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Digital PCR-free technologies for absolute quantitation of nucleic acids at single-molecule level

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ABSTRACT

Ultrasensitive detection of nucleic acids is of great significance for precision medicine. Digital polymerase chain reaction (dPCR) is the most sensitive method but requires sophisticated and expensive instruments and a long reaction time. Digital PCR-free technologies, which mean the digital assay not relying on thermal cycling to amplify the signal for quantitative detection of nucleic acids at the single-molecule level, include the digital isothermal amplification techniques (dIATs) and the digital clustered regularly interspaced short palindromic repeats (CRISPR) technologies. They combine the advantages of dPCR and IATs, which could be fast and simple, enabling absolute quantification of nucleic acids at a single-molecule level with minimum instrument, representing the next-generation molecular diagnostic technology. Herein, we systematically summarized the strategies and applications of various dIATs, including the digital loop-mediated isothermal amplification (dLAMP), the digital recombinase polymerase amplification (dRPA), the digital rolling circle amplification (dRCA), the digital nucleic acid sequence-based amplification (dNASBA) and the digital multiple displacement amplification (dMDA), and evaluated the pros and cons of each method. The emerging digital CRISPR technologies, including the detection mechanism of CRISPR and the various strategies for signal amplification, are also introduced comprehensively in this review. The current challenges as well as the future perspectives of the digital PCR-free technology were discussed.

1. Introduction

Sensitive and accurate detection of nucleic acid is of great importance in many fields, from basic biomedical research to clinical diagnostics [1–3]. Polymerase chain reaction (PCR) is an artificial method for DNA amplification inspired by intracellular DNA replication, with thermal cycling between three temperatures, that enables exponential amplification of target DNA [4]. PCR can be monitored in real-time with fluorescent dyes or probes, and the cycle threshold (Ct) is used to quantify the templates, which is called quantitative real-time polymerase chain reaction (qPCR) [5]. Up to now, the qPCR remains the gold standard for nucleic acid detection due to its versatility, robustness, and sensitivity. However, qPCR requires sophisticated equipment for precise thermal cycling, professional trainees, and complicated operating processes. Therefore, it is labor-intensive and time-consuming, which is not suitable for point-of-care testing (POCT). Furthermore, the quantification results of qPCR rely on the standard curve and are susceptible to variability in amplification efficiency, especially when the initial concentration of the template is low. dPCR enables highly sensitive and accurate quantification of target nucleic acids beyond the traditional qPCR and has attracted much attention in recent years [6]. In dPCR, the sample is divided into tens of thousands of independent partitions, so that each partition contains a few or no target nucleic acids. Each partition acts as an isolated reaction system, and the fluorescence of each unit is recorded and calculated as the outputting signal after PCR, which converts the exponential analog nature of PCR to a linear digital signal, thus eliminating the requirement for standard curves or reference materials [7]. Based on the particular working principle, dPCR shows many notable strengths, such as absolute quantification, single-molecule sensitivity, and low background signal influence. However, dPCR maintains demand for thermal cycling equipment and complicated operating processes.

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To overcome the limitations of the PCR, PCR-free methods, such as alternative isothermal amplification techniques (IATs) have been developed. Representative IATs include loop-mediated isothermal amplification (LAMP) [8], recombinase polymerase amplification (RPA) [9], strand displacement amplification (SDA) [10], nucleic acid sequence-based amplification (NASBA) [11], and rolling circle amplification (RCA) [12]. There have been comprehensive reviews discussing the principles and applications of various IATs [13–16]. One major advantage of IATs is the reduced equipment requirements for temperature control, which lowers the cost and simplifies the operation. Therefore, the IATs hold great promise for low-resource implementation and greatly expand the application range of nucleic acid amplification technology. However, few of the IATs have been approved for clinical applications, because the non-specific amplification and poor reproducibility compromise the detection sensitivity, specificity, and reliability. There is still an unmet need that combine the cost-efficiency and convenience of IATs with the diagnostic accuracy of PCR. dIATs are expected to fulfill this unmet need, which combines the advantages of dPCR and IATs. Compared to conventional IATs, dIATs are able to obtain absolute quantification at the single-molecule level, with better reproducibility and strong tolerance to inhibitors. In addition, dIATs inherit advantages from the IATs, such as fast reaction speed and low instrumentation complexity. Therefore, dIATs have become powerful tools for nucleic acid analysis, especially in POC diagnostic tests.

Recently, the CRISPR and CRISPR-associated (Cas) systems have been demonstrated for the detection of nucleic acids with high sensitivity. The Cas nucleases are guided by CRISPR RNA (crRNA), which enables to detect the target nucleic acid sequence with single-nucleotide target specificity. Similar to the dIATs, CRISPR-based methods could be performed at an isothermal condition, which is beneficial for POC diagnostics. The CRISPR assays could be combined with digital platforms to improve the sensitivity and quantification accuracy. Because these digital assays enable to quantitatively detect the nucleic acids at the single-molecule level such as dPCR, but do not rely on thermal cycling to amplify the signal, we define them as "digital PCR-free technologies". Recently, many digital PCR-free technologies have been developed for the diagnosis of infectious pathogens and other diseases. However, there is still a lack of comprehensive review in this field. In this review, we summarized the various dIATs and the emerging digital CRISPR technology. The current challenges of different detection strategies for their applications in clinical and POCT settings are discussed along with the prospect and the future direction.

2. Digital isothermal amplification technologies (dIATs)

2.1. Digital LAMP (dLAMP)

LAMP is one of the most widely used isothermal amplification methods. As shown in Fig. 1A, two or three pairs of specific primers were used, which recognize distinct regions on the target sequence. The target amplification relies on the Bst DNA polymerase which has high-strand displacement activity and acts as the substitute for the Taq polymerase. During the reaction, the strand-displacement DNA synthesis is continuously auto-cycling, thereby achieving rapid amplification. Usually, the LAMP could be completed in 30 min at 65 °C. By combining with reverse transcription (RT), target RNA could be detected. In this work, based on the ways of partitioning, we classified dLAMP into chambers-based dLAMP and droplets-based dLAMP (Fig. 1B).

2.1.1. Chamber-based dLAMP

The first on-chip dLAMP was developed by Chiu and coworkers on a self-digitization (SD) chip platform (Fig. 1C) [17]. The microfluidics device spontaneously partitions samples into thousands of microchambers based on viscoelastic fluid phenomena driven by the geometric structure. However, the original SD chip needs a long time for sample loading, which limits its practical application. Therefore, the SD chip was further optimized, which significantly improved the loading efficiency, and increased the detection throughput [18,19]. The advanced SD chip was demonstrated to quantify HPV DNA accurately and is favorable for high-throughput and automatic detection. Similarly, a self-priming compartmentalization (SPC) microfluidic chip platform for dLAMP was developed [20]. This chip meets the laboratory's requirements for nucleic acid detection devices: power-free, valve-free, low-cost, and sensitive. It, therefore, has great potential for POCT application for accurate diagnosis of severe infectious pathogens, such as *Vibrio parahemolyticus* [21]. Besides, a 3D multi-chamber-layer chip further increased partitions to achieve the higher throughput dLAMP without increasing the chip size recently [22].

Slipchip is also a common device used in chamber-based dLAMP assays. It consists of two closely contacted plates that have fluidic channels. After the sample loading, the structure-induced self-partition could be achieved through a slip-slide process, which eliminated the cross-contamination and improved the fault tolerance of the operation [23]. Due to its simplicity, the platform was used to assess the performance of dLAMP and was demonstrated to improve efficiency [24,25]. Moreover, it could realize the visual readout of the single-molecule dLAMP with an indicator dye [26]. Another group improved the designs and developed a serial dilution SlipChip. The multistep Slipchip and the "chain of pearls" channel Slipchip enriched the types and further optimized the performance of Slipchips (Fig. 1C). Thus, the limit of detection (LOD) of the dLAMP assay could reach as low as 1 fg/μL [27,28].

Avoiding the complicated process and time consumption of chip fabrication, dLAMP could also be performed with membranes without sacrificing efficiency and sensitivity [29–31]. The micropores in the porous membrane trap the target pathogen *in situ* and each functions as the individual reaction unit, so that the single membrane covers all the processing steps of dLAMP. Moreover, some hydrogel-based dLAMP were also reported [32–35]. In these works, the inside nanostructure of hydrogel could act as a temporary micro-chamber for the reaction.

In summary, in the chamber-based dLAMP, the partition, heating, and analysis could be completed in one microfluidic device, avoiding aerosol pollution. Besides, the amplification process in each chamber could be real-time monitored in these devices. For example, all steps of dLAMP could be integrated into a chip, which eliminated the droplet coalescence during the heating process [36]. Also, this microfluidic device was reusable and easily integrated into a portable instrument, which is beneficial for POCT applications. Even though chamber-based dLAMP has these advantages, complex fabrication processes are needed. It increases the cost, limits the total compartment number, and compromises the practical value of chamber-based dLAMP.

2.1.2. Droplet-based dLAMP

Compared to the chamber-based dLAMP, the droplet-based dLAMP enables partition LAMP mixtures by emulsifying droplets with much simpler structures, without the need for complicated fabrication of microchambers.

In droplet-based dLAMP, the chips with classic T-junction [37] or flow-focusing geometry [38] were commonly used. Some other chips are also reported (Fig. 1D). These assays always relied on the external pump. However, the special design of the chips allowed the droplet generation simpler. For example, Friedrich *et al.* developed a disposable polymer chip and the droplets were generated by centrifuging with standard laboratory devices [39]. Simi-

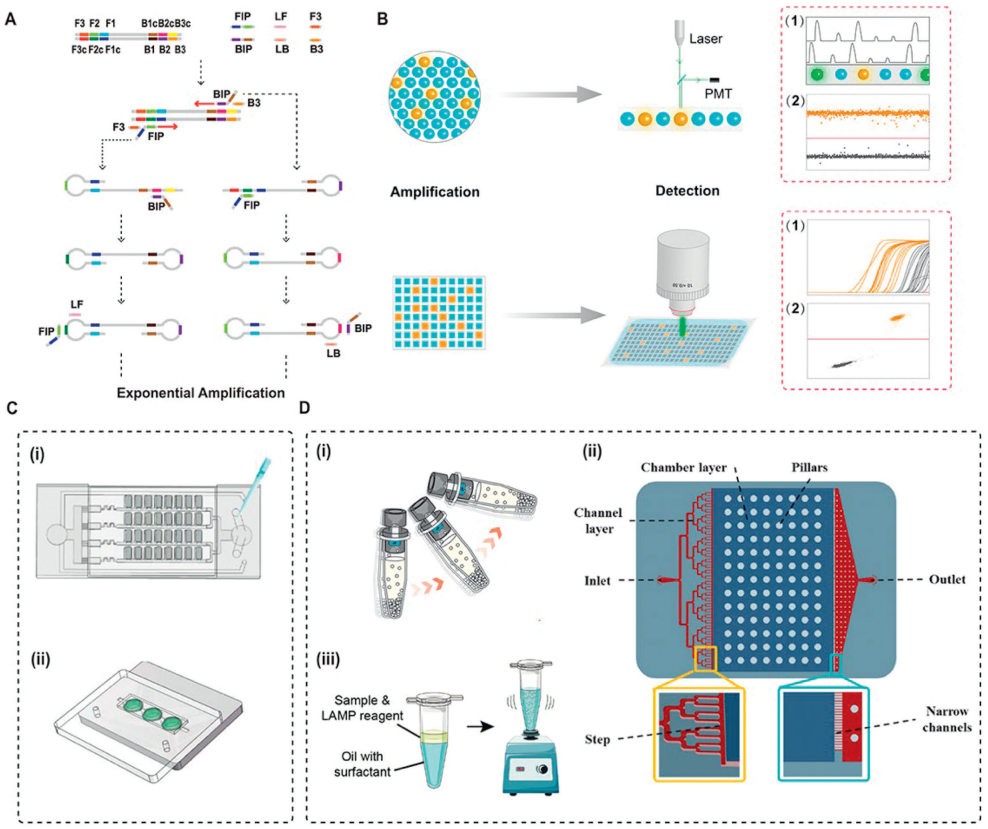

Fig. 1. (A) Schematic amplification principle of LAMP. (B) Schematic diagram of workflow for digital isothermal amplification assay. The sample partition is commonly based on two ways. One is the droplet-based method, which generates water-in-oil droplets relying on the shear force and surface tension. The other is the chamber-based method, which mainly utilizes devices such as microfluidic chips to divide aqueous solutions into different microchambers. The amplification reaction and detection all take place in each individual compartment. (C) Chips utilized in the works of the chamber-based dLAMP: (i) self-digitization chip. Reprinted with permission [19]. Copyright 2021, American Chemical Society. (ii) Slipchip. Reprinted with permission [28]. Copyright 2020, Elsevier. (D) Droplet generation methods performed in the works of the droplet-based dLAMP: (i) centrifugation. Reprinted with permission [41]. Copyright 2001, Royal Society of Chemistry. (ii) multi-layer microfluidic chip. Reprinted with permission [56]. Copyright 2022, Elsevier. (iii) vortexing. Reprinted with permission [45]. Copyright 2022, The Authors.

larly, Peng *et al.* conducted a ddLAMP assay with a centrifugal microfluidic device. This chip could generate and store the droplet which further solved the problem of droplet coalescence [40].

Moreover, some chip-free methods have also emerged. The group of Huang reported a rotational scan dLAMP with low cost, reliability, and facileness, called RS-dLAMP (Fig. 1D) [41]. By controlling centrifugation and a coaxial cylinder with the centrifuge tube, they could perform statistical analysis on the droplets in three dimensions. Zhang *et al.* developed a centrifugal-driven rotating bent capillary and the samples could be segmented into homogeneous droplets [42]. The novel droplet-generator-assisted dLAMP has been demonstrated with a wide dynamic range and a single-copy DNA sensitivity. Du's group developed a cross-interface emulsification (XiE) platform for dLAMP [43]. By using a vibrating capillary, droplets with tunable volume from tens of picoliter to nanoliters can be generated flexibly. This Xie-based dLAMP has been validated for quantifying H5-subtype HPAI viruses with great sensitivity and specificity. Besides, Pan *et al.* reported a strategy using immersed AC electrospray (iACE) for the monodispersed droplet generation [44].

Compared with monodisperse droplet generation methods, the following polydisperse droplet generation method is simpler, more convenient, and more time-saving. Chen *et al.* reported the rapid droplet partition by vortexing and performed the quantitative analysis of polydisperse droplets with deep learning (Fig. 1D) [45]. The deep-dLAMP was validated to provide accurate measurements of nucleic acid concentrations flexibly and efficiently.

Similar to the chamber-based dLAMP, integrated droplet-based microfluidic platforms were also developed recently for the miniaturization of IATs processing [37,46-53]. Rane *et al.* reported a continuous flow dLAMP assay implemented on a microfluidic droplet device [54]. In this work, the continuous flow setting eliminated the limitations on the reaction throughput. Thus, the device has great potential for large screening. Novel strategies have further improved the integrated platforms. For example, Jiang *et al.* utilized magnetic beads to capture the target RNA, which conducted dLAMP with ultrasensitivity, specificity, portability, rapidity and user-friendliness [52]. Besides, smartphone-based imaging systems further simplified the operation and reduced the cost of dLAMP, such as the fast readout strategy [47] and the 3D printed microfluidic platform [55].

2.1.3. A summary of dLAMP

In summary, dLAMP has been widely used in POC diagnosis. However, there are still problems limiting its application. One issue is that dLAMP tends to show false positive signals, which results in the overestimation of quantification. In chamber-based dLAMP, the amplification process in each chamber could be monitored in real-time, and thus false positive signals caused by the primer dimer can be recognized based on the algorithms. On the contrary, the droplet-based dLAMP usually employs end-point readout.

In theory, LAMP requires the primers to anneal in the correct order of FIP/BIP first, then F3/B3, and finally LF/LB. Any other primer hybridization order will affect the amplification speed and

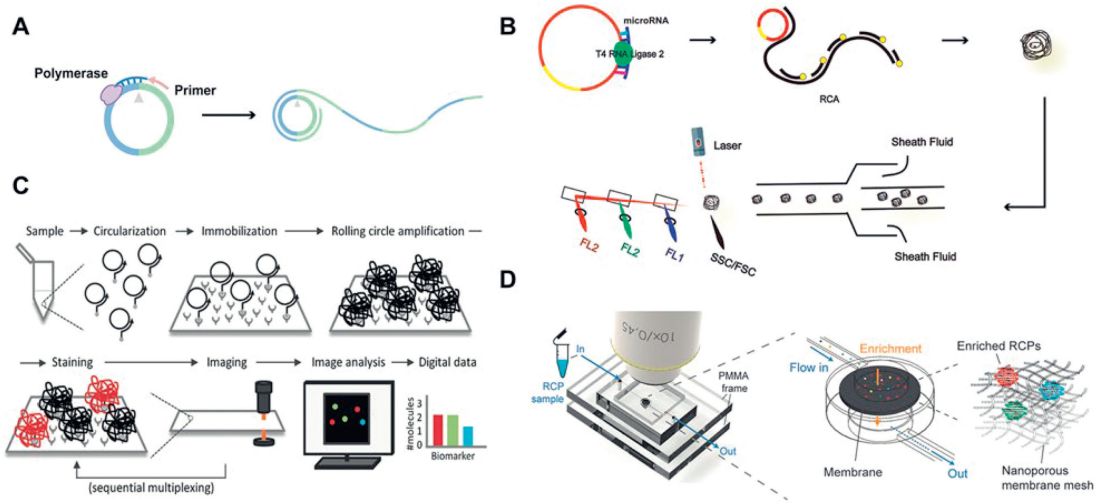

Fig. 2. dRCA for quantitative detection of nucleic acids. (A) Schematic diagram of RCA. (B) The diagram of a digital multiplex microRNA detection procedure. Reprinted with permission [61]. Copyright 1996, Royal Society of Chemistry. (C) Workflow for multiplexed dRCA with fluorescent microscope. Reprinted with permission [62]. Copyright 2020, The Author(s). (D) Single-molecule analysis of DNA with dRCA through a multilayer PDMS microfluidic chip. Reprinted with permission [65]. Copyright 2017, The Author(s).

lead to the time to positive (TTP) being uncertain. In a digital partition, each reaction unit is independent and the amplification rate in each unit is different, thus not every template can generate enough fluorescent signal before the end-point readout [57]. Therefore, dLAMP suffers from low digital efficiency compared to dPCR, which means only part of the templates could be amplified owing to the complex and slow amplification process. To solve the problem, Rustem's group utilized the SlipChip to conduct the LAMP in the correct order [24]. The efficiency of the dRT-LAMP assay could be improved by over 10-fold. Besides, our group has developed a prehybridization-induced enhancement (PIE) strategy which could improve digital efficiency about 2–40 times [57]. In this work, the efficiency of dLAMP was enhanced by improving the annealing of FIP/BIP and did not need any new primer or additional operation.

We believe that more solutions will be proposed to improve the efficiency of dLAMP in the future, and dLAMP will therefore become one of the most crucial technologies for POCT.

2.2. Digital RCA (dRCA)

RCA is a simple and efficient isothermal amplification method. By using a single short primer, a special circular template, and certain polymerases (Phi29, Bst, and Vent exo-DNA polymerase for DNA, and T7 RNA polymerase for RNA), a long single-stranded DNA/RNA sequence can be synthesized by continuously adding nucleotides to a short DNA/RNA primer (Fig. 2A). Different from other digital amplification methods, RCA serves to amplify distinctly countable μm-sized rolling circle products (RCPs). Therefore, unnecessary to be confined in the compartment or droplets, dRCA strategy and the digital enumeration can be achieved by the individual RCPs visualization, such as the work of Jarvius *et al.* [58].

The amplification of the traditional RCA is linear, which is inefficient. To improve the amplification efficiency, digital hyperbranched rolling circle amplification (HRCA), relying on both forward and reverse primers for exponential amplification was developed [59,60]. Furthermore, the multiplexed dRCA can be achieved by using fluorescence flow cytometry (Fig. 2B) [61] or fluorescent microscopic images (Fig. 2C) [62,63]. Therefore, microRNA, short DNA, and RNA could all be detected efficiently with dRCA. Infectious pathogens, such as Ebola, Zika, and Dengue could also be simultaneously detected.

Of course, dRCA was conducted with microfluidic chips, such as a digital microfluidic chip transporting droplets by electrowetting-on-dielectric [64] and a multilayer chip with an embedded membrane (Fig. 2D) [65].

2.3. Digital RPA (dRPA)

RPA is an isothermal amplification method simulating intracellular DNA homologous recombination at a relatively low reaction temperature ranging from 25 °C to 42 °C. Three core enzymes (recombinase, DNA polymerase, and single-stranded binding protein) are involved to coordinate with DNA polymerase and dNTPs, so as to realize the steps of primer pairing, primer substitution, and sequence synthesis, and ultimately allow the exponential accumulation of amplified duplex DNA (Fig. S1A in Supporting information). It is a promising technique with high efficiency, specificity, and cost-effective identification for molecular diagnostics [66].

Thanks to an extremely fast reaction, RPA has enormous potential to be combined with digitization methods for nucleic acid detection [67–69]. Though the RPA reaction normally proceeds at 37 °C, it could amplify the target nucleic acid at room temperature, which leads to the overestimated quantification of dRPA. It could be overcome by using a microfluidic chip which allows the separate step of the activator addition for RPA. For example, the chemical initiator could be mixed with RPA reagents by a sliding step in Slipchip [70], could be pre-patterned on the chip [69], and also could be added during the droplet formation [71]. The SlipChip was able to perform dRPA for quantitative analysis of methicillin-resistant *Staphylococcus aureus* (MRSA) genomic DNA with a LOD of 300 copies/mL [70]. The pre-patterned chip could be applied to the point-of-care analysis for its capability of quantifying nucleic acid directly from the blood and allowing the serial diluted bacterial DNA to be detected (10–10^5 copies/μL) in less than 30 min [69]. The strategy of adding the chemical initiator during the droplet formation showed a high linearity at a series of dilutions [71].

Another way to effectively prevent pre-amplification is the rapid emulsification of the RPA reaction mixture, such as the centrifugal-based ddRPA, which enables quantifying DNA within 15 min [68,72,73]. Recently, the strategy of a multilayer micro-array chip was developed for dRPA assay. The chips not only achieved the rapid partition but also realized the high-throughput and ac-

curate quantification of the nucleic acids (Figs. S1B and C in Supporting information) [74,75].

In summary, compared to other isothermal amplification methods, the quantification accuracy of dRPA could be affected due to the preamplification before the partition. Therefore, the microfluidic chip with a special design allowing the addition of activators to trigger the reaction is necessary. Rapid sample segmentation is another effective strategy to overcome this problem. Of course, more strategies are expected to be proposed for the improvement of dRPA.

2.4. Digital NASBA (dNASBA)

NASBA is one of the earliest IATs for RNA detection with sensitivity and specificity [76]. In a standard reaction system, a pair of primers and three main enzymes (reverse transcriptase, RNase H, and T7 RNA polymerase) are used *in vitro* enzymatic reaction. At a predefined temperature of 41 °C, these three enzymes amplify single-stranded RNA sequences by synergistically mimicking the RNA replication of retroviruses. The template RNA can be amplified by about 10^9 to 10^{12} times in 2 h [77,78].

Wang *et al*. developed dNASBA in the SD chip platform which enables the absolute quantification of RNA [79]. HIV-1 RNA in plasma samples could be directly detected, indicating that dNASBA has great potential in POC diagnosis. dNASBA has many advantages in terms of efficiency, sensitivity, and specificity. It is comparatively more suitable for the detection of RNA. However, three enzymes are required which is high cost and compromises the robustness.

2.5. Digital MDA (dMDA)

Multiple Displacement Amplification (MDA) is an isothermal strand displacement amplification method [80]. By using random hexamer primers and Phi29 DNA polymerase, limited DNA samples can be amplified exponentially under isothermal conditions and finally generate multibranched products [81]. Therefore, MDA has the characteristics of low deviation, less prone to nonspecific binding, and longer products. Therefore, it is suitable for complex genomic analysis, such as next-generation sequencing (NGS) [82]. However, the sequence-dependent amplification bias, such as selective bias, template fragmentation, drift bias, polymerase artifacts, and nontemplated amplification, is inevitable [83]. The digital format gets the reaction mix partitioned into thousands of microdroplets, which reduces the competition among the fragments for primers and polymerase. Droplet or hydrogel-based dMDA platforms were developed to reduce the amplification bias, which is helpful for genome sequencing. Therefore, dMDA is popular in Whole genome amplification (WGA) but is inappropriate for nucleic acid detection.

Blainey's group developed a hydrogel-based virtual microfluidics combining MDA for quantification and single-cell sequencing [84]. MDA clusters were compartmentalized by hydrogel-limited diffusion and then observed as the signal at the endpoint. Kim *et al*. utilized a hand-held syringe and a commercially available splitter to perform ddMDA with efficiency and quality [85]. Qiao *et al*. developed a droplet re-generation platform that re-diluted the generated MDA droplets, controlling the DNA concentration not too high or too low [86]. This work improved the reaction speed and saved more than half of the total reaction time in the conventional dMDA. dMDA is most widely used in WGA. Notably, the future efforts of dMDA are still focusing on the elimination of the non-specific results and the amplification bias.

In summary, the dIATs combine the advantages of dPCR and IATs and represent the next-generation molecular diagnostic technology. It shows great performance in terms of precision and simplicity. The pros and cons and critical performance factors of each method are summarized in Table 1.

3. Digital CRISPR-based assay

CRISPR, a repetitive sequence in the genomes of prokaryotes, is a crucial component of the immune systems in bacteria and archaea. The Cas nucleases guided by crRNA specifically recognize the target nucleic acid sequence and precisely cut the target strand, which has been widely used as a genome editing tool. Recently, the discovery of collateral cleavage from new Cas extended the application of the CRISPR-Cas system in molecular diagnostics due to its specificity, high efficiency, and ease of use [87,88].

So far, Cas12 and Cas13 are the major two types of CRISPR-Cas enzymes for nucleic acid detection. CRISPR-Cas12 includes a group of single crRNA-guided endonucleases that specifically recognize and cut ssDNA or dsDNA, which is termed "*cis*-cleavage" activity [89]. After cutting the target DNA, the Cas12 enzyme remains attached to the targets and cuts other short DNA molecules indiscriminately, which is termed "*trans*-cleavage" activity (Fig. 3A). Similar to Cas12, Cas13 is also single RNA-guided and mediates both "*cis*-cleavage" and "*trans*-cleavage", while its targets are all ssRNA sequences (Fig. 3B). By combining with RPA, the Cas12a-based DETECTR system, and Cas13a-based SHERLOCK system have been developed to detect viruses [90,91]. Though these CRISPR/Cas-based methods are sensitive and specific, they are not able to quantify the target accurately. To improve the quantification capability, digital CRISPR/Cas-based methods for nucleic acid detection have been developed [92,93].

3.1. Digital CRISPR/Cas-based assay combining with isothermal amplification

Conventional CRISPR/Cas assays employ nucleic acid amplification to improve the detection sensitivity, and the RPA was the most commonly used because the reaction temperature of RPA was compatible with CRISPR/Cas. The first digital CRISPR/Cas-assisted method called digitization-enhanced CRISPR/Cas-assisted one-pot virus detection (deCOViD) has been developed for SARS-CoV-2 detection (Fig. 3C) [93]. Combining Cas12a and RPA with a commercially available microfluidic digital chip, deCOViD can quantitatively detect 1 genome equivalent/μL of target RNA in less than 15 min. Yu's group reported a similar droplet-based digital RPA-combined Cas12a method called RADICA [94], which is 4 times faster than conventional dPCR for the detection of the EBV (Epstein-Barr virus). Moreover, Cas13a was also combined with RPA by coupling with T7 RNA polymerase in the water-in-oil droplets, which is named MEDICA (Fig. 3D) [95]. The MEDICA enabled to quantify HPV16/18, and the results were in good agreement with the qPCR. A similar RPA-Cas13a platform PADLOCK-CRISPR was also developed [96], which could detect single molecule HPV16 viral loads within 30 min, and could be applied for HPV16 clinical sample detection. Different from MEDICA, the chemical initiator loading relied on a downstream picoinjector and the amplification commenced only after the MgOAc was dosed. As we discussed in Section 3.3, RPA may amplify the target nucleic acid at room temperature before the sample digitization, resulting in inaccurate quantification. A digital RPA-Cas12a was performed in an integrated adsorption-free self-priming chip for quantitative detection of the *Salmonella* bacteria [97]. A limit of detection of ~0.2 cell/mL could be reached within 30 min. Except for absolute quantification of the target nucleic acid molecules, Zhang *et al*. developed a one-step CRISPR/Cas12a-based digital diagnostic platform targeting mutant alleles [98]. Combining with RPA and a commercial digital

Table 1
Summary of the dIATs.

Method	Temp (°C)	Time	LOD	Advantages	Disadvantages
dLAMP	60–65	30–60 min	0.4 copy/μL	High sensitivity; high amplification efficiency	Cumbersome primer design; non-specific amplification; underestimate the concentration
dRCA	~37	~120 min	1 amol/L	Simple primer design; diversity	The template is circular DNA; RCA is linear amplification
dRPA	25–42	~30 min	1 copy/μL	Fast reaction; simple primer design; run at room temperature	non-specific amplification; overestimate the concentration
dNASBA	37–42	~120 min	1 copy/μL	High sensitivity and specificity; low contamination	Complex reaction system; high cost
dMDA	30	4–16 h	0.1 pg/μL	Good for genome sequencing	Long reaction time; unappropriate for detection

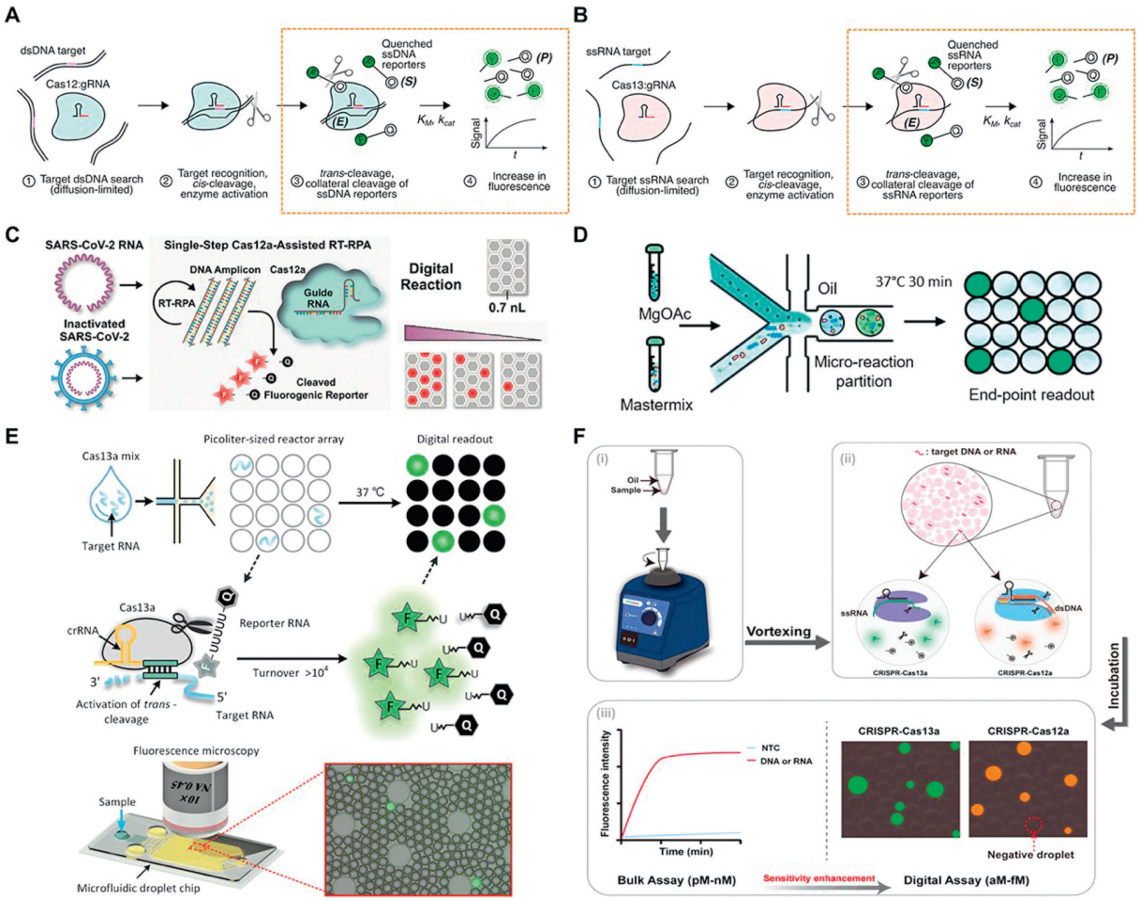

Fig. 3. Digital CRISPR platform for quantitative detection of nucleic acid. (A) Schematic diagram for CRISPR/Cas12 detecting target DNA. Reprinted with permission [108]. Copyright 2021, American Chemical Society. (B) Schematic diagram of the CRISPR/Cas13 detecting target RNA. Reprinted with permission [108]. Copyright 2021, American Chemical Society. (C) Overview of the deCOViD that combined the CRISPR/Cas12a with RPA in a commercially available digital microfluidic chip. Reprinted with permission [93]. Copyright 2021, The Authors. (D) Principle of the MEDICA, which combined the CRISPR/Cas13a with RPA. Reprinted with permission [95]. Copyright 2022, American Chemical Society. (E) Schematic of the amplification-free ultralocalized Cas13a assay. Reprinted with permission [104]. Copyright 2021, American Chemical Society. (F) Principle of the PddCas for amplification-free detection of both target DNA and RNA. Reprinted with permission [109]. Copyright 2023, American Chemical Society.

chip, the platform accurately quantifies the variant allele frequency of EGFR L858R within 1 h at 42 °C and can detect mutant targets as low as 0.498 amol/L in mock multiplex cfDNA samples.

LAMP/RT-LAMP was also employed to combine with the digital CRISPR platform. The optimal reaction temperature of LAMP (60–65 °C) is much higher than that of the CRISPR-Cas12a system (37 °C), thus additional adjustment is needed. Ding *et al.* developed the digital warm-start CRISPR (dWS-CRISPR) by combining CRISPR-Cas12a with RT-DAMP [99], which is a variant of RT-LAMP at a low temperature, that not only improves the detection sensitivity but also generates ultralow nonspecific signals. With a QuantStudio 3D digital chip, the dWS-CRISPR was performed at 52 °C to work for both the LAMP and CRISPR reactions. Wu *et al.* developed a droplet digital CRISPR-based platform combined with LAMP called DropCRISPR [100]. The temperature incompatibility of LAMP and CRISPR-Cas12a is avoided by separating the LAMP amplification and Cas12a assay reaction. The LAMP was performed individually in droplets, and the CRISPR-Cas12a reagent was injected into the droplets containing LAMP products to trigger the Cas12a assay. It avoids the mutual interference between the LAMP amplification reaction and Cas12a. Ultrasensitive detection could thus be achieved at ~fmol/L level. A similar strategy was developed by the group of Shen, who used a parallel multistep digital Slipchip for the quantification of SARS-CoV-2 with a two-step manipulation [101]. The CRISPR-Cas12a reagent can be mixed with LAMP products through a simple slipping operation.

Compared to Cas12a, Cas12b is more resistant to high temperatures and therefore is compatible with LAMP. Our group developed a digital droplet RT-LAMP Enhanced Cas12b-based RNA Detection platform (ddRECD) which combined digital RT-LAMP

with CRISPR/Cas12b for the detection of SARS-CoV-2 RNA [102]. The nucleic acid pre-amplification could be limited in the LAMP-based strategy due to the warm-start characteristic, and the ddRECD is capable of detecting viral RNA down to ~amol/L level. The group of Yu also employed Cas12b to combine with RT-LAMP, which is called Rapid Digital Crispr Approach (WS-RADICA), and demonstrated high sensitivity for both DNA and RNA [103]. Combination of digital CRISPR with isothermal amplification could be an intriguing strategy, because it integrates the high sensitivity of isothermal amplification and the high specificity of the CRISPR-based methods.

3.2. Amplificaion-free digital CRISPR-based assay

The direct CRISPR-based assay could only detect DNA/RNA at sub-pmol/L concentrations, thus usually relying on amplification to improve sensitivity. However, the amplification process may result in amplification bias, premature amplification, and cross-contamination. In the digital assay, local molecular concentration can be enhanced to promote highly efficient reactions due to the confinement effect. In picoliter-sized droplets or chambers, single molecule nucleic acid could be at pmol/L level. Therefore, the digital CRISPR-based assay makes amplification-free single-molecule detection possible.

Zhou's group first demonstrated this concept using Cas13a *via* droplet microfluidics which is called ultralocalized Cas13a assay (Fig. 3E) [104]. This platform enables absolute quantification of RNA at a single-molecule level without the need for amplification, and the sensitivity is enhanced by 10,000 times compared to the bulk assays. It was applied for the quantitative detection of microRNAs, 16S rRNA from uropathogenic, and SARS-CoV-2 RNA. This platform was further extended to Cas12a-based assay [92]. Several important reaction parameters were optimized for Cas12a because the trans-cleavage efficiency of Cas12a is lower than Cas13a. As a result, single-molecule DNA quantification was reached and the clinical samples of several viruses were directly detected. Shinoda and coworkers also developed a Cas13a-based digital format amplification-free detection platform by using microchamber-array technologies [105]. The platform termed CRISPR-based amplification-free digital RNA detection (SATORI), could detect ssRNA at ~fmol/L level in 5 min and the simultaneous use of multiple different guide RNAs enhanced the sensitivity. They further developed an automated platform on SATORI (opn-SATORI), which enables automated detection and greatly improves detection sensitivity [106]. Recently, a similar Cas13a-based amplification-free assay was developed by using an integrated microwell array [107]. Magnetic beads with probes were utilized for the target capture and enrichment which improves the sensitivity of achieving a limit of detection of 2 amol/L.

The digital CRISPR-based methods above need complex microfluidic setups, which are unsuitable for POCT application. To overcome this drawback, an integrated chip was built that allowed the digital CRISPR-Cas12a assay at POCT with a smartphone [110]. The fluorescent signal in the microwell chip could be read out by a smartphone, which enables detecting down to 5 fmol/L of ssDNA. Moreover, the multiplex detection of this platform was validated by quantifying HBV and related DNA markers. The digital CRISPR-based assay relying on microfluidic technology takes a long time to separate the aqueous samples, which may pre-activate the CRISPR/Cas. To simplify the partition of samples, we developed a polydisperse droplet digital CRISPR/Cas-based assay (Pdd-Cas), which utilized a common vortex mixer to generate picoliter-scale polydisperse droplets in several seconds to partition the samples (Fig. 3F). Both Cas12a and Cas13a were used for amplification-free detection of viral DNA and RNA respectively, and the LOD for the target DNA and RNA is approximately 100 amol/L and 10 amol/L respectively, which is about 10^4–10^5 folds more sensitive than corresponding bulk CRISPR assays. SARS-CoV-2 and HPV 18 in clinical samples were successfully detected [109].

4. Conclusions and future perspectives

In this review, we summarized the advance of digital PCR-free technologies for the quantitative detection of nucleic acid. The principles of digital assay and strategies of isothermal amplification were systematically introduced. The pros and cons of each method should be considered for the specific application. As an emerging method for molecular diagnosis, digital CRISPR systems were also described, including the unique amplification mechanism based on the *trans*-cleavage activity of Cas12 and Cas13 enzymes. The application and critical performance factors of each digital CRISPR system were summarized (Table S1 in Supporting information). Given the excellent performance, it is highly likely that digital PCR-free technologies will become the next-generation POCT diagnostic platform for personalized healthcare. dPCR has been developed and commercialized for about two decades. Though dPCR is considered to be the most sensitive method and more reliable for the quantification of nucleic acid, its application in clinics is still limited. The expensive instruments and reagents compromise the benefit-to-cost of this technique, especially when the qPCR has been very mature and common in clinical applications. In addition, IATs look promising for POCT diagnostic. However, the reliability of IATs is limited due to the non-specific amplification and poor reproducibility. The dIATs combines the advantages of dPCR and IATs, which is sensitive and fast, enabling absolute quantification at a single-molecule level, without the requirement for complex instrumentation. Comparatively, dIATs are much more reliable for the quantification of nucleic acid, which could overcome the shortcomings of dPCR and IATs, and are suitable for POCT diagnostic.

Despite great potential, dIATs still face some challenges for clinical application. First, the preparation of dispersed droplets relies on microfluidic devices or microchips, and the fabrication processes of those chips usually are time-consuming and labor-intensive [111]. Though some microfluidic-free platforms are developed, the low-cost and easy-to-use dIATs is still an unmet need. Second, the detection efficiency of dIATs is not as good as dPCR. We demonstrated the quantification results of dLAMP were lower than dPCR, which needed to optimize the operation to improve the quantification accuracy [57]. Third, the characteristics of isothermal amplification lead to the pre-amplification occurring in the process of sample loading and droplet preparation, resulting in inaccurate quantification. The WarmStart polymerase such as Bst 2.0 is used to eliminate the undesired premature amplification [96]. In addition, multistep loading of the reaction initiator is developed to circumvent pre-amplification [95]. A strategy of quick preparation of polydisperse droplets could also reduce the effect of pre-amplification [45,109]. Fourth, dIATs are basically singleplex assays (*i.e.*, assays to measure a single analyte) to date, because they are usually dependent on non-specific intercalating dyes. Recently, multiplexed dLAMP was developed by using scorpion-shaped probes that provide more fluorescent colors [112,113]. Fifth, the throughput of the dIATs is limited and more studies are needed to develop high-throughput platforms for further application. These disadvantages of the dIATs indicate that a lot of work is needed to make the platform more widely applicable.

Now the field of dIATs is still in its infancy and has yet to see any killer commercial application in the market. To overcome the drawbacks above, more and more novel dIATs platforms are being developed. For ASSURED diagnostics purposes (Affordable, Sensitive, Specific, Userfriendly, Rapid, Robust, Equipment-free and Delivered to those who need it), it is still challenging to integrate a

dIATs with both the upstream and downstream processes, to provide a sample-in to answer-out portable device.

Compared to conventional dIATs, digital CRISPR-based methods are particularly promising for POCT diagnosis. (1) CRISPR/Cas works at 37 °C, even at room temperature, thus needs minimum instrumentation. (2) Digital CRISPR system could be amplification-free due to its unique *trans*-cleavage activity, so that avoids carry-on contamination and reduces false positive interference. This is critical for POCT clinical application. (3) Cas12 and Cas13 can detect the DNA and RNA respectively. This unique specificity and preferential cleavage could be used to detect multiple targets. As emerging nucleic acid quantitative analysis systems, these digital PCR-free technologies combine the advantages of dPCR and IATs, which are of unprecedented precision and simplicity, and have a broad prospect in molecular diagnosis. We envision that digital PCR-free technologies can be fast, high-throughput, integrated, multiplex, and automated, which is readily deployed for POC testing as part of an integrated public health surveillance program.

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.

CRediT authorship contribution statement

Xinyi Luo: Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Ke Wang:** Writing – review & editing, Supervision, Resources. **Yingying Xue:** Investigation, Formal analysis, Conceptualization. **Xiaobao Cao:** Validation, Software, Project administration. **Jianhua Zhou:** Writing – review & editing, Funding acquisition, Data curation. **Jiasi Wang:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Supplementary materials

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References

[1] E.A. Pumford, J. Lu, I. Spaczai, et al., Biosens. Bioelectron. 170 (2020) 112674.
[2] Y. Chen, C. Qian, C. Liu, et al., Biosens. Bioelectron. 153 (2020) 112049.
[3] M. Li, F. Yin, L. Song, et al., Chem. Rev. 121 (2021) 10469–10558.
[4] R. Ramesh, A. Munshi, S.K. Panda, Natl. Med. J. India 5 (1992) 115–119.
[5] M.R. Green, J. Sambrook, Cold Spring Harb. Protoc. 2018 (2018) 769–777.
[6] B. Vogelstein, K.W. Kinzler, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 9236–9241.
[7] P.L. Quan, M. Sauzade, E. Brouzes, Sensors 18 (2018) 1271.
[8] Y. Li, P. Fan, S. Zhou, L. Zhang, Microb. Pathogenesis 107 (2017) 54–61.
[9] A. James, J. Macdonald, Expert. Rev. Mol. Diagn. 15 (2015) 1475–1489.
[10] G.T. Walker, M.C. Little, J.G. Nadeau, D.D. Shank, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 392–396.
[11] J.C. Guatelli, K.M. Whitfield, D.Y. Kwoh, et al., Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 1874–1878.
[12] P.M. Lizardi, X. Huang, Z. Zhu, et al., Nat. Genet. 19 (1998) 225–232.
[13] P. Craw, W. Balachandran, Lab Chip 12 (2012) 2469–2486.
[14] P.J. Asiello, A.J. Baeumner, Lab Chip 11 (2011) 1420–1430.
[15] P. Gill, A. Ghaemi, Nucleos. Nucleot. Nucl. 27 (2008) 224–243.
[16] Y. Zhao, F. Chen, Q. Li, et al., Chem. Rev. 115 (2015) 12491–12545.
[17] A. Gansen, A.M. Herrick, I.K. Dimov, et al., Lab Chip 12 (2012) 2247–2254.
[18] J.E. Kreutz, J. Wang, A.M. Sheen, et al., Lab Chip 19 (2019) 1035–1040.
[19] J. Wang, J.P. Staheli, A. Wu, et al., Anal. Chem. 93 (2021) 3266–3272.
[20] Q. Zhu, Y. Gao, B. Yu, et al., Lab Chip 12 (2012) 4755–4763.
[21] B. Pang, X. Ding, G. Wang, et al., J. Agr. Food. Chem. 65 (2017) 11312–11319.
[22] N. Rong, K. Chen, J. Shao, et al., Anal. Chem. 95 (2023) 7830–7838.
[23] D.A. Selck, M.A. Karymov, B. Sun, R.F. Ismagilov, Anal. Chem. 85 (2013) 11129–11136.
[24] B. Sun, F. Shen, S.E. McCalla, et al., Anal. Chem. 85 (2013) 1540–1546.
[25] E.M. Khorosheva, M.A. Karymov, D.A. Selck, R.F. Ismagilov, Nucleic Acids Res. 44 (2016) e10.
[26] J. Rodriguez-Manzano, M.A. Karymov, S. Begolo, et al., ACS Nano 10 (2016) 3102–3113.
[27] M. Yu, X. Chen, H. Qu, et al., Anal. Chem. 91 (2019) 8751–8755.
[28] Z. Yu, W. Lyu, M. Yu, et al., Biosens. Bioelectron. 155 (2020) 112107.
[29] X. Lin, X. Huang, Y. Zhu, et al., ACS Nano 12 (2018) 10281–10290.
[30] X. Lin, X. Huang, K. Urmann, et al., ACS Sens. 4 (2019) 242–249.
[31] Y. Yan, T. Yang, Z. Luo, et al., Food. Chem. 408 (2023) 135226.
[32] L. Cao, X. Guo, P. Mao, et al., ACS Sens. 6 (2021) 3564–3574.
[33] T. Yang, D. Li, Y. Yan, et al., J. Hazard. Mater. 442 (2023) 130050.
[34] C. Yi, Z. Luo, Y. Lu, et al., Biosens. Bioelectron. 184 (2021) 113199.
[35] X. Lin, M. Fang, C. Yi, et al., Biomaterials 280 (2022) 121278.
[36] Y.D. Ma, K. Luo, W.H. Chang, G.B. Lee, Lab Chip 18 (2018) 296–303.
[37] M. Azizi, M. Zaferani, S.H. Cheong, A. Abbaspourrad, ACS Sens. 4 (2019) 841–848.
[38] F. Liu, A. Ge, C. Li, et al., Anal. Chem. 95 (2023) 6672–6680.
[39] S. Friedrich, S. Clara, H. Sebastian, et al., Anal. Methods-UK 8 (2016) 2750–2755.
[40] H. Peng, M. Zhu, Z. Gao, et al., Biomed. Microdevices 22 (2020) 18.
[41] M. Jiang, P. Liao, Y. Sun, et al., Lab Chip 21 (2021) 2265–2271.
[42] Z. Zhang, Y. Cheng, X. Li, et al., Anal. Chem. 95 (2023) 3028–3036.
[43] Y. Hu, P. Xu, J. Luo, et al., Anal. Chem. 89 (2017) 745–750.
[44] Z. Pan, Y. Men, S. Senapati, H.C. Chang, Biomicrofluidics 12 (2018) 044113.
[45] L. Chen, J. Ding, H. Yuan, et al., Adv. Sci. 9 (2022) e2105450.
[46] H. Yuan, Y. Chao, S. Li, et al., Anal. Chem. 90 (2018) 13173–13177.
[47] F. Hu, J. Li, Z. Zhang, et al., Anal. Chem. 92 (2020) 2258–2265.
[48] B. Oliveira, B. Veigas, A.R. Fernandes, et al., Sensors 20 (2020) 1624.
[49] B.J. Coelho, B. Veigas, H. Águas, et al., Sensors 17 (2017) 2616.
[50] L. Wan, T. Chen, J. Gao, et al., Sci. Rep. 7 (2017) 14586.
[51] L. Cao, X. Guo, P. Mao, et al., ACS Sens. 6 (2021) 3564–3574.
[52] L. Jiang, X. Lan, L. Ren, et al., Microsyst. Nanoeng. 9 (2023) 118.
[53] J. Chen, X. Xu, Z. Huang, et al., Chem. Commun. 54 (2018) 291–294.
[54] T.D. Rane, L. Chen, H.C. Zec, T.H. Wang, Lab Chip 15 (2015) 776–782.
[55] S.A. Hsieh, D. Shamsaei, D.R. Eitzmann, J.L. Anderson, Anal. Chem. 94 (2022) 11949–11956.
[56] C. Wu, L. Liu, Z. Ye, et al., Anal. Chim. Acta 1233 (2022) 340513.
[57] Y. Xue, X. Luo, X. Pang, et al., Anal. Biochem. 631 (2021) 114371.
[58] J. Jarvius, J. Melin, J. Göransson, et al., Nat. Methods 3 (2006) 725–727.
[59] L. Mazutis, A.F. Araghi, O.J. Miller, et al., Anal. Chem. 81 (2009) 4813–4821.
[60] K. Wang, X. Bai, Y. Xue, et al., Sensor Actuat. B: Chem. 375 (2023) 132893.
[61] H. Zhian, X. Fujian, S. Gongwei, et al., Chem. Commun. 56 (2020) 5409–5412.
[62] J. Björkesten, S. Patil, C. Fredolini, et al., Nucleic Acids Res. 48 (2020) e73.
[63] S. Ciftci, F. Neumann, S. Abdurahman, et al., J. Mol. Diagn. 22 (2020) 272–283.
[64] M. Kühnemund, D. Witters, M. Nilsson, J. Lammertyn, Lab Chip 14 (2014) 2983–2992.
[65] M. Kühnemund, I. Hernández-Neuta, M.I. Sharif, et al., Nucleic Acids Res. 45 (2017) e59.
[66] R.K. Daher, G. Stewart, M. Boissinot, M.G. Bergeron, Clin. Chem. 62 (2016) 947–958.
[67] Z. Li, Y. Liu, Q. Wei, et al., PLoS One 11 (2016) e0153359.
[68] S. Santiago-Felipe, L.A. Tortajada-Genaro, R. Puchades, Á. Maquieira, Microchim. Acta 183 (2016) 1195–1202.
[69] E.C. Yeh, C.C. Fu, L. Hu, et al., Sci. Adv. 3 (2017) e1501645.
[70] F. Shen, E.K. Davydova, W. Du, et al., Anal. Chem. 83 (2011) 3533–3540.
[71] X. Li, A. Manz, Sensor Actuat. B: Chem. 288 (2019) 678–682.
[72] L.A. Tortajada-Genaro, S. Santiago-Felipe, M. Amasia, et al., RSC Adv. 5 (2015) 29987–29995.
[73] F. Schuler, F. Schwemmer, M. Trotter, et al., Lab Chip 15 (2015) 2759–2766.
[74] J. Yin, Z. Zou, Z. Hu, et al., Lab Chip 20 (2020) 979–986.
[75] Z. Qin, X. Xiang, L. Xue, et al., Microchem. J. 164 (2021) 106050.
[76] B. Deiman, P. van Aarle, P. Sillekens, Mol. Biotechnol. 20 (2002) 163–179.
[77] G. Leone, H. van Schijndel, B. van Gemen, et al., Nucleic Acids Res. 26 (1998) 2150–2155.
[78] J.J. Weusten, W.M. Carpay, T.A. Oosterlaken, et al., Nucleic Acids Res. 30 (2002) e26.
[79] J. Wang, J.E. Kreutz, A.M. Thompson, et al., Lab Chip 18 (2018) 3501–3506.
[80] F.B. Dean, S. Hosono, L. Fang, et al., Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 5261–5266.
[81] L. Huang, F. Ma, A. Chapman, et al., Annu. Rev. Genom. Hum. G 16 (2015) 79–102.

[82] X. Xiang, Y. Shang, J. Zhang, et al., Trac-Trend. Anal. Chem. 149 (2022) 116568-116551.
[83] J. Sabina, J.H. Leamon, Methods. Mol. Biol. 1347 (2015) 15–41.
[84] L. Xu, I.L. Brito, E.J. Alm, P.C. Blainey, Nat. Methods 13 (2016) 759–762.
[85] S.C. Kim, G. Premasekharan, I.C. Clark, et al., Microsyst. Nanoeng. 3 (2017) 17018.
[86] Y. Qiao, W. Liu, N. Lu, et al., Anal. Chim. Acta 1141 (2021) 173–179.
[87] J.S. Gootenberg, O.O. Abudayyeh, J.W. Lee, et al., Science 356 (2017) 438–442.
[88] J.S. Chen, E. Ma, L.B. Harrington, et al., Science 360 (2018) 436–439.
[89] O.O. Abudayyeh, J.S. Gootenberg, S. Konermann, et al., Science 353 (2016) aaf5573.
[90] I.J. Chen, T. Wu, S. Hu, Methods X 5 (2018) 984–990.
[91] M. Patchsung, K. Jantarug, A. Pattama, et al., Nat. Biomed. Eng. 4 (2020) 1140–1149.
[92] H. Yue, B. Shu, T. Tian, et al., Nano. Lett. 21 (2021) 4643–4653.
[93] J.S. Park, K. Hsieh, L. Chen, et al., Adv. Sci. 8 (2021) 2003564.
[94] X. Wu, J.K. Tay, C.K. Goh, et al., Biomaterials 274 (2021) 120876.
[95] F.X. Liu, J.Q. Cui, H. Park, et al., Anal. Chem. 94 (2022) 5883–5892.
[96] J.Q. Cui, F.X. Liu, H. Park, et al., Biosens. Bioelectron. 202 (2022) 114019.
[97] L. Xia, J. Yin, J. Zhuang, et al., Anal. Chem. 95 (2023) 4744–4752.
[98] C. Zhang, Z. Cai, Z. Zhou, et al., Biosens. Bioelectron. 222 (2023) 114956.
[99] X. Ding, K. Yin, Z. Li, et al., Biosens. Bioelectron. 184 (2021) 113218.
[100] H. Wu, X. Cao, Y. Meng, et al., Biosens. Bioelectron. 211 (2022) 114377.
[101] Z. Yu, L. Xu, W. Lyu, F. Shen, Lab Chip 10 (2022) 21886.
[102] X. Luo, Y. Xue, E. Ju, et al., Anal. Chim. Acta 1192 (2021) 339336.
[103] X. Wu, C. Chan, S.L. Springs, et al., Anal. Chim. Acta 1196 (2022) 339494.
[104] T. Tian, B. Shu, Y. Jiang, et al., ACS Nano 15 (2021) 1167–1178.
[105] H. Shinoda, Y. Taguchi, R. Nakagawa, et al., Commun. Biol. 4 (2021) 476.
[106] H. Shinoda, T. Iida, A. Makino, et al., Commun. Biol. 5 (2022) 473.
[107] D. Wang, X. Wang, F. Ye, et al., ACS Nano 17 (2023) 7250–7256.
[108] A. Ramachandran, J.G. Santiago, Anal. Chem. 93 (2021) 7456–7464.
[109] Y. Xue, X. Luo, W. Xu, et al., Anal. Chem. 95 (2023) 966–975.
[110] T. Yu, S. Zhang, R. Matei, et al., Aiche. J. 67 (2021) e17365.
[111] H. Yuan, Y.C. Chao, H.C. Shum, Small 16 (2020) 1904469.
[112] D. Cai, Y. Wang, J. Zou, et al., Adv. Sci. 10 (2023) e2205863.
[113] K. Wu, Q. Fang, Z. Zhao, Z. Li, Anal. Chem. 95 (2023) 5069–5078.