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Detection of *Klebsiella pneumoniae* DNA by PCR-based CRISPR-lbCas12a system

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#The authors wish it to be known that, in their opinion, the first 2 authors should be regarded as joint First Authors.

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Abstract

Klebsiella pneumonia (*K. pneumoniae*) is a Gram-negative bacterium that causes nosocomial infections in the lung, bloodstream, and urinary tract. Therefore, detecting *K. pneumoniae* in early time is important in preventing severe infections. However, clinical detection of *K. pneumoniae* requires a long time of agar plate culture. Nucleic acid detection like qPCR is precise but requires expensive equipment. Recent research reveals that collateral cleavage activity of CRISPR-LbCas12a has been applied in nucleic acid detection. In this study, PCR combined with CRISPR-LbCas12a targeting the *K. pneumoniae* system was established. This system showed excellent detection specificity and sensitivity in both bench work and clinical samples. Due to its advantages, its application can meet different detection requirements in health centers where qPCR is not accessible.

Keywords: CRISPR-Cas, Nucleic acid detection, *Klebsiella pneumonia*

Abbreviations: Polymerase Chain Reaction (PCR), *Klebsiella pneumonia* (*K. pneumoniae)*, Urinary Tract Infection (UTI), bloodstream infections (BSI)

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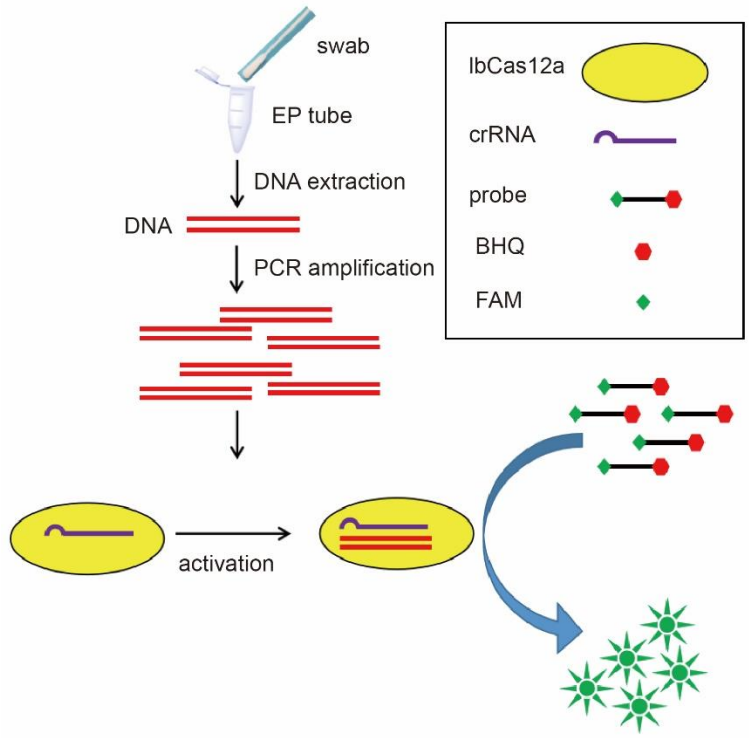
Graphical Abstract



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Introduction

Klebsiella pneumonia (*K. pneumoniae*) is a family of Gram-negative bacteria that causes nosocomial infections in the bloodstream, wound, urinary tract [1]. Hypervirulent *K. pneumoniae* strains such as K1, K2, and K5, have emerged worldwide and caused severe infections, including liver abscess, pneumonia with a mortality rate as high as 20%~30% [2]. Additionally, nearly 44.5% *K. pneumoniae* strains are multi-drug resistant. [3] Due to the risks of mortality and the difficulty in treating, detecting *K. pneumoniae* in patients and ventilators is vital in curing and preventing severe infections.

K. pneumoniae colonies derived from clinical samples under 24-48 hours of plate culture present features that could be diagnosed by well-trained personnel [4]. Despite the low cost and the simplicity of operation, plate culture is time-consuming and sometimes produces false-negative results for the colonies that may not be typical [5,6]. In contrast, nucleic acid detection methods that detect *K. pneumoniae*-specific DNA fragments are more advantageous. Quantitative real-time PCR or qPCR is the most used nucleic acid detection method. It can detect targeted pathogens and even identify subtypes, including K1 and K2 strains that are multidrug-resistant and hypervirulent [7]. However, qPCR requires expensive equipment, which restricts its application in the healthcare center.

The CRISPR-Cas systems have been discovered to cleavage target DNA or RNA under the guidance of crRNA in a base-pairing manner[8]. Recent research shows that Cas13 and Cas12 exhibit collateral cleavage activity that could degrade probes if crRNA perfectly base-pairs targeted RNAs or DNAs [9,10]. Combined with DNA amplification and detection methods, Cas12 and Cas13

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were applied to detect nucleic acid [11]. The detection workflow only requires 37℃ incubation and does not rely on complicated equipment. To date, to combat COVID-19, lots of nucleic acid detection workflows based on Cas13 and Cas12 have been developed [12,13].

Compared to Cas13 system, Cas12 showed advantages in following aspects:

1, Cas12 requires DNA as the target, but Cas13 requires RNA. Because the technologies to directly amplify RNA are not mature enough, the Cas13 detection system needs in vitro transcription process.

2, The detecting probes used in Cas12 are DNA, while Cas13's probes are RNAs that are easily degraded to produce the false-positive effect.

3, The crRNA of Cas13 is as large as 64nt, while that of Cas12 could be as short as 35nt.

The RNA length as short as 35nt could be easily synthesized in the industry[11]. In this study, instead of using crRNA derived from *in vitro.* transcription, which requires complicated processes in enzyme digestion and RNA purification but produces an uncertain amount of RNA, we used synthetic 35nt RNA as crRNA for its stability in quality control and convenience in quantitation. In addition, the slight progression makes the system more stable and more appliable to prepare premix of reagent for clinical use.

Despite these advantages, the CRISPR-Cas12 detection method targeting *K. pneumoniae* has not been reported. We established a sensitive nucleic acid detection method based on CRISPR-Cas12 and PCR to detect *K. pneumoniae*. It produces authentic results in less than 4 hours and requires less expensive equipment. It may help community healthcare centers to accomplish nucleic acid detection.

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Materials and Methods

Nucleic Acid preparation

crRNAs were designed to target 16sRNA, YP_005224572.1 and *IF-2* gene according to protocol [9]. RNA nucleotides were chemically synthesized without 5'-phospohrylation by (Transheep, China). crRNA consists of 19nt of common sequences and 17nt for recognizing target [14]. The RNA sequences are as follows:

crRNA-1：5'-AAU UUC UAC UGU UGU AGA UAC AUC CGA CUU GAC AGA-3'

crRNA-2：5'-AAU UUC UAC UGU UGU AGA UGA AUG CAG UUC CCA GGU-3'

crRNA-3：5'-AAU UUC UAC UGU UGU AGA UUC CAC ACU UCC GGA UAG-3'

crRNA-4：5'-AAU UUC UAC UGU UGU AGA UCC GCU GGU ACC GGC AUC-3'

crRNA-5：5'-AAU UUC UAC UGU UGU AGA UCG UGC UGG AUA GCU UCG-3'

FAM-BHQ labeled DNA probe was synthesized by Sangon Corp (China).

DNA targets for crRNA testing were synthesized by Sangon Corp (China). The sequences are as follows:

Target-1: 5'-GGT CTG TCA AGT CGG ATG TGA AAT C-3'

Target-2: 5'-TCA ACC TGG GAA CTG CAT TCG AAA CTG-3'

Target-3: 5'-GGG CTA TCC GGA AGT GTG GAT AAA CG-3'

Target-4: 5'-GCA GAT GCC GGT ACC AGC GGG AAA AC-3'

Target-5: 5'-TAT CGA AGC TAT CCA GCA CGC TAA AGC-3'

Probe sequence and modification is 5'-FAM-TTTTTT-BHQ-3'.

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Standard DNA carrying *K. pneumoniae* 16sRNA, YP_005224572.1 and IF-2 gene fragments were synthesized and cloned to pUC-19 vector. Full sequences are as follows:

5'-

AGGAAGGCCGGTGAGGTTAATAACCTCTCGATTGACGTTACCCGCAGAAGAAGCACCGG

CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT

GGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAAC

CTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAG

GTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCT

GGACAAAGACTGACGCTCAGGTGCGAAA (16S RNA, 785-1105)

CGAGGTTTACGTCTCAACCGGCTGGGGATCCACCACGAGCGGCTGCCGCCCGGGCGG

CGCACCTCTTATCCACACGCGGAGAGCGATGAGGAAGAGTTCATCTACGTGCTGGAGGG

CTATCCGGAAGTGTGGATAAACGGCTATCTCTGGAAGCTGGAGCCCGGCGACAGCGTGG

GTTTTCCCGCTGGTACCGGCATCTGCCACACCTTTCTCAATAACACCGAGCAGGAAGTTC

GTCTG (RefSeq: YP_005224572.1, 145-384)

CTGGTGGTGGGCGAGGCCAACAAGAAGTACAACCGCATTTATTACCGGCATGATCACCTT

CCTGGATACCCCGGGCCACGCCGCGTTTACCTCCATGCGTGCTCGTGGCGCGCAGGCG

ACGGATATCGTGGTTCTGGTGGTGGCGGCAGACGACGGCGTGATGCCGCAGACTATCGA

AGCTATCCAGCACGCTAAAGCGGCGCAGGTACCGGTGGTAGTGGCGGTGAACAAGATCG

ATAAGCCAGAAGCCGATCCGGATCGCGTGAAGAACGAACTGTCCCAGTACGGCATCCTG

CCGGAAGAGTGGGGCGGCGAGAGCCAGTTCGTCCACGTTTCCGCGAAAGCGGGTACC

GGCATCGACGAC (IF-2 GenBank: CP052761.1; LOCUS: CP052761; gene ID: inf B 1,329-1,647)-3'

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DNA extraction and quantification

Clinical samples were swabs of sputa. According to the manufacturer's protocol, swabs were dipped in cell lysate and processed genomic DNA extraction using the DNA extraction Kit (Tianlong science & technology). Extracted DNA samples were quantified by Nanodrop^{C} and preserved in -80℃ before use.

PCR and qPCR

PCR system was carried in 20 μl reaction system in the 0.2ml PCR tube. Each reaction contains 10 μl of PrimeSTAR (TAKARA) PCR premix, 1 μl of forward primer (10nM) and 1 μl of reverse primer (10nM), 10ng of sample DNA and ddH_2O to supplement the volume to 20 μl. The PCR reactions were processed for 35 cycles on an Eppendorf thermocycler with denaturation at 94°C for 15 seconds, annealing at 58°C for 15 seconds, and extension at 72°C for 20 seconds. DNA electrophoresis was processed in 1% agarose gel in TAE buffer. Primer sequences are KP-F: 5'-TCT CAA TAA CAC CGA GCA-3', KP-R: 5'-TGC TCG GTG TTA TTG AGA-3'.

qPCR reactions were processed using Hieff UNICON Universal Blue qPCR SYBR Green Master Mix (Yeasen) on QuantStudio Dx (ABI). Programed started with a 95℃ for 2min followed by 40 cycles of denaturation 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 15 seconds qPCR. Primers sequences are qKP-F: 5'-TCT CAA TAA CAC CGA GCA-3', qKP-R: 5'-TGC TCG GTG TTA TTG AGA-3'. Each reaction was repeated 3 biologically independent experiments.

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PCR-LbCas12a detection

The LbCas12a detection was carried in 20 μl system. The system contains 2ul Buffer 3 (NEB), 50nM lbCas12a protein, 60nM of crRNA and 30nM labeled probe, and 100ng of purified PCR product. Samples were mixed and then incubated at 37°C, and signals were obtained from QuantStudio Dx (ABI) every minute for 60min. Each reaction was repeated 3 biologically independent experiments.

Study approval

The Luohu Ethnics Comittee approved the project (LLBGS [2021] 020).

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Results

Establishment of PCR-lbCas12

To find the suitable target for nucleic acid detection, we use blast to screen the most suitable gene as a target for nucleic acid detection. Results showed that IF-2, 16S RNA, and YP_005224572.1 are both specific and conservative genome fragments. Therefore, we use these three genes as candidates. By considering the TTTV PAM sequences, we designed five 36nt long crRNAs which share 19nt standards nucleotides and have 17nt unique sequences to base-pair *K. pneumoniae* targets. To quickly test the detecting efficiency of the crRNAs, synthetic single-stranded DNA targets were added to the lbCas12a system. Compared to the negative control, all crRNAs showed a significantly high level of FAM signals (**Fig. 1A**). Among the 5 crRNA sequences, crRNA-2 and crRNA-4 showed the most potent signals (**Fig. 1B**).

On the other hand, the primers to amplify DNA fragments for crRNA-2 and crRNA-4 are tested. The amplification efficiency of IF-2 primers is far more efficient than that of YP_005224572.1. As a result, crRNA-4 and the primers targeting the IF-2 gene are used to establish this system to detect the *K. pneumoniae* strain (**Fig. 1C**).

LbCas12A showed superior sensitivity and specificity in *K. pneumoniae* detection

First, to explore the minimum amount of DNA sample required for nucleic acid detection, serial diluted standard DNA samples were to test PCR, qPCR, and PCR-lbCas12a techniques. Basic PCR and qPCR exhibited positive signals when target DNA was as few as ten copies (**Fig. 2A, B, C**),

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while the LbCas12a system can exhibit signal in 40 min when the copy number is as few as one single copy (**Fig. 2A**). Next, to confirm if the detection system's specificity targets only *K. pneumoniae*, we applied lbCas12a detection in 4 commonly seen bacteria strains such as Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Shigella Castellani, and Salmonella Typhimurium. As a result, *K. pneumoniae* exhibited as strong as 7-folds of fluorescent signals in this system, while the rest bacteria strains did not (**Fig. 2D**). Thus, the results showed that the PCR-LbCas12a detection system is sensitive and specific.

PCR-LbCas12 detecting Clinical samples

We collected 21 positive and seven negative samples diagnosed and confirmed by plate culture examination from the laboratory department to apply this system to clinical use. 21 positive *K. pneumoniae* were also positive in the PCR-LbCas12a detection system **(Fig. 3A, B, C)**. However, one out of the seven negative was tested positive. Basic PCR was processed to confirm this case, and its product was sent for sanger sequencing. Sequencing fully matched the *K. pneumoniae* conservative IF-2 sequence, which means that the sample was indeed positive. A similar Cas12a detecting system named DETECTR used RPA instead of PCR to amplify target DNA to save the thermo-cycling process, which is more appliable to clinical use (Kostyusheva et al. 2021). We also tried this system to detect the same samples. However, compared to our system, their signals were far less significant than satisfactory (**Fig. S1A, B**).

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Discussion

Recent studies revealed that about 73.1% of *K. pneumoniae* are resistant to at least one antibiotic [15]. The rate of colistin resistant strains is still increasing [16]. The infection of *K. pneumoniae* could cause untreatable diseases that could threaten patients' lives. Therefore, the detection of *K. pneumoniae* from patients and ventilators is vital to prevent infections.

The most used detection method is agar well diffusion assay culture [17]. However, the sensitivity and accuracy are only about 56% and 65% [18]. As a comparison, the detection of nucleic acid is more advantageous in stability and accuracy. The golden standard of nucleic acid detection is qPCR. qPCR specifically and accurately identifies *K. pneumoniae* from bacteria like *E. coli* and *S. aureus* [19] and its antibiotic-resistant genes, such as ESBL-encoding and Fosfomycin-encoding genes [20]. However, qPCR needs expensive equipment and skilled workers[21]. Recently, CRISPR-Cas12 mediated trans-collateral activity was widely applied to nucleic acid detection [11,22,23]. The CRISPR-Cas12 detection system is accurate and specific, and it can also combine various readout and amplification technologies [24-26].

In this study, we first established the LbCas12a system to detect *K. pneumoniae* nucleic acid. Before the Cas12 reaction, we used PCR to harvest enough amount of target DNA as substrate. Although the RPA technology can also accomplish this process, however, according to our experiment, PCR is more stable and cheaper (**Fig. S1A, B**). As crRNA could be as short as 36nt, we used chemically synthesized crRNA instead of in vitro. transcribed RNA. After selection and fine-tuning of primers and crRNAs, the PCR-LbCas12a detection system can detect as low as only one copy of *K. pneumoniae*. We also tried the system in samples to explore its possibility in clinical use. Samples were clinically diagnosed by Agar Well Diffusion Assay. After PCR-LbCas12a detection,

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this system successfully checked out all positive samples. To our surprise, one of the negative samples turned out to be a positive case. Additionally, considering the time cost in Agar Well Diffusion Assay culture, which requires 24-48 hours, the PCR-lbCas12a system needs no more than 4 hours. Therefore, this detecting system is a better option in most cases.

The experiment supports that the PCR-LbCas12a system targeting *K. pneumoniae* is a sensitive, accurate and easy-to-use technology to detect *K. pneumoniae* nucleic acid.

FUNDING

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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Figures

Figure.1

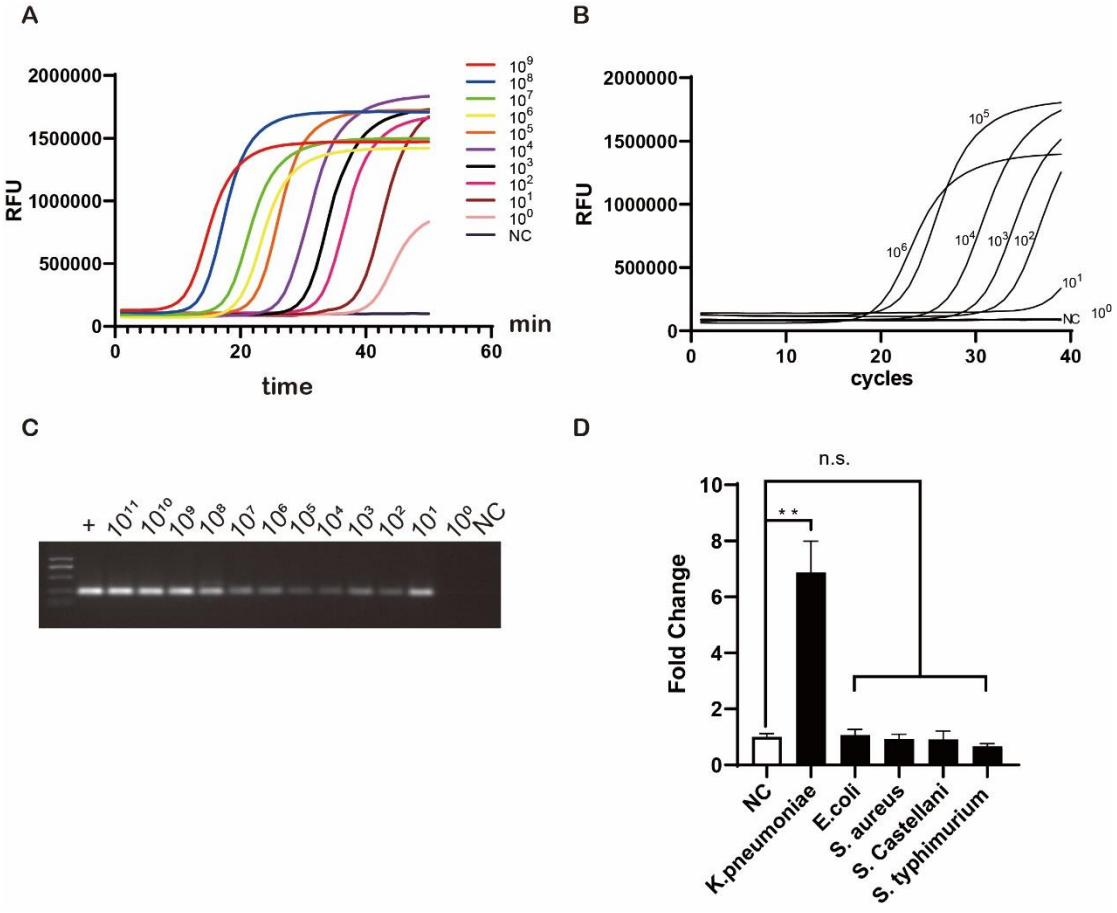

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Figure 2

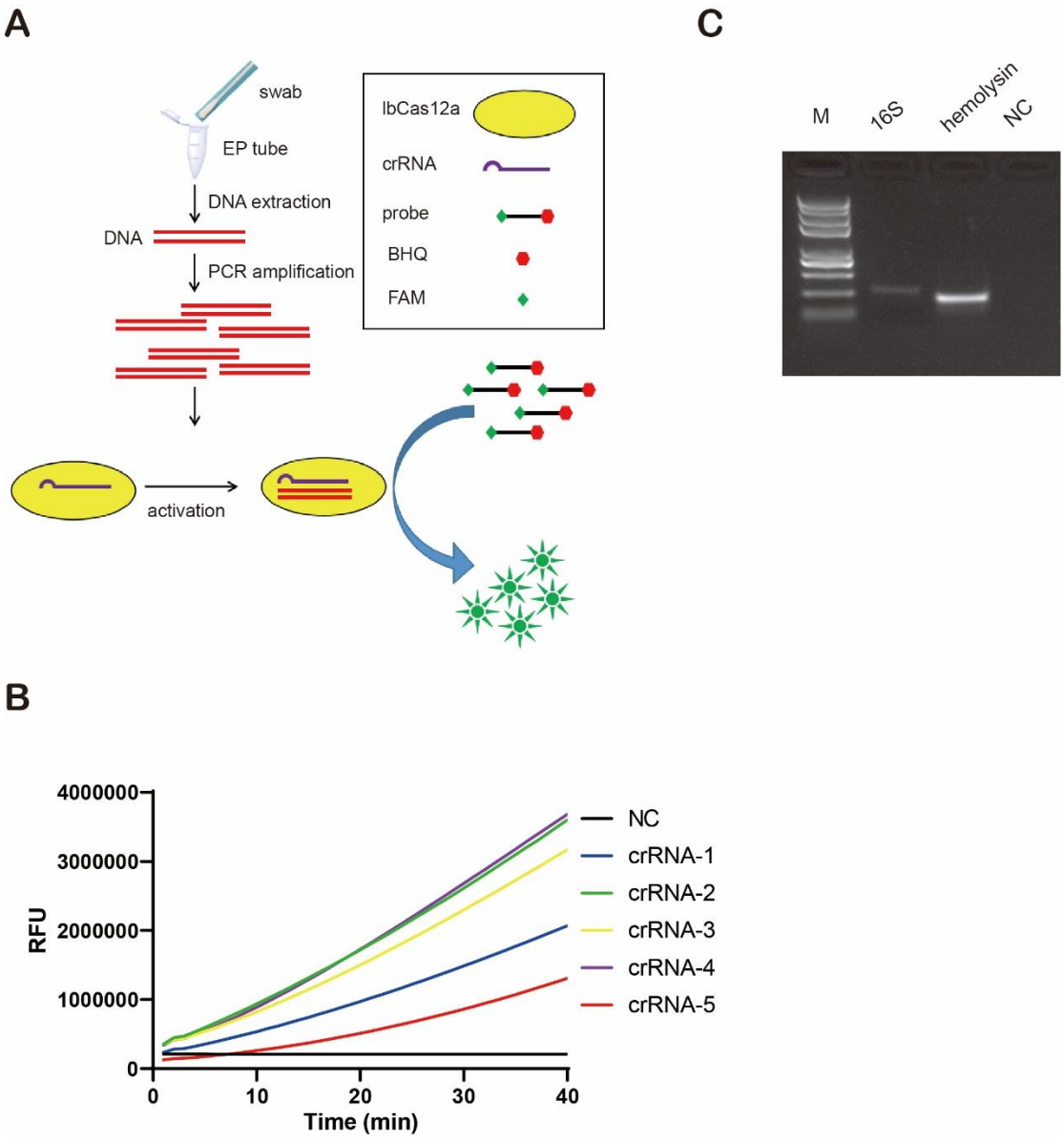

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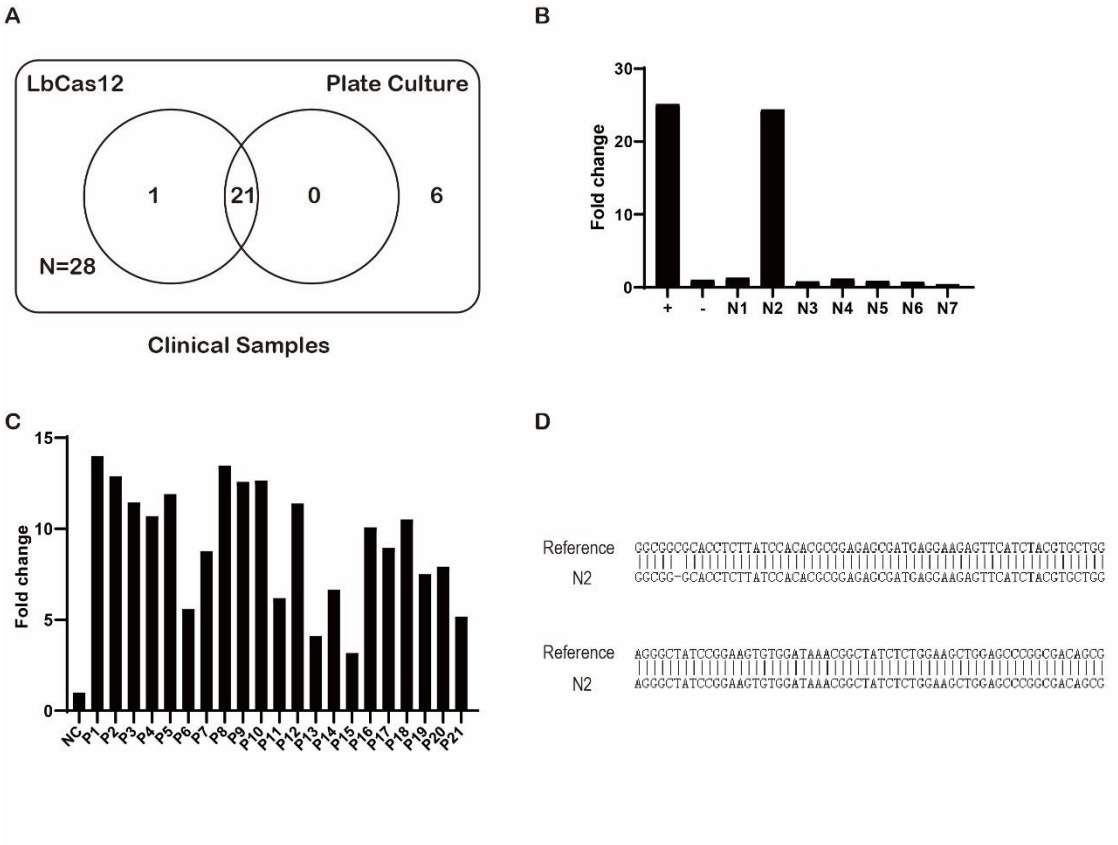
Figure3



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FIGURES LEGENDS

Figure 1. A. Graphical illustration of LbCas12a detecting *K. pneumoniae* nucleic acid. **B**. Tests of 5 crRNA efficiency in LbCas12a detecting system. **C**. Electrophoresis of PCR product of *K. pneumoniae* 16S and YP_005224572.1 gene.

Figure 2. A. The RFU signal generated by the PCR-LbCas12 system detecting the serially diluted standard *K. pneumoniae* DNA samples. **B**. The qPCR results of serial diluted standard DNA samples. **C**. 1% agarose gel electrophoresis of the PCR product from serial diluted standard DNA samples. **D**. Different bacterium was detected by PCR-Cas12 system. Only *K. pneumoniae* was successfully detected. Data are mean ± s. d. of n=3 biological independent experiments.

Figure 3. A. Venn diagrams comparing PCR-LbCas12 system and plate culture in clinically diagnosed samples. **B.** Seven clinical negative samples were tested in the PCR-LbCas12 system, but N2 is positive. **C.** 21 positive samples were all tested positive in the PCR-LbCas12 system. **D.** Sanger sequencing result of N2 was highly consistent with standard *K. pneumoniae* sequences.

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Supplementary information

Figure S1. **A.** seven negative clinical samples were tested in the RPA-LbCas12 system. **B.** 21 positive samples were all tested positive in the RPA-LbCas12 system.