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Clusters Regularly Interspaced Short Palindromic Repeats - Surface-Enhanced Raman Scattering for the detection of the drug resistance gene MacB

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Abstract

Antibiotics have brought many benefits to public health systems worldwide since they were first used in the last century, yet with the overuse of antibiotics in clinical care and livestock farming, new public health issues have arisen.In our previous experiments, we found that the MacB gene in bovine raw milk ranked first among many drug resistance genes.In this paper, we present a strategy for the highly sensitive detection of the drug resistance gene MacB by combining clusters regularly interspaced short palindromic repeats (CRISPR) with surface-enhanced Raman scattering (SERS) technology.This strategy integrates the specific gene identification capabilities of the CRISPR system, the ultra-sensitivity of SERS, the chemometric algorithm analysis and the simple separation properties of nanoparticles, eliminating the need for complex steps such as purification and gene amplification, offering the advantages of rapid, ultra-sensitive and highly specific detection, thus enabling more effective tongue safety.

Keywords: CRISPR/dCas9; Surface-Enhanced Raman Scattering; drug resistance gene MacB; gold nanoparticles.

1 Introduction

Antibiotics have been widely used in the clinical and animal husbandry sectors due to their low cost and efficient pharmacokinetic properties [1]. However, overuse of antibiotics not only causes an increase in bacterial resistance, but also results in excessive residues of antibiotics in food of animal origin, as well as leading to cross-transmission of resistance genes in the human-animal-environment, posing a serious risk to public health.Accurate and sensitive detection of drug resistance genes in food is therefore essential for food safety and human health.Currently, the detection of multiple drug resistance genes is usually performed by polymerase chain reaction (PCR), nucleic acid sequence amplification (NASBA), recombinase-based polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP) and aggregation of regularly interspaced short palindromic repeats (CRISPR)-related nucleases (CRISPR/Cas). Although these amplification methods have high sensitivity and specificity, they often have multiple steps, require long reaction times, complex primer designs, multiple reagents and relevant instruments, etc. In addition, they are prone to contamination and false-positive results during the process, which cannot be completely avoided at present.To overcome the limitations of traditional nucleic acid amplification methods[2, 3], we proposed the following strategy, a detection method based on regularly interspaced clustered short echo repeat

sequences aggregation (CRISPR) combined with surface-enhanced Raman scattering (SERS) technology[4, 5] .

In recent years, the CRISPR/Cas system has become a revolutionary tool for targeted genome editing and this technology has great potential for molecular diagnostic applications[6, 7]. Nucleic acid detection methods based on this approach have the ability to specifically identify gene sequences, i.e. the gene editing protein in this technology is able to bind to the target gene in a specific manner via a single guide RNA (sgRNA) binding site, meaning that the sgRNA can be easily edited to recognise any target gene sequence[8]. Current applications of this method require sample extraction and amplification of nucleic acids[9, 10], [11]making it challenging to use it more simply and accurately for the detection of bacterial resistance genes[12, 13].

Surface-enhanced Raman scattering [14] has been developed as a sensitive method for the preparation of biosensors, which is an optical sensing technique with molecular fingerprinting, sensitivity at the molecular level and retardation to fluorescence bursts. It has therefore been applied to detect bacterial genes or to detect bacterial genes directly without a label, and a variety of SERS label-free rapid detection of bacteria have been reported [15, 16].The tagged SERS assay has improved the sensitivity and stability of the assay [17, 18]. SERS-based genetic detection methods can achieve accurate classification of

bacteria.Combining the sequence-specific recognition capabilities of CRISPR/Cas-based systems with the highly sensitive synergy of SERS will provide a simple, rapid and sensitive method to detect bacterial drug resistance genes.Firstly, the CRISPR/Cas-based systems are mainly detected using fluorescence techniques, which are easy to observe[19, 20]. Moreover, CRISPR/Cas system-mediated SERS sensing methods are less studied, where the dCas9/sgRNA ribonucleoprotein (RNP) complex was designed as a highly specific and efficient targeting DNA probe that acts similarly as a DNA-targeting and labelling antibody.In this study, RNP complexes were combined with gold nanoparticles to form detection probes reacting with the bacterial drug resistance gene MacB gene. Enhanced Raman spectroscopy signals were obtained from the captured detection probes following the incorporation of Raman reporter molecules. This strategy allows us to obtain the ability to detect fM levels without nucleic acid amplification. The method can also be extended to detect a wide range of genes, providing a new detection method in food safety testing.

2 Principles of CRISPR-SERS analysis

In the principle of the CRISPR-SERS technology combination, genomic DNA can be used directly without the need for complex purification and amplification steps for effective detection of bacterial

resistance. Probes formed by gold nanoparticles in complex with RNP and DNA from macrolide antibiotic resistance genomic MacB are mixed and incubated. Since the dCas9/sgRNA complex in the CRISPR system is able to scan the entire genome and is able to capture and bind complementary target gene sequences through specific sites[21, 22], the Raman reporter molecule methylene blue (MB) was later added to the mix and embedded in the target DNA. MB is a 638 nm excitation source and a sensitive Raman reporter molecule used for double-stranded DNA staining. sensitive Raman reporter molecule, which has been successfully applied for DNA quantification in solutions, gels, real-time PCR and cell staining due to its significant increase in fluorescence emission upon interaction with double-stranded DNA [23, 24]. Measurements were completed and Raman spectral signals were obtained by centrifugation of gold nanoparticles bound to the drug-resistant MacB gene following the formation of a probe in complex with RNP.

3 Materials and methods

3.1 Materials and instruments

HNO_3/HCl, tetrachloroalloy acid ($HAuCl_4$) was purchased from Shanghai Aladdin Biochemical Technology Co. Ltd.; 1-ethyl-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich,

nickel chloride ($NiCl_2$) and NHS. Ltd.; MOPs (3-(N-morpholino)propanesulfonic acid) were purchased from Beijing Puyihua Technology Co. Ltd.; gel nucleic acid stain and methylene blue (MB) and all related buffers were purchased from Beijing Bajie Biotechnology Co. Dalian TaKaRa Company); SDS-PAGE precast gel (8%) was purchased from Beijing Biyuntian Biotechnology Co.

TEM images were obtained using the JEM 2100f microscope of Nippon electronics, which operates at 200kV. Mapping analysis was obtained using Oxford X-MAX 80TLE instrument in Oxford, UK. XPS analysis was obtained using semefi 250Xi and monochromatic Al target test (energy 1486.6ev). Raman spectra were obtained by Raman spectrometer (Horiba xplora, France) and excitation source λ= 638 nm, 30 MW neon laser, through 100 × The objective lens is focused on the sample, and the Raman spectrum of the silicon wafer at 520.7cm^{-1} is used to calibrate the instrument.

3.2 Synthesis of Au/NTA-Ni^{2+} nanoparticles

Due to the ability of the precious metal shell to produce a SERS response, a large number of SERS hotspots can be formed when the metal is aggregated[25]; the surface wrapped in precious metal is easily modified with various biomolecules, indicating that nanoparticles can be effectively used in a variety of fields such as bioassay.In this paper we

synthesised gold nanoparticles. First, all glassware was soaked in freshly prepared aqua regia (HNO_3/HCl = 1:3) and thoroughly rinsed and dried in ultrapure water. AuNPs were prepared by sodium citrate reduction according to a previous report [26]:100 mL of 0.01% (w/v) chloroauric acid ($HAuCl_4$) was added to a 250 mL round bottom flask, then the solution was heated to boiling at 140 ° C, 400 rpm and a 1% sodium citrate solution ($NasC_6HsO_7$-$2H_2O$) was quickly added.The colour of the solution changed from light yellow to purple, then to dark red and finally to burgundy within 5min. After continuing to heat for 15min, the solution was cooled to room temperature and the prepared solution was stored in a brown mill-mouth flask at 4 ℃ for backup. The prepared Au nanoparticles were washed 3 times with ultrapure water, dispersed in ultrapure water with MPA, adjusted the pH to 9-11 with alkali, stirred overnight at room temperature, centrifuged and separated, washed 3-5 times with PBS buffer and finally dispersed in PBS buffer to obtain the surface carboxylated Au/MPA nanoparticles(The lye solution is 0.1 mol/L NaOH or KOH solution; the mass ratio of Au nanoparticles to MPA is 1 : 3 to 20).EDC was added to the surface carboxylated Au nanoparticles (Au/MPA) solution made and stirred for 0.5~1h; then NHS was added and stirred for 0.5~1.5h; then NTA dissolved in PBS buffer was added and stirred overnight at room temperature, the reaction was completed with magnetic separation, washed several times with ultrapure water and

dispersed in ultrapure water; finally an excess of $NiCl_2$ solution was added and The reaction was carried out for 2-4h, washed 3 times with binding buffer, and finally dispersed in binding buffer to obtain Au/NTA-Ni^{2+} nanoparticles ($NiCl_2$ solution solubility of 1M; binding buffer containing 50mM MOPs (3-(N-morpholino)propanesulfonic acid), pH 7.9; the mass ratio of Au/MPA, EDC, NHS and NTA was 1 : 2 to 10 : 2 to 10 : 0.1 to 5).

3.3 Approach to CRISPR-SERE technology

For dCas9/sgRNA mobility shift analysis experiments, RNP complexes were constructed by incubating 10ul of sgRNA (10uM) and 40ul of dCas9 protein (5uM) in 50ul of PBS for 10min at room temperature. The incubated RNP complexes were mixed with PCR products (50ng/ul). and incubated in 10ul of 1×FastDigest buffer for 60min at 37°C. After adding 6×DNA loading buffer and mixing, polyacrylamide gel electrophoresis (PAGE) was performed and visualised using the Gel DocTM imaging system.

To construct Au-dCas9/sgRNA probes, we collected the modified gold nanoparticle solutions by centrifugation and then incubated Au-NTA-Ni^{2+} with 60 ul of dCas9/sgRNA in double buffer containing 100 mMPBS and 30 mM HEPES for 30 min and washed away the

unbound RNP complexes with PBS. The formation of Au-dCas9/sgRNA probes was verified by mapping of TEM.

The Au-dCas9/sgRNA probe was bound to the target gene sequence by incubating 10ul of Au-dCas9/sgRNA probe with 100ng of PCR product in 10ul of 1×FastDigest buffer for 60min at 37°C.After completion of the reaction the bound Au-dCas9/sgRNA-DNA was collected by centrifugation, mixed with 6× DNA loading buffer, subjected to polyacrylamide gel electrophoresis (PAGE) and visualised by gel DocTM imaging system.

SERS Raman spectroscopy signals were obtained for analysis. 60 ul of Au-dCas9/sgRNA probe was incubated sequentially with different levels of PCR products in 10 ul of 1×FastDigest buffer at 37°C for 60 min. 10 ul of methylene blue (MB) was added and incubated for 10 min. The reaction was collected by centrifugation with the target Au-dCas9/sgRNA probes bound to the target gene. The collected Au-dCas9/sgRNA-DNA was dropped onto a glass slide and the Raman spectral signal was monitored.

4 Assessment of CRISPR-SERS results

In our preliminary experiments, we found by chance that despite originating from different dairy farms, the abundance of macrolide antibiotics was significantly higher than other types of antibiotics, both in

raw milk and in fresh cow manure, corresponding to a much higher abundance of MacB genes for macrolide antibiotic resistance (Figure S1 in the Supporting Information), which may be related to the fact that dairy farming The high use of macrolide antibiotics in veterinary medicine at the stage of Macrolide antibiotics, including erythromycin, diclofenac and tylosin, are a group of 14 to 16-membered lactone rings and different additional sugars that inhibit protein translation by targeting bacterial ribosomes and are often used in livestock farming as feed additives and oral delivery agents. In bacteria, resistance mechanisms against macrolide antibiotics can be mediated by a variety of mechanisms, such as ribosomal methylation modifications, activated efflux pumps and macrolide antibiotic inactivating enzymes in addition to the MacB gene [27, 28]. As macrolide antibiotics are common in clinical and animal husbandry settings, if consumers continue to acquire resistance genes from ingested food, resulting in the transfer of bacterial resistance to humans via the "farm-to-table" route, there will be a huge economic loss in dealing with infections with resistant pathogens. The rapid detection of MacB genes in milk is therefore important for controlling the transfer of drug-resistant genes from pathogenic bacteria.

To specifically identify the drug resistance gene MacB, we designed sgRNAs for its sequence (Figure S1 in Supporting Information, Table S1) and we used polyacrylamide gel electrophoresis to assess the accuracy of

the designed sgRNAs, which could effectively demonstrate the binding of the RNP complex to the target gene. RNP complexes were prepared by mixing the dCas9 protein and sgRNA. The PCR amplification products containing the target gene sequences were obtained from bacterial genomic DNA extracted from milk samples (Table S2 in the Supporting Information) and the results of the electrophoretic mobility of the mixture of PCR product, RNP complex and PCR product are shown in Figure S2 in the Supporting Information. It can be learned that the electrophoretic bands of the mixture of RNP complex and PCR product are more upward compared to the PCR product, mainly because the binding of the PCR product to the RNP complex results in slower mobility and therefore not as fast as the electrophoretic band migration rate of the PCR product[29]. This result can indicate that the experimentally designed sgRNA can accurately recognize the target gene sequence, and we learned that the use of dCas9/sgRNA complex does not require cleavage of the MacB gene sequence.

The principle of Au-dCas9/sgRNA binding and target gene sequence detection by SERS is shown in Figure 1. In Au-dCas9/sgRNA binding, gold is coupled to the amino group of nitrogen triacetic acid (NTA), which then chelates Ni^{2+} to obtain Au-NTA-Ni^{2+}. After washing and centrifugation, X-ray photoelectron spectroscopy (XPS) was performed to characterise the Au-NTA-Ni^{2+} peaks (Supporting Infographic S3), which

were fitted with a non-linear least squares procedure to produce an S2p (162.6eV) peak, similar to the S2p binding energy of the Au-S bond, which could indicate that the -SH of MPA is bound to the gold nanoparticle surface via Au-S. The high-resolution C1s (284.7eV) peak generated by Au-NTA-Ni^{2+} corresponds to the binding ability of C1s of -(CH2)-[30, 31]. The Ni2p1/2 (874.2eV) and Ni2p3/2 (856.7eV) generated were derived from Ni^{2+} chelates. xPS results indicate the successful preparation of Au-NTA-Ni^{2+} and we know that the dCas9 protein with a 6×His histidine tag is susceptible to binding metal ions during protein purification. We were therefore able to obtain Au-dCas9/sgRNA probes by mixing the prepared Au-NTA-Ni^{2+} with the dCas9/sgRNA complex[32].

To confirm the formation of the Au-dCas9/sgRNA probe, a TEM mapping analysis of the probe was performed (Supporting Infographic S4) and the addition of the dCas9/sgRNA complex resulted in the presence of large amounts of C, O, N and S elements on the surface of the gold. Thus being able to account for the formation of the Au-dCas9/sgRNA probe.

To verify that the Au-dCas9/sgRNA probe specifically recognises the macrolide antibiotic resistance gene MacB, we determined this by comparing the change in polyacrylamide gel electrophoresis bands before and after the addition of the target gene, as shown in Figure 2. After the addition of DNA bound to the Au-dCas9/sgRNA probe, sparser electrophoretic bands could be observed, indicating specific binding of

the Au-dCas9/sgRNA probe to DNA. To be able to capture the Raman spectral signal, we added a methylene blue Raman reporter molecule, which is a dye capable of mosaicking onto the Au-dCas9/sgRNA probe captured by the DNA double strand, as shown in Figure 3. We used the PCR amplification product of the drug resistance gene MacB for the assay. The PCR product (400ng) was reacted with its corresponding Au-dCas9/sgRNA probe in a test tube after adding the Raman dye methylene blue and testing for SERS signal (the SERS image obtained is shown in Supporting Information S5). When no PCR amplification product was added, only Raman spectra with no distinctive spectral peaks could be obtained (black line in Figure 3). Based on these results we can know that this CRISPR-SERS technique can be used for the detection of the drug resistance gene MacB.

In the evaluation of CRISPR-SERS technology, we found that the MacB gene in milk required only 50ng to monitor the Raman signal, and the partial least squares regression PLR algorithm was used to regress the Raman spectra for prediction[33]. After baseline correction of the collected Raman spectral data, the Raman spectral detection provides a wide range of spectral information, which not only contains biological information of the detected objects but also a large amount of redundant information. In order to better ensure the correlation between the characteristic peaks of Raman spectra and the content of PCR products, the characteristic range

of all spectral data was selected, which not only reduces the time to build the model but also improves the This not only reduces the time required to build the model but also improves the accuracy of the model. As the quality of the original spectra was improved after intercepting the spectra, there was still invalid information in the spectra. The normalisation pre-processing method was used to eliminate the drift in the spectral measurements, amplify and separate the overlapping information, improve the signal-to-noise ratio and predict the robustness of the model to obtain more valid spectral information.and PLS modelling was performed on the original and pre-processed spectra. For the acquired Raman spectra after PLS algorithm, the prediction set correlation coefficient Rp was 0.9695, the root mean square error of the prediction set RMSEP was 11.7263 and Bias was 2.6945, as shown in Figure 4(a). This prediction model shows that the intensity of the Raman spectrum increases when the PCR product added increases, as shown in Figure 4(b). The linear fitted equation in Figure 4(c) is $y=9.2125x+543.02$, $R^2=0.9960$. based on LOD=3Sb/m (where Sb is the standard deviation of the SERS response of the blank sample and m is the slope of the calibration curve). The LOD of the macrolide antibiotic resistant MacB gene in milk was therefore 32.5ng, converting the LOD value to 17.4fM, and the complete SERS data is shown in Figure S6 in the Supporting Information. The sensitivity of the system can therefore be investigated by measuring the

intensity of the Raman spectra obtained at different levels of PCR product at room temperature. Therefore, in this experiment the Raman spectral peak intensities were able to effectively differentiate between the MacB content of drug resistance genes and the Au-dCas9/sgRNA probe was able to specifically identify complementary target sequences, allowing accurate detection of drug resistance genes in the samples.

SERS-based nucleic acid biosensors have been widely used and have been applied in various fields due to the hybridization of nucleic acids that can effectively capture and form SERS Raman spectroscopy hotspots, showing great vitality in the biomedical field with high sensitivity[34, 35]. In contrast, the CRISPR/Cas system for nucleic acid detection is currently hot and has received great attention from many scholars, with the characteristic of specific identification of target gene sequences[36]. It is based on the advantages of these two technologies that they are combined. We can know that the combination of CRISPR-SERS technology can contribute to a great extent to the accurate and sensitive detection of target gene sequences. Thus in this experiment we were able to reach the fM level to detect the drug resistance gene MacB, while we did not need to amplify the gene. Overall, our proposed CRISPR-SERS technology can detect the drug resistance gene MacB in milk samples without the need for multiple complex steps such as amplification and purification.

5 Conclusions

In conclusion, we demonstrated that simple, rapid and highly sensitive detection of the drug resistance gene MacB is possible in a system based on CRISPR-SERS technology, with detection limits up to the fM level. The dCas9/sgRNA complex in this system is similar to an antibody in conventional immunisation and acts as a targeting material, acquiring Raman spectroscopic information through the Raman reporter molecule MB. Thus, providing a simple and inexpensive way to obtain the marker. We were able to effectively demonstrate the advantage of higher target specificity of this technique compared to conventional nucleic acid amplification methods[37, 38]. As the target of dCas9/sgRNA can be designed by targeting sequences, the technique is not limited to detecting the MacB sequence of drug resistance genes. It can be widely applied to many other gene target sequences, such as other bacteria, viruses, etc.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests

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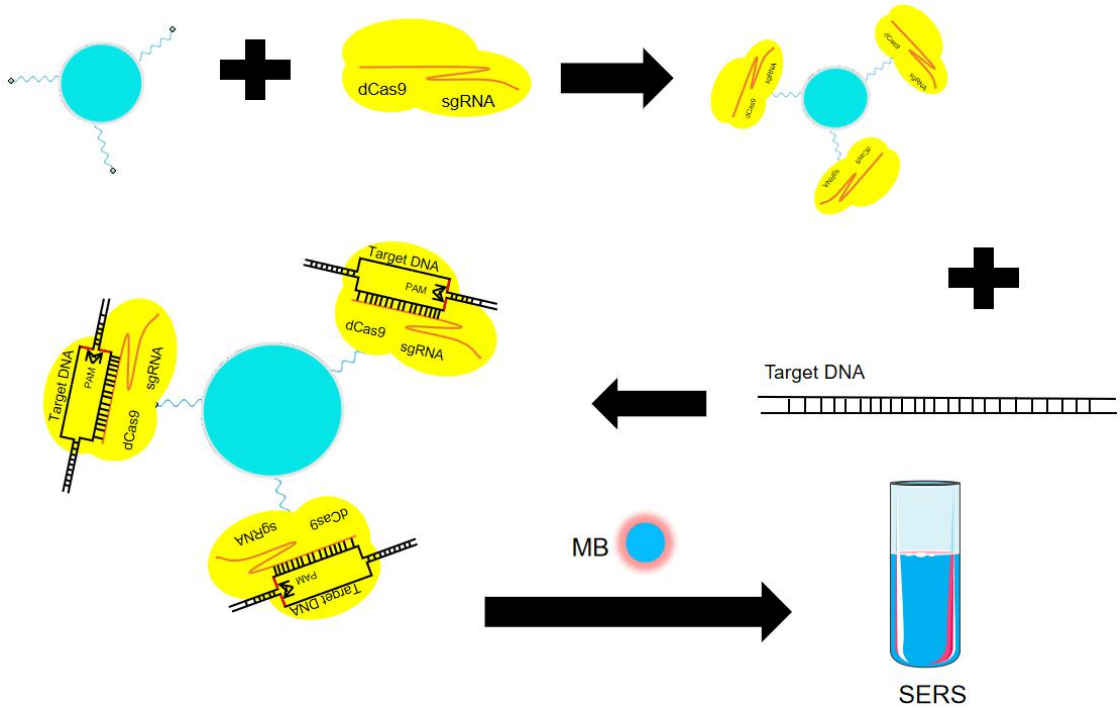


Fig1. Binding of Au-dCas9/sgRNA and detection of target gene sequences by SERS.

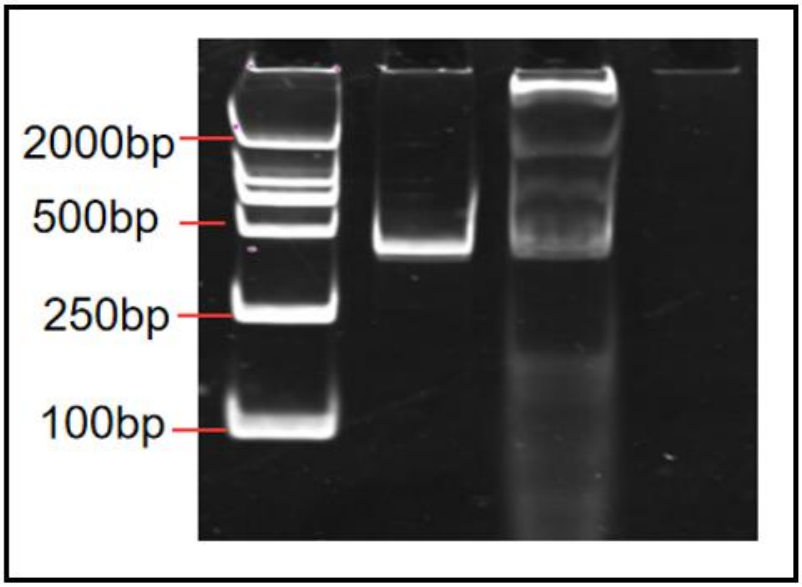


Fig2. polyacrylamide gel electrophoresis images of PCR amplification products before and after reaction with Au-dCas9/gRNA probe (line1: PCR product; line2: mixture of PCR product and Au-dCas9/sgRNA probe).

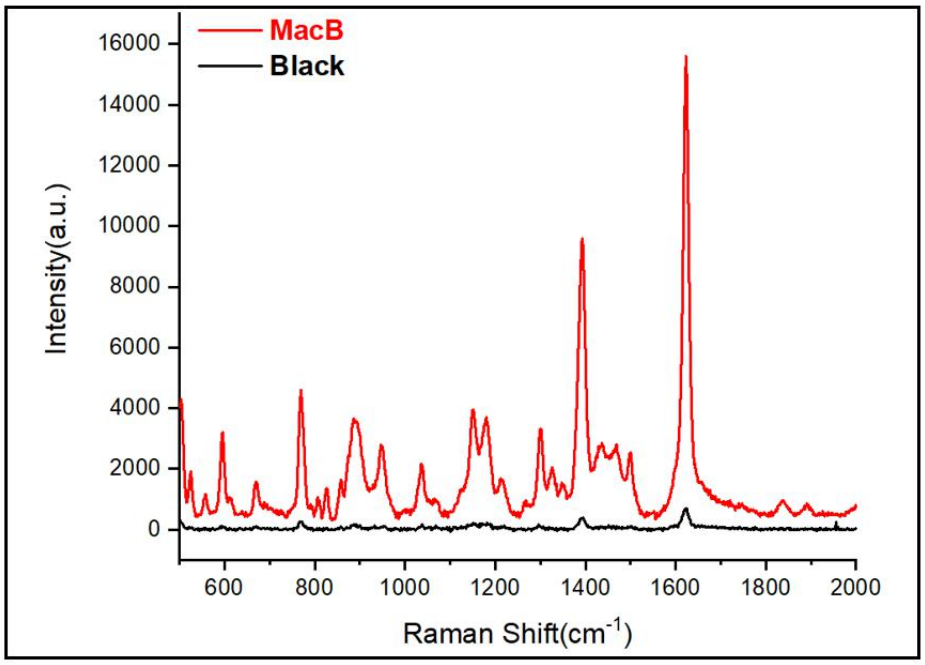
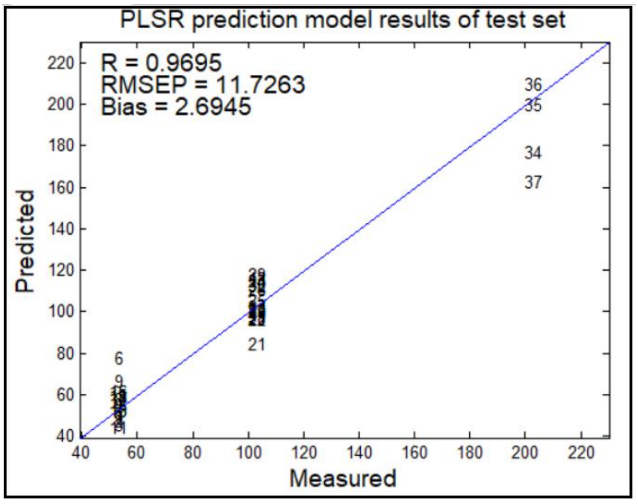
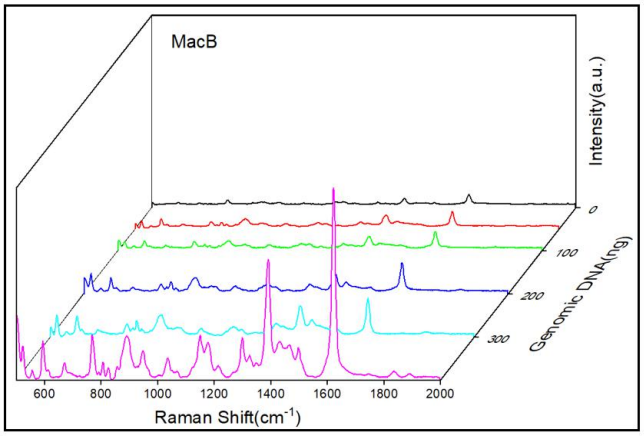


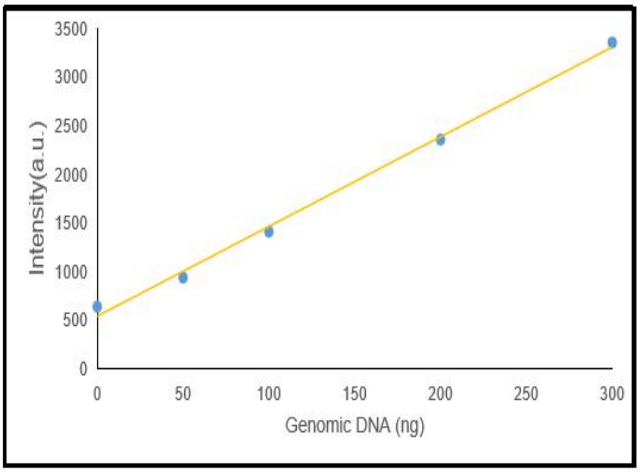
Fig3. Analysis of the PCR amplification product (400ng) by CRISPRP-SER technology.



(a)



(b)



(c)

Fig.4. (a) Results of the PLR prediction model. (b) DNA content of different MacB genomes versus SERS spectra obtained from Au-dcas9/gRNA probe reactions. (c) 1621 cm^{-1} intensity versus the amount of genomic DNA.

Supplementary Files

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