Lab on a Chip

PAPER

View Article Online
View Journal | View Issue

Cite this: *Lab Chip*, 2023, **23**, 1794

A fully integrated nucleic acid analysis system for multiplex detection of genetic polymorphisms related to folic acid metabolism†

Baobao Lin,[a] Zhi Geng,[a] Yanjing Chen,[b] Wu Zeng,[a] Bao Li,[a] Yan Zhang*[b] and Peng Liu *[a]

A sufficient intake of folic acid is essential during pregnancy, but several genetic polymorphisms reduce its absorption, threaten the lives of pregnant women and cause congenital disabilities in newborns. Traditional laboratory detection of genetic variants related to folic acid metabolism is time-consuming and labor-intensive. Microfluidics-based molecular diagnosis integrates sample pre-processing and nucleic acid amplification on-chip to achieve rapid, sensitive, high-throughput, and automated detection. Here, we developed a fully integrated microfluidic system for the detection of genetic polymorphisms related to folic acid metabolism in a "sample in-answer out" style. The system consists of nucleic acid extraction and amplification modules. During nucleic acid extraction, blood cells are lysed, and DNA is captured and eluted through a silica-gel membrane. After that, multiple gene loci are detected using loop-mediated isothermal amplification (LAMP) and the color of the reaction chamber indicates whether genetic mutations are present. The experimental results demonstrate that the system can accurately detect gene polymorphisms associated with folic acid metabolism in blood samples with high sensitivity and no cross-contamination between chambers. The blood samples of five patients were tested for mutant alleles on this system, and the test results were consistent with qPCR and DNA sequencing observations. The operation is fully automated, and the detection is completed in approximately 70 minutes. The proposed system has great potential in prenatal diagnosis and other types of nucleic acid detection.

Received 22nd December 2022,
Accepted 9th February 2023

DOI: 10.1039/d2lc01169g

rsc.li/loc

Introduction

Folic acid is an essential element for synthesizing nucleic acids, a necessary substance for cell growth and tissue repair, and an indispensable nutrient during embryonic development.[1] Folic acid metabolism disorders associated with genetic variation can lead to anemia, high blood pressure, spontaneous abortion, stillbirth, and postpartum hemorrhage in pregnant women.[2] In addition, these variants might also be responsible for congenital disorders in newborns, including neural tube defects,[3] Down syndrome, congenital heart disease,[4] and cleft lip.[5] It is thus necessary to detect the genetic polymorphisms associated with folic acid metabolism and uncover the levels of absorption and utilization of folic acid in pregnant women and, according to test results, recommend an exact folic acid supplement plan and amount to ensure safety during pregnancy and reduce the risk of congenital disorders.[6]

DNA sequencing is the "gold standard" for detecting single nucleotide polymorphisms (SNPs), uncovering individual genes related to disease and helping to prevent and treat diseases in advance.[7] However, the timing and elevated costs of sequencing instruments limit large-scale application, especially in resource-poor areas.[8] Genetic polymorphisms and genes associated with folic acid metabolic enzymes have been studied in clinical practice, which revealed that methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) are the key enzymes of folic acid metabolism that carry genetic variation affecting folic acid absorption.[9] The most common genetic mutation sites of these two enzymes are MTHFR677 G > A, MTHFR1298 T > G, and MTRR 66 A > G. The detection of SNPs in these three genes helps in determining

[a] *Department of Biomedical Engineering, School of Medicine, Tsinghua University, Haidian District, Beijing, 100084, China. E-mail: pliu@tsinghua.edu.cn; Fax: +86 10 62798732; Tel: +86 10 62798732*
[b] *Sports & Medicine Integration Research Center (SMIRC), Capital University of Physical Education and Sports, Haidian District, Beijing, 100191, China. E-mail: zhangyan2021@cupes.edu.cn; Tel: +86 010 82099462*

† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d2lc01169g

the folic acid metabolism in pregnant women.[10,11] The TaqMan probe method is clinically used to detect SNPs related to folic acid metabolism,[12] but this method requires a professional bioassay laboratory and well-trained technicians using multiple types of equipment for analysis in labor-intensive settings. Importantly, establishing accurate, sensitive, and rapid diagnostic procedures is essential to identify SNPs in the genes associated with folic acid metabolism.

Microfluidic technology can integrate multiple operation steps,[13–15] such as sample preparation, nucleic acid enrichment, purification and detection, and cooperate with automated instruments to perform "sample in, answer out" variant detection, eliminating the need for traditional testing laboratories.[16] However, it is difficult to perform PCR reactions on microfluidic chips. Specifically, the temperature cycling of PCR reactions requires complex control and heating systems, and expensive optical detection systems to detect the amplification signals.[17,18] In addition, it is challenging to design a microfluidic chip suitable for PCR, since during the experiment the wall of the reaction chamber must be thin enough and fit tightly to ensure temperature accuracy.[19] The volume expansion of air and liquid during the reaction will generate significant pressure and make it difficult to achieve adequate sealing. Moreover, the chip's material must have a low fluorescence background to achieve effective detection.[20] In comparison, some isothermal amplification techniques,[21] such as loop-mediated isothermal amplification (LAMP),[22] nucleic acid sequence-based amplification (NASBA),[23] and rolling circle amplification (RCA),[24] only require a constant reaction temperature with a simple control system that is appropriate for point of care testing (POCT). Among these methods, LAMP shows high sensitivity and specificity, but is rarely used for detecting SNPs due to the complex primer design. Some studies have helped in improving LAMP amplification to achieve SNP detection,[25] but still require sophisticated optical instruments to detect the fluorescent signals. At present, no LAMP-amplification technology is used to detect SNPs associated with folic acid metabolism. Before amplification, it is necessary to extract nucleic acid from clinical samples, most commonly using magnetic beads.[26] While the efficiency of this extraction method is high, the operation is complicated. Most microfluidic chips that utilize microfluidic valves to serve as magnetic beads during extraction are often complex in structure and difficult to process, requiring precise fluid control and increasing difficulty and costs with no possibility of subsequent system upgrades and/or expansions.[27] Moreover, most fully integrated nucleic acid detection systems can only detect a single target, with increased detection made possible through fully integrated array structures on a single chip or the stacking of multiple control and detection instruments.[28] The aforementioned features highlight the necessity to further improve the detection efficiency and throughput of fully integrated microfluidic systems.

Here, we developed a fully integrated microfluidic system to automatically detect blood samples and determine the genetic polymorphisms associated with folic acid metabolism. Our microfluidic system can be divided into two functional modules: the DNA extraction module and the amplification module. The DNA extraction module utilizes an *EtNa* lysis solution[29] to release nucleic acids from different samples using a silica-gel membrane to capture and elute the DNA, significantly shortening the sample pre-processing time. The amplification module can complete multi-chamber separation of samples and realize multiple-target detection in one injection. Each reaction chamber is independent of the other to avoid cross-contamination. We developed a detection method for genetic polymorphisms related to folic acid metabolism based on AS-LAMP, which does not require complicated optical detection equipment and can be signaled by the changes in reagent color.[30] The detection method can achieve accurate and rapid detection of SNPs with high sensitivity.

We collected whole blood samples from five patients and identified mutated genes using our system. The test results were consistent with the standardized hospital test results and can be completed in less than 70 minutes, demonstrating the great prenatal diagnostic potential of our system.

Materials and methods

Designing the microfluidic cassette

We have previously developed a "3D Extensible" microfluidic design paradigm using a double-sided elastic tape and two plastic blocks to form the essential functional components (Fig. 1A).[31] The position between the port of the block and the through-hole of the flexible tape is misaligned, which creates in and out check valves that can be combined into double-check valves. The on–off valve was developed using the tape's elasticity and cooperating with external pneumatic control. Using this design paradigm, we created a microfluidic cassette for multiplex detection of gene mutations associated with folic acid metabolism (Fig. 1B and C and S1†). The disposable microfluidic cassette consists of nucleic acid extraction and amplification modules. The nucleic acid extraction module is 86 mm long, 28 mm wide, and 38 mm high and contains a 3 mm diameter silica-gel membrane for nucleic acid extraction. The lysis solution, washing solution, elution solution, LAMP mix, and mineral oil were stored in the "3D" block before usage. Fluid transfer during nucleic acid extraction was performed on a "2D" chip with various valves. The nucleic acid amplification module is 42 mm long, 20 mm wide, and 2 mm high and contains multiple reaction chambers for nucleic acid amplification. Double-sided tape (DS tape) with an acrylic foam base (4910 VHB, 3M, Maplewood, MN, USA) was employed to bind the two hard blocks and function as the elastic membrane in the valve.

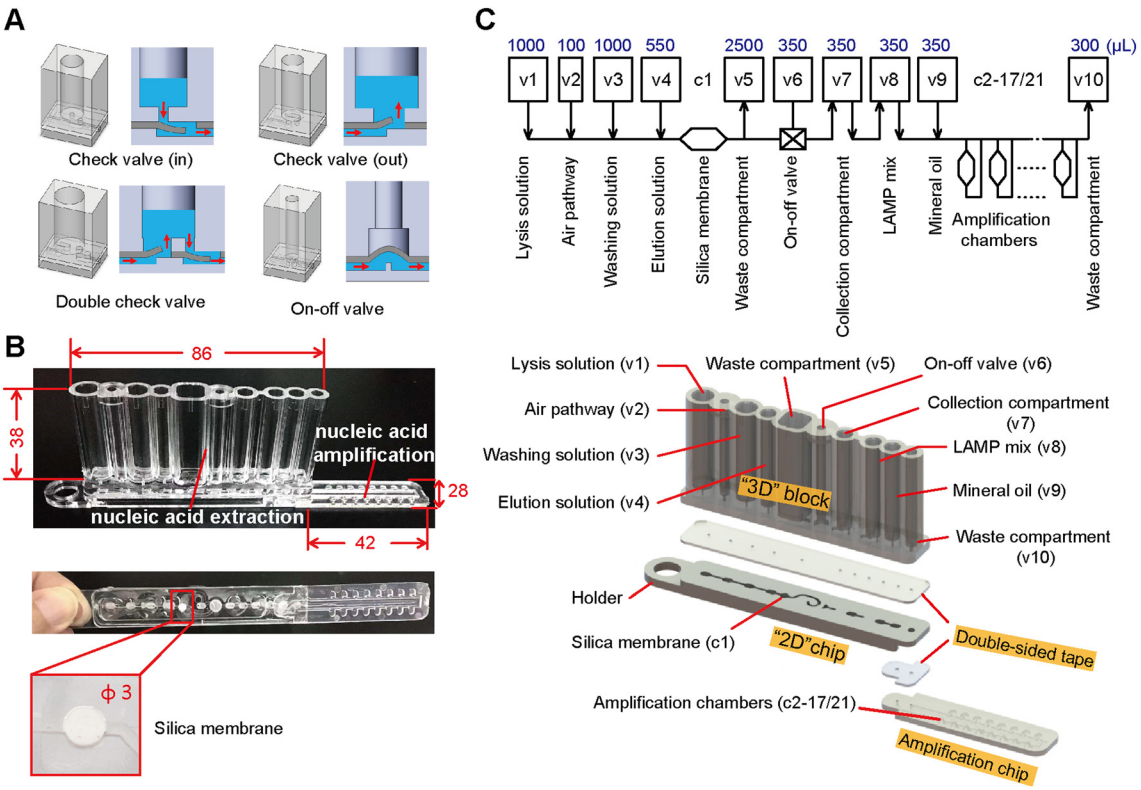

Fig. 1 Design of the microfluidic cassette. (A) The essential elements and the architecture of the cassette. Check valve (in), check valve (out), double check valve, and on–off valve. (B) Photographic images of the microfluidic cassette. The cassette contains two parts: the nucleic acid extraction module and the amplification module. (C) The schematic diagram of the cassette. The "3D" block and the "2D" chip are connected by double-sided tape to form the nucleic acid module and then combined with the nucleic acid amplification module by another double-sided tape.

Construction of the microfluidic cassette

The microfluidic cassette consists of four main parts: a "3D" block, a "2D" chip, an amplification chip, and DS tapes. The "3D" block, "2D" chip, and amplification chip were made of polymethyl methacrylate (PMMA) by injection molding. We improved our previous method of non-adhesive patterning procedure of the DS tape. As shown in Fig. S2,† the patterned double-sided adhesive tape model was designed in AutoCAD (Version 2015, Autodesk, San Rafael, CA, USA). According to the model, two dies were made of steel using milling and drilling techniques. Pieces of release paper (CY9970, Yichuang Electric, Suzhou, China) were cut by the die to achieve the desired pattern, after which a DS tape piece was covered with the patterned release paper as masks from both sides. The second die punched holes in the tape's corresponding position to form the valves. Talc powder was then smeared on the surface of the release paper. Where the release paper did not cover the double-sided tape, the talcum powder came into contact with the adhesive and removed the adhesiveness of the tape. Excess talcum powder was removed using nitrogen. We used double-sided tape to connect the upper and lower PMMA blocks with the help of the alignment tool. After binding, the microfluidic cassette was placed at room temperature for 48 hours to maximize the binding strength.

Microfluidic cassette analyzer

The analyzer was designed using the SolidWorks software and manufactured by the Sujia Technology Company (Shenzhen, China). This analyzer can detect eight cassettes simultaneously, consisting of three modules: a gas path module, a heating module, and an optical scanning module. The three modules are controlled by usb6259 DAQ (NATIONAL INSTRUMENTS). The collected information was uploaded to a computer for further processing (Fig. 2B). When the cassettes are inserted into the analyzer, the pneumatic interface is connected to the top of the cassettes to ensure air tightness (Fig. 2A). A set of air pumps can provide positive and negative pressure and are connected to the waste compartment (v5) and the on–off valve (v6) to control the flow path. Another set of air pumps can provide positive pressure and is connected to other parts as fluid-driven power. The gas flow path is gated through solenoid valves (Fig. 2D). A polyimide electric heating film is used to heat the module, and the temperature is fed back through a K-type thermocouple to maintain 65 °C (Fig. S3†). When the LAMP amplification reaction is over, a stepper motor with a high-definition camera captures images of each cassette. The collected images were processed using the LabVIEW software (NATIONAL INSTRUMENTS). First, the images were grayed and, after this, the appropriate segmentation threshold was

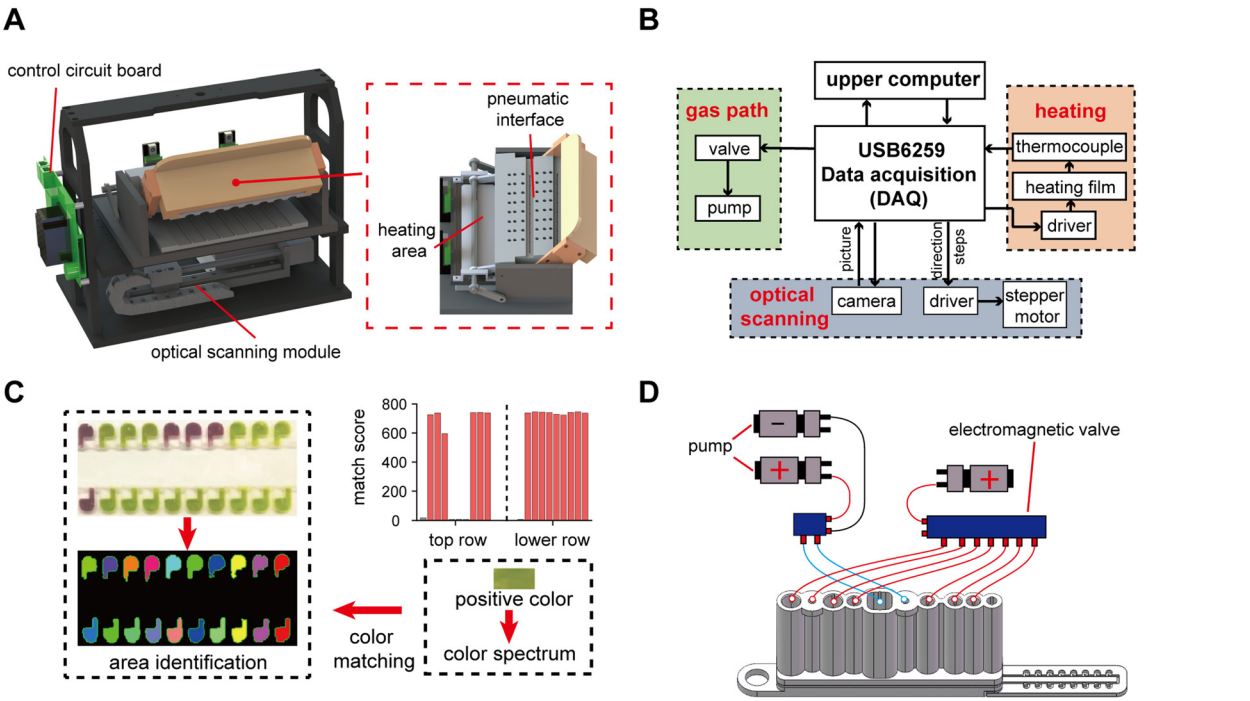

Fig. 2 Design of the microfluidic cassette analyzer. (A) The schematic diagram of the analyzer containing 8 detection channels and integrating the gas path, heating, and optical scanning modules. (B) The control frame of the analyzer (usb6259) is the data acquisition card that uploads the information of the gas path, heating, and optical scanning modules to the upper computer to realize unified processing of information. (C) Quantification of reaction results through region segmentation, color identification, and color matching. (D) Fluid-driven air path for the cassette. Air pumps are connected to the cassette through the pneumatic interface, and the air path is selected through the solenoid valves.

set to divide the image into multiple independent units. Finally, the contour of each unit was extracted and the outline color of each image analyzed. The result of the analysis was compared to the color spectrum of the positive reaction chamber to obtain matching scores, which were used to establish the corresponding threshold and classify as negative or positive (Fig. 2C and S3†).

DNA extraction

The *EtNa* lysate used in this study was referenced from previous work with modifications to adapt to our system.[29] The lysate consisted of 250 mM NaOH, 2.5 mM EDTA, and 75% alcohol. 200 μL of *Escherichia coli* and *Staphylococcus aureus* culture media, along with 50 μL of whole blood samples, were selected for lysis experiments. The FengteBio Corporation (Beijing, China) provided the bacterial samples, primers, and amplification reagents. The bacterial and blood genomic DNA extraction kits were purchased from Tiangen Biochemical Technology (Beijing, China) Co., Ltd. Bacteriophage λ-DNA (Promega, Madison, Wisconsin, USA) was used to check the DNA capture efficiency of the cassette. When λ-DNA was eluted from the silica-gel membrane (Beijing ABigen Biotechnology Co., Ltd.), the eluate was added into tubes for real-time PCR. Each tube contained 50 μL of reaction mixture, which consisted of 1 μL of forward/reverse primers (listed in Table S1†), 25 μL of 2× real-time PCR master mix (Thermo Fisher, Waltham, MA, USA), and 23 μL of deionized water. The thermal cycling protocol included the initial activation of the polymerase at 95 °C for 15 minutes, followed by 50 cycles of 95 °C 30 s, 60 °C 30 s, 72 °C 30 s, and the final extension at 72 °C for 10 minutes.

Loop-mediated isothermal amplification (LAMP) reactions

Unless otherwise indicated, LAMP reactions contained 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 8 mM $MgSO_4$, 1.4 mM dNTPs, 0.1% v/v Tween-20 and 0.2 U μL^{-1} *Bst 2.0* DNA polymerase. The reactions were incubated at 65 °C and all components mixed in water. The indicator dye was purchased from Capitalbio Biological Co., Ltd. Immediately before the reaction, primers (listed in Table S1†) were added or pre-embedded at the following concentrations: 1.6 mM FIP/BIP, 0.2 mM F3/B3, and 0.4 mM LoopF/B. Template DNA was 10 ng λ-DNA for the cross-contamination test. Plasmids with particular sequences were used as templates for sensitivity experiments. All reactions were repeated at least once and were typically performed in triplicate. All the LAMP primers were synthesized by Invitrogen (Shanghai, China).

Clinical blood sample test

All the blood samples were collected at the Fuwai Hospital Chinese Academy of Medical Sciences (CAMS) and approved by the local Ethics Committee. Written informed consent was obtained from the patients and/or their authorized representatives. Blood samples were extracted using the

blood genomic DNA extraction kit (TIANamp Genomic DNA kit) before real-time PCR and sequencing. In real-time PCR, 25 μL of the mixture was composed of 0.5 μL of forward/reverse primers (listed in Table S1†), 12.5 μL of 2× SYBR real-time PCR premix (GoTaq Hot Start Master Mixes), 11.5 μL of DI water, and 1 μL of the template. The thermal cycling protocol included an initial activation of Taq polymerases at 95 °C for 5 min, followed by 50 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension step for 10 min at 72 °C. The DNA sequencing results were obtained by Bioengineering (Shanghai, China) Co., Ltd.

Results and discussion

Workflow of the microfluidic cassette

In this study, the microfluidic cassette was designed to accomplish integrated detection of genetic polymorphisms associated with folic acid metabolism from blood samples of pregnant women. The workflow of the microfluidic cassette is shown in Fig. 3 and S4, and Video S1.† The fluid driving force in the detection process comes from the analyzer matched with the cassette, with the air pumps being used to drive the liquid forward and controlling the opening and closing of the valve. Before the detection, the blood sample and the related reagents for DNA extraction and amplification were manually loaded into the block. The primer pairs used for detecting different genetic mutations were pre-embedded in the reaction chambers of the amplification chip. After the cassette is inserted, the analyzer performs the following procedure steps in less than 70 minutes: (I) cell lysis and DNA capture (10 min). The plasma sample (50 μL) was added to the lysis solution (v1) partition. The sample was mixed with *EtNa* lysate and heated to 60 °C to conduct the lysis. After 5 minutes of lysis, the mixture flowed through the silica-gel membrane (c1) to complete the DNA capture while keeping the on–off valve (v6) closed and then flowed into the waste compartment (v5). (II) Washing (1 min). Positive pressure was applied to the washing solution (v3) partition to make 800 μL of washing solution flow through the silica-gel membrane (c1) while keeping the on–off valve (v6) closed, removing protein and other impurities on the silica-gel membrane (c1). Waste liquid entered the waste compartment (v5). (III) Drying (40 s). Positive pressure was applied to the air pathway (v2) partition while keeping the on–off valve (v6) closed. Air was injected into the pipe for 40 s to completely remove the residual ethanol on the silica-gel membrane. (IV) DNA elution (3 min). Positive pressure was applied to the elution solution (v4) partition while keeping the on–off valve (v6) open, and the waste compartment (v5) closed. The eluate flowed through the membrane, and the eluted DNA solution entered the collection compartment (v7). (V) Mixing (1 min). Positive pressure was applied to the collection compartment (v7) partition to make the DNA solution enter the reagent compartment (v8) partition to dissolve the dry powder of the LAMP reaction reagent. (VI–VII) Distribution (1 min). Positive pressure was applied to the reagent compartment (v8) partition. Reaction reagents entered the amplification chip and automatically filled each chamber, and the remaining reagents entered the waste compartment (v10). (VII) Isolation (20 s). Positive pressure was applied to the mineral oil compartment (v9). The sealing oil entered the amplification chip, exhausted excess gas, and prevented aerosol pollution during the amplification process. The remaining reagents entered the waste compartment (v10). (IX) LAMP reaction (45 min). The reaction chambers of the chip were incubated at 65 °C to perform the LAMP reaction, after which a stepper motor carrying an optical camera captured images of each channel and analyzed the amplification results.

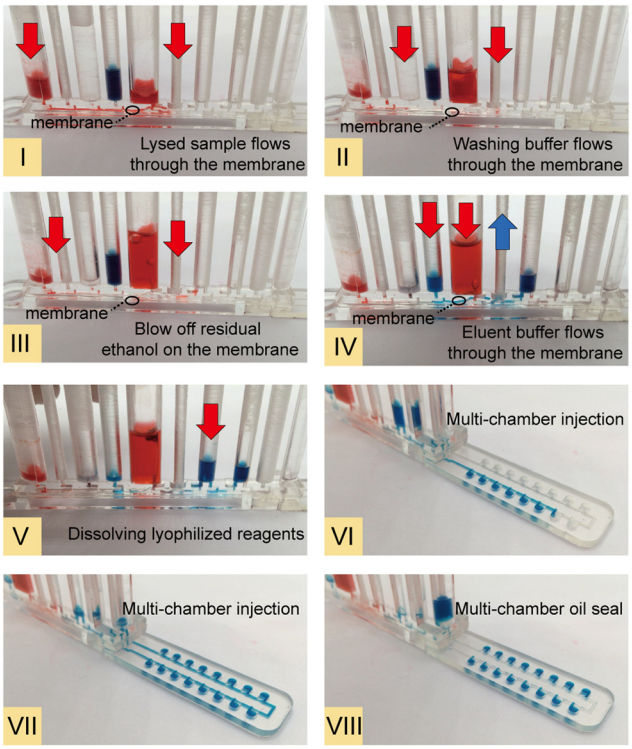

Fig. 3 The cassette processing protocol. Illustration of the entire flow control of the cassette. The automated sample processing sequences inside the cassette: (I) cell lysis and DNA capture; (II) washing; (III) drying; (IV) DNA elution; (V) mixing; (VI–VII) distribution; (VIII) isolation.

The efficiency of DNA extraction

The purity of the extracted DNA is essential for subsequent sensitivity and specificity of the amplification. The DNA extraction on the cassette included cell lysis and DNA capture. We used a lysis method based on EDTA and NaOH (*EtNa*) to conduct the cell lysis and used a silica-gel membrane to capture the DNA. *EtNa* is a fast and low-cost gDNA extraction protocol where it is only necessary to mix the lysate with the sample and heat this mixture at 65 °C for 5 minutes to complete lysis. To prove the efficiency of the lysis method, we compared it to commercial cell lysis kits. Three samples (*E. coli*, *S. aureus*, and human blood cells)

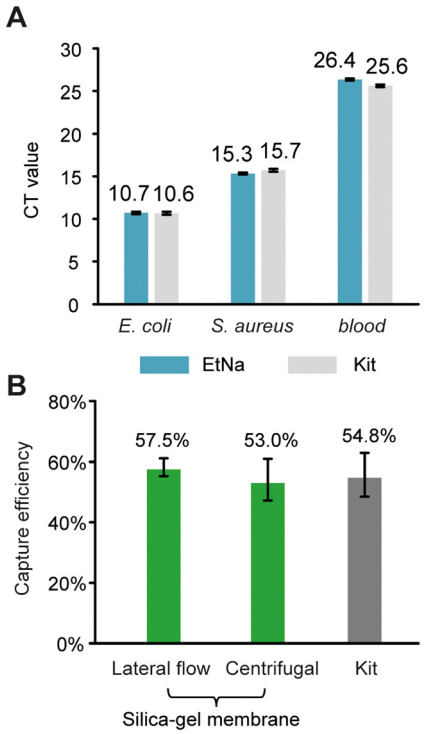

Fig. 4 The efficiency of the DNA extraction. (A) Lysis efficiency of the *EtNa* method for different samples. (B) DNA capture efficiency of the silica-gel membrane mounted in different ways.

were lysed using *EtNa* and commercial kits. Nucleic acid extraction was performed using the same nucleic acid extraction column. As shown in Fig. 4A, the *EtNa* lysis method has a similar efficiency to commercial cell lysis kits and is suitable for blood samples, bacteria, and fungi. This can help further expansion of our system. The solution containing 50 ng λ-DNA passed through the silica-gel membrane in a lateral or centrifugal flow to verify adsorption performance for nucleic acids (Fig. S5†). The lateral flow group employed a syringe pump with an injection speed of 1 mL min^{-1}. The filter membrane drying was achieved by injecting air into the chip for 40 s. The centrifuge and kit groups were operated following the Tiangen kit instructions. After this, the eluate was mixed with PCR reaction reagents for performing real-time fluorescent PCR amplification. As shown in Fig. 4B, the capture efficiency of each group was basically the same, demonstrating that the silica-gel membrane used is similar to the filter membrane employed in the Tiangen nucleic acid extraction kit in terms of capture ability. Simultaneously, this shows that lateral flow capturing of nucleic acids slightly outperforms centrifugation, which can be attributed to its stable and controllable flow rate and complete contact with the silica-gel membrane.

Cross-contamination test

The genetic polymorphisms in genes associated with folate metabolism require simultaneous detection of multiple mutation sites. This requires the reaction solution to be distributed in multiple amplification chambers with different primers pre-embedded in each chamber. The newly developed multi-index amplification microfluidic chip can realize automatic reagent distribution by relying solely on a pressure drive. Fig. 5A and B show the working principle of the chip. The reaction chambers arranged in the array and the injection pipe are connected by a thick and thin pipe. Each reaction chamber contains two valves. During the sample injection process, the reagent faces resistance caused by the liquid's surface tension while flowing through valve 1 and enters the reaction chamber. Since resistance imposed by valve 2 is greater than that of valve 1, the reagent breaks through valve 1 and continues to advance but cannot flow out. As shown in Fig. 5C, reagents enter through the chip entrance and are automatically distributed to each reaction chamber, which are isolated from each other. Furthermore, mineral oil can be continuously injected into the chip to ensure no aerosol contamination between the chambers. To verify whether the reagents in each reaction chamber of the multi-index amplification chip undergo cross-contamination during the sampling and amplification processes, we embedded the LAMP primer mix of λ-DNA in every other chamber of the amplification chip (Fig. S6†). We then injected the LAMP reaction reagent mixture containing the λ-DNA template into the microfluidic chip. After amplification at 65 °C for 40 minutes, the reaction chamber in which amplification occurred turned from dark purple to yellow-green. In contrast, the other reaction chamber remained dark purple. The color-matched scores of the pre-embed chambers were significantly higher than non-embed chambers, demonstrating that no cross-contamination occurred during the reaction.

Detection of genetic polymorphisms related to folic acid metabolism

LAMP is an efficient nucleic acid amplification method that can achieve highly sensitive detection in a short time without temperature cycling. However, this technique is rarely used for genotyping due to the complexity of its primer design. It is thus essential to design a new LAMP amplification method for genotyping, leading to suitable, low-cost, easy-to-use POCT testing. Based on AS-LAMP, two sets of LAMP primers were designed to distinguish between two diverse nucleotides corresponding to the target gene. The mutation site is located at the 3′ end of FIP and BIP. Additional mismatched nucleotides were introduced near the mutation site to enhance the specificity of target nucleotide sequence detection (Fig. S7†). As shown in Fig. 6A, mutant-type primers efficiently amplified the mutant sequence despite additional mismatched nucleotides being introduced near the mutation sites of FIP and BIP. However, the 3′ ends of both genes did not match the wild sequences. Moreover, the introduction mismatched sequences exacerbate the difficulty of their amplification.

We used this method to detect genetic polymorphisms related to folic acid metabolism on the chip. MTHFR and

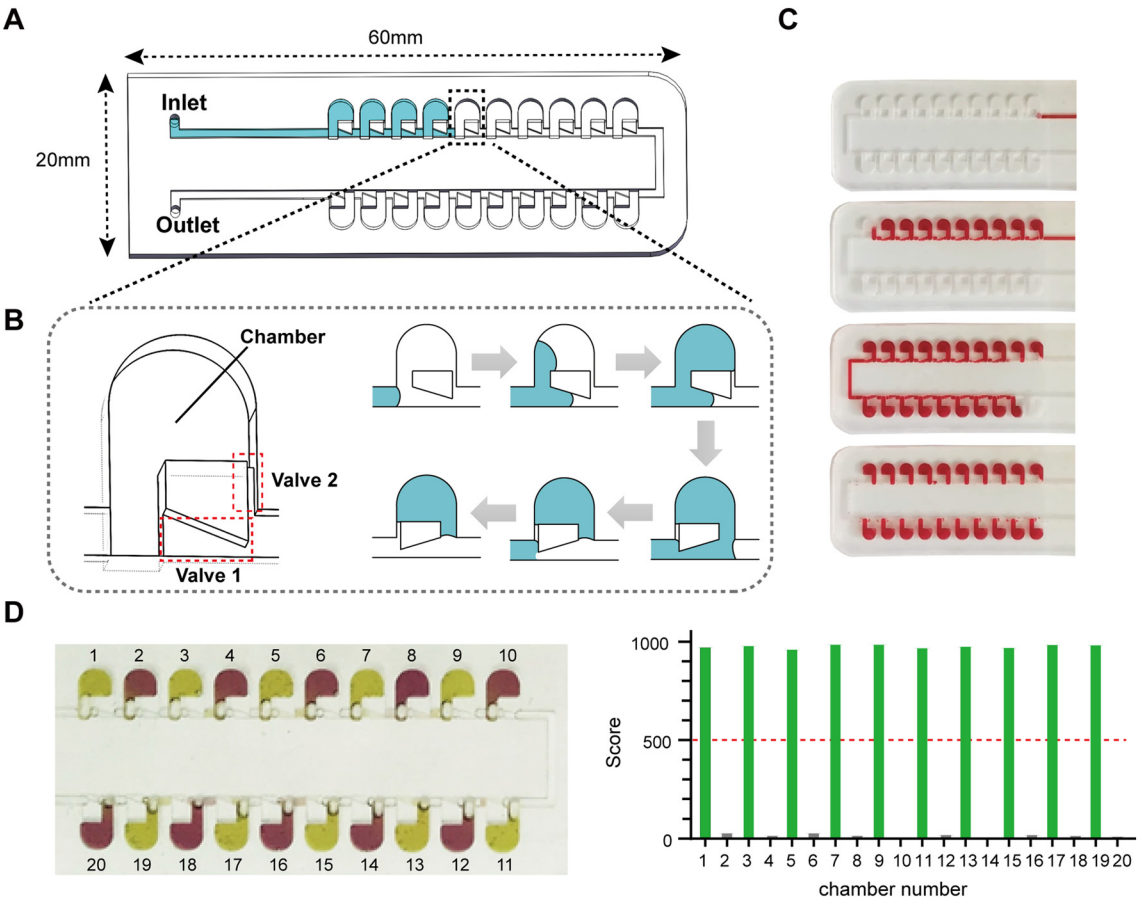

Fig. 5 Cross-contamination tests of the amplification chip. (A) Schematic layout of the amplification chip. (B) The chip processing protocol. Illustration of the flow control of the chip. (C) Photo image of the sample during injection and separation. (D) Cross-contamination tests of the chip. Reaction chambers with pre-embedded primers turn yellow-green after LAMP reaction and their respective color-matching score is higher than the threshold (500) when positive. Reaction chambers without primers are colored dark purple when negative.

MTRR are critical enzymes in folic acid metabolism, and MTHFR677 G > A, MTHFR1298 T > G, and MTRR 66 A > G are common mutations. The sample loaded into our microfluidic system is 50 μL of plasma. Clinically, 100–3000 ng μL^{-1} of genomic DNA can be extracted from this sample, and the corresponding copy number concentration should be greater than 3×10^4 copies per μL. The plasmid template of 3×10^4 copies per μL was used as the initial template concentration of the sensitivity experiment, and the template concentration was diluted 10 times and 100 times for the sensitivity test. The volume of each reaction chamber on the chip was approximately 7 μL, of which 0.8 μL of primer pairs or ultrapure water were added to the reaction chambers of the experimental or the negative control group in advance, respectively. The template solution and the LAMP reaction solution were mixed in a ratio of 1 : 1 and then injected into the chip. After 45 minutes of heating at 65 °C, the reaction chamber in which the amplification occurred turned from dark purple to yellow-green, while the other reaction chamber remained dark purple (Fig. 6B). We processed the amplified images with the LabVIEW software and obtained a color-matching score for each chamber. The experimental results show that our system can achieve stable detection of the three common SNP sites of folic acid metabolism when the initial template is diluted 10 times, and some sites can still be detected after 100 times dilution (Fig. 6C). Therefore, our detection limit is between 300 and 3000 copies per μL. The detection limit of the commercially used qPCR-based folic acid metabolism gene SNP detection kit for these three sites is 2 ng μL^{-1}, which is about 600 copies per μL. The detection limit difference between our detection system and commercial detection kits is within an order of magnitude, which meets the needs of clinical detection, while the visual LAMP detection method used greatly shortens the detection time and the requirements for detection instruments, which is convenient for sample pretreatment system integration.

Evaluation of the system in the analysis of clinical blood specimens

To further demonstrate that our microfluidic system can be used for prenatal diagnosis, we collected blood samples donated by 5 patients and performed SNP detection using

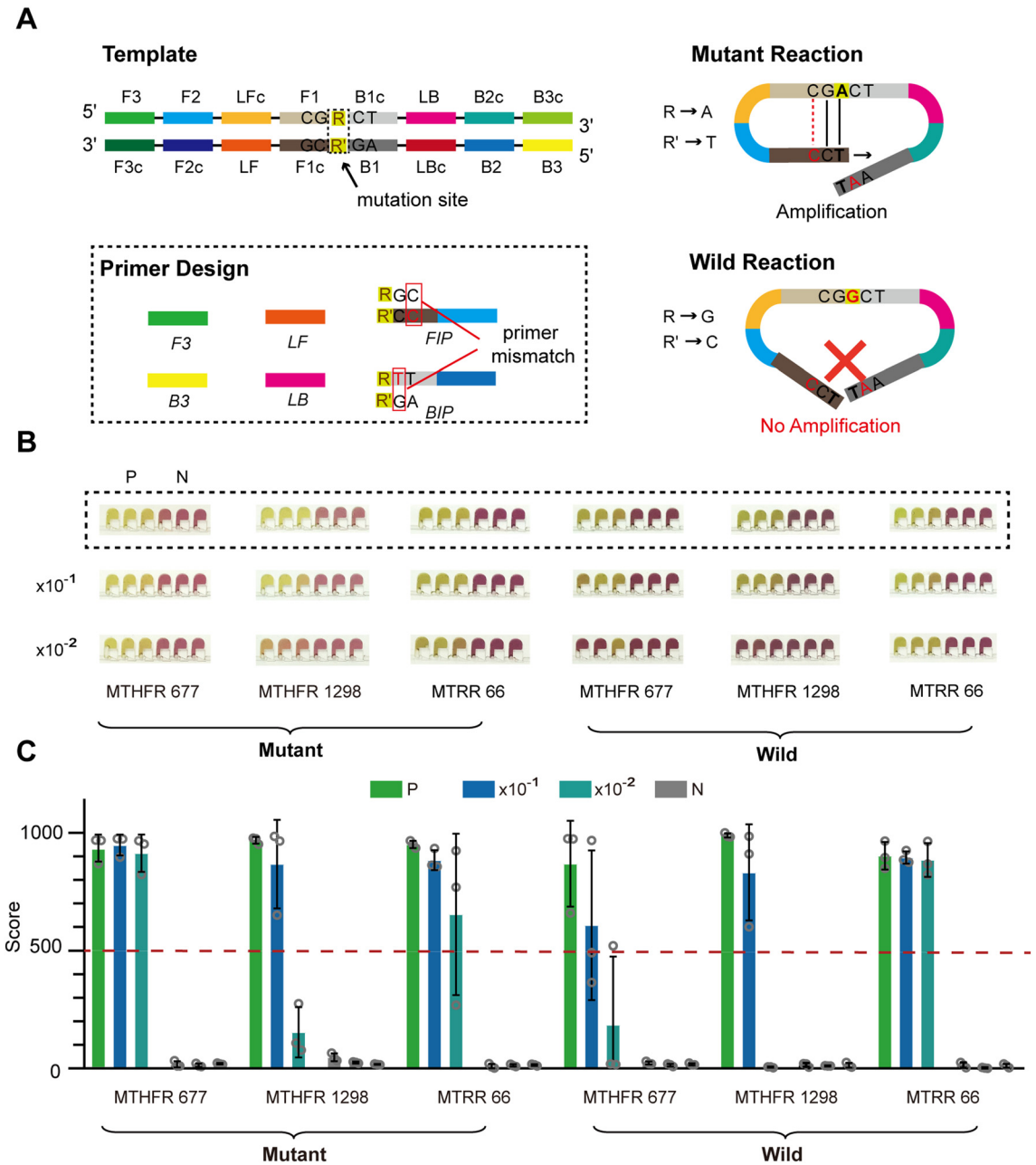

Fig. 6 Detection of SNPs related to folic acid metabolism. (A) Design principle of LAMP primers for detection of mutant genes. (B) Photo images of detection sensitivity of SNPs. (MTHFR 677, MTHFR 1298, MTRR 66). (C) The color matching score associated with detection sensitivity.

our system. At the same time, we extracted nucleic acids from these five samples and performed qPCR detection and sequencing (Fig. 7A and S8†). We designed the amplification chip to detect the wild and mutant types of each of the three sites in the MTHFR677, MTHFR1298, and MTRR66 genes. We repeated the three reaction chambers to ensure the detection accuracy and added two negative controls to confirm the validity of the amplification (Fig. 7B). Our system successfully detected the alleles present in the five samples at these three genetic loci and provided quantitative results using color matching (Fig. 7C). The detection results were completely consistent with the results of qPCR and DNA sequencing, which proved the feasibility and accuracy of the system. In addition, our detection time is only 70 minutes and no manual operation is required, which significantly improves the detection efficiency compared with qPCR and DNA sequencing. Unfortunately, due to limited access to clinical samples, we only tested these 5 samples, which has preliminarily proved its value in clinical application. In the future, we will look for more clinical samples and conduct more comprehensive and extensive tests.

A

The sequencing results				Fluorescence quantitative PCR results			
Sample ID	677	1298	66	Sample ID	677	1298	66
035b	W	W	M	035b	W	W	M
042b	W/M	W	W	042b	W/M	W	W
018b	W/M	W	W/M	018b	W/M	W	W/M
022b	W/M	W	W	022b	W/M	W	W
082b	M	W	W	082b	M	W	W

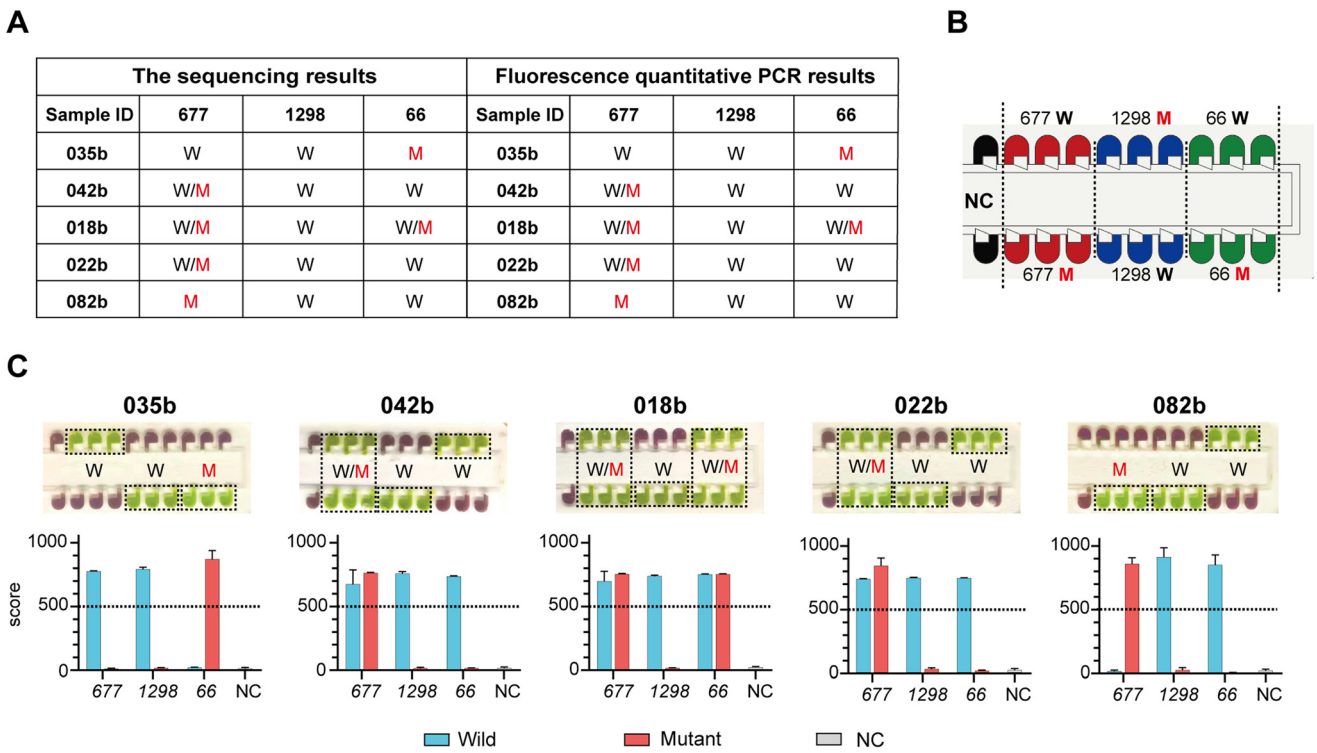

Fig. 7 Detection of genetic polymorphisms related to folic acid metabolism in clinical blood samples. (A) qPCR and sequencing results of clinical samples. (B) Detection of the positional arrangement of the 3 mutant genes on the chip, with each genotype being detected three times. (C) The detection results of 5 samples on the fully integrated nucleic acid analysis system.

Conclusion

We constructed a fully integrated nucleic acid analysis system for multiplex detection of genetic polymorphisms associated with folic acid metabolism in blood samples. The system consists of disposable microfluidic cassettes and a nucleic acid analyzer. Sample lysis, nucleic acid extraction and amplification, and mutation detection were performed inside the microfluidic cassette under the drive and control of the instrument. The entire detection process is fully automated and requires no human involvement. We used the *EtNa* method to achieve the lysis of different samples and combined it with the silica membrane to achieve efficient nucleic acid capture in lateral flow. Compared with extraction methods using magnetic beads, this method is simpler, more efficient and conducive to implementation with a microfluidic chip. We designed a color-change-based LAMP reaction to detect SNPs related to folic acid metabolism. This method has high detection sensitivity and can be stably applied even when sample concentration is diluted 10-fold. By introducing mismatched bases in the FIP and BIP primers, the specificity of LAMP amplification was enhanced and no cross-contamination was present. To further demonstrate that our microfluidic system can be used for prenatal diagnosis, we collected blood samples donated by 5 patients and performed SNP detection using our system. The detection results were matched to qPCR and sequencing results, showing that our system has the potential to detect mutations in genes associated with folic metabolism in pregnant women. According to the test results, the physician can provide the appropriate folic acid supplement plan and quantity to ensure safety during pregnancy and reduce congenital disabilities in newborns. In the future, this system can be easily improved to detect other SNPs or pathogens to provide physicians with early and critical information.

Author contributions

Baobao Lin conducted the experiments and wrote the manuscript; Zhi Geng helped in constructing the cassette and instrument and performed the extraction of nucleic acids; Bao Li and Wu Zeng helped in constructing the cassette and instrument; Yanjin Chen designed the LAMP primers used in the experiment; Peng Liu & Yan Zhang designed the entire study, supervised the work, and wrote the manuscript.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

This work was funded by Beijing Science and Technology Plans (Z190017) from the Beijing Municipal Science & Technology Commission, China.

References

1 V. A. Bjerregaard, L. Garribba, C. T. McMurray, I. D. Hickson and Y. Liu, *Proc. Natl. Acad. Sci. U. S. A.*, 2018, **115**, 13003–13008.
2 N. J. Wald, M. R. Law, J. K. Morris and D. S. Wald, *Lancet*, 2001, **358**, 2069–2073.
3 J. Momb, J. P. Lewandowski, J. D. Bryant, R. Fitch, D. R. Surman, S. A. Vokes and D. R. Appling, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 549–554.
4 J. R. Corona-Rivera, R. Nieto-García, A. S. Gutiérrez-Chávez, L. Bobadilla-Morales, I. M. Rios-Flores, A. Corona-Rivera, G. E. Fabián-Morales, I. Zavala-Cortés, C. Lugo-Iglesias and C. Peña-Padilla, *Am. J. Med. Genet., Part A*, 2019, **179**, 1857–1865.
5 A. Jahanbin, E. Shadkam, H. H. Miri, A. S. Shirazi and M. Abtahi, *J. Craniofac. Surg.*, 2018, **29**, e534–e541.
6 C. Hoyo, A. P. Murtha, J. M. Schildkraut, M. R. Forman, B. Calingaert, W. Demark-Wahnefried, J. Kurtzberg, R. L. Jirtle and S. K. Murphy, *BMC Public Health*, 2011, **11**, 46.
7 J. Shendure, S. Balasubramanian, G. M. Church, W. Gilbert, J. Rogers, J. A. Schloss and R. H. Waterston, *Nature*, 2017, **550**, 345–353.
8 A. Ahmadian and H. A. Svahn, *Lab Chip*, 2011, **11**, 2653–2655.
9 A. Amigou, J. Rudant, L. Orsi, S. Goujon-Bellec, G. Leverger, A. Baruchel, Y. Bertrand, B. Nelken, G. Plat, G. Michel, S. Haouy, P. Chastagner, S. Ducassou, X. Rialland, D. Hémon and J. Clavel, *Cancer Causes Control*, 2012, **23**, 1265–1277.
10 Y. Ménézo, P. Patrizio, S. Alvarez, E. Amar, M. Brack, C. Brami, J. Chouteau, A. Clement, P. Clement, M. Cohen, D. Cornet, B. Dale, G. D' Amato, L. Jacquesson-Fournols, P. Mares, P. Neveux, J. C. Sage, E. Servy, T. M. Huong and G. Viot, *J. Assist. Reprod. Genet.*, 2021, **38**, 2383–2389.
11 B. Lajin, A. Alachkar and A. A. Sakur, *Mol. Cell. Probes*, 2012, **26**, 16–20.
12 G. Q. Shen, K. G. Abdullah and Q. K. Wang, *Methods Mol. Biol.*, 2009, **578**, 293–306.
13 S. Kou, D. Cheng, F. Sun and I. M. Hsing, *Lab Chip*, 2016, **16**, 432–446.
14 L. Zhang, F. Tian, C. Liu, Q. Feng, T. Ma, Z. Zhao, T. Li, X. Jiang and J. Sun, *Lab Chip*, 2018, **18**, 610–619.
15 F. Tian, C. Liu, J. Deng, Z. Han, L. Zhang, Q. Chen and J. Sun, *Sci. China: Chem.*, 2020, **63**, 1498–1506.
16 J. Park, D. H. Han and J. K. Park, *Lab Chip*, 2020, **20**, 1191–1203.
17 C. D. Ahrberg, A. Manz and B. G. Chung, *Lab Chip*, 2016, **16**, 3866–3884.
18 H. Norian, R. M. Field, I. Kymissis and K. L. Shepard, *Lab Chip*, 2014, **14**, 4076–4084.
19 Y. Xu, H. Yan, Y. Zhang, K. Jiang, Y. Lu, Y. Ren, H. Wang, S. Wang and W. Xing, *Lab Chip*, 2015, **15**, 2826–2834.
20 Y. Zhao, X. G. Hu, S. Hu and Y. Peng, *Biosens. Bioelectron.*, 2020, **166**, 112447.
21 P. Craw and W. Balachandran, *Lab Chip*, 2012, **12**, 2469–2486.
22 M. Soroka, B. Wasowicz and A. Rymaszewska, *Cells*, 2021, **10**, 1931.
23 Y. Ju, H. Y. Kim, J. K. Ahn and H. G. Park, *Nanoscale*, 2021, **13**, 10785–10791.
24 R. M. Bialy, A. Mainguy, Y. Li and J. D. Brennan, *Chem. Soc. Rev.*, 2022, **51**, 9009–9067.
25 T. Li, H. Zou, J. Zhang, H. Ding, C. Li, X. Chen, Y. Li, W. Feng and K. Kageyama, *Analyst*, 2022, **147**, 3993–3999.
26 P. Oberacker, P. Stepper, D. M. Bond, S. Höhn, J. Focken, V. Meyer, L. Schelle, V. J. Sugrue, G. J. Jeunen, T. Moser, S. R. Hore, F. von Meyenn, K. Hipp, T. A. Hore and T. P. Jurkowski, *PLoS Biol.*, 2019, **17**, e3000107.
27 Q. Ramadan and M. A. Gijs, *Analyst*, 2011, **136**, 1157–1166.
28 Q. Song, X. Sun, Z. Dai, Y. Gao, X. Gong, B. Zhou, J. Wu and W. Wen, *Lab Chip*, 2021, **21**, 1634–1660.
29 L. Vingataramin and E. H. Frost, *BioTechniques*, 2015, **58**, 120–125.
30 N. A. Tanner, Y. Zhang and T. C. Evans, Jr., *BioTechniques*, 2015, **58**, 59–68.
31 Z. Geng, Y. Gu, S. Li, B. Lin and P. Liu, *Micromachines*, 2019, **10**, 873.