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A Real-time Resistance Measurement for DNA Amplification and Detection

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In this paper, an electrochemical strategy of DNA detection, basing on a specially designed resistance electrode, Loop-mediated isothermal amplification (LAMP), real-time monitor and derivative determination, was introduced. First, a resistive electrode was designed. Then five positive dyes were applied to counteract the negative charges on DNA, which lead to the altering of the resistance value of the reaction liquid. The results show that the Crytal violet was the best out of them. It worked better in 2.0mg.L^{-1} (final concentration). This electrochemical detection of DNA could be completed in 20-40 minutes with the lowest bacterial to 10 CFU.mL^{-1}, good anti-interference ability to hemoglobin, triglyceride, bilirubin and sodium chloride within the sample. Therefore, it was a rapid, sensitive and stable method of DNA detection, could be applied on the gene chips, the pocket instruments, which needed the simple and real-time method of measuring.

Keywords: Electrochemical detection, Real-time resistance measurement, Loop-mediated isothermal amplification (LAMP), DNA detection

1. INTRODUCTION

Today, the polymerase chain reaction (PCR) had become the common method of DNA detection, had been widely applied in diagnosis of viral, bacterial infection, genetic diseases and tumors. With the development of gene chip, it had been paid more attentions to PCR reaction on microfluidic gene chip. Due to small sizes, it was difficulty to meet the requirement of the frequently change of temperature and the real-time measurement of the DNA amplification product. Thus, the method with simple temperature controller, mini and real-time detector were required. The electrochemical detection of DNA maybe becomes the better choice.

Loop-mediated isothermal amplification (LAMP) was invented by Dr. Notomi (Eiken Chemical, Japan) in 2000[1], which was characterized of rapid, high specificity and high sensitivity. LAMP used the bst DNA polymerase in a constant temperature (65℃) and completed DNA amplification in 20-60 minutes. Compared with the traditional PCR, the LAMP was simpler, faster, and no need of expensive equipment. In recent years, LAMP technology had made progress in detections of virus [2-4], bacteria [5-6], parasites [7-9], food safeties [10] and animal embryo sex identifications [11]. It had become a routine detection of the pathogenic microorganism.

There were several determination methods of LAMP. Fluorescent dye method was a common judgment. The amplification products were dyed by fluorescent dye, then been observed under ultraviolet. The positive amplification had a series of graded strips [12]. Rapid electrophoresis and microchip electrophoresis technology promoted the level of the electrophoresis [13]. The new dyes, such as Hydroxy naphthol blue [14], Calcein [15], and SYBR Green I [16] was applied in LAMP as a qualitative indicator. However, electrophoresis, with slow response, strong toxicity of dyes, was restricted in application [17].The turbidimetric method was another common judgment. One of the LAMP products, magnesium phosphate, had large quantity (up to 0.15g.L^{-1}), form a clear white precipitate, could be determinated by a real-time turbidity meter [18-19]. The turbidimetric method was not only quickly and accurately, but also quantitatively. However, it required optical-electrical devices to transformate and output the results, which were expensive and bulky. It was not suitable for the construction on the gene chip and pocket instrument. It also required high transparency of the reaction liquid, which had to increase the preprocessing steps of DNA extraction. [12].

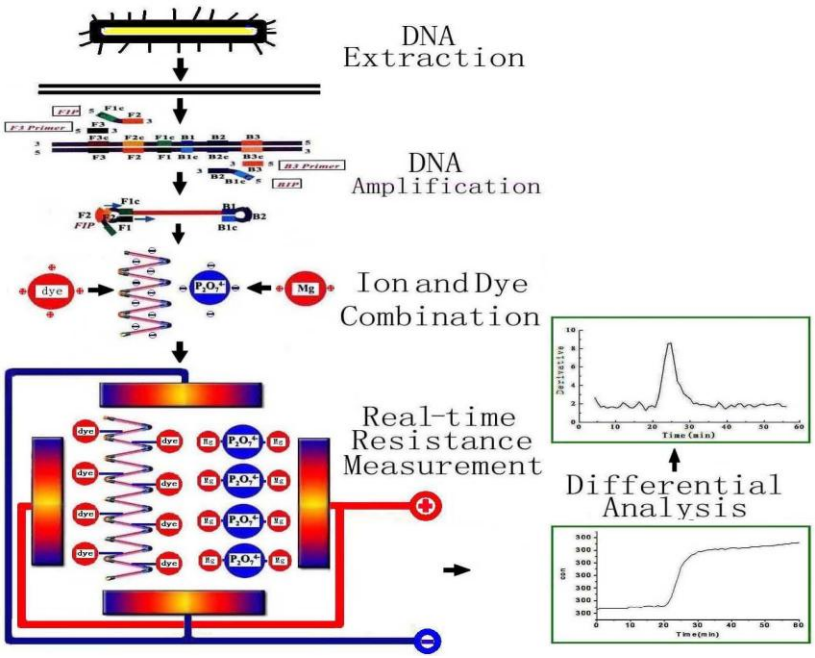


Figure 1. The scheme of real-time resistance measurement of DNA amplification and detection

In this paper, we had proposed an electrochemical detection, real-time resistance measurement, for the amplification and detection of DNA. First, the DNA extracted from specimens was

amplificated through LAMP reaction. The two products of LAMP, DNA and Pyrophosphate, were two kinds of negative ions. They were combined with positive dye (such as Crytal violet [20]) and positive ions (such as Mg^{2+}), which leading to an increase in the resistivity of the reaction liquid. The changes of resistivity were real-time measured by special designed resistance electrode. So the products of DNA amplification were detected. Then five positive dyes (Crytal violet, Methylene blue [21], SYBR green I, Hydroxy naphthol blue, Ethidium bromide [22]) were applied synchronously to be comparised of their reaction characteristics. Then the best dye was found out to test its best working concentration. The change of magnesium's concentration will lead to instable of the LAMP response. In this study , we used a constant concentration of magnesium ($6.0mg.L^{-1}$) which been included in the reagent of DNA-LAMP (Eiken Chemical, Japan). Gradient diluted DNA was used to test the sensitivity of the reaction system. At last, the hemoglobin, triglyceride, bilirubin and sodium chloride were added in the specimens as interfering substances before the DNA extraction and LAMP reaction, to determine the anti-interference ability of the system. The scheme of the experiment was shown in Figure 1.

2. EXPERIMENTAL

2.1. Reagents

Crytal violet, Methylene blue, SYBR green I, Hydroxy naphthol blue, and Ethidium bromide were obtained from Sigma-Aldrich (USA). All of above dyes were dissolved in water at concentration of $100mg.L^{-1}$and stored at 2-8 °C, which were diluted in appropriate concentration prior to use. LAMP reagents were purchased from Eiken Chemical (Japan). DNA extraction reagents were provided by Tiangeng (China).All other reagents were of analytical grade. All solutions were prepared using Millipore-Q water (≥18 MΩ).

2.2. Oligonucleotides

Table 1. Oligonucleotides used in the paper.

Microbe	Target gene	Genbank	LAMP primer （5'→3'）
Escherichia coli	mal B gene	CP002797	F3： GCCATCTCCTGATGACGC B3： ATTTACCGCAGCCAGACG FIP：ATTTTGCAGCTGTACGCTCGCAGCCCATCATGAATGTTGC BIP：CTGGGGCGAGGTCGTGGTATTCCGACAAACACCACGAATT

The maltose operon protein B (mal B) gene was used to design special LAMP primers for *Escherichia coli* by exploring the genebank database. The specificity of primers had been positively searched by BLAST (http://www.ncbi. nlm.nih.gov/blast) (Table 1). Oligonucleotides with the sequences shown in Table 1 were synthesized by Sangong (Shanghai, China). All oligonucleotides were dissolved in trisethylenediaminetetraacetic acid (TE) buffer (10 mM Tris-HCl, 1mM

ethylenediaminetetraacetic acid; pH 8.0) and stored at -20 °C, which were diluted in appropriate buffer prior to use.

2.3. Apparatus and software

The resistance electrode and real-time measure system of DNA amplification were shown in Figure 2.The design goal of electrode was to minimize the deviation of measurement, as well as to avoid the inhibition of DNA amplification efficiency. Thus, the four-trodes electrode was designed as the working electrode, used for real-time resistance monitoring. The two negative and two positive trodes of resistance electrodes were interval from each other (Figure 2-A, B), in order to minimize the voltage interference of resistance measurement.

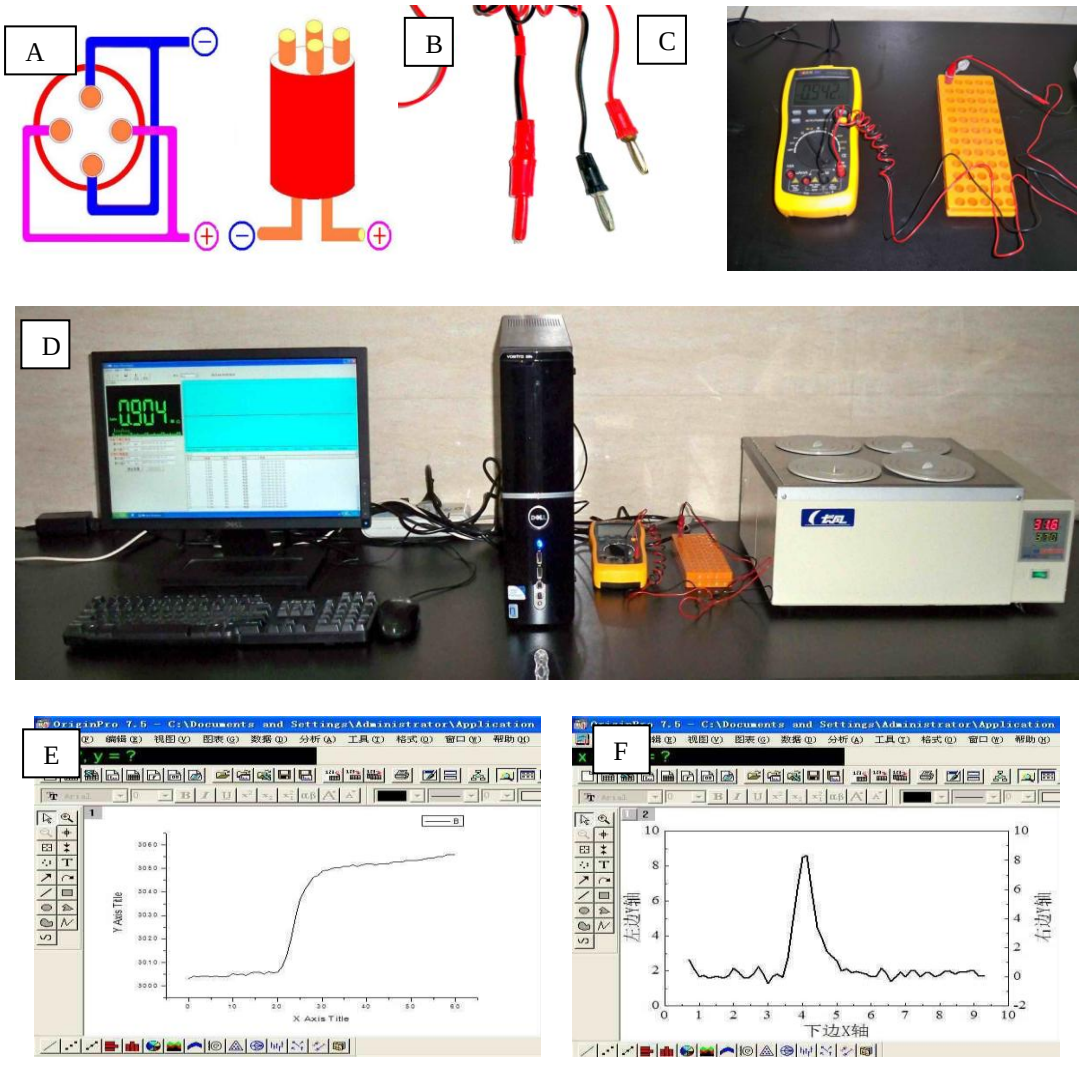


Figure 2. The system of real-time resistance measurement.
(A,B)electrode,(C,D) measure system,(E) real-time curve, (F) derivative analysis

The electrode's surface was electroplated with gold, in order to increase its electrochemical stability in LAMP reaction. The designing and produce of electrodes was assisted by World precision instruments (WPI, USA). All electrochemical measurements were performed on the real-time resistance recorder, VICTOR 86C digital multimeter(VICTOR, China) with a Ag/AgCl electrode as reference, and a resistance electrode as working electrode (Figure 2-C). The DNA amplification was carried out in a constant temperature tank, HWSY21-KP4 (Changfeng, China) and real-time monitored by DMM software (VICTOR, Shengzheng, China) (Figure 2-D). The curve drawing (Figure 2-E) and derivative analysis (Figure 2-F) of test results were completed on OriginPro 7.5 software (OriginLab, USA).

2.4. Preparation of DNA samples

The standard strains of *Escherichia coli*, ATCC 25922（USA）, were grown at 37 °C for 24 h in Columbia blood agar plate （Pangtong, China）. Viable counts were performed by plating 100μL of appropriate 10-fold dilutions in sterile phosphate buffer saline (PBS) onto plate count agar in triplicate and incubating the plate for 24 h at 37 °C. The concentration was estimated by calculating the average number of colony forming units (CFU). 1 mL of each bacterial culture was immediately chilled on ice. After centrifugation at 10,000 rpm for 5 minutes at 4 °C, the supernatant containing genome DNA was carefully transferred to new tubes. DNA of standard *Escherichia coli* strain was extracted as describing in the instructions of the bacteria DNA extraction kit (Tiangen, China). All DNA preparations were stored at -20 °C prior to use. 2 μL liquid per test was used as template DNA for the LAMP.

2.5. Preparation of DNA amplification and detection system

The DNA amplification was carried out in a specific LAMP reaction tube containing 2μL template DNA (*E coli*), 12.5μL reaction reagent, 1.0μL bst DNA polymerase large fragment (Eiken, Japan), 4μL primer mixture (including 4 primers: 10μmol.L^{-1} F3,10μmol.L^{-1} B3, 40μmol.L^{-1} FIP and 40μmol.L^{-1} BIP) and 5.5μL ultra pure water. The mixture in the reaction tube was mixed and incubated isothermally at 65ºC for 60 minutes in constant temperature tank. The resistance electrodes were immersed in the ultra pure water (Millipore-Q water) to avoid oxidation form the air. Before the using, the resistance electrodes must be washes by the ultra pure water and keep balance in reaction liquid at least 30 minutes. All electrochemical measurements were performed on the real-time resistance recorder, VICTOR 86C digital multimeter, with an Ag/AgCl electrode as reference, and a resistance electrode as working electrode. The curve drawing and differential calculation of test results were completed on OriginPro 7.5 software. The DNA amplificated products were analyzed by electrophoresis on 2% agars gels (BIOWEST Co., Ltd; Spain) at a voltage of 100V for 50 min, and visualized in a Kodak Gel Logic 212 PRO Imaging System (Eastman Kodak, USA).

2.6. Optimization of the Real-time Resistance Measurement

The main factors which had the most significant impact on the effects of DNA amplification and detection efficiency were the composition of LAMP reaction liquid, the resistance electrode of real-time detection, and the method of the results' judgment. The optimization of resistance electrode rely on the assistance of electrode manufacturing company and our preliminary tests, including the electrode 's design, detection range, the sampling interval and the discriminant of testing results. Five dyes (Crytal violet, Methylene blue, SYBR green I, Hydroxy naphthol blue, Ethidium bromide) were applied synchronously with the same concentration (1.0 $mg.L^{-1}$) to be comparised of their reaction characteristics. Then the best dye was elected to applied in a series of concentration (0, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0$mg.L^{-1}$). Through the DMM software (VICTOR, Shengzheng, China), the real-time resistance curves were drawn and the results of DNA amplification were made. According to the response threshold of LAMP, the Ct value (the response time of the resistance reaching a threshold, like the cycler time in PCR) was measured. We could draw a standard curve combined with the Ct value and the concentration of template DNA. According to the standard curve, the concentration of DNA in unknown sampler was quantitative determinated. In the same way, the Ct values of differential resistance were calculated by OriginPro 7.5 software (OriginLab, USA), and the standard curve was made too. Then the two methods were compared of its superiority in quantitative determination.

2.7 Preliminary evaluation of the Sensitivity

Solution of *Escherichia coli* standard strains was diluted into a serial of concentrations (from 1×10^{6} to $1\times10^{0}CFU.mL^{-1}$). The DNA amplification was carried out in a specific LAMP reaction tube containing 2μL template DNA , 12.5μL reaction reagent, 1.0μL bst DNA polymerase large fragment (Eiken Chemical, Japan), 4μL primer mixture (including 4 primers: 10$\mu mol.L^{-1}$ F3 and B3, 40$\mu mol.L^{-1}$ FIP and BIP), 1 μL 500$mg.L^{-1}$ MB and 4.5μL ultra pure water. The mixture in the reaction tube was mixed and incubated isothermally at 65ºC for 60 minutes in constant temperature tank. All electrochemical measurements were performed on the real-time resistance recorder, VICTOR 86C digital multimeter, with an Ag/AgCl electrode as reference, and a resistance electrode as working electrode. The curve drawing and differential calculation of test results were completed on OriginPro 7.5 software. The sensitivity of the assay was calculated by regression analysis in Excel 2003 (Mircosoft, USA).

2.8 Study of the anti-interference ability

Four kinds of interference substances, hemoglobin (10, 20, 40, 80$g.L^{-1}$), triglyceride (10, 20, 40, 80$g.L^{-1}$), bilirubin (10, 20, 40, 80$g.L^{-1}$) and sodium chloride (0.2, 0.5, 1.0, 2.0$mol.L^{-1}$) were added in the specimens before the DNA extraction and LAMP reaction, to determine the anti-interference ability of the system. The same parameters as above were applied in DNA extraction, DNA amplification, real-time resistance measurement, differential calculation and result judgment.

3. RESULTS AND DISCUSSION

3.1. Characterization of resistance electrode

The preliminary characterization of resistance electrode was shown in Figure 3. It was shown that the resistance electrode and the real-time resistance recorder were worked stably in 3 hours (Figure 3-A.1: water 65℃, 2: water 25℃,3:Blank Reaction liquid 25℃, 4:Blank reaction liquid 65℃)). The gel electrophoresis of DNA amplificated products displayed that the real-time resistance measurement hadn't obvious effect on the DNA amplification (Figure 3-B, 1:marker, 2:blank and 3,4:*E coli* without measurement, 5:blank and 6,7:*E coli* under measurement). The real-time resistance measurement curve of DNA amplification shown that it could judging the positive amplification by real-time resistance measurement curve, but the basic lines of resistance curve were changeable, and the threshold of DNA (*E coli*,10^5CFU.mL^{-1}) amplification were susceptible to many factors, such as temperature, pH and ion concentration(Figure 3-C). The differential curve shown that it could judging the positive amplification more easily, the basic lines of differential curve were stable , the results were calculated automatically by software, which were more accurate (Figure 3-D).

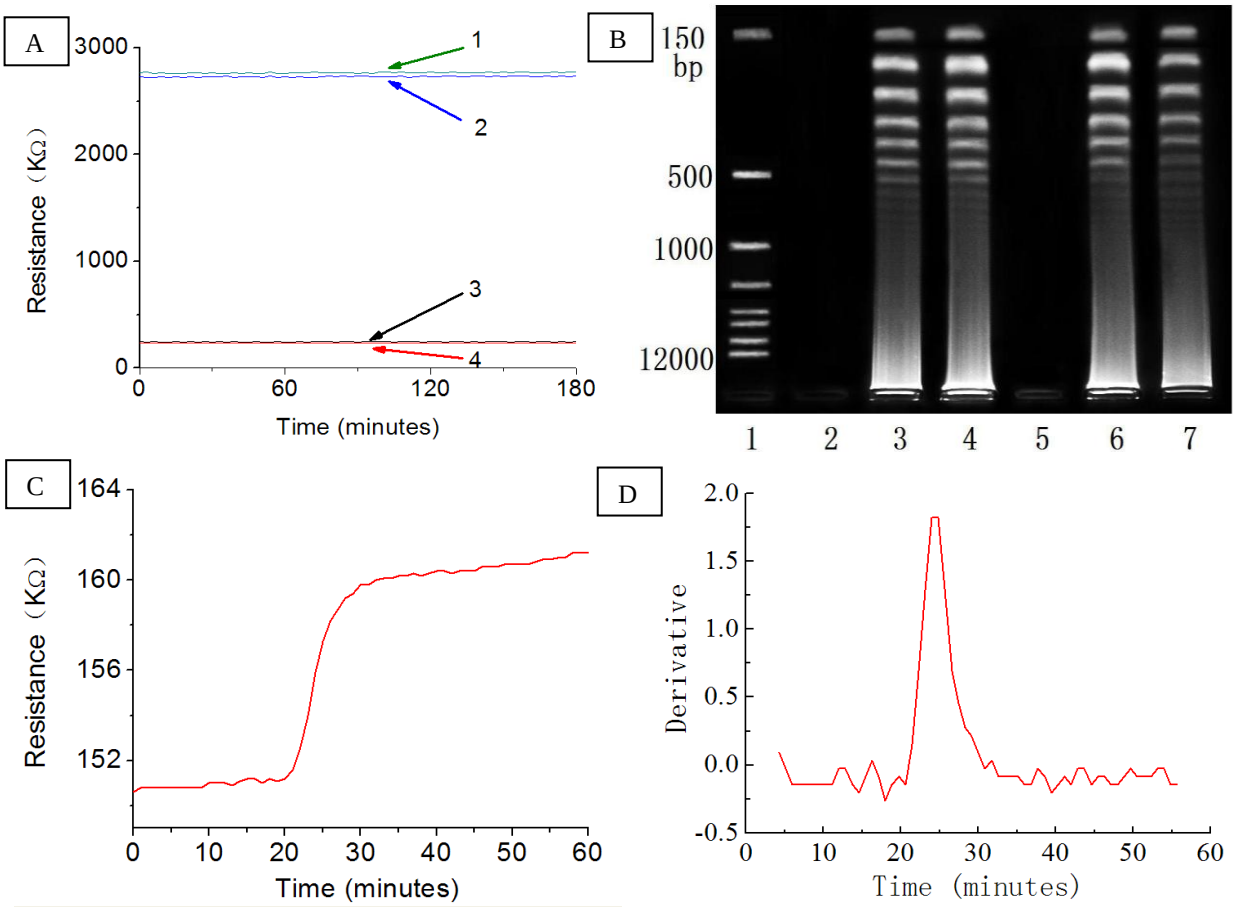
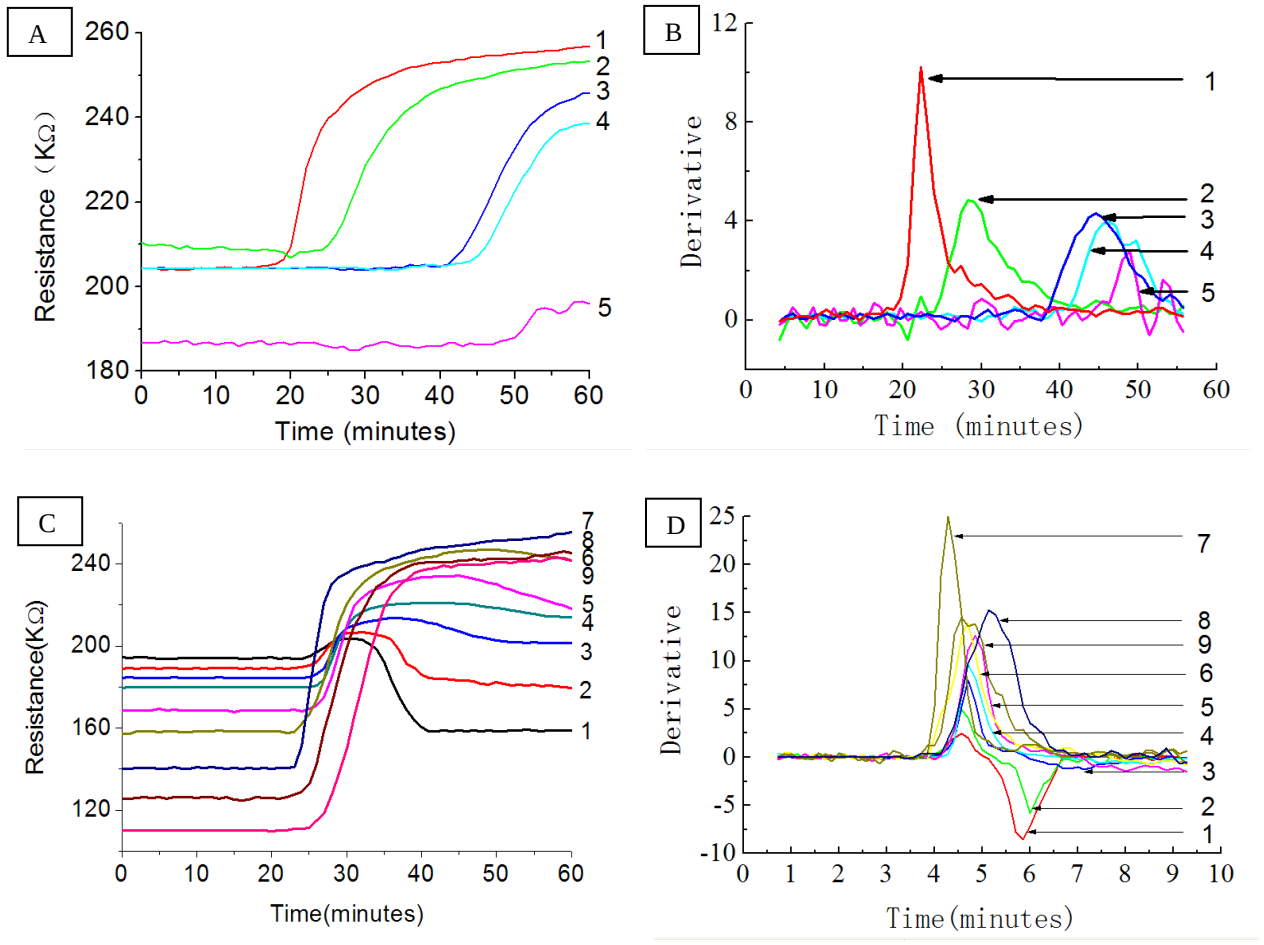


Figure 3. Characterization of resistance electrode (A) stability test,(B)interference test,(C) real-time curve, (D) derivative analysis

3.2. Optimization of the Real-time Resistance Measurement

Five dyes (1: Crytal violet, 2: Methylene blue, 3: SYBR Green I, 4: Hydroxy naphthol blue, 5: Ethidium bromide) were applied in the same concentration (1.0mg.L^{-1}) to compares of their reaction characteristics (Figure 4-A). It shows that the Crytal violet had more efficiency than others. It was more Significant in differential resistance curve than in real-time resistance curve (Figure 4-B). Then the Crytal violet was elected to applied in a series of concentrations (1:blank, 2:0.05, 3:0.1, 4:0.2, 5:0.5, 6:1.0, 7:2.0, 8:5.0, 9:10.0 mg.L^{-1}). It worked best in 2.0mg.L^{-1} (final concentration) (Figure 4-C, D). Gel electrophoresis of DNA amplification produce shown that the applied of real-time Crytal violet in 2.0(6, 7) and 5.0mg.L^{-1}(9, 10) hadn't obvious effect on the DNA amplification (Figure 3-E). Crytal violet (also known as Gentian violet, Methyl violet, or Hexamethyl pararosaniline chloride) is a triarylmethane dye. The Crytal violet is used as a histological stain and in Gram's method of classifying bacteria. When conducting DNA gel electrophoresis, Crytal violet can be used as a non-toxic DNA stain as an alternative to fluorescent, intercalating dyes such as Ethidium bromide. It have been reported that through used of a methyl orange counterstain and a more complex staining method, sensitivity could be improved further to 8 ng of DNA [24]. In this paper, The Crytal violet was elected to the electrochemical detection of DNA.



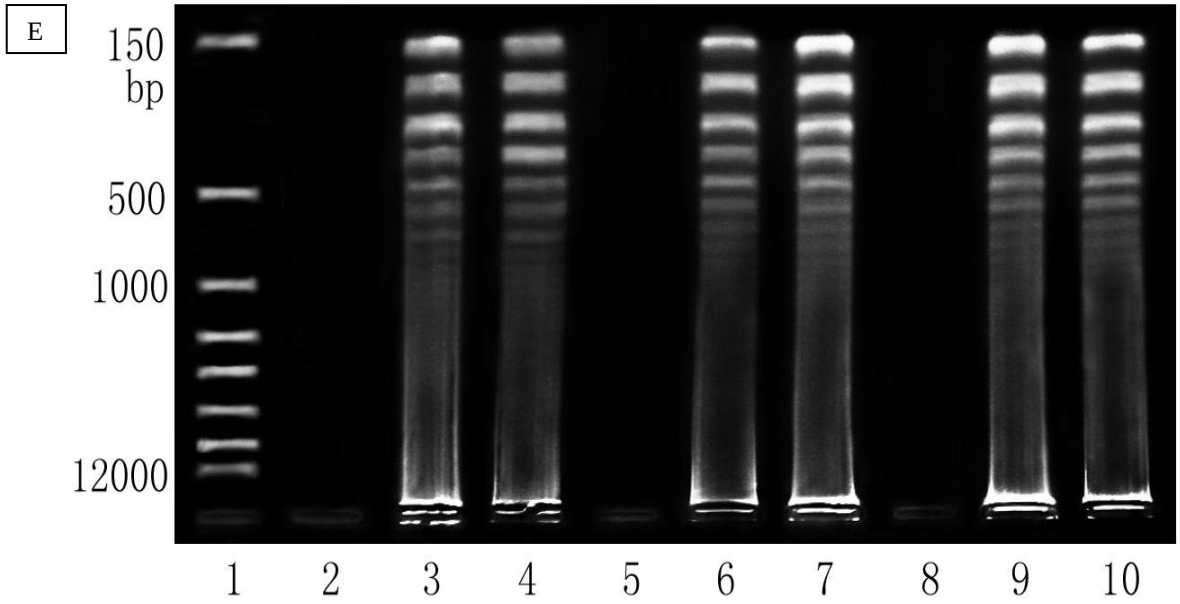
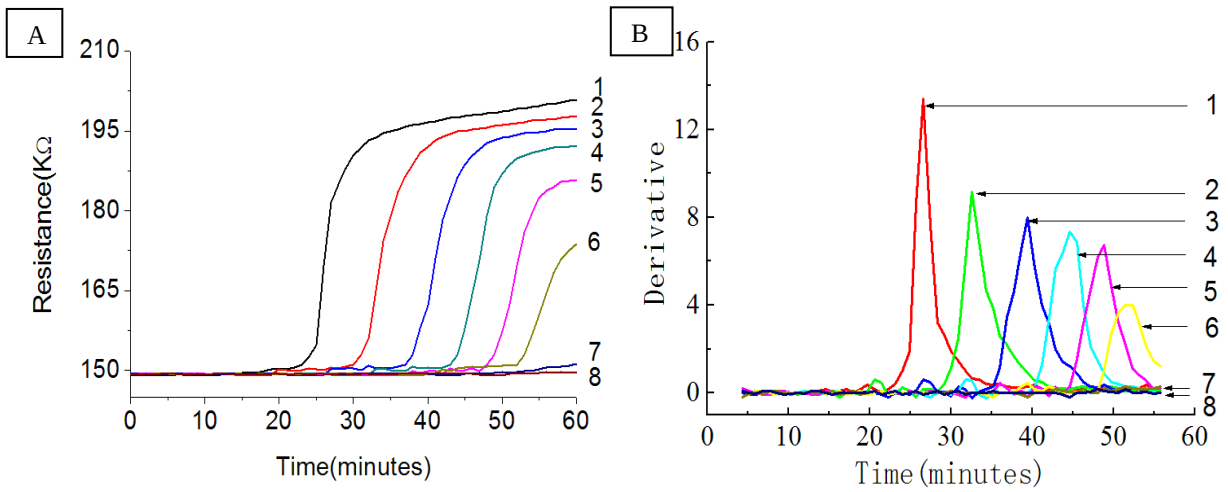


Figure 4. Optimization of the real-time resistance measurement.(A,B) dye select,(C,D) concentration select of Crytal violet,(E) interference of Crytal violet

3.3. Sensitivity of the strategy

Escherichia coli (ATCC25922) (1:10^6, 2:10^5,3: 10^4, 4: 10^3, 5: 10^2, 6:10^1, 7: 10^0CFU.mL^{-1} *E coli*,8:blank) were used to test the sensitivity of the reaction system. It was shown that the DNA detection could complete in 20-40 minutes with the lowest bacterial to 10 CFU.mL^{-1}, had high sensitivity (Figure 5-A, B). The regression analysis of the DNA amplification shown that the *Escherichia coli* had a quite correlation with the discrimination times of derivative analysis ($y = -6.0429x + 63.5$, $R^2 = 0.991$) (Figure 5-C). It may become the quantitative basics of the real-time resistance measurement.



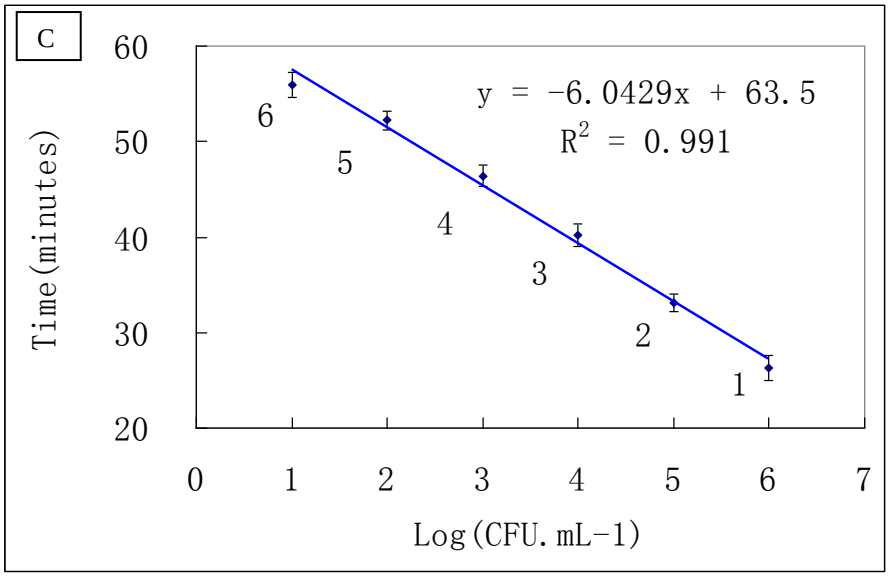
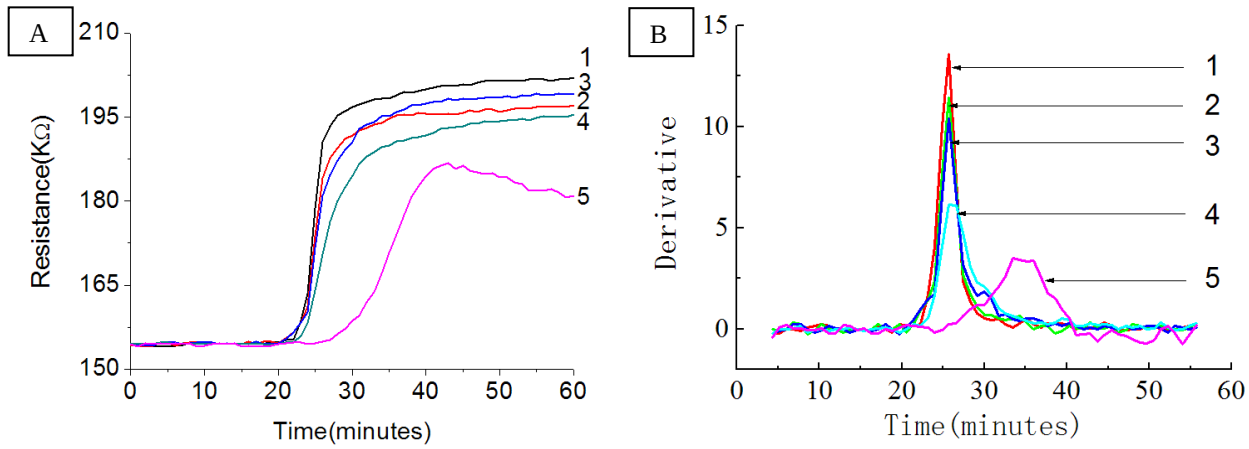


Figure 5. The the real-time resistance measurement(A,B) and regression analysis(C)

3.4. Anti-interference ability of the strategy

In ordinary specimens, the hemolysis, high concentration of lipid and bilirubin were the main interfering factors of DNA amplification and detection. The high concentration of salt could have impact on the determination of the resistance too. Four kinds of interference substances, hemoglobin, triglyceride, bilirubin and sodium chloride were added in the specimens before the DNA extraction and LAMP reaction (Figure 6). It displayed that the real-time resistance measurement had good anti-interference ability to hemoglobin (up to 20g.L^{-1}) (Figure 6-A, 1: 0, 2:5, 3: 10, 4: 20, 5: 40 g.L^{-1}), triglyceride (up to 20g.L^{-1}) (Figure 6-C, 1: 0, 2:5, 3: 10, 4: 20, 5: 40 g.L^{-1}), bilirubin (up to 10g.L^{-1}) (Figure 6-E, 1: 0, 2:5, 3: 10, 4: 20, 5: 40 g.L^{-1}), and sodium chloride (up to 1mol.L^{-1}) (Figure 6-G, 1: 0, 2:0.2, 3: 0.5, 4: 1.0, 5:2.0 mol.L^{-1}). It also shows that the results discrimination was more clearly by derivative analysis than by resistance curves (Figure 6-B, D, F, and H). The main interference effects may have been excluded by the process of DNA extraction and purification. The salt caused the baseline depression, but it could be corrected by differential analysis (Figure 6-H).



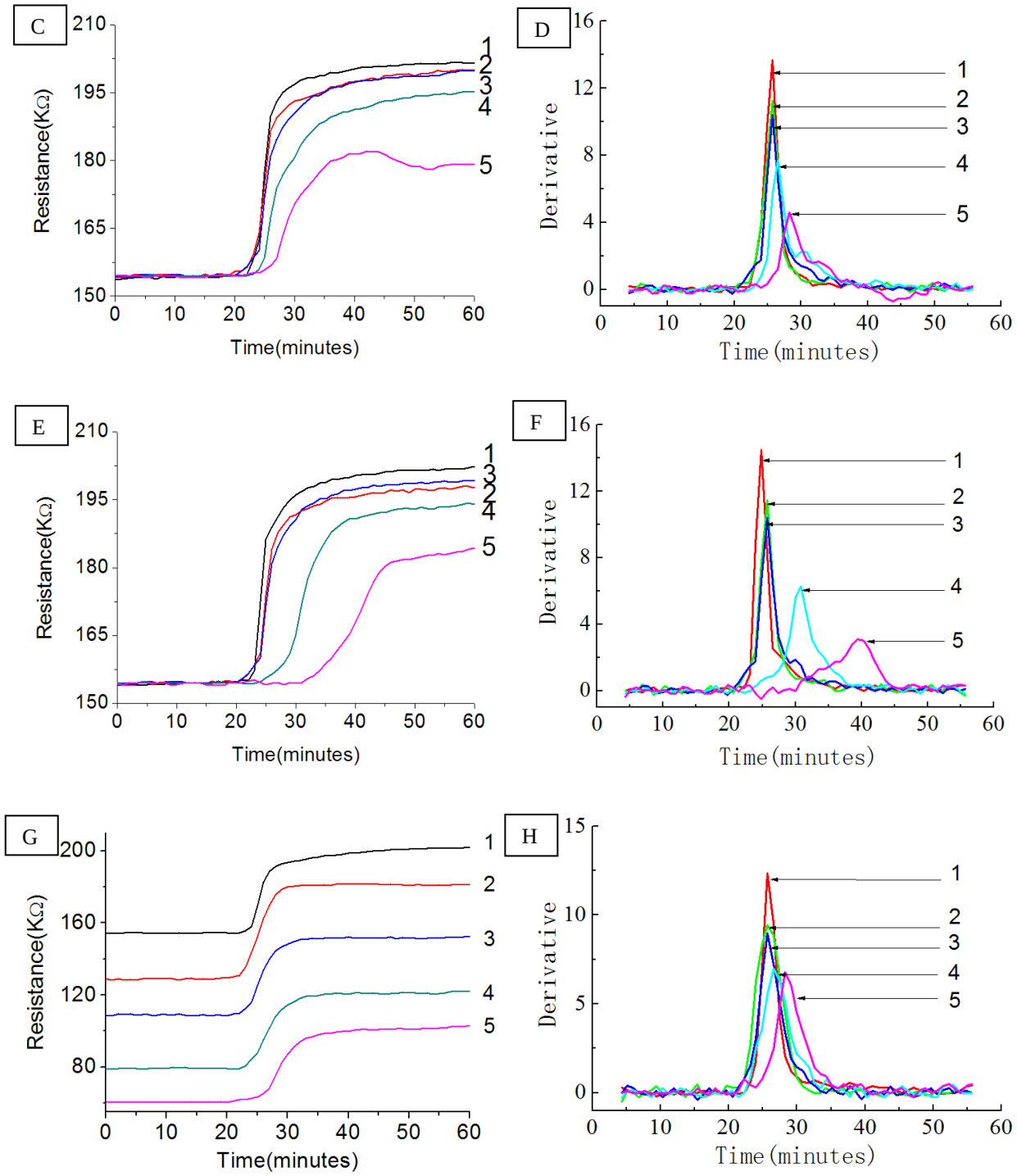


Figure 6. The anti-interference test of real-time resistance measurement to hemoglobin (A,B), triglyceride(C,D), bilirubin(E,F) and sodium chloride (G,H)

4. CONCLUSIONS

The work presented here a strategy of DNA detection, real-time resistance measurement, which basing on LAMP amplification, resistance measurement and differential determination. In the paper, a

special resistive electrode was designed and five dyes were applied in electrochemical detection of DNA. The results show that the Crytal violet was the best dye out of them. Crytal violet worked more effectively in 2mg.L^{-1}. The real-time resistance measurement could be completed in 20-40 minutes, with high sensitivity (lowest to 10 CFU.mL^{-1}), good anti-interference ability to hemoglobin, triglyceride, bilirubin and sodium chloride within the sample. Therefore, the real-time resistance measurement was a rapid, sensitive and stable method. It would provide a powerful tool for DNA detection in biomedical diagnostics, food safety and environmental monitoring，by applied on gene chips, pocket instruments, or other fields which needed the simple, rapid, and real-time methods of measuring.

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