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VIEW POINTS

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Current progress in CRISPR-based diagnostic platforms

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Abstract

The CRISPR-Cas system is a key technology for genome editing and regulation in a wide range of organisms and cell types. Recently, CRISPR-Cas–based diagnostic platform has shown idealistic properties for pathogen detection. Integrating the CRISPR-Cas platform along with lateral flow system allows rapid, sensitive, specific, cheap, and reliable diagnostic. It has the potential to be in frontline for not only pathogen detection during the epidemic outbreak, but also cancer, and genetic diseases.

KEYWORDS

attomolar, Cas13a, clustered regularly interspaced short palindromic repeats-based diagnostic, epidemic diseases, guide-RNA, multiplex

1 | INTRODUCTION

A constant need arises to develop an ideal diagnostic platform for a rapid, sensitive, specific, and cost-effective detection of a wide range of pathogens and diseases. It should be user-friendly and able to give instrument-free readouts for early detection of pathogens.[1] This desperate need was experienced during the Ebola 2014-2016 outbreaks.[2] The second annual review released by World Health Organization (WHO) in February 2018 emphasized on the urgent need for the development of better and improved diagnostic tools for prioritizing the epidemic diseases. The detection of Ebola, Zika, Marburg virus, Middle East respiratory syndrome coronavirus (MERS-CoV) and many more have been prioritized.[3] Recently, CRISPR-based diagnostic (CRISPR-Dx) tool for nucleic acid sequence recognition has productively delineated its potential to fulfill the requirements of an ideal diagnostic tool for pathogens, single-nucleotide polymorphisms (SNPs), and cancer detection.[4]

CRISPR-Cas system found in bacterial system is amongst the many protective strategies that the cell utilizes for the eradication of extracellular genome or to resist the phage infection. Being an adaptive immune response, CRISPR-Cas systems have the ability to memories, adapt and lyse the nucleic acid sequences that have been previously or newly encountered.[5,6] The endonuclease proteins (eg, spCas9, Cpf1) found during the interference event of CRISPR-Cas that are programmed using the mature cr-RNA (commonly known as guide RNA) to target the desired sequence present in cell.[7,8] This has proved to be advantageous for genome editing since it is convenient, rapid, simple, cost-effective, and offers lower chances of off-targeting as compared with alternative tools including zinc finger nucleases and transcription activator-like effector nucleases.[9]

2 | SHERLOCK'S VENTURE INTO DIAGNOSTICS

Cas13a (C2c2) is a programmable RNAse found in CRISPR type-VI class 2 systems. In the presence of a specific guide RNA, it recognizes its target RNA and

Abbreviations: aM, Attomolar; CRISPR, Clustered regularly interspaced short palindromic repeats; EGFR, Epidermal growth factor receptor; gRNA, guide RNA; HUDSON, Heating Un-extracted Diagnostic Samples to Obligate Nuclease; MERS-CoV, Middle East respiratory syndrome coronavirus; RPA, Recombinase polymerase amplification; RT-RPA, Reverse transcriptase-recombinase polymerase amplification; SHERLOCK, Specific High-Sensitivity Enzymatic Reporter UnLOCKing; SNPs, Single-nucleotide polymorphisms; zM, Zetomolar.

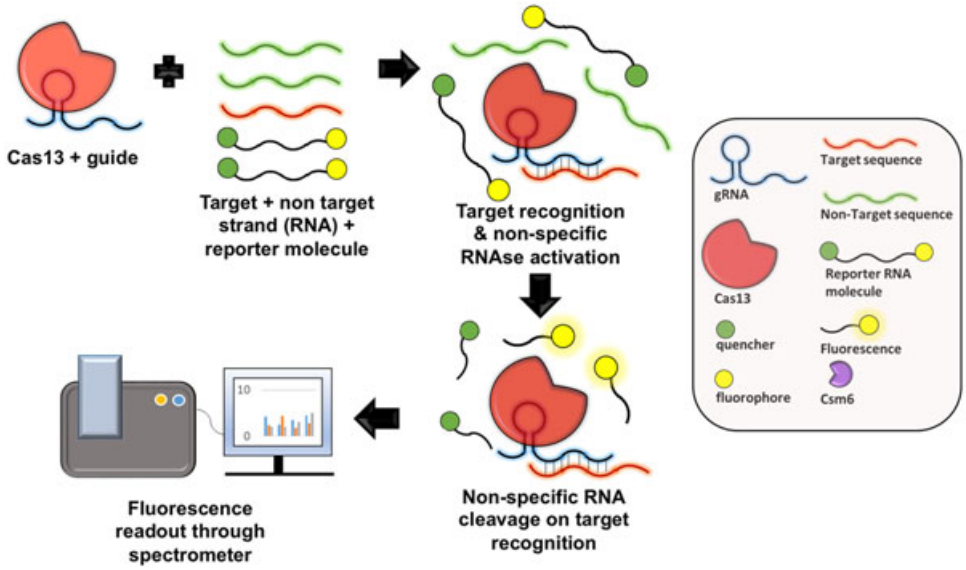

FIGURE 1 Schematic representation of CRISPR-based diagnostic tool, in the presence of the appropriate gRNA, Cas13 recognizes the target RNA sequence that is complementary. On-target recognition, Cas13 activates its nonspecific RNAse activity that can cleave any nearby RNA sequences. In the presence of the fluorescence-quencher RNA-based reporter molecule, the catalytically activated Cas13 could cleave the reporter molecule, resultant emission of fluorescence that can be detected by spectroscopy.[10] gRNA, guide RNA

executes its nonspecific RNA degradation activity.[10] East-Seletsky et al[10] have taken the advantage and demonstrated the use of Cas13 for the detection of 0.01 nM of λRNA with high specificity, using the signals generated from fluorophore-quencher based reporter RNA molecule (Figure 1). In a recent issue of *Science*, two articles reported the development of a CRISPR-based high-sensitive diagnostic platform.[11,12] Gootenberg et al[4] have developed a cost-effective (0.61$ per test) platform for in vitro detection of nucleic acid sequences, which could detect up to attomolar (aM) concentrations without compromising the specificity. This platform has been termed as Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK). In SHERLOCK, the target sequence is amplified with recombinase polymerase amplification for DNA detection (RPA) or reverse transcriptase-recombinase polymerase amplification for RNA detection (RT-RPA), an isothermal, nucleic acid amplification technique that eliminates the use of thermal cycler. The resultant amplified DNA sequence is then subjected to in vitro T7 transcription, and produced RNA molecule is detected by Cas13-guided reporter assay. The fluorescence signal is monitored using the spectroscopy[4] (Figure 2). SHERLOCK has the potential to specifically detect lentiviral particles at a concentration of 2 aM, which harbors Zika and Dengue virus's nucleic acid fragments. It has also the ability to detect Zika virus directly from clinical isolates from serum, urine, and saliva, having a titer as sensitive as 3.2 aM, and through RNA extraction from the same material, a more sensitive detection up to 2.1 aM has been achieved. SHERLOCK is a powerful tool that allows us to distinguish between *Escherichia coli* and *Pseudomonas aeruginosa*; as well as can efficiently figure-out the clinical isolates of *Klebsiella pneumoniae* carrying antibiotic resistance genes.[4] Furthermore, by addition of single mismatch to its synthetic guide-RNA molecule, SHERLOCK can potentially detect SNPs with high specificity, and is also able to detect a clinically relevant range of low-frequency cancer mutations.[4]

3 | SHER"LOCK" IS THE "KEY" FOR MULTIPLEX DETECTION

SHERLOCK's second version (SHERLOCKv2) has been developed and optimized.[11] In this study, Gootenberg et al[11] have unveiled the potential of SHERLOCK for the multiplexed detection of targeted nucleic acid sequences in a single reaction chamber at aM range. The multiplexed detection is achieved by optimizing the cleavage preferences of different Cas13 orthologues towards different sequences of the reporter molecule. In a system containing a pool of different Cas13 orthologues and its guide RNA, a particular target sequence could be recognized by a specific orthologue of Cas13. Cas13 orthologue would prefer to cleave a particular RNA reporter molecule, generating a specific range of fluorescence signals.[11] For improved multiplexed detection, Cas12a has been used along with Cas13 RNAse.[11]

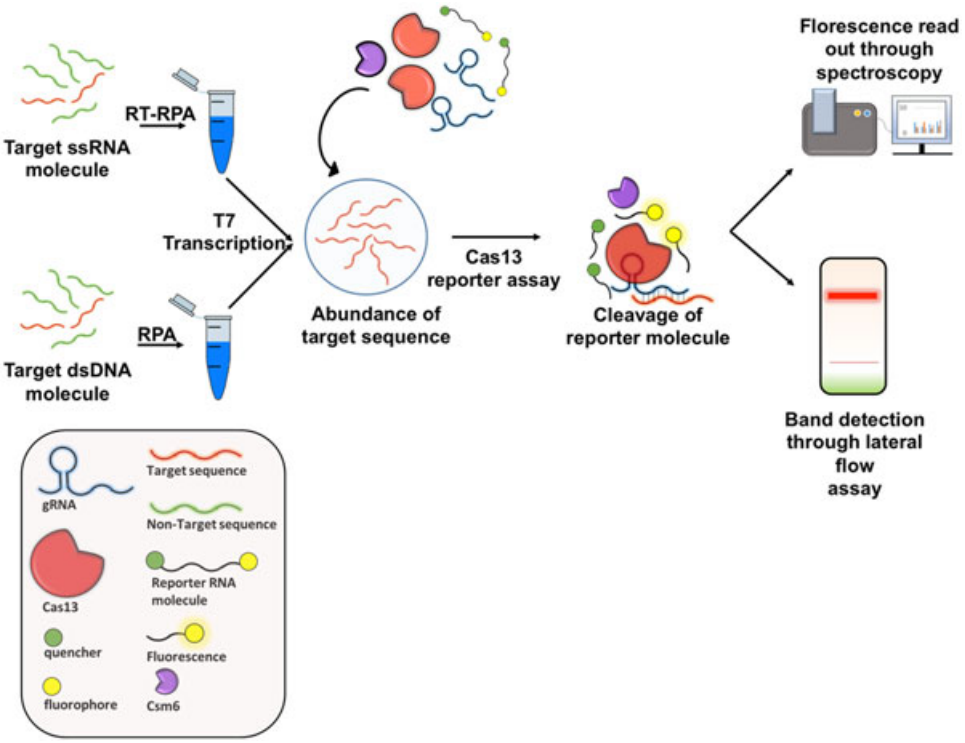

FIGURE 2 Schematic representation of SHERLOCK; In the presence of appropriate RPA primers, the target sequence that needs to be identified is subjected to amplification through the isothermal RPA or RT-RPA. The amplified DNA sequence obtained that is subjected to in vitro T7-transcription. The produced RNA molecule can be recognized via fluorescence signals that are generated by the reporter molecule upon target recognition by the Cas13 RNAse.[4] The cleaved reporter molecule can also be detected in the form of bands through a lateral flow assay. The inclusion of Csm6 could improve the amplification of the output signal[11]

Multiplexing highly specific detection of Zika and Dengue virus was achieved with synthetic double-stranded DNA and single-stranded RNA (ssRNA) molecules.[11] It has also explored for detection of *P. aeruginosa* and *Staphylococcus aureus*. The detection of target ssRNA molecules to the zetomolar (8 zM) range through scale-up of preamplification steps through RPA was also demonstrated.[11]

4 | SIGNAL AMPLIFICATION AND PAPER-BASED READOUT

Amplified fluorescence signal can be obtained after the target recognition using Csm6 nuclease of CRISPR type III. Csm6 nuclease gets activated in the presence of linear adenine homopolymer with 2′3′-cyclic phosphate.[11] It is the nonspecific cleavage via Cas13 RNAse that leads to the formation of 2′3′-cyclic phosphate.[11] Csm6 being nonspecific RNAse upon activation,[13] could help to amply the fluorescence signal via homoadenine 2′3′-cyclic phosphate that was generated by Cas13 on target recognition. Combining this with SHERLOCK has facilitated an easy-to-use lateral flow assay that have shown an instrument free readout in the presence or absence of target sequences at concentrations as low as 2 aM within less than 90 minutes. The details of lateral flow assay have been meticulously described by Bahadır and Sezginturk.[14] Figures 3 and 4 represent an explanatory version of lateral flow assay which has been used by Gootenberg et al.[11] A combination of SHERLOCK with a paper strip lateral flow assay is able to detect non-small cell lung cancer with mutations in the epidermal growth factor receptor. As an added advantage, Csm6 in combination with SHERLOCK-based lateral flow assay gave a strong signal readout.[11]

5 | WHEN HUDSON MEETS SHERLOCK

By combining the Heating Un-extracted Diagnostic Samples to Obligate Nuclease (HUDSON) with SHERLOCK, it is possible to detect viral nucleic acid without the extraction steps.[12] HUDSON is a protocol that uses heat treatment to inactivate the nuclease and lyse the viral particle from the body fluids. It could open a new

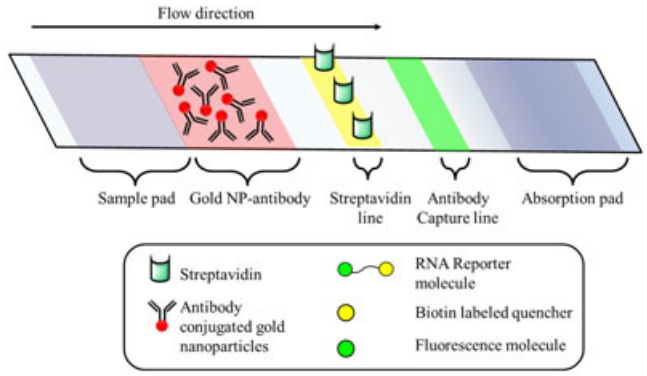

FIGURE 3 Schematic representation of lateral flow test strip. Once the sample is treated with the SHERLOCK reaction mixture, the solution obtained is put on the sample pad of the lateral flow strip. The flow direction would be in the following order; sample pad, antibody conjugated gold nanoparticles (gold NP-antibody), streptavidin line, antibody capture line, and absorption pad. Antibody conjugated gold nanoparticle could bind against florescence molecule. Streptavidin forms a complex with biotin labeled reporter molecule. Antibody capture line traps conjugated antibody complex at a particular position.[11] Absorption pad helps to stop the back flow of the sample[14]

avenue for paper and fluorescence-based readouts directly from the body fluids, hosting the Zika virus at aM concentration limits and that too within less than 2 hours.[12] Additionally, this system could facilitate the detection of Dengue virus directly from the patient's blood or saliva.[12] SHERLOCK alone is capable of discriminating the different types of Dengue virus serotypes and closely related flaviviral species with less than 3.2% and 0.22% off-target fluorescence, respectively. However, Myhrvold et al[12] presented an experimental design, which is rapid and could detect the recently found SNP S139N in the Zika virus from patient samples of a 2015-2016 pandemic with paper-based readouts.[12]

6 | SHERLOCK AN ATTENTION SEEKING TOOL

Currently, the available nucleic acid–based diagnostic platform is sensitive but involves expensive machinery and extensive sample processing. While the antigen-based diagnostic platforms are rapid with minimal demands,[12] it comes with a set of challenges such as poor sample quality and difficulty for analyzing complex data. However, antigen-based diagnostic platforms have often found to be less sensitive and selective.[12] Identification of the causative agent of the infection either bacteria or virus is important, and if it is bacteria, then it is necessary to know its (multi)drug-resistance profile.[15] SHERLOCK is a powerful, ultrasensitive and cost-effective (0.61$ per test) platform without the needs of instrument that is able to discriminate between closely related genetically and antigenically similar flaviviruses as well as serotypes of Dengue virus.[12] It is also able to sort

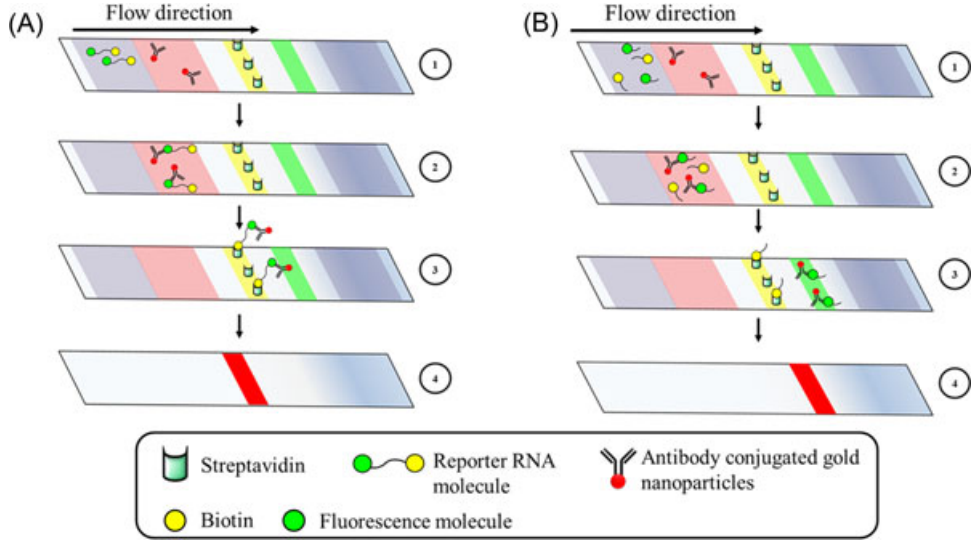

FIGURE 4 Mechanism, analysis, and interpretation of lateral flow test in the presence and absence of target sequences.[11] A, In the absence of target sequence in the sample, the reporter molecule would remain intact (1). The intact reporter molecule would form complex with antibody-conjugated nanoparticles as well as with streptavidin (2,3). The formed complex cannot move further with flow as streptavidin attached to substratum restricts the flow of the complex (3). Therefore, a red colored band would appear at the streptavidin line due to nanoeffects (4). B, In the presence of the target sequence in the sample, the reporter molecule would no longer remain intact (1). The fluorescent molecules of lysed reporter could form complex with antibody-conjugated nanoparticles (2) while biotin would form complex with streptavidin. The antibody-conjugated nanoparticle-fluorescent molecule complex would move further and get trapped in the antibody-capture line (3). Therefore, a red colored band would appear at the antibody-capture line due to nanoeffects (4)[11]

different bacteria and differentiate their antibiotic-resistant strains with exceptional sensitivity and selectivity.[4] SHERLOCK can also detect SNPs and cancer mutations and have shown its adaptability for rapid and reliable development of new tests for recently detected Zika virus.[4,12] So far, SHERLOCK with HUDSON has shown impressive and versatile qualities, it could definitely act as an attention-seeking tool for reliable and cheap diagnostics for infections and genetic disorders.

Overall, SHERLOCK can be further extended to have paper-based single or multiplexed readouts for detection of not just pathogens and cancers, but also genetic disorders, and many other debilitating diseases or infections. In the near future, SHERLOCK may play a vital role for early detection and diagnosis of infection during any epidemic outbreak worldwide.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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