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CRISPR/dCas9-mediated biosensor for detection of tick-borne diseases

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Highlights

- An improved molecular diagnostics tool that utilizes CRISPR/dCas9-mediated biosensor
- It couples a nuclease inactivated Cas9 (dCas9) and single microring resonator biosensor for detection of pathogenic DNA and RNA
- Achieved single molecule sensitivity for the detection of ST (0.54 aM) and SFTS (0.63 aM)
- CRISPR/dCas9-mediated biosensor was able to clearly distinguish between scrub typhus (ST) and severe fever with thrombocytopenia syndrome (SFTS) in serum samples within 20 min.

Abstract

Rapid and highly sensitive detection of biomolecules is greatly needed for pathogen diagnosis in clinical samples, but the method needs to be significantly improved in terms of sensitivity and specificity for actual use in clinical settings. Here, we report the development of an improved molecular diagnostics tool that utilizes CRISPR/dCas9-mediated biosensor that couples a nuclease inactivated Cas9 (dCas9) and single microring resonator biosensor, enables label-free and real-time detection of pathogenic DNA and RNA. We addressed the clinical utility of this CRISPR/dCas9-mediated biosensor in tick-borne illnesses including scrub typhus (ST) and severe fever with thrombocytopenia syndrome (SFTS), whose clinical presentations are too similar to be easily differentiated. By using CRISPR/dCas9-mediated biosensor, we achieved single molecule sensitivity for the detection of ST (0.54 aM) and SFTS (0.63 aM); this detection

sensitivity is 100 times more sensitive than that of RT-PCR assay. Finally, CRISPR/dCas9-mediated biosensor was able to clearly distinguish between ST and SFTS in serum samples within 20 min. We believe that CRISPR/dCas9-mediated biosensor will be useful for rapid and accurate molecular diagnostic tool that is suitable for immediate clinical applications.

Keywords: Clinical diagnosis, CRISPR/Cas system, nuclease inactivated Cas9, SMR biosensor, *in vitro* diagnostics

1. Introduction

Due to the recent increase in the occurrence of emerging infectious diseases such as Zika, Ebola, and MERS-CoV, advances have been made for fast detection of target pathogens [1-3]. To control the spreading of pathogens from one community to another and to provide accurate treatment to patients, correct diagnosis of pathogen in first-line diagnostic places including clinics is crucial. Although many different diagnostic approaches have been introduced, development of a rapid and high sensitive approach for clinical diagnosis still remains a challenge.

Rapid diagnostic testing (RDT) based on antibody detection for infectious diseases produces results within an hour [4-5]. However, the detection sensitivity of RDT is lower than that of sequence-based nucleic acid amplification methods such as PCR [6-8]. Hence, sequence-based nucleic acid testing has many applications in clinical fields such as diagnosis and epidemiology. However, those approaches are limited in that they are long processing time, high-cost, and relatively less sensitive for clinical use [9]. Over the past decade, biosensors combined with electrical, electrochemical, and optical techniques have been developed to improve detection sensitivity [10].

Recently the nucleic acid amplification and detection technologies that enable rapid, simple, and sensitive detection of biomolecules have been developed. The isothermal solid-phase DNA amplification and detection (iSAD) to simultaneously amplify and detect DNA molecules on silicon microring resonator (SMR) biosensor, and the isothermal one-step RNA amplification and detection (iROAD) allows for amplification and detection of RNA after complementary DNA synthesis on SMR biosensor, that offer simple, rapid, label-free, and real-time multiplexed detection of biomolecules near the sensor surface by using refractive index changes [11-13]. For clinical use, however, the sensitivity and specificity of these technologies need to be further improved.

In vivo genome editing technologies have great clinical application potentials [14-15]. Zinc-finger nucleases (ZFNs) or transcription activator-like effectors (TALEs)-based editing tools have been introduced for DNA targeting and regulation, however, these proteins need to be individually designed for

DNA binding, which remains a hurdle for regulating multiple loci [14,16]. In contrast, RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR) associated Cas9 targets specific genomic loci via a single guide RNA (sgRNA), which contains a 20-bp guide sequence followed by a 3-bp protospacer adjacent motif (PAM) and recognizes target DNA through Watson-Crick base pairing [17]. The system uses an endonuclease Cas9 for nucleotide sequence based DNA targeting. Cas9 is guided by the sgRNA that specifically bind to and cleave double-stranded DNA in a site-specific manner [18-21]. Recently, Cas9 nuclease can be used to enrich and detect the small amounts of tumor fragments in the circulating tumor DNA (ctDNA) or Zika virus [22-23]. Also, other types of Cas proteins such as Cas12a or Cas13 are used for rapid detection of nucleic acids [24-27]. In addition to wild-type Cas9 or Cas9 orthologue proteins, catalytically inactivated Cas9 protein (dCas9) has great potential for various biological studies [28-29]. In contrast to the wild-type Cas9, the nuclease-deficient dCas9 have the ability to bind to DNA using sgRNA without cutting. Hence, dCas9 technology is a widely useful tool for *in vivo* and *in vitro* diagnostics [17,28-30].

In this study, we developed an improved diagnostic tool by combining a CRISPR/dCas9 and an isothermal diagnostic approach based on SMR biosensor for simultaneous nucleic acid (RNA and DNA) amplification and detection with speed as well as high sensitivity and specificity. The biosensors transduce the presence of target molecules based on binding-induced changes in the refractive index proximal to the waveguide surface [13]. In the case of combination of dCas9 and isothermal based biosensor, dCas9 can recognize the target DNA or cDNA but not cut the sequences of target. Because of the binding property of dCas9, the refractive index is changed based on the binding with target and dCas9 that could be more enhanced the sensitivity than SMR biosensor alone. On the other hand, Cas9 can recognize the target DNA or cDNA and cut the target sequences. Thus, the binding-induced changes are not affected. We demonstrated the clinical usefulness of this technology by using 3 *Orientia tsutsugamushi*, the causative agent of scrub typhus (ST), and 3 *bunyavirus*, the causative agent of severe fever with thrombocytopenia syndrome (SFTS). ST and SFTS are tick-borne infectious diseases that are common in Eastern Asia, especially in Korea, China, and Japan [28-29]. Moreover, the clinical presentations of these diseases substantially overlap; therefore, a rapid and highly sensitive detection method is greatly needed. Our method clearly distinguished between ST and SFTS within 20 min from serum samples.

2. Material and methods

2.1. Protein purification

For purification of recombinant dCas9 protein, T7 Express BL21 (DE3) *E.coli* were transformed with pET28a-His6-dCas9 plasmid. After culturing *E.coli* in Luria-Bertani (LB) broth at 30 °C, protein expression was induced with 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 16 h at 16 °C. Cell pellet was collected by centrifugation at 5,000 g, and then lysed by sonication in lysis buffer (NaH₂PO₄ 50 mM, NaCl 300 mM, imidazole 10 mM pH 8.0, 1 mM PMSF, 1 mM DTT, 1 mg/mL lysozyme). Soluble lysate was

obtained by centrifugation at 8,000 g and incubated with Ni-NTA agarose beads for 1 - 2 h (Qiagen). Protein bound Ni-nitrilotriacetic acid (NTA) agarose beads were washed (NaH₂PO₄ 50 mM, NaCl 300 mM, imidazole 20 mM pH 8.0) and dCas9 protein was eluted with imidazole containing buffer (NaH₂PO₄ 50 mM, NaCl 300 mM, imidazole 250 mM pH 8.0). With 100 K Amicon centrifugal filter (Millipore), the buffer of eluted protein was exchanged, concentrated, and analyzed with 4-12 % Bis-Tris gels (ThermoFisher).

2.2. *In vitro* cleavage assay

The PCR products (400 ng), which contained each *Orientia Tsutsugamushi* and bunyavirus DNA sequence, were mixed with 5.9 µl of rehydration buffer and 0.5 µl of 280 mM MgAc solution supplied in TwistAmp Basic RT kit. In 10 µl reactions, buffer mixed PCR products were incubated with 1 µg Cas9 protein and 750 ng sgRNA for 1 h at 37 °C as previous study [33]. For the positive control of the cleavage assay, same PCR products were cleaved in 1X NEBuffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 100 µg/ml BSA, New England BioLabs) condition. The RNase A (4 µg) was added to samples to remove sgRNA and the final samples were analyzed with agarose gel electrophoresis.

2.3. *In vitro* binding assay

dsDNA templates were prepared by annealing the 5' biotinylated target DNA strands and the non-biotinylated non-target DNA strands at a 1:1.5 molar ratio. (OT_1_F_biotin: TATAAAGATCTTGTTAAATT GCAGCGTCATGCAGGAATTAGGAAAGC, OT_1_R: GCTTTCCTAATTCCTGCATGACGCTGCAATTTA ACAAGATCTTTATA, SFTS_F_biotin:AAAAATTAGCTGCCCAACAAGAAGAAGATGCAAAGAATCAAGG TGAA, SFTS_R: TTCACCTTGATTCTTTGCATCTTCTTCTTGTGGGCAGCTAATTTTT) 10 nM dsDNA was incubated with 300 nM dCas9 and 1 µM sgRNA in cleavage buffer condition. After 20 min incubation at 37 °C, samples were resolved with 10% TBE gels using 0.5X TBE buffer supplemented with 5 mM MgCl₂. Then, *in vitro* binding status was analyzed by using Chemiluminescent Nucleic Acid Detection Module Kit (ThermoFisher) and Biotinylated Nylon Membrane (ThermoFisher) according to the manufacturer's protocol.

2.4. Operation of CRISPR/dCas9-mediated biosensor and SMR biosensor alone

For simultaneous amplification and detection of target DNA or RNA using SMR biosensor alone, we used the RPA and reverse transcription (RT)-RPA solution for DNA and RNA, respectively. To prepare RPA or RT-RPA solution, 29.5 µL rehydration buffer, 15 µL of RNase inhibitor and H₂O, 2.5 µL of 10µM another primer were mixed. Then, reaction mix was added to freeze-dried enzyme. After that, 2.5 µL of 280 mM magnesium acetate (MgAc) was dissolved into the tube. After mixing, 50 µL of reaction buffer was split into five 10 µL aliquots. To begin the reactions, 5 µL of DNA or RNA extracted from blood serum of the patients and 3 µL of dCas9 RNPs (300 ng of dCas9 and 225 ng of gRNA) were added to 10 µL reaction aliquot. The volume of 18 µl mixture was added into a sensor chip with an acrylic well placed surround the microring sensor area and 10 µl mineral oil added to prevent evaporation the mixture during

amplification. The chip was then placed on a thermal pad to keep a specific temperature (38 °C for DNA and 43 °C for RNA). The wavelength shift was measured every 5 min to monitor the amplification of target DNA or RNA. Relative resonant wavelength shift was calculated by the equation; $\Delta\lambda_{pm} = (\text{target wavelength value, pm}) - (\text{non-target wavelength value, pm})$.

2.5. Clinical specimens

ST and SFTS serum samples were collected from the patients in Asan Medical Center. The study protocol was approved by the Institutional Review Board of Asan Medical Center, and informed consent was obtained from all participants. SFTS was confirmed by detecting viral ribonucleic acid (RNA) by real-time RT-PCR in serum, using a DiaStar 2X OneStep RT-PCR Pre-Mix kit (SolGent, Daejeon, South Korea). A diagnosis of ST was established when we observed either a single positive result of an immunofluorescence assay (IFA; SD Bioline Tsutsugamushi Assay; Standard Diagnostics, Yongin, South Korea), or a $\geq 1:640$ or fourfold rise of IFA titer in successive samples.

3. Results and discussion

3.1. CRISPR/dCas9-mediated biosensor as a molecular diagnostic tool

Figure 1A shows the design of CRISPR/dCas9-mediated biosensor that couples SMR-based isothermal nucleic acid amplification and dCas9 RNP. For simultaneous amplification and detection of nucleic acid, sequence specific primer of target was immobilized to the surface of the SMR biosensor and dCas9 RNP was in reaction chamber with single temperature for isothermal reaction with RPA. For DNA, a primer binds a recombinase enzyme to extend the DNA. For RNA, RT-RPA was used for the formation of complex of a primer and a recombinase enzyme to transcribe cDNA from RNA. The amplified nucleic acid targets were simultaneously detected by monitoring the wavelength shift. During the nucleic acid amplification process with dCas9 RNP on the SMR biosensor, the immobilized primer was hybridized with the target templates; dCas9 RNP, which allows sequence specific binding to the target nucleic acids (nearby the waveguide surface at immobilized forward primer, supplementary figure S1), subsequently caused a dramatic increase as a signal enhancer in the proportion of each resonant wavelength. The SMR biosensors transduce the presence of target molecules based on binding-induced changes in the refractive index proximal to the waveguide surface. dCas9 binds to the amplified product on the surface of the sensor and increases the molecular weight of the sensor surface, thereby increasing the detection sensitivity by increasing the refractive index change. Therefore, the CRISPR/dCas9-mediated biosensor detected the pathogenic nucleic acids with high sensitivity within 20 min compared to the SMR alone (Fig. 1A).

3.2. Assay optimization

To achieve sensitive detection with dCas9 RNP on SMR biosensor, we constructed guide RNAs (gRNAs) targeting two tick-borne pathogens that have substantially overlapping clinical presentations:

Orientia tsutsugamushi, the causative agent of scrub typhus (ST), and *bunyavirus*, the causative agent of severe fever with thrombocytopenia syndrome (SFTS) (Fig. 1B). We observed that Cas9 RNPs induced targeted DNA cleavage and dCas9 RNPs bound target DNA in RPA buffer conditions using an *in vitro* cleavage assay and an electrophoretic mobility shift assay (EMSA), respectively (Fig. 2A, B). To determine whether dCas9 RNP could enhance the detection sensitivity of the SMR biosensor, we amplified DNA fragments from ST clinical samples; as a result, we observed that the signal was higher with ST + dCas9 RNPs than with ST alone or ST + Cas9 RNPs at all time points (from 5 min to 30 min) (Fig. 3A). The relative resonant wavelength shift results of the ST with dCas9 RNPs showed that it is possible to clearly separate the positive and negative than ST alone and ST with Cas9 RNPs. In the case of ST with Cas9 RNPs, because of the cutting property of Cas9 RNPs, the result of relative resonant wavelength shift was reduced and looked like a negative. This improvement is due to the specific binding of dCas9 RNPs with the target fragment on the biosensor, and not a non-specific binding effect (Fig. 3B). Remarkably, dCas9 RNPs improved both detection sensitivity and specificity. We also optimized the concentration of dCas9 RNPs for sensitive and specific detection of pathogenic nucleic acids (Fig. 3C).

3.3. Single molecule detection using CRISPR/dCas9-mediated biosensor

Next, we determined the detection sensitivity of CRISPR/dCas9-mediated biosensor for detection of pathogenic DNA (for ST) or RNA (for SFTS) fragments from *Orientia tsutsugamushi* and *bunyavirus*. We used a RPA- or RT-RPA based CRISPR/dCas9 mediated biosensor for double-stranded DNA and single-stranded RNA amplification, respectively. To test the detection sensitivity of ST and SFTS alone, and with dCas9 RNPs, we used serially diluted samples containing 1×10^0 to 5×10^9 copies of the ST amplicon and T7 transcribed SFTS RNA. As a result, by using dCas9 RNPs, we achieved single-molecule sensitivity for the detection of ST (0.54 aM) and SFTS (0.63 aM) within 30 min (Fig. 4A, B). The detection sensitivity of this CRISPR/dCas9-mediated biosensor (1 copy) was superior to that of the SMR biosensor alone (~10 copies) (Fig. 4A, B) and real-time PCRs (~100 copies) (Fig. 4C, D).

3.4. Utility of CRISPR/dCas9-mediated biosensor in clinical samples

Finally, we investigated the clinical utility of this CRISPR-mediated biosensor for clinical applications that require speed, high sensitivity, and specificity, such as the diagnosis of incipient tick-borne illnesses. We used the CRISPR/dCas9-mediated biosensor to analyze six clinical samples—three from ST patients and three from SFTS patients (Fig. 5). When we employed the CRISPR/dCas9-mediated biosensor with ST primers, we observed elevated signals only in ST samples and not in SFTS samples (Fig. 5A). When we employed the CRISPR-mediated biosensor with SFTS primers, we detected elevated signals only in SFTS samples. Remarkably, the SFTS samples were more clearly detected by the CRISPR/dCas9-mediated biosensor than by the SMR biosensor alone (Fig. 5B). Thus, we demonstrated that the CRISPR-mediated biosensor can clearly distinguish the two pathogens from clinical samples.

4. Conclusion

We developed a CRISPR/dCas9-mediated biosensor that combines a catalytically inactive dCas9 and SMR biosensor based isothermal nucleic acid amplification for rapid and high sensitive detection of pathogens in clinical settings. Using CRISPR/dCas9-mediated biosensor, we achieved single molecule sensitivity for the detection of ST (0.54 aM) and SFTS (0.63 aM). Also, our method clearly distinguished between ST and SFTS within 20 min from serum samples.

We addressed several features for clinical use of this assay. First, CRISPR/dCas9-mediated biosensor enhances detection sensitivity through triple-targeting (primer, target strand, and dCas9). dCas9 shows higher sensitivity and specificity than the conventional method because it requires accurate target recognition of 20 nt of gRNA (Supplementary Table S2). Importantly, the triple-targeting method provides a significant advancement in terms of detection sensitivity from our previous SMR biosensor approach that utilized primer to target strand only [11,13]. By using triple-targeting, we were able to detect a single copy target, which translates into 10 times higher sensitivity than that of SMR biosensor alone (10 copies) and 100 times more sensitive than that of real-time (RT)-PCR methods (100 copies). Second, the readout system of CRISPR/dCas9-mediated biosensor differs from other CRISPR based diagnostic assays (i.e., fluorescence dye) in fundamental ways [24-27]. SMR biosensor based isothermal nucleic acid amplification assay can be simultaneously amplified and detect the target without labeling. In addition, CRISPR/dCas9 enhanced the refractive index change on the sensor surface. Therefore, the readout of sample using our assay can be carried out within 20 min. Third, the results of false-positive or false-negative are minimized by the use of sgRNA, primer, and dCas9. We have also shown that this assay is able to clearly distinguish the two pathogens in clinical samples. Our data on the clinical sensitivity and specificity highlights the utility and practicality of CRISPR/dCas9-mediated biosensor. Fourth, Gootenberg *et al.* developed a CRISPR/Cas13a (previously known as C2c2)-based diagnostic assay [24,25]. Because Cas13a is a programmable RNA-guided ribonuclease, Cas13a-based diagnosis is an RNA-based detection method that exploits the collateral effect of Cas13a. Our complementary approach using CRISPR/dCas9 is a DNA-based detection method that is able to detect nucleic acids during isothermal nucleic acid amplification without *in vitro* RNA transcription.

Finally, our CRISPR/dCas9-mediated biosensor method is being further improved for convenient operation based on the ASSURED criteria (Affordable, Sensitivity, Specificity, User-friendly, Rapid, Equipment-free, Deliverable) in clinical settings [30]. A microfluidic platform is being developed for sample processing that uses non-chaotropic reagent-based thin film techniques. The platform is devised to allow simple, low-cost, rapid, and high-throughput nucleic acids (DNA, RNA) extraction from human body fluids [31,32]. We believe that the final integration of sample preparation and CRISPR/dCas9-mediated biosensors into a single cartridge will significantly facilitate emerging pathogen diagnosis for making timely treatment decision for emerging infectious diseases.

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Figure legends

Figure 1. CRISPR/dCas9-mediated biosensor. (A) Schematic of CRISPR/dCas9-mediated biosensor. SMR biosensor, silicon microring resonator sensor; dsDNA, double stranded DNA. (B) The schematics of gRNA design targeting ST (*Orientia tsutsugamushi*) and SFTS (*Bunyavirus*). The target site is highlighted in blue and the PAM sequence is shown in red.

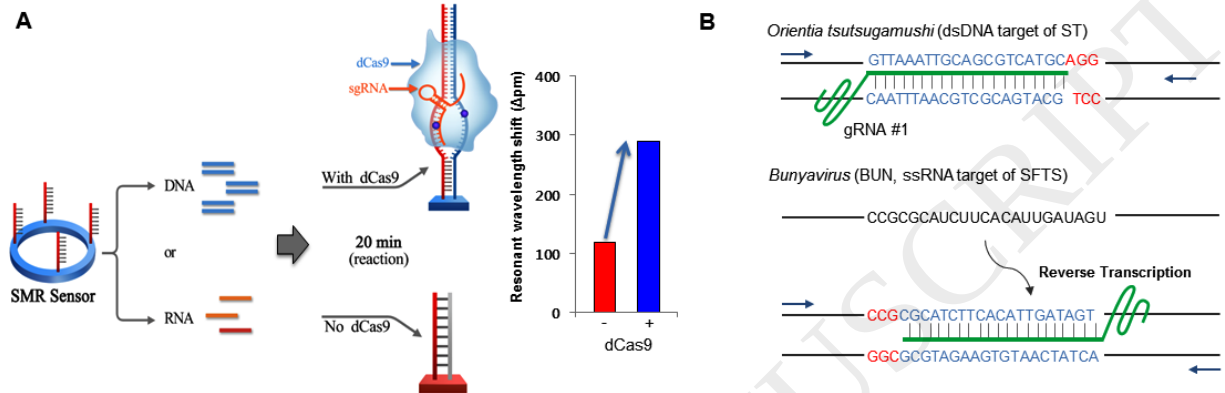


Figure 2. Ability of dCas9 ribonucleoprotein (RNP) in reaction buffer. (A) *in vitro* cleavage assay to investigate the activity of gRNAs in the RPA buffer condition. Cas9 RNP could cleave the PCR products in both the RPA buffer and the NEBuffer 3.1 condition only when gRNAs were matched to the target PCR products. (B) Electrophoretic mobility shift assay (EMSA) using dCas9 RNP and the 5' biotinylated DNA duplexes. The target DNA duplexes were only shifted with the matched gRNAs in both the RPA buffer and the NEBuffer 3.1 condition.

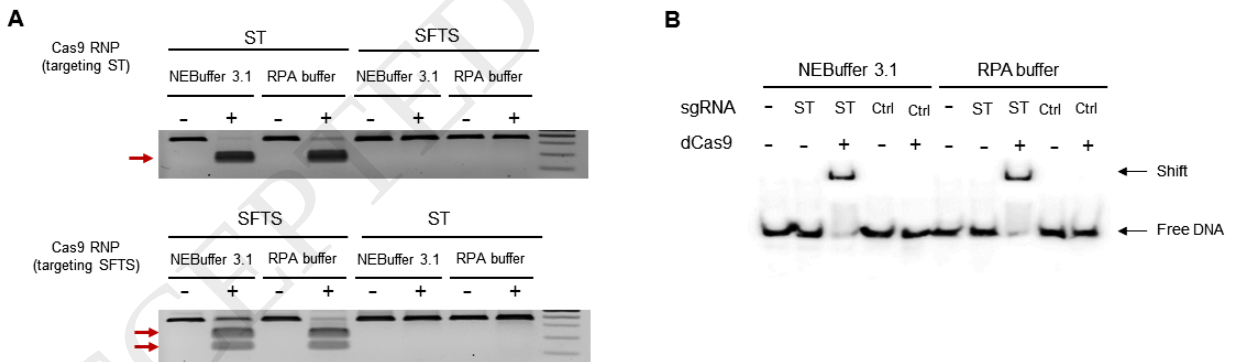


Figure 3. Characterization of CRISPR/dCas9-mediated biosensor. (A) CRISPR/dCas9-mediated biosensor detection of ST-DNA within 30 min. ST (SMR biosensor only, green), ST with dCas9 ribonucleoprotein (RNP) (dCas9 with biosensor, blue), ST with Cas9 RNP (Cas9 with biosensor, red), and negative control (black-X). (B) Resonant wavelength shift of CRISPR/dCas9-mediated biosensor for detection of ST in 15 min. SMR biosensor alone (green), with dCas9 RNP (blue) and with Cas9 RNP (red). (C) Relative resonant wavelength shift of CRISPR/dCas9-mediated biosensor in 30 min. The colors represent the amount of the dCas9 RNP; green (ST without dCas9 RNP), orange (ST with 1x of dCas9 RNP, $p > 0.1$), blue (ST with 3x of dCas9 RNP, $p < 0.001$), and grey (ST with 5x of dCas9 RNP, $p > 0.1$). Error bars indicate standard deviation from the mean, based on at least 3 independent experiments.

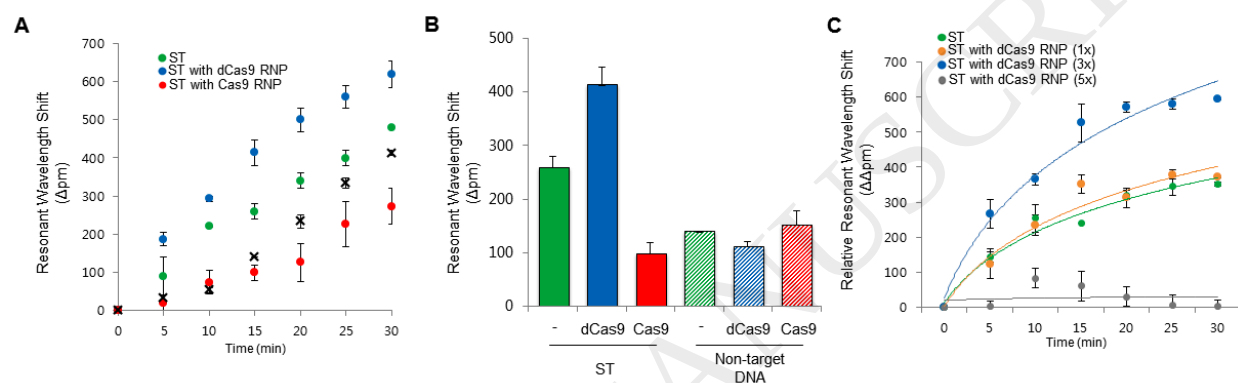


Figure 4. Comparison of detection limit of CRISPR/dCas9-mediated biosensor and real-time PCRs for DNA and RNA. (A) CRISPR/dCas9-mediated biosensor can detect dsDNA of ST at concentration down to 0.54 aM (blue circle), more sensitive than biosensor alone (green circle). (B) CRISPR/dCas9-mediated biosensor detected RNA fragment of SFTS at a concentration of 0.63 aM (blue triangle), which was more sensitive than biosensor alone (green triangle). (C) Linear relationship between the concentration of target DNA and Ct value of fluorescence signal by real-time PCR. The target DNA at low concentration (<100 copies/ml) was not detected (over 40 Ct value). (D) Linear relationship between the concentration of target RNA and Ct value of fluorescence signal by real-time RT-PCR. The RNA target at low concentration (<100 copies/ml) was not detected (over 40 Ct value). Error bars indicate standard deviation from the mean, based on at least 3 independent experiments.

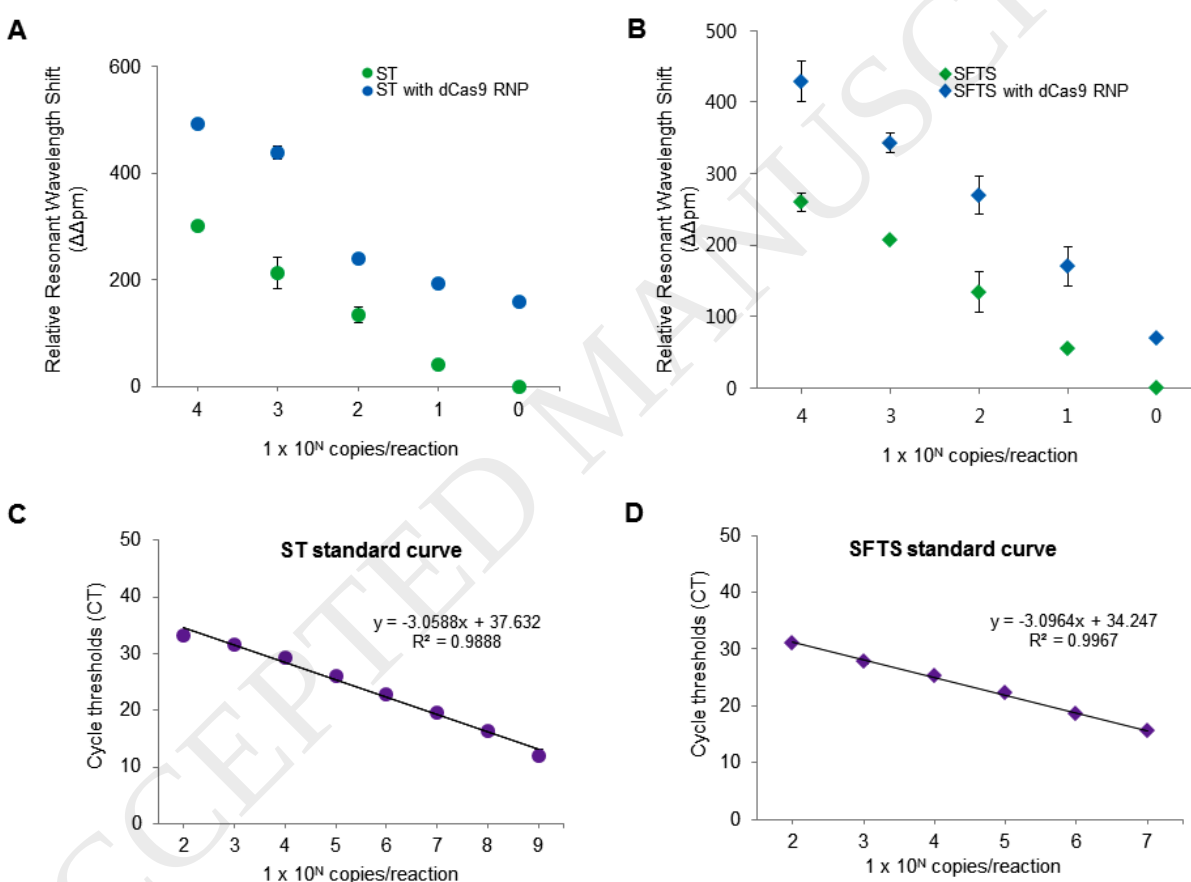


Figure 5. Clinical utility of CRISPR/dCas9-mediated biosensor. (A) Schematic of CRISPR/dCas9-mediated biosensor for ST diagnosis in ST and SFTS samples. (B) Highly sensitive and specific detection of ST-DNA with CRISPR/dCas9-mediated biosensor (blue circle) in six clinical samples compared to that of SMR biosensor alone (green circle). P1-3, human sera from ST patients as positives; N1-3, human sera from severe fever with thrombocytopenia syndrome (SFTS) patients as negatives. (C) Schematic of CRISPR/dCas9-mediated biosensor for SFTS diagnosis in ST and SFTS samples. (D) Highly sensitive and specific detection of SFTS-RNA with CRISPR/dCas9-mediated biosensor (blue triangle) in six clinical samples compared to that of biosensor alone (green triangle). P1-3, human sera from SFTS patients as positives; N1-3, human sera from ST patients as negatives. Error bars indicate standard deviation from the mean, based on at least three independent experiments.

