

inevitably suffer from trade-offs among sensitivity, specificity, simplicity, and speed. For example, qPCR approaches are sensitive but are expensive and rely on complex instrumentation, limiting usability to highly trained operators in laboratory settings. As nucleic acid diagnostics become increasingly relevant for a variety of healthcare applications, detection technologies that provide high specificity and sensitivity at low cost would be of great utility in both clinical and basic research settings.

[0006] Many nucleic acid amplification approaches are available with various detection platforms. Among them, isothermal nucleic acid amplification methods have been developed for amplification without drastic temperature cycling and complex instrumentations. These methods include nucleic-acid sequenced-based amplification (NASBA), recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), strand displacement amplification (SDA), helicase-dependent amplification (HDA), or nicking enzyme amplification reaction (NEAR). These isothermal amplification approaches, however, may still require an initial denaturation step and multiple sets of primers. Furthermore, novel approaches combining isothermal nucleic acid amplification with portable platforms (Du et al., 2017; Pardee et al., 2016), offer high detection specificity in a point-of-care (POC) setting, but have somewhat limited applications due to low sensitivity.

SUMMARY

[0007] In one aspect, the present disclosure provides a method of amplifying and/or detecting a target double-stranded nucleic acid, comprising: (a) combining a sample comprising the target double-stranded nucleic acid with an amplification reaction mixture, the amplification reaction mixture comprising: (i) an amplification CRISPR system, the amplification CRISPR system comprising a first and second CRISPR/Cas complex, the first CRISPR/Cas complex comprising a first CRISPR/Cas enzyme and a first guide molecule that guides the first CRISPR/Cas complex to a first strand of the target nucleic acid, and the second CRISPR/Cas complex comprising a second CRISPR/Cas enzyme and a second guide molecule that guides the second CRISPR/Cas complex to a second strand of the target nucleic acid; (ii) a helicase; (iii) a primer pair comprising a first and second primer, wherein the first primer comprises a portion that is complementary to the first strand of the target nucleic acid and the second primer comprises a portion that is complementary to the second strand of the target nucleic acid; and (iv) a polymerase; (b) amplifying the target nucleic acid by opening R-loops

of the target nucleic acid using the first and second CRISPR/Cas complexes, unwinding the first and the second strand of the target nucleic acid using the helicase, annealing and extending the strands using the primer pair and the polymerase; and (c) further amplifying the target nucleic acid by repeated opening, unwinding, annealing and extension under isothermal conditions; and (d) optionally detecting the amplified target nucleic acid.

[0007a] In another aspect, the present disclosure provides a method for amplifying and/or detecting a target single-stranded nucleic acid, comprising: (a) converting the single-stranded nucleic acid in a sample to a target double-stranded nucleic acid; and (b) performing the steps as described herein, optionally wherein the target single-stranded nucleic acid is an RNA molecule, optionally wherein the RNA molecule is converted to the double-stranded nucleic acid by a reverse-transcription and amplification step, optionally wherein the target single-stranded nucleic acid is selected from the group consisting of single-stranded viral DNA, viral RNA, messenger RNA, ribosomal RNA, transfer RNA, microRNA, short interfering RNA, small nuclear RNA, synthetic RNA, synthetic single-stranded DNA, long non-coding RNA, pre-microRNA, viral dsRNA, and non-viral dsRNA.

[0007b] In another aspect, the present disclosure provides a system for amplifying and/or detecting a target double-stranded nucleic acid in a sample, the system comprising: a) an amplification CRISPR system, the amplification CRISPR system comprising a first and second CRISPR/Cas complex, the first CRISPR/Cas complex comprising a first CRISPR/Cas enzyme and a first guide molecule that guides the first CRISPR/Cas complex to a first strand of the target nucleic acid, and the second CRISPR/Cas complex comprising a second CRISPR/Cas enzyme and a second guide molecule that guides the second CRISPR/Cas complex to a second strand of the target nucleic acid; b) a helicase; c) a primer pair comprising a first and second primer, wherein the first primer comprising a portion that is complementary to the first strand of the target nucleic acid and the second primer comprising a portion that is complementary to the second strand of the target nucleic acid; d) a polymerase; and optionally e) a detection system for detecting amplification of the target nucleic acid; optionally wherein the CRISPR/Cas enzyme is a dead CRISPR/Cas enzyme selected from a dead Cas9 enzyme or a dead Cas12 enzyme, optionally wherein the dead Cas12 enzyme is a dead Cas12a, Cas12b, or Cas12c enzyme, or wherein the CRISPR/Cas enzyme is a Cas9 or Cas12 enzyme, optionally wherein the Cas12 enzyme is a Cas12a, Cas12b, or Cas12c enzyme, optionally wherein the polymerase is selected from the group consisting of *Bst* 2.0 DNA polymerase, *Bst* 2.0 WarmStart DNA polymerase, *Bst* 3.0 DNA polymerase, full length *Bst* DNA polymerase, large

fragment *Bst* DNA polymerase, large fragment *Bsu* DNA polymerase, phi29 DNA polymerase, T7 DNA polymerase, Gst polymerase, Taq polymerase, Klenow fragment of *E. coli* DNA polymerase I, KlenTaq NDA polymerase, Pol III DNA polymerase, T5 DNA polymerase, and Sequenase DNA polymerase, and Sau LF DNA polymerase, optionally wherein the helicase is selected from the group consisting of UvrD helicase, CRISPR-Cas3 helicase, Rep helicase, PcrA helicase, *E. coli* helicase I, *E. coli* helicase II, *E. coli* helicase III, *E. coli* helicase IV, Rep helicase, DnaB helicase, PriA helicase, PcrA helicase, T4 Gp41 helicase, T4 Dda helicase, SV40 Large T antigen, yeast RAD helicase, RecD helicase, RecQ helicase, thermostable *T. tengcongensis* UvrD helicase, thermostable *T. thermophilus* UvrD helicase, thermostable *T. aquaticus* DnaB helicase, Dda helicase, papilloma virus E1 helicase, archaeal MCM helicase, eukaryotic MCM helicase, and T7 Gp4 helicase, optionally wherein the helicase is Tte-UvrD D409A/D410A, optionally further comprising one or more additives selected from a crowding agent, a co-factor, single-strand binding protein, and/or assisting proteins, optionally wherein the crowding agent is polyethylene glycol 20K molecular weight, optionally provided at 2.5% to 6.5%, optionally wherein the co-factor is ATP, optionally provided at 0.75 to 5 mM, optionally wherein the single-strand binding protein is selected from T4 gp32 or thermophilic SSB (ET-SSB), optionally wherein the assisting protein is dCpf1 and/or UvsX Recombinase, optionally wherein the CRISPR/Cas enzyme, the helicase, and the polymerase perform under the same temperature, in some instances, at about 37 °C, optionally wherein the detection system comprises a CRISPR system comprising a Cas 12 or Cas 13 effector protein and a guide RNA designed to bind to a corresponding target molecule; and a masking construct, optionally wherein the detection system comprises Cas 13 enzyme LwaCas13, optionally further comprising an additional amplifier primer comprising a promoter sequence identity with the first primer of the primer pair.

[0007c] In yet another aspect, the present disclosure provides a system for amplifying and/or detecting a target single-stranded nucleic acid in a sample, the system comprising: reagents for converting the target single-stranded nucleic acid to a double-stranded nucleic acid; b) components as described herein; optionally wherein the detection system comprises a CRISPR system comprising a Cas 12 or Cas 13 effector protein and a guide RNA designed to bind to a corresponding target molecule; and a masking construct, optionally wherein the detection system comprises Cas 13 enzyme LwaCas13, optionally further comprising reagents for purifying the single-stranded nucleic acid in the sample.

[0007d] In one example, the disclosure provides a method of amplifying and/or detecting a target double-stranded nucleic acid. The method comprises (a) combining a sample comprising the target double-stranded nucleic acid with an amplification reaction mixture, (b) amplifying the target nucleic acid, and (c) further amplifying the target nucleic acid by repeated opening, unwinding, annealing and extension under isothermal conditions. The method optionally comprises a step of detecting the target nucleic acid.

[0008] The amplification reaction mixture may include (i) an amplification CRISPR system, (ii) a helicase, (iii) a primer pair comprising a first and second primer, and (iv) a polymerase. The amplification CRISPR system may comprise a first and second CRISPR/Cas complex. The first CRISPR/Cas complex may comprise a first CRISPR/Cas enzyme and a first guide molecule that guides the first CRISPR/Cas complex to a first strand of the target nucleic acid. The second CRISPR/Cas complex may comprise a second CRISPR/Cas enzyme and a second guide molecule that guides the second CRISPR/Cas complex to a second strand of the

target nucleic acid. The first and second CRISPR/Cas enzymes may be active or dead. The first primer may comprise a portion that is complementary to the first strand of the target nucleic acid and the second primer may comprise a portion that is complementary to the second strand of the target nucleic acid. The target nucleic acid may be amplified by opening R-loops of the target nucleic acid using the first and second CRISPR/Cas complexes, unwinding the first and the second strand of the target nucleic acid using the helicase, and annealing and extending the strands using the primer pair and the polymerase.

[0009] In some examples, the dead CRISPR/Cas enzyme may be selected from the group consisting of dead Cas9, dead Cas12a, dead Cas12b, and dead Cas12c. In some examples, the dead CRISPR/Cas enzyme may be a dead Cas9 protein which comprises a mutation in the HNH and RuvC domain.

[0010] The dead CRISPR/Cas enzyme may be a dead Cas9 protein which comprises a mutation corresponding to D10A and N863A in SpCas9, or D10A and H840A in SpCas9. The dead CRISPR/Cas enzyme may be a dead Cas9 protein derived from a bacterial species selected from the group consisting of *Streptococcus pyogenes*, *Staphylococcus aureus*, *Streptococcus thermophilus*, *S. mutans*, *S. agalactiae*, *S. equisimilis*, *S. sanguinis*, *S. pneumoniae*, *C. jejuni*, *C. coli*, *N. salmophilus*, *N. tergastris*, *S. auricularis*, *S. carnosus*, *N. meningitidis*, *N. gonorrhoeae*, *L. monocytogenes*, *L. ivanovii*, *C. botulinum*, *C. difficile*, *C. tetani*, *C. sordellii*, *Francisella tularensis* 1, *Prevotella albensis*, *Lachnospiraceae* bacterium MC2017 1, *Butyrivibrio proteoclasticus*, *Peregrinibacteria bacterium* GW2011_GWA2_33_10, *Parcubacteria bacterium* GW2011_GWC2_44_17, *Smithella* sp. SCADC, *Acidaminococcus* sp. BV3L6, *Lachnospiraceae* bacterium MA2020, *Candidatus Methanoplasma termitum*, *Eubacterium eligens*, *Moraxella bovoculi* 237, *Leptospira inadai*, *Lachnospiraceae* bacterium ND2006, *Porphyromonas crevioricanis* 3, *Prevotella disiens* and *Porphyromonas macacae*.

[0011] In some examples, the dead CRISPR/Cas enzyme may be a dead Cas12 protein. The dead Cas12 protein may comprise a mutation in the RuvC domain. The dead Cas12 enzyme may be a dead Cas12a, Cas12b, or Cas12c. The dead Cas12a protein may comprise a mutation corresponding to D908A or E993A in AsCpf1.

[0012] The dead Cas12 protein may be derived from a bacterial species selected from the group consisting of *Francisella tularensis*, *Prevotella albensis*, *Lachnospiraceae* bacterium, *Butyrivibrio proteoclasticus*, *Peregrinibacteria bacterium*, *Parcubacteria bacterium*, *Smithella* sp., *Acidaminococcus* sp., *Lachnospiraceae* bacterium, *Candidatus Methanoplasma*

termitum, *Eubacterium eligens*, *Moraxella bovoculi*, *Leptospira inadai*, *Porphyromonas crevioricanis*, *Prevotella disiens* and *Porphyromonas macacae*, *Succinivibrio dextrinosolvens*, *Prevotella disiens*, *Flavobacterium branchiophilum*, *Helcococcus kunzii*, *Eubacterium sp.*, *Microgenomates (Roizmanbacteria) bacterium*, *Flavobacterium sp.*, *Prevotella brevis*, *Moraxella caprae*, *Bacteroidetes oral*, *Porphyromonas cansulci*, *Synergistes jonesii*, *Prevotella bryantii*, *Anaerovibrio sp.*, *Butyrivibrio fibrisolvens*, *Candidatus Methanomethylophilus*, *Butyrivibrio sp.*, *Oribacterium sp.*, *Pseudobutyrovibrio ruminis* and *Proteocatella sphenisci*.

[0013] The dead Cas12 enzyme may be a dead Cas12b protein which comprises a mutation in the Nuc domain. The dead Cas12b may comprise a mutation corresponding to D570A, E848A, or D977A in AacC2c1.

[0014] The dead Cas12b protein may be derived from a bacterial species selected from the group consisting of *Alicyclobacillus acidoterrestris*, *Alicyclobacillus contaminans*, *Alicyclobacillus macrosporangiidus*, *Bacillus hisashii*, *Candidatus Lindowbacteria*, *Desulfovibrio inopinatus*, *Desulfonatronum thiodismutans*, *Elusimicrobia bacterium RIFOXYA12*, *Omnitrophica WOR_2 bacterium RIFCSPHIGO2*, *Opitutaceae bacterium TAV5*, *Phycisphaerae bacterium ST-NAGAB-D1*, *Planctomycetes bacterium RBG_13_46_10*, *Spirochaetes bacterium GWB1_27_13*, *Verrucomicrobiaceae bacterium UBA2429*, *Tuberibacillus calidus*, *Bacillus thermoamylovorans*, *Brevibacillus sp. CF112*, *Bacillus sp. NSP2.1*, *Desulfatirhabdium butyrivorans*, *Alicyclobacillus herbarius*, *Citrobacter freundii*, *Brevibacillus agri* (e.g., BAB-2500), and *Methylobacterium nodulans*.

[0015] In some examples, the first dead CRISPR/Cas enzyme and the second dead CRISPR/Cas enzyme may be the same. In alternative examples, the first dead CRISPR/Cas enzyme and the second dead CRISPR/Cas enzyme may be different.

[0016] In some examples, the first and second CRISPR/Cas enzyme are catalytically active enzyme. In some instances, the CRISPR/Cas enzyme is a Cas9, Cas12, Cas 12b or Cas 12c enzyme.

[0017] In some examples, the polymerase may be selected from the group consisting of Bst 2.0 DNA polymerase, Bst 2.0 WarmStart DNA polymerase, Bst 3.0 DNA polymerase, full length Bst DNA polymerase, large fragment Bst DNA polymerase, large fragment Bsu DNA polymerase, phi29 DNA polymerase, T7 DNA polymerase, Gst polymerase, Taq polymerase, Klenow fragment of E. coli DNA polymerase I, KlenTaq NDA polymerase, Pol III DNA polymerase, T5 DNA polymerase, and Sequenase DNA polymerase.

[0018] In some examples, the helicase may be selected from the group consisting of UvrD helicase, CRISPR-Cas3 helicase, *E. coli* helicase I, *E. coli* helicase II, *E. coli* helicase III, *E. coli* helicase IV, Rep helicase, DnaB helicase, PriA helicase, PcrA helicase, T4 Gp41 helicase, T4 Dda helicase, SV40 Large T antigen, yeast RAD helicase, RecD helicase, RecQ helicase, thermostable *T. tengcongensis* UvrD helicase, thermostable *T. thermophilus* UvrD helicase, thermostable *T. aquaticus* DnaB helicase, Dda helicase, papilloma virus E1 helicase, archaeal MCM helicase, eukaryotic MCM helicase, and T7 Gp4 helicase. In some instances, the helicase is substituted to increase helicase processivity and or activity.

[0019] The amplification of the target nucleic acid may be performed at about 37°C-65°C. The amplification of the target nucleic acid may be performed at about 50°C-59°C. The amplification of the target nucleic acid may be performed at about 60°C-72°C. The amplification of the target nucleic acid may be performed at about 37°C. The amplification of the target nucleic acid may also be performed at room temperature. Room temperature may vary from 20°C-25°C. Amplification of the target nucleic acid may be performed at a constant temperature. Amplification may be performed under mesophilic or thermophilic isothermal conditions.

[0020] In some instances, the methods disclosed herein are performed under mesophilic isothermal conditions, and requires use of a mesophilic polymerase. In some instances, the mesophilic polymerase is Sau LF polymerase. Similarly, when amplification is performed under mesophilic isothermal conditions, the helicase utilized performs with sufficient activity under mesophilic conditions. In some instances, the helicase is a UvrD-like helicase, wild-type, or a mutant with super helicase mutations. Super helicase mutations are typically DD to AA substitutions that increase activity of the helicase. In some instances, the helicase is an *E. coli* UvrD helicase with. Substitutions at D403A and D404A. In some examples, the helicase is a pcrA (UvrD)-type helicase, for example, Tte-UvrD helicase with mutations at D409/D410A. In some instances, the helicase is an ATP dependent helicase, and an ATP co-factor can be used in conjunction with the helicase. In some cases, the helicase is PcrA helicase from *Thermoanaerobacter ethanolicus* with mutations D403A/D404A, PcrA helicase from *Bacillus* sp. FJAT-27231 with mutations D407A/D408A, PcrA helicase from *Bacillus megaterium* with mutations D415A/D416A, PcrA helicase from *Bacillus simplex* with mutations D407A/D408A, or PcrA helicase from *Paenibacillus sordellii* with mutations D402A/D403A.

[0021] The target nucleic acid sequence may be about 20-30, about 30-40, about 40-50, or about 50-100 nucleotides in length. The target nucleic acid sequence may be about 100-200, about 100-500, or about 100-1000 nucleotides in length. The target nucleic acid sequence may be about 1000-2000, about 2000-3000, about 3000-4000, or about 4000-5000 nucleotides in length. In some instances, when the target nucleic acid is comprised within genomic DNA, a CRISPR/Cas enzyme can be used that is catalytically active and cuts smaller target nucleic acid sequences for further amplification.

[0022] Detection of the amplified nucleic acid may be performed by a method selected from the group consisting of gel electrophoresis, intercalating dye detection, PCR, real-time PCR, fluorescence, Fluorescence Resonance Energy Transfer (FRET), mass spectrometry, lateral flow assay, colorimetric assays, and CRISPR-based detection system. The first or the second primer may comprise an RNA polymerase promoter. The amplified nucleic acid may be detected by a Cas13-based system, a CRISPR Cas12-based system, or a combination thereof. In some examples, the LwCas13 is used for detection.

[0023] The target nucleic acid may be detected at attomolar sensitivity. The target nucleic acid may be detected at femtomolar sensitivity.

[0024] The target nucleic acid may be selected from the group consisting of genomic DNA, mitochondrial DNA, viral DNA, plasmid DNA, circulating cell free DNA, environmental DNA, synthetic double-stranded DNA, and RNA.

[0025] In examples where the target nucleic acid is RNA, the RNA may be reverse transcribed into cDNA prior to amplification. Exemplary amplification methods include recombinase polymerase amplification (RPA). In some examples, T7 RNA polymerase is used for in vitro transcription.

[0026] In some examples, the method of amplification and detecting can comprise one or more additives, including crowding agents, co-factors, single-strand binding proteins, and/or assisting proteins. Additives can be selected, for example, based on measurement of signal to background ratio. In some instances, ATP can be used as a co-factor. Crowding agents can include polymers such as polyethylene glycol (PEG). In some examples, the PEG has a molecular weight of 20K, however, the molecular weight and level of branching can be selected on the complexity of the system in which the methods are applied.

[0027] Single-strand binding proteins are also contemplated for use in some methods and systems presently disclosed. For example, T4 gp32 or thermophilic SSB (ET-SSB). Can be

used. Assisting proteins for complex templates can also be utilized, particularly for complex templates. In some examples, dCpf1 and/or UvsX Recombinase can be used.

[0028] The sample may be a biological sample or an environmental sample. The biological sample may be a blood, plasma, serum, urine, stool, sputum, mucous, lymph fluid, synovial fluid, bile, ascites, pleural effusion, seroma, saliva, cerebrospinal fluid, aqueous or vitreous humor, or any bodily secretion, a transudate, an exudate, or fluid obtained from a joint, or a swab of skin or mucosal membrane surface. The sample may be blood, plasma or serum obtained from a human patient. The sample may be a plant sample. The sample may be a crude sample. The sample may be a purified sample.

[0029] In another example, the disclosure provides a method for amplifying and/or detecting a target single-stranded nucleic acid. The method comprises converting the single-stranded nucleic acid in a sample to a target double-stranded nucleic acid prior to performing the steps described so far. The target single-stranded nucleic acid may be an RNA molecule. The RNA molecule may be converted to the double-stranded nucleic acid by a reverse-transcription and amplification step.

[0030] The target single-stranded nucleic acid may be selected from the group consisting of single-stranded viral DNA, viral RNA, messenger RNA, ribosomal RNA, transfer RNA, microRNA, short interfering RNA, small nuclear RNA, synthetic RNA, and synthetic single-stranded DNA, long non-coding RNA, pre-microRNA, viral dsRNA, and non-viral dsRNA.

[0031] In instances where the target nucleic acid is RNA, the RNA may be reverse transcribed into cDNA prior to amplification. Amplification may then be done by any suitable amplification method known in the art, such as any described herein. One exemplary amplification method may include recombinase polymerase amplification (RPA).

[0032] In another example, the disclosure provides a system for amplifying and/or detecting a target double-stranded nucleic acid in a sample. The system may comprise an amplification CRISPR system, a helicase, a primer pair comprising a first and second primer, a polymerase, and optionally a detection system for detecting amplification of the target nucleic acid.

[0033] In some examples, the method further comprises mediating a first step of amplifying with the primer pair, and subsequently mediating the steps of amplifying with an amplifier primer that comprises sequence identity to a portion of the first primer of the primer pair. In some instances, the amplifier primer and the first primer of the primer each comprise a T7 promoter sequence.

[0034] The amplification CRISPR system may comprise a first and second CRISPR/Cas complex. The first CRISPR/Cas complex may comprise a first CRISPR/Cas enzyme and a first guide molecule that guides the first CRISPR/Cas complex to a first strand of the target nucleic acid. The second CRISPR/Cas complex may comprise a second CRISPR/Cas enzyme and a second guide molecule that guides the second CRISPR/Cas complex to a second strand of the target nucleic acid. The CRISPR/Cas enzyme may be active or dead. The first primer may comprise a portion that is complementary to the first strand of the target nucleic acid and the second primer may comprise a portion that is complementary to the second strand of the target nucleic acid.

[0035] The dead CRISPR/Cas enzyme may be a dead Cas9 enzyme or a dead Cas12 enzyme. The dead Cas12 enzyme may be a dead Cas12a, Cas12b, or Cas12c enzyme.

[0036] The polymerase may be selected from the group consisting of Bst 2.0 DNA polymerase, Bst 2.0 WarmStart DNA polymerase, Bst 3.0 DNA polymerase, full length Bst DNA polymerase, large fragment Bst DNA polymerase, large fragment Bsu DNA polymerase, phi29 DNA polymerase, T7 DNA polymerase, Gst polymerase, Taq polymerase, Klenow fragment of E. coli DNA polymerase I, KlenTaq DNA polymerase, Pol III DNA polymerase, T5 DNA polymerase, and Sequenase DNA polymerase.

[0037] The helicase may be selected from the group consisting of UvrD helicase, CRISPR-Cas3 helicase, E. coli helicase I, E. coli helicase II, E. coli helicase III, E. coli helicase IV, Rep helicase, DnaB helicase, PriA helicase, PcrA helicase, T4 Gp41 helicase, T4 Dda helicase, SV40 Large T antigen, yeast RAD helicase, RecD helicase, RecQ helicase, thermostable T. tengcongensis UvrD helicase, thermostable T. thermophilus UvrD helicase, thermostable T. aquaticus DnaB helicase, Dda helicase, papilloma virus E1 helicase, archaeal MCM helicase, eukaryotic MCM helicase, and T7 Gp4 helicase. The dead CRISPR/CAS enzyme, the helicase, and the polymerase may perform under the same temperature.

[0038] In another example, the disclosure provides a system for amplifying and/or detecting a target single-stranded nucleic acid in a sample. In addition to the components described in the previous example, the system may comprise reagents for converting the target single-stranded nucleic acid to a double-stranded nucleic acid.

[0039] In another example, the disclosure provides a kit for amplifying and/or detecting a target double-stranded nucleic acid in a sample. In addition to all the components described in the previous examples, the kit may also comprise a set of instructions for use. The kit may further comprise reagents for purifying the double-stranded nucleic acid in the sample.

[0040] In yet another example, the disclosure provides a kit for amplifying and/or detecting a target single-stranded nucleic acid in a sample. The kit may comprise components described in previous examples and a set of instructions for use. The kit may further comprise reagents for purifying the single-stranded nucleic acid in the sample.

[0041] These and other examples, objects, features, and advantages of the example examples will become apparent to those having ordinary skill in the art upon consideration of the following detailed description of illustrated example examples.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] An understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention may be utilized, and the accompanying drawings of which:

[0043] **FIG. 1** is a schematic describing the HDA workflow.

[0044] **FIG. 2** is a schematic describing the CRISPR-HDA workflow.

[0045] **FIGs. 3A-3G** are graphs illustrating the sensitivity of a 2-step thermophilic CRISPR HDA SHERLOCK isothermal amplification method for single molecule detection. The individual graphs show detection at (FIG. 3A) 2 pM, (FIG. 3B) 200fM, (FIG. 3C) 20 fM, (FIG. 3D) 2 fM, (FIG. 3E) 200 aM, (FIG. 3F) 2 aM, and (FIG. 3G) 0 aM concentrations.

[0046] **FIG. 4** is a graph showing that dAsCpf1 improves 2-step thermophilic HDA (tHDA) SHERLOCK sensitivity.

[0047] **FIG. 5** is a graph showing the effect is caused by the dAsCpf1:crRNA complex.

[0048] **FIG. 6** is a graph showing that both Cas12a and Cas12b improve 2-step tHDA-SHERLOCK.

[0049] **FIG. 7** is charts selection of mesophilic helicases, including range of temperature, left, and size (aa), right.

[0050] **FIG. 8** depicts an exemplary isothermal helicase reporter assay

[0051] **FIG. 9** shows Alanine substitutions for D403 and D404 in E.Coli UvrD increase Helicase processivity and activity. Consensus identity is provided, with sites conserved among selected UvrD helicases, boxed (SEQ ID NO:1-14).

[0052] **FIG. 10** shows selected pcrA (UvrD)-type helicase have a broad activity spectrum, ‘Super helicase’ DD-> AA substitutions increase activity.

CLAIMS

What is claimed is:

1. A method of amplifying and/or detecting a target double-stranded nucleic acid, comprising:
 - (a) combining a sample comprising the target double-stranded nucleic acid with an amplification reaction mixture, the amplification reaction mixture comprising:
 - (i) an amplification CRISPR system, the amplification CRISPR system comprising a first and second CRISPR/Cas complex, the first CRISPR/Cas complex comprising a first CRISPR/Cas enzyme and a first guide molecule that guides the first CRISPR/Cas complex to a first strand of the target nucleic acid, and the second CRISPR/Cas complex comprising a second CRISPR/Cas enzyme and a second guide molecule that guides the second CRISPR/Cas complex to a second strand of the target nucleic acid;
 - (ii) a helicase;
 - (iii) a primer pair comprising a first and second primer, wherein the first primer comprises a portion that is complementary to the first strand of the target nucleic acid and the second primer comprises a portion that is complementary to the second strand of the target nucleic acid; and
 - (iv) a polymerase;
 - (b) amplifying the target nucleic acid by opening R-loops of the target nucleic acid using the first and second CRISPR/Cas complexes, unwinding the first and the second strand of the target nucleic acid using the helicase, annealing and extending the strands using the primer pair and the polymerase; and
 - (c) further amplifying the target nucleic acid by repeated opening, unwinding, annealing and extension under isothermal conditions; and
 - (d) optionally detecting the amplified target nucleic acid.
2. The method of claim 1, wherein the CRISPR/Cas enzyme is a dead CRISPR/Cas enzyme selected from the group consisting of dead Cas9, dead Cas12a, dead Cas12b, and dead Cas12c, optionally wherein the dead CRISPR/Cas enzyme is a dead Cas9 protein which comprises a mutation in the HNH and RuvC domain, or wherein the dead CRISPR/Cas enzyme is a dead Cas9 protein which comprises a mutation corresponding to D10A and N863A in SpCas9, or D10A and H840A in SpCas9, optionally wherein the dead CRISPR/Cas enzyme is a dead Cas9 protein derived from a bacterial species selected from the group consisting of

Streptococcus pyogenes, *Staphylococcus aureus*, *Streptococcus thermophilus*, *S. mutans*, *S. agalactiae*, *S. equisimilis*, *S. sanguinis*, *S. pneumoniae*; *C. jejuni*, *C. coli*; *N. salsuginis*, *N. tergarctus*; *S. auricularis*, *S. carnosus*; *N. meningitidis*, *N. gonorrhoeae*; *L. monocytogenes*, *L. ivanovii*; *C. botulinum*, *C. difficile*, *C. tetani*, *C. sordellii*, *Francisella tularensis* 1, *Prevotella albensis*, *Lachnospiraceae* bacterium MC2017_1, *Butyrivibrio proteoclasticus*, *Peregrinibacteria* bacterium GW2011_GWA2_33_10, *Parcubacteria* bacterium GW2011_GWC2_44_17, *Smithella* sp. SCADC, *Acidaminococcus* sp. BV3L6, *Lachnospiraceae* bacterium MA2020, *Candidatus Methanoplasma termitum*, *Eubacterium eligens*, *Moraxella bovoculi* 237, *Leptospira inadai*, *Lachnospiraceae* bacterium ND2006, *Porphyromonas crevioricanis* 3, *Prevotella disiens* and *Porphyromonas macacae*, or wherein the dead CRISPR/Cas enzyme is a dead Cas12 protein, optionally wherein the dead Cas12 protein comprises a mutation in the RuvC domain, optionally wherein the dead Cas12 enzyme is a dead Cas12a, Cas12b, Cas12c, optionally wherein the dead Cas12a comprises a mutation corresponding to D908A or E993A in AsCpf1, optionally wherein the dead Cas12 protein is derived from a bacterial species selected from the group consisting of *Francisella tularensis*, *Prevotella albensis*, *Lachnospiraceae* bacterium, *Butyrivibrio proteoclasticus*, *Peregrinibacteria* bacterium, *Parcubacteria* bacterium, *Smithella* sp., *Acidaminococcus* sp., *Lachnospiraceae* bacterium, *Candidatus Methanoplasma termitum*, *Eubacterium eligens*, *Moraxella bovoculi*, *Leptospira inadai*, *Porphyromonas crevioricanis*, *Prevotella disiens* and *Porphyromonas macacae*, *Succinivibrio dextrinosolvens*, *Prevotella disiens*, *Flavobacterium branchiophilum*, *Helcococcus kunzii*, *Eubacterium* sp., *Microgenomates* (*Roizmanbacteria*) bacterium, *Flavobacterium* sp., *Prevotella brevis*, *Moraxella caprae*, *Bacteroidetes* oral, *Porphyromonas cansulci*, *Synergistes jonesii*, *Prevotella bryantii*, *Anaerovibrio* sp., *Butyrivibrio fibrisolvens*, *Candidatus Methanomethylophilus*, *Butyrivibrio* sp., *Oribacterium* sp., *Pseudobutyrvibrio ruminis* and *Proteocatella sphenisci*, or wherein the dead Cas12 is a dead Cas12b protein which comprises a mutation in the Nuc domain, optionally wherein the dead Cas12b comprises a mutation corresponding to D570A, E848A, or D977A in AacC2c1, optionally wherein the dead Cas12b protein is derived from a bacterial species selected from the group consisting of *Alicyclobacillus acidoterrestris*, *Alicyclobacillus contaminans*, *Alicyclobacillus macrosporangioides*, *Bacillus hisashii*, *Candidatus Lindowbacteria*, *Desulfovibrio inopinatus*, *Desulfonatronum thiodismutans*, *Elusimicrobia* bacterium RIFOXYA12, *Omnitrophica* WOR_2 bacterium RIFCSPHIGHO2, *Opitutaceae* bacterium TAV5, *Phycisphaerae* bacterium ST-NAGAB-D1, *Planctomycetes* bacterium RBG_13_46_10, *Spirochaetes* bacterium GWB1_27_13, *Verrucomicrobiaceae* bacterium UBA2429,

Tuberibacillus calidus, *Bacillus thermoamylovorans*, *Brevibacillus* sp. CF112, *Bacillus* sp. NSP2.1, *Desulfatirhabdium butyrativorans*, *Alicyclobacillus herbarius*, *Citrobacter freundii*, *Brevibacillus agri* (e.g., BAB-2500), and *Methylobacterium nodulans*.

3. The method of any of the preceding claims, wherein the first dead CRISPR/Cas enzyme and the second dead CRISPR/Cas enzyme are the same, or wherein the first dead CRISPR/Cas enzyme and the second dead CRISPR/Cas enzyme are different, optionally wherein the dead CRISPR/Cas enzyme is a dCas9, dCas12a, dCas12b, dCas12c, or dCas14, optionally wherein the polymerase is selected from the group consisting of *Bst* 2.0 DNA polymerase, *Bst* 2.0 WarmStart DNA polymerase, *Bst* 3.0 DNA polymerase, full length *Bst* DNA polymerase, large fragment *Bst* DNA polymerase, large fragment *Bsu* DNA polymerase, phi29 DNA polymerase, T7 DNA polymerase, Gst polymerase, Taq polymerase, Klenow fragment of *E. coli* DNA polymerase I, KlenTaq DNA polymerase, Pol III DNA polymerase, T5 DNA polymerase, and Sequenase DNA polymerase, optionally wherein the helicase is selected from the group consisting of UvrD helicase, CRISPR-Cas3 helicase, *E. coli* helicase I, *E. coli* helicase II, *E. coli* helicase III, *E. coli* helicase IV, Rep helicase, DnaB helicase, PriA helicase, PcrA helicase, T4 Gp41 helicase, T4 Dda helicase, SV40 Large T antigen, yeast RAD helicase, RecD helicase, RecQ helicase, thermostable *T. tengcongensis* UvrD helicase, thermostable *T. thermophilus* UvrD helicase, thermostable *T. aquaticus* DnaB helicase, Dda helicase, papilloma virus E1 helicase, archaeal MCM helicase, eukaryotic MCM helicase, and T7 Gp4 helicase.

4. The method of any of the preceding claims, wherein amplification of the target nucleic acid is performed at 37°C-65°C, at 50°C-59°C, at 60°C-72°C, or at room temperature.

5. The method of claim 1, performed under mesophilic isothermal conditions, optionally wherein the polymerase is a mesophilic polymerase, optionally wherein the mesophilic polymerase is Sau LF Polymerase, optionally wherein amplification of the target nucleic acid is performed at about 37°C, optionally wherein the helicase is a *E. Coli* UvrD helicase, optionally with two or more substitutions of D to A, for example at D403 and D404, or wherein the helicase is a pcrA (UvrD)-type helicase comprising one or more mutations corresponding to D409A and D410A, optionally wherein the helicase is Tte-UvrD D409A/D410A.

6. The method of any of the preceding claims, wherein the first or the second primer comprises an RNA polymerase promoter.

7. The method of any of the preceding claims, comprising one or more additives selected from a crowding agent, a co-factor, single-strand binding protein, and/or assisting proteins, optionally wherein the crowding agent is polyethylene glycol, optionally wherein the co-factor is ATP, optionally wherein the single-strand binding protein is selected from T4 gp32 or thermophilic SSB (ET-SSB), optionally wherein the assisting protein is dCpf1 and/or UvsX Recombinase.

8. The method of any of the preceding claims, further comprising detecting the amplified nucleic acid by a method selected from the group consisting of gel electrophoresis, intercalating dye detection, PCR, real-time PCR, fluorescence, Fluorescence Resonance Energy Transfer (FRET), mass spectrometry, lateral flow assay, colorimetric assays, and CRISPR-based detection system, optionally wherein the amplified target nucleic acid is detected by a CRISPR Cas13-based system, a CRISPR Cas12-based system, or a combination thereof, optionally wherein the amplified target nucleic acid is detected by a CRISPR Cas-13 based system comprising LwaCas13 enzyme.

9. The method of any of the preceding claims, wherein the target nucleic acid is selected from the group consisting of genomic DNA, mitochondrial DNA, viral DNA, plasmid DNA, circulating cell free DNA, environmental DNA, synthetic double-stranded DNA, and RNA, optionally wherein the target nucleic acid is RNA, and wherein the RNA is reverse transcribed into cDNA prior to amplification, optionally wherein the amplification is recombinase polymerase amplification (RPA).

10. The method of any of the preceding claims, wherein the sample is a biological sample or an environmental sample, optionally wherein the biological sample is a blood, plasma, serum, urine, stool, sputum, mucous, lymph fluid, synovial fluid, bile, ascites, pleural effusion, seroma, saliva, cerebrospinal fluid, aqueous or vitreous humor sample, or any bodily secretion, a transudate, an exudate, or fluid obtained from a joint, or a swab of skin or mucosal membrane surface, preferably wherein the sample is blood, plasma or serum obtained from a human patient, or wherein the sample is a plant sample, optionally wherein the sample is a crude sample, or wherein the sample is a purified sample.

11. A method for amplifying and/or detecting a target single-stranded nucleic acid, comprising:

(a) converting the single-stranded nucleic acid in a sample to a target double-stranded nucleic acid; and

(b) performing the steps of claim 1, optionally wherein the target single-stranded nucleic acid is an RNA molecule, optionally wherein the RNA molecule is converted to the double-stranded nucleic acid by a reverse-transcription and amplification step, optionally wherein the target single-stranded nucleic acid is selected from the group consisting of single-stranded viral DNA, viral RNA, messenger RNA, ribosomal RNA, transfer RNA, microRNA, short interfering RNA, small nuclear RNA, synthetic RNA, synthetic single-stranded DNA, long non-coding RNA, pre-microRNA, viral dsRNA, and non-viral dsRNA.

12. The method of claim 1 further comprising an amplifier primer that comprises a portion contained in the first primer of the primer pair; and comprising the steps of the first primer of the primer pair mediates a first step of amplifying, and the amplifier primer together with the second primer of the primer pair mediating the subsequent steps of amplifying, optionally wherein the amplifier primer and the first primer of the primer pair each comprise a T7 promoter sequence, optionally wherein the amplifier primer comprises a length of 23 to 35 nucleotides.

13. A system for amplifying and/or detecting a target double-stranded nucleic acid in a sample, the system comprising:

- a) an amplification CRISPR system, the amplification CRISPR system comprising a first and second CRISPR/Cas complex, the first CRISPR/Cas complex comprising a first CRISPR/Cas enzyme and a first guide molecule that guides the first CRISPR/Cas complex to a first strand of the target nucleic acid, and the second CRISPR/Cas complex comprising a second CRISPR/Cas enzyme and a second guide molecule that guides the second CRISPR/Cas complex to a second strand of the target nucleic acid;
- b) a helicase;
- c) a primer pair comprising a first and second primer, wherein the first primer comprising a portion that is complementary to the first strand of the target nucleic acid and the second primer comprising a portion that is complementary to the second strand of the target nucleic acid;
- d) a polymerase; and optionally

e) a detection system for detecting amplification of the target nucleic acid;

optionally wherein the CRISPR/Cas enzyme is a dead CRISPR/Cas enzyme selected from a dead Cas9 enzyme or a dead Cas12 enzyme, optionally wherein the dead Cas12 enzyme is a dead Cas12a, Cas12b, or Cas12c enzyme, or wherein the CRISPR/Cas enzyme is a Cas9 or Cas12 enzyme, optionally wherein the Cas12 enzyme is a Cas12a, Cas12b, or Cas12c enzyme, optionally wherein the polymerase is selected from the group consisting of *Bst* 2.0 DNA polymerase, *Bst* 2.0 WarmStart DNA polymerase, *Bst* 3.0 DNA polymerase, full length *Bst* DNA polymerase, large fragment *Bst* DNA polymerase, large fragment *Bsu* DNA polymerase, phi29 DNA polymerase, T7 DNA polymerase, Gst polymerase, Taq polymerase, Klenow fragment of *E. coli* DNA polymerase I, KlenTaq NDA polymerase, Pol III DNA polymerase, T5 DNA polymerase, and Sequenase DNA polymerase, and Sau LF DNA polymerase, optionally wherein the helicase is selected from the group consisting of UvrD helicase, CRISPR-Cas3 helicase, Rep helicase, PcrA helicase, *E. coli* helicase I, *E. coli* helicase II, *E. coli* helicase III, *E. coli* helicase IV, Rep helicase, DnaB helicase, PriA helicase, PcrA helicase, T4 Gp41 helicase, T4 Dda helicase, SV40 Large T antigen, yeast RAD helicase, RecD helicase, RecQ helicase, thermostable *T. tengcongensis* UvrD helicase, thermostable *T. thermophilus* UvrD helicase, thermostable *T. aquaticus* DnaB helicase, Dda helicase, papilloma virus E1 helicase, archaeal MCM helicase, eukaryotic MCM helicase, and T7 Gp4 helicase, optionally wherein the helicase is Tte-UvrD D409A/D410A,

optionally further comprising one or more additives selected from a crowding agent, a co-factor, single-strand binding protein, and/or assisting proteins, optionally wherein the crowding agent is polyethylene glycol 20K molecular weight, optionally provided at 2.5% to 6.5%, optionally wherein the co-factor is ATP, optionally provided at 0.75 to 5 mM, optionally wherein the single-strand binding protein is selected from T4 gp32 or thermophilic SSB (ET-SSB), optionally wherein the assisting protein is dCpf1 and/or UvsX Recombinase, optionally wherein the CRISPR/Cas enzyme, the helicase, and the polymerase perform under the same temperature, in some instances, at about 37 °C,

optionally wherein the detection system comprises a CRISPR system comprising a Cas 12 or Cas 13 effector protein and a guide RNA designed to bind to a corresponding target molecule; and a masking construct, optionally wherein the detection system comprises Cas 13 enzyme LwaCas13,

optionally further comprising an additional amplifier primer comprising a promoter sequence identity with the first primer of the primer pair.

14. A system for amplifying and/or detecting a target single-stranded nucleic acid in a sample, the system comprising:

a) reagents for converting the target single-stranded nucleic acid to a double-stranded nucleic acid;

b) components of claim 13;

optionally wherein the detection system comprises a CRISPR system comprising a Cas 12 or Cas 13 effector protein and a guide RNA designed to bind to a corresponding target molecule; and a masking construct, optionally wherein the detection system comprises Cas 13 enzyme LwaCas13, optionally further comprising reagents for purifying the single-stranded nucleic acid in the sample.

15. The method of claim 1 or the system of claim 13, wherein the helicase is PcrA helicase from *Thermoanaerobacter ethanolicus* with mutations D403A/D404A, PcrA helicase from *Bacillus* sp. FJAT-27231 with mutations D407A/D408A, PcrA helicase from *Bacillus megaterium* with mutations D415A/D416A, PcrA helicase from *Bacillus simplex* with mutations D407A/D408A, or PcrA helicase from *Paenibacillus sordellii* with mutations D402A/D403A.