ABSTRACT: We report a novel design of chamber-based digital polymerase chain reaction (cdPCR) chip structure. Using a wet etching process and silicon-glass bonding, the chamber size can be adjusted independently of the process and more feasibly in a normal lab. In addition, the structure of the chip is optimized through hydrodynamic computer simulations to eliminate dead space when the sample is injected into the chip. The samples will be distributed to each separated microchambers for an isolated reaction based on Poisson distribution. Due to the difference in expansion coefficients, isolation of the sample in the microchambers by the oil phase on top ensures homogeneity and independence of the sample in the microchambers. The prepared microarray cdPCR chip enables high-throughput and high-sensitivity quantitative measurement of the SARS-CoV-2 virus gene and the mutant lung cancer gene. We applied the chip for the detection of different concentrations of the mix containing the open reading frame 1ab (ORF1ab) gene, the most specific and conservative gene region of the SARS-CoV-2 virus. In addition to this, we also successfully detected the fluorescence of the epidermal growth factor receptor (EGFR) mutant gene in independent microchambers. At a throughput of 46 200 microchambers, solution mixtures containing both genes were successfully tested quantitatively, with a detection limit of 10 copies/μL. Importantly, the chips are individually inexpensive and easy to industrialize. In addition, the microarray can provide a unified solution for other viral sequences, cancer marker assay development, and point-of-care testing (POCT).

1. INTRODUCTION

Coronavirus Disease 2019 (COVID-19) is a highly infectious respiratory illness caused by the SARS-CoV-2, which has spread around the world in just a few months and became a worldwide pandemic. So far, many scientific researchers around the world are analyzing and exploring the new coronavirus, hoping to get more information and treatment possibilities. ORF1ab is the most specific and conservative gene region of the SARS-CoV-2 virus, which can encode a nonstructural protein RNA-dependent RNA polymerase (RdRp). It is a key enzyme in the SARS-CoV-2 virus’s biological cycle, responsible for viral replication and transcription, and currently considered to be an important target for SARS-CoV-2 detection. Quick and accurate detection of the ORF1ab gene is very important for the diagnosis of infected patients and controlling transmission.

In addition to infectious diseases, cancer is one of the most important topics to be tackled. Lung cancer is one of the fastest growing malignancies in terms of incidence and mortality, and one of the most threatening to the health and lives of the population. EGFR is a protein on the surface of cells in the body. When the EGFR gene is mutated, the EGFR gene expresses the EGFR protein and assembles on the cell membrane surface, resulting in too many epidermal growth factor receptors on the cell membrane surface. This means that...
the epidermal growth factor can bind to a large number of receptors and accelerate the promotion of abnormal cell growth and division, which eventually leads to the birth of tumors. EGFR gene mutations can be detected by genetic testing for ultra-early detection and targeted therapy of lung cancer.6,7

Polymerase chain reaction (PCR) microchip is a powerful technique commonly used for biological sample amplification, especially for the rapid detection of the above genes. Since the PCR technology has been invented by American scientist Kary Mullis in 1985,8 this technique experienced the first generation of gel electrophoresis PCR technology and the second generation of real-time fluorescence quantitative PCR technology, and the third generation of digital PCR technology has been developed to date.9−11 PCR can be used for the analysis of gene mutation, gene expression research, miRNA expression analysis, single-cell gene expression analysis, etc.12

Digital PCR is a method for the absolute quantification of nucleic acid molecules. Using microfluidic chip technology to integrate sample preparation, reaction, separation, and detection into a single chip, you can directly count the number of DNA molecules.13−15 Unlike quantitative PCR, which requires reliance on standard curves or reference genes to measure nucleic acid amounts, digital PCR is more convenient, more accurate, easier to perform, and more easily developed for POCT applications.16,17

Due to these excellent characteristics, it excels in the detection of very small nucleic acid samples, the identification of small differences in expression, and the detection of rare mutations. It also has promising applications in pathogen detection, food testing, efficacy assessment, detection of rare mutations in cancer markers, single-cell gene expression, diagnosis of fetal developmental disorders, and many other areas.18−22

With the development of various microassembly technologies, microelectromechanical systems (MEMS) combined with biodetection can produce digital PCR chips with a large specific surface area and high integration. However, there are still some defects in many aspects, such as insufficient sealing of the chip, which may lead to sample loss and sample contamination. In addition, the high cost of the chip, the lack of integration and sensitivity, and the insufficient simplicity of operation are to be improved.23−25 Currently there are two main forms of dPCR, chip-based dPCR and droplet-based dPCR(ddPCR), but the basic principle is to disperse a large amount of diluted nucleic acid solution into microreactors or microdroplets on a chip, with less than or equal to 1 nucleic acid template per reactor. Compared to ddPCR, cdPCR generates microdroplets of uniform volume, with higher stability and less influence between systems.26−29

In this article, we present a novel cdPCR chip structure design. Utilizing the wet etching process and silicon-glass bonding, the chamber dimension can be adjusted independently without being affected by the process. When the premix is dropped into the inlet, the fluid directly filled the chamber through capillarity. Due to the different expansion coefficients between the mineral oil and the sample, the sample may be blocked in the microchambers. This results in a homogeneous and independent premix in the microchannel and helps prevent sample contamination. Based on this strategy, a low-cost, high-throughput, high-sensitivity, and high-precision digital PCR chip for target gene testing can be fabricated.

2. RESULTS AND DISCUSSION

2.1. Design of the Microarray to Detect Target Genes.

The working principle of the chip we designed is shown in Figure 1. First, the PCR premix is injected at the inlet port and

![Figure 1. Strategy of the Designed In Silico Digital PCR Chip for Detecting Target Genes.](https://doi.org/10.1021/acsomega.1c05082)
oxide layer thickness of about 5000 Å as a sacrificial layer (Figure 2g). The microchamber pattern is formed after photolithography development by dumping the photoresist on the front side of the substrate (Figure 2h,i). The silicon oxide layer was etched with a BOE etchant and the microchamber pattern on the photoresist was transferred to the oxide layer after development (Figure 2j). After debinding, the silicon layer was etched anisotropically by wet etching with 30% KOH etching solution at 50°C. The microchambers were prepared by controlling the etching rate and etching time (Figure 2k,l). The remaining silicon oxide layer was etched with the BOE etchant after sulfuric acid cleaning (Figure 2m). A BYF33 glass substrate that can be bonded to the silicon wafer was selected. We used an electrostatic bonding process, which first cleans the silicon wafer 312 and then uses acetone ultrasound. Alignment bonding was performed after megasonic cleaning. A 1–2 mm diameter injection hole was punched by laser punching at a set positioning point for the injection of the sample to be measured and the mineral oil. The silicon-glass bonding process, as shown in Figure 2n, was used to obtain this silicon-based digital PCR chip.

2.3. Water and Oil Containment Test. The silicon wafer has good microfabrication properties and the silicon oxide wafer with a single polished surface of (100) can be anisotropically etched along the (111) crystal surface by a wet etching process with potassium hydroxide, resulting in a microchamber with an inverted quadrilateral shape, neatly arranged and uniform in size. The (100) crystalline silicon wafer is selected as the chip substrate, the front side is etched with a concave cell and internal microvials, and the top is bonded with BF33 borosilicate glass, the entire chip forms a closed chamber, and the glass has been punched with laser perforation for sample access and mineral oil access.

The optical image of the chip, the bright-field and dark-field microscopy images, are shown in Figure 3A–C. No surface hydrophilic modification is required and the injection process is introduced spontaneously by capillary action. Moreover, injection often takes only a few seconds to complete, making it convenient and fast. Since the density of mineral oil is lower than the density of the sample, injection from the inlet port into the chip drains excess sample from the reservoir inside the chip. All samples are blocked in the microchamber array, allowing samples to be stored uniformly in each microchamber for independent PCR reactions. This ensures the cleanliness and independence of the reaction process.

2.4. Optimization of the Digital PCR Chip Structure. 2.4.1. Chip Edge Structure Optimization. The premix can be added dropwise during the injection process and flow spontaneously into the microchamber due to the capillary phenomenon. The mineral oil oil-phase injection process requires the connection of an external injection pump to fill the chip. Both of these steps have the potential to cause bubbles to remain in the dead space of the injection, resulting in a change in bubble volume during PCR in the closed chamber to compress the flow of liquid in the microchamber, ultimately leading to experimental failure. Therefore, the dead space at the edge of the chip structure needs to be optimized.

In optimization by COMSOL fluid pressure simulation, there is a step of pressure release from the microchannel of the inlet to the microchamber platform. The liquid phase simulates the flow direction from left to right. If the radius of the rounded corner at the edge is too small, it will result in the liquid not reaching the most corner of the edge of the chip inlet microchannel, as shown in Figure 4. We set the laminar flow of cdPCR chip microprocessing (a) growing the oxide layer, (b) spin coating the photoresist, (c) photolithography, (d) etching the oxide layer, (e) KOH etching, (f) removing the oxide layer, (g) growing a new oxide layer, (h) spin coating the photoresist, (i) photolithography, (j) KOH etching, (k) etching the oxide layer, (l) KOH etching, (m) deoxidizing layer, and (n) bonding glass.

Figure 3. Optical image of the cdPCR silica-based glass chip (1 × 2 cm²), photo by author Sun Yimeng (A) rhodamine feed sealed by mineral oil, bright-field (B), and dark-field (C) images of the chip.
flow interface to model and use the line average feature to evaluate the relative pressure at the inlet. In a state that approximates the actual pressure field, the right-angled edges on both sides of the input platform from the pipe cannot be filled when the oil phase is introduced. We optimized the dead-end region along the predicted trajectory to avoid this situation. The exit platform will also have a dead corner of the inlet, which will lead to bubbles in the chip. The optimized structure of the flowing chip avoids this problem.

2.4.2. Microchamber Etching Depth Optimization. In a microchamber array, the volume that can be accommodated in the microchamber is proportional to the square etching depth. In contrast, the KOH etching process on the silicon (100) crystal plane is proportional to the square depth of the wet etching microchamber. To obtain a high-throughput microchamber array, the volume that can be accommodated in the inlet, which will lead to bubbles in the chip. The optimized structure of the flowing chip avoids this problem.

So we made mask plates with microchamber side lengths of 10, 20, 30, 40, and 50 μm to explore the optimal size (Figure 6A,B). We have designed microchambers of different diameters on the same silicon wafer. This size is mainly restricted by two aspects: On the one hand, the larger the diameter of the microchambers, the smaller the number of microchambers in the chip of the same size. On the other hand, as the diameter becomes smaller, the microchambers obtained by wet etching are too shallow for complete PCR. So we prepared the diameter gradient to get the solution with the optimal size. In the experiment, it was found that when the diameter was less than 20 μm, PCR could not be carried out.

We think this is because the anisotropic corrosion of wet etching limits the volume of the sample stored in the cavity. Because the sample volume is too small, premix is not enough to support the reaction. Since the microchamber with 10 μm edge length was not deep enough to be fed by the water/oil method, the microchamber with 20 μm edge length was finally determined as the optimal size of the chip. The bright and dark fields after PCR amplification are shown in Figure 7A,B. The bright-field image on the left is a local orthomosaic microscope photograph of a 20 × 20 μm² digital PCR chip, with the microchambers arranged in an inverted pyramidal array, and the overall uniform distribution in the octant slot. By calculation, it can be obtained that just 48 nL of the sample can fill all of the microchambers of the entire chip.

2.5. Gene Quantitative Assay. 2.5.1. ORF1ab Gene Quantitative Assay. We further applied the chip for the detection of different concentrations of the mix containing the ORF1ab gene of the SARS-CoV-2 virus. The quantitative test results are shown in Figure 8. The image magnification is 20 times and the exposure time is 2 s.

When a high-concentration sample solution was added (Figure 8A), the number of nucleic acid molecules is larger than the total number of microchambers, and it can ensure that all microchambers have nucleic acid templates and present positive signals. With the dilution of the sample solution, the nucleic acid templates are not enough to fill all microchambers, and the positive signal will show an obvious Poisson distribution. The number of positive microchambers can show a significant gradient change with the change of the dilution factor.

When the sample solution with a template concentration of 10⁵ cp/μL is added, because of the large number of nucleic acid molecules in the unit volume of sample solution, all of the microchambers theoretically contain at least one target molecule, and the whole field of vision shows all positive signals. In accordance with the expected value, our actual results also show the strong fluorescence signal of all microchambers. When a sample solution with a template concentration of 10³ cp/μL was added, the number of theoretically positive microchambers was 3–4 and the number of positive signals we actually tested was 4, which basically corresponded to the theoretical value. Continuous dilution of the sample solution, as shown in Figure 8C, when the sample solution with a template concentration of 10³ cp/μL is added, according to the Poisson distribution principle, theoretically, the number of positive microchambers is 0 or 1. In fact, the number we measured is 1, which is also consistent with the theoretical
value. When the sample solution is continuously diluted, because the number of nucleic acid molecules in unit volume is very small, it is difficult to observe a positive signal in injection detection. We also set a blank control, which did not show a positive signal as expected (Figure 8D). So for now, the lowest detection concentration of $10^1 \text{cp}/\mu \text{L}$ can be achieved in this microchamber environment. The results show that the chip can be successfully used for the qualitative and quantitative detection of the ORF1ab gene of the SARS-CoV-2 virus, and has great advantages in detection flux, sensitivity, accuracy, and reagent consumption.

2.5.2. EGFR Gene Quantitative Assay. The injection process takes advantage of the difference in the expansion coefficient between mineral oil and sample to block the sample to be tested in the microchambers, which is uniformly independent and helps prevent sample contamination to produce a high-throughput, highly sensitive, high-precision digital PCR chip for target gene testing.

According to the principle of Poisson distribution, the copy number of target molecules in the reaction system can be calculated by the equation

$$A = -\ln \left( \frac{N - X}{N} \right) \times N$$

(number of chambers—$N$, number of positive reaction systems—$X$), thus solving the possibility of multiple target molecules existing

Figure 6. Optimization process diagram of the microchamber size. Bright-field (A) and dark-field (B) images after sampling of a cdPCR chip with different microchamber edge lengths.

Figure 7. Bright-field (A) and dark-field (B) images after sampling of the cdPCR chip with a microchamber edge length of 20 μm.

Figure 8. ORF1ab gene quantitative test results (A) template concentration is $10^5 \text{cp}/\mu \text{L}$, (B) concentration is $10^3 \text{cp}/\mu \text{L}$, (C) concentration is $10^1 \text{cp}/\mu \text{L}$, and (D) blank control.
in a single droplet. As the number of positive reaction system \( X \) continues to increase, the uncertainty of the digital PCR results also increases. Generally speaking, the number of digital PCR positive systems should not exceed 80% of the total number of systems. On the other hand, the increase of \( N \) will make the entire digital PCR system have a larger linear range, so it is necessary to increase the number of chambers and the number of droplets to be distributed while the cost is controllable.

As shown in Figure 9A–D, the wild-type fluorescence fully fills the microchamber with 100% filling after sample addition at a concentration of around 10⁵ copies/μL. With the dilution of concentration, the fluorescence of mutant genes fills the microchamber with the Poisson distribution pattern in a 10⁻² dilution state. When the dilution reaches 10⁻⁴, the microchamber inside the chip is sufficient to fill all mutant genes independently and the detection limit is reached, which is around 10 copies/μL. We subjected the above experiments to a linear gradient test, and the measured linearity of the dilution gradient reached \( R^2 = 0.99942 \). This means that the chip can guarantee both high-throughput detection and quantitative detection, i.e., digital detection.

3. CONCLUSIONS
We fabricated high-throughput digital PCR chips based on microfabrication techniques using silicon wafers as the substrate material. The chips were fabricated by photolithography, wet etching, and other process steps. And the structure was completed by silicon-glass bonding by laser punching on the coverslip. The chip structure was optimized by computer simulation to eliminate the dead space of sample feeding. During the feeding process, different expansion coefficients of mineral oil and the sample inhibited the storage of samples in the microchambers and ensured uniform independence of samples in the microchambers. We also tested the ORF1ab gene of the SARS-CoV-2 virus and the EGFR mutated gene of lung cancer using the designed and fabricated cdPCR chip. The test of these two real samples yielded satisfactory results for independent mutation detection. In addition, the prepared cdPCR chip is easy to operate, low cost, and highly sensitive, and has good utility for ultra-early cancer detection, as well as for the detection and control of the epidemic.

4. MATERIAL AND METHODS

4.1. Reagents and Instruments. Poly(dimethylsiloxane) (PDMS) was purchased from Dow Corning. Mineral oil and double-distilled water were procured from Sigma-Aldrich. Upstream primers, downstream primers, Minor Groove Binder (MGB) probe, and pGEM plasmid for the EFGR gene exon 21 gene sequence were procured from Shanghai Biosign Biotechnology Co. The upstream primers, downstream primers, and probes for the ORF1ab gene sequence of the SARS-CoV-2 virus were purchased from Shanghai Zhanbiao Biotechnology Co., and the pUC57 plasmid for the ORF1ab gene was procured from Sangon Biotech (Shanghai) Co., Ltd.

The PCR in situ amplifiers used for cdPCR chip reactions were purchased from Eppendorf, Germany. The chip injection pneumatic pump was purchased from Suzhou Wenhao Co. The microscope for observing the injection process was IX73, and the fluorescence imaging system was a IX51 microscope and a DP80 CCD image sensor, both purchased from Olympus, Japan. The image processing was done using imageJ software.

4.2. Preparations of the PCR Premix. Probes and primers used for cdPCR reactions were diluted to 10 μM with double-distilled water. Primers included upstream primers and downstream primers, and the probes for the EFGR gene were ROX-labeled MGB hydrolysis probes, as shown in Table 1.

| Primer   | MGB Probe Sequence for the EFGR Gene |
|----------|--------------------------------------|
| forward  | AGCATGCAAGATCAGAGTTT                 |
| reverse  | CCTCTTCGATGTGATTTTC                  |
| MGB probes | ROX-TCTTCCGACCCAGC-MGB              |

The reaction system was a 10 μL system containing 5 μL of the 2X LightCycler 480 Probe Master, 0.4 μL of primers, 0.3 μL of the probe, and 1 μL of the DNA template. Primers included upstream primers and downstream primers, and the probes for the ORF1ab gene were FAM-labeled MGB hydrolysis probes, as shown in Table 2. The reaction system was a 10 μL system containing 5 μL of the 2X LightCycler 480 Probe Master, 0.45

![Figure 9. EGFR gene quantitative test results: (A) template concentration is 10⁵ cp/μL, (B) concentration is 10³ cp/μL, (C) concentration is 10¹ cp/μL, and (D) blank control. Dilution gradient plot for EGFR gene quantification assay with a dilution factor of 1–10⁻⁴.](https://doi.org/10.1021/acsomega.1c05082)
μL of primers, 0.2 μL of the probe, and 1 μL of the DNA template.

| Table 2. Primer and MGB Probe Sequence for the ORF1ab Gene |
| --- |
| reactants | sequence (5′-3′) |
| forward primer | TAGCTAATGAGTGCTGCTGAATTT |
| reverse primer | GTTGTGGCATCTCGTGATGAG |
| MGB probes | FAM-TGTGATGCGGTTGACTAT-MGB |

4.3. Fabrication of the Microchip. The steps of the chip fabrication method are shown in Figure 5. First, a layer of 3000 Å silicon oxide is made on the silicon wafer surface as a sacrificial layer. The organic and inorganic impurities on the surface of the silicon oxide layer are removed by cleaning and dried by dehydration hot baking. Then, the groove pattern of the mask is transferred to the sacrificial layer by photolithography, and the groove is etched on the wafer by the wet etching process. Finally, the inlet and outlet holes are punched into the Borosilicate33 (BF33) glass cover sheet and the silicon-glass bonding is performed. After scribing, our cdPCR chip is obtained.

4.4. Validation Experiment of the cdPCR Chip. To be able to confirm the correctness of the fabrication process, we performed validation experiments of the microchips using Sudan red fluorescent dye instead of biological samples. On the basis of this, we also fabricated chips with different pore sizes by the flow-through process. The pore size of the micro-chambers was determined by simulating the sample feed with the dye.

4.5. Quantitative EGFR and ORF1ab Gene Testing. The quantitative assay experiment used the EGFR exon 21 gene and the ORF1ab gene as the target genes, which were used to check the performance of the microarray assay. The sample used for the EGFR gene detection is the pGEM plasmid solution embedded with the target gene, and the stock solution is diluted into multiple concentration samples at the ratio of 1:10^{-1}:10^{-2}:10^{-3}:10^{-4}:10^{-5} for the assay. The sample used for ORF1ab gene detection is the pUC57 plasmid solution embedded with the target gene, and the stock solution (10^5 copies/μL) is diluted into multiple concentration samples of 10^3 and 10^2 copies/μL for the assay, and another is set as a blank control.

ASSOCIATED CONTENT

Supporting Information
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Notes
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