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Biosensors and Bioelectronics 177 (2021) 112952

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: http://www.elsevier.com/locate/bios

Fully integrated microfluidic devices for qualitative, quantitative and digital nucleic acids testing at point of care

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ARTICLE INFO

Keywords:
Paper microfluidics
Microfluidic chips
Sample preparation
Isothermal amplification
Digital amplification
Commercial products

ABSTRACT

Benefiting from emerging miniaturized and equipment-free nucleic acid testing (NAT) technologies, fully integrated NAT devices at point of care (POC) with the capability of "sample-in-answer-out" are proceeding at a break-neck speed to eliminate complex operations and reduce the risk of contamination. Like the development of polymerase chain reaction (PCR) technology (the standard technique for NAT), the detection signal of fully integrated NAT devices has evolved from qualitative to quantitative and recently to digital readout, aiming at expanding their extensive applications through gradually improving detection sensitivity and accuracy. This review firstly introduces the existing commercial products, and then illustrates recent fully integrated microfluidic devices for NAT at POC from the aspect of detection signals (*i.e.*, qualitative, quantitative and digital). Importantly, the key issues of existing commercial products and the main challenges between scientific research and product development are discussed. On this basis, we envision that the **MARCHED** (**m**iniaturized, **a**utomatic, **r**eagent-preloaded, **c**ommercializable, **h**igh-throughput, **e**nvironment-independent and **d**isposable) NAT devices are expected to be realized in the near future.

1. Introduction

Nucleic acid testing (NAT) has found widespread applications in disease diagnosis (Chen et al., 2019; Park 2018), food safety analysis (Aissa et al., 2017; Oh et al., 2016; Tang et al., 2017), environment monitoring (Choi et al., 2016b; Gong et al., 2019) and forensic identification (Jung et al., 2018). Especially, we have witnessed the continuous spreading of novel coronavirus Disease (COVID-19) since 2019, which has caused serious economic losses and social burdens worldwide (Ali and Alharbi 2020; Gong et al., 2020). The timely detection of novel coronavirus is crucial for preventing the COVID-19 pandemic spread, and NAT is currently used as the standard method. For example, the Chinese Center for Disease Control and Prevention recommends to detect N gene and ORF1ab gene of the novel coronavirus genome for COVID-19 patient screening. Generally, three separate steps are involved in NAT, *i.e.*, NA extraction, NA amplification and signal readout. There are a lot of equipment-based technologies in central laboratories to realize these three steps, such as NA extraction kit-based method for NA extraction, polymerase chain reaction (PCR) for NA amplification, and real-time fluorescent quantitative method for signal readout (Choi et al., 2016a; Ganguli et al., 2017; Nzelu et al., 2016). However, these equipments used for NAT are costly, bulky, complicated-to-operate, and with limited access at resource-poor settings. Thus point-of-care (POC) NAT technologies with specific characteristics of being affordable, portable, rapid and user-friendly are highly expected. Besides, the need for well-trained operators and the potential

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https://doi.org/10.1016/j.bios.2020.112952
Received 14 October 2020; Received in revised form 16 December 2020; Accepted 28 December 2020
Available online 31 December 2020

issue of contamination of nucleic acid aerosol may negatively affect the accuracy of detection results. Thus, fully integrated NAT technologies with the capability of "sample-in-answer-out" are highly demanded to eliminate complex operations and reduce the risk of contamination.

An ideal fully integrated POC microfluidic device should be ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users), enabling NAT implemented by non-professional personnel away from central laboratories in a short time. Recently, with the development of emerging miniaturized and equipment-free NAT technologies (Li et al., 2020), various microfluidic devices consisting of NA extraction, NA amplification and signal readout (Goller et al., 2018; Jovanovich et al., 2015) have been proposed to realize fully integrated NAT, which can be mainly classified by paper-based microfluidics (Choi et al., 2016b; Lafleur et al., 2016; Seok et al., 2017; Tang et al., 2017) and chip-based microfluidics (Ganguli et al., 2017; Hajian et al., 2019; Oh et al., 2016; Yeh et al., 2017). For instance, the development of membrane-based NA extraction techniques, such as FTA card and FTA Elute card (Whatman), enables the integration of NA extraction process on chips (Chen et al., 2019; Trinh et al., 2019, 2020). The emerging isothermal amplification technologies featured by no need of thermal cycler (e.g., nuclear acid sequence-based amplification (NASBA) (Compton 1991), rolling circle amplification (RCA) (Fire and Xu 1995), loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000) and recombinase polymerase amplification (RPA) (Piepenburg et al., 2006)) make NA amplification on chip easy to access. As for signal readout, sample-in-qualitative-answer-out strategy based on lateral flow assay (LFA) has been well developed, which however is associated with limited sensitivity (Choi et al., 2016c). Then, fluorescence-based (Liao et al., 2016; Sun et al., 2020) and electrochemical (Abi et al., 2018; Slouka et al., 2015) signals have been integrated on chips for quantitative answer out with superior sensitivity. Now, with the increasing demand for low abundance NA detection, sample-in-digital-answer-out strategy is ongoing (Cao et al., 2017).

With the outbreak and extension of COVID-19 pandemic, the translation of POC NAT devices from academic research into viable products has gained considerable attentions. Meanwhile, a lot of newly-developed POC NAT devices emerge continuously to address new technical challenges caused by the pandemic (Tian et al., 2020; Ye et al., 2020; Zhou et al., 2020). Although there exist several reviews focusing on microfluidic systems for NAT (Ahrberg et al., 2016; Shrivastava et al., 2020; Yin et al., 2019; Zhang et al., 2017), they mostly focus on the development of nucleic acid amplification technologies, lacking in detailed discussion on translation. Following PCR technology (the standard technique for NAT) goes from qualitative to quantitative to recent digital manner in aspect of signal readout during last decades, the detection signal for fully integrated NAT devices has also evolved from qualitative to digital readout to improve detection sensitivity and accuracy and to meet various demands for clinical diagnostics. In this review, we firstly review commercial products and successively summarize recent fully integrated microfluidic devices for NAT at POC from the aspect of detection signals (*i.e.*, qualitative, quantitative and digital) (Fig. 1). Importantly, the key issues of existing commercial products and the main challenges between scientific research and product development are discussed, which is the thrust of this paper. Finally, conclusions and future perspectives are discussed.

2. Commercial integrated NAT devices

Recently, with the increasing demand for NAT for infectious diseases testing, genetic testing and antibiotic resistance testing, commercially available fully integrated NAT devices have been well developed, such

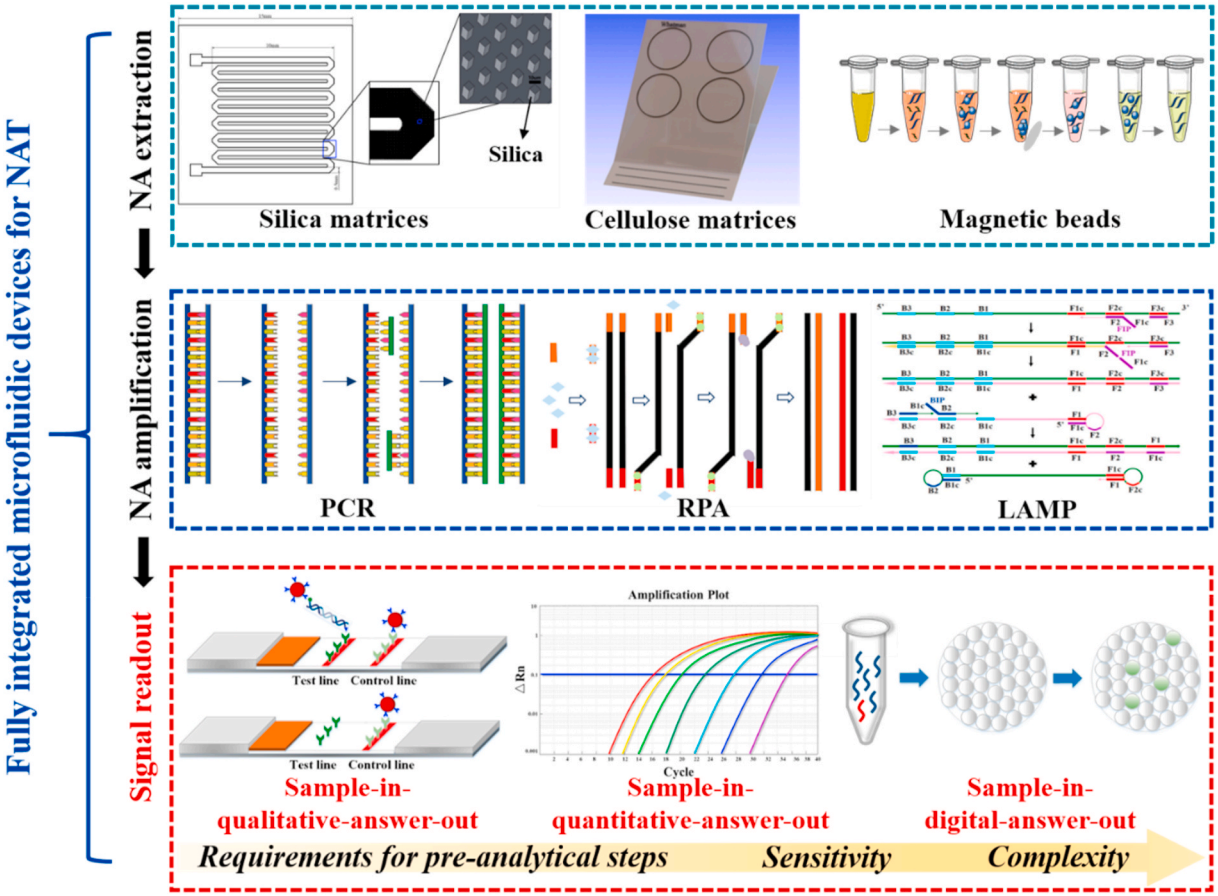

Fig. 1. Development of fully integrated microfluidic devices for nucleic acids testing at point of care.

as ID NOW® by Abbott, GeneXpert® by Cepheid, and FilmArray® by Biofire. A significant feature of these commercial products is that most products consist of a disposable cartridge filled with preloaded reagents and a portable instrument to automatically perform mechanical action, heating and signal readout. In addition, it is remarkable that since fluorescent signal offers the capability of quantification of NAs with relatively low requirements on technologies and instruments, almost all commercial integrated instruments adopt quantitative fluorescent signal output. Products from different companies vary in cost, analysis time, detection throughput, and their detailed information is shown in Table 1. These commercial products generally involve three steps for NAT, i.e., NA extraction, NA amplification, and fluorescent signal readout. NA extraction on these systems is mostly based on solid phase extraction (SPE) methods, represented by GeneXpert® (Fig. 2a) and FilmArray® (Fig. 2b) with silica-coated magnetic beads-based NA extraction method. For NA amplification, PCR as the gold standard method is widely employed on commercial integrated devices. For example, iCubate® adopts traditional quantitative PCR strategy to amplify target NAs. A limitation to this strategy is its long amplification time caused by slow thermal cycling. To address this, ultrafast PCR has been developed through improving thermal cycling (You et al., 2020). As an illustration, Cobas® Liat® PCR system (Fig. 2c) adopts three different temperature zones to replace traditional Peltier-based thermal cycling, which can dramatically shorten the amplification time (within 20 min). Besides, emerging isothermal amplification methods enable commercial NAT devices to own rapid turnaround time, good portability and energy saving (Zhao et al., 2015). For example, ID NOW® by Abbott uses nicking enzyme amplification reaction (NEAR) to amplify target NA (Fig. 2d), while the all-in-one test kit® for COVID-19 diagnosis delivered by Lucira Health uses LAMP as its amplification method (Fig. 2e). So far, most reported commercial integrated NAT products can detect multiple samples at the same time, where high throughput detection is usually realized through introducing multiple cartridges simultaneously.

Under the background of COVID-19 pandemic, most of these commercial products have shown their capability for the novel coronavirus detection. Especially, the all-in-one test kit® was designed for self-detection of COVID-19 at home. Besides, such products have potential for other common application scenarios. GeneXpert® system mainly focuses on infectious diseases, such as group A streptococcus (GAS), influenza virus A/B, respiratory syncytial virus (RSV) and mycobacterium tuberculosis (MTB). Novodiag® delivered by Mobidiag and GeneXpert® system can perform penicillin and myxomycetin resistance test and rifampicin (RIF) resistance test respectively, so as to carry out personalized medical treatment. In addition, another application area is genetic testing, such as cancer early screening, obstetrics and gynecology examination. For instance, Idylla® developed by Biocartis contains an analyzer and 1–8 cartridges, which can detect epidermal growth factor receptor (EGFR) mutations within 90–150 min. For different application scenarios, these single-use consumables need to be modified with new reagents accordingly.

Although a certain number of integrated NAT products exist on the market, there is still plenty of room for improvement. Firstly, the detection cost on these NAT products is still relatively high, taking tens to hundreds of dollars, due to high cost of reagents and consumables. Secondly, most commercial products suffer from insufficient capacity for quantitative signal readout, especially those relying on isothermal amplification technologies. Thirdly, the high throughput detection on such products is based on introducing multiple parallel consumables with preloaded reagents, which will increase additional consumables cost and operation complexity. The multiple detection on such devices is expected to be performed in a single consumable. Fourthly, most products still rely on skilled operators and central laboratories due to their multiple operation steps with bulky analyzers, except the all-in-one test kit®. The desired operation on such products necessitates all the procedures performed in an enclosed and automated instrument.

3. Sample-in-qualitative-answer-out

The main challenge for NAT is to realize inexpensive, high-throughput, automatic NA detection from raw samples (e.g., whole blood), which can be addressed by the fully integrated microfluidics devices that are easy to manufacture and use. The fully integrated NA detection is paramount, in which sample-in-qualitative-answer-out strategy is relatively easy to achieve due to the low requirements for purity of NA extraction and efficiency of NA amplification. Thus, it is very suitable for NA detection with “yes” or “no” results such as Acquired Immune Deficiency Syndrome (AIDS) window test (Phillips et al., 2019) and genotyping (Chang et al., 2015). Benefiting from emerging isothermal amplification technologies and chromogenic materials, NA detection signals on qualitative integrated microfluidic devices mainly include colorimetric (Trinh et al., 2020) and fluorescent signals (Sayad et al., 2018).

The results of colorimetric signals can be directly observed by naked eyes without additional instruments, which is user-friendly and cost-

Table 1
Commercially available sample-in-answer-out detection systems.

System	Company	Targets/Diseases	Time (min)	Amplification methods	Cost	Throughput
GeneXpert	Cepheid (cepheid.com)	GAS/Influenza A&B/RSV/MTB/RIF	20	Real-time PCR	$ 42.7/test; € 56,000 for instrument	1–16
Filmarray	Biofire (biofiredx.com)	Respiratory tract/Blood infection	60	Nested real-time PCR	$ 125/test; $ 49,000 for analyzer	1
Cobas Liat	Roche (diagnostics.roche.com)	GAS/Influenza A&B/RSV	20	Real-time PCR	$ 30/test; $ 20,000 for analyzer	1
ID NOW	Abbott (abbott.com)	GAS/Influenza A&B/RSV/Novel coronavirus	13	NEAR/EXPAR	$ 100/test;	1
COVID-19 all-in-one test kit	Lucira Health (lucirahealth.com)	Novel coronavirus/Influenza A&B	30	RT-LAMP	$ 50/test	1
Automated NA analytical system	Ustar (bioustar.com)	MTB/Respiratory tract diseases/ Reproductive tract diseases	30	CPA and fluorescent detection	¥ 200,000 for analytical system	1–2
RTisochipTM-A	Capitalbio (cn.capitalbio.com)	Food-born microbe/respiratory tract pathogen	50	LAMP and fluorescent detection	¥ 100,000–420,000 for analyzer	1–24
Novodiag	Mobidiag (mobidiag.com)	CarbaR+/Novel coronavirus/C. difficile	60	Multiple RT-PCR and microarray detection	€45/test; €45,000 for analyzer	1–4
Idylla	Biocartis (biocartis.com)	Cancer-associated mutations	90–150	Real-time PCR	£115–395/test	1–8
ePlex	GeneMark Dx (genmarkdx.com)	Respiratory/Blood/Central nervous system infections	90	PCR and electrochemical detection	$ 90/test;	1–24
MDx platform	iCubate (icubate.com)	Non-tuberculosis Mycobacterium	300	Arm-PCR and fluorescent detection	¥ 700,000 for terminal instruments	1–4

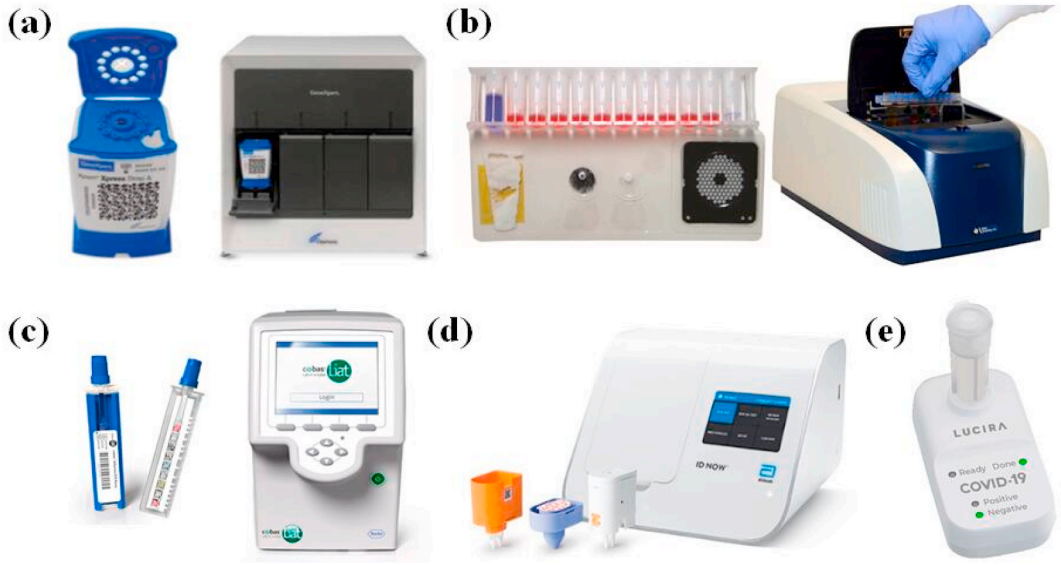

Fig. 2. Fully integrated commercial microfluidic devices for detection of nucleic acids. (a) GeneXpert® system developed by Cepheid. (b) Filmarray® developed by Biofire. (c) Cobis Liat® developed by Roche. (d) ID NOW® developed by Abbott. (e) COVID-19 all-in-one test kit® delivered by Lucira Health.

effective. For colorimetric signal readout, paper-based and chip-based fully integrated NAT devices are widely used. For instance, Chen et al. recently reported a simple and fully integrated paper chip composed of various types of paper for detecting gene mutations, where DNA extraction was completed with an FTA card and DNA detection was performed on an attached LFA with colorimetric signal readout based on aggregation of gold nanoparticles (Fig. 3a) (Chen et al., 2019). The whole detection can be completed in 90 min, in which LAMP reaction takes 60 min. This system is truly low-cost because all the materials involved in fabrication are affordable and no advanced instruments are needed during use with manual rotation of FTA card to different functional zones. A limitation to this proposed chip is lacking in the capability to perform multiplexed detection. To address this issue, parallel multiple testing is usually adopted. Trinh et al. developed a slidable and integrated paper/plastic hybrid chip with capability of detecting three common pathogens simultaneously (Trinh et al., 2019). In this chip, six embedded FTA cards in parallel were adopted for successive DNA extraction from bacteria, LAMP reaction, and colorimetric detection through manual sliding. Here, a novel fuchsin-mediated chromogenic method was employed for colorimetric detection of LAMP amplicons in the reaction chamber. Noteworthily, these paper-based integrated devices show excellent capacity for long-term storage of reagents, including amplification reagents and colorimetric indicators, which is crucial for promoting "sample-in-answer-out" integration where only sample needs to be added during testing. On the other hand, the manual operations including rotation, sliding and folding still need to be

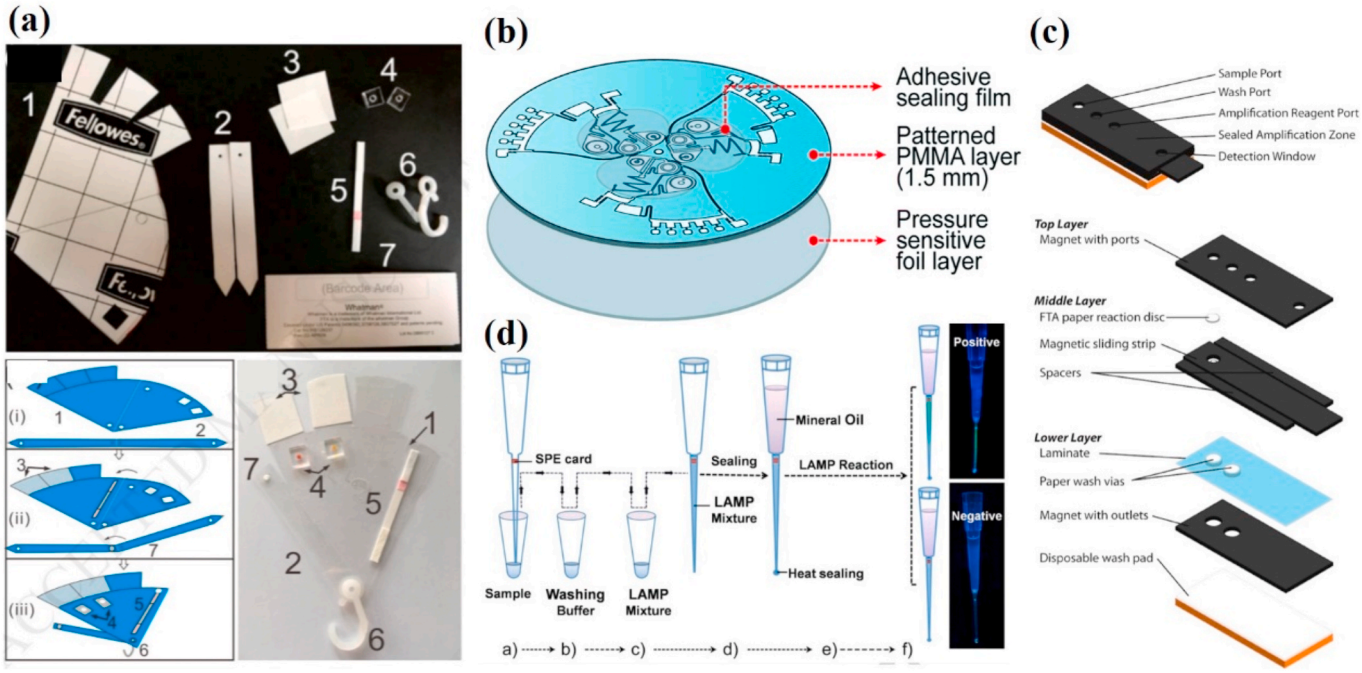

Fig. 3. Fully integrated microfluidic devices for qualitative detection of nucleic acids. (a) A fully integrated paper chip for EGFR mutation detection. (b) An integrated centrifugal microfluidic disc for multiple targets detection. (c) A "Paper Machine" for *Escherichia coli* detection. (d) A micro-pipette tip-based NAT in crude samples. Images reproduced from (Chen et al., 2019; Connelly et al., 2015; Lu et al., 2016; Oh et al., 2016).

addressed in paper-based devices. To increase automation, centrifugal chip-based devices with portable instruments for automatically implementing microfluidic operations (e.g., liquid flow, mixing, separation) are widely applied. As an illustration, an integrated centrifugal microfluidic disc together with a miniaturized rotary instrument was developed with the function of microbead-assisted NA extraction, LAMP-based amplification and eriochrome black T (EBT)-mediated colorimetric signal readout (Fig. 3b) (Oh et al., 2016). Different fluids involved were automatically released on the disc through rotating at different directions and speeds. Compared to paper-based devices, these devices possess higher automation and detection throughput. For example, Sayad et al. developed a centrifugal microchip with capability of performing 30 colorimetric analyses simultaneously (Sayad et al., 2018). Additionally, both liquid and solid reaction reagents (e.g., enzymes, primers, buffers) are easy to be integrated into these microchips, leading to high integration level. There are also other colorimetric indicators, such as silver nitrate (Trinh et al., 2020), calcein (Sayad et al., 2018), and pH indicator (Kadimisetty et al., 2018), which can be adopted for colorimetric signal readout. The major challenge for colorimetric signal-based NATs is its unsatisfactory detection limit.

To address the limited sensitivity of colorimetric detection, fully integrated NAT devices based on fluorescent signal readout have been developed. For instance, Connelly et al. presented a "Paper Machine" to detect *Escherichia coli* in human plasma (Fig. 3c) (Connelly et al., 2015). This system consists of a sliding-strip, a hand-held UV source and smartphone, which provides the functions of FTA-based extraction, LAMP-based amplification and SYBR Green-based end-point fluorescent signal readout. The detection process can be completed by sliding the FTA-contained strip to different areas. However, the throughput of the "Paper Machine" is unsatisfactory and the reaction reagents need to be added manually. To address this challenge, a micro-pipette tip-based NAT in crude samples was developed with the capability of high-throughput sample-to-answer detection of DNA or RNA (Fig. 3d) (Lu et al., 2016). With the help of pipette and tips, NA extraction based on FTA card was completed within 30 min. The process of LAMP-based amplification and calcein-based fluorescent signal readout was performed in the tips. The limit of detection (LOD) of this platform is 8 CFU of Ebola virus in *Escherichia coli*. Both the calcein-based signal and intercalating dye-based signal are the main forms for end-point fluorescent qualitative detection.

In short, the fully integrated microfluidic devices for NA qualitative testing have been well developed and widely used in recent years due to their excellent characteristics of being small, easy-to-operate and suitable for early diagnosis. The colorimetric signal of integrated devices can be read by naked eyes without any instrument, which reduces the requirements of cost and resources. By comparison, the fluorescent detection of integrated devices has lower LOD than the colorimetric ones. With the miniaturization of fluorescent readout devices, the sample-in-fluorescent-answer-out devices show good prospect for NA qualitative detection. The main challenge for commercialization of these qualitative devices is insufficient user-friendliness caused by multiple manual interventions during detection. And the detection sensitivity and stability need to be further improved to meet clinic demands.

4. Sample-in-quantitative-answer-out

For the applications where accurate detection results are required, sample-in-quantitative-answer-out technologies can provide accurate information for optimum clinic intervention. To realize this purpose, a variety of quantitative detection technologies based on electrochemistry, fluorescence, absorbance, and surface plasmon resonance (SPR) have been proposed.

Electrochemical microchip based on patterned electrodes holds the capability for integrated quantitative NA detection (Hsieh et al., 2015; Koo et al., 2018). For example, Kevin et al. reported an integrated electrochemical microchip for circulating tumor DNA (ctDNA) detection, containing a central cell lysis zoom and four branched amplification and detection zooms (Fig. 4a) (Koo et al., 2018). The NA extraction was completed through dc 5 V loading in 60 s, the NA amplification was performed through RPA, and the quantitative readout was obtained through chronoamperometry. Similarly, Andrew et al. also developed an electrokinetic NA extraction method called isotachophoresis (ITP) on paper substrate from serum and whole blood, combined with in situ RPA technology to fulfill NA extraction, amplification and results readout (Fig. 4b) (Bender et al., 2018). This paper-based ITP exhibits highly efficient separation of NA from complex samples with the aid of a discontinuous buffer system together with an electric field, showing outstanding simplicity and portability for POC applications due to its minimal intervention, accessible raw materials and good capability for large sample processing. The obtained detection range of human immunodeficiency virus-1 (HIV-1) DNA in serum was from 10^4 to 10^7 copies/mL, meeting the needs for clinical testing.

Fluorescent signals based on dye molecule labels have been widely introduced on POC devices for quantitative readout through image processing software or portable APP. Thus, combined with isothermal amplification methods, microfluidic chips with the capability of fluorescent signal output are able to realize sample-to-quantitative-answer-out for NA detection. For instance, Sun et al. developed a disposable eight-channel cyclic olefin copolymer (COC) microfluidic chip together with a reusable peripheral control and analysis system for quantitative detection of *Salmonella* in food (Sun et al., 2015). Eight channels on chip hold the capability of handling eight samples simultaneously, as NA extraction, amplification and detection of one sample is completed in a single channel. Here, real-time LAMP based on SYTO-62 dye in eight channels was carried out through ESE log detector (Qiagen, Germany) for quantitative signal readout, where threshold time similar to the threshold cycle in real-time PCR was recorded as an indication of NA concentration. However, the reagents involved in the assay including washing buffers and LAMP mixtures were stored in PCR tubes connected to the chip through plastic tubes, which may limit its field use. To address this limitation, the most effective way is to increase the integration and miniaturization of peripheral system. For instance, Kadimisetty et al. developed a four-channel microfluidic chip embedded with a NA isolation membrane in each channel for NA extraction (Fig. 4c) (Kadimisetty et al., 2018). Compared to magnetic beads-based NA extraction method, membrane-based ones are much easier to integrate into chips without the need for magnetic field loading. Here, the authors developed a miniaturized 3D printed battery-operated reactor composed of a portable heater module for LAMP reaction and a USB fluorescence microscope for real-time fluorescence analysis through Eva Green dye. This proposed device exhibits good compatibility with different samples, such as plasma and cerebrospinal fluid.

Absorbance is another quantitative signal, which can be combined with isothermal amplification methods for quantitative NAT. Recently, Wang et al. proposed an integrated chip with on-chip reagents storage for quantitative detection of *Salmonella* relying on smartphone-based real-time LAMP turbidity monitoring (Fig. 4d) (Wang et al., 2020). The viable bacteria DNA was extracted through propidium monoazide treated magnetic nanoparticles. The real-time quantitative detection data was transferred to smartphone through Bluetooth module during LAMP reaction. As a result, the feasibility of this chip was verified by detecting viable *Salmonella* in spiked chicken meat with the LOD of 14 CFU/mL in 1.5 h. Although the chip is highly integrated with the capability of sample-in-answer-out, the main possible obstacle for hindering its practical application lies in the high complexity of peripheral system, such as light source, light photodiodes, rotatable magnetic fields and precise pumps.

SPR, characterized by its label-free biomolecular quantification, is promising for POC nucleic acid testing through combination with microfluidic chip coated with plasmonic nanomaterials, exhibiting favorable features of excellent sensitivity and real-time information output (Bonyár 2020). The detection principle is based on monitoring

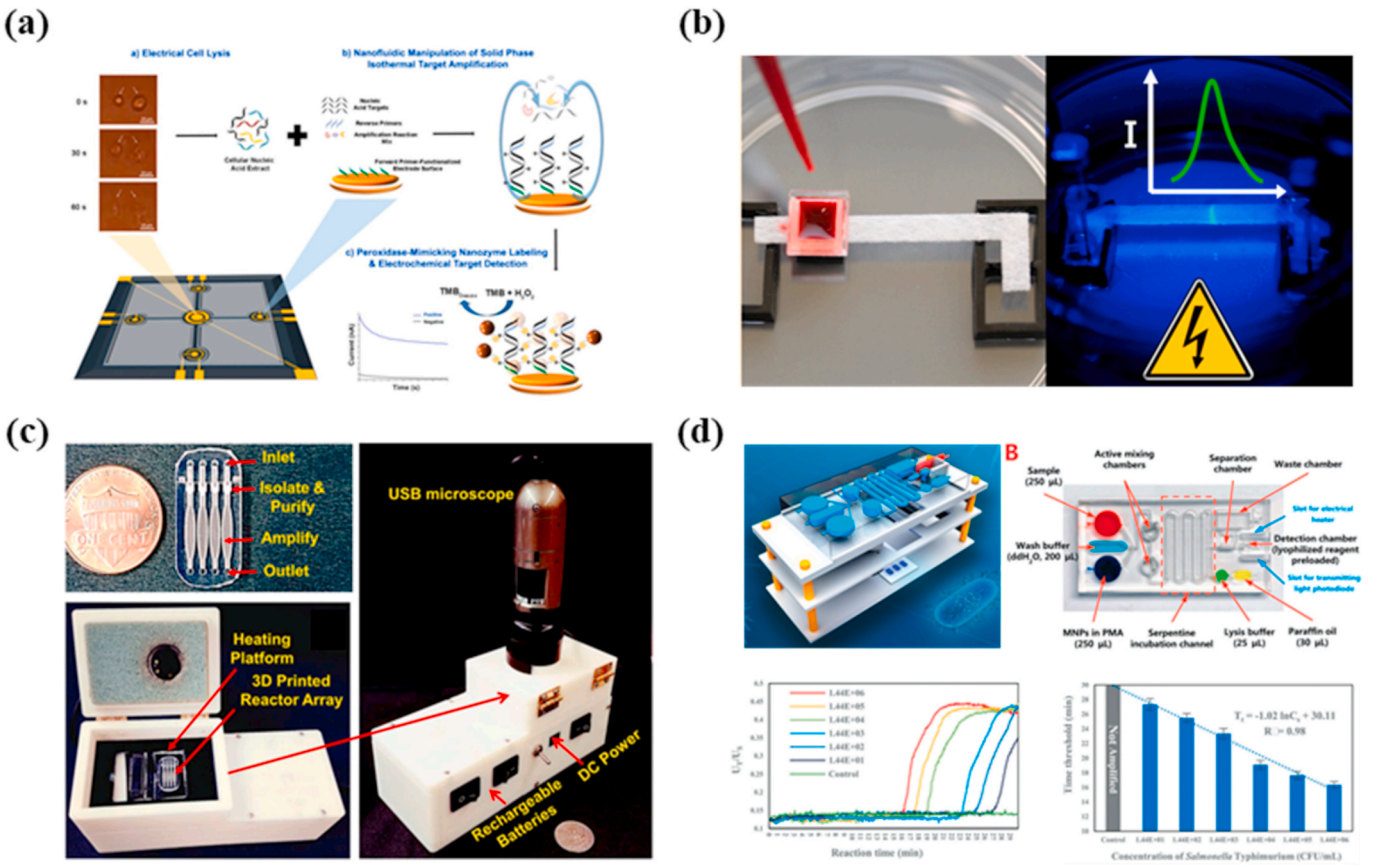

Fig. 4. Fully integrated microfluidic devices for quantitative detection of nucleic acids. (a) An integrated electrochemical chip for NA detection. (b) A paper-based integrated NAT device based on combination of ITP and RPA. (c) A miniaturized 3D printed battery-operated reactor composed of a portable heater module and a USB fluorescence microscope for chip analysis. (d) An integrated chip for viable *Salmonella* detection based on real-time turbidity LAMP monitoring. Images reproduced from (Bender et al., 2018; Kadimisetty et al., 2018; Koo et al., 2018; Wang et al., 2020).

the refractive index change on the chip surface. For instance, Xue et al. developed a SPR-based miRNA sensor by employing antimonene nanosheets onto Au film with LOD of 10 aM (Xue et al., 2019). Compared to traditional PCR-based detection techniques, SPR holds the superiority of elimination of time-consuming and laborious amplification process. Recently, a portable POC nanoplasmonic biosensor for direct detection of pseudovirus based on SPR was developed (Adegoke et al., 2017). Owing to elimination of nucleic acid extraction and amplification, the detection can be completed in 15 min with LOD of 4000 virus particles. Such POC device could facilitate rapid diagnosis of viral infectious diseases. However, the SPR-based POC device is still in its infancy, mainly because of lacking clinical validation of real samples, in which complex components such as cell debris may pose a big challenge to detection specificity. Thus, further efforts are still urgently needed to increase the sensing selectivity and miniaturize optical setups.

Different from qualitative detection, quantitative detection with better sensitivity can provide the ability of dynamic monitoring and disease classification, thus showing great potential for wide clinical application. Similar to the classical real-time PCR for quantitative NA detection, real-time isothermal amplification (e.g., real-time LAMP and real-time RPA) methods based on periodic acquisition of fluorescence or absorbance signal have been widely applied for quantitative results output. But these current quantitative techniques need complex peripheral signal processing systems, including signal capture, analysis and transfer modules. So most existing sample-in-quantitative-answer-out technologies turn into the format of a disposable chip together with a reusable instrument. For practical POC applications, instrument independent detection manner is preferred, especially for resource-poor areas. Thus, the miniaturization of analysis instrument is significant to increase portability and save energy, making it well suitable for field use. Meanwhile, the transformation of computer-based data processing into mobile terminal would also promote commercialization. On the other hand, existing portable commercially available quantitative devices, such as glucometer (Gu et al., 2018) and pressuremeter (Liu et al., 2017), have been introduced for indirect NA quantification, showing great potential to commercialize instrument-free integrated quantitative NA detection.

5. Sample-in-digital-answer-out

As discussed above, fully integrated NAT technologies with qualitative and quantitative readout have attracted considerable interests during the past decades and shown impressive capability in biomedical applications. But for the case where the target NA concentration is particularly low, such as detection of cell-free NAs associated with tumors, the sensitivity of these methods is hardly satisfactory. Benefiting from the development of emerging digital PCR technology, one of the most sensitive methods for NAT, researchers are now devoted to integrating digital detection signal readout into POC NAT microfluidic devices to make these devices suitable for absolute quantification of low abundance NA.The integrated microfluidic devices with digital answer out can be classified into microchamber-based ones and droplet-based ones.

The main challenge to realize digital signal readout on such POC microfluidic devices is the dispersion of NA copies, which often requires complicated microstructures, external driving force and power supply. To address this, Lee's group proposed a self-powered integrated microfluidic chip to realize automatic dispersion of NA samples with the aid of

an integrated vacuum battery on chip, which eliminates the need of pumping system and power sources (Fig. 5a) (Yeh et al., 2017). In this work, NA amplification was performed in plasma samples by RPA without need of NA extraction. When the blood sample mixed with RPA reagents was added, blood plasma was separated and dispersed into hundreds of microchambers precoated with amplification initiator for digital RPA, while blood cells were trapped in flow channel to avoid hemoglobin interference. After 30 min of isothermal amplification on a reusable and portable heater, digital answer out was obtained. This integrated digital RPA method is easy-to-use, rapid-to-analyze and equipment independent, showing great potential for on-site absolutely quantitative NAT. However, the number of microchambers for NA dispersion is only 224 probably due to the limited pressure source supplied by the vacuum battery, which may cause negative effect on detection sensitivity and detection range. Besides, the capability for multiplex NAT on this platform is also inadequate. To address this, Mu's group developed an integrated multiplex digital RPA chip with the capability of simultaneously detecting three targets, creating a "sample-in-multiplex-digital-answer-out" strategy (Fig. 5b) (Yin et al., 2020). This chip consists of two functional parts: NA extraction and digital amplification. With cooperation of screw valves and vacuum-based pumping, the NA extraction was completed in 15 min by manual movement of magnetic beads for cell lysis, washing and elution on chip, and the successive digital RPA was implemented after self-priming compartmentalization of extracted NA copies into microchambers with a total number of 12,800. It is worth noting that the reagents for RPA reaction including primers, probes and enzymes were lyophilized on chip, enabling the chip for field application. Although these microchamber-based digital integrated devices have exhibited good detection performance and the NA extraction and amplification can be completed on chip without need of any equipment, the integration and miniaturization of fluorescent images acquisition and analysis system still need to be addressed.

Compared to microchamber-based digital readout strategies, droplet-based ones offer the advantage of flexibility in designing the number of droplets for NA dispersion (range from tens of thousands to millions), thus leading to superior detection sensitivity and detection range. The main difference is generating water-in-oil microdroplets instead of constructing microwells on chip. Considering that the digital signal readout strategy is end point detection, smartphone-based visual readout manner is well suited to capture and analyze fluorescence image through phone camera and designed APP, finally giving expected numerical detection value. Hu et al. developed a droplet-based digital LAMP integrated chip based on smartphone imaging and analyzing (Fig. 5c) (Hu et al., 2019). The chip consists of immiscible phase filtration-based NA extraction, cross channel-based droplet formation, and tilling cavity-based droplet storage for digital LAMP amplification. The whole detection including NA extraction from spiked plasma can be completed in 60 min. But this method suffers from complicated manual operations, such as reagents preparation and adding, hand-held magnet movement, and the need for a negative pressure pump for microdroplets formation. Furthermore, to reduce manual operations, a fully integrated digital droplet PCR microdevice for detecting ctDNA mutation was developed recently with the aid of sophisticated control and detection system designed to improve automation (Fig. 5d) (Geng et al., 2020). The detection sensitivity for EGFR Gene T790 M mutation by using this device can reach to 1% mutant from 2 mL of plasma sample, even taking ctDNA extraction efficiency in account.

Although fully integrated sample-in-digital-answer-out strategies have been developed and shown great promise in absolute

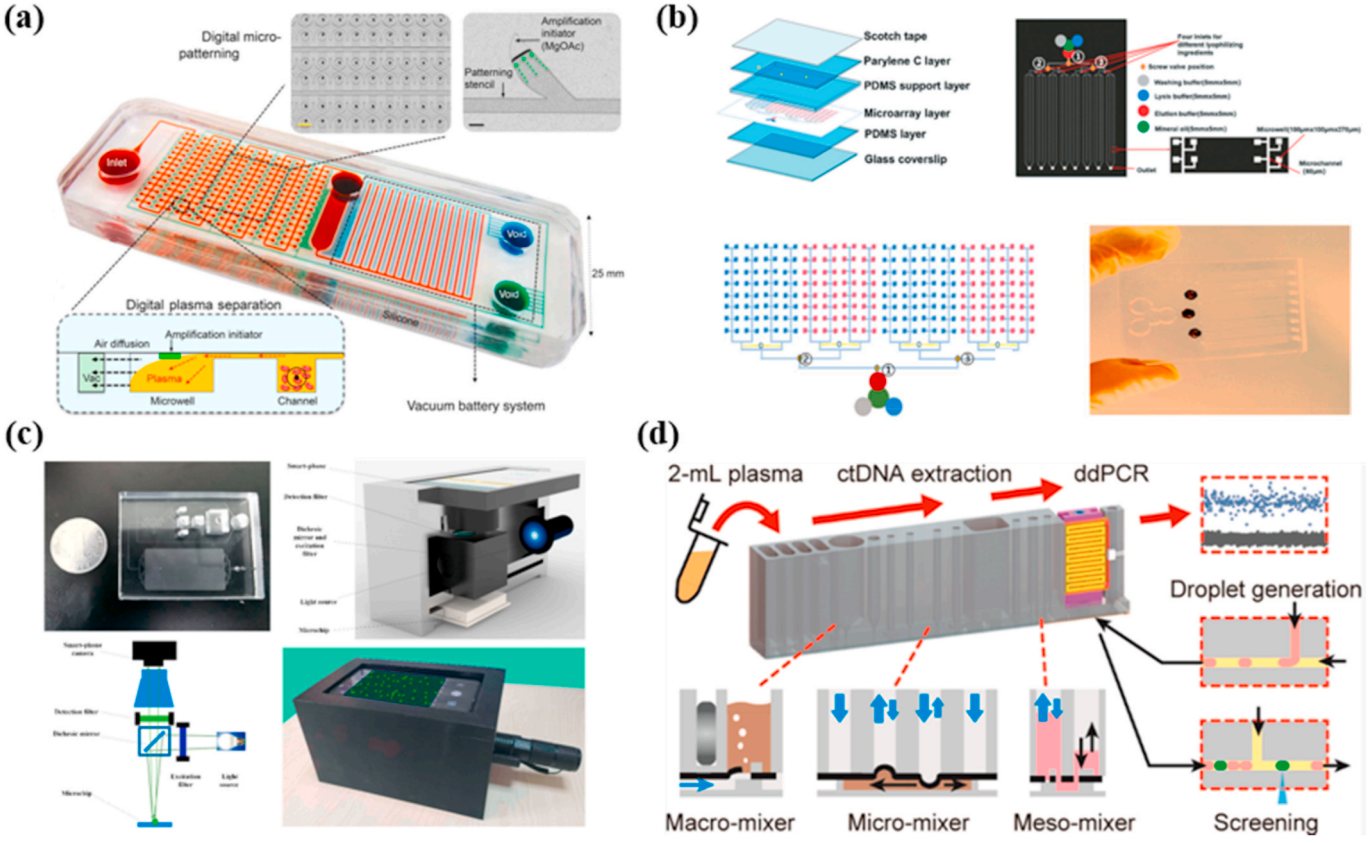

Fig. 5. Fully integrated microfluidic devices for digital detection of nucleic acids. (a) Self-powered integrated microfluidic point-of-care low-cost enabling (SIMPLE) chip for detecting DNA directly from human plasma. (b) A "sample-in-multiplex-digital-answer-out" chip for fast detection of pathogens. (c) An integrated droplet-based digital LAMP device based on smartphone imaging and analyzing. (d) An integrated droplet-based digital PCR device for ctDNA detection. Images reproduced from (Geng et al., 2020; Hu et al., 2019; Yeh et al., 2017; Yin et al., 2020).

quantification of low abundance NA, some challenges still exist for their commercialization. It is hard to realize minimal manual operation (e.g., just sample adding) under the premise of compact control and detection system. Especially for NA extraction from complex sample on chip, magnetic beads-based extraction methods are widely used, where manual movement of beads between different zones is inevitable. Additionally, the extracted NA samples need to mix with reaction mixture for amplification, which is hard to automatically operate within miniaturized instruments. By comparison, microchamber-based strategies are more suitable for constructing integrated digital POC devices than droplet-based ones, as the driving force supplied by portable vacuum-based pumping system is sufficient for NA dispersion into microchambers, while droplet-based strategies usually need additional injection pumps. The next optimization of microchamber-based strategies should focus on increasing the number of microchambers with small footprint. The detection robustness for digital NAT still needs to be improved, as its detection accuracy is easily disturbed by operator-based variations (e.g., sample adding).

6. Conclusion and future perspectives

Global health, especially in developing countries, has gained great concern due to the high morbidity and mortality from infectious diseases such as AIDS, dengue, malaria, Zika and more recent COVID-19. Because abnormal nucleic acids changes occur at early stage of the disease, NAT has been widely used in various applications. Compared to lab-based NAT methods, the attractive features of the fully integrated microfluidic devices at POC are low-cost, time-saving and portable at resource-limited areas. In this review, we have introduced fully integrated microfluidic devices for qualitative, quantitative and digital NAT of pathogens, oncogene, genetic diseases and so forth (Table 2). These devices integrate the steps of NA extraction, NA amplification and signal readout, where the basic detection principle is transforming the presence of targets to readable signals, such as optical, electrochemical and magnetic signals. Although many research groups have reported various NAT devices for POC, there are still some challenges to be addressed (Fig. 6): (a) high requirements for professional level of users; (b) complex conditions for reagents storage and transportation; (c) environment-dependent reactions and materials; (d) low throughput; (e) large and electric-powered analyzers. In addition to scientific researches, we also presented the well-known commercial devices for NAT. Most of the architecture of these platforms consists of a disposable cartridge and an analyzer.

Fig. 6. Future perspectives of integrated microfluidic devices for nucleic acids testing at point of care.

Under the current background of COVID-19 pandemic and possible normalization of pandemic in future, integrated POC NAT devices would play a crucial role in rapid disease screening due to their rapid turnaround time, elimination of professional and centra labs. Such devices could be manufactured at large batch and distributed in pandemic area quickly, enabling people respond quickly when an outbreak is detected.

Table 2
Summary of detection nucleic acids on the fully integrated microfluidic devices.

Targets	Samples	LOD	Time (min)	Amplification	Signal	Readout	Refs.
$EGFR_{L858R}$	Lung adenocarcinoma cells	3 cells	90	LAMP	Colorimetry-LFA	Qualitative	Chen et al. (2019)
E. coli/S. Typhimurium /V. parahaemolyticus /L. monocytogenes	Milk	10 bacteria	65	LAMP	Colorimetry-EBT		Oh et al. (2016)
E. coli	Spiked plasma samples	5 cells	60	LAMP	Fluorescence-SYBR Green I		Connelly et al. (2015)
E. coli/Ebola virus plasmids/CK19 mRNA in cancer cells	Potatoes	8 CFU for *E. coli* 2 copies for *Ebola*	90–160	LAMP	Fluorescence-Calcein		Lu et al. (2016)
Salmonella	Pork meat	50 cells per test	40	LAMP	Fluorescence	Quantitative	Sun et al. (2015)
Plasmodium falciparum/Neisseria meningitides	Plasma/Cerebrospinal fluid	100 fg 50 CFU	50	LAMP	Fluorescence		Kadimisetty et al. (2018)
HIV-1 DNA	Human serum	10^4 copies/mL	20	RPA	Fluorescence-Turbidity		Bender et al. (2018)
Salmonella	Chicken meat	14 CFU/mL	90	LAMP			Wang et al. (2020)
Methicillin-resistant S. aureus	Human blood	10 copies/μL	30	dRPA	Fluorescence-exo probe	Digital	Yeh et al. (2017)
E. coli/S. enterica /L. monocytogenes	Milk	10 bacteria	45	dRPA	Fluorescence-exo probe		Yin et al. (2020)
EGFR L858R mutation	Plasma	10 copies/μL	60	dLAMP	Fluorescence-Calcein		Hu et al. (2019)
ctDNA of T790M mutation in cancer cells	Plasma	1% mutation	210	dPCR	Fluorescence-SYBR Green I		Geng et al. (2020)

For the application in pandemic, such devices should be designed into enclosed formation to protect operator and environment. And easy sampling and pretreatment matching with such devices should be taken into consideration, as these integrated devices are expected to be used outside of central labs with non-skilled operator with basic precautions, even at home. The detection throughput and time are also key factors for pandemic application, as rapid detection with high throughput can dramatically increase detection efficiency during a certain time. Besides, household NAT devices are promising during a pandemic. Especially, the all-in-one test kit® developed by Lucira Health for COVID-19 testing has just been approved by Food and Drug Administration (FDA), which is the first NAT device approved for self-COVID-19 testing at home. The cost of such household devices is the key to popularization and application. In short, affordable, rapid, high-throughput, enclosed and highly integrated NAT devices are expected to cope with possible pandemic in the future.

Benefiting from the emerging technologies such as isothermal amplification methods (Giuffrida and Spoto 2017; Ye et al., 2018), clustered regularly interspaced short palindromic repeats-associated proteins (CRISPR-Cas)-based assays (Broughton et al., 2020; Ding et al., 2020; Shen et al., 2020), freeze drying technologies (Deng and Jiang 2019; Kumar et al., 2020) and daily gadget-based technologies (Michael et al., 2020), the fully integrated microfluidic devices at POC will be further developed to realize NAT with better performance and be alternative choices to replace traditional NAT protocols in the future. Therefore, **m**iniaturized, **a**utomatic, **r**eagent-preloaded, **c**ommercializable, **h**igh-throughput, **e**nvironment-independent and **d**isposable (**MARCHED**) NAT devices are expected. Besides, the development of NAT devices will be toward personalized medicine, gene screening and intensive care, and might even combine with artificial intelligence (AI) (Liang et al., 2019; Mei et al., 2020; Yu et al., 2018) in the future. We believe that the fully integrated microfluidic devices can provide the precious opportunities for early, accurate, rapid and low-cost NAT at both developed and developing countries.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Key R&D Program of China (2018YFC1707700), the National Natural Science Foundation of China (21904104, 11761161004), the Key Program for Science and Technology Innovative Research Team in Shaanxi Province of China (2017KCT-22), the General Financial Grant from the China Postdoctoral Science Foundation (2020M673418) the Opening Project of Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Research, College of Stomatology, Xi'an Jiaotong University (2019LHM-KFKT005 and 2019LHM-KFKT006) and the Fundamental Research Funds for the Central Universities (xzy012019067, 1191329125).

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