

# Rapid Identification of Pathogens in Severe Pneumonia by Species-specific Bacterial Detector (SSBD)

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## Abstract

Fast and accurate detection of pathogenic bacterial infection in patients with severe pneumonia is significant to its treatment. The traditional culture method currently used by most medical institutions relies on a time-consuming culture process (over two days) that is unable to meet clinical needs. Rapid, accurate, and convenient species-specific bacterial detector (SSBD) has been developed to provide timely information on pathogenic bacteria. The SSBD was designed based on the fact that Cas12a indiscriminately cleaves any DNA following the binding of the crRNA-Cas12a complex to the target DNA molecule. SSBD involves two processes, starting with PCR of the target DNA using primers specific for the pathogen, followed by detection of the existence of pathogen target DNA in the PCR product using the corresponding crRNA and Cas12a protein. Compared to the culture test, the SSBD can obtain accurate pathogenic information in only a few hours, dramatically shortening the detection time and allowing more patients to benefit from timely clinical treatment.

**Keywords:** Severe pneumonia, Pathogen detection, Cas12a, Species-specific bacterial detector, Bacterial infections

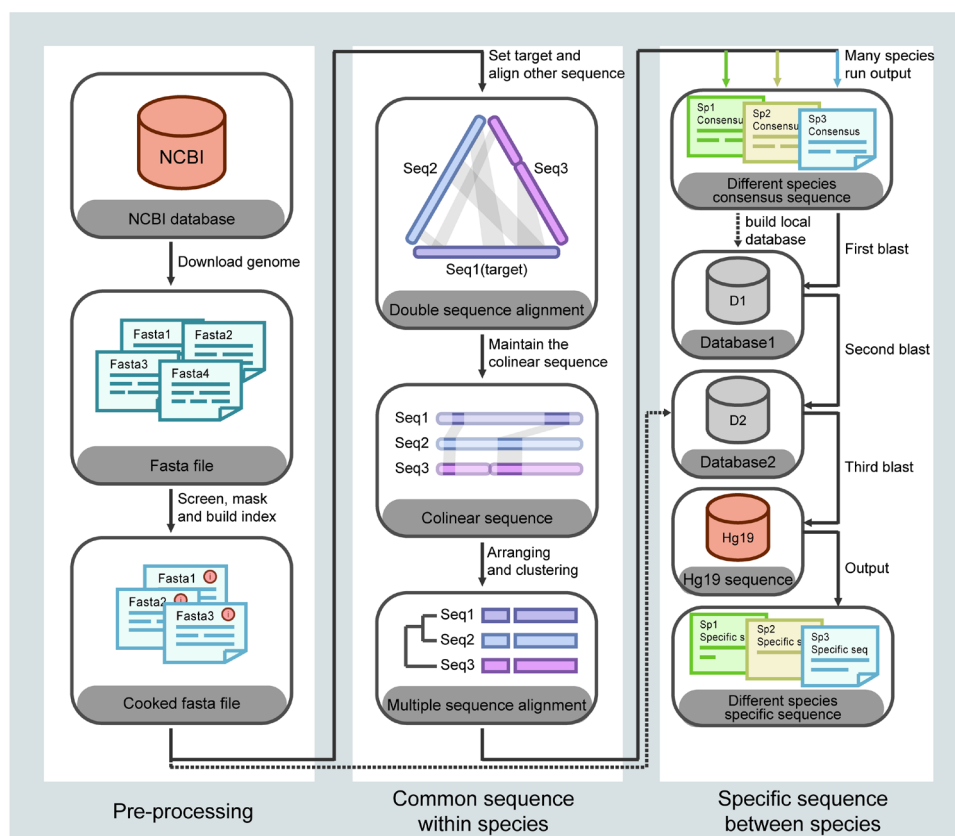
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## Background

Severe pneumonia is a progressive inflammation of the lungs caused by pathogenic bacterial infection, leading to systemic infection if not treated promptly. The types of pathogens that cause severe pneumonia are complex and diverse, and antibacterial drugs used for different pathogen infections have great differences (De Pascale et al., 2012; Hansen et al., 2016). Therefore, the detection of early pathogenesis is not only the basis for precise diagnosis, but also necessary for accurate treatment and rational drug selection. However, the traditional culture method used by most medical institutions often relies on the process of pathogen culture, which takes several days (Lazcka et al., 2007). Inability to provide timely information about pathogen infection affects the clinical need to treat critically infected patients. Therefore, it is critical to accurately and rapidly identify the pathogens that cause severe pneumonia to enhance the diagnosis and treatment of such severe diseases to the greatest degree feasible.

The CRISPR/Cas system, widely used in the gene editing field, performs endonuclease functions on target nucleic acid molecules by Cas proteins and the guide RNA complementary to the target sequence. Recently, some Cas proteins (e.g., Cas12a and Cas13a) have been found to exhibit nuclease activity that indiscriminately cleaves arbitrary nucleic acids following specific binding to target molecules (Gootenberg et al., 2017; Chen et al., 2018; Li et al., 2018). Thus, by introducing single-stranded reporter nucleic acids that can generate fluorescent signals after cleavage, the detection of target nucleic acid molecules can be enabled by detecting fluorescent signals. Based on this principle, we have developed an accurate and rapid species-specific bacterial detector (SSBD) system (Wang et al., 2022). In previous studies, we have proven through clinical trials that SSBD can accurately detect ten of the most common clinical pathogens, namely *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Stenotrophomonas maltophilia*, and *Staphylococcus capitis*.

The detection target for SSBD needs to be selected for each species. In summary, we identify the detection target through two processes. The first process utilizes bioinformatics to align the 1,791 high-quality genomes of 232 microorganism species (each species includes multiple isolated strains) and screen species-specific DNA fragments of each species. In the second process, the species-specific DNA fragments are manually selected and experimentally validated to identify the optimal DNA targets. For the first bioinformatics analysis process (Figure 1), we first performed genome sequence alignment of multiple strains belonging to the same species to filter the common DNA fragments of the species. Then, these common DNA fragments were aligned with other species' genomes to remove similar DNA fragments present in other species, with the remaining DNA fragments being the species-specific DNA fragments. The screened species-specific DNA fragments of each pathogen had the following features: (1) intra-species conservation (these DNA fragments are present in multiple isolated strains belonging to the same species), and (2) inter-species specificity (these DNA fragments are not present in other species). For each species, dozens to thousands of specific DNA fragments were screened, but not all of these fragments were ideal targets for Cas12a detection. Therefore, the bioinformatics data were then artificially filtered based on the following three principles: DNA length preferably longer than 200 bp to facilitate primer design, absence of repetitive sequences, and the existence of PAM sequences (5'-TTTN-3') of Cas12a. Then, for 10 common clinical pathogens, several species-specific DNA fragments were randomly selected as the candidate target for detection. Primers and crRNAs were designed according to the selected candidate target DNA fragments sequence of each pathogen. Finally, the specificity and reliability of these primers and crRNAs were verified by crossover validation experiments, and the best-performing primer and crRNA for each species were selected based on the experimental results.



**Figure 1. Schematic diagram of screening species-specific DNA fragments by bioinformatics.** Reference from our published articles (Wang et al., 2022).

The advantage of the SSBD system is mainly reflected on detection accuracy and speed. The accuracy of the SSBD is reflected by the fact that the target of the detection is the species-specific DNA fragments (intra-species conserved and inter-species specificity) of each pathogen (DNA targets of the 10 pathogens in this protocol are shown in Table 5), as screened by the bioinformatics analysis in our previous study (Wang et al., 2022). The corresponding PCR amplification primers and crRNAs guiding Cas12a for targeted cleavage were designed according to the species-specific DNA fragments. SSBD can be completed in just a few hours, as it does not include a culture process and only involves three steps of sample DNA extraction, PCR amplification, and Cas12a testing. Meanwhile, the method is easy to be replicated, as DNA extraction and PCR amplification are the fundamental experimental assays in many molecular testing departments. Additionally, Cas12a protein and crRNA can be ordered from biotech companies. In conclusion, SSBD has great advantages as a new rapid detection method for pathogens, showing potential to even replace traditional culture methods, making it well worth to be extended and applied in the clinical diagnosis of patients with severe pneumonia. Meanwhile, the SSBD is based on species-specific DNA targets of pathogenic microbes, which means it can be expanded to any species other than bacteria.

## Materials and Reagents

1. 384-well microplate (Corning, catalog number: 3701)
2. 1.5 mL nuclease-free tube (Eppendorf, catalog number: 0030125150)
3. 0.2 mL PCR tube (Eppendorf, catalog number: 0030124332)
4. 10 µL tips (SANFICO, catalog number: TFR810)
5. 10 µL extended tips (SANFICO, catalog number: TFR810-L)

6. 200 µL tips (SANFICO, catalog number: TFR8200)
7. 1,000 µL tips (SANFICO, catalog number: TFR81000)
8. Quick-DNA/RNA Pathogen Miniprep kit (ZYMO RESEARCH, catalog number: R1042)
9. TIANSeq HiFi Amplification Mix (TIANGEN, catalog number: NG219)
10. NEBuffer™ 3.1 (New England Biolabs, catalog number: B7203)
11. Nuclease-free water (Thermo Fisher, catalog number: 10977015)
12. Cas12a protein

*Note: In our experiments, the Cas12a protein expression and purification was performed as described (Li et al., 2018). We also recommend purchasing Cas12a from a biological company (e.g., New England Biolabs).*

13. Primers for PCR amplification (primer sequences are shown in Table 1)

*Note: The sequence of primers was published in our previous article in Appendix 1, Table 1 (Wang et al., 2022). The primers can be ordered as PAGE-purified DNA oligonucleotide from a DNA synthesis company (e.g., GenScript Biotech or Sangon Biotech).*

**Table 1. Primers sequences**

Species	Primers sequences (5' > 3')	
<i>A. baumannii</i>	forward primer	CACAGCGTTTAACCCATGCC
	reverse primer	TATCGCCACCTGCACAGAAG
<i>E. coli</i>	forward primer	GTTCTGACTATCTGGCGGG
	reverse primer	GCTTCTGACTCCAGACACC
<i>K. pneumoniae</i>	forward primer	CATGGGCATATCGACGGTCA
	reverse primer	CCTGCAACATAGGCCAGTGA
<i>S. aureus</i>	forward primer	AGGTGCAGTAGACGCATAGC
	reverse primer	CATTCGCTGCGCCAATACAA
<i>P. aeruginosa</i>	forward primer	TCTCTCTATCACGCCGGTCA
	reverse primer	TCGCATCGAGGTATTCCAGC
<i>S. epidermidis</i>	forward primer	CACGCATGGCACTAGGTACA
	reverse primer	CGAAAAAGAGTTGTCCTTGTGA
<i>S. capitis</i>	forward primer	GGTTCAGTCATCCCCACGTT
	reverse primer	CAGCTGCGACAACCTGCTTAC
<i>E. faecalis</i>	forward primer	CGGCAAGTTTGGAAGCAGAC
	reverse primer	CAGCGCCTAGTCCTTGTGAT
<i>E. faecium</i>	forward primer	ATCGGAAATCGGTGTGGCTT
	reverse primer	TCAAATGCATCCCTGTGCCT
<i>S. maltophilia</i>	forward primer	CGCCTCCCGTTTACAGATTA
	reverse primer	TCGGCTCCACCACATACAC

14. crRNA used in Cas12a reaction (crRNA sequences are shown in Table 2)

*Note: The RNA can be ordered as a desalted RNA oligonucleotide or as PAGE-purified RNA oligonucleotide from an RNA synthesis company (e.g., GenScript Biotech or Sangon Biotech).*

**Table 2. crRNA sequences**

Species	crRNA sequences (5' > 3')
<i>A. baumannii</i>	UAAUUUCUACUAAGUGUAGAUGUAAAAGAAGAAUUACUUGAA
<i>E. coli</i>	UAAUUUCUACUAAGUGUAGAUACGGUCGCUAUGAUGGCAAG
<i>K. pneumoniae</i>	UAAUUUCUACUAAGUGUAGAUACUGCGGUAAUCGCCAUCUU
<i>S. aureus</i>	UAAUUUCUACUAAGUGUAGAUACAACGUGAACUUGCUGAGG
<i>P. aeruginosa</i>	UAAUUUCUACUAAGUGUAGAUUUGUAAACACACAUGAGGAG
<i>S. epidermidis</i>	UAAUUUCUACUAAGUGUAGAUUAAUUAUUAUCAAUUAGAGA
<i>S. capitis</i>	UAAUUUCUACUAAGUGUAGAUAAUCACCUUAAUUAUCAAUA
<i>E. faecalis</i>	UAAUUUCUACUAAGUGUAGAUAGCACCACAAUAAACACAGCUGA

<i>E. faecium</i>	UAAUUUCUACUAAGUGUAGAUACUACCUGAACUUAUUAUACA
<i>S. maltophilia</i>	UAAUUUCUACUAAGUGUAGAUACUACCUGACACCUGCGACCAGG

*Note: The backbone sequence of crRNA is 5'-UAAUUUCUACUAAGUGUAGAU-3', and the following sequence is used to recognize the target DNA.*

- ssDNA-reporter (synthesized by Sangon Biotech)  
*Note: Sequence of ssDNA-reporter: 5'-FAM-TTATT-BHQ1-3'. The feature of the ssDNA-reporter is that a fluorescent molecule that can generate a fluorescent signal (e.g., FAM) is coupled at one DNA end, and a quencher molecule (e.g., BHQ1) that can block the fluorescent molecule producing a fluorescent signal is coupled at the opposite DNA end.*
- Ethanol (Sangon Biotech, catalog number: A500737-0500)

## Equipment

- Centrifuge (Eppendorf, Centrifuge 5418 R)
- Centrifuge for 0.2 mL PCR tube (Scilogex, catalog number: 914041419999)
- PCR thermocycler (LongGene, T20D)
- Fluorescence plate reader (Austria Tecan, Infinite M200 Pro)
- NanoDrop One (Thermo Scientific, catalog number: ND-ONE-W)
- Biological safety cabinet (Thermo Scientific, catalog number: 51028226)
- Vortexer (Scilogex, catalog number: 821200049999)
- 1–10 µL pipettes (Eppendorf, catalog number: 3123000020)
- 2–20 µL pipettes (Eppendorf, catalog number: 3123000098)
- 20–200 µL pipettes (Eppendorf, catalog number: 3123000055)
- 100–1,000 µL pipettes (Eppendorf, catalog number: 3123000063)

## Software

- Primer design software, NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>)
- CrRNA design software, CHOPCHOP (<https://chopchop.cbu.uib.no/>)
- Software to control the fluorescence plate reader, Tecan i-control (Austria Tecan)
- Software for data analysis, Microsoft Excel

## Procedure

### A. Detection targets design

*Note: The primers and crRNAs used in this protocol for 10 pathogens (Table 1) have been experimentally and clinically validated with excellent effectiveness.*

- Select detection targets. Manual screening of species-specific DNA data from bioinformatics; the screening principles are:
  - To facilitate primer design, DNA length is preferably longer than 200 bp.
  - DNA fragment without repetitive sequences.
  - DNA fragment contains PAM sequences (5'-TTTN-3') of Cas12a.
- Design crRNA.
  - Find PAM sequences (5'-TTTN-3') within the DNA target region.
  - Select the sequence 20 bp downstream of the PAM sequence as the target of crRNA. Replace the T-

base in the 20 bp target sequence with a U-base. Place the target sequence downstream of the crRNA backbone sequence (5'-UAAUUUCUACUAAGUGUAGAU-3').

*Note: The base distribution of the target sequence should be balanced, with a GC content of 40%–80%. We also recommend using crRNA design software, e.g., CHOPCHOP (<https://chopchop.cbu.uib.no/>).*

3. Use NCBI Primer-BLAST primer design software to select primers.
  - a. The input sequence is the upstream and downstream sequence of the crRNA target site.
  - b. Set the *Primer parameters* as default parameters.
  - c. Set *Primer Pair Specificity Checking Parameters* Database as Refseq representative genomes, and *Organism* as *Homo sapiens* (taxid: 9606).
  - d. Pick a primer pair predicted without non-specific amplification.
4. Experimental validation  
For example, for the 10 sets of primers and crRNAs used in this protocol, each set of primers and crRNAs needs to ensure that no non-specific signals are detected for the remaining pathogens and human genomic DNA.

## B. DNA extraction from bronchoalveolar lavage fluid (BALF) sample

*Biosafety notes: DNA extraction procedure should be performed in a biological safety cabinet to ensure user safety. Wear biological protecting clothing, gloves, and masks during operation.*

DNA extraction was performed referring to the Quick-DNA/RNA Pathogen Miniprep kit instructions. The procedure is as follows:

1. Add 800  $\mu$ L of DNA/RNA shield to each 200  $\mu$ L of BALF sample and mix well by using vortexer.
2. Centrifuge at 16,000 $\times$  g for 1 min. Transfer up to 200  $\mu$ L of the supernatant into a 1.5 mL nuclease-free tube.
3. Add 2  $\mu$ L of proteinase K and mix well.
4. Add 400  $\mu$ L of pathogen DNA/RNA buffer. Mix well and incubate at room temperature for 5 min.
5. Transfer the mixture into a Zymo-Spin IIC column in a collection tube and centrifuge at 16,000 $\times$  g for 30 s. Discard the flowthrough.
6. Add 500  $\mu$ L of pathogen DNA/RNA wash buffer to the Zymo-Spin IIC column and centrifuge at 16,000 $\times$  g for 30 s. Discard the flowthrough.
7. Repeat the previous step.
8. Add 500  $\mu$ L of ethanol (95%–100%) to the Zymo-Spin<sup>TM</sup> IIC column and centrifuge at 16,000 $\times$  g for 1 min to ensure that the ethanol is removed. Discard the collection tube and transfer the Zymo-Spin<sup>TM</sup> IIC column into a new nuclease-free 1.5 mL tube.
9. Add 25–50  $\mu$ L of nuclease-free water at 65  $^{\circ}$ C directly to the matrix of the column and centrifuge at 16,000 $\times$  g for 1 min to elute the DNA.
10. Measure DNA concentration using a NanoDrop and adjust DNA concentration to 100 ng/ $\mu$ L.

*Note: The DNA yield extracted from 200  $\mu$ L of BALF is 8–43  $\mu$ g. Depending on the patient's situation and operating procedure, typically 200  $\mu$ L of BALF is sufficient for subsequent analysis. If the DNA yield is low, centrifuge the BALF sample at 12,000 $\times$  g for 5 min, discard the supernatant, and retain 200  $\mu$ L of bottom liquid.*

## C. PCR amplification enrichment of target DNA sequences to be tested

1. For one BALF sample DNA, prepare 10 PCR reactions using amplification primers, one for each of the 10 pathogenic bacteria.
2. Setting up the positive and negative controls: the templates for the positive and negative controls are the genomic DNA of the corresponding pathogen and nuclease-free water, respectively.
3. Prepare the PCR reactions in a 0.2 mL PCR tube.

Component	50 $\mu$ L reaction
2 $\times$ HiFi amplification mix	25 $\mu$ L

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- |  |                   |  |
|--|-------------------|--|
| Forward and reverse primers (10 $\mu$ M) | 2 $\mu$ L of each |  |
| Template DNA (100 ng/ $\mu$ L)           | 1 $\mu$ L         |  |
| Nuclease-free water                      | 20 $\mu$ L        |  |
- Mix well and pulse-spin in a microcentrifuge.
  - Place a 0.2 mL PCR tube in a PCR thermocycler.
  - PCR procedure:
 

98 $^{\circ}$ C	2 min	
98 $^{\circ}$ C	10 s	
52 $^{\circ}$ C	10 s	38 cycles
72 $^{\circ}$ C	15 s	
72 $^{\circ}$ C	5 min	
4 $^{\circ}$ C	Forever	
  - After the PCR reaction, without the PCR purification step, directly use the PCR reaction solution as the detection substrate for the Cas12a reaction.

#### D. Detection of PCR products using Cas12a

- Prepare the reactions in a 384-well microplate on ice:
 

Component	20 $\mu$ L reaction
ssDNA-reporter (10 $\mu$ M)	1 $\mu$ L
PCR reaction solution	2 $\mu$ L
10 $\times$ NEBuffer 3.1	2 $\mu$ L
Cas12a (10 $\mu$ M)	1 $\mu$ L
crRNA (10 $\mu$ M)	1 $\mu$ L
Nuclease-free water	13 $\mu$ L

*Note: The PCR reaction solution for each pathogen requires the use of the corresponding crRNA. Preparation of premixes is recommended to improve experimental efficiency.*
- With a pipette set at 15  $\mu$ L and keeping the plate on ice, mix the reaction by pipetting the entire solution up and down 10 times.
 

*Note: Pipetting 10 times can ensure sufficient mixing of the reaction solution. Avoid excessive bubbles in the reaction solution; any trace bubbles will clear after 30 min of incubation at 37  $^{\circ}$ C in the next step.*
- Place the plate in a fluorescence plate reader. Incubate at 37  $^{\circ}$ C for 30 min, then measure the fluorescence values of each reaction well (excitation wavelength: 485 nm; emission wavelength: 535 nm).

#### Data analysis

*Note: The data analysis of this protocol is applied to the detection of pathogenic bacteria in patients with severe pneumonia in the ICU. For other studies, the analysis standards require adjustments accordingly.*

- For each sample, analyze the fluorescence values for each pathogen separately.
- For each pathogen detected, the positive control (PC) is the genomic DNA of corresponding pathogen, and the negative control (NC) is nuclease-free water.
- The fluorescence value of BALF sample (shown as F) is two-fold larger than the negative control, indicating that the SSBD method detected the corresponding pathogen.
- In our previous article, the application of SSBD was for pathogen detection in ICU patients, so the degree of pathogenic infection is required to guide drug usage. The pathogen strength is determined to some extent by the fluorescence signal (Table 3). Set I (interval) as the point of distinction.  $I = (PC - 2NC)/3$ . When  $2NC < F < 2NC+I$ , the pathogen strength level is defined as level I. When  $2NC+I < F < 2NC+2I$ , the pathogen strength level is defined as level II. When  $F > 2NC+2I$ , the pathogen strength level is defined as level III.



**Table 3. Determining the presence of bacteria by fluorescence signal**

Fluorescence value	Test results	Bacterial DNA molarity
$F < 2NC$	Undetected	N.A.
$2NC < F < 2NC+I$	Detected level I	$10^{-15}$ M– $10^{-14}$ M
$2NC+I < F < 2NC+2I$	Detected level II	$10^{-14}$ M– $10^{-13}$ M
$F > 2NC+2I$	Detected level III	over $10^{-13}$ M

NC: Value<sub>negative control</sub>; F: Value<sub>Sample</sub>; I = (Value<sub>positive control</sub> - 2 Value<sub>negative control</sub>)/3

## Example results

- Using the data of two samples from our previously published article, we calculated the values of 2NC, 2NC+I, and 2NC+2I according to the fluorescence values of PC and NC, respectively. The fluorescence values of the samples are shown in Table 4.

**Table 4. Fluorescence values of two samples**

Species	Fluorescence value							Detection result	
	PC	NC	Sample 1	Sample 2	2NC	2NC+I	2NC+2I	Sample 1	Sample 2
<i>A. baumannii</i>	9788	903	9300	9398	1806	4467	7127	Detected Level III	Detected Level III
<i>E. coli</i>	8435	821	657	630	1642	3906	6171	Undetected	Undetected
<i>K. pneumoniae</i>	8285	1791	1718	1730	3582	5150	6717	Undetected	Undetected
<i>S. aureus</i>	8316	1008	1018	1003	2016	4116	6216	Undetected	Undetected
<i>P. aeruginosa</i>	9323	1170	1164	1069	2340	4668	6995	Undetected	Undetected
<i>S. epidermidis</i>	9309	1237	1210	1183	2474	4752	7031	Undetected	Undetected
<i>S. capitis</i>	8832	1633	1949	1772	3266	5121	6977	Undetected	Undetected
<i>E. faecalis</i>	8241	614	512	505	1228	3566	5903	Undetected	Undetected
<i>E. faecium</i>	8432	1936	1692	1700	3872	5392	6912	Undetected	Undetected
<i>S. maltophilia</i>	8194	1620	1549	1579	3240	4891	6543	Undetected	Undetected

- The fluorescence value of *A. baumannii* in these two samples was higher than 2NC+2I. Therefore, the presence of *A. baumannii* in these two samples was detected, with a level III bacterial strength. For the rest of the pathogens, the fluorescence values of the samples were lower than 2NC, which indicates that the rest of the pathogens were not detected.

## Notes

- Optimizing PCR reactions can significantly avoid the generation of non-specific signals. When designing primers, BLAST the primers and select primers with excellent specificity. The suitable PCR reaction program is confirmed by experiment. Moreover, it is basically impossible for crRNA recognition sequences to exist in the non-specific amplification products generated during PCR. Therefore, combining the crRNA-Cas12a reaction with the PCR reaction greatly ensures the specificity of the detection. It is noteworthy that if crRNA is synthesized by in vitro transcription with RNA polymerase, the DNA used as in vitro transcription template needs to be removed to avoid contaminating crRNA (since the DNA templates can bind to crRNA to activate Cas12a).
- Features considered important while selecting target:
  - Target sequences are present in multiple strains of this species' genomes.
  - Target sequences are not found in other species' genomes.
  - Target without repetitive sequences.
  - Target including PAM sequences.



**Table 5. Information of target DNA region used in the protocol**

Species	GenBank of genome	Target sequences (5' > 3')	Corresponding gene
<i>A. baumannii</i>	CP115629.1	CACAGCGTTTAACCCATGCCATTGGTAAAGCTAAAGCAATGGAAATGTGCCTTACCGCTCGTCAAATGG GTGCAGTAGAGGCAGAACAAAGTGGTTTGGTTGCACGTGTGTTTAGTAAAGAAGAATTACTTGAACAA ACCTTGCAAGCCGCTGAAAAAATCGCAGCACGGTCTTAACTGCAAACATGATGCTCAAAGAAACCATT AATCGAGCTTTTGAGGTGAATCTCACCGAAGGTTTACGTTTGAACGCCGCATGTTCCACTCCATTTTGTG CAACTGCGGACCAAAAAGAAGGCATGCAGGCATTTGTTGAAAAACGGCAGGCAAAATTTAAAAATCAG TAAGAGATCAATAAAATGAATACAGAAAACTACTTAATGATTGAGCGACAAGGTAAGCTTGGCGTGAT CACCTTAGATCGTGTGACTCATCTCAATGCATGTGCGTAGATATGATTGAAGGAATTGGCGCCCAATT GGAGTTGTGGCGAAATGATGCTGCTGTTACGGCGATCTTAATCAAATCAAATAGCCCAAAAGCCTTCTG TGCAGGTGGCGATA	enoyl-CoA hydratase/isomerase family protein
<i>E. coli</i>	CP115173.1	GTTCTGACTATCTGGCGGGGAATGGTGTGGTTTATCAAACCAGTGATGTGAAGTATGTGATTGCCAAC AACAACTTGTGGGCCAGCCCCTGGATCAACAGTTGCGCAACACCCTGGTTGCCAACCTGAGTACGCAA CTGCCCCGGCTGGGTGGTTGCCTCCCAGCCTCTGGGAAGCGCCAGGACACGCTCAATGTTACCGTAACG GAGTTAACGGTCGCTATGATGGCAAGGTCATTGTCAGTGGTGAGTGGCTGTTGAACCACCAGGGACAA CTGATCAAACGTCCGTTCCGTCTGGAAGGAGTGCAAACTCAGGATGGTTACGATGAGATGGTTAAAGTG CTGGCCGGTGTCTGGAGTCAGGAAGC	membrane integrity-associated transporter subunit PqiC
<i>K. pneumoniae</i>	CP115714.1	CATGGGCATATCGACGGTCACCGATTGCCCCGGACGCAGGCGAACATCTTTTTCCGCCACCCGGTCCGC CTGCAGCGCCTGGCCATCGCCGGCAAAACAGCGACGGGTAGTCAGTATTGTCGAACGCTTGCCGGTCTT CAGCTGATAAATACGCACCACCGTCGCCAGGGAGGCGCCTTTGGCGTTGTTATTCACGCCCTCCCGGC GCGAAGATCCAGATGCAGGGTTTTACCTGGGGGTAAAAAATGGACTGGGTACGGAACGGCGCCGT CTTTCACGGTCTGCGTCAGGCCGACGCCGTTAAGACGGTAACCATGAGGAATGCCAGCAACCTGGCG GAGGGTTTAACTGCGGTAATCGCCATCTTCATCGCTCTCCCTGCGGTGGATATTTCCCGGACGCGCTGA TAGCGCCCCAGATAAATCGTGACTCTGTGTCAGCCTGTTTCTGCGCATCCAGAGGACGCAGCACAGCG GTACGGCCGAGCTGGACGGCATGCTCCTGCTGGCAGCAAAGTTGCGCATCCGGCAGTAAATGGCGGGC GACGCAAAAGCTGCAGCCGCACGTCAAGATGAGATCCGAGCCAGACGTGCAGAAGCGCCATCAGGTGCG TGAACAGTTCGCCGCCGGGCAGCCAGCCGCAACCTCATCAGGCTTTTCCGTGGCCAGCTGCAGCAATA CCTGACCGTTACATCCGTGGCGTGGGTTCCTCATCTGCGCTATGTTGCAGG	type VI secretion system baseplate subunit TssG; type VI secretion system lipoprotein TssJ
<i>S. aureus</i>	CP063802.1	AGGTGCAGTAGACGCATAGCATCATCATATTGAATAGTAAAAACAAATAAAACATAGTAACGTGATTC AGTCGATGTAACAGTCGATAATGAGTCACGTTTTTTTATAGAAAAATATAAGACATAAAAAATGTCATAA TTTATAGTCGACAAATATCATACTGTATAAACATTTATCATTTTCTCAAGTGCCTTTTACACGATGGAAT GAACTTACTTTTACGAAATTATGCGTATTTTATAAACAAATATCATTGATATAACGGTAAATGTAAGCG TTTACAACAGAAATAACTGCATGTACGATATTTTGTAAATTCACAGTATTCAAGTATTTAAGTCAATA TGAGGAGGGATGTTATGAGCGATTCTGAGAAAGAAATTTAAAAAGAATTAAAGATAATCCGTTTATTT	winged helix-turn-helix transcriptional regulator

		CACAACGTGAACTTGCTGAGGCAATTGGATTATCTAGACCCAGCGTAGCAAAACATTATTTTCAGGATTAA TACAAAAGGAATATGTTATGGGAAAGGCATATGTTTTAAATGAAGATTATCCTATTGTTTGTATTGGCG CAGCGAATG	
<i>P. aeruginosa</i>	CP080405.1	TCAGTGACATGAAGCCCGTCCCCGCGGCCAGGCTGCGCAGTTGCGCCTACGAGATGGGTTTTCTCTCT ATCACGCCGGTCAATGCTTGCGCGTGGTTCTCTAGGTTGACCGAATCAAGAGTGTGTTTTTCAGCAAAA TAATGATAGTTTGTGTAAACACACATGAGGAGGTCGTCATGAGCGCTCTCATCAAGGAACGTCCCAGC GCCGATGCCGTCTGGCCAAGGCCGTCTGGCCGCGCGGAGCAATTGGGGCTGACGCAGCTCGAACT GGCCGGCATCGTCGCGCTCGATCGCAGCGCCATCAGCCGCTGGAAGACCCAGGGCTGCGGGTGGACA GCAAGACCGGCGAGCTGGCTCTGCTGCTGGTGCGAGTCTATCGCGCACTGTATGCCCTGTTTCGGCGGGC AGCAGGAGGACATGCGCCACTTCTGCGCACTCCCAACCATCACCTGGCGGGCGAGCCGCTGGCACTG ATGGGACAGGTGCAGGGCCTGGTTCATGTGCTGGAATACCTCGATGCGA	DUF2384 domain-containing protein; FAD-dependent oxidoreductase
<i>S. epidermidis</i>	CP106834.1	CACGCATGGCACTAGGTACAAATCCAGTATAAACAGCGTAGATTAATAATAGTAAATAGTTACACCTT TAATGAGAACTAGAGGTATATTTAAACGTACTAACAGTTGATAAACGAGGACAATAATTGTACCAACAT GTGTACCACTTATAGCTAGTAAATGGTAAATACCTATATCTTTGATGTTAGATTTGTAATATTCATCAAT TAGAGAGGTATCACCTGTTATCAACGCTAATATCTTTTCAGGATGCGTGACCCAGATTTATGAATTATT TTGGTGATATAATTTTGATGATGATATATAGGAGTGAAAATATTGTTTTCTTGGAAGACTTAAATTGAA CTGTTGAAATATTCAACAAGGACAACCTTTTTTCG	DNA internalization-related competence protein ComEC/Rec2
<i>S. capitis</i>	AP014956.1	GGTTCAGTCATCCCCACGTTTTGATCTTCAATATTCATGTTATAATTTTCTCCTTTTTAAAAGGTTTCATT TACCATTGTTTACTACAGTAAAGGTTGTTTTAAACATATTCGTTTTAATATTTTTAGAGTGAAGATTCTA AAACGTGTGATAAAGGTGGAATATACACATAAAAAATTTCCGATATAGAAAAATAAAAAGCTTTCAATC ACCTTATTAATCAATAAAAAGTGATTAAAAGCTTTAAATTAAGAGGATAAACATCAATTCATATCTC AAACGCTTCTAACATTTTTGAGTTAGATGACTGAATTTGAACTGTGTTTATATCAAGCTTTTGTCTATTCT AGCCATCTTAGAAAACTATATAGAATGAATTCCTATTTAAGAAATCAATGCAGTATTTCTTTAAACTTCT GTTACTTCGACTTCGATGTGTTCGATGCGATGATATGCACCATGATCAACAGGTCCTACAAATTCATTCA TTTTCCAACCGTTTTTGATTGCAGATGTACGAAAGCTTTAGCAGCGATGACTGCTTCTCTTGAGATTT GCCGTTAGCTAGGTAAGCAGTTGTCGCAGCTG	putative pyridoxine kinase; FAD binding superfamily protein
<i>E. faecalis</i>	CP046113.1	CGGCAAGTTTGGAAGCAGACAAAGGTATTTTTGCAGTATTGAATGTCATCATTGGGATTAATGGTTTTG TTTGGAATGGTACATTGGCTATCGCTGGTTTGATTTTCGCTTTTCTTGGGGTATAACTTAGCGAAAGC ATACAATGTGAATGAGCTAGCTGGTGGGATTGTCAGTTTAGCTACCTTAATTCAGGTGTTGCTTTTGCA CCAAATAACACAGCTGAATTAGCTGTAAAAGTGCCAGAAAAGATTGCCAACGCAATTAATGGGGCAGA AATTGGTGCGTCAATTGCTAATAATCAATTGACTGTCAATCCATGGGGTTGGTTAAATCTCAACCATCTA AATGGTAACGCTTTCTTTACGGTGATGATTATGGGCGCTTATCAACCATTAATTTTGTAAATTAATGC AAGCGGATTTAACAATAAAAAATGCCTGATTCTGTACCACCAGCAGTTTCAAAAGCGTTTTCGGCAATTT TACCAGCAACAATCGCATTGTATGTCGTAGCAATTATTAACCTTACGGTGTCAAAACCTTTCAGGCGGTC	PTS sugar transporter subunit IIC

		AATTATTAATTGACTTAATTCAAAAAATATATCGCGGAACCGTTCCTTGGGTATCACAAGGACTAGGCGCT	
<i>E. faecium</i>	CP112862.1	ATCGGAAATCGGTGTGGCTTATGCGAAAGGCATCCAGTGATCGGCTTATATACTGATACTCGACAGCAAGGCGGAACCCACCCGAAAAAATCGCTGCACTACAAGAAACAGCTGAAAATCAATTTCACCTGACTTATATACAATAGGTTTGATCAAATTGAACGGAAGAGTGGTTTCTTCAGAAATTGAGTTGCTTTTCAGAAAGTAAAAAGATTTTATAGATGGAGGGACTTTCAGTGATTAAGAAATCAAAAAAGGTGCAAATAGCTTTACTTGCTTTTGGTGTATTCGTTGTCTGCTATAATTTTATGAATTTATCACACAAAAATATTCGACTTCTCAAGGAATCACATTTATCGTGGAATCTTTATTAGGGATTGCATTGATTTTCATGCCACAAGTCATTTTGAAAGTTTTCAAACCTAAAAATACCAGCAGCAATCGTTTGTCTTACTGGTTTTCTTGTTTCATCTCTGTCTTTTAGGCACAGGGATGCATTTGA	nucleoside 2-deoxyribosyltransferase; hypothetical protein
<i>S. maltophilia</i>	CP040440.1	CCCGCGAGAAGCTGGATGCGCTGGAAACCGCCGTGCGCGAGCTGGAAGGGCGCGGCCGCGCAGAATGA AAAAAGGAGGCGCACCGCGCTCCCGTTTACAGATTATTTCCCGGATGGGGCCTTGCAGCAAGGCCACCGGGCGCCCTAGACTCGCCGGCCTGTCGATCCGGCAGCACAGAGAGGGGCAGTGCCGATGGCGCAGGACACCGAATGGACACCCGTTTCGCATGACACCTGCGACCAGGTGCGCGGGCCGTTCTATCACGGCACCCGCGCTGACCTGGCGGTGGGCGAGCTGCTGAGCGCGGGCTTCCGCTCCAACATATCGCGACAGCGTGGTGATGAACCACATCTACTTCACCACGATTGCCAAGGGCGCCGGTTGGCTGCGGAGATGGCCCCGGGTGAAGGGCGACCGCGCGTGTATGTGGTGGAGCCGA	accessory factor UbiK family protein; NAD(+)-rifampin ADP-ribosyltransferase

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## Competing interests

The authors declare no competing interests.

## Ethics

This study was registered in English at <https://clinicaltrials.gov/> (NCT04178382) in November 2019.

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