in high-throughput screens and multiple assays with fast analysis, small sample volume, low power consumption, and efficient control and manipulation; however, LOCC devices are so delicate that it is also difficult to fabricate, package, and interface, while multiplexing and reuse are of great challenge [96]. Compared with other platforms, LOCC exhibits several exclusive merits in maximum application diversity and best compatibility for technologies, while drawbacks are also obvious in the high complexity and weak reproducibility. The dependence on external pumps, which are usually huge and expensive, further constrains its usage for POCT.

During the COVID-19 outbreak, a large amount of attention has been paid to LOCC. Meanwhile, some novel chips integrated with various techniques emerged. For example, smartphones are now widely available as portable analytic devices and have great potential to integrate with LOCC. Sun et al. [21] fabricated a microfluidic chip that can multiply amplify specific nucleic acid sequences of five pathogens, including SARS-CoV-2 by LAMP, and detected them at the end of the reactions by a smartphone in 1 h. As another example, Sundah et al. [112] formed a molecular switch [catalytic amplification by the transition-state molecular switch (CATCH)], which can directly and sensitively detect SARS-CoV-2 RNA targets with a smartphone. CATCH is compatible with portable LOCC and achieves superior performance (approximately 8 RNA copies/μl; < 1 h at room temperature) [112]. In addition, some driving forces are also used in LOCC equipment for molecular diagnostics, such as vacuum, stretching, and electric fields. Kang et al. [113] demonstrated an ultrafast and real-time nanoplasmonic on-chip PCR to rapidly and quantitatively diagnose COVID-19 on site using the vacuum-driven plasmofluidic PCR chip. Li et al. [114] subsequently developed a stretching-driven microfluidic chip that realized the diagnosis of COVID-19. The platform adopted a RT-LAMP amplification system to decide whether the sample qualitatively tested positive or negative. Subsequently, Ramachandran et al. [115] achieved a proper electric field gradient utilizing isotachophoresis (ITP), a selective ionic focusing technique-to implement in microfluidics. Through ITP, target RNA within raw nasopharyngeal swab samples can be automatically purified. Then, Ramachandran et al. [115] incorporated this ITP purification with LAMP and the ITP-enhanced CRISPR assay for SARS-CoV-2 detection in approximately 35 min from both contrived and clinical nasopharyngeal swab samples. Additionally, new ideas are being launched all the time. Jadhav et al. [116] proposed a diagnostic protocol based on surface-enhanced Raman spectroscopy coupled with microfluidic devices that contain integrated microchannels functionalized with vertically-aligned aurum/argentum-coated carbon nanotubes or with disposable electrospun micro/nano-filter membranes. This device adsorbs viruses from various biological fluids/secretions, such as saliva, the nasopharynx, and tears. Therefore, the viral titer is enriched and the viruses can be accurately identified from the Raman signatures.

Lab on a disc (LOAD)

LOAD is a centrifugal microfluidic platform, in which all the processes are controlled by the frequency protocol of a rotating micro-structured substrate [110]. The LOAD device is characterized by utilizing centrifugal forces as significant driving forces. Fluids are also controlled by capillary, Euler, and Coriolis forces. With a centrifugal unit, assays are conducted by sequential liquid operations from radial inward-to-outward positions, leaving out extra external tubes, pumps, actuators, and active valves. Briefly, the sole control method eases manipulation. The forces on liquids at the same distance from the center of the LOAD and in the identical microfluidic channel are

Table 1 Comparison of the detection methods for infectious diseases based on microfluidics

Detecting methods	Microfluidic systems	Amplification methods	Extracting methods	Analyte	Performance	Pros and cons	References
Fluorescence	μPAD	RCA	CNAEK	SARS-CoV-2 RNA	0.7 aM 15 min	#Superior sensitivity, low cost, easy to operate, rapid analysis *High background noise	[4]
	LOCC	LAMP	CNAEK	Virus DNA	74 copies/μl 30 min		[21]
	LOCC	RCA	Silica beads	Influenza RNA	0.5 fM 40 min		[25]
	LOAD	–	Silica beads	*E. coli* O157:H7 DNA	60% EE		[27]
	–	SEA	Silica membrane	*Vibrio parahaemolyticus* DNA	10 CFU/g		[30]
	LOCC	RT-qPCR	Silicon micropillars	Virus RNA	95% EE		[32]
	μPAD	PCR	Chitosan porous membranes	Bacteria DNA	89% EE		[40]
	–	LAMP	Microcapillary	Blood DNA	0.2 μl sample 150 min		[41]
	μPAD	RT-PCR	FTA membranes	HIV RNA	3 copies/μl 5 min		[50]
	LOAD	RT-PCR	CNAEK	SARS-CoV-2 RNA	10 copies/μl 15 min		[53]
	LOAD	RT-PCR	CNAEK	SARS-CoV-2, influenza A and B RNA	10 copies/μl 57 min		[60]
	LOAD	PCR	CNAEK	β-actin DNA	70.5 pg/channel 10 min		[64]
	LOAD	RT-LAMP	CNAEK	SARS-CoV-2 RNA	2 copies/μl 70 min		[72]
	LOAD	RT-LAMP	CNAEK	Virus RNA	10 copies/μl 40 min		[74]
	LOAD	RT-LAMP	CNAEK	SARS-CoV-2 RNA	15 copies/μl 45 min		[75]
	μPAD	RT-RPA	CNAEK	HIV RNA	5 copies/μl 45 min		[78]
	LOAD	RPA	CNAEK	*S. aureus* DNA	3 CFU/μl 60 min		[79]
Electrochemistry	–	–	Silicon nitride nano filter	*E. coli* O157:H7 DNA	–	#Rapid detection, easy to fabricate, low cost, portable and self-controlled *Unstable and susceptible	[28]
	–	–	Silicon nitride nano filter	Micro RNA	30 min		[29]
	LOCC	LAMP	Magnetic beads	Bacteria DNA	10 copies/μl 15 min		[33]
	LOCC	RT-LAMP	Magnetic beads	SARS-CoV-2 RNA	470 copies/μl 60 min		[38]
	LOCC	RT-PCR	Magnetic beads	Virus RNA	40 copies/μl 100 min		[43]
	LOCC	PCR	CNAEK	*E. coli* O157:H7 DNA	100 CFU/ml 60 min		[61]
Colorimetry	μPAD	LAMP	FTA card	Blood DNA	90 min 3 copies/μl	#Portable, low cost, easy to prepare, naked eye readout *Unable to quantitatively detect, limited sensitivity	[44]
	–	PCR	FTA card	Leishmaniasis DNA	60 min		[47]
	–	PCR	FTA card	Plasmodium DNA	3 parasites/μl 45 min		[48]
	LOAD	RT-LAMP	CNAEK	SARS-CoV-2 RNA	0.1 copy/μl 10 min		[73]

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Table 1 (continued)

Detecting methods	Microfluidic systems	Amplification methods	Extracting methods	Analyte	Performance	Pros and cons	References
Magnetic	LOCC	–	Magnetic beads	*H. pylori* DNA	DNA purification and 40-fold pre-concentration within 7 min	#Low cost, efficient, rare sample preparation, negligible magnetic background *Hard to read out magnetic signals with miniaturized systems	[37]
	LOAD	RT-LAMP	Magnetic beads	SARS-CoV-2 RNA	8 samples in parallel 30 min		[39]

Performance includes the limit of detection, testing time (min), extraction efficiency (EE), and other characteristics (# refers to advantage and * refers to disadvantage). "–" means the information is not officially available from public data or is too complicated to be noted; *LOCC* lab on a cartridge chip, *LOAD* lab on a disc, *μPAD* microfluidic paper-based analytical device, *RCA* rolling circle amplification, *LAMP* loop-mediated isothermal amplification, *SEA* strand exchange amplification, *RT-qPCR* reverse-transcription quantitative polymerase chain reaction, *PCR* polymerase chain reaction, *RT-LAMP* reverse-transcription loop-mediated isothermal amplification, *RT-RPA* reverse-transcription recombinase polymerase amplification, *CNAEK* commercial nucleic acid extraction kits, *FTA* Finders Technology Associates, *aM* 10^{-18} mol/L, *fM* 10^{-15} mol/L, *CFU* colony-forming units, *SARS-CoV-2* severe acute respiratory syndrome coronavirus 2, *E. coli Escherichia coli*, *S. aureus Staphylococcus aureus*, *H. pylori Helicobacter pylori*

equal, making the repeats of channel structure possible. Thus, it is easier and more economical to design and fabricate LOAD devices than conventional LOCC ones, while reactions are highly independent and parallelized; however, because of the high mechanical strength of the centrifugal equipment, the available materials of chips are limited and small volumes are hard to be performed. Simultaneously, most LOAD devices are for one-time use only, which is high-cost for large-scale assays [96, 117–119].

LOAD is regarded as one of the most promising microfluidic devices and has received great attention from researchers and manufacturers in recent decades. As a result, LOAD has been widely accepted and utilized in molecular diagnostics of infectious pathogens [120–124], especially during the outbreak of COVID-19. For example, at the end of 2020 Ji et al. [60] showed the direct RT-qPCR assay to detect SARS-CoV-2, and influenza A and B viral infections in parallel from pharyngeal swab samples rapidly and automatically. Then, Xiong et al. [74] presented a disk-like microfluidic platform integrated with LAMP for rapid, accurate, and simultaneous detection of seven human respiratory coronaviruses, including SARS-CoV-2, within 40 min. In early 2021, de Oliveira et al. [73] displayed a polystyrene-toner centrifugal microfluidic chip manually controlled by a fidget spinner for molecular diagnostics of COVID-19 by RT-LAMP. Subsequently, Dignan et al. [39] revealed an automated, portable, centrifugal microdevice to purify SARS-CoV-2 RNA directly from buccal swab cuttings. Xiong et al. [53] presented a small-volume rotating microfluidic fluorescence chip-integrated aerosol SARS-CoV-2 sampling system with a detection limit of 10 copies/μl and the shortest cycle threshold of 15 min. Soares et al. [75] recently reported the development of an integrated modular centrifugal

microfluidic platform to detect SARS-CoV-2 RNA by LAMP directly from heat-inactivated nasopharyngeal swab samples. These examples demonstrate a huge advantage in applying LOAD in molecular diagnostics of COVID-19 and good prospects for growth.

Microfluidic paper-based analytical devices (μPADs)

In 1945, Müller and Clegg [125] first introduced the microfluidic channel on paper by using filter paper and paraffin. In 2007 the Whitesides group [126] created the first functional paper platform to test protein and glucose. The paper has become an ideal substrate for microfluidics. Papers have intrinsic properties, such as a hydrophilic and porous structure, excellent biocompatibility, lightweight, flexibility, fold ability, low cost, ease of use, and availability. Classic μPADs are composed of hydrophilic/hydrophobic structures built on paper substrates. Based on the three-dimension structure, μPADs can be classified into two dimensional (2D) and three dimensional (3D) μPADs. 2D μPADs are produced by patterning hydrophobic borders to form microfluidic channels, while 3D μPADs are usually made from stacking of 2D microfluidic paper layers, and sometimes by paper folding, slip techniques, open channels, and 3D-printing [96]. Aqueous solutions or biological fluids on μPADs are mainly controlled by capillary forces without external power sources, thus facilitating reagent pre-storage, sample manipulation, and multiplex detection. Nevertheless, precise control of flow and multiple assays are blocked while lacking detection speed, sensitivity, and reusability [96, 127–130].

As an extraordinary microfluidic platform, μPADs have been greatly promoted and developed for molecular diagnostics of infectious diseases, such as HCV, HIV, and SARS-CoV-2 [131, 132]. To detect HCV selectively and

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sensitively, Teengam et al. [133] developed a novel fluorescent paper-based biosensor employing a highly specific pyrrolidinyl peptide nucleic acid probe. The nucleic acid was covalently immobilized onto partially oxidized cellulose paper through reductive alkylation between the amine and aldehyde groups, while the detection was based on fluorescence. The signals can be read out by a custom-made portable fluorescent camera gadget combined with a cellphone camera. Subsequently, Lu et al. [134] constructed a flexible paper-based electrode based on a nickel metal–organic framework composite/aurum nanoparticles/carbon nanotubes/polyvinyl alcohol for target HIV DNA detection by DNA hybridization using methylene blue as a redox indicator. Recently, Chowdury et al. [135] proposed a hypothetical design of a μPADs point-of-care platform for COVID-19 analyte detection using unprocessed patient-derived saliva, combined with LAMP and a handheld image acquisition technique.

Lateral flow assay (LFA) chips

Lateral flow tests drive liquids by capillary forces and control fluid movement by the wettability and characteristic structure of the porous or micro-structured substrate. The lateral flow device consists of sample, conjugate, incubation and detection, and absorbent pads. Nucleic acid molecules in a LFA recognize specific conjugates pre-stored on the conjugate pad and combined as complexes. When the fluid pass through the incubation and detection pad, the complexes will be captured by the capture molecules located on the test and control line, showing results that can be read directly by the unaided eye. Typically, LFA can be completed in 2–15 min, which is faster than traditional assays. Due to its special mechanism, LFA requires few operations and omits extra equipment, which is user-friendly. It is convenient for fabrication and miniaturization, while paper-based substrate also has a low cost. Yet, it is only for qualitative analysis and has great difficulty for quantitative detection, while multiplexing capability and throughput are so limited that only one kind of nucleic acid that is sufficient can be tested at a time [96, 110, 127].

Even though most applications of LFA are focused on immunoassay, applying LFA for molecular diagnostics in microfluidic chips is also efficient and popular [136]. Using hepatitis B virus, HIV, and SARS-CoV-2 LFA as examples, Gong et al. [137] presented an upconversion nanoparticle-based LFA platform and demonstrated the universality of this miniaturized and portable platform by sensitively and quantitatively detecting several targets, such as hepatitis B virus nucleic acids. Furthermore, Fu et al. [138] showed a novel surface-enhanced Raman spectroscopy-based LFA for the quantitative analysis of low concentration HIV-1 DNA. To rapidly and sensitively detect SARS-CoV-2, Liu et al. [85] developed the microfluidic-integrated lateral flow RPA assay, combining the RT-RPA and a universal lateral flow dipstick detection system into a single microfluidic system.

The applications of different microfluidic platforms are varied in specific research, taking advantage of the platform capabilities and merits. LOCC is the most inclusive platform for application diversity and technology compatibility because of available valves, pumps, and channels. Therefore, we hope and suggest that the most novel research be conducted in LOCC as a first attempt and that conditions are optimized. In addition, more efficient and accurate approaches are expected to be discovered and utilized in the system. LOAD succeeded in precisely controlling liquids from available LOCC devices and showed unique advantages in the solo driver by centrifugal forces without an external actuator, while parallel reactions could be individual and synchronized. Thus, LOAD will be the mainstream of future microfluidic platforms with decreased artificial operations, requiring more mature and automatic techniques. The μPAD platforms integrate the advantages of both LOCC and paper material, and are suitable for inexpensive and one-time diagnosis. Therefore, future development should focus on technologies that are convenient and well-developed. Furthermore, LFA is a highly suitable for unaided eye detection, which is expected to reduce sample consumption and accelerate testing speed. The detailed comparison of the platforms is shown in Table 2.

Digital nucleic acid assay

A digital assay partitions a sample into many microreactors, and each contains a discrete number of target molecules [139, 140]. A digital assay offers significant advantages for performing absolutely quantitative assays by simultaneously and individually conducting thousands of parallel biochemical experiments in micrometer-sized compartments instead of the continuous phase. Reactions in compartments can reduce sample volumes, improve reaction efficiency, and easily integrate with other analytic techniques without the need for networks of channels, pumps, valves, and compact design compared to traditional microfluidics [141–147]. The following two approaches are used for the digital assay to accomplish uniform and precise compartmentalization of solutions, including reagents and samples, such as cells, nucleic acids, and other particles or molecules: (1) droplet emulsions exploiting the interfacial instability of liquids; and (2) array separation through the geometric constraints of the device. In the former method, droplets containing reagents and samples in microchannels can be generated by passive methods, such as co-flow, cross-flow,

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Table 2 Comparison of microfluidic platforms for diagnosis

Platforms	Features compared with other platforms	Driving forces	Advantages	Disadvantages	References
LOCC	Maximum application diversity Best compatibility for technologies High complexity Weak reproducibility	Pressure gradient Capillary effects Electric fields Magnetic fields Lorentz forces Acoustic wave	High-throughput, multiple, fast analysis Small sample volume Low power consumption Efficient control and manipulation	Difficulty of fabricating, packaging interfacing Difficulty of multiplexing and reuse	[21, 112–116]
LOAD	Sole controlled by centrifugal forces Highly independent and parallelized reactions	Centrifugal forces Capillary forces Euler forces Coriolis forces	Easy to control Easy and economical to design and fabricate Multiple, independent and parallelized reactions	Limited available materials Hard for small volumes Difficulty of reuse	[39, 53, 60, 73–75]
µPADs	Sole controlled by capillary forces Hydrophilic and porous nature Fold ability Low-cost Ease of use	Capillary forces	Biocompatibility with various substrates Lightweight, flexibility, fold ability, ease of use and availability Low-cost	Imprecise and solo control of flow Lack of detection speed and sensitivity Difficulty of multiplexing and reuse	[133–135]
LFA	Sole controlled by capillary forces Pre-stored chemicals Naked eye read out by color change Low-cost	Capillary forces	Fast Low-cost Easy to operate Equipment-independent Easy to fabricate and miniaturize	Solo assay Hard for quantitative detection Difficulty of multiplexing and reuse Low throughput	[85, 137, 138]

LOCC lab on a cartridge chip, *LOAD* lab on a disc, *µPAD* microfluidic paper-based analytical device, *LFA* lateral flow assay

flow-focusing, step emulsification, microchannel emulsification, and membrane emulsification through viscous shear forces and variations of channel confinement [143, 145, 146, 148, 149], or by active methods with the aid of additional energy input through electrical, magnetic, thermal, and mechanical controls [150, 151]. In the latter method, better uniformity of liquid volume in microfluidic chambers is partitioned by restricting to spatial structures of the same size, for example, microwell and surface arrays [152–154]. Notably, droplets are the mainstream partitions and can also be generated and manipulated on an array of electrodes, which is based on digital microfluidics (DMF). Electrowetting on dielectric is one of the most intensively studied theories in DMF because electrowetting on dielectric is able to control over fluid shape and flow through asymmetric electrical signals on different sides, making precise manipulations of single droplets possible [141, 144]. Basic manipulations of droplets in DMF include sorting, splitting, and merging [151, 155, 156], which can be applied to various analytic fields, especially in molecule detection [157–159].

The digital nucleic acid assay is third-generation technology of molecular diagnostics after conventional PCR and quantitative real-time PCR (qPCR), parallel to high-throughput sequencing and liquid biopsies. The digital nucleic acid has developed quickly in the field of molecular diagnostics to target infectious pathogens in the last two decades [160–162]. The absolute quantification of digital nucleic acid assay begins with packaging samples and reagents into divided compartments to ensure that every target sequence has the same probability to enter every discrete partition. Theoretically, every partition can be assigned a few target sequences or none as an independent micro-reaction system. Through the many kinds of sensing mechanisms discussed above, compartments with the target sequences of microorganisms producing signals above a particular threshold can be visualized by the unaided eye or machines and labeled as positive, while the other compartments producing signals below the threshold are labeled as negative, which makes the signal of every partition Boolean. Therefore, by calculating the number of compartments generated and the positive rate after the reaction, the original copies of the tested samples can be reconciled through the Poisson distribution formula without a standard curve, as is necessary for conventional quantitative detection, like qPCR [163]. Compared with traditional molecular diagnostic technology, the digital nucleic acid assay is much more automatic and integrated with higher analysis velocity and sensitivity, fewer reagents, and lower possibility of pollution, while also easier to design and fabricate. For these reasons, the application of digital assay, especially droplet-based method in molecular diagnostics combining amplification and signal read-out techniques, is well-studied during the crucial outbreak of SARS-CoV-2. For example, Yin et al. [164] combined droplet digital and

rapid PCR techniques to detect ORF1ab, N, and RNase P genes in SARS-CoV-2 in a microfluidic chip. Notably, the system can identify a positive signal within 115 s, which is more rapid than conventional PCR, suggesting its efficiency for point-of-care detection (Fig. 7a). Dong et al. [165], Suo et al. [157], Chen et al. [166], and Alteri et al. [167] also applied droplet digital PCR (ddPCR) in microfluidic systems to detect SARS-CoV-2 and achieved impressive research results. To further improve detection speed, Shen et al. [168] realized chip imaging based on ddPCR in just 15 s without applying stitching technology for images, which speeds up the lab-to-application process ddPCR technology. Not only thermal amplification technologies, like PCR, but also isothermal amplification techniques are applied for simplified reaction conditions and rapid response time. Lyu et al. [71] designed a droplet assay SlipChip that is capable of producing droplets of various sizes at high density with a single slipping step and quantifying SARS-CoV-2 nucleic acids via digital LAMP (Fig. 7b). As a rapidly growing technology, CRISPR can also play an important role in the digital nucleic acid assay for its convenient colorimetric visualization without additional nucleic acid dyes. Combinatorial arrayed reactions for multiplexed evaluation of nucleic acids were developed by Ackerman et al. [158] to detect 169 human-associated viruses, including SARS-CoV-2, in droplets containing CRISPR-Cas13-based nucleic acid detection reagents in a microwell assay (Fig. 7c). Moreover, isothermal amplification and CRISPR technologies can be utilized in a system to integrate the advantages of both. Park et al. [169] developed a CRISPR/Cas12a-assisted digital assay in commercial microfluidic chips to detect both extracted and heat-inactivated SARS-CoV-2 based on single-step RT-RPA, which outperforms its bulk counterpart with a shorter detection time, higher signal-to-background ratio, wider dynamic range, and better sensitivity (Fig. 7d). Some descriptions of these examples are shown in Table 3.

The digital nucleic acid assay is developing at high speed in infectious pathogen diagnosis, although some challenges deserve better solutions. First, the generation of partitions, especially droplets, is supposed to be rapid, stable, and uniform, which calls for an efficient and easy-producing method. Methods that depend on complex external pumps and tubes to compartmentalize are doomed to be replaced by convenient methods. Second, adding some surfactants is necessary to stabilize droplets in microfluidic devices, causing additional business costs. Therefore, a less expensive stabilizer or method is required to ensure stabilization in droplet reactions. Third, the measurement of original copies is based on signal read-out technologies, which involve algorithms to identify positive compartments. Program optimization and algorithm innovation are essential processes to achieve fast and accurate results. Our team created a novel Monte Carlo-based statistic modeling for absolute quantification of pathogenic nucleic acids via digital LAMP, the results of which agree with the proposed mathematical model [170]. Lastly, digital assay can cooperate with DMF to perform individual and parallel reactions, leaving a huge space for development. Overall, we are expecting integrated and automatic digital nucleic acid assays applied in infectious pathogen diagnosis to conduct sample-to-result testing and POCT.

Commercial microfluidic POCT devices

Microfluidic POCT devices exhibit many advantages in in vitro molecular diagnostics, especially in developing areas. Compared with laboratory testing, the operations of microfluidic POCT devices are integrated into a single microfluidic chip, cartridge, and tube, from sample purification-to-nucleic acid amplification and pathogen measurement, while results are easy and rapid to achieve at a comparatively low financial cost [96, 171, 172]. More and more interest has been drawn to microfluidic POCT devices from worldwide manufacturers because of the automatic tests and limited required reagents [19, 173]. Therefore, microfluidic POCT devices show a bright future to molecular diagnostics in urgent or daily situations and deserve further study. Herein we present some typical and current commercial microfluidic POCT devices for molecular diagnostics to show the current state of development.

Biological manufacturers have developed commercial devices to be applied in various fields, such as food security, agricultural product testing, medical diagnosis, animal industry, and environmental testing. Among these fields, medical diagnosis, especially molecular diagnostics, is of the greatest relevance to mankind, making the application much more popular. Using SARS-CoV-2 as an example and since the COVID-19 outbreak, microfluidic devices or newly-designed chips targeting the virus have been launched, such as FilmArray® Biofire® [Biofire (Salt Lake City, UT, U.S.)] [174], GenPlex® [BOHUI (Beijing, China)] [175], Vivalytic [BOSCH (Waiblingen, BW, German); Randox (Antrim, N.IRE, UK)] [176], RTisochip™-A (CapitalBio) [177], RTisochip™-W (CapitalBio) [178, 179], DxLab-2A (CapitalBio) [180], Cue™ [Cue health (San Diego, CA, U.S.)] [181], Simplexa™ [Focus Diagnostics (Cypress, CA, U.S.)] [182], QuanPLEX [IntelliBio (Qingdao, SD, China)] [183], a microchip based real-time PCR analyzer AriaDNA [Lumex Instruments (Mission, BC, Canada)] [184], Novodiag® [Mobidiag (Espoo, Finland)] [185], Cobas® Liat® [Roche (Indianapolis, IN, U.S.)] [186], iGeneTec MA3000 [Superchip technology (Shanghai, China)] [187], BINAS [Tsinghua University
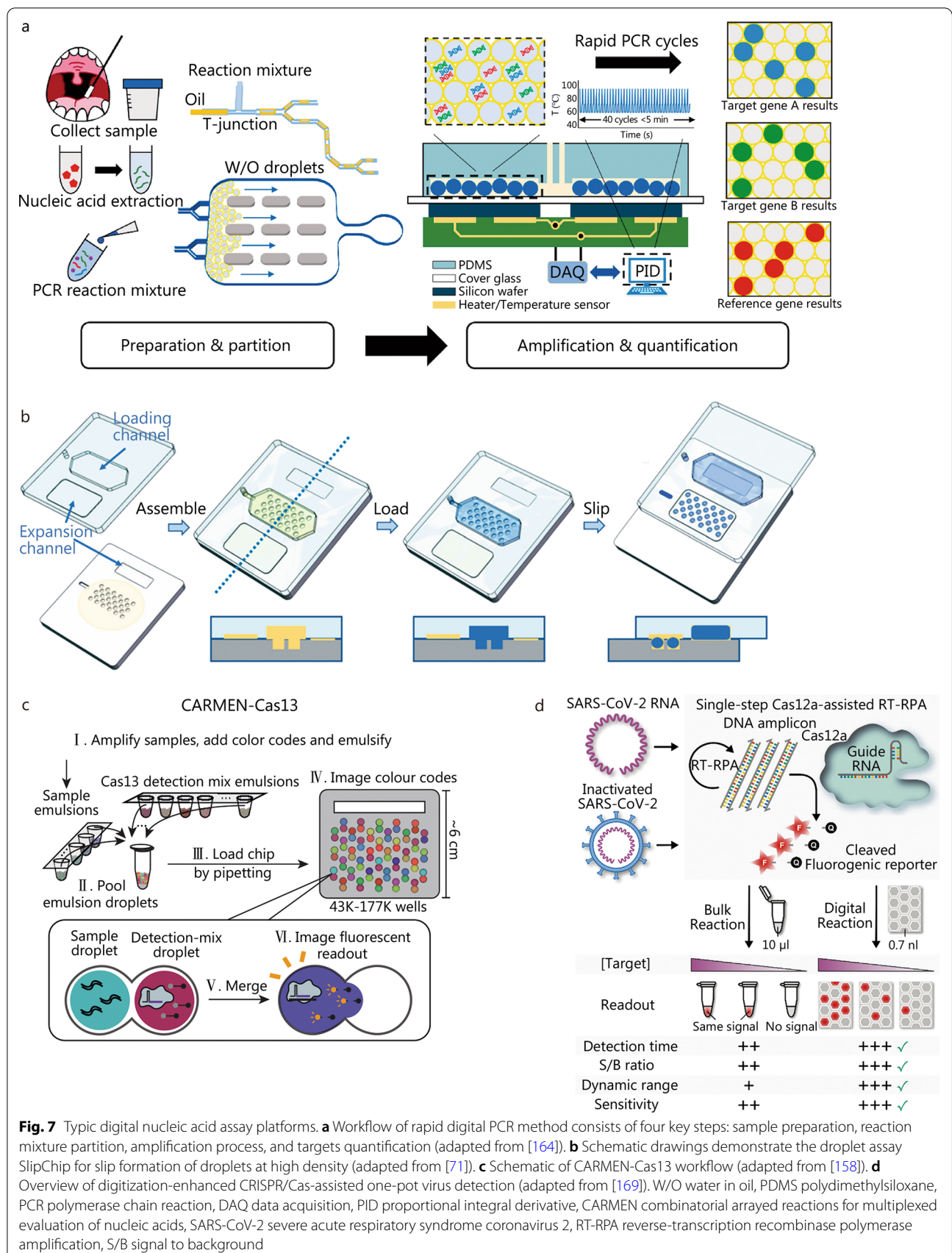
Fig. 7 Typic digital nucleic acid assay platforms. **a** Workflow of rapid digital PCR method consists of four key steps: sample preparation, reaction mixture partition, amplification process, and targets quantification (adapted from [164]). **b** Schematic drawings demonstrate the droplet assay SlipChip for slip formation of droplets at high density (adapted from [71]). **c** Schematic of CARMEN-Cas13 workflow (adapted from [158]). **d** Overview of digitization-enhanced CRISPR/Cas-assisted one-pot virus detection (adapted from [169]). W/O water in oil, PDMS polydimethylsiloxane, PCR polymerase chain reaction, DAQ data acquisition, PID proportional integral derivative, CARMEN combinatorial arrayed reactions for multiplexed evaluation of nucleic acids, SARS-CoV-2 severe acute respiratory syndrome coronavirus 2, RT-RPA reverse-transcription recombinase polymerase amplification, S/B signal to background

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Table 3 Applications of the digital nucleic acid assay in detecting SARS-CoV-2

Compartmentalization methods	Analyte	Amplification methods	Performance	Detecting methods	References
Crossflow/T-junction	SARS-CoV-2 *ORF1ab*, *N* and *RNase P* genes	RT-qPCR	5 copies/test in 5 min	Fluorescent probes	[164]
Flow-focusing by QX200™ Droplet Digital™ PCR System, Bio-Rad (Pleasanton, California, U.S.)	SARS-CoV-2 *ORF1ab*, *N* and *E* genes	RT-qPCR	2 copies/reaction in 4 h	TaqMan Hydrolysis probe and EvaGreen	[165]
Flow-focusing by QX200™ Droplet Digital™ PCR System	SARS-CoV-2 *ORF1ab* and *N* genes	RT-qPCR	1.4 copies/reaction	TaqMan PCR reaction mixture	[157]
By head-flattened pipette tips	SARA-CoV-2 *ORF1ab* and *N* genes	RT-qPCR	3.8 copies/reaction in 1.5 h	Thermo Scientific TaqMan 2019-nCoV Assay Kit v1	[166]
Flow-focusing by QX200™ Droplet Digital™ PCR System	SARS-CoV-2 *RdRp* and *RNase P* genes	RT-PCR	2.9 copies /reaction	GeneFinder™ COVID-19 Plus RealAmp Kit, ELITech and Allplex™ 2019-nCoV Assay, Seegene	[167]
SlipChip	SARS-CoV-2 NA extracted from the COVID-19 pseudo virus	LAMP	344 copies/ml	LAMP fluorescent dye	[71]
Microwell arrays	Synthetic SARS-CoV-2 targets	CRISPR-Cas13-based SHERLOCK technology	10 copies/ml	Single-stranded DNA fluorogenic reporters	[158]
By QuantStudio chips (ThermoFisher) with 0.7 nl digital reaction wells	SARS-CoV-2 N gene	CRISPR/Cas12a based RT-RPA	1 GE/µl of SARS-CoV-2 RNA and 20 GE/µl of heat-inactivated SARS-CoV-2, qualitative detection in 15 min and quantitative detection in 30 min	Single-stranded DNA fluorogenic reporters	[169]

RT-qPCR reverse-transcription quantitative polymerase chain reaction, *LAMP* loop-mediated isothermal amplification, *RT-PCR* reverse-transcription polymerase chain reaction, *CRISPR* clustered regularly interspaced short palindromic repeats, *SHERLOCK* specific high sensitivity enzymatic reporter unlocking, *RT-RPA* reverse-transcription recombinase polymerase amplification, *GE* genome equivalent, *COVID-19* corona virus disease 2019, *SARS-CoV-2* severe acute respiratory syndrome coronavirus 2

(Beijing, China)]) [188], Visby Medical™ [Visby Medical (San Jose, CA, U.S.)] [189] and WizDx™ F-150 Real-time PCR Systems [Wizbiosolutions (Seongnam-si, Republic of Korea)] [190]. Most of these devices also have focused on other respiratory viruses, such as influenza, before the epidemic. Sexually transmitted diseases are increasingly gaining attention from the public, leading to the creation of associated equipment. For example, an IO single module system [Binx health (Boston, MA, U.S.)] [191, 192] can detect *Chlamydia trachomatis* that may cause sexually transmitted diseases in just 30 min and Visby Medical™ can detect three kinds of sexual pathogens at the same speed, while GenPlex® and Vivalytic can also test for vaginal pathogens, such as human papilloma virus. As an immunologic challenge in human medicine, the detection of HIV in POCT devices is meaningful, so Abbott launched Alere™ Q [Abbott (Des Plaines, IL, U.S.)] [193] and matched HIV-1/2 detection chips. The test can be completed in 52 min, requiring only 25 µl of peripheral blood or plasma. Biocartis focus on tumors and launched Idylla™ [Biocartis (Mechelen, Belgium)] to target clinically-significant test sites of genes, such as *BRAF*, *KRAS*, *NRAS*, and *EGFR* [194]. Because of the similarity of molecular diagnostic methodologies, many microfluidic devices perform multiplex detection through concurrent testings and even more testing items may be added by product upgrades. For example, iChip-400 [195] and Onestart-1000 [196] from Baicare (Beijing, China), BD MAX™ [197] from BD (Sparks, MD, U.S.), FilmArray® Biofire® from Biofire, GeneXpert® Infinity Systems from Cepheid (Sunnyvale, CA, U.S.) [198], Unyvero A50 [199] from Curetis (West Boylston, MA, U.S.), Revogene® [200] from GenePOC (Quebec, PQ, Canada), ePlex [201] from GenMark (Carlsbad, CA, U.S.), AriaDNA from Lumex Instruments, Novodiag® from Mobidiag, Verigene® [202] from Nanosphere (Beverly, MA, U.S.), and Visby Medical™ from Visby Medical. These devices greatly increase the efficiency of the screening and diagnosis. More details of all the aforementioned devices are shown in Table 4.

Commercial microfluidic POCT devices are attempting to keep up with the modern medical tests conducted at hospitals and laboratories on test sensitivity and specificity. Commercial microfluidic POCT devices are showing exclusive advantages, especially in detecting diversity and degree of integration. In an epidemic, commercial

Table 4 Comparison of microfluidic POCT devices for molecular diagnostics

Approval time	Devices	Manufacturers	Regulator	Detection technology	Targets	LOD	Detection time	References
2007.08	Unyvero A50	Curetis	CE	Multiplex PCR	Hospitalized pneumonia, blood culture, intra-abdominal infection, urinary tract infection, implant & tissue infection	–	< 5 h	[199]
2007.09	Verigene®	Nanosphere	FDA	RT-PCR	*C. diff*, enteric pathogens, respiratory pathogens, Gram bacteria	–	2–2.5 h	[202]
2010.05	Simplexa™	Focus Diagnostics	FDA	RT-PCR	SARS-CoV-2, influenza A/B & RSV, HSV 1 & 2, influenza A, H1N1	242–500 copies/ml	< 80 min	[182]
2011.08	Cobas® Liat®	Roche	FDA	Multiplex real-time RT-PCR	SARS-CoV-2, influenza A/B, RSV, Cdiff and strep A	10^{-3}–10^{-1} $TCID_{50}$/ml	< 20 min	[186]
2012.01	BD MAX™	BD	FDA	RT-PCR	GBS, MRSA, *C. diff*, SA, vaginal pathogens, enteric bacterial and parasite, CT/GC/TV	–	45–90 min	[197]
2012.11	GeneXpert® Infinity Systems	Cepheid	FDA	RT-PCR	GBS, MRSA, gastrointestinal pathogens, MTB, TV, respiratory viruses and so on	–	–	[198]
2013.02	FilmArray® Biofire®	Biofire	FDA	Nested Multiplex PCR	Respiratory, blood, gastrointestinal and meningitis infection and so on	1000 $TCID_{50}$/ml	45–60 min	[174]
2014	Idylla™	Biocartis	CE	RT-PCR	*BRAF*, *KRAS*, *NRAS*, *EGFR* genes	–	90–180 min	[194]
2015	Alere™ Q	Abbott	CE	RT-PCR	HIV	–	< 52 min	[193]
2016.02	IO single module system	Binx health	CE	PCR	CT, NG and so on	–	< 30 min	[191, 192]
2017.05	Revogene®	GenePOC	FDA	RT-PCR	*C. diff*, GBS, GAS and CRE	–	< 70 min	[200]
2017.06	ePlex	GenMark	FDA	RT-PCR	Bloodstream infections and respiratory pathogens	–	< 90 min	[201]
2017.07	RTisochip™-A	CapitalBio Technology	NMPA	Isothermal amplification	19 respiratory viruses and 8 pathogenic bacteria	10–10^3 copies/run	< 50 min	[177]
2018.09	iChip-400	Baicare	NMPA	LAMP	16 pathogenic bacteria	–	< 1 h	[195]

Table 4 (continued)

Approval time	Devices	Manufacturers	Regulator	Detection technology	Targets	LOD	Detection time	References
2018.11	GenPlex®	BOHUI	NMPA	Multiplex PCR	24 HPV and 18 respiratory viruses	–	–	[175]
2019.07	RTisochip™-W	CapitalBio Technology	NMPA	NASBA	19 respiratory viruses and 8 pathogenic bacteria	50 copies/run	20–50 min	[178, 179]
2020.01	WizDx™ F-150 Real-time PCR System	Wizbiosolutions	CE	Ultra-fast RT-PCR	SARS-CoV-2	20 copies/run	< 40 min	[190]
2021.01	Onestart-1000	Baicare	NMPA	RT-PCR	60 pathogenic bacteria	10^3 copies/ml	< 1.5 h	[196]
2021.02	DxLab-2A	CapitalBio Technology	NMPA	Nested PCR	SARS-CoV-2	500 copies/ml	< 45 min	[180]
2021.03	Cue™	Cue health	FDA	Isothermal amplification	SARS-CoV-2	20 copies/test	20 min	[181]
2021.06	iGeneTec MA3000	Superchip technology	NMPA	Isothermal amplification	SARS-CoV-2	500 copies/ml	< 45 min	[187]
2021.08	Visby Medical™	Visby Medical	FDA	PCR	SARS-CoV-2, chlamydia, NG, TV	–	< 30 min	[189]
–	Vivalytic	BOSCH, Randox	–	End-point PCR	Respiratory and sexually transmitted infections viruses	–	30–150 min	[176]
–	QuanPLEX	IntelliBio	–	qPCR	7 respiratory viruses	–	–	[183]
–	AriaDNA	Lumex Instruments	–	RT-PCR	SARS-CoV-2, african swine fever, cattle pathogens, fish pathogens and avian pathogens	9000 copies/ml	< 50 min	[184]
–	Novodiag®	Mobidiag	–	RT-PCR	SARS-CoV-2, *C. diff*, enteric pathogens and so on	–	< 1 h	[185]
–	BINAS	Tsinghua University	–	Nested isothermal amplification	SARS-CoV-2	< 400 copies/ml	< 30 min	[188]

"–" means the information is not officially available from public data or is too complicated to be noted; *LOD* limits of detection, *NMPA* national medical products administration; *CE* conformite europeenne, *FDA* U.S. Food & Drug Administration, *C. diff Clostridium difficile, CRE Carbapenem-resistant Enterobacteriaceae, GAS Group A streptococcus; GBS Group B* streptococcus, *HPV human papilloma virus, HSV herpes simplex virus, MRSA* methicillin-resistant *Staphylococcus aureus, MTB Mycobacterium tuberculosis*, *NG Neisseria gonorrhoeae, RSV respiratory syncytial virus, SA Staphylococcus aureus, TV Trichomonas vaginalis, SARS-CoV-2* severe acute respiratory syndrome coronavirus 2, *PCR polymerase chain reaction, qPCR* quantitative real-time polymerase chain reaction, *LAMP* loop-mediated isothermal amplification, *RT-PCR* reverse-transcription polymerase chain reaction, *NASBA* nucleic acid sequence-based amplification, $TCID_{50}$ 50% tissue culture infective dose

microfluidic devices are of great importance in diagnosis because of efficient technologies, optimized reaction conditions, and convenience without the need for a large-scale laboratory or testing center. Because commercial microfluidic POCT devices were developed during a disease outbreak in a short time by transplanting conventional steps, there is still much room for development with respect to the level of efficiency, automation, integration, sensitivity, specificity, portability, and affordability, thus making the industry in its early stage of application. We are expecting qualified techniques combined with elaborately designed and fabricated chips to get sample-to-result instruments.

Conclusions

Infectious diseases are posing problems for public medical systems and attracted much attention from the public and scientists. Microfluidics is one of the best

technologies to conduct molecular diagnostics for infectious diseases and has made great achievements, especially during the outbreak of COVID-19. In this review we presented the applications of microfluidics-based strategies for infectious disease detection. In the first part, we systematically discussed the common processes of molecular testing based on microfluidics, including sample preprocessing (silicon-, magnetic-, and porous materials-based strategies), nucleic acid amplification (PCR, isothermal amplification, and CRISPR-based amplification-free methods), and signal reading-out (electrochemical, fluorescence, colorimetric, chemiluminescence, surface plasmon resonance-based, and magnetic-based biosensors). Next, various microfluidic platforms, including LOCC, LOAD, μPADs, and LFA, were compared to highlight the features, advantages, and disadvantages. We further discussed and emphasized the novel applications of the digital nucleic acid assay for absolute quantification. Subsequently, we investigated 27 commercial microfluidics-based POCT devices for molecular diagnostics from a decade ago and displayed the targeting objectives and performances.

There is still ample room for the development of microfluidics to deal with the severe, ongoing pandemic. More significantly, new infectious diseases may emerge in the near future. The traditional technologies are mature and optimized, but require multiple steps and frequent transfers of samples between platforms. These sophisticated processes lead to unnecessary pollution and complicated manual operations. Thus, the trend of fully integrated microfluidics is unstoppable, which combines sampling, sensing, and signaling modules. In the sampling module, a large quantity of molecules is expected to be extracted from the limit sample, therefore providing efficient cleavage enzymes, nucleic acid transport carriers, and cleaning agents. In the sensing module, the false-negative results caused by low-sensitive detection usually lead to misdiagnosis and create a burden in the public medical system. Prevention of pandemics calls for high-throughput testing that can precisely detect very few nucleic acids. In practical applications, the diagnostic requirements are so diverse that multiplex diagnostics are more suitable for future tests with expended testing items. In the signaling module, great efforts have been made to accurately identify signals transformed from amplified molecules by algorithms incorporating artificial intelligence, avoiding errors and limitations of manual judgments. There are also some novel strategies still in their early stages; for example, target molecules can be directly tested from sample solutions omitting the pretreatment process. Therefore, nucleic acids must be specifically distinguished between cluttered background molecules, which is challenging. In addition, detecting nucleic acids

bypassing sensing modules demands more sensitive testing methods, such as CRISPR, which can respond significantly to individual molecules.

Moreover, the industrialization of microfluidics is still in the start-up phase, reflected in complex channel design, expensive substrate materials, necessary optimization of reactions, liquid leakage, valve failure, and difficulty in reproduction and recyclability. These issues are the main barriers for large-scale adoption, so further improvement is needed to build a convenient chip-design platform, combined with material science to find less expensive substrate substitutes, enhance functional modularity, and push automation of chip producing. Fortunately, in dealing with infectious diseases currently and in the future, microfluidic-based molecular diagnostic strategies are indispensable and receiving more attention from frontier scientists. Microfluidics-based molecular diagnostic strategies will become the mainstream of large-scale detection, utilizing rarely required samples, to conduct diagnosis automatically at a low cost.

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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