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Integrated and Rapid Detection of SARS-CoV-2 and a Variety of Respiratory Viruses

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Abstract

Because of the rapid spread of respiratory viruses and the sharp increase in the number of infections, the most efficient way is to detect positive patients as quickly as possible. In this paper, we have developed an all-in-one integrated microsystem for the diagnosis of multiple respiratory viruses. The integrated system is based on magnetic bead method for nucleic acid extraction and adopts isothermal amplification and fluorescence detection system, which can be applied for point-of-care detection of respiratory viruses. The entire detection process from sample collection, nucleic acid extraction, cassette equipment, fluorescence detection and result output can be completed within one hour. From the analysis of the results, the detection limit could reach 100 copies/μL, and there was no non-specific amplification detected by five sets of primers, implying that the integrated detection system had high sensitivity and specificity. The extraction and detection processes were optimized and compared with commercial instruments. The results demonstrated that the device could be applied to the detection of a variety of respiratory pathogens without aerosol contamination in the process of sample amplification.

Keywords: Respiratory viruses; Point-of-care testing; Integration

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Introduction

Since the end of 2021, the large-scale outbreak of SARS-CoV-2 has caused social panic due to its rapid onset and extremely high morbidity. There are numerous types of respiratory viruses, viruses are easily to mutate, and are easy to infect and induce pandemics [1,2]. Acute respiratory infections included viruses, bacteria, fungi and atypical pathogens, among which viral infections accounted for the highest proportion, and influenza A virus, influenza B virus, respiratory syncytial virus and adenovirus were the most common in daily life [3-6]. Respiratory pathogens are usually transmitted by contact, droplets or aerosols [7]. Because the symptoms of respiratory viruses are comparable, different medications should be selected for different viruses. The development of combined testing for respiratory pathogens is extremely significant and can result in significant cost savings and a reduction in the probability of hospital-acquired viral infections [8-10].

At present, the laboratory methods employed in clinical laboratories for the diagnosis of respiratory pathogens include virus culture, nucleic acid amplification-based testing, antigen-antibody detection based testing, and high-throughput sequencing [9]. Virus culture method takes a long time and demands a lot of manpower and material resources, which cannot be used to detect the results swiftly and effectively. Antigen-antibody tests have weak sensitivity and cannot detect patients carrying low concentrations of virus [11,12]. High-throughput sequencing is expensive and time-consuming, and is mainly used in studies to detect novel pathogens. Nucleic acid amplification test is widely used in clinical laboratories due to its advantages of short detection time, high sensitivity and strong specificity [13]. Compared with Polymerase Chain Reaction (PCR) -based assays, other assays can achieve faster analysis times and lower costs. Loop-Mediated Isothermal Amplification (LAMP) is a recently developed gene amplification method, which combines the characteristics of rapidity, simplicity and high specificity. LAMP can be utilized for early disease

detection and identification of microorganisms. With the assistance of *Bst* DNA polymerase, a maximum amplification of 109 to 1010 times can be attained within 2 h, and the target gene can be amplified to a concentration that can be detected by traditional fluorescence methods.

Point of Care Testing (POCT) products are widely used in clinical testing, major epidemic detection, food safety monitoring, drug testing, alcohol testing and other public health fields, and can also be used in individual health management [14,15]. Compared with traditional department or laboratory diagnosis, POCT retains the core steps of "sample-analysis-quality control-output", which greatly reduces the diagnosis time [16,17]. By decreasing the necessity for manual operations, automation also reduces reproducible errors for operators, laboratories, and even countries in the face of epidemics [18]. In POCT, immunodiagnosis and molecular diagnosis are clearly distinguished by their sensitivity and detection time. Immunodiagnosis shows high sensitivity, while molecular diagnosis shows high specificity [19]. Padoan et al. demonstrated that the use of saliva could decrease analysis time and enhance the effectiveness of contact tracking, thereby developing a device for SARS-CoV-2 molecular diagnostics that can be used in point-of-care testing but lacks the high sensitivity and lower detection limit of Real-time Quantitative PCR Detecting System (qPCR) [20].

In this experiment, by utilizing an integrated detection instrument, based on the magnetic bead extraction method and real-time fluorescent LAMP detection method, For SARS-CoV-2, Influenza A virus (H1N1), Influenza B virus (Flu B) Five common respiratory pathogens, Adenovirus-7 (ADV-7) and *Mycoplasma pneumoniae* (MP), were detected simultaneously. It can decrease the time to diagnosis of respiratory pathogens, prevent large-scale infection, and put pressure on human and material resources, which is a cost-effective testing strategy. This detection method has the advantages of high sensitivity and specificity, which is more in line with the current requirements for clinical detection. Additionally, the instrument is small and simple to carry, which matches the requirements for aerosol pollution in the experiment, lowers the requirements for the environment and personnel technology, and can be used for rapid diagnosis of infectious diseases in poor medical areas.

Materials and Methods

Synthesized standard DNA

B By comparing the sequencing results of the latest SARS-CoV-2 genome (GenBank accession No. NC_045512.2) in the NCBI database, the conserved sequence of N gene was filtered as the target sequence for detection. The target sequence was combined with pUC57 vector, and the recombinant plasmid vector obtained was synthesized into a fragment with a total length of 3889 bp (Shanghai LogenBio, China). To compute the copy number of the positive standard applied in subsequent LAMP and PCR experiments, the formula $(6.02\times10^{23}\times(X\ ng/\mu L\times10^{-9})/(DNA\ length\times660) = copies/\mu L$ (where X is OD value), The concentration of the synthesized fragment was 2.25×10^{10} copies/μL.

The HA sequence of H1N1, NP sequence of Flu B, Hexon gene sequence and MP gene sequence of ADV7 were selected from the GenBank nucleic acid sequence database established by NCBI. The GenBank numbers are: MH329147.1, OQ463058.1, AB330088.1, CP039787.1, DNAMAN was used to compare multiple sequences of different subtypes of the identical virus and find relatively conserved gene fragments, which were verified by NCBI-BLAST. The verified gene fragments were loaded into plasmid PUC57 to prepare plasmid standards and transported to the positive standards synthesized for each virus as above (Shanghai LogenBio Co., Ltd., China). The copy number of the positive standard was calculated as above and diluted to 10^5, 10^4, 10^3, 10^2, 10^1, 10^0, 10^{-1} copies/μL for the subsequent test of sensitivity and specificity.

LAMP primer design

According to the synthesized viral positive standard sequences of SARS-CoV-2, H1N1, Flu B, ADV7 and MP, LAMP primers of five viruses were designed using Primer Explorer V5, and the corresponding values were adjusted to meet the amplification conditions of *Bst* DNA polymerase. The primer sets of four inner and outer primers (FIP and BIP were the inner primers, F3 and B3 were the outer primers) suitable for amplification were aligned, and the corresponding two loop primers (LF and LB) were obtained according to the primer sets. The primer sets were then put into Blast for sequence alignment, hoping that all sequences were species specific, and the primer sets (Sangon Biotech (Shanghai) Co., Ltd., China) were sent for synthesis. The associated LAMP sequences are shown in Table S1.

LAMP reaction

The LAMP kit reaction system for the test sample, positive control, and negative control was prepared. The total reaction system for the test sample was 25 μL, including 5 μL of the test sample, 2 μL of 8U *Bst* DNA polymerase (Meridian Bioscience, United States), and 5 μL of the test sample. 10× Reaction Buffer 2.5 μL (Meridian Bioscience, United States), 10 mM $MgSO_4$ 1.5 μL (Wuhan Hzymes Biotechnology Co., Ltd., China), 10 mM dNTPs 3.5 μL (Wuhan Hzymes Biotechnology Co., Ltd., China), 0.5×LAMP Fluorescent Dye 0.5 μL (New England Biolabs Co., Ltd., United States), primer mixture 6 μL, adding ddH_2O supplementation to 25 μL; The concentration of F3/B3 and FIP/BIP was 10 μM and 40 μM, respectively. At the same time, 5 μL was added to the reaction system of positive control and negative control. The constructed 10^4 copies/μL plasmid standard was used as positive control, and DEPC water was used as negative control. The cells were put into LightCycler 96 (Roche, Switzerland) and heated at 65°C for 45 min. The fluorescence was collected every minute by FAM channel, and the corresponding amplification curve was obtained after the reaction.

Optimization of LAMP system

This part was optimized utilizing the components of the LAMP experiment, and only one element was selected for each experiment for comparison. The optimal value in the experiment was selected by the Cycle threshold (Ct) value and curve shape of the amplification curve after the reaction. $MgSO_4$, *Bst* DNA polymerase, inner and outer primer concentration ratio, DNA concentration, dye concentration, and temperature were selected in this experiment.

In the LAMP reaction, Mg^{2+} is the active center of *Bst* DNA polymerase, so Mg^{2+} can increase the activity of *Bst* DNA polymerase and thus improve the specificity of LAMP product and the reaction progress. Moreover, the combination of Mg^{2+} and dNTP could accelerate the LAMP reaction. With the increase of Mg^{2+} concentration, the catalytic ability of the enzyme was gradually enhanced, and the amplification products increased. However, when the product concentration increased to a certain concentration, it would inhibit the enzyme or even inactivate the enzyme, which would inhibit the

amplification reaction. When the concentration of magnesium ion was reduced, the non-specific amplification of negative control was significantly reduced and the background signal was reduced. Therefore, $MgSO_4$ in the reaction system should be optimized. In this optimization. Using 100 mM $MgSO_4$, the optimization was started from an initial volume of 1.5 μL and increased or decreased by 0.5 μL up and down. Only one component was changed in each optimization, so 0.5 μL ~ 2.5 μL was used for comparative experiments. After the reaction, the fluorescence amplification map generated from the instrument was used to determine the experiment, and the agarose gel electrophoresis was used for verification to select the optimal $MgSO_4$ concentration.

Bst DNA polymerase is a key enzyme in LAMP reaction. It has strong thermal stability, strand displacement and polymerase activity. *Bst* DNA polymerase can quickly, efficiently and specifically amplify templates under constant temperature conditions, without the need for PCR thermal denaturation amplification cycle. As the enzyme concentration increases, the LAMP reaction will produce more amplification products and the amplification time will be shortened. However, exceeding a certain concentration of enzyme does not improve the reaction sensitivity and the reaction time does not change, so it is necessary to find the optimal value for the amplification reaction in the range of 0.5 μL to 2.5 μL. Consistent with the above, no other components in the reaction system were changed, only the enzyme concentration was changed. The optimal *Bst* DNA polymerase concentration was determined by fluorescence amplification maps and agarose gel electrophoresis.

LAMP amplification reactions use four to six primers to identify several specific regions in the target DNA. Using four specific primers and relying on a strand-displacement DNA polymerase, the synthesis of strand-displacement DNA can consistently replicate itself and attain a substantial amount of amplification. The concentration ratio of primers will influence the sensitivity and specificity of the amplification reaction. As the primer concentration ratio increases, the binding between the primer and the template DNA will also be better. However, due to the increase of primers, more primer dimers are also formed, which reduces the specificity of identifying the target sequence and impacts the recognition of the amplification curve, so it is more necessary to verify from the agarose gel electrophoresis map. In this optimization, we merely changed the primer concentration ratio for experiments, and configured primer sets with the inner and outer primer ratios of 1:1, 2:1, 4:1, 6:1, 8:1, and 10:1, respectively. The optimal primer concentration ratio was selected by validating the reaction in a fluorescence amplicon and agarose gel electrophoresis.

The template DNA concentration is a vital step in the LAMP amplification reaction. The amount of DNA added from the template can change the sensitivity of the reaction, as well as the reaction time. As the concentration of template DNA increases, the time of the reaction decreases. Since there is a certain amount of primer bound to it, an excessive concentration of template DNA does not make the reaction faster. Therefore, it demands to be optimized based on the initial concentration. The most appropriate template DNA concentration was selected from 1 μL to 5 μL by using the amplification curve in the LAMP amplification reaction.

Chimeric fluorescent dye is a commonly used DNA-binding dye in fluorescence quantitative PCR and LAMP amplification. It can nonspecifically bind to double-stranded DNA and emit fluorescence. Therefore, the selection of fluorescent dye concentration plays a crucial role in amplification efficiency. The more fluorescent dye was added, the more efficient the amplification was. However, since the amount of template DNA is fixed, too much fluorescent dye will not affect the amplification experiment, and too much fluorescent dye will exceed the fluorescence detection range of the instrument, resulting in abnormal amplification results and deviation in the heat map displayed. Therefore, it is necessary to choose a suitable concentration of fluorescent dye.

Temperature is also a crucial step in the amplification reaction, which enables *Bst* DNA polymerase to begin the strand displacement effect, while also maintaining the enzyme activity, so that more amplification products are generated and amplification efficiency is enhanced. The Tm value is the melting temperature of the DNA, so temperature also affects how well the primer binds to the template DNA. Too high a temperature will inactivate the enzyme, and too low a temperature will make the primer and template DNA binding amplification reaction incomplete or even no reaction.

LAMP system testing of five viruses

Based on the optimized system, it was applied to these five viruses to test the sensitivity and specificity of the system. By adding the positive standard substance diluted according to the concentration gradient to the system, a total of 25 μL of the sample reaction system to be tested was prepared, which was heated at 65°C for 45 min in the fluorescence quantitative amplification instrument. After the detection results were generated, the detection limit on the fluorescence amplification curve was determined.

Each of the five viruses was amplified individually with the other viruses for a total of five reactions, and ddH_2O was applied as a negative control. Amplification was performed by LAMP on a real-time fluorescence quantitative PCR instrument. After the reaction, the amplification products of the LAMP test were subjected to agarose gel electrophoresis. Agarose gel electrophoresis exhibited evident trapezoidal bands due to the mixture of DNA with different lengths, different numbers of stem-loop structures and inverted repeats after LAMP amplification.

Testing process of the integrated system

In this experiment, we used magnetic bead extraction method and real-time fluorescent LAMP detection method to carry out the whole experimental process in an integrated system (Shenzhen LemnisCare Medical Technology Co., Ltd., China). Such an integrated design combined with the application of disposable card box can avoid aerosol pollution in the amplification experiment, and reduce manual operation to avoid experimental errors in the experiment.

The integrated detection process is divided into two parts: Extraction and detection. The extraction part was added to the cassette carrier adapted to the integrated instrument by the reagent in the commercial extraction kit, which was divided into lysis Wells, cleaning Wells and elution Wells. Using the magnetic bead-based extraction method, the nucleic acid can be extracted efficiently in a shorter time through the process of lysis, cleaning and elution by transferring magnetic beads. In the detection part, the LAMP detection kit of the optimized system was used, and the amplification curve was displayed in real time through fluorescence amplification. The identification was performed by reading Time threshold (Tt) value and fluorescence amplification curve. The holes of the reaction tray in the integrated instrument card box are divided into the extraction stage and the detection stage, and different holes

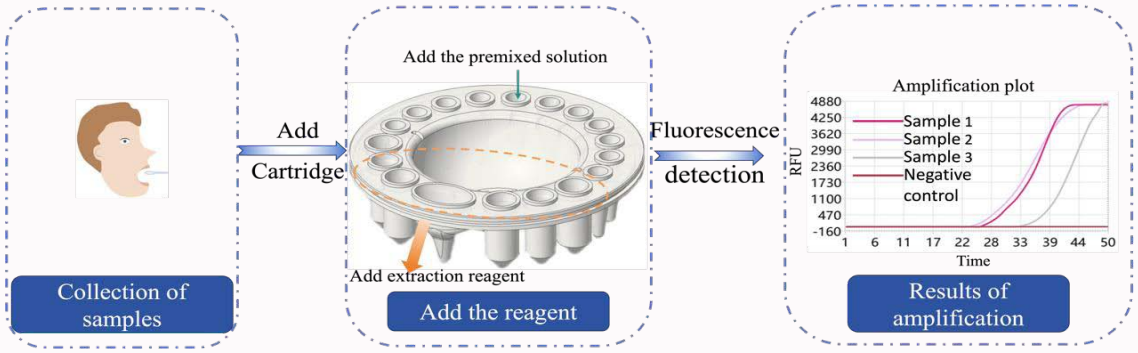

Figure 1: Flow chart of integrated detection.

correspond to different reagents. First, the pre-packaged reagent was put into the reaction tray of the card box and assembled with the suction and discharge liquid assembly, and 200 μL of the sample was put into the lysis hole, and then put into the integrated instrument. The template with a pre-set program was selected for detection. The flow of integrated detection is shown in Figure 1.

In the detection part, the LAMP amplification detection kit was used to display the real-time amplification curve through fluorescence amplification, and the Tt value and fluorescence amplification curve were read for identification. For positive samples, when the cycle threshold Tt value was smaller, the number of cycles demanded was less, which reflected that the virus content in the sample was relatively high and the virus was easier to be detect. The inverse is simple. If the fluorescence amplification curve displayed a similar standard S-type curve compared with the positive control, it was judged as positive. If the sample was consistent with the positive control, no drop occurred, and the threshold was 0, it was judged as negative.

Extraction optimization of integrated detection system: As a necessary step in nucleic acid detection, nucleic acid extraction plays a decisive role in the amount of DNA amplified by nucleic acid. Therefore, optimizing nucleic acid extraction is a necessary step. Magnetic beads method was used to extract the samples. The surface of Magnetic Nanoparticles (MNPs) has a negative charge, and the negatively charged phosphate groups in the nucleic acid molecules can form ion Bridges with positively charged salt ions to achieve specific binding. The extraction time of one sample can realize the processing of multiple samples, the manual operation time is short, and the extraction of trace DNA is very efficient. In this experiment, we optimized the selection of magnetic bead particle size, the temperature and time of lysis and elution stages.

Magnetic beads have the ability to adsorb free nucleic acid DNA, and cannot bind proteins and other impurities, which affects the amount of DNA bound in magnetic beads and the binding ability of binding buffer. As a magnetic bead for nucleic acid extraction, it can be used as a solid phase carrier for DNA enrichment. It possesses the basic properties of good adsorption, short magnetic reaction time, and high adsorption stability. Therefore, the particle size of magnetic beads has a great influence on the DNA enrichment. In order to test this performance, we selected 200 nm, 500 nm, and 1000 nm magnetic beads for experiments. Due to the require for vibration in the extraction process, samples with high concentrations are prone to aerosol pollution, so we selected medium concentrations for extraction tests. 10^4 copies/μL of SARS-CoV-2 positive standard was added for extraction optimization test, and then the extracted samples were amplified by qPCR experiment, and the Ct value in the fluorescence amplification map was applied for judgment.

The lysate can be cleaved by the addition of proteinase K to the outer shell of the cell or pathogen, releasing the nucleic acid inside. The main factors influencing lysis are the temperature and time of lysis. If the temperature of lysis is too high or the reaction time is too long, the nucleic acid DNA will be degraded. If the temperature is too low or the reaction time is too short, the shell of the pathogen will be incompletely lysed and the nucleic acid will not be completely exposed, which will impact the amount of DNA in nucleic acid amplification. In order to optimize the lysis stage, 10^4 copies/μL of SARS-CoV-2 positive standard was added for extraction optimization test, and we used 84°C, 87°C, 90°C, 93°C, and 96°C to screen the lysis temperature. For the lysis time, we selected 3 min, 4 min, 5 min, 6 min, and 7 min for the reaction, and just one variable was altered for each reaction. Then the extracted samples were amplified by qPCR experiment, and the Ct value in the fluorescence amplification map was applied to judge.

The ability of the eluate to disrupt the high-salt environment, the ability of the nucleic acid to shed from the magnetic beads and undergo preservation in the low-salt environment. We usually choose to use enzyme-free water or TE as the eluent. As in the lysis stage, the temperature and time of elution need to be screened. 50°C, 53°C, 56°C, 59°C, 62°C and 1 min, 2 min, 3 min, 4 min, and 5 min were selected, and only one variable was changed for each reaction to find the optimal value suitable for the amplification reaction.

After the optimization of the extraction of each component is completed, we require to conduct a comparative experiment between the extraction stage in the integrated instrument and the commercial extractor. We used a commercial qPCR kit (Zhuhai Biori Biotechnology Co., Ltd., China), adding 10^4 copies/μL SARS-CoV-2 positive control and negative control, and the same extraction parameters were set for both sets, and the test was repeated three times. The extracted samples obtained by the two instruments were put into a commercial fluorescence quantitative qPCR instrument, and the average Tt value, Standard Deviation (SD) and Coefficient of Variation (CV) were calculated through the fluorescence amplification map and Tt value to test its stability and extraction efficiency.

Test of integrated detection system: After the above extraction optimization, the optimal value most suitable for the amplification reaction was obtained, and the parameters in the extraction process were adjusted for detection experiments. We pre-added the lysate, washing solution, eluent, silicone oil, and the detection reagents

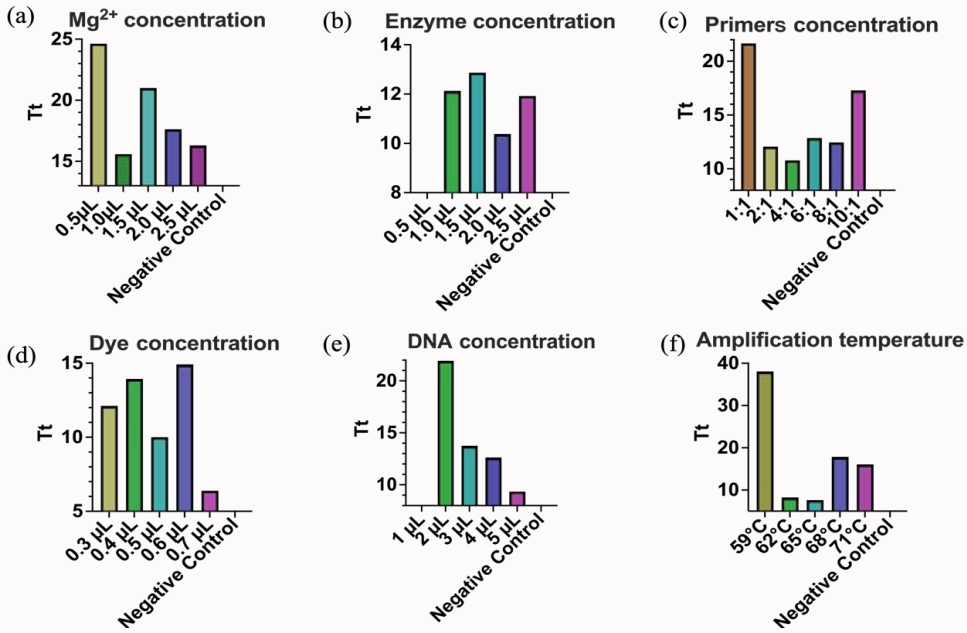

Figure 2: Optimization results of LAMP system for five viruses. (a) is the comparison of Mg^{2+} concentration; (b) is the comparison of Bst DNA polymerase concentration; (c) is the comparison of inner and outer primer concentrations; (d) is the comparison of dye concentration; (e) is the comparison of DNA concentration; (f) shows the comparison of different amplification temperatures.

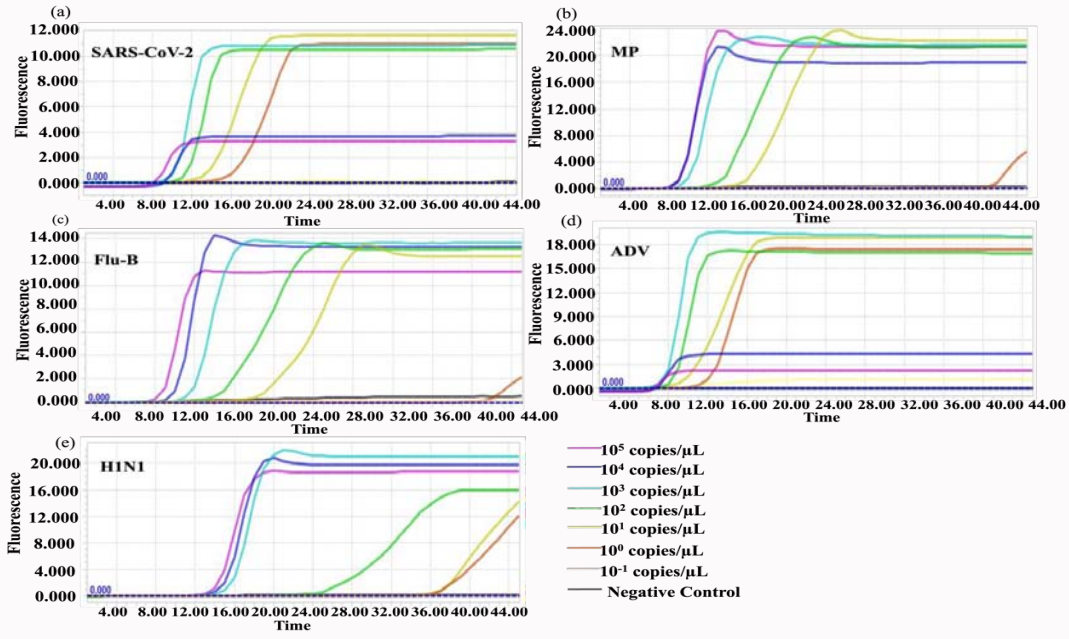

Figure 3: Detection limit amplification results of five viruses (a) shows the amplification pattern of SARS-CoV-2; (b) is the amplification map of MP; (c) shows the amplification pattern of Flu-B; (d) shows the amplification pattern of ADV; (e) shows the amplification plot of H1N1.

needed to detect the reaction to the corresponding Wells. 20 μL proteinase K and 200 μL sample were added to the lysis Wells, 5 μL sample was allocated to each reaction well, positive control and negative control were added to the positive control and negative control Wells, respectively, and the reaction temperature of 65°C was set for amplification for 45 mins. We tested the stability of the instrument amplification for sensitivity, homogeneity, and reproducibility.

The sensitivity was tested by SARS-CoV-2 positive standard. The concentration gradients of 10^{-1}, 10^{0}, 10^{1}, 10^{2}, 10^{3}, 10^{4}, 10^{5} copies/μL were added to the above LAMP system for fluorescence detection, and the sensitivity of the instrument was compared with that of commercial fluorescence quantitative extractor. The pre-packed detection reagent was added to the reaction tray of the disposable card box, and then the samples were added to each reaction well one by one. The test was for the detection part of the integrated system.

The homogeneity was determined by testing 11 reaction Wells for the same sample, and the consistency of the results was judged by

calculating the average Tt value, SD and CV through the fluorescence amplification map and Tt value obtained by the integrated instrument. The test is for the integrated system from extraction to detection part, the pre-packed extraction reagent and detection reagent are added to the reaction tray of the disposable card box, and then the test sample is added to the lysis hole to test the uniformity of the Tt value of each reaction hole.

The repeatability was tested three times with three high, medium and low concentrations of 10^5, 10^3, 10^1 copies/μL. The average Tt value, SD and CV were calculated according to the fluorescence amplification map and Tt value obtained by the integrated instrument to judge the repeatability of different concentration amplification. The test is aimed at the integrated system from the extraction to the detection part. Each concentration was extracted and fluorescence collected, three wells were replicated three times, and the values were then calculated to test the reproducibility for high and low concentrations.

Integrated rapid detection of five respiratory viruses

Virus-positive standards of SARS-CoV-2, MP, Flu B, ADV7, and H1N1 were used as simulated samples for testing. There are 11 reaction wells in the reaction tray in the integrated detection system, and the detection premixes of the five viruses were put into each of them and tested in duplicate wells, and the negative sample was the positive standard of SARS-CoV-2, and a total of five experiments were tested. In the first experiment, mixed samples of positive standards of SARS-CoV-2, MP, Flu B, ADV7 and H1N1 were put into the lysis wells, and extracted and fluorescence detected by magnetic bead method. In the second experiment, mixed samples of SARS-CoV-2, MP, Flu B and ADV7 positive standards were put into the lysis wells, in the third experiment, mixed samples of SARS-CoV-2, MP and Flu B positive standards were put into the lysis wells, in the fourth experiment, mixed samples of SARS-CoV-2 and MP positive standards were put into the lysis wells and in the fifth experiment, mixed samples of SARS-CoV-2 positive standards were put into the lysis wells for simulation, in order to obtain multiple samples of H1N1 positive standards In the fourth experiment, a mixture of SARS-CoV-2 and MP-positive standards was placed, and in the fifth experiment, a SARS-CoV-2-positive standard was placed as a simulated sample, with the aim of obtaining the experimental results of the multi-tube multiplex test.

Results

LAMP system for the five viruses

Optimization of LAMP system: The positive standard of SARS-CoV-2 was selected as the object of optimization, and the optimized system can be applied to numerous other viruses. A positive standard of 10^5 copies/μL was selected as the template for amplification, and experiments were performed from the variables of $MgSO_4$ concentration, *Bst* DNA polymerase concentration, inner and outer primer ratio, dye concentration, sample concentration, and amplification temperature. These variables are shown on the abscissa and Tt values on the ordinate in Figure 2. When the Tt value is smaller, the concentration of the amplified sample is higher, and when the Tt value is larger, the concentration of the amplified sample is lower, so that the best optimization results of each variable can be selected. According to this principle, it can be seen from the figure that the optimal LAMP system can be obtained by adding 1 μL $MgSO_4$, 2 μL *Bst* DNA polymerase, 0.7 μL dye concentration and 5 μL sample concentration, and then applying the inner and outer primer concentration ratio of 4:1 and selecting the temperature of 65°C for amplification. And in each optimization, the negative control group did not take off, indicating that there was no nonspecific amplification. We therefore applied this system to the remaining four viruses for subsequent testing.

Sensitivity test of five viruses: The optimized LAMP system was applied to the positive standards of SARS-CoV-2, MP, Flu B, ADV7 and H1N1. After 10-fold dilution of each standard, 10^{-1}, 10^0, 10^1, 10^2, 10^3, 10^4, 10^5 copies/μL were selected to test the sensitivity. Figure 3 shows that each virus amplifies well, and no nonspecific amplification occurs. As can be seen in each figure, the detection limit was 100 copies/μL. Therefore, the optimized LAMP system can be applied to the five kinds of viruses, and the detection limit is within a reasonable range with good sensitivity.

Specificity test of the five viruses: After specific amplification of several viruses, the amplified products were tested by agarose gel electrophoresis. A voltage of 120 V and a current of 100 mA were selected to operate for 30 min. As can be seen in Figure 4, all the five viruses have apparent trapezoid electrophoresis bands, each set of primers does not show non-specific amplification, and the negative control also shows no bands, which can prove that the primers designed by each virus have good specificity and will not affect the subsequent integrated amplification results.

Test results of the extraction part of the integrated system

Optimization results of extraction part of integrated system: SARS-CoV-2 positive standards were selected as optimized objects for the integrated system testing process. In the optimization, 10^4 copies/μL of positive standard was selected as the template for amplification, and the optimization was performed from the size of different magnetic bead particle sizes, lysis time, lysis temperature, elution time and elution temperature. Each optimization changes only one component and continues the results of the previous optimization. Using qPCR system, 5 μL samples that had been extracted in accordance with the requirements were added to the reaction solution, and 5 μL ddH_2O was added as negative control. The extracted samples were then placed into a commercial real-time fluorescence amplification.

As can be seen in Figure 5(a), when the particle size of magnetic beads is 500 nm, more products are enriched and the concentration is higher, so the Ct value is lower. It can be seen from Figure 5(b) that too long lysis time causes a reduction in the amount of DNA, while a low lysis time causes inadequate extraction. When the lysis time was 5 min, the Ct value was the smallest, indicating that the viral load was the largest at this time and could be used as the best lysis time. Figure 5(c) shows that the lysis temperature has a great influence on the extraction. Too high or too low temperature will lead to insufficient DNA extraction, resulting in too low viral load. It is obvious from the figure that when the lysis temperature is 90°C, the Ct value is the lowest and the viral load is the highest, so we choose 90°C as the optimal lysis temperature. From Figure 5(d), we can clearly see that when the elution time is 4 min, the Ct value is the smallest, indicating that the viral load is the largest at this time, so 4 min is selected as the best elution time. As shown in Figure 5(e), the extraction temperature has a great influence on the enrichment of nucleic acid, too high or too low temperature will reduce the amount of DNA. When the extraction temperature is 56°C, the Ct value is the lowest and the viral

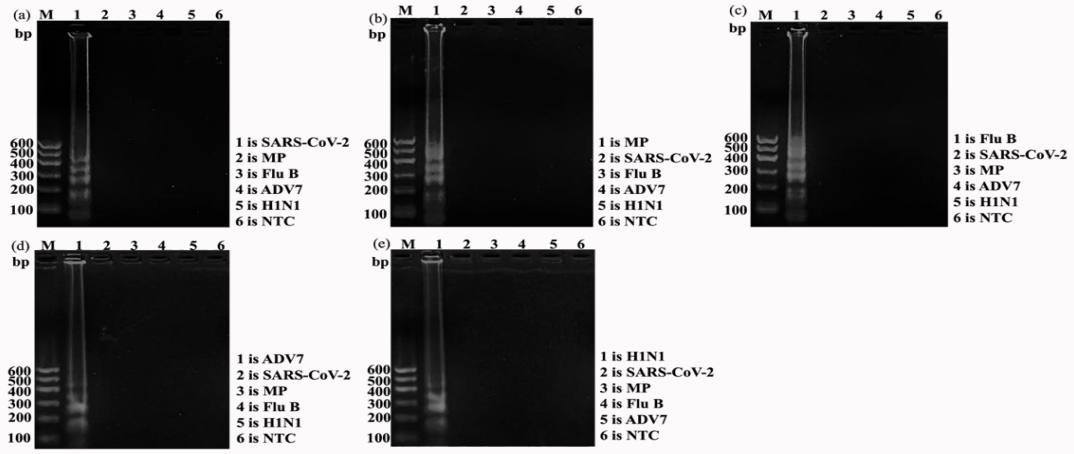

Figure 4: Electrophoretogram of the specificity analysis of the five viruses (a) shows the electrophoretogram of SARS-CoV-2; (b) is the electrophoresis pattern of MP; (c) shows the electrophoresis pattern of Flu-B; (d) shows ADV electrophoresis pattern; (e) shows the electrophoretogram of H1N1.

Table 1: Integrated system and commercial extractor repeatability test.

Figure	Average Ct		SD		CV	
	Integrated system	Commercial extractor	Integrated system	Commercial extractor	Integrated system	Commercial extractor
(a)	24.40	24.08	0.17	0.19	16.90%	1.83%
(b)	24.32	23.54	0.43	0.01	2.70%	0.30%
(c)	25.17	23.72	0.19	0.05	1.70%	0.97%

Table 2: Repeatability test results of extraction at high, medium and low concentration.

Concentration (copies/μL)	Average Tt			SD	CV
10^1	25.493	26.810	26.862	0.395	1.51%
10^3	10.350	11.671	11.005	0.395	3.56%
10^5	6.719	6.718	7.040	0.156	2.29%

load is the highest, so 56°C is selected as the best elution temperature.

The integrated system extracts repeatability tests: After extraction optimization, we performed a repeatability test on the extraction part of the integrated system. Using qPCR system, 10^4 copies/μL of SARS-CoV-2 positive samples and negative controls were added, and the same extraction parameters were set for both machines, and the test was repeated three times. Figure S1 shows the qPCR amplification diagram of the extraction repeatability test of the integrated system. (a), (b) and (c) correspond to the results after each extraction by the integrated system and the commercial extractor, respectively. It can be seen that the Ct values of the two extraction methods are close. The extraction of the integrated system from Table 1 is slightly behind the Ct value of the commercial extractor. Due to the differences in the design of the two instruments, the integrated system needs to use the same pipetting head for extraction and detection due to the structural limitations and different requirements, and the pipetting method should be selected to facilitate the detection of the extracted liquid. However, the commercial extractor needs to do batch extraction, so the mobile magnetic beads should be selected. Therefore, the enrichment of DNA on magnetic beads is better and more stable than that of the integrated system.

Test results of the detection part of the integrated system

After the above extraction optimization, the optimal value most suitable for the amplification reaction was obtained, and the parameters in the extraction process were adjusted for detection experiments. We tested the stability of the instrument amplification for sensitivity, homogeneity, and reproducibility.

Integrated system sensitivity test: The six concentrations of 10^{-1}, 10^0, 10^1, 10^2, 10^3, 10^4, 10^5 copies/μL of the five viruses were put into the reaction tray of the integrated system card box. As can be seen from Figure 5, the detection limit is 100 copies/μL, and each concentration has a good amplification. The Tt values of the high and medium concentrations were basically the same, and the Tt values of the low concentration were slightly delayed. Due to the nucleic acid extraction, the enrichment of the low concentration samples was not as good as that of the positive standard, but the overall trend was that the Tt value increased with the decrease of the concentration logarithm, and the difference did not affect the judgment of the results, which could be used for subsequent integrated detection experiments Figure 6.

Homogeneity test of integrated system: The same concentration of 10^5 copies/μL samples of 11 SARS-CoV-2 positive standards were put into the integrated instrument, and the results were obtained by fluorescence detection after extraction. As shown in Figure 7, from the morphology of the curves, the 11 curves have a high degree of overlap. According to the Tt value, the experimental results showed that the average Tt value was 5.81, the SD was 0.38, and the CV was 6.61%, which indicated that the overall homogeneity of the sample amplification was good, and the results obtained from the reaction Wells of the integrated instrument were basically the same.

Integrated system repeatability test: Three high, medium and low concentrations of 10^5, 10^3, 10^1 copies/μL were tested for three times, and the Wells were repeated for three times. The repeatability of amplification of the three concentrations was judged by the fluorescence amplification map obtained by the integrated system.

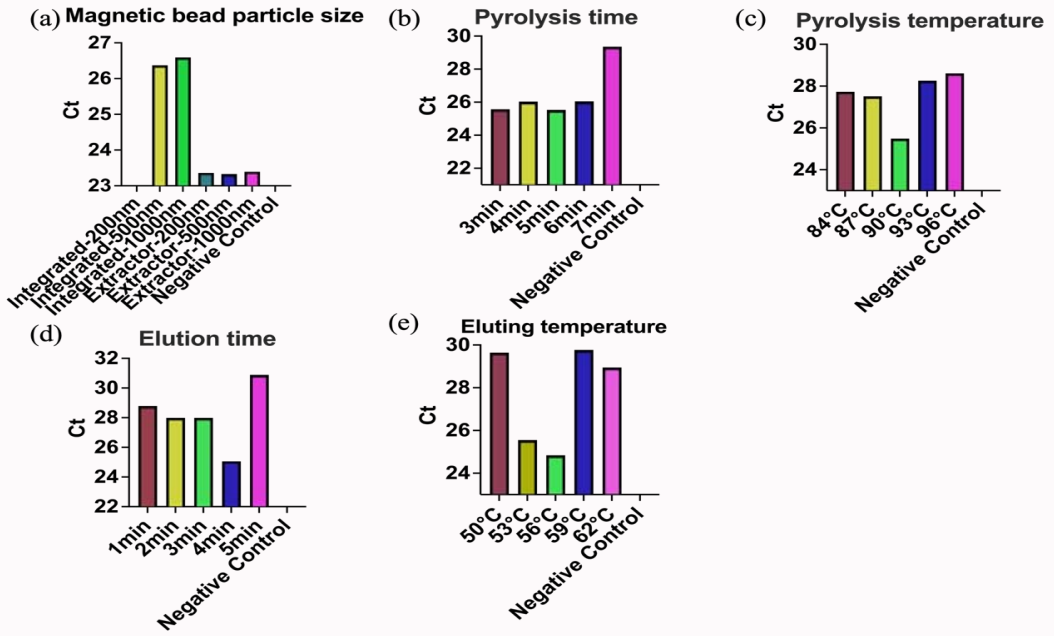

Figure 5: Analysis of extraction optimization results in the integrated system (a) shows the comparison between the integrated system and the commercial extractor in magnetic beads with different particle sizes; (b) is the comparison of lysis time; (c) is the comparison of lysis temperature; (d) is the comparison of elution time; (e) shows the comparison of elution temperatures.

Figure 6: The detection results of the five viruses in the integrated system. (a) is the amplification map of SARS-CoV-2. (b) is the amplification map of MP; (c) shows the amplification pattern of Flu-B; (d) shows the amplification pattern of ADV; (e) shows the amplification plot of H1N1.

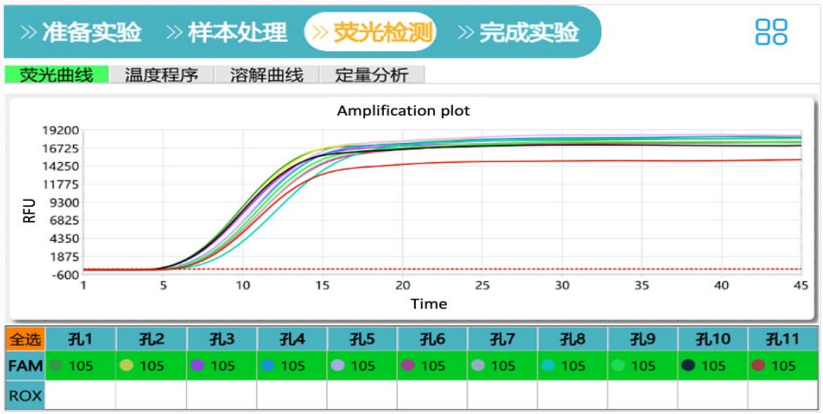

Figure 7: The results of the homogeneity test in the integrated system.

The average Tt value, SD and CV were calculated according to the Tt value. As can be seen from Table 2, the average Tt values of high, medium and low concentrations are close, and the values of SD and CV are small, indicating that the results of high, medium and low concentrations are close, which can verify the good repeatability of the instrument results.

Integrated rapid detection of five respiratory viruses

The positive standards of SARS-CoV-2, MP, Flu B, ADV7 and H1N1 were used as simulated samples for testing. The negative samples were positive standards for SARS-CoV-2 and were tested for a total of five experiments. Figure S2(a) shows the test results of SARS-CoV-2 positive standard samples in the first experiment. Figure S2(b) shows the test results of mixed samples with SARS-CoV-2 and MP positive standard samples in the second experiment. Figure S2(c) shows the detection results of mixed samples with SARS-CoV-2, MP and Flu B positive standards in the third experiment, and Figure S2(d) shows the detection results of mixed samples with SARS-CoV-2, MP, Flu B and ADV7 positive standards in the fourth experiment. Figure S2(e) shows the results of mixed samples with positive standards for SARS-CoV-2, MP, Flu B, ADV7 and H1N1 in the fifth experiment. As can be seen from the figure, each mixed sample was amplified as expected, and the mixing of multiple samples did not affect the test results. Moreover, the difference from extraction to detection was similar from the multiple holes. Therefore, the integrated system was feasible for the detection of a variety of respiratory tract, and the detection time from configuration of the system to extraction to fluorescence detection was less than 60 min.

Conclusion and Prospect

In this thesis, we have developed an integrated microsystem for the diagnosis of multiple respiratory viruses. This integrated microsystem uses magnetic beads for nucleic acid extraction and isothermal amplification with fluorescence detection for point-of-care detection of respiratory viruses. The entire detection process from sample collection, nucleic acid extraction, cassette equipment, fluorescence detection and result output can be completed within one hour. The sensitivity and specificity of this method were tested. From the analysis of the results, the detection limit could reach 100 copies/μL, and there was no non-specific amplification detected by five sets of primers. The integrated detection system has high sensitivity and specificity. As the detection process is divided into two steps, extraction and detection, the two processes were optimized and compared with commercial instruments. From the results, it can be determined that the instrument can be applied to the qualitative detection of a variety of respiratory pathogens without aerosol contamination in the process of sample amplification. Cost-effective testing strategies can be implemented to reduce the time for diagnosis of respiratory pathogens and prevent large-scale infections. Because the instrument is compact and easy to carry, it reduces the requirements of environment and personnel experimental operation technology, and can be used for rapid diagnosis of infectious diseases in poor medical areas.

Because different primer sets need to be added to the premix solution during the experiment, this step will increase the operation time, so freeze-drying technology can be introduced to ensure the detection limit and at the same time, the experimental operation can be completed conveniently and quickly. In practice, a single sample will be used for multiple respiratory pathogens in a cartridge-box device. Thus, the corresponding freeze-dried assay premix can be configured in each test well. We expect that this instrument will provide a promising solution for rapid field detection and mutation monitoring of pandemic viruses.

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