

CRISPR-CAS12A BASED TARGET DETECTION**FIELD OF THE INVENTION**

[0001] The present invention belongs to the field of molecular diagnostics with a specific focus on CRISPR-Cas based diagnostics for the detection of nucleic acids associated with diseases. The present invention particularly relates to the field of brucellosis diagnosis.

BACKGROUND

[0002] Brucellosis is a disease that poses significant global challenges in livestock and human health. Brucellosis is the most widespread zoonotic disease globally, yet it is listed by the World Health Organization (WHO) as one of the seven most neglected diseases. Brucella infection not only jeopardizes the breeding industry but also disrupts international livestock and meat trade, resulting in substantial economic losses and social burdens. Even in developed countries, the persistence of Brucella in wildlife complicates complete eradication efforts. Current diagnostic methods heavily rely on serological detection, particularly the Rose Bengal Test (RBT), which, although rapid and sensitive, exhibits limitations due to potential cross-reactivity with other pathogens, leading to false positives. Additionally, RBT faces challenges in detecting Brucella during the window period of infection. Polymerase chain reaction (PCR) offers practical and sensitive nucleic acid amplification but is hindered by cost, equipment requirements, and a lack of standardized diagnostic protocols, particularly in resource-limited regions.

[0003] The present disclosure addresses a critical need in the field of brucellosis diagnosis. The present disclosure introduces a novel CRISPR-Cas12a based detection method designed for Point-of-Care Testing (POCT) of Brucella. This innovative approach offers convenience, simplicity, high sensitivity, and robust specificity. Notably, the combination of Cas12a enzymes in combination with novel crRNAs for Brucella nucleic acid assay yields results in approximately 30 minutes, enabling the rapid POCT detection of Brucella nucleic acid. This swift and efficient process is particularly advantageous in remote pastures, providing a practical solution for real-time monitoring and intervention. These features position the presently disclosed CRISPR-Cas12a based detection package as a cutting-edge tool for precise and expeditious brucellosis diagnosis in diverse settings.

SUMMARY

[0004] In one aspect, the present disclosure refers to a Cas12a protein having an amino acid sequence selected from the group consisting of SEQ ID NO: 3 (CrmCas12a) and SEQ ID NO: 4 (SrmCas12a) or a Cas12a protein of the same clade as the Cas12a protein
5 having an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4; wherein the Cas12a protein comprises a maltose-binding protein (MBP) tag having an amino acid sequence of SEQ ID NO: 72.

[0005] In another aspect, the present disclosure refers to a CRISPR RNA (crRNA) for detecting Brucella, characterized in that the crRNA is selected from the group consisting

10 of the following sequences:

S-BC1 – sequence shown in SEQ ID NO: 8;

S-BP1 – sequence shown in SEQ ID NO: 9;

S-OM1 – sequence shown in SEQ ID NO: 10;

C-BC1 – sequence shown in SEQ ID NO: 11;

15 C-BP1 – sequence shown in SEQ ID NO: 12;

C-OM1 – sequence shown in SEQ ID NO: 13;

S-BC2 – sequence shown in SEQ ID NO: 14;

S-BC3 – sequence shown in SEQ ID NO: 15;

S-BC4 – sequence shown in SEQ ID NO: 16;

20 S-BC5 – sequence shown in SEQ ID NO: 17;

S-BC6 – sequence shown in SEQ ID NO: 18;

S-BC7 – sequence shown in SEQ ID NO: 19;

S-BC8 – sequence shown in SEQ ID NO: 20;

C-BC2 – sequence shown in SEQ ID NO: 21;

25 C-BC3 – sequence shown in SEQ ID NO: 22;

C-BC4 – sequence shown in SEQ ID NO: 23;

C-BC5 – sequence shown in SEQ ID NO: 24;

C-BC6 – sequence shown in SEQ ID NO: 25;

C-BC7 – sequence shown in SEQ ID NO: 26;

30 C-BC8 – sequence shown in SEQ ID NO: 27;

S-BP2 – sequence shown in SEQ ID NO: 28;

S-BP3 – sequence shown in SEQ ID NO: 29;

- S-BP5 – sequence shown in SEQ ID NO: 30;
S-BP6 – sequence shown in SEQ ID NO: 31;
S-BP7 – sequence shown in SEQ ID NO: 32;
C-BP2 – sequence shown in SEQ ID NO: 33;
5 C-BP3 – sequence shown in SEQ ID NO: 34;
C-BP5 – sequence shown in SEQ ID NO: 35;
C-BP6 – sequence shown in SEQ ID NO: 36;
C-BP7 – sequence shown in SEQ ID NO: 37;
S-OM2 – sequence shown in SEQ ID NO: 38;
10 S-OM3 – sequence shown in SEQ ID NO: 39;
S-OM4 – sequence shown in SEQ ID NO: 40;
C-OM2 – sequence shown in SEQ ID NO: 41;
C-OM3 – sequence shown in SEQ ID NO: 42;
C-OM4 – sequence shown in SEQ ID NO: 43;
15 S-BP8 – sequence shown in SEQ ID NO: 44;
S-BP9 – sequence shown in SEQ ID NO: 45;
C-BP8 – sequence shown in SEQ ID NO: 46; and
C-BP9 – sequence shown in SEQ ID NO: 47;
wherein the crRNA comprises a direct repeat (DR) and a guide domain (protospacer),
20 wherein the DR and the protospacer are connected in the 5' to 3' direction of the crRNA,
and the crRNA is configured to target a nucleic acid of Brucella with a predetermined
target sequence and form a complex with the Cas12a protein as disclosed herein.
[0006] In another aspect, the present disclosure refers to a detection kit for Brucella,
wherein the detection kit comprises the crRNA as disclosed herein.
25 [0007] In another aspect, the present disclosure refers to a method of detecting a
nucleic acid of Brucella in a sample using the detection kit as disclosed herein, wherein
the method comprises the following steps: incubating the sample containing the nucleic
acid of Brucella with the Cas12a protein and the crRNA, wherein when the nucleic acid
of Brucella is present in the sample, collateral cleavage activity of the Cas12a protein is
30 induced to generate a detectable signal when the probe nucleic acid is cleaved.
[0008] In another aspect, the present disclosure refers to a CRISPR-Cas12a
composition comprising the Cas12a protein as disclosed herein, and a CRISPR RNA

(crRNA), wherein the crRNA comprises a direct repeat (DR) and a guide domain (protospacer), wherein the DR and the guide domain are connected in the 5' to 3' direction of the crRNA, and the crRNA is configured to target a nucleic acid of a predetermined target sequence and form a complex with the Cas12a protein as disclosed herein.

- 5 [0009] In another aspect, the present disclosure refers to a method of detecting a nucleic acid having a predetermined target sequence in a sample using the CRISPR-Cas12a composition as disclosed herein, comprising: incubating the sample with the CRISPR-Cas12a composition, wherein when the nucleic acid having the predetermined target sequence is present in the sample, the collateral cleavage activity of the CRISPR-
- 10 Cas12a composition is induced to generate a detectable signal when the probe nucleic acid is cleaved.

BRIEF DESCRIPTION OF THE DRAWINGS

- 15 [0010] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

- [0011] **Figure 1** shows (A) The solubility of SrmCas12a (left) and CrmCas12a (right) with and without MBP tag. (1) total protein fraction with MBP, (2) soluble protein fraction with MBP fraction, (3) total protein fraction without MBP, (4) soluble protein fraction without MBP. (B) Ion Exchange Chromatography concentrated fractions of CrmCas12a and SrmCas12a with and without MBP tag.
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- [0012] **Figure 2** shows trans-cleavage activity of CrmCas12a and SrmCas12a with and without MBP tag for three target genes: *bp26*, *Omp2a*, and *BCSP31*. End-point fluorescence signal was measured after 60 min. Values are shown in the graphs as means $\pm$ SD (n = 3).
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- [0013] **Figure 3** shows results of crRNA screening for *BCSP31*. (A-G) SrmCas12a and CrmCas12a enzymes were used for S-BC and C-BC crRNAs, respectively. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means $\pm$ SD (n = 3).
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[0014] **Figure 4** shows results of crRNA screening for *bp26*. (A-E) SrmCas12a and CrmCas12a enzymes were used for S-BP and C-BP crRNAs, respectively. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means±

5 SD (n = 3).

[0015] **Figure 5** shows results of crRNA screening for *Omp2a*. (A-C) SrmCas12a and CrmCas12a enzymes were used for S-OM and C-OM crRNAs, respectively. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means±

10 SD (n = 3).

[0016] **Figure 6** is a bar graph showing the impact of protospacer sequence on trans-cleavage activity of SrmCas12a and CrmCas12a enzymes. End-point fluorescence signal was measured after 60 min. Values are shown in the graphs as means ± SD (n = 3).

[0017] **Figure 7** is a bar graph showing the impact of crRNA length on trans-cleavage activity of SrmCas12a and CrmCas12a enzymes. The protospacer length of crRNAs S-BC3 and C-BC3 were extended from 14 to 32 nucleotides in steps of two, resulting in crRNAs S-BP3-1 to BP3-10. End-point fluorescence signal was measured after 60 min. Values are shown in the graphs as means ± SD (n = 3).

[0018] **Figure 8** is a line graph showing the impact of crRNA combination on SrmCas12a trans-cleavage activity for targeting *bp26* gene. crRNAs S-BP3 and S-BP7 were used separated and in combination. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means± SD (n = 3).

[0019] **Figure 9** is a line graph showing the impact of crRNA combination on SrmCas12a trans-cleavage activity for targeting *BCSP31* gene. crRNAs S-BP1 and S-BL1 were used separated and in combination. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means± SD (n = 3).

[0020] **Figure 10** is a line graph showing the influence of combining CrmCas12a and SrmCas12a enzymes on augmenting the fluorescence signal in trans-cleavage activity for targeting the *bp26* gene. crRNAs C-BP5 and S-BP7 were used in this study. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of

real-time background subtracted fluorescence output are shown in the graphs as means $\pm$ SD (n = 3).

[0021] **Figure 11** is a line graph showing the influence of combining CrmCas12a and SrmCas12a enzymes on augmenting the fluorescence signal in trans-cleavage activity for targeting the *bp26* gene. crRNAs C-BP5 and S-BP3 were used in this study. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means $\pm$ SD (n = 3).

[0022] **Figure 12** is a line graph showing the influence of combining CrmCas12a and SrmCas12a enzymes on augmenting the fluorescence signal in trans-cleavage activity for targeting the *BCSP31* gene. crRNAs C-BC5 and S-BC1 were used in this study. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means $\pm$ SD (n = 3).

[0023] **Figure 13** is a line graph showing the influence of combining CrmCas12a and SrmCas12a enzymes on augmenting the fluorescence signal in trans-cleavage activity for targeting the *BCSP31* gene. crRNAs C-BC5 and S-BCL1 were used in this study. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means $\pm$ SD (n = 3).

[0024] **Figure 14** is a bar graph showing the performance of SrmCas12a and CrmCas12a enzymes at 20, 25, 30, 35, 40, 50, and 60 °C. End-point fluorescence signal was measured after 60 min. Values are shown in the graphs as means $\pm$ SD (n = 3).

[0025] **Figure 15** are line graphs showing temperature-dependent activity of (A) SrmCas12a compared to (C) LbaCas12a and (B) CrmCas12a. The figure (A-C) shows the activity profiles of SrmCas12a, CrmCas12a, and LbaCas12a, and at 60°C, followed by a reduction to 37°C for 30 minutes, mimicking a one-pot diagnostic system for LAMP amplification and Cas12a detection. At 60°C, LbaCas12a exhibited high activity, which increased further when the temperature was reduced to 37°C. CrmCas12a showed low activity at 60°C that gradually increased and then significantly improved after the temperature reduction. In contrast, SrmCas12a exhibited minimal activity at 60°C, but its activity increased rapidly after 30 minutes at 37°C. The fluorescence was measured in 1

min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means $\pm$ SD (n = 3).

[0026] **Figure 16** are line graphs showing temperature-dependent activity of (A) SrmCas12a compared to (C) LbaCas12a and (B) CrmCas12a. The figure (A-C) shows the activity profiles of SrmCas12a, CrmCas12a, and LbaCas12a, and at 65°C, followed by a reduction to 37°C for 30 minutes, mimicking a one-pot diagnostic system for LAMP amplification and Cas12a detection. LbaCas12a showed relatively high initial activity, which further increased after the temperature was reduced to 37°C, although the overall activity was lower than at 60°C. CrmCas12a's activity at 65°C remained low, with only a slight increase observed when the temperature dropped to 37°C for two crRNAs. SrmCas12a, similar to its behavior at 60°C, exhibited very low activity at 65°C, but its activity increased sharply after the temperature was reduced to 37°C. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means $\pm$ SD (n = 3).

[0027] **Figure 17** is a bar graph showing the effect of tween 20 on SrmCas12a and CrmCas12a activity. Graph showing enzymatic activities of SrmCas12a and CrmCas12a at varying Tween 20 concentrations (2.5, 5, 10, 25, and 50 mM), compared to the control. Activity is enhanced at lower concentrations (2.5 and 5 mM) but decreases at higher concentrations. End-point fluorescence signal was measured after 60 min. Values are shown in the graphs as means $\pm$ SD (n = 3).

[0028] **Figure 18** are bar graphs showing the effect of (A) DMSO, (B) glycerol, (C) MgCl₂, and (D) betaine on SrmCas12a and CrmCas12a activity. DMSO, glycerol, MgCl₂, and betaine were tested at various concentrations. No substantial enhancement in enzymes' activity was observed for any compound. End-point fluorescence signal was measured after 60 min. Values are shown in the graphs as means $\pm$ SD (n = 3).

[0029] **Figure 19** is a line graph showing the activity of SrmCas12a at varying probe concentrations. The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means $\pm$ SD (n = 3).

[0030] **Figure 20** is a line graph showing the activity of CrmCas12a at varying probe concentrations. The fluorescence was measured in 1 min intervals for a total of 60 min.

The measurement of real-time background subtracted fluorescence output are shown in the graphs as means $\pm$ SD (n = 3).

[0031] **Figure 21** are line graphs showing real-time fluorescence signals of (A) SrmCas12a and (B) CrmCas12a at varying DNA concentrations. Real-time PCR signals were monitored for both SrmCas12a and CrmCas12a across different target DNA concentrations (20, 2, 0.2, 0.1, and 0.02 copies/ μ L). The fluorescence was measured in 1 min intervals for a total of 60 min. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means $\pm$ SD (n = 3).

[0032] **Figure 22** is a bar graph showing end-pointy signals of SrmCas12a and CrmCas12a across different target DNA concentrations (20, 2, 0.2, 0.1, and 0.02 copies/ μ L). End-point fluorescence signal was measured after 60 min. Values are shown in the graphs as means $\pm$ SD (n = 3).

[0033] **Figure 23** is a picture showing the sensitivity of the combination of CRISPR-Cas12a enzyme method was assessed using plasmid copy numbers ranging from 0-20 copies/ μ L of fu plasmid. In the representation, C is the quality control line, and T indicates the positive line.

DETAILED DESCRIPTION

[0034] Existing technologies for Brucella detection in animals face substantial challenges, with large number of animals being infected worldwide, threatening the breeding industry, human health, and global livestock and meat trade while highlighting the shortcomings of current detection systems. The current reliance on serological detection, primarily based on the detection of livestock-specific antibodies, presents limitations due to variations in individual immune responses and potential cross-reactivity, leading to false-positives. PCR-based methods, despite their high sensitivity, encounter challenges such as the cost of instruments, the requirement for matching standard reagents, dependence on high-standard laboratories and professional technicians, and the lack of unified diagnostic standards. PCR's limitations are particularly pronounced in developing countries and rural areas with high brucellosis morbidity, where the scarcity of laboratory equipment and professional technicians hinders its implementation. Additionally, PCR is time-consuming and not suitable for

rapid on-site screening, further emphasizing the need for alternative and efficient diagnostic methods.

[0035] The urgent demand for faster and more effective methods for assaying nucleic acids in the field underscores the necessity for innovative solutions. The CRISPR-Cas12a based Point-of-Care Testing (POCT) detection system as disclosed herein is
5 transformative solution, providing enhanced sensitivity, specificity, speed, cost-effectiveness, and on-site testing capabilities.

[0036] The present disclosure integrates advanced molecular biology techniques, particularly CRISPR-Cas technology, to provide accurate and efficient diagnostics in the
10 context of animal health.

[0037] In one aspect, the present disclosure refers to a Cas12a protein having an amino acid sequence selected from the group consisting of SEQ ID NO: 3 (CrmCas12a) and SEQ ID NO: 4 (SrmCas12a) or a Cas12a protein of the same clade as the Cas12a protein having an amino acid sequence selected from the group consisting of SEQ ID NO: 3 and
15 SEQ ID NO: 4; wherein the Cas12a protein comprises a maltose-binding protein (MBP) tag having an amino acid sequence of SEQ ID NO: 72.

[0038] In general, the CRISPR system (Clustered Regularly Interspaced Short Palindromic Repeats) is a powerful tool for gene editing that allows scientists to precisely alter DNA sequences and modify gene function. It was originally discovered as a natural
20 defense mechanism in bacteria and archaea, where it helps these organisms fend off viral infections. CRISPR works via the following steps: (1) Recognition: When a virus invades a bacterial cell, the bacterium incorporates a segment of the viral DNA into its own genome at the CRISPR locus. This segment is known as a "spacer." (2) Transcription: The CRISPR locus is transcribed into a long RNA molecule, which is then processed into
25 shorter "guide RNAs" (gRNAs) that include sequences complementary to the viral DNA. (3) Targeting: The guide RNA forms a complex with a CRISPR-associated (Cas) protein. This complex can recognize and bind to the corresponding viral DNA sequence. (4) Cleavage: The Cas protein acts as a molecular scissor, cutting the viral DNA at the targeted location, thereby neutralizing the threat. Applications of CRISPR system
30 includes: (1) Gene Editing: CRISPR can be used to add, remove, or alter specific DNA sequences in the genome of living organisms. This has vast implications for research, medicine, and agriculture. (2) Gene Therapy: CRISPR is being explored as a potential

treatment for genetic disorders by correcting mutations at the DNA level. (3) Functional Genomics: Researchers use CRISPR to study gene function by creating targeted mutations and observing the resulting phenotypes. In the context of the present disclosure, CRISPR is used to detect target nucleic acid of a particular pathogen for pathogen or disease detection.

[0039] The CRISPR-Cas12a system, also known as Cpf1, is a CRISPR/Cas system that is known to have target-specific cleavage activity against double-stranded nucleic acids, and thus has high utility for gene editing in eukaryotic cells. The CRISPR-Cas12a system shows that 1) Cas12a protein has only one type of nucleic acid cleavage domain (RuvC domain, responsible for cleaving the target DNA), 2) guide RNA consists only of crRNA—Unlike the Cas9 system, which requires both crRNA and tracrRNA, the Cas12a system uses a single guide RNA (crRNA) to direct the Cas12a protein to the target DNA, and 3) generally, a few nucleotides (4-5) nucleotides adjacent to the protospacer adjacent motif (PAM) are required for the target sequence cleavage. It is characterized by cleaving nucleic acids in the form of a sticky end that causes an overhang. Cas12a protein can be largely divided into REC (RECOgnition) lobe (which is involved in recognizing and binding the guide RNA) and NUC (NUClease) lobe (which contains several domains responsible for DNA cleavage). NUC lobe is further divided into RuvC domain (which catalyzes the cleavage of the target DNA strand), NUC domain (involved in the cleavage process), WED domain (which includes subdomains WED II and WED III, which play a role in recognizing the PAM sequence), PI (PAM Interacting) domain (which is critical for recognizing the PAM sequence), and BH (Bridge Helix) domain (which plays a structural role in maintaining the integrity of the protein). The WED II, WED III, REC1, and PI domains are important for recognizing the PAM sequence, which is essential for the Cas12a protein to bind to the target DNA. The RuvC domain and the NUC domain are primarily responsible for cleaving the nucleic acid of the target sequence. The guide RNA of the CRISPR-Cas12a system, unlike the guide RNA of the CRISPR/Cas9 system, is composed of a single RNA molecule, which is called crRNA. The guide RNA (crRNA) in the CRISPR-Cas12a system includes a direct repeat (DR) and a guide domain. Specifically, the DR and the guide domain are sequentially connected from the 3' end to the 5' end of the guide RNA. The DR is a part involved in the interaction of the guide RNA with the Cas12a protein to form a complex, and the guide domain binds to the

nucleic acid of the target sequence so that the CRISPR-Cas12a system can achieve target-specific cleavage activity and incidental cleavage activity. The guide domain of the crRNA binds to the nucleic acid of the target sequence. It is responsible for directing the Cas12a protein to the specific DNA sequence that needs to be cleaved.

- 5 [0040] The CRISPR-Cas12a system has a target-specific nucleic acid cleavage activity, and two conditions are required to exhibit this target-specific nucleic acid cleavage activity: (1) Recognition of a Specific Nucleotide Sequence: The Cas12a protein must recognize a nucleotide sequence of a certain length in the nucleic acid. This sequence typically includes a protospacer adjacent motif (PAM) that is essential for the
- 10 initial binding of the Cas12a protein. (2) Complementary Binding of the Guide RNA: The guide RNA (crRNA) must have a sequence that can complementarily bind to the target sequence around the recognized nucleotide sequence. This binding ensures that the Cas12a protein is directed to the correct location on the nucleic acid. When the above two conditions are satisfied, 1) the Cas12a protein recognizes the nucleotide sequence of a
- 15 certain length, and 2) the guide domain complementarily binds to a portion of the sequence surrounding the nucleotide sequence of the certain length, nucleic acid cleavage activity is exhibited. At this time, the nucleotide sequence of a certain length recognized by the Cas12a protein is referred to as a Protospacer Adjacent Motif (PAM) sequence. The PAM sequence is a unique sequence determined according to the Cas12a protein.
- 20 Knowing the PAM sequence for a specific Cas12a protein allows researchers to design a CRISPR-Cas12a system that targets a specific nucleic acid sequence near the PAM. This enables precise gene editing by ensuring that the Cas12a protein can recognize and bind to the correct location on the DNA.

- [0041] The CRISPR-Cas12a system has a collateral cleavage activity in addition to
- 25 the target-specific nucleic acid cleavage activity. When the CRISPR-Cas12a system recognizes and binds to a target nucleic acid, it activates the Cas12a protein, which then indiscriminately cleaves single-stranded nucleic acids in the vicinity. This secondary cleavage activity can be utilized to detect the presence of a target nucleic acid in a sample, making the CRISPR-Cas12a system a powerful tool for diagnostic applications.

- 30 [0042] CRISPR detection is generally divided into 2 parts. The first step is usually the amplification of a target sequence, which specifically comprises the steps of amplifying target nucleic acid by utilizing the technologies of PCR, RT-PCR, RPA, LAMP and the

like; the second step is a trans-cleavage reaction of the Cas protein: adding a reporter, such as a non-specific single-stranded DNA or RNA reporter (ssDNA-reporter or ssRNA-reporter) with a fluorescent emitting group at one end and a fluorescent quenching group at one end into the system, and exciting the cleavage activity of the single-stranded DNA or RNA fluorescent reporter after the Cas protein-crRNA complex is combined with the target DNA, thereby generating a free fluorescent emitting group and emitting detectable fluorescence. In addition to this, CRISPR-Cas detection can be performed in a one-pot system, where target amplification, CRISPR-based recognition, and signal detection occur within a single reaction vessel without the need for multiple handling steps.

5 [0043] As used herein, the terms "Cas12a protein", "Cas12a", "Cpf1" and "Cas" used herein are used interchangeably to refer to RNA-guided nucleases including Cas12a protein or functionally active fragments thereof. Cas12a protein is an RNA-guided endonuclease protein of the CRISPR-Cas12a genome editing system. It forms a complex with a CRISPR RNA (crRNA) which guides it to a specific target sequence. Upon binding to the target sequence, Cas12a introduces a double-stranded break. DNA double-strand breaks can activate the inherent repair mechanisms in cells, non-homologous end joining (NHEJ) and homologous recombination (homologous recombination, HR), thereby repairing DNA damage in cells. During repair, site-specific editing is performed on that specific target sequence. Besides gene editing, Cas12a also detects target DNA sequence by binding to a specific target sequence on the DNA molecule guided by crRNA. Once bound, Cas12a activates a collateral cleavage activity, to indiscriminately cut single-stranded DNA nearby, including reporter molecules that can be designed to signal the presence of the target DNA when cleaved, thus enabling sensitive detection of the target sequence. In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. A target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. Cas12a can detect dsDNA or ssDNA directly. For RNA, it usually goes either through synthesis of double strand DNA then identification (RT-Cas12a one-pot detection) or through RT-RPA/LAP-T7 Cas12a detection (two pots detection). In some embodiments, a target sequence is located in the nucleus or cytoplasm

of a cell. In some embodiments, the target sequence may be within an organelle of a eukaryotic cell, for example, mitochondrion or chloroplast.

[0044] The present disclosure discloses a novel CRISPR-Cas12a system. The novel CRISPR-Cas12a system consists of a novel Cas12a protein, and a programmable guide RNA (crRNA) for the novel Cas12a protein. The present disclosure discloses an ortholog of the Cas12a protein that has not been previously reported.

[0045] As used herein, CrmCas12a and SrmCas12a refer to Cas12a protein variants. Both CrmCas12a and SrmCas12a can be used in the context of CRISPR technology for precise genome editing, diagnostics, and other biotechnological applications.

[0046] In one example, the Cas12a protein is CrmCas12, which has an amino acid sequence of SEQ ID NO: 3:

MKSSHHHHHHHHHGGSSMKIEEGKLVWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDII
FWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSIIYKDLLPNPPKTWEEIPALDKEL
KAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVNAGAKAGLTFLVDLIKHKHMNADTDYSIAEAAF
NKGETAMTINGPWAWSNIDTSKVNYGTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNK
DKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNN
NNNNNNNNLGIEENLYFQSNAMNLDSTGKYKLAKTLRFELRPVGRTLTFKEKFFEGDKRRDEDPYIVKELLDDGEHK
ALLERALSPPGELDWTALAAQHEDYRTSDKSKEAKDTLVAKQAEFRKKIVALFKKDVAFSSLTAPTPKDIFAAMRRK
YEDAGEEVPEALVTFSRFAGFFKGYQENRRNIYSDEAQAATAANRAVNENFPKFLEDVRIFRHIADTYPAILSDAEKEL
ETLLKGRTLASLFDPAAYGSFLSQSNIDFFNSVLGGFVPGEGEKTGKINEFVNLVYQQHEDGRTDRALSSLHPLYKQIL
SDRETQSLVPRALTEDEDVLAASRAMFEERLLGLATESGRVNVLLDQLALLSKIPAADGIWIDGAEISRVSMDDLGSW
NALGSLMESAAEVRFASESTEKKRLAAIDKWMKRSVYALSELADLRQETDTGSMIVDVSKLWKGPVAVQRFDAARA
AVAVVPLLSPPVENGPKLRERKDDVGIKAALDAVLVLFVFKPLHAGEGLDRDEAFYGAFDLSLYASLDGFFVPLYN
KVRNYLTKKPGETERIKLMFDNPTLADGWDQNKEDNTCVLFCRDGLYYLVGMNPKETDFSKLSGPETPGCYRKM
VYKLLPGPNKMLPHVFFSKKGVETFHPSNLSLEKYHVGAYKKGSGFDLAFCKRLIDFYKASISAHPDWWSKDFRFSPT
ASFNGIDEFYREVSEQGYRISFENIPYETVDRLVEEGKLCFLQWKNDFSKASTGRPNLHTQYWNVAVFAPENLQDVVIK
LNGEAEIFYRPSIKENVFRHKVGEKVMVNSRGADGSPMPESVHGELFRHFNGSKEPLSDEAKWWIQSGNLLVVKDVA
HEIVKDRRYTEDKFSFHVPLTINFKQPDAPARFNEQVRDFLRGNDVNVIGIDRGERNLIYTLVDQRGNLLEQRSFNVV
SRTRRDGVVEVQTDYQSKLAQAEKDRAAARLTWSEIGAIDKLEGYLSAVVHEIAKMMVERNAIVLEDLNFGFKRGRF
RIERQVYQKFEKALIDKLVFKDRGMAEPGGTLRGYQLTDAFESFERIGKQTGFLFYVPAGYTSKIDPTTGFTNLFN
TKKCTNAAGVRDFFTAFDISRWDAAARHAFADFYNFKTSQESHRTAWTVYSANRRLVFDKDARTEKEIDPTAILLD
ALKKRGVSVTDGFDLKALLADTEPSKANAGFFRDVYVFDRTLQMRNSRAGEDYIESPVNLGGQKFFDSRKADATLP
KDADANGAYHIALKGVLLEENIAENRTANGSADLKIEHRDWFRFAQELAARKFK

[0047] In another example, the Cas12a protein is SrmCas12, which has an amino acid sequence of SEQ ID NO: 4:

MKSSHHHHHHHHHGGSSMKIEEGKLVWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDII
FWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSIIYKDLLPNPPKTWEEIPALDKEL
KAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVNAGAKAGLTFLVDLIKHKHMNADTDYSIAEAAF
NKGETAMTINGPWAWSNIDTSKVNYGTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNK
DKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNN
NNNNNNNNLGIEENLYFQSNAMKTIEKFCGQKNGYSITLRNRLVPIGKTEENILKLKLLDKDMERARAYNEVKKLIDD
FHRVFIEDVLTNVFEWGPLYDQFDLFQTEKDKQKNKIKKELEQLQSLMRKKIVEAFKKDKRFEKLFKKELLTELVPA

VIKNDETGTITDKESALEIFKGFATYFTGFHQNRQNMYSGEAQSTAISNRIVNENFPKFYANVKVFEYLNNNYPEINETE
 KSLAGFLRGKKLADIFSSEAFNSVMSQSGIDFYNTVIGGIADAGSKKIQGLNEIINLTSQQLPSEEEKYKLKKKMTILYKQ
 ILSDRNTASFIPVSFEKSEDVYESVKHFKEEILDKVITNTKKLFDSVDYDLGQIYVPAKEVTEFSLKVFGNWSIIHNGLFLL
 EQDMAKKALTEKQIEALKKEIAKKDFPLTEIQNAYERWTKENDVTVEKNVKDYFKLVELRTDEKSKEKISVDILSNIDRL
 5 YSKINFENKENLIQEKDAATPIKNLLDEVQNLHYHLKLVDRGEEQKDTDFYSKYDEIMQVLSEIPLYNKVRNFVTKKP
 NDIKKVKLNFDCPTLANGWDLNKESSNDAILRKKGIYYLGIFNPKDKPKFEQCTVEDSCYEKMIYKLLPGANKMLPKV
 FFSTKGRETFQPPEDLILGYEEGKHKKGDDFDKAFMHKLIDWFKYAINQHEDWKNFNFKFSPTESYEDMSGFYNEVEL
 QGYKITFSKVSEKCINSLVDSGKLFQIYNKDYSAGKDGNGATGKKNLHTLYWENLFSEENLRNVCFKLNGEAELEF
 10 WRDANPNVKAVCHKKDSVLVNRTRDGSIPPEIYQEIYKYKNPEKQEKDFTLSKEAKELLESGETTVCKKAKFDITKDR
 HFTQQTYLHFCPITMNFKAAEITGRKFNERVQEILRNNEPVKIGLDRGERHLIYLSLINQKGEIELQKTLNIVEQIRNDKT
 VSVNYQEKLQKEGERDRARKSWQAISNIKEGYSNIVHEIAQLMVENNAIVVMEDLNFGFKRGRFPVERQVYQK
 FENMLIEKLNLYVFKGKNVTEPGGVNLAYQLADKAANVSDVGKQCGWIFYPASYTSKIDPKTGANLFYTAGLTNIEK
 KKDDFFDKFDSILFDRKLDSEFVSFDYSNFSNADFNKKWNVYSRGERLIFSKEAESTISVNPTENLTLFDKQGIWNSE
 15 ENFIDQIHAVQAERDNVPFYDGLYRSFTVILQMRNSIPNSSRQEDDYLSVMAEDGNFYDSRVEAAKGKDEKGKWISK
 LPVDADANGAYHIALKGLYLLKNNFNLDKGFENISNADWFRFAQEKEYAK

[0048] In SEQ ID NO: 3 and SEQ ID NO: 4 shown above, the underlined part denotes 10HIS, 10-histidine affinity tag for protein purification through affinity chromatography. It is a sequence of ten histidine residues used in protein purification processes. The histidine tag binds to nickel or cobalt ions, allowing for easy separation of the tagged protein from a mixture. The bold part denotes Maltose Binding Protein (MBP), which is often used as a fusion partner to enhance the solubility of recombinant proteins and facilitate their purification. The underlined and italic part denotes Tobacco Etch Virus Protease (TEV) which is a protease recognition site. The bold and italic part denotes Cas12a gene.

[0049] As used herein, the term "clade" refers to a group of organisms (or genes or proteins, in this case) that have evolved from a common ancestor. Specifically, it means that the Cas12a proteins as disclosed herein (such as CrmCas12a and SrmCas12a) and any other Cas12a proteins within the same clade share a common evolutionary origin and thus have similar genetic and structural characteristics.

[0050] As used herein, "Maltose binding protein (MBP)" or "MBP tag" refers to a protein expression tag, used to significantly enhance the solubility of many proteins. MBP is one of the most well-known and accomplished means of tagging proteins expressed in microbes. Fusion of a target protein to MBP permits its one-step purification using amylose resin. The MBP-fusion protein will bind to amylose resin while other proteins flow through. The MBP-protein fusion can then be eluted from the resin with maltose. The purified fusion protein can then be treated with a specific protease to cleave the MBP-

tag from the target protein and the target protein can be separated from the MBP tag by affinity chromatography.

[0051] The Cas12a protein as disclosed herein comprises the MBP tag as disclosed herein. In one example, the MBP tag comprises an amino acid sequence of SEQ ID NO:

5 72:

MKIEEGKLVINGDKGYNGLAIEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIFWAHDFGGYAQSGLLAIEIT
PDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIA
ADGGYAFKYENGKYDIKDVGDNAGAKAGLTFLVDLIKHKHMNADTDYSIAEAFNKGETAMTINGPWAWSNIOTSKVN
YGVTVLPTFKGQPSKPFVGLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATME
10 NAQKGEIMPNIQMSAFWYAVRTAVINAASGRQTVDEALKDAQT

[0052] Advantageously, the incorporation of the MBP tag into Cas12a enzymes in this study not only improved enzyme solubility but also increased the final yield without a significant reduction in enzyme activity. Notably, the observed comparable activity between enzymes with and without the MBP tag allows streamlining the enzyme
15 production process by omitting the MBP removal step, resulting in significant savings in both time and resources.

[0053] The DNA sequence encoding SEQ ID NO: 3 is SEQ ID NO: 1. The DNA sequence encoding SEQ ID NO: 4 is SEQ ID NO: 2. The DNA sequence encoding SEQ ID NO: 72 is SEQ ID NO: 71. These DNA and amino acid sequences are shown in **Table**

20 1.

[0054] As used herein, the terms "polypeptide", "peptide", and "protein" used herein are used interchangeably in this application to refer to a polymer of amino acid residues. The term applies to amino acid polymers in which one or more amino acid residues are an artificial chemical analog of the corresponding naturally occurring amino acid, and to
25 naturally occurring amino acid polymers. The terms "polypeptide", "peptide", "amino acid sequence" and "protein" may also include modified forms including, but not limited to, glycosylation, lipid linkage, sulfation, gamma carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation. Unless otherwise stated, amino acid sequences disclosed herein are written in the N-terminal to C-terminal direction using one-letter
30 notation. For example, when expressed as RNVP, it means a peptide in which arginine, asparagine, valine, and proline are sequentially connected from the N-terminal to the C-terminal. The method of notation of each amino acid is as follows: Alanine (Ala, A); Arginine (Arg, R); Asparagine (Asn, N); Aspartic acid (Asp, D); Cysteine (Cys, C); Glutamic acid (Glu, E); Glutamine (Gln, Q); Glycine (Gly, G); Histidine (His, H);

Isoleucine (Ile, I); Leucine (Leu, L); Lysine (Lys K); Methionine (Met, M); Phenylalanine (Phe, F); Proline (Pro, P); Serine (Ser, S); Threonine (Thr, T); Tryptophan (Trp, W); Tyrosine (Tyrosine; Tyr, Y); and Valine (Val, V).

[0055] As used herein, the terms "polynucleotide", "nucleic acid sequence",
5 "nucleotide sequence" or "nucleic acid fragment" are used interchangeably and are single-
or double-stranded RNA or DNA polymers, optionally containing synthetic unnatural, or
altered nucleotide bases. Nucleotides are referred to by their single letter designations as
follows: "A" for adenosine or deoxyadenosine (for RNA or DNA, respectively), "C" for
cytidine or deoxycytidine, "G" for guanosine or deoxyguanosine, "U" means uridine, "T"
10 means deoxythymidine.

[0056] In another aspect, the present disclosure refers to a CRISPR RNA (crRNA) for
detecting Brucella, characterized in that the crRNA is selected from the group consisting
of the following sequences:

- S-BC1 – sequence shown in SEQ ID NO: 8;
- 15 S-BP1 – sequence shown in SEQ ID NO: 9;
- S-OM1 – sequence shown in SEQ ID NO: 10;
- C-BC1 – sequence shown in SEQ ID NO: 11;
- C-BP1 – sequence shown in SEQ ID NO: 12;
- C-OM1 – sequence shown in SEQ ID NO: 13;
- 20 S-BC2 – sequence shown in SEQ ID NO: 14;
- S-BC3 – sequence shown in SEQ ID NO: 15;
- S-BC4 – sequence shown in SEQ ID NO: 16;
- S-BC5 – sequence shown in SEQ ID NO: 17;
- S-BC6 – sequence shown in SEQ ID NO: 18;
- 25 S-BC7 – sequence shown in SEQ ID NO: 19;
- S-BC8 – sequence shown in SEQ ID NO: 20;
- C-BC2 – sequence shown in SEQ ID NO: 21;
- C-BC3 – sequence shown in SEQ ID NO: 22;
- C-BC4 – sequence shown in SEQ ID NO: 23;
- 30 C-BC5 – sequence shown in SEQ ID NO: 24;
- C-BC6 – sequence shown in SEQ ID NO: 25;
- C-BC7 – sequence shown in SEQ ID NO: 26;

- C-BC8 – sequence shown in SEQ ID NO: 27;
 S-BP2 – sequence shown in SEQ ID NO: 28;
 S-BP3 – sequence shown in SEQ ID NO: 29;
 S-BP5 – sequence shown in SEQ ID NO: 30;
 5 S-BP6 – sequence shown in SEQ ID NO: 31;
 S-BP7 – sequence shown in SEQ ID NO: 32;
 C-BP2 – sequence shown in SEQ ID NO: 33;
 C-BP3 – sequence shown in SEQ ID NO: 34;
 C-BP5 – sequence shown in SEQ ID NO: 35;
 10 C-BP6 – sequence shown in SEQ ID NO: 36;
 C-BP7 – sequence shown in SEQ ID NO: 37;
 S-OM2 – sequence shown in SEQ ID NO: 38;
 S-OM3 – sequence shown in SEQ ID NO: 39;
 S-OM4 – sequence shown in SEQ ID NO: 40;
 15 C-OM2 – sequence shown in SEQ ID NO: 41;
 C-OM3 – sequence shown in SEQ ID NO: 42;
 C-OM4 – sequence shown in SEQ ID NO: 43;
 S-BP8 – sequence shown in SEQ ID NO: 44;
 S-BP9 – sequence shown in SEQ ID NO: 45;
 20 C-BP8 – sequence shown in SEQ ID NO: 46; and
 C-BP9 – sequence shown in SEQ ID NO: 47;
 wherein the crRNA comprises a direct repeat (DR) and a guide domain (protospacer),
 wherein the DR and the protospacer are connected in the 5' to 3' direction of the crRNA,
 and the crRNA is configured to target a nucleic acid of *Brucella* with a predetermined
 25 target sequence and form a complex with the Cas12a protein as disclosed herein.
 [0057] As used herein, the term "CRISPR RNA (crRNA)" refers to a crucial
 component of the CRISPR-Cas system. crRNA is transcribed from the CRISPR locus,
 which contains a series of short, repetitive DNA sequences (direct repeat) interspaced
 with unique sequences called protospacers or spacers. These spacers are derived from
 30 previous infections by viruses or other mobile genetic elements. crRNA consists of a
 repeat-derived sequence (direct repeat) and a spacer sequence (protospacer). The repeat-
 derived sequence is conserved, while the spacer sequence is complementary to the target

DNA sequence. The degree of complementarity between a crRNA and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting examples of which include the Smith- Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows- Wheeler Transform (e.g. the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies, ELAND (Illumina, San Diego, Calif.), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). The primary role of crRNA is to guide the Cas nuclease (such as Cas12a) to the target DNA during the interference step of the CRISPR-Cas immune response. The crRNA forms a complex with the Cas protein (such as Cas12a), and this complex then binds to the target DNA sequence, leading to its cleavage and subsequent degradation.

[0058] As used herein, the term “specific binding” or “binding” can refer to non-covalent physical association of a first and a second moiety (such as a crRNA and a target sequence, or a CRISPR-Cas12a complex and a target sequence, or hybridization between complementary nucleic acids) wherein the association between the first and second moieties is at least 2 times as strong, at least 5 times as strong as, at least 10 times as strong as, at least 50 times as strong as, at least 100 times as strong as, or stronger than the association of either moiety with most or all other moieties present in the environment in which binding occurs. Binding of two or more entities may be considered specific if the equilibrium dissociation constant, K_d , is 10^{-3} M or less, 10^{-4} M or less, 10^{-5} M or less, 10^{-6} M or less, 10^{-7} M or less, 10^{-8} M or less, 10^{-9} M or less, 10^{-10} M or less, 10^{-11} M or less, or 10^{-12} M or less under the conditions employed, e.g., under physiological conditions such as those inside a cell or consistent with cell survival. In some embodiments, specific binding can be accomplished by a plurality of weaker interactions (e.g., a plurality of individual interactions, wherein each individual interaction is characterized by a K_d of greater than 10^{-3} M).

[0059] Protospacer is the sequence within the crRNA that is complementary to the target sequence. It is derived from the DNA of previous viral invaders and is used to recognize and bind to the target sequence during subsequent infections. In one example, the protospacer has 20-26 nucleotides. In another example, the protospacer has 20-22, 21-

23, 22-24, 23-25, 24-26, 20, 21, 22, 23, 24, 25, or 26 nucleotides. In one example, the protospacer has 20-24 nucleotides. In another example, the protospacer has 20 nucleotides. In another example, the protospacer has 21 nucleotides. In another example, the protospacer has 22 nucleotides. In another example, the protospacer has 23
5 nucleotides. In another example, the protospacer has 24 nucleotides.

[0060] Advantageously, the incorporation of the present novel CrmCas12a and SrmCas12a enzymes into the experimental framework has facilitated a pivotal advancement in the field of disease detection, such as Brucella detection. Specifically, the introduction of innovative protospacers and crRNAs designed for Brucella detection
10 represents a significant leap forward, demonstrating superior activity when compared to previously reported crRNAs. This strategic molecular refinement translates into a noteworthy improvement in the precision and sensitivity of the present detection system. By harnessing the enhanced capabilities of the present CrmCas12a and SrmCas12a enzymes, the standard for pathogen detection methodologies has been elevated,
15 promising heightened accuracy and efficacy in identifying pathogens such as Brucella.

[0061] In addition, advantageously, the present disclosure showcases a significant technical advancement, revealing a substantial augmentation in the trans-cleavage activity of CrmCas12a and SrmCas12a enzymes through the elongation of protospacers from 20 to 24 during Brucella detection. This deliberate modification imparts a
20 heightened efficacy to the CRISPR-Cas12a system, representing a valuable improvement for achieving superior diagnostic accuracy and sensitivity in molecular detection methodologies.

[0062] In one example, one of the crRNAs disclosed herein (having sequences of SEQ ID NO: 8-47) is used for forming complex with the Cas12a protein to detect Brucella. In
25 another example, the combination of more than one, for example, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, or more crRNAs are used for forming complex with the Cas12a protein to detect Brucella. Advantageously, the innovative use of combination of crRNAs for detection of Brucella substantially elevated the signal for the target genes. This
30 enhancement, achieved by cleaving an increased number of ssDNA probes, holds promise for eliminating the need for pre-amplification, particularly in scenarios where target DNA

concentrations are low. No existing CRISPR-Cas based detection system uses combined crRNAs for the detection of *Brucella*.

[0063] The term "complex" or "CRISPR-Cas12a complex" as used herein refers to the complex formed by the combination of CRISPR RNA (crRNA) and the Cas12a protein, i.e. a ribonucleoprotein complex. The crRNA can hybridize with the target sequence and thus the complex recognizes and cleaves polynucleotides that hybridize to the crRNA.

[0064] Thus, in the context of the formation of a CRISPR-Cas12a complex, "target sequence" refers to a polynucleotide such as DNA or RNA targeted by a crRNA, e.g., a polynucleotide sequence complementary to the crRNA, wherein the hybridization between the target sequence and the crRNA will promote Cas12a to exert its activity, such as the activity of cutting the target sequence. Full complementarity is not required, as long as there is sufficient complementarity to cause hybridization and facilitate Cas12a to exert its activity. A target sequence can include any polynucleotide, such as DNA or RNA. In some examples, the target sequence is located in the nucleus or cytoplasm of a cell. In some examples, the target sequence may be located in an organelle of a eukaryotic cell such as the mitochondria or chloroplast. In some examples, the target sequence can be a sequence encoding a gene product (e.g. protein) or a non-coding sequence (e.g. regulatory polynucleotide or dummy DNA). For the crRNA comprising SEQ ID NO: 8-47 as disclosed herein, they can bind to a nucleic acid of *Brucella* with a predetermined target sequence and form a complex with the Cas12a protein as disclosed herein. In one example, the nucleic acid of *Brucella* is DNA. In another example, the nucleic acid of *Brucella* is RNA. Cas12a can detect dsDNA or ssDNA directly. For RNA, it usually goes either through synthesis of double strand DNA then identification (RT-Cas12a one-pot detection) or through RT-RPA/LAP-T7 Cas12a detection (two pots detection).

[0065] The detection system as disclosed herein comprising the Cas12a protein and crRNAs as disclosed herein, can be used for detection of various *Brucella* species comprising for example, *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella canis*, *Brucella ovis*, *Brucella neotomae*, *Brucella microti*, *Brucella ceti*, *Brucella pinnipedialis* or a combination thereof.

[0066] In one example, the nucleic acid of *Brucella* to be detected by the CRISPR-Cas12a complex as disclosed herein comprises a sequence selected from the group consisting of SEQ ID NO: 5 (BSCP31), SEQ ID NO: 6 (bp26), and SEQ ID NO: 7

(Omp2a). These are examples of target sequences of *Brucella*, which can be bound by the CRISPR-Cas12a complex formed by the Cas12a protein and crRNA as disclosed herein, to therefore trigger the activity of Cas12a protein to cut the target nucleic acid sequence of *Brucella* as disclosed herein. While previously reported detection methods have employed bp26 and Omp2a genes (Xu J et al, 2022; Dang S et al, 2023), there exists no prior development of a CRISPR-Cas based detection specifically designed for Bcsp31, a gene uniquely specific to *Brucella* species.

[0067] In one example, only CrmCas12a comprising the amino acid sequence of SEQ ID NO: 3 is used to form complex with the one or more crRNAs disclosed herein to detect *Brucella*. In another example, only SrmCas12a, comprising the amino acid sequence of SEQ ID NO: 4 is used to form complex with the one or more crRNAs disclosed herein to detect *Brucella*. In another example, both CrmCas12a comprising the amino acid sequence of SEQ ID NO: 3 and SrmCas12a comprising the amino acid sequence of SEQ ID NO: 4 are used to form complex with the one or more crRNAs disclosed herein to detect *Brucella*.

[0068] CrmCas12a, SrmCas12a, or the combination of both CrmCas12a and SrmCas12a achieves a low detection limit for the *Brucella* nucleic acid. In one example, the nucleic acid of *Brucella* is DNA and the DNA to be detected by the Cas12a and crRNA disclosed herein is 10 copies/ μ L or less, for example, as low as about 0.1 copies/ μ L (which is the Limit of detection, LOD). In another example, the *Brucella* DNA to be detected by the Cas12a and crRNA disclosed herein is 0.1-10 copies/ μ L, 0.1-2 copies/ μ L, 0.5-2.5 copies/ μ L, 1-3 copies/ μ L, 1.5-3.5 copies/ μ L, 2-4 copies/ μ L, 2.5-4.5 copies/ μ L, 3-5 copies/ μ L, 3.5-5.5 copies/ μ L, 4-6 copies/ μ L, 4.5-6.5 copies/ μ L, 5-7 copies/ μ L, 5.5-7.5 copies/ μ L, 6-8 copies/ μ L, 6.5-8.5 copies/ μ L, 7-9 copies/ μ L, 7.5-9.5 copies/ μ L, 8-10 copies/ μ L, less than 10 copies/ μ L, less than 15 copies/ μ L, less than 20 copies/ μ L, less than 30 copies/ μ L, less than 40 copies/ μ L, less than 50 copies/ μ L, less than 60 copies/ μ L, less than 70 copies/ μ L, less than 80 copies/ μ L, less than 90 copies/ μ L, less than 100 copies/ μ L, or less than 150 copies/ μ L. In another example, the *Brucella* DNA to be detected by the Cas12a and crRNA disclosed herein is about 0.1 copies/ μ L, about 0.2 copies/ μ L, about 0.3 copies/ μ L, about 0.4 copies/ μ L, about 0.5 copies/ μ L, about 0.6 copies/ μ L, about 0.7 copies/ μ L, about 0.8 copies/ μ L, about 0.9 copies/ μ L, about 1 copies/ μ L, about 1.5 copies/ μ L, about 2 copies/ μ L, about 2.5 copies/ μ L, about 3

copies/ μ L, about 3.5 copies/ μ L, about 4 copies/ μ L, about 4.5 copies/ μ L, about 5 copies/ μ L, about 5.5 copies/ μ L, about 6 copies/ μ L, about 6.5 copies/ μ L, about 7 copies/ μ L, about 7.5 copies/ μ L, about 8 copies/ μ L, about 8.5 copies/ μ L, about 9 copies/ μ L, about 9.5 copies/ μ L, about 10 copies/ μ L, about 11 copies/ μ L, about 12 copies/ μ L, about 13 copies/ μ L, about 14 copies/ μ L, about 15 copies/ μ L, about 16 copies/ μ L, about 17 copies/ μ L, about 18 copies/ μ L, about 19 copies/ μ L, about 20 copies/ μ L, about 30 copies/ μ L, about 40 copies/ μ L, about 50 copies/ μ L, about 60 copies/ μ L, about 70 copies/ μ L, about 80 copies/ μ L, about 90 copies/ μ L, about 100 copies/ μ L, about 110 copies/ μ L, about 120 copies/ μ L, about 130 copies/ μ L, about 140 copies/ μ L, about 150 copies/ μ L, or more. In another example, no *Brucella* DNA amplification is required before detection using the disclosed CRISPR-Cas12a-based detection system and method.

[0069] In another example, the nucleic acid is an RNA or a DNA from a pathogen such as *Brucella*. In another example, the RNA is reverse-transcribed to complementary DNA (cDNA), and then isothermally amplified using methods such as Recombinase Polymerase Amplification (RPA) or Loop-Mediated Isothermal Amplification (LAMP), before subjected to the detection method as disclosed herein. The loop-mediated isothermal amplification (LAMP) is a simple, fast, precise and low-cost gene amplification method, which can amplify nucleic acid in a short time (usually within one hour) under the isothermal condition (60-65 °C). In another example, the nucleic acid is a cDNA. In another example, the target DNA is selected from the group consisting of single-stranded DNA, double-stranded DNA, or a combination thereof. In another example, the target DNA is a synthetic or naturally occurring DNA. In another example, the target DNA comprises a wild-type or mutant DNA. In another example, the target DNA includes DNA obtained by reverse transcription or amplification of RNA, such as cDNA.

[0070] Advantageously, the present disclosure showcases superior detection of low concentrations of *Brucella* genes using combined enzymes (both CrmCas12a and SrmCas12a), a pivotal feature in the present amplification-free CRISPR-Cas12a-based detection system. The combination of two enzymes for detection of *Brucella* significantly amplifies the detection signal such as a fluorescence signal and holds promise for eliminating the need for pre-amplification, particularly in scenarios where target DNA

concentrations are low. No other existing CRISPR-Cas based detection system used combination of two enzymes for single target/disease detection.

[0071] In another aspect, the present disclosure refers to a detection kit for Brucella, wherein the detection kit comprises the crRNA as disclosed herein.

5 [0072] As used herein, "kit" means a collection of at least one component constituting the kit. Together, the components constitute a functional unit for a given purpose. Individual member components may be physically packaged together or separately. For example, a kit comprising an instruction for using the kit may or may not physically include the instruction with other individual member components. Instead, the instruction
10 can be supplied as a separate member component, either in a paper form or an electronic form which may be supplied on computer readable memory device or downloaded from an internet website, or as recorded presentation.

[0073] In one example, the detection kit may further comprise the Cas12a protein as disclosed herein.

15 [0074] In another example, the detection kit may further comprise a detecting agent comprising a probe nucleic acid, wherein the detecting agent generates a detectable signal when the probe nucleic acid is cleaved by the Cas12a protein. In one example, the probe nucleic acid is a fluorescent probe, wherein the 5' end of the fluorescent probe sequence is labeled with a fluorescent group, and the 3' end of the fluorescent probe sequence is
20 labeled with a quenching group. In some examples, the fluorescent group is selected from the group consisting of FAM, VIC, HEX, TRT, Cy3, Cy5, ROX, JOE and Texas Red, and the quenching group is selected from the group consisting of IABkFQ, DABCYL, MGB, BHQ-1, BHQ-2 and BHQ-3. The effect of the fluorescent probe in the CRISPR detection system is that after the probe is nonspecifically cut by Cas12a, the
25 fluorescent group and the quenching group are separated, the quenching group removes the blocking effect on the fluorescent group, and the change of fluorescence intensity can be detected by means of a fluorescent signal detection device under the condition of light irradiation with specific wavelength. In another example, /56-FAM/CCCCCCCC/3IABkFQ/ is used as a fluorescent probe. A fluorescence signal
30 generated upon cutting of a fluorescent probe can be detected by means of a fluorescence detection equipment such as a fluorescence spectrometer, fluorometer, fluorescence

microscopes, microplate reader, real-time PCR, flow cytometry, and fluorescence scanners.

[0075] In another example, the probe nucleic acid is a colorimetric probe. In some examples, the colorimetric probe comprises FAM and Biotin. In another example, the colorimetric probe can be detected by a lateral flow strip. Lateral flow strips use gold nanoparticles or colored latex beads conjugated with detection molecules. Cleavage events by CRISPR-Cas lead to a visible color change on the strip. In another example, /56-FAM/CCCCCCCC/3Biotin/ is used as a colorimetric probe. A colorimetric signal can be directly visually detected by naked eyes without an instrument, or with a colorimetric reader such as a colorimeter, spectrophotometer, color analyzer, chromameter, etc.

[0076] In another example, CRISPR-Cas cleavage events can be transduced into an electrochemical signal using modified electrodes or nanoparticle-based sensors.

[0077] In another example, the detection kit further comprises nucleic acid amplification primers, wherein the nucleic acid amplification primers are PCR, RPA or LAMP primer pairs.

[0078] The disclosed CRISPR-Cas12a based detection system is compatible with lateral flow strips for visual detection of the detectable signal, making it highly suitable for point-of-care testing applications. This combination of high sensitivity, specificity, and ease of use makes the method particularly valuable for detecting low-abundance nucleic acids in diagnostics and other applications.

[0079] In another example, the Cas12a protein and the crRNA are in the form of a ribonucleoprotein complex. In another example, the concentration of the Cas12a protein used to form ribonucleoprotein complex is 200-3200 nM, for example, about 200 nM, about 250 nM, about 300 nM, about 350 nM, about 400 nM, about 450 nM, about 500 nM, about 550 nM, about 600 nM, about 650 nM, about 700 nM, about 750 nM, about 800 nM, about 850 nM, about 900 nM, about 950 nM, about 1000 nM, about 1050 nM, about 1100 nM, about 1150 nM, about 1200 nM, about 1250 nM, about 1300 nM, about 1350 nM, about 1400 nM, about 1450 nM, about 1500 nM, about 1550 nM, about 1600 nM, about 1650 nM, about 1700 nM, about 1750 nM, about 1800 nM, about 1850 nM, about 1900 nM, about 1950 nM, about 2000 nM, about 2050 nM, about 2100 nM, about 2150 nM, about 2200 nM, about 2250 nM, about 2300 nM, about 2350 nM, about 2400 nM, about 2450 nM, about 2500 nM, about 2550 nM, about 2600 nM, about 2650 nM,

about 2700 nM, about 2750 nM, about 2800 nM, about 2850 nM, about 2900 nM, about 2950 nM, about 3000 nM, about 3050 nM, about 3100 nM, about 3150 nM, or about 3200 nM. In another example, the concentration of crRNA used to form ribonucleoprotein complex is 200-3200 nM, for example, about 200 nM, about 250 nM, about 300 nM, about 350 nM, about 400 nM, about 450 nM, about 500 nM, about 550 nM, about 600 nM, about 650 nM, about 700 nM, about 750 nM, about 800 nM, about 850 nM, about 900 nM, about 950 nM, about 1000 nM, about 1050 nM, about 1100 nM, about 1150 nM, about 1200 nM, about 1250 nM, about 1300 nM, about 1350 nM, about 1400 nM, about 1450 nM, about 1500 nM, about 1550 nM, about 1600 nM, about 1650 nM, about 1700 nM, about 1750 nM, about 1800 nM, about 1850 nM, about 1900 nM, about 1950 nM, about 2000 nM, about 2050 nM, about 2100 nM, about 2150 nM, about 2200 nM, about 2250 nM, about 2300 nM, about 2350 nM, about 2400 nM, about 2450 nM, about 2500 nM, about 2550 nM, about 2600 nM, about 2650 nM, about 2700 nM, about 2750 nM, about 2800 nM, about 2850 nM, about 2900 nM, about 2950 nM, about 3000 nM, about 3050 nM, about 3100 nM, about 3150 nM, or about 3200 nM. In another example, the concentration of the Cas12a protein and the concentration of crRNA used to form ribonucleoprotein complex is each about 800 nM.

[0080] In another aspect, the present disclosure refers to a method of detecting a nucleic acid of *Brucella* in a sample using the detection kit as disclosed herein, wherein the method comprises the following steps: incubating the sample containing the nucleic acid of *Brucella* with the Cas12a protein and the crRNA, wherein when the nucleic acid of *Brucella* is present in the sample, collateral cleavage activity of the Cas12a protein is induced to generate a detectable signal when the probe nucleic acid is cleaved.

[0081] As used herein, collateral cleavage activity of a Cas12a protein refers to its ability to indiscriminately cleave single-stranded nucleic acids in the vicinity once it has been activated by recognizing and binding to a specific target nucleic acid sequence. This activity is also known as trans-cleavage activity.

[0082] In one example, the nucleic acid of *Brucella* in the sample can be amplified using a technology selected from the group consisting of Polymerase Chain Reaction (PCR), Recombinase Polymerase Amplification (RPA), Loop-Mediated Isothermal Amplification (LAMP), and Rolling Circle Amplification (RCA) prior to carrying out the detection method as disclosed herein. In another example, the method of detecting a

nucleic acid of *Brucella* in a sample as disclosed herein does not comprise pre-amplifying the nucleic acid of *Brucella* in the sample.

[0083] In another example, the method of detecting a nucleic acid of *Brucella* in a sample further comprising using a detection method selected from the group consisting of a colorimetric detection method, a fluorescent detection method and an electrochemical detection method to detect the detectable signal which is a colorimetric signal, a fluorescent signal, or an electrochemical signal. In another example, the colorimetric detection method uses a lateral flow strip and a colorimetric probe comprising FAM and Biotin. In another example, the fluorescent detection method uses a fluorescent probe, wherein the fluorescence signal is measured using a fluorometer, a microplate reader, or Real-Time PCR. In another example, the electrochemical detection method transduces CRISPR-Cas cleavage event to an electrochemical signal using a modified electrode or a nanoparticle-based sensor.

[0084] In one example, the Cas12a protein having an amino acid sequence of SEQ ID NOs: 3 (CrmCas12a) is used to detect *Brucella*. In another example, the Cas12a protein having an amino acid sequence of SEQ ID NO: 4 (SrmCas12a) is used to detect *Brucella*. In another example, both the Cas12a protein having an amino acid sequence of SEQ ID NOs: 3 (CrmCas12a) and the Cas12a protein having an amino acid sequence of SEQ ID NO: 4 (SrmCas12a) are used to detect *Brucella*.

[0085] In one example, the method of detecting a nucleic acid of *Brucella* in a sample as disclosed herein is performed at a temperature of 20-55 °C. In another example, the method of detecting a nucleic acid of *Brucella* in a sample as disclosed herein is performed at a temperature selected from the group consisting of 20-25 °C, 25-30 °C, 30-35 °C, 35-40 °C, 40-45 °C, 45-50 °C, 50-55 °C, about 20 °C, about 25 °C, about 30 °C, about 35 °C, about 40 °C, about 45 °C, about 50 °C, and about 55 °C. In another example, the method of detecting a nucleic acid of *Brucella* in a sample as disclosed herein uses CrmCas12a protein at a temperature of 20-55 °C, 25-30 °C, 30-35 °C, 35-40 °C, 40-45 °C, 45-50 °C, 50-55 °C, for example, about 20 °C, about 25 °C, about 30 °C, about 35 °C, about 40 °C, about 45 °C, about 50 °C, or about 55 °C. In another example, the method of detecting a nucleic acid of *Brucella* in a sample as disclosed herein uses SrmCas12a protein at a temperature of 20-55 °C, 25-30 °C, 30-35 °C, 35-40 °C, 40-45 °C, 45-50 °C, 50-55 °C, for example, about 20 °C, about 25 °C, about 30 °C, about 35 °C, about 40

°C, about 45 °C, about 50 °C, or about 55 °C. Advantageously, when SrmCas12a is used, it maintains a substantial proportion (80.3%) of its peak activity at 25 °C, which is close to room temperature. When CrmCas12a is used, it maintains a substantial proportion (72.5%) of its peak activity at 25 °C, which is close to room temperature. This characteristic is especially advantageous for point-of-care diagnostics performed in resource-limited settings, where precise temperature control may not be feasible. The enzyme's robust activity at 25 °C highlights its adaptability, ensuring reliable performance in environments without temperature regulation.

[0086] As disclosed herein, the nucleic acid of Brucella in the sample can be pre-amplified using a technology such as Loop-Mediated Isothermal Amplification (LAMP) in a one-pot system of both amplification and CRISPR-Cas12a based detection. The Cas12a protein as disclosed herein has low activity at about 65 °C. The low activity at 65 °C and substantial increase after 30 minutes when the temperature is reduced to 37 °C offers several distinct advantages in the context of the one-pot LAMP-Cas12a system. At 60-65 °C, the low activity of the Cas12a protein as disclosed herein such as SrmCas12a ensures that a minimal amount of target DNA is cleaved. By keeping the cleavage activity low during the high-temperature phase, the system preserves a larger pool of intact target DNA, which remains available for the subsequent LAMP amplification. This is particularly beneficial because the LAMP reaction relies on an ample amount of target DNA for effective amplification. With more intact substrate available for amplification, the LAMP reaction can proceed with greater efficiency, leading to higher yields of the amplified product. This ensures that the amplification step has enough substrate to generate detectable levels of DNA, which is crucial for the overall sensitivity and robustness of the assay. In contrast, enzymes with higher activity at elevated temperatures, such as commercial LbaCas12a, would cleave more target DNA during the high-temperature phase, potentially reducing the amount of substrate available for LAMP. This could lead to inefficient amplification or suboptimal assay performance. By delaying Cas12a activation and allowing more substrate to accumulate before cleavage occurs, Cas12a protein as disclosed herein such as SrmCas12a ensures that the one-pot system maximizes both amplification and detection, offering a more efficient and reliable diagnostic process.

[0087] In another example, the Cas12a protein as disclosed herein can detect nucleic acid of *Brucella* which comprises a sequence selected from the group consisting of SEQ ID NO: 5 (BSCP31), SEQ ID NO: 6 (bp26), and SEQ ID NO: 7 (Omp2a). These are examples of target sequences of *Brucella*, which can be bound by the CRISPR-Cas12a complex formed by the Cas12a protein and crRNA as disclosed herein, to therefore trigger the activity of Cas12a protein to cut the target sequence. While previously reported detection methods have employed bp26 and Omp2a genes, there exists no prior development of a CRISPR-Cas based detection specifically designed for Bcp31, a gene uniquely specific to *Brucella* species.

[0088] In one example, the nucleic acid of *Brucella* to be detected by the CRISPR-Cas12a detection method as disclosed herein is DNA and the DNA is 10 copies/ μ L or less, for example, as low as about 0.1 copies/ μ L. In another example, the DNA to be detected by the Cas12a and crRNA disclosed herein is 0.1-10 copies/ μ L, 0.1-2 copies/ μ L, 0.5-2.5 copies/ μ L, 1-3 copies/ μ L, 1.5-3.5 copies/ μ L, 2-4 copies/ μ L, 2.5-4.5 copies/ μ L, 3-5 copies/ μ L, 3.5-5.5 copies/ μ L, 4-6 copies/ μ L, 4.5-6.5 copies/ μ L, 5-7 copies/ μ L, 5.5-7.5 copies/ μ L, 6-8 copies/ μ L, 6.5-8.5 copies/ μ L, 7-9 copies/ μ L, 7.5-9.5 copies/ μ L, 8-10 copies/ μ L, less than 10 copies/ μ L, less than 15 copies/ μ L, less than 20 copies/ μ L, less than 30 copies/ μ L, less than 40 copies/ μ L, less than 50 copies/ μ L, less than 60 copies/ μ L, less than 70 copies/ μ L, less than 80 copies/ μ L, less than 90 copies/ μ L, less than 100 copies/ μ L, or less than 150 copies/ μ L. In another example, the DNA to be detected by the Cas12a and crRNA disclosed herein is about 0.1 copies/ μ L, about 0.2 copies/ μ L, about 0.3 copies/ μ L, about 0.4 copies/ μ L, about 0.5 copies/ μ L, about 0.6 copies/ μ L, about 0.7 copies/ μ L, about 0.8 copies/ μ L, about 0.9 copies/ μ L, about 1 copies/ μ L, about 1.5 copies/ μ L, about 2 copies/ μ L, about 2.5 copies/ μ L, about 3 copies/ μ L, about 3.5 copies/ μ L, about 4 copies/ μ L, about 4.5 copies/ μ L, about 5 copies/ μ L, about 5.5 copies/ μ L, about 6 copies/ μ L, about 6.5 copies/ μ L, about 7 copies/ μ L, about 7.5 copies/ μ L, about 8 copies/ μ L, about 8.5 copies/ μ L, about 9 copies/ μ L, about 9.5 copies/ μ L, about 10 copies/ μ L, about 11 copies/ μ L, about 12 copies/ μ L, about 13 copies/ μ L, about 14 copies/ μ L, about 15 copies/ μ L, about 16 copies/ μ L, about 17 copies/ μ L, about 18 copies/ μ L, about 19 copies/ μ L, about 20 copies/ μ L, about 30 copies/ μ L, about 40 copies/ μ L, about 50 copies/ μ L, about 60 copies/ μ L, about 70 copies/ μ L, about 80 copies/ μ L, about 90 copies/ μ L, about 100 copies/ μ L, about 110

copies/ μ L, about 120 copies/ μ L, about 130 copies/ μ L, about 140 copies/ μ L, about 150 copies/ μ L, or more. In another example, no DNA amplification is required before detection using the disclosed CRISPR-Cas12a-based detection system and method.

[0089] Advantageously, the present disclosure showcases superior detection of low concentrations of *Brucella* genes using combined enzymes (both CrmCas12a and SrmCas12a), a pivotal feature in the present amplification-free CRISPR-Cas12a-based detection system. The combination of two enzymes for detection of *Brucella* significantly amplifies the detection signal such as a fluorescence signal and holds promise for eliminating the need for pre-amplification, particularly in scenarios where target DNA concentrations are low. No other existing CRISPR-Cas based detection system used combination of two enzymes for single target/disease detection.

[0090] In another example, the *Brucella* to be detected by the method as disclosed herein is selected from the group consisting of *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella canis*, *Brucella ovis*, *Brucella neotomae*, *Brucella microti*, *Brucella ceti*, *Brucella pinnipedialis* or a combination thereof.

[0091] In another example, the sample comprising the *Brucella* is a biological sample obtained from a tissue sample, saliva, blood, plasma, milk, sera, stool, urine, sputum, mucous, lymph, synovial fluid, cerebrospinal fluid, ascites, pleural effusion, seroma, pus, nasal swab specimen, or swab of skin or a mucosal membrane surface or a combination thereof. In another example, the biological sample is a crude sample. In another example, the target molecule (such as nucleic acid (e.g. DNA) of *Brucella*) is not purified or amplified from the biological sample.

[0092] In another example, the method of detecting a nucleic acid of *Brucella* in a sample as disclosed herein is performed in a buffer composition comprising an osmolyte and protein stabilizer (such as betaine), a detergent (such as Tween), a metal ion (such as $MgCl_2$), and an organic co-solvent (such as DMSO and glycerol). In another example, the concentration of the detergent such as Tween 20 in the buffer is 2.5–5 mM, for example, about 2.5 mM, about 3 mM, about 3.5 mM, about 4 mM, about 4.5 mM, or about 5 mM.

[0093] In another aspect, the present disclosure refers to a CRISPR-Cas12a composition comprising the Cas12a protein as disclosed herein, and a CRISPR RNA (crRNA), wherein the crRNA comprises a direct repeat (DR) and a guide domain (protospacer), wherein the DR and the guide domain are connected in the 5' to 3' direction

of the crRNA, and the crRNA is configured to target a nucleic acid of a predetermined target sequence and form a complex with the Cas12a protein as disclosed herein.

[0094] The CRISPR-Cas12a composition can target and detect other target sequences besides those of Brucella, by using different crRNAs which are complementary to the target sequences. In one example, the crRNA is the crRNA for detecting Brucella as disclosed herein, comprising SEQ ID NO: 8-47.

[0095] In another example, the CRISPR-Cas12a composition further comprises a detecting agent comprising a probe nucleic acid, wherein the detecting agent generates a detectable signal when the probe nucleic acid is cleaved. In another example, the probe nucleic acid is a fluorescent probe, wherein the 5' end of the fluorescent probe sequence is labelled with a fluorescent group, and the 3' end is labelled with a quenching group; or a colorimetric probe. In some examples, the fluorescent group is selected from the group consisting of FAM, VIC, HEX, TRT, Cy3, Cy5, ROX, JOE and Texas Red, and the quenching group is selected from the group consisting of IABKFQ, DABCYL, MGB, BHQ-1, BHQ-2 and BHQ-3. In some examples, the colorimetric probe comprises FAM and Biotin, and is for detection by a lateral flow strip.

[0096] In another example, the probe nucleic acid has a concentration of 400-3200 nM, for example about 400 nM, about 500 nM, about 800 nM, about 1000 nM, about 1200 nM, about 1600 nM, about 2000 nM, about 2400 nM, about 2800 nM, about 3000 nM, or about 3200 nM.

[0097] In another example, the Cas12a protein and the crRNA are in the form of a ribonucleoprotein complex.

[0098] In another aspect, the present disclosure refers to a method of detecting a nucleic acid having a predetermined target sequence in a sample using the CRISPR-Cas12a composition as disclosed herein, comprising: incubating the sample with the CRISPR-Cas12a composition, wherein when the nucleic acid having the predetermined target sequence is present in the sample, the collateral cleavage activity of the CRISPR-Cas12a composition is induced to generate a detectable signal when the probe nucleic acid is cleaved.

[0099] In one example, the method as disclosed above comprises pre-amplifying the nucleic acid having a predetermined target sequence in the sample, using a technology selected from the group consisting of Polymerase Chain Reaction (PCR), Recombinase

Polymerase Amplification (RPA), Loop-Mediated Isothermal Amplification (LAMP), and Rolling Circle Amplification (RCA). In another example, the method as disclosed above does not comprise pre-amplifying the nucleic acid having a predetermined target sequence in the sample.

5 [00100] In another example, the method as disclosed above further comprises using a detection method selected from the group consisting of a colorimetric detection method, a fluorescent detection method and an electrochemical detection method to detect the detectable signal which is a colorimetric signal, a fluorescent signal, or an electrochemical signal. In another example, the colorimetric detection method uses a lateral flow strip and
10 a colorimetric probe comprising FAM and Biotin. In another example, the fluorescent detection method uses a fluorescent probe, wherein the fluorescence signal is measured using a fluorometer, a microplate reader, or Real-Time PCR. In another example, the electrochemical detection method transduces CRISPR-Cas cleavage event to an electrochemical signal using a modified electrode or a nanoparticle-based sensor.

15 [00101] In another example, one crRNA is used to detect the nucleic acid having a predetermined target sequence. In another example, more than one crRNAs are used to detect the nucleic acid having a predetermined target sequence, for example, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, or more crRNAs are used to detect the nucleic acid
20 having a predetermined target sequence.

[00102] The crRNA can be designed to specifically bind to a predetermined target sequence of a pathogen, including but not limited to Brucella. The following adaptations of the crRNA and/or Cas12a protein can be explored to suit the purpose of detecting various types of pathogens (including but not limited to Brucella):

- 25
- The sequence of protospacers in crRNAs is adaptable and can be modified to target alternative locations within a gene of a pathogen (not limited to Brucella), providing flexibility to enhance trans-cleavage activity of the enzymes.
 - The sequence of protospacers in crRNAs can be further customized to target alternative genes of a pathogen, allowing for versatile adaptation to different
30 genetic regions.

- Adjusting the length of protospacers in crRNAs (for example, 14-32 nucleotides, preferably 20-24 nucleotides) is possible to optimize and improve the trans-cleavage activity of the enzymes, offering tunability for enhanced performance.
- Modification of the Direct Repeat sequence in crRNAs is an option to fine-tune and boost the trans-cleavage activity of the enzymes, providing an additional parameter for optimization.
- Utilizing the existing protospacer sequences to design crRNAs for other Cas12a enzymes enables compatibility and flexibility for diverse Cas12a variants, expanding the range of detectable *Brucella* strains.
- Different combinations of crRNAs can be employed for detection purposes, allowing for customization based on specific diagnostic requirements.
- The system allows for the use of single or multiple Cas12a enzymes in combination with single or multiple crRNAs for the detection of *Brucella* or other pathogens in one reaction, offering versatility and higher sensitivity in experimental design.
- Other isothermal enzymes can be substituted for RPA without compromising the functionality of the CRISPR-Cas12a system, providing alternatives for amplification.
- The CRISPR-Cas12a system presented here is adaptable to work both with and without pre-amplification of the target, offering versatility in experimental setups and facilitating streamlined procedures for rapid on-site detection.

[00103] In another example, both the Cas12a protein having an amino acid sequence of SEQ ID NOs: 3 (CrmCas12a) and the Cas12a protein having an amino acid sequence of SEQ ID NO: 4 (SrmCas12a) are used to detect the nucleic acid having a predetermined target sequence. In another example, the nucleic acid having a predetermined target sequence is DNA. In another example, the DNA is 10 copies/ μ L or less, for example, as low as 0.1 copies/ μ L. In another example, the DNA to be detected by the Cas12a and crRNA disclosed herein is 0.1-10 copies/ μ L, 0.1-2 copies/ μ L, 0.5-2.5 copies/ μ L, 1-3 copies/ μ L, 1.5-3.5 copies/ μ L, 2-4 copies/ μ L, 2.5-4.5 copies/ μ L, 3-5 copies/ μ L, 3.5-5.5 copies/ μ L, 4-6 copies/ μ L, 4.5-6.5 copies/ μ L, 5-7 copies/ μ L, 5.5-7.5 copies/ μ L, 6-8 copies/ μ L, 6.5-8.5 copies/ μ L, 7-9 copies/ μ L, 7.5-9.5 copies/ μ L, 8-10 copies/ μ L, less than 10 copies/ μ L, less than 15 copies/ μ L, less than 20 copies/ μ L, less than 30 copies/ μ L, less

than 40 copies/ μ L, less than 50 copies/ μ L, less than 60 copies/ μ L, less than 70 copies/ μ L, less than 80 copies/ μ L, less than 90 copies/ μ L, less than 100 copies/ μ L, or less than 150 copies/ μ L. In another example, the DNA to be detected by the Cas12a and crRNA disclosed herein is about 0.1 copies/ μ L, about 0.2 copies/ μ L, about 0.3 copies/ μ L, about 0.4 copies/ μ L, about 0.5 copies/ μ L, about 0.6 copies/ μ L, about 0.7 copies/ μ L, about 0.8 copies/ μ L, about 0.9 copies/ μ L, about 1 copies/ μ L, about 1.5 copies/ μ L, about 2 copies/ μ L, about 2.5 copies/ μ L, about 3 copies/ μ L, about 3.5 copies/ μ L, about 4 copies/ μ L, about 4.5 copies/ μ L, about 5 copies/ μ L, about 5.5 copies/ μ L, about 6 copies/ μ L, about 6.5 copies/ μ L, about 7 copies/ μ L, about 7.5 copies/ μ L, about 8 copies/ μ L, about 8.5 copies/ μ L, about 9 copies/ μ L, about 9.5 copies/ μ L, about 10 copies/ μ L, about 11 copies/ μ L, about 12 copies/ μ L, about 13 copies/ μ L, about 14 copies/ μ L, about 15 copies/ μ L, about 16 copies/ μ L, about 17 copies/ μ L, about 18 copies/ μ L, about 19 copies/ μ L, about 20 copies/ μ L, about 30 copies/ μ L, about 40 copies/ μ L, about 50 copies/ μ L, about 60 copies/ μ L, about 70 copies/ μ L, about 80 copies/ μ L, about 90 copies/ μ L, about 100 copies/ μ L, about 110 copies/ μ L, about 120 copies/ μ L, about 130 copies/ μ L, about 140 copies/ μ L, about 150 copies/ μ L, or more.

[00104] In another example, the sample comprising the predetermined target sequence is a biological sample obtained from a tissue sample, saliva, blood, plasma, milk, sera, stool, urine, sputum, mucous, lymph, synovial fluid, cerebrospinal fluid, ascites, pleural effusion, seroma, pus, nasal swab specimen, or swab of skin or a mucosal membrane surface or a combination thereof. In another example, the biological sample is a crude sample. In another example, the target molecule comprising predetermined target sequence is not purified or amplified from the sample.

[00105] In another example, the nucleic acid having the predetermined target sequence in the sample belongs to a microbe selected from a bacterium, a virus, an archaea, a protozoa, a fungi, an algae, a slime mode, a lichen, and a prion. In another example, the nucleic acid having the predetermined target sequence in the sample belongs to a plant, an animal, an insect, a microbe as disclosed herein, or a combination thereof.

[00106] In another example, the virus is selected from a group consisting of a double-stranded DNA virus, a single-stranded DNA virus, a double-stranded RNA virus, a positive sense RNA virus, a negative sense RNA virus, and a retrovirus.

- [00107] In another example, the virus is selected from a group consisting of a coronavirus, an Ebola virus, measles, SARS, Chikungunya virus, Marburg, MERS, Dengue, Lassa, influenza, rhabdovirus, HIV, a hepatitis virus (including hepatitis A, B, C, D, or E), an influenza virus (including an influenza A or influenza B), a human
- 5 respiratory syncytial virus, Sudan ebola virus, Bundibugyo virus, Tai Forest ebola virus, Reston ebola virus, Achimota virus, Aedes flavivirus, Aguacate virus, Akabane virus, Alethinophid reptarenavirus, Allpahuayo mammarenavirus, Amapari mmarenavirus, Andes virus, Apoi virus, Aravan virus, Aroa virus, Arumwot virus, Atlantic salmon paramyxovirus, Australian bat lyssavirus, Avian bomavirus, Avian metapneumovirus,
- 10 Avian paramyxoviruses, penguin or Falkland Islandsvirus, BK polyomavirus, Bagaza virus, Banna virus, Bat herpesvirus, Bat sapovirus, Bear Canon mammarenavirus, Beilong virus, Betacoronavirus, Betapapillomavirus 1-6, Bhanja virus, Bokeloh bat lyssavirus, Boma disease virus, Bourbon virus, Bovine hepacivirus, Bovine parainfluenza virus 3, Bovine respiratory syncytial virus, Brazoran virus, Bunyamwera virus,
- 15 Caliciviridae virus, California encephalitis virus, Candiru virus, Canine distemper virus, Canine pneumovirus, Cedar virus, Cell fusing agent virus, Cetacean morbillivirus, Chandipura virus, Chaoyang virus, Chapare mammarenavirus, Colobus monkey papillomavirus, Colorado tick fever virus, Cowpox virus, Crimean-Congo hemorrhagic fever virus, Culex flavivirus, Cupixi mammarenavirus, Dengue virus, Dobrava-Belgrade
- 20 virus, Donggang virus, Dughe virus, Duvenhage virus, Eastern equine encephalitis virus, Entebbe bat virus, Enterovirus A-D, European bat lyssavirus 1-2, Eyach virus, Feline morbillivirus, Fer-de- Lance paramyxovirus, Fitzroy River virus, Flaviviridae virus, Flexal mammarenavirus, GB virus C, Gairo virus, Gemycircularvirus, Goose paramyxovirus SF02, Great Island virus, Guanarito mammarenavirus, Hantaan virus,
- 25 Hantavirus Z10, Heartland virus, Hendra virus, Hepatitis A/B/C/E, Hepatitis delta virus, Human bocavirus, Human coronavirus, Human endogenous retrovirus K, Human enteric coronavirus, Human genital-associated circular DNA virus- 1, Human herpesvirus 1-8, Human mastadenovirus A-G, Human papillomavirus, Human parainfluenza virus 1-4, Human paraechovirus, Human picomavirus, Human smacovirus, Ikoma lyssavirus, Ilheus
- 30 virus, Influenza A-C, Ippy mammarenavirus, Irkut virus, J-virus, JC polyomavirus, Japanese encephalitis virus, Junin mammarenavirus, KI polyomavirus, Kadipiro virus, Kamiti River virus, Kedougou virus, Khujand virus, Kokobera virus, Kyasanur forest

disease virus, Lagos bat virus, Langat virus, Lassa mammarenavirus, Latino mammarenavirus, Leopards Hill virus, Liao ning virus, Ljungan virus, Lloviu virus, Louping ill virus, Lujo mammarenavirus, Luna mammarenavirus, Lunk virus, Lymphocytic choriomeningitis mammarenavirus, Lyssavirus Ozemoe, MSS12Y225

5 virus, Machupo mammarenavirus, Mamastrovirus 1, Manzanilla virus, Mapuera virus, Marburg virus, Mayaro virus, Measles virus, Menangle virus, Mercadeo virus, Merkel cell polyomavirus, Middle East respiratory syndrome coronavirus, Mobala mammarenavirus, Modoc virus, Moijang virus, Mokolo virus, Monkeypox virus, Montana myotis leukoencephalitis virus, Mopeia lassa virus reassortant 29, Mopeia

10 mammarenavirus, Morogoro virus, Mossman virus, Mumps virus, Murine pneumonia virus, Murray Valley encephalitis virus, Nariva virus, Newcastle disease virus, Nipah virus, Norwalk virus, Norway rat hepacivirus, Ntaya virus, O'nyong-nyong virus, Oliveros mammarenavirus, Omsk hemorrhagic fever virus, Oropouche virus, Parainfluenza virus 5, Parana mammarenavirus, Parramatta River virus, Peste-des-petits-

15 ruminants virus, Pichande mammarenavirus, Picomaviridae virus, Pirital mammarenavirus, Piscihepevirus A, Porcine parainfluenza virus 1, porcine rubulavirus, Powassan virus, Primate T-lymphotropic virus 1-2, Primate erythroparvovirus 1, Punta Toro virus, Puumala virus, Quang Binh virus, Rabies virus, Razdan virus, Reptile bomavirus 1, Rhinovirus A-B, Rift Valley fever virus, Rinderpest virus, Rio Bravo virus,

20 Rodent Torque Teno virus, Rodent hepacivirus, Ross River virus, Rotavirus A-I, Royal Farm virus, Rubella virus, Sabia mammarenavirus, Salem virus, Sandfly fever Naples virus, Sandfly fever Sicilian virus, Sapporo virus, Sathuperi virus, Seal anellovirus, Semliki Forest virus, Sendai virus, Seoul virus, Sepik virus, Severe acute respiratory syndrome-related coronavirus, Severe fever with thrombocytopenia syndrome virus,

25 Shamonda virus, Shimoni bat virus, Shuni virus, Simbu virus, Simian torque teno virus, Simian virus 40-41, Sin Nombre virus, Sindbis virus, Small anellovirus, Sosuga virus, Spanish goat encephalitis virus, Spondweni virus, St. Louis encephalitis virus, Sunshine virus, TTV-like mini virus, Tacaribe mammarenavirus, Taila virus, Tamana bat virus, Tamiami mammarenavirus, Tembusu virus, Thogoto virus, Thottapalayam virus, Tick-

30 borne encephalitis virus, Tioman virus, Togaviridae virus, Torque teno canis virus, Torque teno douroucouli virus, Torque teno felis virus, Torque teno midi virus, Torque teno sus virus, Torque teno tamarin virus, Torque teno virus, Torque teno zalophus virus,

Tuhoko virus, Tula virus, Tupaia paramyxovirus, Usutu virus, Uukuniemi virus, Vaccinia virus, Variola virus, Venezuelan Vesicular stomatitis Indiana virus, WU Polyomavirus, Wesselsbron virus, West Caucasian bat virus, West Nile virus, Western equine encephalitis virus, Whitewater Arroyo mammarenavirus, Yellow fever virus, Yokose virus, Yug Bogdanovac virus, Zaire ebolavirus, Zika virus, or Zygosaccharomyces bailii virus Z viral sequence, Canine Parvovirus, Canine Distemper Virus, Canine Coronavirus, Canine Influenza Virus, Feline Herpesvirus, Feline Calicivirus, Feline Leukemia Virus, Feline Immunodeficiency Virus, Canine Adenovirus - Type 1 and Type 2, Canine Herpesvirus, Tobacco Mosaic Virus, Potato Virus Y, Tomato Spotted Wilt Virus, Cucumber Mosaic Virus, Potato Virus X, Barley Yellow Dwarf Virus, Bean Common Mosaic Virus, Maize Dwarf Mosaic Virus, Soybean Mosaic Virus, Citrus Tristeza Virus, Rice Yellow Mottle Virus, Plum Pox Virus, Turnip Mosaic Virus, Aphid-Transmitted Yellow Dwarf Viruses, Cotton Leaf Curl Virus, Papaya Ringspot Virus, Cassava Mosaic Virus, Rice Tungro Virus, Apple Mosaic Virus, Sugarcane Mosaic Virus or a combination thereof.

[00108] In another example, the bacteria is a Gram-positive bacterium or a Gram-negative bacterium selected from the group consisting of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus anthracis*, *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Clostridium difficile* and *Clostridium tetani*, *Corynebacterium diphtheria*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Chlamydia trachomatis*, *Yersinia pestis*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Proteus mirabilis*, *Enterobacter cloacae*, *Serratia marcescens*, *Helicobacter pylori*, *Salmonella enteritidis*, *Salmonella typhi*, and *Vibrio cholera*, *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella canis*, *Brucella ovis*, *Brucella neotomae*, *Mycoplasma mycoides*, *Clostridium perfringens*, *Mycobacterium avium* *Salmonella* spp., *Pasteurella multocida*, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Yersinia pestis*, *Francisella tularensis*, *Bacillus anthracis*, *Coxiella burnetii*, *Listeria monocytogenes*, *Clostridium chauvoei*, *Campylobacter* spp., *Chlamydia psittaci*, *Anaplasma marginale*, *Leptospira* spp. Causes leptospirosis,

Bordetella bronchiseptica, *Clostridium perfringens*, *Staphylococcus intermedius*, *Bartonella henselae*, *Chlamydophila felis*, *Mycoplasma* spp, *Pasteurella multocida*, *Erwinia amylovora*, *Xanthomonas* spp., *Xanthomonas axonopodis*, *Ralstonia solanacearum*, *Pectobacterium* and *Dickeya* spp., *Agrobacterium tumefaciens*,
 5 *Pseudomonas syringae*, *Pseudomonas syringae* pv. *lachrymans*, *Xanthomonas campestris* pv. *campestris*, *Xanthomonas oryzae*, *Clavibacter michiganensis*, *Xylella fastidiosa*, *Pantoea* spp., *Burkholderia glumae*, *Xanthomonas citri*, *Xanthomonas translucens*, *Xanthomonas fragariae*, *Xanthomonas hortorum*, *Clavibacter michiganensis*, *Pseudomonas syringae* or a combination thereof.

- 10 [00109] In another example, the nucleic acid is a RNA or a DNA from a virus as disclosed herein, or a RNA or a DNA from a bacteria as disclosed herein; the RNA is reverse-transcribed to complementary DNA (cDNA), and then isothermally amplified using methods such as Recombinase Polymerase Amplification (RPA) or Loop-Mediated Isothermal Amplification (LAMP), before subjected to the method as disclosed herein. In
 15 another example, the nucleic acid is a cDNA. In another example, the target DNA is selected from the group consisting of single-stranded DNA, double-stranded DNA, or a combination thereof. In another example, the target DNA is a synthetic or naturally occurring DNA. In another example, the target DNA comprises a wild-type or mutant DNA. In another example, the target DNA includes DNA obtained by reverse
 20 transcription or amplification of RNA, such as cDNA.

[00110] In another example, the method is used for identifying and characterizing genetic variations selected from the group consisting of single nucleotide polymorphism (SNP) analysis and allelic discrimination.

[00111] **Industrial Applicability**

- 25 [00112] It will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.
 [00113] The CRISPR-Cas12a enzymes presented here can be used for a wide range of
 30 nucleic acid detections including bacterial and virus detections, for examples, detection of *Brucella*.

[00114] As used in this application, the singular form “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a primer” includes a plurality of primers, including mixtures and combinations thereof.

[00115] As used herein, the term “comprising” means “including.” Variations of the word “comprising”, such as “comprise” and “comprises,” have correspondingly varied meanings. Thus, for example, a composition “comprising” X may consist exclusively of X or may include one or more additional unrecited components.

[00116] As used herein, the term “about” in the context of concentration of a substance, size of a substance, length of time, or other stated values means +/- 5% of the stated value, or +/- 4% of the stated value, or +/- 3% of the stated value, or +/- 2% of the stated value, or +/- 1% of the stated value, or +/- 0.5% of the stated value.

[00117] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[00118] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms “comprising”, “including”, “containing”, etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions

embodied herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00119] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form
5 part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00120] Unless otherwise indicated, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the
10 invention belongs.

[00121] Other embodiments are within the following claims and non-limiting examples.

EXAMPLES

15 [00122] Non-limiting examples of the disclosure will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the disclosure.

[00123] Example 1: Nucleic acid preparation

[00124] The efficient production of recombinant proteins on a large scale with superior
20 quality continues to pose a bottleneck, impeding progress in biochemical and functional studies as well as industrial applications. To address this, the open reading frames of Cas12a enzymes CrmCas12a and SrmCas12a were subjected to codon optimization according to codon usage in the E. coli using GenSmart™ Codon Optimization (GenScript).

25 [00125] In addition, Cas12a proteins were fused to the prokaryotic maltose-binding protein (MBP) to improve solubility, stability, proper folding of proteins. CrmCas12a and SrmCas12a sequences were N-terminally tagged with a His10-MBP-TEV (His10, 10-histidine affinity tag for protein purification through affinity chromatography) and MBP and TEV protease recognition site. The nucleic acid and protein sequences are provided
30 in **Table 1**.

[00126] **Table 1.** The sequence of 10HIS-MBP-TEV-Cas12a Gene

SEQ ID NO: 1	CrmCas 12a	ATGAAAAGTTACATCACCAACCACCACCATCACCATCACACGGTTCTAGCATGAAAATCGA AGAAGGCAAACCTGGTCATTTGGATCAATGGCGATAAAGGTTACAATGGCTGGCGGAGGT GGGTAAGAAATTTGAGAAAGACACAGGTATCAAGGTGACGGTGGAGCACCCGGACAAGC TGGAGGAAAAGTTTCGCGAGGTGGCGGCGACCCGGCGATGGTCCGGATATTATCTTTTGGG CGCACGACAGGTTTCGGCGGTTACGCTCAATCGGGCCTACTGGCAGAGATCACCCCGGAT AAGGCGTTTCAGGATAAACTATACCCGTTTACCTGGGATGCGGTTTCGGTATAACCGTAAAT TAATCGCGTACCCGATCGCGGTGGAAGCATTGTCCCTGATCTACAACAAGGACTTGCTCC CGAATCCACCGAAAAAGTGGGAAGAGATCCCGGCTTGGATAAGGAACTAAAAGCGAAG GGTAATCTGCATTGATTTTAACCTGCAGGAACCTTATTTACCTGGCCGTTAATTCGG CGGACGGCGGTTATGCTTTCAAGTACGAAAACGGTAAGTATGATATCAAGGACGTGGGCG TTGACAACGCTGGTGCAGAAAGCTGGCCCTCACCTTTTGGTGGACCTGATCAAGAATAAGC ACATGAATGCGGACACAGACTATTCTATCGCGGAAGCGGCTTCAACAAGGTTGAGACA GCAATGACCATTATGGCCCTTGGGCATGGTCCAACATTGATACCAAGCAAGGTGAACCTAC GGCCTTACCGTTCTCCGACCTTCAAAGGTCAGCCATCCAAGCCGTTTGTGGTGTGA GTGCCGGCATTAAACGCAGCTTCCCAAACAAGGAGCTGGCCAAAGAATTTCTGGAGAACT ATCTGCTGACCGACGAGGCTTGGAGGCGGTGAACAAGGATAAACCGCTGGGTGCGGTA GCGCTGAAGAGCTACGAAGAGGAGCTGGCGAAGGACCCGCGCATCGCTGCCAACGATGTA AAAACGCCCCAGAAAGGTGAGATCATGCCGAACATCCCGCAAATGAGCGCATTTTGGTATG CCGTCCGTACCCGCTGATTAAACGCCGCGAGTGGTCTGTCAGACCGCTGGATGAAGCGCTG AAAGACGCGCAAACCAAGCTCAAGCAACAACAACAATAACAACAACAACAACCTGG GCATCGAGGAGAACTGTACTTCCAGTCAATGCTATGAACCTGGATAGCTTACCGGAA GTACAAATTAGCGAAGAGCTGCGCTTCAACTGCGTCCGTTGGCGGATGCGTGAAC CTTTAAGGAGAAATTTTGGAGGGTGATAAGCGTAGAGACGAGGATTACCCGATTGTTAAG GAACGTGTAGACGGCGAACAACAAGCACTGCTGGAAGAGCGCTGTGTACCCACCGCGG TGAGTTAGACTGGACCGCACTGGCTCAAGCGCATGAGGACTACCGTACGAGCGATAAGA GCAAGGAGGCGAAGGACACTTAGTAGCCAAGCAGGACAGATTCGTAAGAAAAATTTGTG CGTTGTTTAAAAAGATGTGGCGTTCTCGTCCCTGACGGCCCCGACCCCAAAGGACATCT TCGCGGCTATGCGTAAGCGTTATGAAGACGCTGGTGAAGAAAGTCCCGGAAGCGTTAGTGA CGTTTAGCGGTTTCGCGGTTTCTTTAAGGGTACCAAGAAAACCGCGCAATATCTATTTC CGATGAAGCGCAAGCGACCGCGCTGCGAACCCTGCTGTAACGAAATTTTCCGAAGTT CCTGGAGGATGTTGCGATCTTTCGTCATATCGCAGACACGATACCGGCAATTTCTGAGCGAT GCGGAAAAAGAAATGGAGACGCTCCTTAAGGGCGCTACCTTGGCGAGCGCTGTTCCGACC GGCCCGCTTACGGTTCTGTTCTGAGCCAACTAATCATCGACTTTTTCAACAGCGTTCTAGGC GGTCTTTGTTCCGGGTGAGGGCGAAAAGACCCGTGGGATTAAAGATTGTTAATCTGTAC AGGCAGCAGCATGAGGATGGTGTGACGGACCGCGGCTTATCCTCCTGCACCGCGTGTGA AAGCAGCATTTCTTCCGATCGCGAACCAGAGCCTGGTCCGAGAGCCCTGGAGACTG ACGAAGATGTTCTGGCAAGCCTGCGTGCAATGTTTGAAGAGCGCCTCCTGGGTCTGGCGA CCGAAAGCGGCGGTGTTAAGCTTCTGGATGATCTTCAAGCGCTTCTGTCCAAAATTCGGG CGCGCGATGGGATCTGGATCGACGGCGCAGAGATCTCCCGTGTCTATGGATGCTGTGG GCAGCTGGAATGCAATTAGGTAGCCTTATGGAAGCGCGCGGGAAGTTCGTTTTCGAAGCG AGAGCACCGAATAAAGCTGTGGCGGCCATCGACAAGTGGATGAACCTGAGCGTGTGAC GCGTTGTCTGAATTGGCGGACCTGCGCCAAGAAACGGATACGGGACGATGATTGTGGA CGTCTCGAAATATGGAAGGACCGGTTGCAAGTTCAGCGTTTCGACGCGCGCTGTTGCTGCG AGTGGCCGTTGTCTCGCGCTGCTGTCCAGCCCTGTGCCGAAACCGTCCCAAGCTGCG TGAACGTGAAGGACGACGTTGGTATTATCAAAGCAGCCCTCGACGCGGTGCTCGATGTTCT GCACTTGTGAACCGCTGCAACGACGCGAAGGTCTCGACGCGCAGGCTTCTATG TGCGTTGCACTCCCTGTACGCGAGCTTGGATGGGTTTGTTCGCTGTACAATAAGGTGCG CAACTACCTGACAAAGAAGCCTGGCGAGACCGAGCGTATCAAATTGATGTTTCGACAACCC GACGCTAGCGGATGGTTGGGATCAAAACAAAGAAAGGACAATACCTGTGTTTATTCTG CCGTGATGCGCTGTACTATCTGGGTGTATGAACCCAAAGGAGAAGACCGATTTTCAGCAA ACTGAGCGGTCCAGAAACCCCGGTTGTTATCGTAAGATGGTTTATAAATTGCTGCCGGG TCCAAATAAAATGCTGCCGATGTTTTTTTTTCCAAAGAGGGGGTGAACAGCTTCCATCCG TCTAACAGCTTGCTGGAGAAATACCAGTGGGCGCGTACAAAAGGGGACGCGGTTTCGAT CTGGCGTTCTGCCGCAAAATTGATTGATTTTACAAAGCGTCCATTAGCGCGACCCCGGACT GGAGCAAATTCGACTTCCGTTTCAGTCCGACCGCGAGCTTCAACGGTATTGACGAGTTCT ACCGTGAAGTGAGCGAACAGGTTATCGTATCAGCTTCGAGAACATTCCGGTGGAAACCG TTGACCGTCTGGTTGAGGAAGGTAACTGTGCGTGTTCAACTGTGGAACAGGACTTCA GCAAAGCATACCCGCGCTCCAAATTTGCACACCCAGTATTGGAACCGAGTATTTCGCC CTGAGAACCTGCAAGATGTGGTGATCAAATTGAATGGTGAGGCGCAACTTTCTACCGTC CGAGATCTATCAAGGAGAAGCTGTTCCGCCACAAAGTAGGTGAGAAGATGGTTAACAGCC GTGGGCGCGACGGGTCCGCGATGCCGGAATCTGTGCATGGTGAGCTGTTCCCGCACTTTA ATGGTAGCAAGGAACCCCTGTCCGACGAAGCCAAATGGTGGATTAGAGTGGCAACCTT GTGGTGAAGACGTTGCACATGAGATCGTCAAAGACCGCGCTTACACCGAGGACAAGTTC TCGTTCCACGTACCGCTGACGATTAAATTTAAACAACCGGACGCTCCGGCGCGTTTAAACG AACAGTGCGTGACTTTTTCGTTGGCAATCCGGATGTCAACGTGATCGGCATTGACAGAG GCGAACGCAATTTGATTTATTTGACCTGTTGATCGCCAAGGCAATTTGCTAGAGCAGCG TAGCTTTAACGTCGTCTCTGCTACTCGCCGTGATGGCGTCGAGGTACAGACTGACTACCA GAGTAACTGGCACAAAGCCGAGAAGGACCGCGCGCGCGCTGACCTGGTCAGAA ATCGGTGCGATCAAGGATCTCAAGGAGGCTATCTGAGCGCGGTGTTATGAAATTGCT AAAATGATGGTGAACGCAACGCGATTGTTGTGCTGGAGGACCTGAAATTTGGTTTCAAG CGTGGCCGTTTCCGTATTGAAAGACAGGTTTACCAAAAATTCGAAAAAGCTCTGATTGATA AACTGAACACTTGGTGTCAAGGATCGGGGCGATGGCAGAGCCGGGTGGTACCTTGGC GGTATCAGCTGACTGACGCGTTTCGAGAGCTTCGAGCGCATTTGGCAAGCAGACCGGTTTC
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SEQ ID NO: 2	SrmCas 12a	ATGAAAAGTTACATCACCACCACCACCATCACCATCACCACGGTTCTAGCATGAAAATCGA AGAAGGCAAACTGGTCATTTGGATCAATGGCGATAAAGGTTACAATGGTCTGGCGGAGGT GGGTAAGAAATTTGAGAAAAGACACAGGTATCAAGGTGACGGTGGAGCCACCGGACAAAGC TGGAGGAAAAGTTTCGCGAGGTGGCGGCGACCGGCGATGGTCCGGATATTATCTTTTGGG CGCACGACAGGTTTCGGCGTTACGCTCAATCGGGCTACTGGCAGAGATCACCCGGAT AAGGCGTTTCAGGATAAACTATACCCGTTTACCTGGGATGCGGTTCCGGTATAACGGTAAAT TAATCGCGTACCCGATCGCGGTGGAAGCATTTGCTCCTGATCTACAACAAGGACTTGCTCC CGAATCCACCGAAAACGTGGGAAGAGATCCCGGCTTGGATAAGGAATTAAGACGAAAG GGTAAATCTGCATTGATGTTTAACTGACGGAACCTTATTTACCTGGCGGTTAATTCGCG CGGACGGCGGTTATGCTTTCAAGTACGAAAACGGTAAGTATGATATCAAGGACGTGGGCG TTGACAACGCTGGTGCAGAAAGCTGGCCTCACCTTTTGGTGGACCTGATCAAGAATAAGC ACATGAATGCGGACACAGACTATTCTATCGCGGAAGCGGCTTCAACAAGGCTGAGACA GCAATGACCATTATGGCCCTTGGGCATGGTCCAACATTGATACCGCAAGGTGAACCTAC GGCGTTACCGTTCTCCCGACCTTCAAGGTGAGCCATCCAAGCGGTTTGTGGTGTGTTGA GTGCGGCGATTAAACGAGCTTCCCAAACAAGGAGCTGGCCAAAAGAAATTTCTGGAGAACT ATCTGCTGACCGACGAGGCTGAGAGCGGTGAACAAGGATAAACCGCTGGGTGCGGTA GCGCTGAAGAGCTACGAAGAGGAGCTGGCGAAGGACCCGCGCATCGCTGCAACGATGG AAAACGCCCGAAGAGGTGAGATCATGCCGAACATCCGCAATGAGCGGATTTGGGTATG CCGTCCGTACCGCGGTGATTAAACGCCGCGAGTGGTCTGAGACCGTGGATGAAGCGCTG AAAGACCGCGCAAAACCAAGCTCAAGCAACAACAACAATAACAACAACAACAACCTGG GCATCGAGGAGAAATCTGTACTTCCAGTCAATGCTATGAAAACAATTGAGAAATTTTGTGG TCAGAAAATGGTTATTCACGTTCTATTACATTAAGGAACAGACTTGTCCAATAGGAAAA ACAGAAAGAAAATTTTAAACTAAACTTCTTGATAAAGACATGGAGCGGGCAAGGGCA TATAATGAAGTTAAAAAATTTATGATGACTTTATCTGCTGATTATAGAAAGCGTACTTTT CACAGTAAATTTTGAATGGGGACCTTTATATGACCAAGTTTGATTATTTTCAGACAGAAAA GACAAGCAGAGAAAAATAAATAAAAAAGAACTAGAACAGTTGCAAGGTTTAAATGAGA AAAAAATAGTAGGCAATTTAAAAAGATAAACGTTTGA AAAAATTTTAAAGAAAGAAC TTCTTACAGAAATGGTTCCTGCTGTTATTAAGAATGATGAAACAGGAATATTACTGACAA AGAATCAGCGCTTGAATTTTAAAGGATTTGCAACATATTTACGGGTTTTCATCAAAACA GGCAGAATATGTATAGCGGAGAGCTCAGTCTACAGCTATAAGCAACAGGATTTGTTAATG AAAAATTTCCAAAATTTTATGCGAATGTAAAAGTATTTGAATTTTAAATAAATATTATCCA GAAATAATAATGAGACGGA AAAAATCATTGGCAGGATTTTAAAGGGGAAAAAATCTGGC GATATATTTTCATCAGAAGCTTTTAAATTCGGTCATGAGCCAGAGTGAATTTGATTTTACAA TACTGTCAATGGTGGTATTCAGACGAGGCGGTTCAAAAAAGATTACGGGACTTAATGA GATAATAAATCTTACGAGTCAGCAATTTGCCCTCAGAGGAAAAATATAAACTAAAAAGAA AATGACAATCTTTTCAAGCAGATTTTGAGTGACAGAAATACAGCATCTTTTATTCGGGTC AGCTTTGAAAAAAGTGAAGATGTTTATGAATCTGTGAAACATTTTAAAGGAAGAAATCTGG ATAAGGTTATTAATAACAAAAAACTTTTGAATCTGTGTTGATTATGATTTAGGTCAAAATTT ATGTACCTGTAAAGAAAGTAACAGAGTTTCTTTGAAAGTTTGGTAAGTGGTCAATTATT CATAATGGGTGTTTTTTCCTTGAACAGGATATGGCAAAAAAGCATTGACTGAAAAACAAA TCGAAGCACTTAAAAAGAAATTTGCAAGAAAGATTTCCCTCTAAGTGAATACAGAAATG CTTACGAGCGCTGGACAAAAGAAATGATGTAAACAGTAGAAAAAATGTAAGAGGATTATT TTAAGCTTTGTTGAGCTTCGATCAGATGAAAAATCAAAAGAAAAAATCTGTTGATATTTTA AGCAATATAGACAGGTTATATCAAAAAATAATTTGAAAAATAAGAGAACCTAATCCAGG AGAAAGATGCGGCGACTCCTATTAAGAATCTTTAGATGAAGTTCAAGAAATTTGATCATT TTTAAATTAAGTTGATTATCGTGGTGAAGAACAAAAGGATACAGATTTCTATTCAAAATATG ATGAAATAATGCAGG TCCTTTCTGAAATAATCCGCTGTATAATAAAGTTTCGTAACTTTGTAACAAAAGACCGGAAT GATATTA AAAAGGTAAAGTTGAATTTGATTGTCCGACTTATGCTAATGGGTGGGATTTAA ATAAGGAATCTTCAATGATGCAATTAATCTGCGAAAAAAGGAATATATTATTAGGTATT TTCAATCCTAAAGACAAGCCAAAGTTTGAACAATGCACTGTTGAAGATTCTTGTATGAAA AGATGATTATAACTTCTCCAGGAGCTAACAAAAATGCTTCTTAAAGTTTCTTTTCAACT AAAGGTGAGAAACTTTTCAACCACCAAGAGATTGATATTGGGATATGAAGAGGAAAG CATAAAAAGGGAGATGATTTTGATAAAGCGTTTCATGCATAAAATTAATTGACTGGTTAAGT ATGCAATTAATCAGCATGAGGACTGGAAAAATTTAATTTTAAATTTTACCCGACCGAATCT TACGAAGATATGAGTGGATTTTATAACGAAGTTGAATTCAGGGATATAAAATTAACCTTCA GTAAGGTATCGGAAAAATGCATCAATTCCTTGTAGATTCCGGTAAACTTTTCTTTTTCAG ATTTATAACAAAGATTATTCCGCTGGTAAAGATGGTGGAAATGGAGCGACAGGTA AAAA AATCTCCATACTCTTTATTGGGAAAACTTTTTAGTGAAGAGAAATCTTCGTAATGTTTGTCTT

		AAGCTTAATGGCGAAGCAGAACTTTTCTGGAGGGATGCAAAATCCAAATGTTAAGGCAGTG TGC CATAAAAAAGATTTCAGTACTCGTAAATAGAACAAACAAGAGATGGAAAAATCTATACCGGAA GAAATCTATCAGGAAATTTATAAATACAAGAATCCGGAAAAAGCAGGAAAAAGACTTTACA CTTTCAAAAGAGCAAAAGAACTTTTGGAAAGTGGAAGTGTGTTTGTAAAAAGCAAAAT TTGATATTACAAAAGACAGACATTTTACACAGCAGACATATCTGTTCCATTGTCCAATTAAC AATGAATTTTAAGGCAGCGGAAATTACGGGAAGAAAAGTTCAACGAGCGGGTTCAAGAGAT TCTTAGAAAAATCCCGAAGTAAAAATTATTGGACTTGACCGAGGTGAGCGCTCATTGATT TATCTATCACTTATAAATCAGAAGGGTGAATTTGAATTGCAAAAACTCTAAATATTGTTGA ACAGATTCTGAATGACAAAAACAGTTTCTGTTAATTATCAGGAAAAAACTGTTTCAAGAGGAA GGTGAGAGGGATAGAGCTCGTAAAAGCTGGCAGGCTATCAGTAATATCAAAGAACTTAAG GAAGGTTATCTTTCAAATATTGTTTCATGAAATTGCACAATTAATGGTGAAAAATAATGCAAT TGTTGAATGGAAGATTTGAATTTTGGCTTTAAGCGAGGGCGGTTTCTGTTGAAAGACAA GTTTATCAGAAGTTTGAATAATGCTTATTGAAAACTTAACATCTGTTTAAAAAGTGGCAA GAATGTTACAGAGCCTGGCGGAGTATTAATGCATATCAGCTGGCAGATAAGGCTGCTAA TGTTAGTGATGTTGGAAAACAGTGTGGCTGGATTTTATATTCCTGCGTCATATCTCGA AAATAGATAAAAAACCGGTTTTCAAAATTTATTCTATCTGAGGCTTACCAATATAGA AAAGAAAAAGATTTTGTATAAGTTTGATTCAATCCTGTTTGACAGAAAGCTTGATTGATT TTGTTTTCAGTTTGTATTATCAAACTTTTCAGAAAAATGCAGATTTCATAAAAAAGTGGAA GTCTATTCTCGCGGGGAAAAGACTGATTTTGTAGTAAAGCAGAAAAATCAACAATAAGTGTA ACCTTACTGAAATCTTAAAACTTTATTGACAAGCAGGGTATTATATGGAATCTCGAAGA AACTTTATAGACCAGATTCATGCTGTTCAAGCAGAAAGAGATAATGTTCTTCTTATGAT GGTCTTTATCGTTGTTTACAGTATTCTTCAAATGAGAAATTCATTCCAAATCTTCAAG GCAGGAAGATGATTATTAATTTCTCTGTAATGGCTGAGGATGGAAGTGAATTCATGACAGC CGTGTGAGCAGCTAAAGGAAAAGATGAAAAAGGAAAATGGATTTCAAACCTGCTGTT GATGCTGATGCAAAATGGTGTCTTACCACATGCTCTGAAAGGCTGTATTGCTAAAGAAATA ATTTAATCTGAATGATAAGGTTTCATTGAGAATATTTCCAATGCAGATTGGTTTCAGGTTT GCTCAGGAGAAAAGATATGCAAAATAA
SEQ ID NO: 3	CrmCas 12a	MKSSHHHHHHHHHSSMKIEEGKLVINGDKGYNGLAEVGKKFEKDTGKIVTVEHPDKLEE KFPQVAATGDPDIIFWAHDFRGGYAGSGLLAEITPDKAFQDKLYPFTWDVAVRYNGKLIAYPIA VEALSLIYNKDLLPNPPKTWEEIPALDKELKAGKKSALMFNLQEPYFTWPLAADGGYAFKYEN GKYDIKDVGVNDAGAKAGLTFVLDIKKNHMANADTDYSIAEAFAFNKGETAMTINGPWAWNSID TSKVNYGVTVLPTFGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKKDKPL GAVALKSYEEELAKDPRIAATMENAQKGEIMPNIQMSAFWYAVRTAVINAASGRQTVDALK DAQTNSSSNNNNNNNNNLIEENLYFQSNAMNDSFTGKYKLAKTLRFELRPVGRLETTFKE KFFEGDKRRDEDYPIVKELLDGEHKKALLERALSNNPPGELDWTALAAQAHEDYRTSDKSAEKD TLVAKQAEFRKKIVALFKKDVAFFSLTAPTPKDIFAAAMRKRYEDAGEEVPALVTFSRFAFFK GYQENRRNIYSDEAQTAAANRAVNENFPKFLEDVRIFRHIADTYPAISLDAEKELETLKGRTL ASLFDPAAYGSLFSGSNIDFFNSVLGGFVPGEGEKTGTRINEFVNLVYRQHQHEDGRTDRALSLLH PLYKQILSDRETQSLVPRALETDDEVLASLRAMFEERLLGLATESGRVNVLDLQALLSKIPAA DGIWIDGAESRVSMDLLGSWNALGSLMESAAEVRFASESTEKKRLAAIDKWMKRSVYALSEL ADLRQETDGTSMIVDVSLLWKGPVAVQRFDAARAAVAVVPLLSSPVPENGPKLREKDDVG IKAALDAVLVLHFKPLHAGEGLDRDEAFYGAFDLSYASLDGFVPLYNKVRNLYLTKKPGETE RIKLMDFNPTLADGWDQNKKEKDNCTVLCFRDGLYYLGVMMNPKETDFSLSGPEPTGCRYRM VYKLLPGPNKMLPHVFFSKGVETFHPSNSLLEKYHVGAAYKKGSGFDLAFCKRLIDFYKASISA HPDWSKFDFFSPTASFNGIDEFYREVSEQQYRISFENIPVETVDRLVEEGKLCFLQLWNKDFS KASTGRPNLHTQYWNNAVFAPELQDVVILNGEAELEFYRPSIKENVFRHKVGEKMMVNSR GADGSPMPESVHGELEFRHFNKSKEPLSDEAKWWIQSGNLVVKDVAHEIVKDRRYTEDKFSFH VPLTINFKQPDAPARFNEQVRDRLRGNPDVNVIGIDRGERNLILYTLVDRQGNLLEQSRFNVVS RTRRDGVEVQTDYQSKLAQAEKDRAAARLTWSEIGAIDKLEGYLSAVVHEIAKMMVERNAIV VLEDLNFQFKRGRFRIEROVYQKFEKALIDKLNLYL VFKDRGMAEPGGTLRGYQLTDAFESFERI GKQTGFLFYVPAGYTSKIDPTTGFTNLNTKCTNAAGVRDFTAFDSIRWDAARHAFADF YRNFKTSQESHRTAWTVYSANRRLVFDKDATEKEIDPTAILLDALKRGVSVTDGFDLKALL ADTEPSKANAGFFRDVYVFDRTLQMRNSRAGEDYIESPVNLNGQGKFDSRKADATLPKDAD ANGAYHIALKGVLLEENIAENRTANGSADLKIEHRDWFRFAQELAARKFK
SEQ ID NO: 4	SrmCas 12a	MKSSHHHHHHHHHSSMKIEEGKLVINGDKGYNGLAEVGKKFEKDTGKIVTVEHPDKLEE KFPQVAATGDPDIIFWAHDFRGGYAGSGLLAEITPDKAFQDKLYPFTWDVAVRYNGKLIAYPIA VEALSLIYNKDLLPNPPKTWEEIPALDKELKAGKKSALMFNLQEPYFTWPLAADGGYAFKYEN GKYDIKDVGVNDAGAKAGLTFVLDIKKNHMANADTDYSIAEAFAFNKGETAMTINGPWAWNSID TSKVNYGVTVLPTFGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKKDKPL GAVALKSYEEELAKDPRIAATMENAQKGEIMPNIQMSAFWYAVRTAVINAASGRQTVDALK DAQTNSSSNNNNNNNNNLIEENLYFQSNAMKTIEKFCGQKNGYSRSITLRNLVPIGKTEEN ILKLKLLDKDMERARAYNEVKKLIDDFHRVFIEDVLTSTVNFEGWGLYDQDFLQTEKDKQKKNK IKKELEQLQSLMRKKIVEAFKKDKRFEKLFKCELLTELVPVAVIKNDETGTITDKESALEIFKGFAT YFTGFHQRNQNMYSGEAQSTAISNRIVNENFPKFYANVKVFEYLNNNYPEIINETEKSLAGFLR GKKLADIFSSAEFNSVMSQSIDFYNTVIGGIADEAGSKKIQGLNEIINLT SQQLPSEEKYKLKK KMTILYKQILSDRNTASFIPVSFEKSEDVYESVKHFEKIEILDKVITNTKLLFDSVDYDLGQYVPA KEVTEFSLKVFGNWSIIHNGLFLLEQDMAKKALTEKQIEALKKEIAKKDFPLTEIGNAYERWTKE NDVTVEKNVVDYFKLVELRTDEKSKEKISVDILSNIDRLYSKINFENKLENLQEKDAATPIKINLLD EVQNLHYHLKLVDRGEEQKDDTFYSKYDEIMQVLSEIPLYNKVRNFTVTKPNDIKKVK

		<i>LNFD CPTLANGWDLNKESSNDAILRKKGIYYLGI FNP KDKPKFEQCTVEDSCYEKMIYKLLPG ANKMLPKVFFSTKGRETFOPPEDLILGYEEGKHKKGDDFDKAFMHKLIDWFKYAINQHEDWK NFNFKFSPTESYEDMSGFYNEVELQGYKITFSKYSEKCINSLVDSGKFLFQIYNKDY SAGKDG GNGATGKKNLHTLYWENLFSEENLRNVCFKLNGEAE LFWRDANPNVKA VCHKKDSVLVNRT TRDGKSIPEEIYQEIYKYKNPEKQEKDFTL SKEAKELLES GTVVCKKAKFDITKDRHFTQQTYLF HCPITMNFKA AEITGRKFNERVQEI LRNNPEVKI GLDRGERHLYLSLINQKG EIELQKTLNIVEQI RNDKTVSVNYQEKLVQKEGERDRARKSWQAISNIKELKEGYLSNIVHEIAQLMVENNAIVVMED LNFGFKRGRFPVERQVYQKFENMLIEKLNLYLVFKGKNVTEPGGVLNAYQLADKAANVSDVGK QCGWIFYIPASYTSKIDPKTG FANLFYTAGLTNIEKKKDFDFKDSILFDRKLD SFSVFSFYDYSNFS ENADFNKKWNVYSRGERLIFS KAEKSTISVNPTENKTLFDKQGIWNSEENFIDQIHAVQAE R DNVPFYDGLYRSFTVILQMRNSIPNSSRQEDDYLISPVM AEDGNFYDSRVEAAKGKDEKGKWI SKLPVDADANGAYHIALKGLYLLKNFNLNDKGF IENISNADWFRFAQEKEYAK</i>
SEQ ID NO: 71	MBP (DNA sequen ce)	<i>ATGAAAATCGAAGAAGGCAAACTGGTCATTGGGATCAATGGCGATAAAGGTTACAATGGTCT GGCGGAGGTGGGTAAGAAATTTGAGAAAGACACAGGTATCAAGGTGACGGTGGAGCACCC GGACAAGCTGGAGGAAAAGTTCCCGAGGTGGCGGCGACCGGCGATGGTCCGGATATTAT CTTTTGGCGGCACGACAGGTTCCGGCGGTACGCTCAATCGGGCCTACTGGCAGAGATCACG CCGGATAAGGCGTTTCAGGATAAACTATACCCGTTTACCTGGGATGGGTTCCGGTATAACG GTAAATTAATCGCGTACCCGATCGCGGTGGAAGCATTGTCCCTGATCTACAACAGGACTTG CTCCGAATCCACCGAAAACGTGGGAAGAGATCCCGGCTTGGATAAGGAACATAAAGCGA AGGGTAAATCTGCATTGATGTTTAACTCGCAGGAACCTTATTTACCTGGCCGTTAATTGCG GCGGACGGCGGTTATGCTTTCAAGTACGAAAACGGTAAGTATGATATCAAGGACGTGGGCG TTGACAACGCTGGTGCGAAAGCTGGCCTCACCTTTTGGTGACCTGATCAAGATAAGCA CATGAATGCGGACACAGACTATTCTATCGCGGAAGCGGCCTTCAACAGGGTGAGACAGCA ATGACCATTAATGGCCCTTGGGCATGGTCCAACATTGATACCAGCAAGGTGAACACTACGGCG TTACCGTTCTCCGACCTTCAAAGGTGAGCCATCCAAGCCGTTTGTGGTGTGTTGAGTGCC GGCATTAAACGAGCTTCCCAACAGGAGCTGGCCAAAGAAATTTCTGGAGAACTATCTGCT GACGACGAGGGTCTGGAGGCGGTGAACAAGGATAAACCGCTGGGTGCGGTAGCGCTGAA GAGCTACGAAGAGGAGCTGGCGAAGGACCCGCGCATCGCTGCAACGATGGAAGACGCCCA GAAAGGTGAGATCATGCCGAACATCCCGCAAATGAGCGCATTTTGGTATGCCGTCCGTACC GCCGTGATTAAACGCCGCGAGTGGTCTGACACCGTGGATGAAGCGCTGAAAGACGCGCAA ACC</i>
SEQ ID NO: 72	MBP (amino acid sequen ce)	<i>MKIEEGKLVINGDKGYNGLAEVGGKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAH DRFGGYAQSGLLAEITPDKAFQDKLYPFTWDVRYNGKLIAYPIAVEALSLYNNKDLLPNPPKTWE EIPALDKELKAKGSALMFNLQEPYFTWPLIADGGYAFKYENGKYDIKDVGV DNAGAKAGLTFL VDLIKNNHMANADTDYSIAEAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFV GVL SAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKS YEELAKDPRIATMENA QKGEIMPNPQMSAFWYAVRTAVINAASGRQTVDEALKDAQT</i>

[00127] **Example 2: Expression and purification of Cas12a enzymes**

[00128] **Methods**

[00129] His10-Cas12a and His10-MBP-TEV-Cas12a were synthesised and cloned between NdeI and NotI restriction endonuclease site of pET21a(+) by Genscript company (USA). After dilution of the lyophilized synthetic vector and agarose gel analysis, 50 ng of the pET-21a(+) plasmid containing His10-MBP-TEV-Cas12s was transformed into E. coli (BL21) (DE3). Subsequently, the expression levels and solubility were assessed using 10% SDS-PAGE. Initially, the expression was conducted on a small scale (20 ml Terrific broth (TB)) in a 100 cc flask to identify optimal conditions for soluble protein expression.

Cells were acrobically cultured in a shaker at 37°C with 200 rpm until reaching an optical density of 0.6. Cas12s production was induced by adding 0.5 mM IPTG, and the culture was maintained for 18 hours at 16°C. Following this, the conditions optimized in the prior step were scaled up to cultivate a significant biomass in 2 L of Terrific Broth (TB) media, guided by the identified expression levels. Cells were subsequently harvested and resuspended in a lysis buffer (20 mM HEPES pH 7.5, 500 mM KCl, 20 mM Imidazole,

1 mM TCEP, 10% Glycerol, 0.5 mM PMSF, EDTA-free protease inhibitor (Roche)). Additionally, 0.04 mg/ml lysozyme and 400 mg/L protamine sulfate were added to the suspension, followed by vortexing the sample. The mixture was then incubated on ice for 30 minutes, gently agitating the cell suspension every 5 minutes. Subsequently, sonication
5 (Hielscher, UP100H) was performed on ice at 60% amplitude, 0.5 cycle, and a sonication pulse rate of 600 seconds ON. The lysate was then centrifuged at 14,000g for 30 minutes at 4°C to pellet cellular debris. The resulting cell lysate supernatant, containing the soluble fraction of the recombinant protein, was retained. Invisible cell debris was eliminated by passing the lysate through a 0.22-micron filter, and the filtered cell lysate was applied to
10 a column containing 1 cc Ni-NTA resin (Sigma) per 10 cc of filtered cell lysate (equivalent to 1/10 of the volume of cell supernatant). Cas12s was dialyzed into Buffer (20 mM HEPES pH 7.5, 100 mM KCl, 10% glycerol, 1 mM TCEP, and 0.5 mM EDTA) for overnight (OV) at 4°C and subsequently applied onto a 5 ml HiTrap SP HP Sepharose column (Cytiva). After washing with three column volumes of Buffer A, Cas12s was
15 eluted using buffer (20 mM HEPES pH 7.5, (100 mM-2M) KCl, 5% glycerol, 1 mM TCEP) containing a linear KCl gradient from 100 mM to 2M over 2 column volumes. The Cas12a protein fractions were mixed and concentrated using an Amicon® Ultra-15 Centrifugal Filter (50K device). Cas12a Storage Buffer (20 mM HEPES pH 7.5, 300 mM KCl, 10% glycerol, 1 mM TCEP) was added to the concentrated protein, which was then
20 flash-frozen with liquid nitrogen and stored at -80°C for subsequent SDS-PAGE analysis. The concentrated fractions were analyzed on a 10% SDS gel.

[00130] **Results**

[00131] The findings indicate that the solubility of CrmCas12a and SrmCas12a proteins were low when expressed without the MBP tag. In contrast, the presence of the MBP
25 protein significantly enhanced their solubility, as illustrated in **Figure 1A**.

[00132] In addition, the Hi-Trap protein purification results revealed a purity exceeding 95% (**Figure 1B**). This high level of protein purity is crucial for conducting accurate comparisons of protein activity. Consistency in protein concentration is essential for each collateral activity comparison test, emphasizing the significance of achieving such purity
30 levels.

[00133] **Example 3: Target preparation**

[00134] The complete DNA sequences of Bcsp31, bp26, and Omp2a from *Brucella melitensis* (Table 2) were obtained from NCBI and then synthesized and cloned into the pUC19 vector (Genscript, USA). The resulting three pUC19 plasmids carrying *BCSP31*, *bp26*, and *Omp2a* genes were transformed into DH5-alpha-competent *E. coli* cells. Plasmid extraction was performed following the protocol described in the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific Inc, USA). A 5µl aliquot of the purified plasmids was subjected to electrophoresis on a 1% agarose gel. The concentrations of the plasmids were determined using the Qubit 4 Fluorometer (Thermo Fisher Scientific Inc, USA).

[00135] **Table 2.** DNA sequences of Bcsp31, bp26, and Omp2a from *Brucella melitensis*

SEQ ID NO:5	<i>Bcsp31</i> (Accession #M20404.1)	ATGAAATTCGGAAGCAAAATCCGTCGCTTGGCTGTTGCGGCGGTGGCGGGCGCA TTGCGTTGGGAGCGAGCTTTGCGGTTGCACAGGCCCGACATTTTCGGTATCGG CACTGGCGGCACAGCCGGAACCTATTATCCGATTGGTGGTCTGATCGCGAACCGG ATTTCCGGCGCAGCGCAAAAGGGCGTGCCGGGTCTCGTCGCGACGGCCGTTTCG TCGAATGGCTCGGTTGCCAATATCAATGCGATCAAGTCGGGGCGCTCTGGAGTCCG GCTTTACGCAGTCAGACGTTGCCATTTGGGCTATAACGGCACCGGCCCTTTATGAT GGCAAGGGCAAGGTGGAAGATTTGCGCCTTCTGGCGACGCTTTACCGGGAACGA TCCATATCGTTGCGCGTAAGGATGCAAAACATCAATCGGTGCGACACCTGAAAGGC AAGCGCGTTTCGCTGGATGAGCCGGGTTCTGGCACCATCGTTCGATCGCGTATCG TTCTTGAAGCCTACGGCCTCACGGAAGACGATATCAAGGCTGAACACCTGAAGCC GGGACCGGCAGCGAGAGGCTGAAAGATGGTGGCTGGACGCCATTTCTTTGTG GGCGGCTATCCGACGGGCGCAATCTCGGAACCTGGCCATCTCGAACGGTATTTCCG TCGTTCCGATCTCCGGGCGCGAAGCGGACAAAGATTCTGGAGAAATATTCTCTTC TCGAAGGATGTGGTTCTGCGGAGCCTATAAGGACGTGGCGGAAACACCGACCC TTGCCGTTGCCGACAGTGGGTGACGAGCGCCAAGCACCGGACGACCTCATCTA TAACATCAACCAAGTTCTCTGGAACGAGGATACACGCAAGGCACTCGATGCGGGC CATGCCAAGGGCAAGCTCATCAAGCTCGATAGTGCGACGAGCAGCCTCGGTATTC CGCTGCATCCCGGCGCAGAACGCTTTTACAAGGAAGCGGGCGTGCTGAATAA
SEQ ID NO: 6	<i>bp26</i> (Accession # OQ378327)	ATGAACACTCGTGTAGCAATTTCTCGCAGCCTCATTTTCCAAATCATGTCTGCTC GGCGCTTTCAGCCTGCCCGCTTTCGCACAGGAGAATCAGATGACGACGCGAGCCG CGCGCATCGCGTCACCGGGGAAGGCATGATGACGGCTTCGCCGATATGGCCA TTCTCAATCTCTCGGTGCTACGCCAGGCAAGACCGCGCGCGAAGCCATGACCGC GAATAATGAAGCCATGACAAAAGTGCTCGATGCCATGAAGAAGGCCGCGCATCGAA GATCGCGATCTCCAGACAGGCGGCATCAATATCCAGCCGATTTATGTCTATCTGA CGACAAGAACAACCTGAAAGAGCCTACCATCACCGGCTATTCTGTATCCACCAATC TCACGGTTTCGCGTGCAGCACTGGCCAATGTTGGAATAATTTGGATGAATCCGCT ACGCTCGGTGTTAATCAGGGCGGTGATTGAACCTGGTCAATGATAATCCCTCCGC CGTGATCAACGAGCGCGCAAGCGCGCAGTGCCCAATGCCATTGCCAAGGCGAA GACGCTTGGCGACGCTGCAGGCGTGGGCTTGGCGGTGTGGTGGAAATCAATGA ACTGAGCGCGCCCGCCATGCCGATGCCAATTGGCGCGGACAGTTCAAGACCATG CTAGCAGCGCACCGGACAATCCGTGCCGATTGCGCAGCGCAAAACAGCTATA ACGTATCGGTCAATGTGCTTTTGAATCAAGTAA
SEQ ID NO:7	<i>Omp2a</i> (Accession # U26440.1)	ATGAACATCAAGAGCCTTCTCCTTGGCTCCGCCGAGCTCTGGTGCAGCTCCG GCGCTCAGGCTGCCGACGCAATCGTCGCGCCAGAGCCCGAAGCGCTTGAATATGT CCGCGTTTTCGACGCTTACCGCGCTGGCTACTTCTACATTCGGGCGACCGAAACC TGCCTGCGCGTCCATGGTTACGTCGTTACGACGTAAGGGCGCGCATGACGTTT ACTCCGGTACCGACCGCAATGGCTGGGACAAGGGCGCTCGTTTCGCACTCATGTT CAACACGAATTCGGAACCGAATCGGCACACTCGGCACCTATACTCAGCTGCCG TTCAACTACACGACGAACAATTCACGTCATGATGGCCAATACGGCGATTTACGCGA TGATCGTGATGTCGCTGATGGCGCGTAAGCACCGGCACCGATCTGCAGTTTGA TATATCACGCTTGGTGGTTTCAAGTTGGTATCGACGAATCCGAATCCATACCTTC ACCGGTTACCTCGGTGATGTCATCAACGATGATGTCGTCGCTGCTGGCTCTACCG CACCGGCAAGATCGCTACACCTTACCGGCGGAAACGGCTTCTCGGCTGTGATC GCTCTGAACAGGGTGGCGAAGACGTTGACAACGATTACACGATCGACGGTTACA TGCCGACGTTGTTGGCGGCTGAAATATGCTGGCGGCTGGGGTTTCATCGCTGG

		TGTTGTTGCCTATGACTCGGTCATCGAAGAATGGGCTACAAAGGTTCTGTGGCGACG TCAACATCACCGACCGGTTCTCGGTATGGCTGCAGGGCGCATATTCGTCCGCAGC GACGCCGAACGAGAACTACGGTCAGTGGGGCGGCGATTGGGCTGTCGTGGGGTGG TGCAAAGTTCAATTGCCACCGAAAAGGCAACCTTCAATCTGCAGGCTGCCGATGACG ACTGGGGCAAGACCGCAGTTACCGCCAACGTCGCTTATCAGCTCGTTCCCGGATT CACCATACGCCGGAAGTTTCCTACACCAATTTGGTGGCGAGTGAAAGACACCG TTGCTGAAGACAATGCCTGGGGCGGTATCGTTCTTCCAGCGCTCGTTCTAA
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[00136] **Example 4: Comparing the activity of CrmCas12a and SrmCas12a with and without MBP tag**

[00137] The findings from Example 2 revealed a substantial enhancement in the solubility of Cas12a enzymes upon incorporating the MBP protein tag. Nevertheless, it is crucial to highlight that the removal of the MBP tag from the enzyme necessitates an extra step and incurs additional costs, especially when upscaling production for industrial applications. In this investigation, whether the inclusion of the tag had any noticeable effect on the activity of Cas12a enzymes was explored.

[00138] **Methods**

[00139] Collateral cleavage assays involving CrmCas12a and SrmCas12a enzymes were conducted in a final reaction volume of 20 µL. Initially, Cas12a proteins and crRNAs were combined to form ribonucleoprotein (RNP) complexes by mixing 400 nM purified Cas12a with 400 nM crRNA (**Table 3**) in 2 µL of 10X NEBuffer™ r2.1, followed by incubation at room temperature for 15 min, unless stated otherwise. Subsequently, 400 nM of the assembled RNP was mixed with 1.7 nM of the target and 200 nM ssDNA reporter (/56-FAM/CCCCCCCC/3IABkFQ/) from Integrated DNA Technologies, and the reactions were incubated for 1 h at 37 °C. The end-point fluorescence signal was measured using the Step One ABI Real-time PCR system (Applied Biosystems, CA, USA). To facilitate comparisons across different conditions, fluorescence from background conditions (no target) was subtracted from samples, yielding background-subtracted fluorescence. The graphs present the mean background-subtracted fluorescence output with standard deviation (n = 3).

[00140] **Table 3-** crRNA sequences (5' to 3'). The protospacer regions have been highlighted in grey.

Enzyme	Target gene	Sequence (5' to 3')	Name of crRNA's
SrmCas12a	<i>BCSP31</i>	AAAUUUUCUACUGUUGUAGAU CGCCUUCUGGCCAGGCCUUUA	S-BC1 (SEQ ID NO: 8)
	<i>bp26</i>	AAAUUUUCUACUGUUGUAGAU GCACAGGAGAAUCAGAUGAC	S-BP1 (SEQ ID NO: 9)
	<i>Omp2a</i>	AAAUUUUCUACUGUUGUAGAU GCUGCAGGGUGCAUAUCCGCCG	S-OM1 (SEQ ID NO: 10)
CrmCas12a	<i>BCSP31</i>	UCAAUUUUCUACUGUUGUAGAU CGCCUUCUGGCCAGGCCUUUA	C-BC1 (SEQ ID NO: 11)
	<i>bp26</i>	UCAAUUUUCUACUGUUGUAGAU GCACAGGAGAAUCAGAUGAC	C-BP1 (SEQ ID NO: 12)
	<i>Omp2a</i>	UCAAUUUUCUACUGUUGUAGAU GCUGCAGGGUGCAUAUCCGCCG	C-OM1 (SEQ ID NO: 13)

[00141] **Results**

5 [00142] The results indicated that there was no significant difference between CrmCas12a and SrmCas12a with and without the MBP tag (**Figure 2**). Consequently, for subsequent studies, CrmCas12a and SrmCas12a are chosen to be utilized with the MBP tag. Therefore, in the subsequent examples, CrmCas12a and Sarasa denote enzymes with the MBP tag.

10 [00143] **Example 5: Comparing the activity of CrmCas12a and SrmCas12a using novel crRNAs**

[00144] To develop a sensitive Cas12-based direct detection assay for Brucella that facilitates point-of-care testing, the optimization of Cas12a activation was undertaken, which involved crRNA selection.

15 [00145] The *Bcsp31*, *bp26*, and *Omp2a* were chosen, considering their high conservation and widespread utilization as diagnostic targets.

[00146] **Methods**

[00147] A total of 7, 6, and 3 crRNAs were designed to target *Bcsp31*, *bp26*, and *Omp2a* regions, respectively (**Table 4-6**). To optimize the sensitivity, the efficiency of all 16 crRNAs was evaluated. The experimental conditions are described in Example 4. Real-time fluorescence measurements were collected on Step One ABI Real-time PCR (Applied Biosystems, CA, USA) at 1 min intervals for a total of 60 min. To allow comparisons between different conditions, fluorescence for background conditions (no target) were subtracted from samples to generate background subtracted fluorescence.

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25 The measurement of real-time background subtracted fluorescence output are shown in the graphs as means (n = 3).

[00148] **Table 4-** crRNA sequences (5' to 3') to detect *Brucella* BCSP31 gene. crRNAs with similar BC number have similar protospacer sequence. The protospacer regions have been highlighted in grey.

Enzyme	Protospacer	Sequence (5' to 3')	Name
SrmCas12a	BC2	AAAAUUUCUACUGUUGUAGAU CGGUUGCAGGCCCGGACA	S-BC2 (SEQ ID NO: 14)
	BC3	AAAAUUUCUACUGUUGUAGAU CGGAGUCAGCGUUGCCUAG	S-BC3 (SEQ ID NO: 15)
	BC4	AAAAUUUCUACUGUUGUAGAU CAAGGAAGCGGGCGUGGUGA	S-BC4 (SEQ ID NO: 16)
	BC5	AAAAUUUCUACUGUUGUAGAU UGAUUGGCAAGGCCAAGGUGG	S-BC5 (SEQ ID NO: 17)
	BC6	AAAAUUUCUACUGUUGUAGAU CAUCCUACGGCGCAACGUAU	S-BC6 (SEQ ID NO: 18)
	BC7	AAAAUUUCUACUGUUGUAGAU CGCGGAACCGAUCCAUUCCG	S-BC7 (SEQ ID NO: 19)
	BC8	AAAAUUUCUACUGUUGUAGAU UCCGAAUGGGUCGCGUUGCCG	S-BC8 (SEQ ID NO: 20)
CrmCas12a	BC2	UCAAUUUCUACUGUUGUAGAU CGGUUGCAGGCCCGGACA	C-BC2 (SEQ ID NO: 21)
	BC3	UCAAUUUCUACUGUUGUAGAU CGGAGUCAGCGUUGCCUAG	C-BC3 (SEQ ID NO: 22)
	BC4	UCAAUUUCUACUGUUGUAGAU CAAGGAAGCGGGCGUGGUGA	C-BC4 (SEQ ID NO: 23)
	BC5	UCAAUUUCUACUGUUGUAGAU UGAUUGGCAAGCGCAAGGUGG	C-BC5 (SEQ ID NO: 24)
	BC6	UCAAUUUCUACUGUUGUAGAU CAUCCUACGGCGCAACGUAU	C-BC6 (SEQ ID NO: 25)
	BC7	UCAAUUUCUACUGUUGUAGAU CGCGGAACCGAUCCAUUCCG	C-BC7 (SEQ ID NO: 26)
	BC8	UCAAUUUCUACUGUUGUAGAU UCCGAAUGGGUCGCGUUGCCG	C-BC8 (SEQ ID NO: 27)

5 [00149] **Table 5-** crRNA sequences (5' to 3') to detect *Brucella* *bp26* gene. crRNAs with similar BC number have similar protospacer sequence. The protospacer regions have been highlighted in grey.

Enzyme	Protospacer	Sequence (5' to 3')	Name
SrmCas12a	BP2	AAAAUUUCUACUGUUGUAGAU UGUCUAGCCUGACGACAAGA	S-BP2 (SEQ ID NO: 28)
	BP3	AAAAUUUCUACUGUUGUAGAU CCUUGCGUAGCCACCGAGAGA	S-BP3 (SEQ ID NO: 29)
	BP5	AAAAUUUCUACUGUUGUAGAU AGGUUUGUUCUUGUCUACGG	S-BP5 (SEQ ID NO: 30)
	BP6	AAAAUUUCUACUGUUGUAGAU CAACAUUGGGCAGUUGCGCG	S-BP6 (SEQ ID NO: 31)
	BP7	AAAAUUUCUACUGUUGUAGAU UCAAUGGGCUCAUAUAUUGCGG	S-BP7 (SEQ ID NO: 32)
CrmCas12a	BP2	UCAAUUUCUACUGUUGUAGAU UGUCUAGCCUGACGACAAGA	C-BP2 (SEQ ID NO: 33)
	BP3	UCAAUUUCUACUGUUGUAGAU CCUUGCGUAGCCACCGAGAGA	C-BP3 (SEQ ID NO: 34)
	BP5	UCAAUUUCUACUGUUGUAGAU AGGUUUGUUCUUGUCUACGG	C-BP5 (SEQ ID NO: 35)
	BP6	UCAAUUUCUACUGUUGUAGAU CAACAUUGGGCAGUUGCGCG	C-BP6 (SEQ ID NO: 36)
	BP7	UCAAUUUCUACUGUUGUAGAU UCAAUGGGCUCAUAUAUUGCGG	C-BP7 (SEQ ID NO: 37)

[00150] **Table 6-** crRNA sequences (5' to 3') to detect *Brucella* *Omp2a* gene. The protospacer regions have been highlighted in grey.

Enzyme	Protospacer	Sequence (5' to 3')	Name
SrmCas12a	OM2	AAAAUUUCUACUGUUGUAGAU CGACGGCUUACGGGCGUGGCG	S-OM2 (SEQ ID NO: 38)
	OM3	AAAAUUUCUACUGUUGUAGAU CGAUCCGAGCGGGUGUAGAC	S-OM3 (SEQ ID NO: 39)
	OM4	AAAAUUUCUACUGUUGUAGAU CGGUUACGGGACGUAGCCG	S-OM4 (SEQ ID NO: 40)
CrmCas12a	OM2	UCAAUUUCUACUGUUGUAGAU CGACGGCUUACGGGCGUGGCG	C-OM2 (SEQ ID NO: 41)
	OM3	UCAAUUUCUACUGUUGUAGAU CGAUCCGAGCGGGUGUAGAC	C-OM3 (SEQ ID NO: 42)
	OM4	UCAAUUUCUACUGUUGUAGAU CGGUUACGGGACGUAGCCG	C-OM2 (SEQ ID NO: 43)

[00151] **Results**

[00152] In the comprehensive experimental investigation, the polypeptides CrmCas12a and SrmCas12a, as disclosed in the invention, exhibited distinct activities when paired with diverse crRNAs targeting crucial Brucella genes, namely *BCSP31*, *bp26*, and *Omp2a* (Figure 3-5). The intricate interplay between these Cas12a variants and the selected crRNAs revealed nuanced variations in their trans-cleavage activities, shedding light on the intricate molecular dynamics governing their interactions. The observed differences in activities across these target genes suggest a potential for tailored applications, allowing for the fine-tuning of the CRISPR-Cas12a system to specific genetic targets within the Brucella species. These findings pave the way for the development of a highly versatile and adaptable detection platform, with implications for precision diagnostics and targeted pathogen detection strategies.

[00153] **Example 6- The impact of protospacer sequence on the trans-cleavage activity of Cas12a enzymes**

[00154] Here, the impact of protospacer sequence on trans-cleavage activity of SrmCas12a and CrmCas12a was compared. The results of the novel protospacers and crRNAs in examples 4 and 5 with two of best protospacers previously reported for bp26 by Dang et al. (BMC Veterinary Research (2023) 19:202), were included (Table 7).

[00155] **Table 7-** crRNA sequences (5' to 3') to detect Brucella *bp26* gene. The protospacer regions have been highlighted in grey.

Enzyme	Protospacer	Sequence (5' to 3')	Name
SrmCas12a	BP8	AAAAUUUCUACUGUUGUAGAUAAACUUGGUCAAUGAAUAUCC	S-BP8 (SEQ ID NO: 44)
	BP9	AAAAUUIICUACUGUUGUAGAUCAUGAAUCCGUCACGCUCCG	S-BP9 (SEQ ID NO: 45)
CrmCas12a	BP8	UCAAUUUCUACUGUUGUAGAUAAACUUGGUCAAUGAAUAUCC	C-BP8 (SEQ ID NO: 46)
	BP9	UCAAUUUCUACUGUUGUAGAUCAUGAAUCCGUCACGCUCCG	C-BP9 (SEQ ID NO: 47)

[00156] The findings underscore the critical role of the protospacer sequence in modulating the trans-cleavage activity of SrmCas12a and CrmCas12a enzymes during the detection of diverse Brucella genes (Figure 6). Importantly, the present study revealed heightened trans-cleavage activities associated with several newly designed protospacers when juxtaposed with those previously reported by Dang et al. (2023) for BP8 and BP9. This comparison highlights the superior performance and efficacy of the present novel protospacers, reinforcing their potential as key elements in advancing the field of Brucella detection.

[00157] The specific crRNAs such as BC1, BC4, BP3, and BP7 for SrmCas12a enzyme, and BC5, BC6, BP3, and BP5 for CrmCas12a enzyme, exhibited a remarkable increase in fluorescence signal during the trans-cleavage activity assessment. The robust performance of these specific crRNAs underscores their effectiveness in facilitating a more sensitive and accurate detection of Brucella, offering promising prospects for the advancement of CRISPR-Cas12a-based diagnostic assays.

[00158] **Example 7- Optimizing the length of crRNA enhance the signal**

[00159] Studies indicate that the length of crRNA plays a pivotal role in shaping the efficiency and specificity of Cas12a collateral cleavage. The optimization of crRNA length becomes imperative to strike the desired equilibrium between specificity and efficiency in the context of Cas12a-mediated collateral cleavage.

[00160] Here, whether SrmCas12a and CrmCas12a enzymes are sensitive to changes in protospacer and crRNA length was investigated.

[00161] **Methods**

[00162] The length of the protospacer sequence of crRNAs SrmCas12a-BP3 and CrmCas12a-BP3 was systematically varied. The protospacer lengths were adjusted incrementally from 14 to 32 nucleotides in steps of two (14, 16, 18, ..., 32) (Table 8). The experimental conditions are described in Example 4. The end-point fluorescence signal was measured using the Step One ABI Real-time PCR system (Applied Biosystems, CA, USA). To facilitate comparisons across different conditions, fluorescence from background conditions (no target) was subtracted from samples, yielding background-subtracted fluorescence. The graphs present the mean background-subtracted fluorescence output with standard deviation ($n = 3$).

[00163] **Table 8-** crRNA sequences (5' to 3'). The protospacer regions have been highlighted in grey.

Enzyme	Protospacer (nt)	Sequence (5' to 3')	Name
SrmCas12a	14	AAAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	S-BP3-1 (SEQ ID NO: 48)
	16	AAAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	S-BP3-2 (SEQ ID NO: 49)
	18	AAAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	S-BP3-3 (SEQ ID NO: 50)
	20	AAAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	S-BP3-4 (SEQ ID NO: 29)
	22	AAAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	S-BP3-5 (SEQ ID NO: 51)
	24	AAAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	S-BP3-6 (SEQ ID NO: 52)
	26	AAAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	S-BP3-7 (SEQ ID NO: 53)
	28	AAAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	S-BP3-8 (SEQ ID NO: 54)
	30	AAAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	S-BP3-9 (SEQ ID NO: 55)
	32	AAAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	S-BP3-10 (SEQ ID NO: 56)
CrmCas12a	14	UCAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	C-BP3-1 (SEQ ID NO: 57)
	16	UCAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	C-BP3-2 (SEQ ID NO: 58)
	18	UCAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	C-BP3-3 (SEQ ID NO: 59)
	20	UCAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	C-BP3-4 (SEQ ID NO: 34)
	22	UCAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	C-BP3-5 (SEQ ID NO: 60)
	24	UCAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	C-BP3-6 (SEQ ID NO: 61)
	26	UCAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	C-BP3-7 (SEQ ID NO: 62)
	28	UCAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	C-BP3-8 (SEQ ID NO: 63)
	30	UCAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	C-BP3-9 (SEQ ID NO: 64)
	32	UCAAUUUCUACUGUUGUAGAUCCUGGCGUAGCACC	C-BP3-10 (SEQ ID NO: 65)

[00164] **Results**

5 [00165] The effect of protospacer length on the activity of SrmCas12a and CrmCas12a was evaluated using systematically varied crRNA protospacer lengths ranging from 14 to 32 nucleotides.

[00166] SrmCas12a demonstrated robust activity across a wide range of protospacer lengths, maintaining higher activity levels compared to CrmCas12a throughout the tested
10 range (**Figure 7**). For protospacer lengths of 20 to 24 nucleotides, SrmCas12a exhibited its peak activity. While activity slightly decreased at a protospacer length of 26 nucleotides, it remained relatively stable with only minor declines as the length increased up to 32 nucleotides.

[00167] In contrast, CrmCas12a exhibited its highest activity specifically within the 20
15 to 24 nucleotide range, with significantly lower activity observed for both shorter and longer protospacer lengths. This indicates that CrmCas12a has a narrower optimal range for protospacer lengths compared to SrmCas12a (**Figure 7**).

[00168] These findings highlight key differences in the tolerance and efficiency of SrmCas12a and CrmCas12a with varying protospacer lengths. The broad activity range
20 observed for SrmCas12a suggests it may be more adaptable to diverse target sequences

with varying spacer lengths, making it a potentially versatile tool for CRISPR-based diagnostics.

[00169] **Example 8- The combination of crRNAs augmented the trans-cleavage signal at low target concentrations**

- 5 [00170] In contrast to a single crRNA system, recent years have seen the development of crRNA combination strategy. It aims to enhance the fluorescence signal when the concentration of target DNA is low, eliminating the necessity for pre-amplification.

[00171] **Methods**

- [00172] The experimental conditions closely resembled those described in Example 4,
10 with two modifications. Firstly, the target concentration was reduced from 1.7 nM to 1 pM. Secondly, for the single crRNA, RNA complexes were formed by combining 800 nM purified Cas12a with 800 nM crRNA. For the double crRNA, a mixture of 800 nM purified Cas12a with 400 nM of each crRNA (totalling 800 nM crRNA) was utilized. Real-time fluorescence measurements were acquired at 1-minute intervals over a total
15 duration of 60 minutes. To facilitate comparisons across diverse conditions, fluorescence values for background conditions (absence of target) were subtracted from the samples, generating background-subtracted fluorescence. The graphs depict the means ($n = 3$) of real-time background-subtracted fluorescence output.

[00173] **Results**

- 20 [00174] The detection of low concentrations of *Brucella* genes *bp26* and *BCSP31* was investigated using crRNA combinations, aiming for an amplification-free CRISPR-Cas12a-based detection system. Two of best crRNAs for *bp26* and *BCSP31* genes identified in this study were included for this experiment.

- [00175] The present results showed that the combination of crRNAs substantially
25 increased the signal for both genes (**Figure 8 and 9**).

[00176] With an increased number of ssDNA probes being cleaved in crRNA combination system, the fluorescence signal is enhanced which may eliminate the necessity of pre-amplification specially when the concentration of target DNA is low.

[00177] **Example 9- The combination of enzymes enhanced the signal**

- 30 [00178] Consistent with the approach outlined in Example 8, it was postulated that, unlike a single enzyme system, combination of enzymes for single target detection in one reaction could amplify the cleavage of probes and subsequently boost the fluorescence

signal. The methodology behind this approach entails the utilization of combination of enzymes for pathogen detection. The objective is to augment the fluorescence signal, particularly when the target DNA concentration is low, thereby eliminating the need for pre-amplification.

5 [00179] **Methods**

[00180] The experimental conditions closely resembled those described in Example 4, with two modifications. Firstly, the target concentration was reduced from 1.7 nM to 1 pM. Secondly, for the single crRNA experiment, RNA complexes were formed by combining 800 nM purified SrmCas12a or 800 nM purified CrmCas12a with 800 nM of corresponding crRNA. In the double enzyme experiment, a mixture of 400 nM purified CrmCas12a and 400 nM purified SrmCas12a with 400 nM of each corresponding crRNA (totaling 800 nM crRNA) were utilized. Real-time fluorescence measurements were acquired at 1-minute intervals over a total duration of 60 minutes. To facilitate comparisons across diverse conditions, fluorescence values for background conditions (absence of target) were subtracted from the samples, generating background-subtracted fluorescence. The graphs depict the means ($n = 3$) of real-time background-subtracted fluorescence output.

[00181] **Results**

[00182] The detection of low concentrations of *Brucella* genes *bp26* and *BCSP31* was investigated by employing strategies with combined enzymes, aiming for an amplification-free CRISPR-Cas12a-based detection system. Two of best crRNAs for *bp26* and *BCSP31* genes identified in this study were included for this experiment.

[00183] The present results showed that the combination of enzymes substantially increased the signal for both genes (**Figures 10-13**). This strategic advancement holds promise for simplifying and expediting the detection process, particularly in resource-limited settings or when dealing with samples containing low concentrations of the target DNA.

[00184] **Example 10- Temperature Robustness Assessment**

[00185] CRISPR-Cas diagnostics with the ability to operate across a wide temperature range offer significant advantages for flexible and reliable molecular testing. This adaptability ensures consistent performance in diverse environments, from resource-limited areas without temperature control to one-pot systems. By maintaining high

sensitivity and specificity under varying thermal conditions, these diagnostics address a key limitation of traditional molecular tests that rely on strict temperature regulation. Such versatility is essential for field deployment, rapid outbreak responses, and decentralized healthcare solutions. In this regard, we evaluated the performance of SrmCas12a and CrmCas12a enzymes at 20, 25, 30, 35, 40, 50, and 60 °C, further exploring their potential for robust and temperature-tolerant diagnostic applications.

[00186] **Methods**

[00187] Collateral cleavage assays using CrmCas12a and SrmCas12a enzymes were performed in a 20 µL reaction volume. To assemble ribonucleoprotein (RNP) complexes, 400 nM purified Cas12a and 400 nM crRNA (BP3) were combined in 2 µL of 10X NEBuffer™ r2.1 and incubated at room temperature for 15 minutes unless specified otherwise. Following this, 400 nM of the RNP complex was mixed with 1.7 nM of the target and 200 nM ssDNA reporter (/56-FAM/CCCCCCCC/3IABkFQ/) obtained from Integrated DNA Technologies. The reaction mixtures were then incubated under various temperature conditions (20, 25, 30, 35, 40, 50, and 60 °C) for a total of 60 minutes. The end-point fluorescence signal was measured using the Step One ABI Real-time PCR system (Applied Biosystems, CA, USA). To facilitate comparisons across different conditions, fluorescence from background conditions (no target) was subtracted from samples, yielding background-subtracted fluorescence. The graphs present the mean background-subtracted fluorescence output with standard deviation (n = 3).

[00188] **Results**

[00189] The activity of SrmCas12a and CrmCas12a was evaluated across a range of temperatures (20, 25, 30, 35, 40, 50, and 60 °C) by measuring fluorescence output as an indicator of collateral cleavage activity.

[00190] SrmCas12a consistently outperformed CrmCas12a in fluorescence output across all tested temperatures except at 60 °C, where both enzymes showed minimal activity. SrmCas12a exhibited its peak activity at 35 °C, retaining 80.3% of this activity at 25 °C. In contrast, CrmCas12a demonstrated its maximum activity at 35 °C, maintaining 72.5% of this activity at 25 °C (**Figure 14**).

[00191] The results demonstrate that SrmCas12a exhibits superior performance and a broader functional temperature range compared to CrmCas12a, making it particularly suitable for diagnostic applications under variable conditions. Notably, SrmCas12a

maintained a substantial proportion (80.3%) of its peak activity at 25 °C, which is close to room temperature. This characteristic is especially advantageous for point-of-care diagnostics performed in resource-limited settings, where precise temperature control may not be feasible. The enzyme's robust activity at 25 °C highlights its adaptability, ensuring reliable performance in environments without temperature regulation. CrmCas12a, while exhibiting a similar temperature profile, showed lower activity overall, particularly at room temperature (72.5% retention of peak activity at 25 °C), limiting its utility in such scenarios.

[00192] **Example 11. Enhanced high-temperature functionality of SrmCas12a compared to commercial LbaCas12a**

[00193] The ability of the Cas12a enzyme to function effectively across a range of temperatures, including elevated temperatures between 60°C and 65°C, provides significant advantages for nucleic acid-based diagnostics. This temperature range aligns with isothermal amplification methods such as Loop-Mediated Isothermal Amplification (LAMP), facilitating seamless integration of amplification and detection processes. The performance of Cas12a at these temperatures improves specificity and sensitivity by minimizing secondary structure formation and nonspecific binding, while also increasing resistance to contaminants like DNases or RNases. These attributes make the Cas12a enzyme a robust and versatile tool for point-of-care diagnostics and field applications, offering compatibility with a wide range of amplification methods and reliable performance under diverse conditions.

[00194] To evaluate this capability, the activity of SrmCas12a and CrmCas12a enzymes alongside commercial LbaCas12a was tested by mimicking a one-pot system where LAMP preamplification and Cas12a detection occur in a single tube. Specifically, the reactions were incubated for 30 minutes at 60°C or 65°C, followed by a temperature reduction to 37°C for an additional 30 minutes. One-pot systems offer significant advantages by streamlining the workflow, reducing the risk of contamination, and minimizing hands-on time. These benefits are especially critical for point-of-care diagnostics, where simplicity, speed, and reliability are paramount.

[00195] **Methods**

[00196] To evaluate the high-temperature functionality of Cas12a enzymes in a one-pot diagnostic workflow, two SrmCas12a and CrmCas12a enzymes and commercial

LbaCas12a were tested under conditions mimicking a combined LAMP preamplification and Cas12a detection system. Reaction mixtures were prepared in a single tube, containing all necessary components for both LAMP amplification and Cas12a detection. [00197] Collateral cleavage assays using CrmCas12a, SrmCas12a, and LbaCas12a enzymes were performed in a 20 µL reaction volume. To assemble ribonucleoprotein (RNP) complexes, 400 nM purified Cas12a and 400 nM crRNA were combined in 2 µL of 10X NEBuffer™ r2.1 and incubated at room temperature for 15 minutes unless specified otherwise. Following this, 400 nM of the RNP complex was mixed with 1.7 nM of the target and 200 nM ssDNA reporter (/56-FAM/CCCCCCCC/3IABkFQ/) obtained from Integrated DNA Technologies. Each reaction was incubated at 60°C or 65°C for 30 minutes. Following this high-temperature incubation, the reaction temperature was reduced to 37°C and maintained for an additional 30 minutes to allow optimal Cas12a-mediated target detection.

[00198] All three enzymes were evaluated with a range of CRISPR RNAs (crRNAs) to assess their robustness across diverse crRNAs and target sequences. The tested crRNAs included those listed in **Table 9** for LbaCas12a, S-BP1, S-BP2, S-BP3, S-BP7, and S-BP9 for SrmCas12a, and C-BP1, C-BP2, C-BP3, and C-BP9 for CrmCas12a.

[00199] Real-time fluorescence measurements were collected on Step One ABI Real-time PCR (Applied Biosystems, CA, USA) at 1 min intervals for a total of 60 min. To allow comparisons between different conditions, fluorescence for background conditions (no target) were subtracted from samples to generate background subtracted fluorescence. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means (n = 3).

[00200] **Table 9-** crRNA sequences (5' to 3') to detect *Brucella bp26* gene. The protospacer regions have been highlighted in grey.

Enzyme	Protospacer	Sequence (5' to 3')	Name
LbaCas12a	BP1	UAAUJUCUACUAAGUGUAGAU GCACAGGAGAAUCACAUCA	L-BP1 (SEQ ID NO: 66)
	BP2	UAAUJUCUACUAAGUGUAGAU UGUCUAUCCUGACGACAAGA	L-BP2 (SEQ ID NO: 67)
	BP9	UAAUJUCUACUAAGUGUAGAU GAUGAAUCCGUCACGCGCGG	L-BP9 (SEQ ID NO: 68)

[00201] **Results**

[00202] At 60°C, LbaCas12a exhibited high activity, which further increased when the temperature was reduced to 37°C. CrmCas12a displayed low activity at 60°C, which gradually increased, and its activity increased substantially when the temperature was

reduced to 37°C. In contrast, SrmCas12a showed the lowest activity at 60°C, but its activity increased rapidly after the temperature was reduced to 37°C (**Figure 15**).

[00203] At 65°C, LbaCas12a showed relatively high activity during the first 30 minutes at 65°C, which further increased when the temperature was reduced to 37°C. However, the activity was substantially lower than what was observed at 60°C. CrmCas12a's activity was significantly affected at 65°C. It remained low at 65°C and showed a slight increase for only two crRNAs when the temperature was reduced to 37°C. In contrast, SrmCas12a's response was similar to that observed at 60°C. It exhibited very low activity at 65°C, but its activity increased rapidly after 30 minutes when the temperature was reduced to 37°C (**Figure 16**).

[00204] This pattern of activity of SrmCas12a (very low activity at 65°C and substantial increase after 30 minutes when the temperature was reduced to 37°C) offers several distinct advantages in the context of the one-pot LAMP-Cas12a system. At 65°C, the low activity of SrmCas12a ensures that a minimal amount of target DNA is cleaved. By keeping the cleavage activity low during the high-temperature phase, the system preserves a larger pool of intact target DNA, which remains available for the subsequent LAMP amplification.

[00205] This is particularly beneficial because the LAMP reaction relies on an ample amount of target DNA for effective amplification. With more intact substrate available for amplification, the LAMP reaction can proceed with greater efficiency, leading to higher yields of the amplified product. This ensures that the amplification step has enough substrate to generate detectable levels of DNA, which is crucial for the overall sensitivity and robustness of the assay.

[00206] In contrast, enzymes with higher activity at elevated temperatures, such as LbaCas12a, would cleave more target DNA during the high-temperature phase, potentially reducing the amount of substrate available for LAMP. This could lead to inefficient amplification or suboptimal assay performance. By delaying Cas12a activation and allowing more substrate to accumulate before cleavage occurs, SrmCas12a ensures that the one-pot system maximizes both amplification and detection, offering a more efficient and reliable diagnostic process.

[00207] **Example 12. Enhancement of cas12a collateral cleavage activity via buffer optimisation**

[00208] The activity of Cas12, similar to other enzymes, is known to be influenced by various factors, including the buffer composition. In this study, the effects of various compounds on Cas12a activity were tested, including osmolytes and protein stabilizers (betaine), detergents (Tween), metal ions (MgCl₂), and organic co-solvents (DMSO, glycerol), to optimize enzyme stability and performance.

[00209] The objective of testing these compounds were to identify conditions that influence Cas12a activity, aiming to optimize its performance by assessing potential enhancement or inhibition effects. By systematically varying the type and concentration of compounds in the reaction buffer, it was aimed to delineate the optimal conditions that support the enzymatic activity of Cas12.

[00210] **Method**

[00211] Collateral cleavage assays involving CrmCas12a and SrmCas12a enzymes were conducted in a final reaction volume of 20 µL. Initially, Cas12a proteins and crRNAs (Table 3) were combined to form ribonucleoprotein (RNP) complexes by mixing 400 nM purified Cas12a with 400 nM crRNA (BP3) in 2 µL of 10X NEBuffer™ r2.1 (base reaction buffer), followed by incubation at room temperature for 15 min, unless stated otherwise. Subsequently, 400 nM of the assembled RNP was mixed with 1.7 nM of the target and 200 nM ssDNA reporter (/56-FAM/CCCCCCCC/3IABkFQ/) from Integrated DNA Technologies, and the reactions were incubated for 1 h at 37 °C. The end-point fluorescence signal was measured using the Step One ABI Real-time PCR system (Applied Biosystems, CA, USA). To facilitate comparisons across different conditions, fluorescence from background conditions (no target) was subtracted from samples, yielding background-subtracted fluorescence. The graphs present the mean background-subtracted fluorescence output with standard deviation (n = 3). The base reaction buffer was NEBuffer™ r2.1, containing 50 mM NaCl, 10 mM Tris-HCl (pH 7.9 at 25°C), 10 mM MgCl₂, and 100 µg/ml recombinant albumin.

[00212] A range of concentrations were tested for each compound: 2.5, 5, 10, 25, and 50 mM for Tween 20; 5, 10, 25, 50, and 100 mM for DMSO, glycerol, and MgCl₂; and 0.05, 0.10, 0.25, 0.5, and 1 M for betaine. These compounds were separately added to the

base reaction buffer. The final concentrations of each compound were systematically varied to assess the influence of ionic strength and salt composition on Cas12a activity.

[00213] The experimental conditions are described in Example 4. The end-point fluorescence signal was measured using the Step One ABI Real-time PCR system (Applied Biosystems, CA, USA). To facilitate comparisons across different conditions, fluorescence from background conditions (no target) was subtracted from samples, yielding background-subtracted fluorescence. The graphs present the mean background-subtracted fluorescence output with standard deviation (n = 3).

[00214] **Result**

[00215] The addition of various compounds to the reaction buffer had distinct impacts on the activity of SrmCas12a and CrmCas12a. These effects varied depending on the compound and its concentration.

[00216] Tween 20: The results indicate that Tween 20 at low concentrations (2.5–5 mM) enhances the enzymatic activities of both SrmCas12a and CrmCas12a (**Figure 17**). At 2.5 mM, SrmCas12a activity increased 2.22-fold compared to the control, and CrmCas12a activity increased 2.05-fold compared to the control. At 5 mM, SrmCas12a activity remained elevated at 2.04-fold, while CrmCas12a activity was enhanced by 1.47-fold. This enhancement is attributed to Tween 20's surfactant properties, which reduce non-specific interactions and stabilize the enzymes by maintaining an optimal ionic and structural environment.

[00217] At concentrations above 5 mM, Tween 20 significantly inhibits enzymatic activity. For example, at 10 mM, SrmCas12a activity dropped to 30.6% of the control, and CrmCas12a activity dropped to 15.4% of the control. Near-total loss of function was observed at 25 mM (SrmCas12a: 0.23% of the control, CrmCas12a: 0.09% of the control) and 50 mM (SrmCas12a: 0%, CrmCas12a: 0.22% of the control). This inhibition is likely caused by excessive surfactant disrupting enzyme-substrate interactions or destabilizing the structural integrity of the enzymes.

[00218] DMSO, glycerol, betaine, and MgCl₂: The results indicate that the effects of DMSO, glycerol, betaine, and MgCl₂ on the enzymatic activities of both SrmCas12a and CrmCas12a were minimal (**Figure 18**). Across all tested concentrations, the enzyme activities showed either no significant enhancement or a slight decrease. These findings

suggest that these compounds do not substantially enhance the enzymatic activity of SrmCas12a and CrmCas12a within the tested concentration range.

[00219] **Example 13- Probe concentration**

[00220] The activity of enzymes can be influenced by various factors, including the concentration of the probes used in the reaction. In this study, the enzymatic activity of SrmCas12a and CrmCas12a under varying probe concentrations (200, 400, 800, 1600, and 3200 nM) was evaluated. The objective was to determine the optimal probe concentration that maximizes the activity of SrmCas12a and CrmCas12a enzymes, providing insights for their efficient application in diagnostic and molecular biology assays.

[00221] **Methods**

[00222] The procedure was as described in Example 4. The cleavage assay was performed with probe concentration of 00, 400, 800, 1600, and 3200 nM. Real-time fluorescence measurements were acquired at 1-minute intervals over a total duration of 60 minutes. To facilitate comparisons across diverse conditions, fluorescence values for background conditions (absence of target) were subtracted from the samples, generating background-subtracted fluorescence. The graphs depict the means ($n = 3$) of real-time background-subtracted fluorescence output.

[00223] **Results**

[00224] The enzymatic activity of SrmCas12a exhibited a clear concentration-dependent enhancement with increasing probe concentrations (**Figure 19**). At 400 nM, the activity increased by approximately 267% compared to the baseline at 200 nM. This trend continued at 800 nM, with an approximately 63% increase relative to 400 nM. A substantial enhancement was observed at 1,600 nM, where activity increased by 76% compared to 800 nM. The highest activity was recorded at 3,200 nM, representing a further 23% increase relative to 1,600 nM.

[00225] The enzymatic activity of CrmCas12a showed a notable concentration-dependent increase up to 1,600 nM. At 400 nM, the activity increased by approximately 104% compared to the baseline at 200 nM (**Figure 20**). A further enhancement of about 86% was observed at 800 nM relative to 400 nM. At 1,600 nM, the activity increased by approximately 31% compared to 800 nM, marking the highest recorded activity.

However, at 3,200 nM, the activity decreased by about 7% compared to 1,600 nM, indicating a potential inhibitory effect at higher probe concentrations.

[00226] These results highlight that SrmCas12a is more robust and responsive to increasing probe concentrations, achieving higher relative activity levels across all tested concentrations. In contrast, CrmCas12a displayed a more moderate response, with its activity peaking at 1,600 nM before declining at the highest probe concentration. This suggests that SrmCas12a may perform more effectively under a broader range of probe concentrations.

[00227] **Example 14- Limit of detection (LOD)**

[00228] The limit of detection (LOD) is a critical evaluation of CRISPR-Cas12a sensitivity in identifying target nucleic acids. This assessment highlights CrmCas12a and SrmCas12a enzymes' potential for sensitive diagnostics. By testing a series of diluted target samples, the LOD test determines the smallest concentration of the target that consistently generates a distinguishable signal from the background.

[00229] **Methods**

[00230] In this experiment, the *Bp26* gene was selected as the target sequence and then Oligo7 software was used to design RPA forward and reverse primers (Table 10).

[00231] **Table 10. RPA Primer sequences (5' to 3')**

Gene	Name	Sequence (5' to 3')
BP26	F	GACGGCCGTTTCGTCGAATGGCTCGGTTGCC (SEQ ID NO: 69)
	R	ATCGACGATGGTGCCAGAACCCGGCTCATCC (SEQ ID NO: 70)

[00232] The target plasmid was prepared as Example 4. The concentration of plasmid was estimated by Qubit4 fluorometer (Invitrogen) as its instruction manual.

[00233] The plasmid copy number was calculated using the following formula: plasmid copy number (copies/ml) = $[6.02 \times 10^{23} \times \text{plasmid concentration (ng/ml)} \times 10^{29}] / (\text{plasmid length} \times 660)$.

[00234] The sensitivity of the reaction was assessed using the RPA reaction system. Various concentrations of positive plasmid were employed as templates, while RNase-free water served as the negative control for each dilution.

[00235] The plasmid gradient dilution was from zero to 1×10^3 copies/ μ l (negative control (0), 10^0 , 0.5×10^1 , 10^1 , 10^2 , 10^3 copies/ μ l). RPA sensitivity was measured by the

RPA reaction system, with each diluted concentration of positive plasmid as a template and RNase-free water as a negative control.

[00236] RPA reaction assay was performed in a 50 μ L final reaction volume, with the plasmid DNA concentrations in the reaction ranging from 20 copies/ μ L to 0.02 copies/ μ L, based on the dilution of the plasmid stock solution and the addition of 1 μ L of plasmid to the final reaction mixture. 2.4 μ L (of 10 μ M/ μ L) of each RPA forward -and-reverse-direction primers F and R (**Table 8**), 29.5 μ L of primer-free rehydration buffer, 2.5 mM of MgOAc, 1 μ L of target DNA, and RNase-free water was replenished to 50 μ L. The mixture was incubated in a conventional water bath at 39 °C for 30 min.

[00237] Collateral cleavage assays involving CrmCas12a and SrmCas12a enzymes were conducted in a final reaction volume of 20 μ L. Initially, Cas12a proteins and crRNAs were combined to form ribonucleoprotein (RNP) complexes by mixing 400 nM purified Cas12a with 400 nM crRNA (BP3) in 2 μ L of 10X NEBuffer™ r2.1 with 2.5mM Tween 20, followed by incubation at room temperature for 15 min, unless stated otherwise. Subsequently, 400 nM of the assembled RNP was mixed with 1 μ L of the RPA amplified product and 1600 nM and 1600 nM ssDNA reporter (/56-FAM/CCCCCCCC/3IABkFQ/) from Integrated DNA Technologies, and the reactions were incubated for 1 h at 37 °C. Real-time fluorescence measurements were collected on Step One ABI Real-time PCR (Applied Biosystems, CA, USA) at 1 min intervals for a total of 60 min. To allow comparisons between different conditions, fluorescence for background conditions (no target) were subtracted from samples to generate background subtracted fluorescence. The measurement of real-time background subtracted fluorescence output are shown in the graphs as means (n = 3).

[00238] **Results**

[00239] The sensitivity RPA amplification coupled with combination of Cas12a enzymes was tested here using a series of diluted positive quality controls. (**Figures 21 and 22**).

[00240] Both SrmCas12a and CrmCas12a demonstrated strong sensitivity, with fluorescence signals at 0.1 copies/ μ L significantly higher than the background (0 copies/ μ L). However, at 0.02 copies/ μ L, the signal approached the background, indicating that the LOD for both enzymes is approximately 0.1 copies/ μ L under these

conditions. This highlights its ability to reliably detect low DNA concentrations with clear separation from background noise.

[00241] However, SrmCas12a exhibited a much faster response in terms of signal generation compared to CrmCas12a, reaching plateau levels within a significantly shorter period of time. Specifically, at a concentration of 20 copies/ μ L, SrmCas12a achieved its plateau signal within just 5 minutes, while CrmCas12a required approximately 13 minutes to reach the same plateau. Furthermore, for all other tested concentrations, SrmCas12a demonstrated higher signal intensities at earlier time points compared to CrmCas12a. This faster signal accumulation is particularly important for diagnostic applications because it enables more rapid detection of target DNA. In a clinical or field-based setting, where time is often a critical factor, the ability to achieve reliable results more quickly can improve the efficiency of testing processes. Faster reaction times can lead to shorter assay durations, enabling quicker diagnosis and potentially reducing patient wait times, which is crucial for time-sensitive conditions, especially when rapid intervention is needed.

[00242] At lower concentrations (0.2 and 0.1 copies/ μ L), SrmCas12a showed substantially higher end-point fluorescence signals compared to CrmCas12a. This enhanced signal at low copy numbers is critical for applications that require detection of minimal amounts of DNA, such as pathogen detection in environmental samples, or in cases where sample volume is limited. The higher end-point signal further supports the conclusion that SrmCas12a is not only faster in signal generation but also more sensitive, making it an excellent candidate for low-concentration diagnostics, where precise detection of minimal DNA is essential.

[00243] SrmCas12a's ability to generate higher signals in shorter periods and its superior sensitivity at low copy numbers provide significant advantages for diagnostic applications, allowing for faster, more reliable, and sensitive detection of target DNA, which can improve both the throughput and accuracy of diagnostic assays.

[00244] **Example 15- Detection using lateral flow strips**

[00245] Utilizing strips for CRISPR-Cas-based detection of Brucella in Point-of-Care Testing (POCT) provides rapid results, simplicity in operation, and portability, making them well-suited for immediate on-site testing, particularly in resource-limited or remote settings. The visual readout on the strips and the cost-effectiveness of strip-based assays

enhances their accessibility for widespread use, aligning with the principles of POCT of *Brucella*.

[00246] **Methods**

[00247] The plasmid preparation and RPA reaction was performed as described in Example 1, except that 1 copy/ μ L was also included.

[00248] Collateral cleavage assay was performed in a 100 μ L final reaction volume using both CrmCas12a and SrmCas12a enzymes as described in Example 14. Briefly, 10 μ L of 10x NEBr2.1 buffer, 2000 nM purified CrmCas12a, 2000 nM purified SrmCas12a, 2000 nM crRNA C-BC5, 2000 nM crRNA S-BCL1, 1000nM Probe (56-FAM/CCCCCCCC/3Biotin/), 10 μ L of the RPA amplified product, refilling to final volume with RNase-free water to 100 μ L. The prepared reaction tube was vortex and centrifuged at 4,000 rpm/min for 30 seconds. The mixture was incubated in a conventional water bath at 37 °C for 20 min.

[00249] The samples were transferred to a safe area after the Cas12a cleavage reaction and test strip MGH1 (Milenia Biotec GmbH) was inserted below the liquid level for 15 minutes. After the test strip had infiltrated completely, the test strip was removed. The photo was taken by camera of A73 Samsung Smartphone.

[00250] **Results**

[00251] The sensitivity of RPA amplification, coupled with the combination of SrmCas12a and CrmCas12a enzymes, was tested across different target DNA concentrations (20, 2, 1, 0.2, 0.1, and 0.02 copies/ μ L). The results demonstrated that both enzymes were capable of detecting target DNA at concentrations as low as 0.1 copies/ μ L when using lateral flow strips for detection (**Figure 23**). The use of lateral flow strips in this system is particularly significant for Point-of-Care Testing (POCT), as it enables rapid, easy-to-read results without the need for complex laboratory equipment. This approach is well-suited for on-site diagnostics, offering high sensitivity and simplicity, which are essential for the timely and accurate detection of low-abundance targets in diverse settings.

[00252] Surprisingly, it was observed that CrmCas12a produced a stronger positive band than SrmCas12a at low DNA concentrations. Despite using similar probe sequences, biotinylated probe was used for lateral flow assay. CrmCas12a may cleave the biotinylated probe more efficiently, leading to more detectable fragments. The

biotinylated probe, once cleaved, binds strongly to streptavidin on the lateral flow strip, which amplifies the signal and results in a stronger band. This suggests that CrmCas12a may have a higher or faster collateral cleavage activity with the biotinylated probe, leading to a stronger band at lower target DNA concentrations.

References:

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- Xu J, Ma J, Li Y, Kang L, Yuan B, Li S, Chao J, Wang L, Wang J, Su S, Yuan Y. A general RPA-CRISPR/Cas12a sensing platform for Brucella spp. detection in blood and milk samples. Sensors and Actuators B: Chemical. 2022 Aug 1;364:131864.

Claims

1. A Cas12a protein having an amino acid sequence selected from the group consisting of SEQ ID NO: 3 (CrmCas12a) and SEQ ID NO: 4 (SrmCas12a) or a Cas12a protein of the same clade as the Cas12a protein having an amino acid sequence selected from
5 the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4; wherein the Cas12a protein comprises a maltose-binding protein (MBP) tag having an amino acid sequence of SEQ ID NO: 72.
2. The Cas12a protein of claim 1, wherein the Cas12a protein is encoded by a DNA sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2.
- 10 3. The Cas12a protein of claim 1, wherein the MBP tag is encoded by a DNA sequence of SEQ ID NO: 71.
4. A CRISPR RNA (crRNA) for detecting Brucella, characterized in that the crRNA is selected from the group consisting of the following sequences:
S-BC1 – sequence shown in SEQ ID NO: 8;
15 S-BP1 – sequence shown in SEQ ID NO: 9;
S-OM1 – sequence shown in SEQ ID NO: 10;
C-BC1 – sequence shown in SEQ ID NO: 11;
C-BP1 – sequence shown in SEQ ID NO: 12;
C-OM1 – sequence shown in SEQ ID NO: 13;
20 S-BC2 – sequence shown in SEQ ID NO: 14;
S-BC3 – sequence shown in SEQ ID NO: 15;
S-BC4 – sequence shown in SEQ ID NO: 16;
S-BC5 – sequence shown in SEQ ID NO: 17;
S-BC6 – sequence shown in SEQ ID NO: 18;
25 S-BC7 – sequence shown in SEQ ID NO: 19;
S-BC8 – sequence shown in SEQ ID NO: 20;
C-BC2 – sequence shown in SEQ ID NO: 21;

- C-BC3 – sequence shown in SEQ ID NO: 22;
C-BC4 – sequence shown in SEQ ID NO: 23;
C-BC5 – sequence shown in SEQ ID NO: 24;
C-BC6 – sequence shown in SEQ ID NO: 25;
5 C-BC7 – sequence shown in SEQ ID NO: 26;
C-BC8 – sequence shown in SEQ ID NO: 27;
S-BP2 – sequence shown in SEQ ID NO: 28;
S-BP3 – sequence shown in SEQ ID NO: 29;
S-BP5 – sequence shown in SEQ ID NO: 30;
10 S-BP6 – sequence shown in SEQ ID NO: 31;
S-BP7 – sequence shown in SEQ ID NO: 32;
C-BP2 – sequence shown in SEQ ID NO: 33;
C-BP3 – sequence shown in SEQ ID NO: 34;
C-BP5 – sequence shown in SEQ ID NO: 35;
15 C-BP6 – sequence shown in SEQ ID NO: 36;
C-BP7 – sequence shown in SEQ ID NO: 37;
S-OM2 – sequence shown in SEQ ID NO: 38;
S-OM3 – sequence shown in SEQ ID NO: 39;
S-OM4 – sequence shown in SEQ ID NO: 40;
20 C-OM2 – sequence shown in SEQ ID NO: 41;
C-OM3 – sequence shown in SEQ ID NO: 42;
C-OM4 – sequence shown in SEQ ID NO: 43;
S-BP8 – sequence shown in SEQ ID NO: 44;
S-BP9 – sequence shown in SEQ ID NO: 45;
25 C-BP8 – sequence shown in SEQ ID NO: 46; and

- C-BP9 – sequence shown in SEQ ID NO: 47;
wherein the crRNA comprises a direct repeat (DR) and a guide domain (protospacer), wherein the DR and the protospacer are connected in the 5' to 3' direction of the crRNA, and the crRNA is configured to target a nucleic acid of
- 5 Brucella with a predetermined target sequence and form a complex with the Cas12a protein of any one of claims 1-3.
5. The crRNA of claim 4, wherein the protospacer has 20-26 nucleotides.
6. The crRNA of claim 4 or 5, wherein the nucleic acid of Brucella comprises a sequence selected from the group consisting of SEQ ID NO: 5 (BSCP31), SEQ ID NO: 6
- 10 (bp26), and SEQ ID NO: 7 (Omp2a).
7. A detection kit for Brucella, wherein the detection kit comprises the crRNA of any one of claims 4-6.
8. The detection kit of claim 7, further comprising the Cas12a protein of any one of claims 1-3.
- 15 9. The detection kit of claim 8, further comprising a detecting agent comprising a probe nucleic acid, wherein the detecting agent generates a detectable signal when the probe nucleic acid is cleaved.
10. The detection kit of claim 9, wherein the probe nucleic acid is a fluorescent probe, wherein the 5' end of the fluorescent probe sequence is labeled with a fluorescent
- 20 group, and the 3' end is labeled with a quenching group; or a colorimetric probe.
11. The detection kit of claim 10, wherein the fluorescent group is selected from the group consisting of FAM, VIC, HEX, TRT, Cy3, Cy5, ROX, JOE and Texas Red, and the quenching group is selected from the group consisting of IABKFQ, DABCYL, MGB, BHQ-1, BHQ-2 and BHQ-3.
- 25 12. The detection kit of claim 10, wherein the colorimetric probe comprises FAM and Biotin, and is for detection by a lateral flow strip.

13. The detection kit of any one of claims 7-12, wherein the Cas12a protein and the crRNA are in the form of a ribonucleoprotein complex.
14. A method of detecting a nucleic acid of *Brucella* in a sample using the detection kit of any one of claims 7-13, wherein the method comprises the following steps:
- 5 incubating the sample containing the nucleic acid of *Brucella* with the Cas12a protein and the crRNA, wherein when the nucleic acid of *Brucella* is present in the sample, collateral cleavage activity of the Cas12a protein is induced to generate a detectable signal when the probe nucleic acid is cleaved.
15. The method of claim 14, further comprising amplifying the nucleic acid of *Brucella* in the sample, using a technology selected from the group consisting of Polymerase Chain Reaction (PCR), Recombinase Polymerase Amplification (RPA), Loop-Mediated Isothermal Amplification (LAMP), and Rolling Circle Amplification (RCA).
- 10 in the sample, using a technology selected from the group consisting of Polymerase Chain Reaction (PCR), Recombinase Polymerase Amplification (RPA), Loop-Mediated Isothermal Amplification (LAMP), and Rolling Circle Amplification (RCA).
16. The method of claim 14, wherein the method does not comprise amplifying the nucleic acid of *Brucella* in the sample.
- 15 nucleic acid of *Brucella* in the sample.
17. The method of claim 14, further comprising using a detection method selected from the group consisting of a colorimetric detection method, a fluorescent detection method and an electrochemical detection method to detect the detectable signal which is a colorimetric signal, a fluorescent signal, or an electrochemical signal.
- 20 18. The method of claim 17, wherein the colorimetric detection method uses a lateral flow strip and a colorimetric probe comprising FAM and Biotin.
19. The method of claim 17, wherein the fluorescent detection method uses a fluorescent probe, wherein the fluorescence signal is measured using a fluorometer, a microplate reader, or Real-Time PCR.
- 25 20. The method of claim 17, wherein the electrochemical detection method transduces CRISPR-Cas cleavage event to an electrochemical signal using a modified electrode or a nanoparticle-based sensor.

21. The method of claim 14, wherein one or more of crRNAs of SEQ ID NO: 8-47 are used to detect *Brucella*.
22. The method of any one of claims 14-21, wherein both the Cas12a protein having an amino acid sequence of SEQ ID NOs: 3 (CrmCas12a) and the Cas12a protein having an amino acid sequence of SEQ ID NO: 4 (SrmCas12a) are used to detect *Brucella*.
23. The method of claim 14, wherein the nucleic acid of *Brucella* comprises a sequence selected from the group consisting of SEQ ID NO: 5 (BSCP31), SEQ ID NO: 6 (bp26), and SEQ ID NO: 7 (Omp2a).
24. The method of claim 23, wherein the nucleic acid of *Brucella* is DNA, and the DNA is 10 copies/ μ L or less.
25. The method of any one of claims 14-24, wherein the *Brucella* is selected from the group consisting of *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella canis*, *Brucella ovis*, *Brucella neotomae*, *Brucella microti*, *Brucella ceti*, *Brucella pinnipedialis* or a combination thereof.
26. The method of claim 14, wherein the sample is a biological sample obtained from a tissue sample, saliva, blood, plasma, milk, sera, stool, urine, sputum, mucous, lymph, synovial fluid, cerebrospinal fluid, ascites, pleural effusion, seroma, pus, nasal swab specimen, or swab of skin or a mucosal membrane surface or a combination thereof.
27. The method of claim 26, wherein the biological sample is a crude sample and/or wherein the target molecule is not purified or amplified from the sample.
28. A CRISPR-Cas12a composition comprising the Cas12a protein of any one of claims 1-3, and a CRISPR RNA (crRNA), wherein the crRNA comprises a direct repeat (DR) and a guide domain (protospacer), wherein the DR and the guide domain are connected in the 5' to 3' direction of the crRNA, and the crRNA is configured to target a nucleic acid of a predetermined target sequence and form a complex with the Cas12a protein of any one of claims 1-3.
29. The CRISPR-Cas12a composition of claim 28, wherein the crRNA is the crRNA for detecting *Brucella* of any one of claims 4-6.

30. The CRISPR-Cas12a composition of claim 28, further comprising a detecting agent comprising a probe nucleic acid, wherein the detecting agent generates a detectable signal when the probe nucleic acid is cleaved.
31. The CRISPR-Cas12a composition of claim 30, wherein the probe nucleic acid is a
5 fluorescent probe, wherein the 5' end of the fluorescent probe sequence is labelled with a fluorescent group, and the 3' end is labelled with a quenching group; or a colorimetric probe.
32. The CRISPR-Cas12a composition of claim 31, wherein the fluorescent group is selected from the group consisting of FAM, VIC, HEX, TRT, Cy3, Cy5, ROX, JOE
10 and Texas Red, and the quenching group is selected from the group consisting of IABKFQ, DABCYL, MGB, BHQ-1, BHQ-2 and BHQ-3.
33. The CRISPR-Cas12a composition of claim 31, wherein the colorimetric probe comprises FAM and Biotin, and is for detection by a lateral flow strip.
34. The CRISPR-Cas12a composition of any one of claims 28-33, wherein the Cas12a
15 protein and the crRNA are in the form of a ribonucleoprotein complex.
35. A method of detecting a nucleic acid having a predetermined target sequence in a sample using the CRISPR-Cas12a composition of any one of claims 28-34, comprising: incubating the sample with the CRISPR-Cas12a composition, wherein
20 when the nucleic acid having the predetermined target sequence is present in the sample, the collateral cleavage activity of the CRISPR-Cas12a composition is induced to generate a detectable signal when the probe nucleic acid is cleaved.
36. The method of claim 35, further comprising amplifying the nucleic acid having a predetermined target sequence in the sample, using a technology selected from the group consisting of Polymerase Chain Reaction (PCR), Recombinase Polymerase
25 Amplification (RPA), Loop-Mediated Isothermal Amplification (LAMP), and Rolling Circle Amplification (RCA).
37. The method of claim 35, wherein the method does not comprise amplifying the nucleic acid having a predetermined target sequence in the sample.

38. The method of claim 35, further comprising using a detection method selected from the group consisting of a colorimetric detection method, a fluorescent detection method and an electrochemical detection method to detect the detectable signal which is a colorimetric signal, a fluorescent signal, or an electrochemical signal.
- 5 39. The method of claim 38, wherein the colorimetric detection method uses a lateral flow strip and a colorimetric probe comprising FAM and Biotin.
40. The method of claim 38, wherein the fluorescent detection method uses a fluorescent probe, wherein the fluorescence signal is measured using a fluorometer, a microplate reader, or Real-Time PCR.
- 10 41. The method of claim 38, wherein the electrochemical detection method transduces CRISPR-Cas cleavage event to an electrochemical signal using a modified electrode or a nanoparticle-based sensor.
42. The method of any one of claims 35-41, wherein one or more crRNAs are used to detect the nucleic acid having a predetermined target sequence.
- 15 43. The method of any one of claims 35-42, wherein both the Cas12a protein having an amino acid sequence of SEQ ID NOs: 3 (CrmCas12a) and the Cas12a protein having an amino acid sequence of SEQ ID NO: 4 (SrmCas12a) are used to detect the nucleic acid having a predetermined target sequence.
44. The method of claim 43, wherein the nucleic acid is DNA.
- 20 45. The method of claim 44, wherein the DNA is 10 copies/ μ L or less.
46. The method of claim 35, wherein the sample is a biological sample obtained from a tissue sample, saliva, blood, plasma, milk, sera, stool, urine, sputum, mucous, lymph, synovial fluid, cerebrospinal fluid, ascites, pleural effusion, seroma, pus, nasal swab specimen, or swab of skin or a mucosal membrane surface or a combination thereof.
- 25 47. The method of claim 46, wherein the biological sample is a crude sample and/or wherein the target molecule is not purified or amplified from the sample.

48. The method of claim 35, wherein nucleic acid in the sample belongs to a microbe selected from a bacterium, a virus, an archaea, a protozoa, a fungi, an algae, a slime mode, a lichen, and a prion.
49. The method of claim 48, wherein the virus is selected from a group consisting of a
 5 double-stranded DNA virus, a single-stranded DNA virus, a double-stranded RNA virus, a positive sense RNA virus, a negative sense RNA virus, and a retrovirus.
50. The method of claim 49, wherein the virus is selected from a group consisting of a coronavirus, an Ebola virus, measles, SARS, Chikungunya virus, Marburg, MERS, Dengue, Lassa, influenza, rhabdovirus, HIV, a hepatitis virus (including hepatitis A,
 10 B, C, D, or E), an influenza virus (including an influenza A or influenza B), a human respiratory syncytial virus, Sudan ebola virus, Bundibugyo virus, Tai Forest ebola virus, Reston ebola virus, Achimota virus, Aedes flavivirus, Aguacate virus, Akabanc virus, Alethinophid reptarenavirus, Allpahuayo mammarenavirus, Amapari
 15 mmarenavirus, Andes virus, Apoi virus, Aravan virus, Aroa virus, Arumwot virus, Atlantic salmon paramyxovirus, Australian bat lyssavirus, Avian homavirus, Avian metapneumovirus, Avian paramyxoviruses, penguin or Falkland Islands virus, BK polyomavirus, Bagaza virus, Banna virus, Bat herpesvirus, Bat sapovirus, Bear Canon mammarenavirus, Beilong virus, Betacoronavirus, Betapapillomavirus 1-6, Bhanja virus, Bokcloh bat lyssavirus, Boma disease virus, Bourbon virus, Bovine
 20 hepatic virus, Bovine parainfluenza virus 3, Bovine respiratory syncytial virus, Brazoran virus, Bunyamwera virus, Caliciviridae virus, California encephalitis virus, Candiru virus, Canine distemper virus, Canine pneumovirus, Cedar virus, Cell fusing agent virus, Cetacean morbillivirus, Chandipura virus, Chaoyang virus, Chapare mammarenavirus, Colobus monkey papillomavirus, Colorado tick fever virus,
 25 Cowpox virus, Crimean-Congo hemorrhagic fever virus, Culex flavivirus, Cupixi mammarenavirus, Dengue virus, Dobrava-Belgrade virus, Donggang virus, Dugbe virus, Duvenhage virus, Eastern equine encephalitis virus, Entebbe bat virus, Enterovirus A-D, European bat lyssavirus 1-2, Eyach virus, Feline morbillivirus, Ferde- Lance paramyxovirus, Fitzroy River virus, Flaviviridae virus, Flexal
 30 mammarenavirus, GB virus C, Gairo virus, Gemycircularvirus, Goose paramyxovirus SF02, Great Island virus, Guanarito mammarenavirus, Hantaan virus, Hantavirus

Z10, Heartland virus, Hendra virus, Hepatitis A/B/C/E, Hepatitis delta virus, Human bocavirus, Human coronavirus, Human endogenous retrovirus K, Human enteric coronavirus, Human genital-associated circular DNA virus- 1, Human herpesvirus 1-8, Human mastadenovirus A-G, Human papillomavirus, Human parainfluenza virus

5 1-4, Human paracchovirus, Human picomavirus, Human smacovirus, Ikoma lyssavirus, Ilheus virus, Influenza A-C, Ippy mammarenavirus, Irkut virus, J-virus, JC polyomavirus, Japanese encephalitis virus, Junin mammarenavirus, KI polyomavirus, Kadipiro virus, Kamiti River virus, Kedougou virus, Khujand virus, Kokobera virus, Kyasanur forest disease virus, Lagos bat virus, Langat virus, Lassa

10 mammarenavirus, Latino mammarenavirus, Leopards Hill virus, Liao ning virus, Ljungan virus, Lloviu virus, Louping ill virus, Lujo mammarenavirus, Luna mammarenavirus, Lunk virus, Lymphocytic choriomeningitis mammarenavirus, Lyssavirus Ozemoe, MSS12Y225 virus, Machupo mammarenavirus, Mamastrovirus 1, Manzanilla virus, Mapuera virus, Marburg virus, Mayaro virus, Measles virus,

15 Menangle virus, Mercadeo virus, Merkel cell polyomavirus, Middle East respiratory syndrome coronavirus, Mobala mammarenavirus, Modoc virus, Moijang virus, Mokolo virus, Monkeypox virus, Montana myotis leukoencephalitis virus, Mopeia lassa virus reassortant 29, Mopeia mammarenavirus, Morogoro virus, Mossman virus, Mumps virus, Murine pneumonia virus, Murray Valley encephalitis virus, Nariva

20 virus, Newcastle disease virus, Nipah virus, Norwalk virus, Norway rat hepacivirus, Ntaya virus, O'nyong-nyong virus, Oliveros mammarenavirus, Omsk hemorrhagic fever virus, Oropouche virus, Parainfluenza virus 5, Parana mammarenavirus, Parramatta River virus, Peste-des-petits- ruminants virus, Pichande mammarenavirus, Picomaviridae virus, Pirital mammarenavirus, Piscihepevirus A, Porcine

25 parainfluenza virus 1, porcine rubulavirus, Powassan virus, Primate T-lymphotropic virus 1-2, Primate erythroparvovirus 1, Punta Toro virus, Puumala virus, Quang Binh virus, Rabies virus, Razdan virus, Reptile bomavirus 1, Rhinovirus A-B, Rift Valley fever virus, Rinderpest virus, Rio Bravo virus, Rodent Torque Teno virus, Rodent hepacivirus, Ross River virus, Rotavirus A-I, Royal Farm virus, Rubella virus, Sabia

30 mammarenavirus, Salem virus, Sandfly fever Naples virus, Sandfly fever Sicilian virus, Sapporo virus, Sathuperi virus, Scal ancillovirus, Semliki Forest virus, Sendai virus, Seoul virus, Sepik virus, Severe acute respiratory syndrome-related

- coronavirus, Severe fever with thrombocytopenia syndrome virus, Shamonda virus, Shimoni bat virus, Shuni virus, Simbu virus, Simian torque teno virus, Simian virus 40-41, Sin Nombre virus, Sindbis virus, Small anellovirus, Sosuga virus, Spanish goat encephalitis virus, Spondweni virus, St. Louis encephalitis virus, Sunshine virus,
- 5 TTV-like mini virus, Tacaribe mammarenavirus, Taila virus, Tamana bat virus, Tamiami mammarenavirus, Tembusu virus, Thogoto virus, Thottapalayam virus, Tick-borne encephalitis virus, Tioman virus, Togaviridae virus, Torque teno canis virus, Torque teno douroucouli virus, Torque teno felis virus, Torque teno midi virus, Torque teno sus virus, Torque teno tamarin virus, Torque teno virus, Torque teno zalophus virus, Tuhoko virus, Tula virus, Tupaia paramyxovirus, Usutu virus,
- 10 Uukuniemi virus, Vaccinia virus, Variola virus, Venezuelan Vesicular stomatitis Indiana virus, WU Polyomavirus, Wesselsbron virus, West Caucasian bat virus, West Nile virus, Western equine encephalitis virus, Whitewater Arroyo mammarenavirus, Yellow fever virus, Yokose virus, Yug Bogdanovac virus, Zaire ebolavirus, Zika virus, or Zygosaccharomyces bailii virus Z viral sequence, Canine Parvovirus, Canine Distemper Virus, Canine Coronavirus, Canine Influenza Virus, Feline Herpesvirus, Feline Calicivirus, Feline Leukemia Virus, Feline Immunodeficiency Virus, Canine Adenovirus - Type 1 and Type 2, Canine Herpesvirus, Tobacco Mosaic Virus, Potato Virus Y, Tomato Spotted Wilt Virus, Cucumber Mosaic Virus, Potato Virus X, Barley
- 20 Yellow Dwarf Virus, Bean Common Mosaic Virus, Maize Dwarf Mosaic Virus, Soybean Mosaic Virus, Citrus Tristeza Virus, Rice Yellow Mottle Virus, Plum Pox Virus, Turnip Mosaic Virus, Aphid-Transmitted Yellow Dwarf Viruses, Cotton Leaf Curl Virus, Papaya Ringspot Virus, Cassava Mosaic Virus, Rice Tungro Virus, Apple Mosaic Virus, Sugarcane Mosaic Virus or a combination thereof.
- 25 51. The method of claim 48, wherein the bacterium is a Gram-positive bacterium or a Gram-negative bacterium selected from the group consisting of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus anthracis*, *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Clostridium difficile* and *Clostridium tetani*,
- 30 *Corynebacterium diphtheria*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Chlamydia trachomatis*,

- Yersinia pestis*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Proteus mirabilis*, *Enterobacter cloacae*, *Serratia marcescens*, *Helicobacter pylori*, *Salmonella enteritidis*, *Salmonella typhi*, and *Vibrio cholera*,
- 5 *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella canis*, *Brucella ovis*, *Brucella neotomae*, *Mycoplasma mycoides*, *Clostridium perfringens*, *Mycobacterium avium* *Salmonella* spp., *Pasteurella multocida*, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Yersinia pestis*, *Francisella tularensis*, *Bacillus anthracis*, *Coxiella burnetii*, *Listeria monocytogenes*, *Clostridium chauvoei*, *Campylobacter*
- 10 spp., *Chlamydia psittaci*, *Anaplasma marginale*, *Leptospira* spp. Causes leptospirosis, *Bordetella bronchiseptica*, *Clostridium perfringens*, *Staphylococcus intermedius*, *Bartonella henselae*, *Chlamydomydia felis*, *Mycoplasma* spp, *Pasteurella multocida*, *Erwinia amylovora*, *Xanthomonas* spp., *Xanthomonas axonopodis*, *Ralstonia solanacearum*, *Pectobacterium* and *Dickeya* spp., *Agrobacterium tumefaciens*,
- 15 *Pseudomonas syringae*, *Pseudomonas syringae* pv. *lachrymans*, *Xanthomonas campestris* pv. *campestris*, *Xanthomonas oryzae*, *Clavibacter michiganensis*, *Xylella fastidiosa*, *Pantoea* spp., *Burkholderia glumae*, *Xanthomonas citri*, *Xanthomonas translucens*, *Xanthomonas fragariae*, *Xanthomonas hortorum*, *Clavibacter michiganensis*, *Pseudomonas syringae* or a combination thereof.
- 20 52. The method of claim 35, wherein the nucleic acid is a RNA or a DNA from a virus of claim 49 or 50, or a RNA or a DNA from a bacteria of claim 51; wherein the RNA is reverse-transcribed to complementary DNA (cDNA), and then isothermally amplified using methods such as Recombinase Polymerase Amplification (RPA) or Loop-Mediated Isothermal Amplification (LAMP), before subjected to the method of claim
- 25 35.
53. The method of claim 35, wherein the method is used for identifying and characterising genetic variations selected from the group consisting of single nucleotide polymorphism (SNP) analysis and allelic discrimination.