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SHERLOCK: nucleic acid detection with CRISPR nucleases

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Rapid detection of nucleic acids is integral to applications in clinical diagnostics and biotechnology. We have recently established a CRISPR-based diagnostic platform that combines nucleic acid pre-amplification with CRISPR–Cas enzymology for specific recognition of desired DNA or RNA sequences. This platform, termed specific high-sensitivity enzymatic reporter unlocking (SHERLOCK), allows multiplexed, portable, and ultra-sensitive detection of RNA or DNA from clinically relevant samples. Here, we provide step-by-step instructions for setting up SHERLOCK assays with recombinase-mediated polymerase pre-amplification of DNA or RNA and subsequent Cas13- or Cas12-mediated detection via fluorescence and colorimetric readouts that provide results in <1 h with a setup time of less than 15 min. We also include guidelines for designing efficient CRISPR RNA (crRNA) and isothermal amplification primers, as well as discuss important considerations for multiplex and quantitative SHERLOCK detection assays.

Introduction

Rapid nucleic acid detection is an important part of many applications in human health and biotechnology, including the sensing of infectious agents, agricultural pathogens, or circulating DNA or RNA associated with disease. Standard methods to amplify nucleic acids for detection (such as PCR) are effective but require instrumentation that is not portable, precluding their deployment in the field. Demand for instrument-free nucleic acid detection technologies has driven the development of multiple methods for isothermal amplification. However, common approaches for isothermal amplification, such as recombinase polymerase amplification (RPA)[1], require optimization and cannot typically discriminate between single-base-pair differences in target sequences, a distinction that can have important consequences for pathogenicity. Recently, enzymes from CRISPR–Cas systems have been adapted for the specific, rapid, sensitive, and portable sensing of nucleic acids[2–4]. These approaches rely on Cas13 (refs. [5,6]) or Cas12 (ref. [7]), both of which exhibit nonspecific endonuclease activity after binding to a specific target via programmable crRNAs[6,8,9]. By combining the programmable specificity of Cas13 or Cas12 with a reporter molecule that is activated upon target recognition, these enzymes result in a specific and sensitive indication of the presence or quantity of a nucleic acid.

Here, we describe a protocol for SHERLOCK nucleic acid detection (using RPA and CRISPR–Cas13) with instructions for reagent preparation, including recombinant *Leptotrichia wadei* Cas13 (LwaCas13a) protein expression and purification, as well as in vitro transcription (IVT) of crRNAs and sample extraction from various starting materials. Experimental design and downstream analysis considerations are also discussed.

Development of the protocol

Cas13, which encompasses four divergent family members (Cas13a–d), is an RNA-guided RNase that produces multiple cleavage sites in single-stranded areas of an RNA target with specific base preferences[5,6,10–13] (Fig. 1a). Cas13 also exhibits target-dependent promiscuous RNase activity, leading to *trans* cleavage of bystander RNA molecules, an effect termed 'collateral activity'[6]. It was recently discovered that Cas effectors from other types of CRISPR–Cas systems also display collateral activity, including subfamilies of Cas12 (Box 1; refs. [8,9]). The discovery of collateral activity, which links highly

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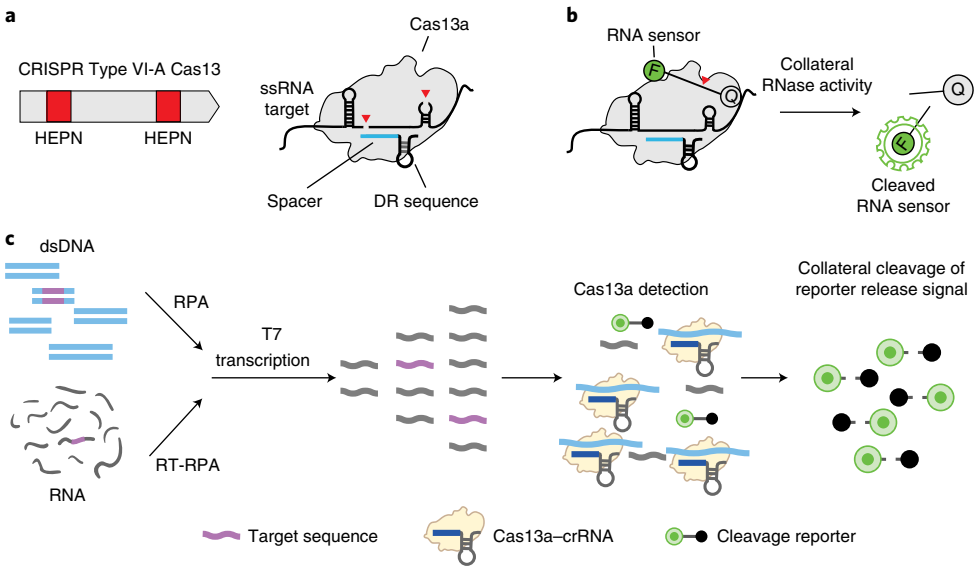


Fig. 1 | Cas13 complex and collateral activity. a, CRISPR–Cas13 RNA targeting complex components. CRISPR–Cas13 enzymes are programmed by a crRNA, which is composed of a DR sequence flanked by a target-complementary spacer sequence (shown in blue). RNA cleavage is mediated by two higher eukaryotic and prokaryotic nuclease domains (HEPNs; shown as red boxes) within a typical Type VI-A Cas13. **b**, Reporter unlocking via CRISPR–Cas13 collateral RNase activity. The CRISPR–Cas13–RNA complex is activated by binding to a complementary target RNA. The activation triggers collateral cleavage of a nonspecific RNA reporter in *trans*. The fluorescently labeled reporter (fluorophore (F)) is quenched (by a quencher (Q)) when intact and emits fluorescence when cleaved by the activated CRISPR–Cas13 complex. **c**, SHERLOCK detection assay. Schematic of SHERLOCK assay steps, starting with pre-amplification of either a DNA or RNA target input. Amplified targets are converted to RNA via T7 transcription and are then detected by Cas13–crRNA complexes, which activate and cleave fluorescent RNA reporters. For Cas12 detection, the T7 transcription step is omitted, allowing direct detection of amplified targets. ssRNA, single-stranded RNA.

specific target recognition to a subsequent general RNase activity that can cleave a reporter molecule (Fig. 1b), made it possible to develop a new approach for nucleic acid detection[6,10,14].

Nucleic acid detection with collateral activity can be achieved using a variety of reporter molecules and methods, including: (i) gel-based detection with fluorescently labeled reporters[6], (ii) fluorometer detection with quenched fluorescent reporters[10,14], (iii) visual detection through modulation of solution turbidity via liquid–liquid phase separation[15], and (iv) lateral flow detection with antigen-labeled reporters[16]. Although Cas13 orthologs have been identified that can detect as little as 50 fM of target RNA input (~600,000 molecules)[14], they cannot reach single-molecule detection, a necessity for many clinical applications. In addition, a versatile detection platform for clinical diagnostics would need to detect DNA as well as RNA.

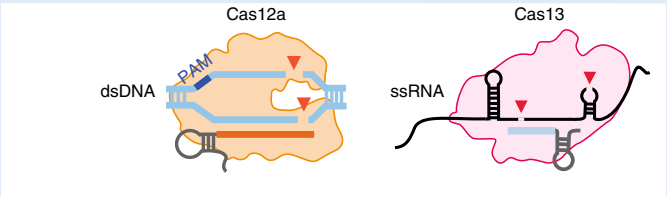
Several platforms have been developed to boost Cas13 detection. We focus here on SHERLOCK, which includes a pre-amplification step, typically RPA, that can amplify either RNA or DNA and introduce a T7 RNA polymerase promoter, allowing RNA transcription and subsequent detection by Cas13 (Fig. 1c)[14]. Building on the original SHERLOCK technology, we developed additional features (including multiplex fluorescence-based detection and visual readout on lateral flow strips) to create SHERLOCKv2 (ref. [16]). These additional features are also described in the protocol.

Overview of the procedure

The four main stages of the SHERLOCK procedure are reagent preparation (Steps 1–41), sample extraction (Step 42), isothermal target nucleic acid pre-amplification (Steps 43–48), and CRISPR–Cas13 nucleic acid detection (Steps 49–52), as outlined in Fig. 2. The first two stages (LwaCas13a protein purification and crRNA IVT) are optional because both products can be purified by a contract research organization (CRO) or obtained commercially. Although this protocol focuses mostly on LwaCas13a purification, it can also be used and adapted for Cas13b purification. The next two stages are the pre-amplification reaction, most often with RPA, and the CRISPR collateral detection reaction, which cleaves a nucleic acid reporter and generates a detectable signal (Fig. 1c).

Box 1 | Cas13 and Cas12 orthologs for CRISPR diagnostics

To date, both the Cas13 and Cas12 protein families of CRISPR systems have been shown to have collateral activity, making them useful for nucleic acid detection applications[6,8,9]. The schematic diagrams and table below show the key differences between the Cas13 and Cas12a enzymes.



PAM required	Yes	No
PAM identity	TTTV	Not applicable
Cleavage	Single staggered cut	Many cleavage sites
Target type	ssDNA, dsDNA	ssRNA only
Collateral	Yes	Yes

Many of the Cas13 subtypes and orthologs have different base preferences, cleaving at specific dinucleotide motifs[16]. In addition, Cas13 subtypes differ in CRISPR RNA (crRNA) structure, DR sequence, and size (see table below). Although Cas13 has a PAM-like sequence motif called the protospacer flanking site (PFS) that restricts activity to only certain target sites, there are a number of very active Cas13 orthologs, such as LwaCas13a, that show no PFS. Lack of a PFS is a distinguishing feature of these orthologs that enables them to target any possible sequence or mutation.

Cas12a has weak collateral activity, enabling nucleic acid detection with low sensitivity (low nanomolar range)[9,16]. When combined with pre-amplification, Cas12a-mediated detection can detect 2 aM concentrations[14,16].

Comparison of Cas13 and Cas12 orthologs

	LwaCas13a	LbaCas13a	CcaCas13b	PsmCas13b	AsCas12a
Organism	*L. wadei*	*L. bacterium* NK4A179	*C. canimorsus*	*P.* sp. MA2016	*A.* sp. BV3L6
Target	ssRNA	ssRNA	ssRNA	ssRNA	ssDNA/dsDNA
DR orientation	5′	5′	3′	3′	5′
Motif preference	Poly U/AU	Poly A/AC	Poly U/UA/UC	Poly A/GA	NA
Spacer length (nt)	28	28	30	30	20
Sensitivity	~5e^7 aM[16]	~1e^9 aM[33]	~5e^7 aM[16]	~5e^9 aM[16]	~5e^{10} aM[16]

NA, not applicable.

These two reactions can either be run sequentially, with a transfer step in between (two-step detection procedure; Steps 49–52 of main procedure), or as a single combined mixture (one-pot detection procedure; Box 2).

SHERLOCK is compatible with multiple readouts, either fluorescence detection or lateral flow detection, depending on reporter molecule choice. Although either Cas12 or Cas13 enzyme can be used for detection, this protocol focuses on Cas13, which requires the introduction of a T7 RNA polymerase promoter during pre-amplification and a T7 RNA polymerase during the detection reaction to generate RNA for Cas13 collateral activation (Fig. 1c). Fluorescence detection can be performed either as an endpoint readout or in real time with a plate reader or another compatible fluorometer. Lateral flow detection is an endpoint assay, with lateral flow strips exposed to the reaction mixture post incubation.

Alternative methods

SHERLOCK joins a large array of other portable and isothermal methods for nucleic acid detection, including traditional RPA[1], loop-mediated isothermal amplification (LAMP)[17], and helicase-

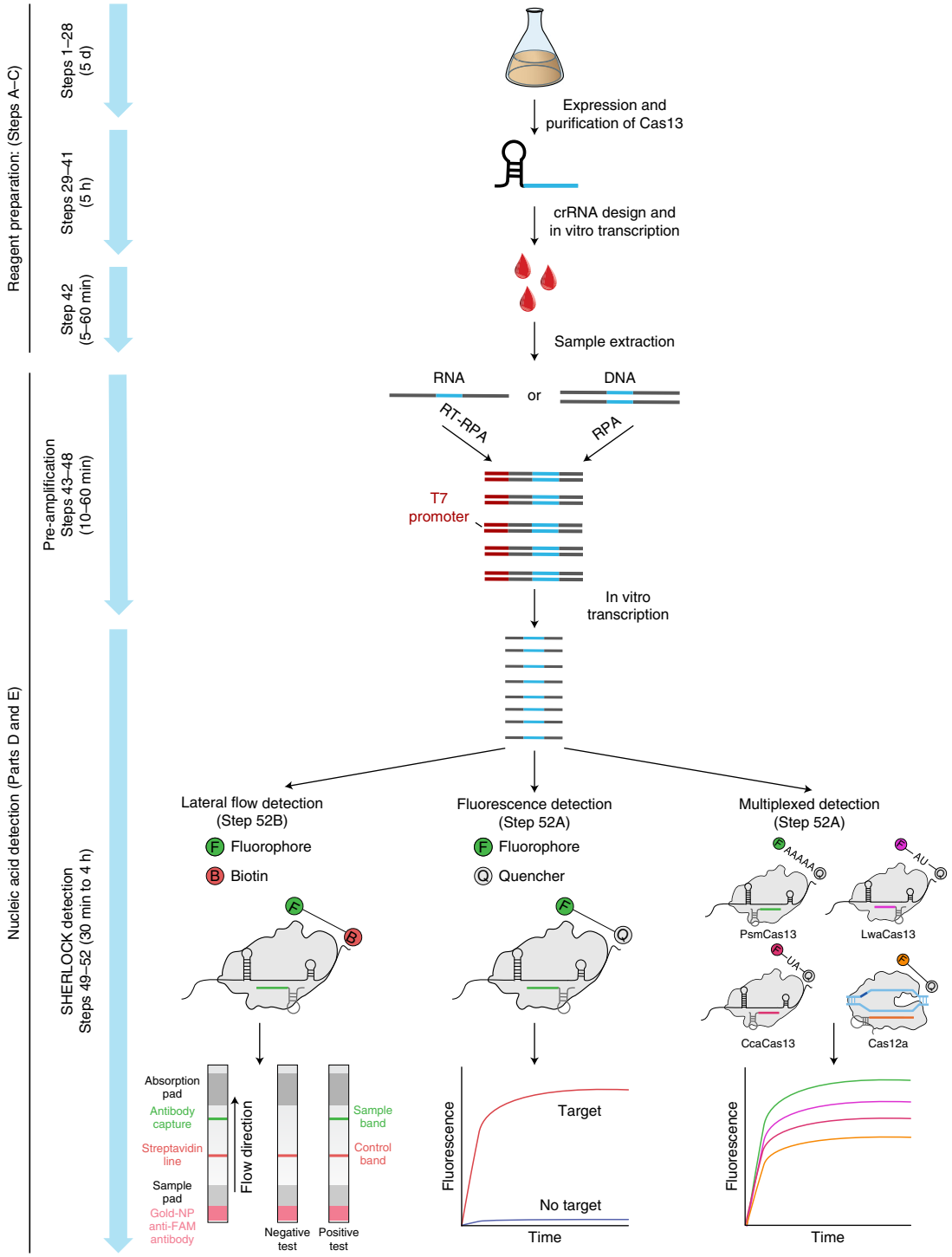


Fig. 2 | Complete SHERLOCK experimental workflow. First, LwaCas13a is recombinantly expressed and purified in *Escherichia coli* (Steps 1–28). After crRNA design and in vitro transcription (Steps 29–41), sample extraction is performed to yield the target nucleic acid (Step 42). This sample is then used for RPA-based pre-amplification (Steps 43–48) and detection by Cas13 (Steps 49–52). Detection can be performed as fluorescence-based single or multiplex SHERLOCK reactions (Step 52A) or a single-plex colorimetric lateral flow reaction (Step 52B). Multiple targets can be detected within the same reaction using CRISPR–Cas13 enzymes with orthogonal cleavage preferences or by combining Cas13 with Cas12 in the same assay.

dependent amplification (HDA)[18]. These methods all provide varying degrees of speed, sensitivity, portability, and ease of design. In the case of traditional RPA, risk of nonspecific amplification requires the screening of numerous primer sets to find robust pairs. Alternatively, RPA can be

Box 2 | One-pot Cas13-SHERLOCK nucleic acid detection ● Timing 1–4 h

▲ **CRITICAL** The recommended procedures for RNA and DNA pre-amplification are nearly identical. However, important distinctions are summarized in the 'Experimental design' section. If multiplexed nucleic acid detection or quantification is desired, the reader should follow the guidance provided in the 'Experimental design' section.

1 Pre-heat the fluorescence plate reader to 37 °C.
2 Prepare the pre-amplification area by wiping down the work surface and pipettors with RNase Away.
3 Thaw normalized crRNA (at 300 ng/μL), SHERLOCK RNaseAlert reporter (2 μM), and an aliquot of Cas13 protein (2 mg/mL, 14.44 μM) on ice, covered with aluminum foil to protect from light exposure.
4 Dilute LwaCas13a to 50× (348 ng/mL) by adding 19 μL of protein SB to 4 μL of LwaCas13a (2 mg/mL).
5 Dilute the crRNA to 25 ng/μL by adding 11 μL of UltraPure water to 1 μL of crRNA (300 ng/μL).
6 Prepare the following Cas13-SHERLOCK master mix by adding the following components to an Eppendorf tube in top-down order. The master mix given below is enough for one sample with three to four technical replicates. Scale up proportionally for the number of samples.

Component	Volume per reaction (μL)
Forward primer (100 μM)	0.48
Reverse primer (100 μM)	0.48
Rehydration Buffer, from TwistAmp Basic kit	59
LwaCas13a in SB (348 ng/mL)	2
crRNA (25 ng/mL)	0.17
RNaseAlert v2 (2 μM)	6.83
Murine RNase inhibitor, 40 U/μL	2.73
rNTP solution mix	8
T7 RNA polymerase, 5 U/μL	2
$MgCl_2$, 1 M	0.5
Magnesium acetate, from TwistAmp Basic kit	5
UltraPure water	7.81
Total	95

7 Add 95 μL of master mix without sample to a single pellet aliquot and carefully resuspend the mixture on ice. Then transfer the entire reconstituted reaction back to the initial Eppendorf or PCR master mix tube.
▲ **CRITICAL** One pellet yields approximately three individual 20-μL one-pot reactions. If a larger number of reactions is needed, scale up the master mix volume and pellets accordingly. When doing so, it is critical not to change the pipette between transfers of resuspended pellets, as the stickiness of RPA reaction components could result in a substantial loss of reaction enzymes.
8 Transfer 71.25 μL of master mix to a PCR-strip tube or 96-well PCR plate. This is the amount for one condition and three technical replicates.
9 Spin down the samples (in a minifuge at room temperature for 5 s), carefully open the tubes, and transfer 3.75 μL of each sample to 71.25 μL of master mix on ice. Briefly vortex and spin down (in a plate spinner or for 500*g*, 22 °C, 15 s) in a plate to collect the entire reaction mix in the well.
10 Carefully open the reaction tubes and transfer 20 μL per technical replicate and condition to a 384-well, round black-well, clear-bottom plate. To group technical replicates, pipette the remaining technical replicates beside and below, to form a square of 2 × 2 grouped reactions. Note the lower right quadrant of this grid will be empty because the one-pot reactions yield only three technical replicates.
11 Briefly spin down (500*g*, 22 °C, 15 s) the plate to remove potential bubbles and place it into a 37 °C pre-heated BioTek plate reader, or equivalent.
▲ **CRITICAL** Following plate preparation, quickly place it into the fluorescence plate reader because SHERLOCK Cas13 reactions may begin as soon as they are removed from ice.
12 Start data acquisition by monitoring fluorescence over 3 h at 37 °C with a 5-min interval between well-data acquisitions.
? TROUBLESHOOTING

combined with probe-based methods, such as those involving exo-probes, to increase the stringency of detection. However, these probes require multiple modifications, such as fluorophores and quenchers, and are expensive to order. LAMP suffers from similar limitations, although a higher temperature of operation (65 °C) can reduce nonspecific effects. However, this high temperature can make heating difficult in situations without electricity, as in applications for use in the field. Furthermore, primer design with LAMP requires four different primer sets, leading to a substantial degree of complexity that limits the programmability of LAMP for certain users. In the case of HDA, commercial methods require high temperatures, long incubation times, and often fail to reach single-molecule detection thresholds.

In addition, portable versions of more-conventional methods for nucleic acid detection, such as PCR (e.g., TaqMan qPCR) and next-generation sequencing (NGS), have been developed for rapid or point-of-care detection[19–21]. However, these methods require specialized instrumentation that may not be practical in many low-resource settings. Although some portable NGS devices are commercially available, they are better suited for unbiased nucleic acid discovery, rather than sensitive and targeted detection of known targets. Thus, portable NGS and SHERLOCK are complementary approaches in nucleic acid diagnostics.

Since the details of SHERLOCK were first published, additional CRISPR-based diagnostic (CRISPR-Dx) platforms have been developed, including DETECTR and HOLMES[9,22]. Whereas SHERLOCK refers to the method using a CRISPR enzyme for collateral detection with any pre-amplification of RNA or DNA, DETECTR refers to the specific instance of using Cas12a collateral detection after pre-amplification by RPA[9]. The HOLMES method, similar to DETECTR, uses Cas12a for DNA or RNA detection after pre-amplification with rapid PCR amplification[22]. HOLMESv2 is a modified version of HOLMES that allows one-pot detection using thermophilic Cas12b with LAMP[23]. Catalytically inactive Cas9 (dCas9) has also been harnessed for a digital chip-based nucleic acid detection platform[24], enabling femtomolar detection without pre-amplification, but this approach, referred to as CRISPR-Chip, requires bespoke instrumentation (a graphene field-effect transistor on which Cas9 has been immobilized) that is currently not widely available.

Advantages and limitations

SHERLOCK is highly sensitive and specific. It is capable of single-molecule detection in 1-μL sample volumes (2 aM) of both DNA and RNA targets. In addition, by scaling up the pre-amplification volume, it is possible to achieve single-molecule detection in large sample input volumes (up to 540 μL; 8 zM)[16]. SHERLOCK leverages the specificity of Cas13 (refs. [6,14,25]) and Cas12 enzymes[7,26,27] to enable single-nucleotide mismatch distinction in target sequences. Cas13 does not catalytically activate when there are two or more mismatches in the crRNA:target duplex. As a result, sequences of similar viruses, such as dengue virus and Zika virus, can easily be distinguished by SHERLOCK[14]. In cases in which detection of the target requires single-base distinction, such as viral and human genotyping or the detection of cancer-associated mutations in circulating nucleic acids, the specificity of Cas13 can be enhanced by the introduction of a 'synthetic mismatch' into the crRNA[14,16]. The Cas13 enzyme used in SHERLOCK does not require strict sequence preferences at the target site, whereas Cas12 enzymes require a protospacer adjacent motif (PAM) for cleavage. This allows more flexibility and a broader target range for SHERLOCK as compared to DETECTR and HOLMES.

An attractive feature of SHERLOCK is the rapid nature of the assay. Usually, RPA is performed for 5–10 min as an initial reaction, and part of this solution is transferred to the Cas13 detection reaction as a two-step reaction, which can then detect the target in as little as 5 min. As with sensitivity, RPA primer and crRNA design are important determinants for the kinetics of target detection. It is important to screen designs, because choosing the right primer and crRNA combination can markedly increase speed of detection. RPA can also be combined in the same reaction as Cas13 detection for a one-pot assay, although the viscosity of the mixture can reduce robustness of detection, leading to a potentially higher false-negative rate and less sensitivity (Table 1)[14]. Analogous to one- and two-step reverse-transcription (RT)-qPCR, combining pre-amplification and detection in a one-pot assay has both benefits and disadvantages (Table 1). One-pot SHERLOCK is faster and simpler than two-step SHERLOCK. By combining the pre-amplification and Cas13/Cas12 detection into a single one-pot reaction, it is possible to achieve detection in as little as 10–15 min for targets in the femtomolar concentration range and in less than an hour for targets in the attomolar concentration range. As well as being faster, there is also less risk of contamination and it is easier to obtain quantitative results (owing to real-time detection) with one-pot SHERLOCK. However, it is also less sensitive than two-step SHERLOCK, and experiment optimization is often more challenging. In general, one-pot SHERLOCK is more useful for time-sensitive, high-throughput, or quantitative applications, as well as applications with increased contamination risk (e.g., repeated testing, large target amounts), whereas two-step SHERLOCK is more useful for applications with challenging sample inputs (e.g., quick extractions from body fluids such as urine or saliva).

Another advantage of the SHERLOCK platform over other detection platforms (such as TaqMan qPCR) is the low cost of its components. A typical single-plex reaction is estimated to cost as little as $0.60 (ref. [14]). This is partly because the quencher has a fixed sequence and does not need to be redesigned and ordered for each new target. In addition, the crRNA is a short RNA sequence that is

Table 1 | Comparison of two-step and one-pot SHERLOCK formats

Aspect	Two-step SHERLOCK	One-pot SHERLOCK
Experimental time	30–60 min	15–30 min
Protocol optimization	Easy (each step can be individually optimized)	Difficult (more challenging compared to two-step)
Contamination risk	Increased risk of surface contamination with pre-amplification reaction	Decreased risk because of single tube reaction
Quantitation	Difficult (depends on finding time point at which separation is possible)	Easy (owing to real-time detection)
Sensitivity	Zeptomolar range (single molecule per milliliter)	Femto- to attomolar range (1–1,000 molecule(s) per microliter). Variability in sensitivities has been observed, which we largely attribute to the viscous RPA sample composition, which can influence Cas13 detection
Appropriate applications	• End-point binary detection • Applications with challenging sample inputs (e.g., quick extractions from unpurified samples)	• Time-sensitive applications (<30 min) • Quantitative applications • High-throughput applications • Applications with increased contamination risk (repeated testing, large target amounts)

cheap to synthesize, and the enzyme components can be produced in bulk. Because the majority of reagents are used by all three methods (SHERLOCK, DETECTR, and HOLMES), the associated costs are similar.

Despite its advantages over existing detection technologies, SHERLOCK has several caveats that can make it unacceptable for certain cases. SHERLOCK currently involves the preparation and testing of reaction components, some of which require expertise in protein purification and RNA biology. Moreover, pre-designed assays, including reaction mixtures and RNA/DNA oligonucleotides, are currently not commercially available for SHERLOCK. Because validated target assays exist for established PCR-based methods, routine laboratory applications might benefit from such simplified solutions and can minimize the optimization process as compared to that for SHERLOCK. Existing standard detection technologies may also be more appropriate for applications that do not demand the speed or portability of SHERLOCK, such as oncology assays.

Another potential limitation of SHERLOCK is the multi-step nucleic acid amplification process, which may affect precise target quantification. Although we recently demonstrated the quantitative detection of nucleic acids with SHERLOCK, absolute digital quantification such as in digital droplet PCR is currently not possible, and small differences in target quantity (<2× changes) may not be detected. SHERLOCK may therefore be less useful for precise gene expression profiling.

Applications

Because the SHERLOCK assay described in this protocol forms the basis of a reprogrammable amplification system, it can be applied in any situation that requires sensitive detection of a DNA or RNA target. SHERLOCK has been used for the detection and genotyping of bacterial and viral infectious disease agents, including distinguishing single-nucleotide variants and finding antibiotic resistance genes[14]. For example, SHERLOCK has been applied to detect Zika and dengue virus directly from patient urine and serum samples, demonstrating the field-deployable nature of the technology. Furthermore, SHERLOCK can distinguish between Zika strains from Honduras, the Dominican Republic, and the United States, even in regions that differ only by a single nucleotide[28]. The single-nucleotide specificity of SHERLOCK has also been applied to genotyping of patient samples and detection of cancer-associated mutations from circulating cell-free DNA, even in samples in which the target is present at an abundance of just 0.1% compared to background[14,16].

Experimental design

Sample format

An initial consideration for choosing a nucleic acid amplification or detection method is the concentration of RNA or DNA, which is dependent on sample extraction and removal of any inhibitory or confounding components. The appropriate method of extraction depends on the abundance of nucleic acids in the sample, the difficulty of releasing DNA or RNA from any cells or viral particles,

and the presence of amplification inhibitors or nucleases that would interfere with downstream reactions. In resource-limited settings such as point-of-care applications, even 'standard' laboratory equipment (such as centrifuges) may be unavailable. Thus, selecting a method for isolating nucleic acids is context dependent and the method may have to be optimized for special cases. Common commercial methods for isolating and concentrating DNA or RNA from samples include column- or bead-based purification methods, which allow extraction of pure concentrated nucleic acids but require equipment and power, as well as substantial time investment from trained operators.

Simpler methods, which do not extract pure nucleic acids but instead rely on mechanical force or chemical disruption of free DNA or RNA for detection, are alternatives in low-resource settings. However, raw nucleic acid extractions may result in contamination by nucleases or PCR/amplification inhibitors. Nuclease carryover to SHERLOCK reactions may degrade the reporter and lead to false-positive signals. For RNA samples, nucleases may also degrade the target RNA, leading to false-negative signals. The addition of RNase inhibitor is therefore essential to prevent such unwanted sources of variation. Additionally, pre-treatments such as HUDSON (heating unextracted diagnostic samples to obliterate nucleases) have been developed to inactivate nucleases commonly found in body fluids[28]. Other commonly known PCR/amplification inhibitors such as ethanol, detergents, strong acids, or bases may lead to failed SHERLOCK reactions and false negatives. For purified nucleic acids, target detection with Cas13 or Cas12 is almost identical for RNA and DNA, because both inputs are pre-amplified as DNA first. However, in the case of RNA, a reverse transcriptase needs to be included during pre-amplification.

SHERLOCK is compatible with many different sample formats, including crude biological matrices[14,28]. However, it is imperative to remove or inactivate all resident RNases, because they will cleave reporter molecules and contribute to background signal. HUDSON[28] relies on heating and reducing reagents to prepare samples for input into SHERLOCK reactions. Heating viral particles releases enclosed nucleic acids, whereas additional treatment with the reducing agent Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) inactivates RNases that are present in the sample matrix. HUDSON is compatible with human blood, serum, urine, and saliva and allows instrument-free direct sampling of viruses[28].

Multiplexing

Multiplexing with SHERLOCK can be performed either spatially, allowing up to 384 small reactions to be performed in a single reaction plate, or spectrally, allowing simultaneous detection of up to four targets in a single reaction. The most basic form of SHERLOCK with LwaCas13a, an active ortholog selected from a screen of Cas13a orthologs in bacterial cells, allows detection of only a single target per reaction via a fluorescence channel or a line on a lateral flow strip. Characterization of additional Cas13 and Cas12 orthologs has allowed multiplexed detection of up to four targets in a single reaction in separate fluorescent color channels[16]. Biochemical profiling of these orthologs showed diverse cleavage preferences, allowing for collateral cleavage activity of different enzymes to be read out by reporters with different nucleotide motifs and attached fluorophores. Owing to spectral limitations, it may be difficult to multiplex more than four target and enzyme pairs, but so far, five orthogonal cleavage preferences have been discovered for Cas12a from *Acidaminococcus* sp. BV3L6 (AsCas12a) and Cas13s from *L. wadei* (LwaCas13a), *Capnocytophaga canimorsus* Cc5 (CcaCas13b), *Prevotella* sp. MA2016 (PsmCas13b), and *Lachnospiraceae bacterium* NK4A179 (LbaCas13a) (Box 1)[16]. To combine multiplexed Cas13 and Cas12 detection with pre-amplification, multiple primer pairs must be designed, unless universal primers are being used for a variable internal site or multiple targets along a longer amplicon are being sensed. Owing to inherent base bias of the recombinases in RPA for specific primer sequences, the use of compatible primer pairs can be difficult. Multiple primer pairs must be designed, and, upon experimental testing, the primer concentrations must be adjusted depending on these biases to allow even amplification of all the amplicons. Alternative pre-amplification strategies that have less primer bias, such as HDA or nicking enzymatic amplification reactions, could also be used[29]. However, RPA has been demonstrated to be more robust and more sensitive than other isothermal amplification methods such as nucleic acid sequence-based amplification (NASBA)[14,30,31]. The reaction temperature of RPA also allows the combination of pre-amplification with detection in one pot SHERLOCK assays.

Primer design

For pre-amplification with RPA, two primers (forward and reverse) must be designed (Fig. 2a). To maximize amplification success, the primer lengths should be ~25–35 nt, and the total amplicon size

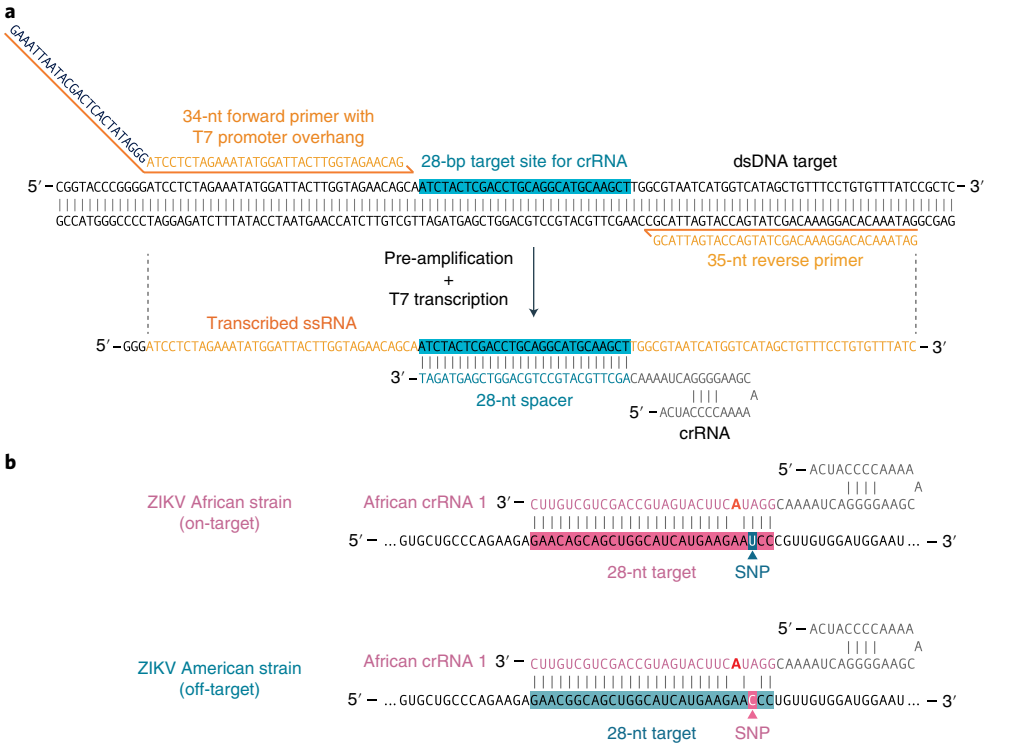


Fig. 3 | Considerations for primer and crRNA design. a, RPA primer and LwaCas13a crRNA design for target detection. Schematic of LwaCas13a crRNA detection of target RNA transcribed from an RNA amplicon. Sequences of the RPA primers and crRNA are shown to highlight how these should be designed relative to the target DNA and transcribed RNA. A T7 promoter should be added to the 5′ end of the forward RPA primer, and the crRNA sequence should be the reverse complement of the target site in the transcribed RNA. The DR of the LwaCas13a crRNA is on the 5′ end of the spacer sequence. **b**, Synthetic mismatch crRNA design for single-nucleotide specificity. Schematic of crRNA design for sensing a single-nucleotide mismatch difference between African and American strains of the Zika virus. The single-nucleotide polymorphism (SNP) site should be placed in the third position of the spacer sequence and the synthetic mismatch (highlighted in red) should be placed in the fourth or fifth position of the spacer sequence.

should be 80–140 bp[1]. Primers are typically designed with melting temperatures between 54 and 67 °C. Primers can be designed with NCBI Primer-BLAST[32], using the custom parameters discussed above. Although the RPA manufacturer recommends designing ~96 primer pairs in order to find an optimal pair that is specific, only a few primer pairs need to be designed and tested for SHERLOCK, because specificity is encoded by the crRNA and most primer pairs will result in high amplification rates. Owing to the additional specificity of the CRISPR detection, it is not necessary to evaluate primer sets using gel electrophoresis to look for specific amplicons. For detection reactions involving Cas13, a T7 RNA polymerase promoter must be appended to the 5′ end of one of the primers to allow T7 transcription (Fig. 3a). We recommend appending the promoter sequence to the forward primer and designing crRNAs complementary to the sense strand (Fig. 3a).

crRNA design

The most important consideration of crRNA design for Cas13 detection is the lack of overlap with RPA primers, because crRNA overlap with primers may result in detection of the primer sequence in off-target amplification products, increasing background (Fig. 3a). The crRNA sequence should be the reverse complement of the target site in the transcribed RNA. A spacer sequence (28 nt, in the case of LwaCas13a) can be placed anywhere within the resulting pre-amplified RPA product. Avoidance of targeting the target RNA secondary structure has been shown to increase RNA targeting with LwaCas13a (ref. [25]). The spacer sequence is then joined with a 5′ direct repeat (DR) sequence to generate a complete crRNA.

crRNA can be generated from DNA templates via IVT or produced as chemically synthesized oligonucleotides via phosphoramidite synthesis providers. IVT methods are typically less expensive

and can be used to produce large amounts of crRNA at scale, but the generation of crRNA and subsequent purification takes longer. In addition, chemically synthesized crRNAs are purer, resulting in a stronger detection signal and increased sensitivity. When generating crRNA via IVT, an additional T7 RNA polymerase promoter is placed upstream of the crRNA sequence to allow T7 transcription, and the entire sequence is ordered as the DNA reverse complement.

When enzymes other than LwaCas13a are used (Box 1), more considerations apply. For the use of additional Cas13 variants, such as Cas13b orthologs[16], the spacer length must be changed (Cas13b orthologs use spacer lengths of 30 nt), the DR sequence must be modified to correspond to the ortholog, and the position of the DR sequence may need to be moved from the 5′ to the 3′ end of the spacer, because Cas13b crRNAs have a 3′ DR sequence. When using Cas12a for detection, the spacer length (20 nt for AsCas12a) and DR sequence position (5′) should be considered. When Cas12a targets single-stranded DNA (ssDNA), it does not require a PAM, but when targeting double-stranded DNA (dsDNA), such as in combination with RPA, the crRNA must be placed next to a PAM (commonly 5′ TTTV for AsCas12a)[9].

Owing to the inherent specificity of Cas13, which will not cleave sequences with two or more mismatches, SHERLOCK is capable of specific sequence detection. To design crRNA for single-base pair specificity, the base to be distinguished is placed in the third position of the crRNA, and an additional synthetic mismatch is placed in either the fourth or fifth position of the crRNA (Fig. 3b). This synthetic mismatch generates a bubble of two base pairs when the base pair of interest is not present, resulting in more stringent discrimination at the targeted position. In addition, by placing the region where a small deletion occurs in the seed region of the crRNA (positions ~5–15), deletions can be sensed[16].

Readout formats

Typically, the SHERLOCK reaction readout uses a fluorescent reporter involving a short RNA oligonucleotide with a fluorophore on one end and a quencher on the other[14]. These assays are easy to set up in a multiwell plate and can be measured using any standard fluorescence plate reader or qPCR machine. Given the right setup, these reactions can also be monitored using handheld fluorometers, allowing portable fluorescence detection. Fluorescence readout also allows continuous monitoring, enabling real-time quantitation.

For even simpler instrument-free and portable detection, lateral flow readouts can be used[16]. The lateral flow reporter uses an RNA reporter flanked by a fluorescein and biotin on separate ends. On the lateral flow strips, a line of streptavidin will bind to biotin, capturing all the intact probe. Anti-fluorescein antibodies labeled with gold nanoparticles (NPs) will bind the fluorescein end of the reporter and form a dark purple color at this first line. When RNA reporters are cleaved because of target presence and collateral activity, gold NP–labeled antibodies will flow over to a second line of anti-species secondary antibody, capturing all the antibodies and forming a dark purple color at the second line that indicates the presence of target. The lateral flow readout takes only a few minutes and is usually performed after 5–10 min of pre-amplification and 20–30 min of Cas13 detection.

Quantitation

For many nucleic acid detection applications, it is sufficient to simply distinguish between the presence and absence of a target, but some situations—such as measuring the fraction of cancer-associated cell-free DNA or determining viral load—may call for quantitation of target levels. The activation of Cas13 is quantitative with respect to RNA target abundances, and increased levels of target will result in increased activation and faster accumulation of signal as measured by fluorescence output. In cases in which RPA and T7 transcription are used, these additional steps add complexities to quantitation. T7 transcription is a linear amplification and does not introduce many barriers to quantitation, but RPA, as with many isothermal amplification methods, undergoes asynchronous amplification with the potential to saturate, which can prevent accurate quantitation[14]. To overcome this barrier, the concentration of primer in the RPA reaction can be tuned to find a quantitative window. In published experiments, a primer concentration of 240 nM resulted in quantitative SHERLOCK activity over multiple orders of magnitude[16], but quantitation should be optimized for each target. Generally, we recommend optimizing primer concentration and pre-amplification times for individual targets, in both single-plex and multiplex detection formats. Synthetic versions of the desired nucleic acid target may help in establishing robust reaction conditions.

Table 2 | Example sequences for testing SHERLOCK

Name	Sequence	Purpose
American ZIKV synthetic RNA		
Zika American synthetic RNA	GACACCGGAACUCCACACUGGAACAACAAAGAAGCAC UGGUAGAGUUCAAGGACGCACAUGCCAAAAGGCAAAC UGUCGUGGUUCUAGGGAGUCAAGAAGGAGCAGUUCAC ACGGCCCUUGCUGGAGCUCUGGAGGCUGAGAUGGAUG GUGCAAAGGGAAGGCUGUCCUCUGGC	American ZIKV synthetic RNA
Zika American strain RPA forward primer	GAAATTAATACGACTCACTATAGGGCGGAACTCCACAC TGGAACAACAAA	RPA primer for pre-amplification of American ZIKV
Zika American strain RPA reverse primer	TGCACCATCCATCTCAGCCTCCAGAGCTCC	RPA primer for pre-amplification of American ZIKV
Zika American LwaCas13a crRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAA ACACUCCCUAGAACCACGACAGUUUGCCUU	American ZIKV LwaCas13a detection crRNA
Zika American LwaCas13a crRNA IVT template	AAGGCAAACTGTCGTGGTTCTAGGGAGTGTTTTAGTC CCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGA GTCGTATTAATTTC	American ZIKV LwaCas13a detection crRNA IVT template
Synthetic DNA 1		
Synthetic DNA 1	GGCCAGTGAATTCGAGCTCGGTACCCGGGGATCCTCTAG AAATATGGATTACTTGGTAGAACAGCAATCTACTCGACC TGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTG TTTCCTGTGTTTATCCGCTCACAATTCCACACAACATACG AGCCGGAAGCATAAAG	Synthetic DNA 1
Synthetic DNA 1 RPA forward primer	AATTCTAATACGACTCACTATAGGGATCCTCTAGAAATAT GGATTACTTGGTAGAACAG	RPA primer for pre-amplification of DNA 1
Synthetic DNA 1 RPA reverse primer	GATAAACACAGGAAACAGCTATGACCATGATTACG	RPA primer for pre-amplification of DNA 1
Synthetic DNA 1 LwaCas13a crRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAAC CGCCAAGCUUGCAUGCCUGCAGGUCGAG	DNA 1 LwaCas13a detection crRNA
Synthetic DNA 1 LwaCas13a crRNA IVT template	CTCGACCTGCAGGCATGCAAGCTTGGCGGTTTTAGTCC CCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGAGT CGTATTAATTTC	DNA 1 LwaCas13a detection crRNA IVT template
T7-3G IVT primer	GAAATTAATACGACTCACTATAGGG	T7 promoter annealing oligonucleotide for IVT

ZIKV, Zika virus.

Trial run

Readers should attempt a trial run on synthesized DNA or RNA, or in vitro transcribed RNA. Example sequences for American ZIKV synthetic RNA or synthetic DNA 1 can be found in Table 2. A trial run will familiarize the experimenter with the procedure without potentially wasting extracted nucleic acid material. It also provides a quality control assessment of reagents, because SHERLOCK nucleic acid detection is performed with RNA probes that undergo enzymatic hydrolysis and therefore require sensitive handling.

Materials

Biological materials

- Rosetta 2(DE3)pLysS Singles competent cells (Millipore, cat. no. 71401-3) **! CAUTION** The cells used in your research should be regularly checked to make sure that they are authentic and are not infected with mycoplasma.

Reagents

- TwinStrep−SUMO−LwaCas13a expression plasmid (pC013−TwinStrep−SUMO−huLwCas13a; Addgene, plasmid no. 90097)
- SOC outgrowth medium (25 mL; New England Biolabs, cat. no. B9020S)
- Luria broth (LB; ready-made powder, 250 g; Thermo Scientific, cat. no. J75854.30)
- Terrific broth powder (TB; VWR, cat. no. 89126-196)
- Glycerol (≥99.5% (wt/vol); Sigma-Aldrich, cat. no. G9012-1GA)
- Ampicillin, sodium salt (AmericanBio, cat. no. AB00115-00025; CAS no. 69-52-3)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, cat. no. I6758)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S7653)

- DL-Dithiothreitol solution (DTT; BioUltra, for molecular biology, 1 M in H_2O; Sigma–Aldrich. cat. no. 43816-50ML)
- Tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP; 0.5 M, pH 7.0; Sigma-Aldrich, cat. no. 646547-10X1ML)
- β-Mercaptoethanol (BME; Sigma-Aldrich, cat. no. M6250-100ML) **! CAUTION** BME is considered toxic. Wear gloves and work in a well-ventilated area.
- Agar (Affymetrix, cat. no. 75851)
- Tris-HCI buffer (1 M, pH 7.5 and 8.0; Invitrogen UltraPure, cat. nos. 15-568-027 and 15-568-025)
- cOmplete Ultra Tablets, Mini, EDTA-free (Sigma-Aldrich, cat. no. 05892791001)
- Lysozyme from chicken egg white (Sigma-Aldrich, cat. no. L6876-10G)
- Benzonase nuclease (Sigma-Aldrich, cat. no. E1014-25KU)
- Bolt 4–12% Bis-Tris Plus Gels for SDS-Page electrophoresis (Thermo Fisher Scientific, cat. no. NW04125BOX)
- Bolt sample-reducing agent (10×; Thermo Fisher Scientific, cat. no. B0004)
- Bolt LDS sample buffer (4×; Thermo Fisher Scientific, cat. no. B0007)
- Bolt MES SDS running buffer (20×; Thermo Fisher Scientific, cat. no. B000202)
- Strep-Tactin Superflow Plus resin (50%; Qiagen, cat. no. 30004) **▲ CRITICAL** We have found superior performance using Strep-Tactin Superflow Plus resin from Qiagen as compared with resin from other vendors.
- SUMO protease (250 U; Thermo Fisher Scientific, cat. no. 12588018)
- Octylphenoxy poly(ethyleneoxy)ethanol (branched, IGEPAL CA-630; Sigma-Aldrich, cat. no. I8896-50ML) or NP-40 Surfact-Amps Detergent Solution (50 mL; Thermo Fisher Scientific, cat. no. 85124)
- HEPES powder (Sigma-Aldrich, cat. no. H4034-1KG)
- HEPES buffer (1 M; Thermo Fisher Scientific, cat. no.15630-080)
- Magnesium chloride ($MgCl_2$; 0.2 μm filtered, 1 M; Thermo Fisher Scientific, cat. no. AM9530G)
- HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, cat. no. E2050S)
- Sodium hydroxide solution (NaOH; 10 M; Sigma-Aldrich, cat. no. 72068-100ML) **! CAUTION** NaOH is a strong base and should be handled with appropriate personal protective equipment.
- EDTA (0.5 M, pH 8.0; RNase free; Invitrogen, cat. no. AM9260G)
- NTP buffer mix, T7 RNA polymerase mix (from HiScribe T7 Quick High Yield RNA Synthesis Kit; New England Biolabs, cat. no. E2050S)
- Standard *Taq* Buffer (10×; New England Biolabs, cat. no. B9014S)
- UltraPure DNase/RNase-free distilled water (Invitrogen, cat. no. 10977015)
- Agencourt RNAClean XP Kit (Beckman Coulter, cat. no. A63987)
- Absolute ethanol (EtOH; anhydrous, 200 proof/100% (vol/vol); VWR, cat. no. 89125-170) **! CAUTION** Absolute ethanol is highly flammable. Keep away from open flames and work in a well-ventilated area.
- Novex TBE-urea Gels (10%; Thermo Fisher Scientific, cat. no. EC68755BOX)
- SYBR Gold nucleic acid gel stain (10,000× concentrate in dimethyl sulfoxide; Thermo Fisher Scientific, cat. no. S11494)
- TwistAmp Basic (TwistDx, cat. no. TABAS03KIT)
- RNase inhibitor (murine; New England Biolabs, cat. no. M0314S)
- NxGen T7 RNA polymerase (125,000 U; Lucigen, cat. no. 30223-2)
- RNaseAlert Lab Test Kit v2 (Invitrogen, cat. no. 4479768)
- Ribonucleotide solution mix (rNTP solution mix; New England Biolabs, cat. no. N0466L)
- Milenia HybriDetect 1 (TwistDx, cat. no. MILENIA01)
- P1 buffer (Qiagen, cat. no. 19051)
- Isopropanol (Sigma, cat. no. I9516)
- RNase Away (Thermo Fisher Scientific, cat. no. 10328011)
- Sequence-specific reporters (LwaCas13a (/5TEX615/T*A*rArUG*C*/3IAbRQSp/), CcaCas13b (/5Cy5/T*A*rUrAG*C*/3IAbRQSp/), PsmCas13b (/56-FAM/rArArArArA/3IABkFQ/), and AsCas12a (available in DNaseAlert Kit; IDT, cat. no. 11-02-01-04)). Asterisks indicate phosphorothioate bonds. These sequences can be obtained as custom orders from IDT
- LF-RNA reporter 1: /56-FAM/rUrUrUrUrU/3Bio/ (Integrated DNA Technologies, cat. no. custom order from IDT)
- (Optional, needed only if not using eStain L1 Protein Staining System) Coomassie Brilliant Blue R-250; Bio-Rad, cat. no. 1610400)
- (Optional, needed only if not using eStain L1 Protein Staining System) Destain solution (GenScript, cat. no. L00659)

- UltraPure deionized water (Thermo Fisher Scientific, cat. no. 15230162)
- PBS (Thermo Fisher Scientific, cat. no. 10010023)
- Triton X-100 (Sigma, cat. no. 11332481001)
- T7-3G oligonucleotide: GAAATTAATACGACTCACTATAGGG (Integrated DNA Technologies, custom order)

Equipment

- Water bath (VWR, cat. no. 76308-834)
- Constant temperature incubator (Quincy Lab, model no. 12-140E)
- Bacteriological Petri dish (100 mm × 15 mm; not tissue culture treated; Corning, cat. no. 351029)
- Culture flasks (1 L; PYREX wide-mouth flasks; Corning, cat. no. 5100-1L)
- Biological shaker (Eppendorf, model no. Innova S44i)
- Fisherbrand disposable cuvettes (polystyrene; Fisher Scientific, cat. no. 14-955-127)
- Floor centrifuge (4-L capacity; Avanti J-E; Beckman Coulter, model no. 369001)
- Gel electrophoresis tank and power source (Mini Gel Tank; Invitrogen, model no. A25977; 250-V power source; VWR, cat. no. 93000-746)
- Microfluidizer (Microfluidics, model no. LM20)
- Polypropylene centrifuge tubes (250 mL; Corning, cat. no. 05-538-53)
- Tube rotator (Scilogex MX-RL-Pro; Scilogex, model no. 824222019999)
- Plate shaker (Mini Shaker; VWR, model no. 12620-938)
- Chromatography column (glass Econo-Column; Bio-Rad, cat. no. 7374252)
- eStain L1 Protein Staining System (GenScript, model no. L00657)
- Fast protein liquid chromatography (FPLC) instrument (ÄKTA pure; GE Healthcare Life Sciences, model no. 29018228)
- LightCycler 480 Instrument II (Roche Diagnostics, model no. 05015243001)
- QX200 droplet generator (Bio-Rad, cat. no. 186-4002)
- QX200 droplet reader (Bio-Rad, cat. no. 186-4003)
- Benchtop centrifuge (Beckman Coulter, model no. Allegra X-15)
- Axygen 8-strip PCR tubes (Fisher Scientific, cat. no. 14-222-250)
- Axygen PCR plates (96 well; VWR, cat. no. PCR-96M2-HS-C)
- Optical plate (384-well; LightCycler 480 multiwell plate 384; Roche, cat. no. 5102430001)
- Axygen boil-proof microcentrifuge tubes (1.5 mL; VWR, cat. no. 10011-702)
- Falcon tubes (polypropylene, 15 and 50 mL; Corning cat. nos. 352097 and 352070)
- Eppendorf Safe-Lock microcentrifuge tubes (1.5 mL; Eppendorf, cat. no. T9661-1000EA)
- Filtered sterile pipette tips (Rainin, cat. nos. RT-LTS-A-1000μL-/F-768/8, RT-LTS-A-200μL-/F-960/10, and RT-LTS-A-10μL-/F-960/10)
- Corning bottle-top vacuum filter system (0.22 μM; Sigma-Aldrich, cat. no. CLS431098)
- Stericup filter unit (0.45 μM; Millipore, cat. no. SCHVU02RE)
- Amicon Ultra-15 centrifugal filter unit (with Ultracel-50 membrane; Sigma-Aldrich, cat. no. UFC905024)
- Thermocycler (with programmable temperature-stepping functionality; 96 well; Veriti; Applied Biosystems, cat. no. 4375786)
- Desktop microcentrifuges (Eppendorf, cat. no. 5424)
- Digital gel imaging system (GelDoc EZ; Bio-Rad, model no. 170-8270) and white sample tray (Bio-Rad, cat. no. 170-8272)
- UV spectrophotometer (NanoDrop; Thermo Fisher Scientific, model no. 2000c)
- Plate spectrophotometer (Synergy Neo2 multi-mode microplate reader; BioTek, model no. NEO2SM)
- Small-scale size exclusion chromatography column (Superdex 200 Increase 10/300 GL; GE Healthcare Life Sciences, cat. no. 28990944)
- HiTrap SP HP cation exchange column (5 mL; GE Healthcare Life Sciences, cat. no. 17115101)
- Parafilm (VWR, cat. no. 52858-076)
- Clear reclosable bags (VWR, cat. no. 89005-284)
- Centrifugal spin filters (15 mL; 50 MWCO; EMD Millipore, cat. no. UFC905008)
- Magnetic stand (Thermo Fisher Scientific, cat. no. 12027)
- (Optional) QIAamp DNA Blood Mini Kit (Qiagen, cat. no. 51104)
- (Optional) QIAamp Viral RNA Mini Kit (Qiagen, cat. no. 52904) or RNeasy Plus Mini Kit (Qiagen, cat. no. 74134)

Software

- Gel quantification software (Bio-Rad ImageLab or open-source ImageJ from the National Institutes of Health (NIH); available at http://rsbweb.nih.gov/ij/)

Reagent setup

LB agar plates

Reconstitute the LB with agar at a concentration of 35 g/L in deionized water and swirl to mix. Autoclave to sterilize at 121.0 °C for 20 min. Allow the LB agar to cool to 55 °C before adding ampicillin to a final concentration of 100 μg/mL and swirling to mix. On a sterile bench area, pour ~20 mL of LB agar per 100-mm Petri dish. Place the lids on the plates and allow them to cool at room temperature (22 °C) for 30–60 min until solidified. Invert the plates and let them sit for several more hours or overnight. Agar plates can be stored in plastic bags or sealed with Parafilm at 4 °C for up to 3 months.

TB medium (1 L, ampicillin)

Reconstitute TB medium by adding 50.8 g of TB powder to a 2-L flask, adding 4 mL of 100% (wt/vol) glycerol, and filling up with deionized water to a 1-L final volume. Heat at 50 °C with repeated stirring to dissolve completely; then autoclave at 121.0 °C for 15 min. Let cool for several hours, then add ampicillin to a 100 μg/mL final concentration. TB medium is stable at 4 °C for 6 months or for 1 month at room temperature.

IPTG

Dissolve 1.19 g of IPTG by adding it to 8 mL of deionized water and then vortexing. Add deionized water to bring the volume to 10 mL and filter-sterilize with a 0.22-μm syringe filter. Store at −20 °C for up to 6 months.

Lysis buffer

Combine 40 mL of Tris-HCl (pH 8.0, 1 M), 200 mL of NaCl (5 M), and 2 mL of DTT (1 M), and bring the final volume to 2 L with UltraPure water. Use within 48 h of preparation. For longer storage, prepare the buffer without the reducing agent (DTT) and keep at 4 °C for up to 2 weeks.

Supplemented lysis buffer

Add 12 cOmplete Ultra EDTA-free tablets, 600 mg of lysozyme, and 6 μL of benzonase to 600 mL of lysis buffer. Prepare fresh and use within 24 h following preparation. Keep at 4 °C when not in use **! CAUTION** Follow the handling instructions in the material safety data sheet to minimize risk when using hazardous reducing agents **▲ CRITICAL** The lysis buffer contains the reducing agent DTT, which can be replaced with TCEP or BME. DTT and BME are less stable in solution, so the lysis buffer should be freshly prepared for optimal results.

SUMO protease cleavage solution

Supplement 15 mL of lysis buffer with 250 μL of SUMO protease and 22.5 μL of NP-40. This should be prepared fresh.

Buffer A

Combine 40 mL of Tris-HCl (pH 7.5, 1 M), 200 mL of glycerol (50% (wt/vol)), and 2 mL of DTT (1 M), and bring the final volume to 2 L with UltraPure water. Filter through a 0.22-μm vacuum filter and store at 4 °C for up to 48 h. For storage of up to 2 weeks, prepare the buffer without the reducing agent (DTT) and add this fresh when performing FPLC.

Buffer B

Combine 20 mL of Tris-HCl (pH 7.5, 1 M), 100 mL of glycerol (50% (wt/vol)), 400 mL of NaCl (5 M), and 1 mL of DTT (1 M), and bring the final volume to 1 L with UltraPure water. Filter through a 0.22-μm vacuum filter and store at 4 °C for up to 48 h. For storage of up to 2 weeks, prepare the buffer without the reducing agent (DTT) and add this fresh when performing FPLC.

S200 size-exclusion buffer

Combine 10 mL of HEPES (pH 7, 1 M), 5 mL of $MgCl_2$ (1 M), 200 mL of NaCl (5 M), and 2 mL of DTT (1 M), and bring the volume to 1 L with UltraPure water. Filter through a 0.22-μm vacuum filter

and store at 4 °C for up to 48 h. For storage of up to 2 weeks, prepare the buffer without the reducing agent (DTT) and add this fresh when performing FPLC.

LwaCas13a protein storage buffer

Combine 2.5 mL of Tris-HCl (pH 7.5, 1 M), 6 mL of NaCl (5 M), 2.5 mL of glycerol, and 100 µL of DTT (1 M), and bring the final volume to 50 mL with UltraPure water. Filter through a 0.22-µm, 50-mL vacuum filter and store at 4 °C for up to 48 h. For storage of up to 1 year, make aliquots of the buffer and store at −20 °C. Avoid repeated freeze–thaw cycles of the buffer ▲ **CRITICAL** Prepare the buffer under nuclease-free conditions.

SDS-PAGE sample buffer

Add 4 µL of 10× Bolt sample-reducing agent and 10 µL of 4× Bolt LDS sample buffer to 16 µL of UltraPure water. The buffer mixture can be stored for up to 2 weeks at 4 °C. When running SDS-PAGE, 10 µL of sample is added to the above mixture and heated to 95 °C for 5 min. If the sample volume needs to be changed, adjust the sample buffer component volumes accordingly to obtain a final volume of 40 µL, including the sample.

(Optional) Plant extraction buffer

Prepare 10 mL of alkaline plant extraction buffer by adding 0.5 mL of 10 M NaOH and 0.2 mL of 0.5 M EDTA to 9.3 mL of nuclease-free water. Prepare 1-mL aliquots of the buffer in 1.5-mL plastic Eppendorf tubes and store at −20 °C for up to 6 months.

Procedure

Recombinant expression and purification of LwaCas13a ● Timing 5 d

▲ **CRITICAL** The workflow for transforming bacteria with the appropriate expression constructs for large-scale expression and protein purification is shown in Fig. 4a.

Large-scale expression of LwaCas13a ● Timing 3 d

1 Thaw one vial of Rosetta 2(DE3)pLysS competent cells on ice for 30 min, and then add 1 µL of 50 ng/µL of LwaCas13a expression plasmid. Incubate on ice for 5 min.

2 Heat-shock the cells by placing the vial into a 42 °C pre-heated water bath for 45 s, and then cold-shock the cells on ice for 2 min.

3 Add 200 µL of SOC medium to the cells and plate 100 µL of cell suspension on a pre-warmed LB agar plate containing 100 µg/mL ampicillin. Incubate the plate overnight in a 37 °C incubator.
? TROUBLESHOOTING

4 The next day, inoculate 25 mL of TB medium containing 100 µg/mL ampicillin with a single colony and incubate the culture overnight at 37 °C in a biological shaker at 300 r.p.m.

5 Inoculate 4–12 L of TB medium, containing 100 µg/mL ampicillin, with a 5 mL/L starter culture and determine the optical density (OD, 600 nm). The amount of starter culture depends on the downstream expression scale. We recommend starting with 5 mL of starter culture for every 1 L of large-scale culture. Shake cultures at 37 °C, 300 r.p.m.

6 Monitor the OD every hour until the cells reach an OD of 0.4–0.6, and then transfer the flasks to 4 °C for 30 min to allow them to cool before induction. Take an aliquot of uninduced culture for SDS-PAGE analysis.
▲ **CRITICAL STEP** For optimal expression, it is important to strictly adhere to the indicated OD value of 0.4–0.6 at the time point of induction.

7 Induce expression by adding 1 mL/L 0.5 M IPTG and shake the cultures for 16 h at 300 r.p.m. in a pre-chilled 21 °C biological shaker.

8 Harvest the cells by spinning the culture down at (5,200*g*) for 15 min at 4 °C. Take a small aliquot and resuspend it in 500 µL of P1 buffer. Run together with the uninduced culture aliquot on a Bolt 4–12% Bis-Tris Plus SDS-PAGE gel in 1× Bolt MES SDS running buffer for 20 min at 200 V. Stain gel with the eStain L1 protein staining system and visualize the gel on the BioRad Digital gel imaging system.
? TROUBLESHOOTING
■ **PAUSE POINT** The remaining cells can be directly used for purification or stored at −80 °C for up to 1 year. Cells are routinely stored as spread paste in clear reclosable bags, which enables future expression testing and preparation of aliquots by breaking the frozen paste.

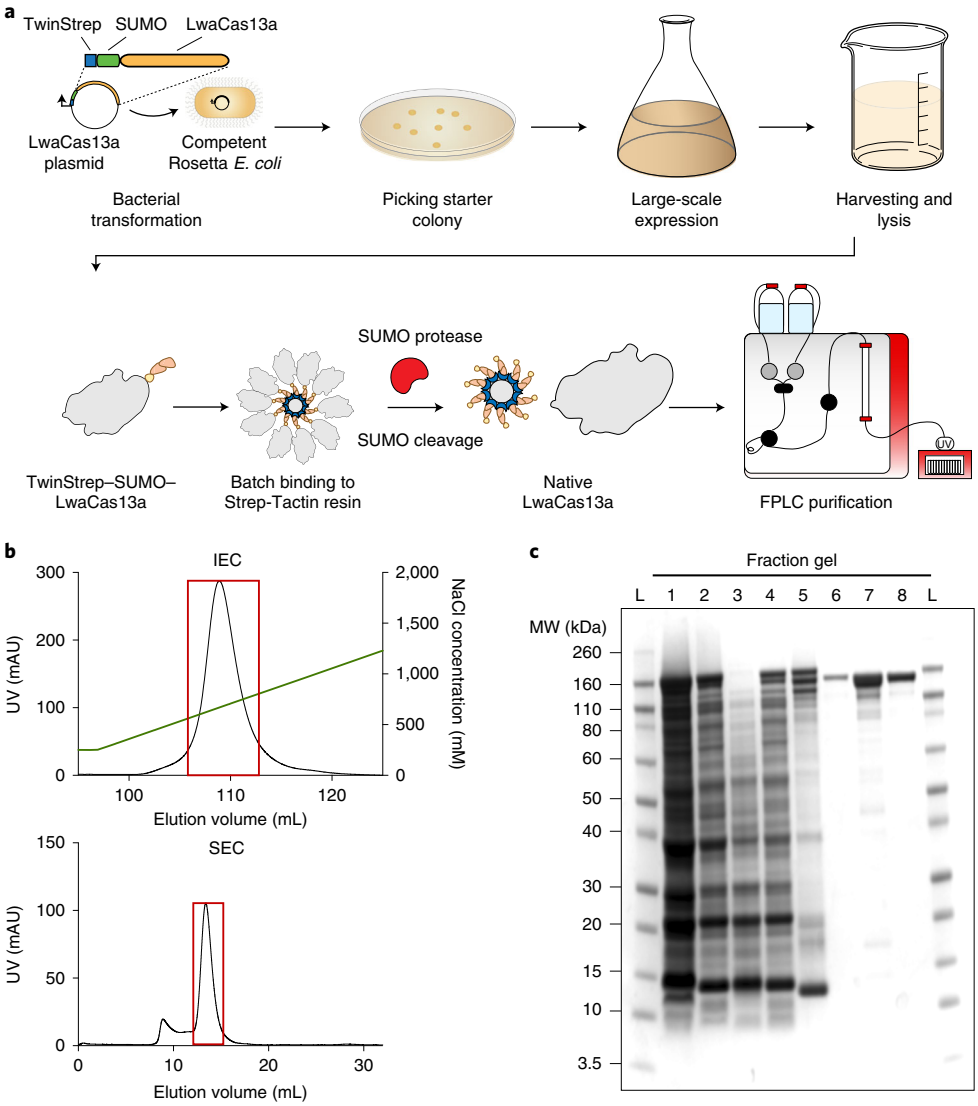


Fig. 4 | Protein purification workflow and expected results. a, LwaCas13a protein expression and purification workflow. The TwinStrep–SUMO–LwaCas13a protein expression plasmid is first transformed into competent Rosetta *E. coli* cells. After antibiotic selection and initial growth, expression is induced by IPTG. Following growth, cells are harvested and lysed. The recombinant protein is subsequently enriched from the total cell protein by affinity Strep-Tactin purification. The TwinStrep–SUMO tag is then cleaved by SUMO protease to obtain native LwaCas13a protein. The enzyme is further purified by ion-exchange and size-exclusion chromatography on an FPLC system. **b**, FPLC chromatograms: a representative chromatogram of ion exchange (IEC, top image) and a size-exclusion chromatogram (SEC, bottom image) for LwaCas13a. The UV absorbance in milli-arbitrary units (mAU) is plotted against the elution volume in milliliters. The NaCl gradient for IEC is shown by the green line. The red boxes indicate the protein-containing fractions that were pooled and concentrated. **c**, Coomassie-stained SDS-PAGE gel of protein fraction. The progress of protein purification is shown on a Coomassie-stained SDS-PAGE gel. The fractions are L, ladder; 1, cell lysate; 2, cleared cell lysate; 3, cell pellet after clearing of lysate; 4, flow-through following Strep-Tactin batch binding; 5, Strep-Tactin resin before SUMO protease cleavage; 6, eluted fraction post SUMO protease cleavage; 7, concentrated sample post IEC; 8, final product after SEC. AU, arbitrary units.

Purification of LwaCas13a ● Timing 1.5 d

▲ **CRITICAL** Perform all steps at 4 °C and do not let the Strep-Tactin Superflow Plus resin run dry. In steps where a working environment of 4 °C cannot be achieved, try to keep the sample near 4 °C by cooling on ice.

9 Crush and resuspend the frozen pellet in 4× (wt/vol) supplemented lysis buffer (e.g., 20 g of pellet in 80 mL of buffer) by stirring the mixture at 4 °C for 30 min on a magnetic stir table.

▲ **CRITICAL STEP** To ensure optimal lysis downstream, monitor resuspension progress until a homogeneous mixture is obtained.

10 Lyse the cells by passing the cell suspension once through a pre-chilled LM20 Microfluidizer system at 27,000 p.s.i. Alternatively, cells can be ruptured on ice using sonication with an amplitude setting of 100% for 1 s on and 2 s off; a total of 10 min of sonication time is recommended to avoid heat-induced denaturation of the lysed protein. Collect a 100-µL fraction for SDS-PAGE analysis.

11 Clear the lysate by centrifugation for 1 h at 10,000 r.p.m. at 4 °C.

12 Decant the cleared supernatant into a conical 250-mL tube and collect a 100-µL fraction for SDS-PAGE analysis. With a 1,000-µL pipette tip, streak an aliquot of the insoluble fraction and resuspend it in 100 µL of lysis buffer for SDS-PAGE.

13 Add 5 mL of Strep-Tactin Superflow Plus resin to the supernatant. Batch-bind the recombinant protein to the resin for 2 h by gentle shaking at 4 °C.

14 Meanwhile, prepare a 50-mL Bio-Rad glass Econo-Column by washing the column with 2× 50 mL of cold lysis buffer; then add 20 mL of cold lysis buffer to equilibrate the column bed. Drain the column immediately before sample application.
▲ **CRITICAL STEP** Do not use supplemented lysis buffer, because the presence of protease inhibitor will affect downstream cleavage of the Strep-Tactin–SUMO tag.

15 Pour the resin–sample suspension over the prepared column and collect the flow-through. Collect a 100-µL fraction for SDS-PAGE analysis. Then wash the collected resin three times with 25 mL of cold lysis buffer. With a 200-µL pipette tip, take a small aliquot of the resin and resuspend it in 100 µL of lysis buffer for SDS-PAGE analysis.

16 Add 15 mL of SUMO protease cleavage solution to the resin, close the glass column with the provided cap, and allow SUMO protease cleavage to proceed overnight at 4 °C under gentle shaking.
▲ **CRITICAL STEP** Avoid vigorous shaking to prevent foam formation and extensive coating of the column glass surface with protein-bound resin.

17 The next day, drain the column and collect the cleavage solution into a separate 50-mL Falcon tube. Then wash the remaining sample three times with 5 mL of lysis buffer to ensure complete transfer of the cleaved protein. Collect a 100-µL aliquot of the cleaved fraction for SDS-PAGE analysis.
▲ **CRITICAL STEP** The resin bound with the Twin-Strep–SUMO tag will remain in the column, whereas the collected fraction should contain untagged, native LwaCas13a. To ensure cleavage is complete, take a small aliquot of the resin with a 200-µL pipette and resuspend it in 100 µL of lysis buffer for SDS-PAGE analysis.

18 Perform SDS-PAGE analysis of all collected protein fractions (listed in the table below) to confirm successful cleavage by SUMO protease. To do so, add 10 µL of sample to 30 µL of SDS-PAGE sample buffer, heat to 95 °C for 5 min and run SDS-PAGE on a Bolt 4–12% Bis-Tris Plus SDS-PAGE gel in 1× Bolt MES SDS running buffer for 20 min at 200 V. Stain gel on the eStain L1 protein staining system, and visualize the gel on the BioRad Digital gel imaging system.

Step	Sample
10	Cell lysate
12	Cleared cell lysate
12	Cell pellet after clearing of lysate
15	Flow-through following Strep-Tactin batch binding
15	Strep-Tactin resin before SUMO protease cleavage
17	Eluted fraction post SUMO protease cleavage

? TROUBLESHOOTING

FPLC tandem purification ● Timing 0.5 d

▲ **CRITICAL** Although the sample taken at Step 17 might appear to be a clear, single band in SDS-PAGE analysis, we recommend further purification of the enzyme via FPLC tandem purification (Steps 19–28) to remove unwanted contaminants such as SUMO protease and nucleases.

19 Using an ÄKTA pure or similar FPLC system, wash a 5-mL SP HP column with 5 column volumes (25 mL) of buffer B, followed by 5 column volumes (25 mL) of buffer A, using a system flow rate of 5 mL/min. Then equilibrate the column with 5 column volumes (25 mL) of a mixture of 12.5% buffer B in buffer A (250 mM NaCl) at a flow rate of 5 mL/min.
▲ **CRITICAL STEP** Use freshly prepared and filtered buffers for operating the FPLC system to avoid column or system damage.

20 Dilute the collected sample twofold by adding 30 mL of buffer A.
▲ CRITICAL STEP The dilution step is important in lowering the NaCl concentration to 250 mM, in order to efficiently bind the sample to the equilibrated column during sample application.

21 Start sample application and cation exchange chromatography by running the program described below at 5 mL/min.

Step	Program	Volume
1	Sample application	Continuous application until entire volume is run through column
2	Column wash: 12.5% buffer B	5 column volumes (25 mL)
3	Gradient elution: 12.5% buffer B to 100% buffer B	10 column volumes (50 mL)
4	Column wash: 100% buffer B	10 column volumes (50 mL)
5	Equilibration: 12.5% buffer B	5 column volumes (25 mL)

22 Collect 100 µL of peak-containing fractions for SDS-PAGE analysis. An example FPLC chromatogram of the ion exchange chromatography result is shown in Fig. 4b.
? TROUBLESHOOTING

23 Pool fractions containing LwaCas13a protein into a 15-mL centrifugal spin filter (50 MWCO) and concentrate to a <1-mL final volume by centrifugation for 15 min at 4,000*g* at 4 °C.
▲ CRITICAL STEP Sample concentration is important to reducing the sample loading volume for downstream size-exclusion chromatography, which is done to ensure a high-resolution separation of potential protein aggregates and other proteins from monomeric LwaCas13a.
? TROUBLESHOOTING

24 Equilibrate a Superdex 200 Increase 10/300 GL size-exclusion chromatography column with 5 column volumes (120 mL) of S200 size-exclusion buffer at 0.75 mL/min.

25 Apply the sample and start size-exclusion chromatography by running the program described below at 0.75 mL/min.

Step	Program	Volume
1	Sample application	0.5-mL or 2-mL sample loop
2	Elution	1 column volume (24 mL)
3	Wash	1 column volume (24 mL)
4	Equilibration	2 column volumes (48 mL)

26 Collect 100 µL of peak-containing fractions for SDS-PAGE analysis. An example FPLC chromatogram of the size-exclusion chromatography result is shown in Fig. 4b. Protein gel results for samples from the steps leading up to the size-exclusion chromatography are shown in Fig. 4c.
? TROUBLESHOOTING

27 Pool and concentrate the fractions containing LwaCas13a by centrifugation at 4,000*g* for 15 min at 4 °C in a 15-mL (50 MWCO) centrifugal spin filter. Then add protein storage buffer (SB) to the same centrifugal filter to bring the volume to 15 mL and repeat the centrifugation step.
▲ CRITICAL STEP Buffer exchange into protein SB is very important for storage and downstream SHERLOCK. Therefore, for optimal results, do not change the formulation of the protein SB.
? TROUBLESHOOTING

28 Determine the final protein concentration, dilute to 2 mg/mL in protein SB, and store as 5-µL aliquots in PCR strip tubes at −80 °C until use. Aliquots are stable for at least 6 months at −80 °C.
■ PAUSE POINT The purified protein should be of high quality and ready for Cas13-based nucleic acid detection. A 5-µL aliquot is sufficient for ~50 individual SHERLOCK reactions or 12.5 individual SHERLOCK conditions with four technical replicates for each condition.

IVT and purification of Cas13 crRNAs ● Timing 5 h or overnight

▲ CRITICAL crRNA quality is instrumental to the performance of CRISPR diagnostics. An RNase-free bench and nuclease-free reagents and consumables are therefore strongly recommended for IVT of crRNAs.

29 Prepare the following annealing reaction for each sample in a PCR tube:

Component	Volume to add (µL)
crRNA template, 100 µM (use sequences shown in Table 2, or user must custom design for their application)	1
T7-3G oligonucleotide, 100 µM	1
Standard *Taq* buffer, 10×	1
UltraPure water	7

30 Anneal the crRNA template and T7-3G oligonucleotide by performing a 5-min denaturation, followed by slowly cooling the reaction to 4 °C in a PCR thermocycler. For slow cooling, adjust the ramp rate to 0.08 °C/s (or use a 2.5% ramp rate on most thermocyclers with a 3.2 °C/s maximum ramp rate).

31 Add the following IVT mix to the annealing reactions:

Component	Volume to add (µL)
Annealing reaction	10
NTP buffer mix	10
T7 RNA polymerase mix	2
UltraPure water	17

32 Incubate the reaction for 4 h at 37 °C, and then cool to 4 °C in a PCR thermocycler until purification.

■ **PAUSE POINT** IVT reactions can either be stored at −20 °C (for up to 2 weeks) or directly purified.

33 Move Agencourt RNAClean XP beads to room temperature.

▲ **CRITICAL STEP** Agencourt RNAClean XP beads must be brought to room temperature for optimal use. To do this, let the entire bottle or an aliquot rest on the bench for 30 min.

34 Per 30 µL of sample, add 100 µL of Agencourt RNAClean XP magnetic beads and 90 µL of isopropanol.

35 Mix by pipetting up and down five times, and then incubate the mixture for 5 min at room temperature.

36 Place the reaction on a magnetic stand for 5 min. The beads will accumulate near the magnetic surface.

37 While keeping the reaction on the magnetic stand, pipette off the clear supernatant and discard. Then add 200 µL of 85% EtOH to the beads, wait for 30 s, and then discard the ethanol solution. Repeat the washing step and discard the ethanol solution.

38 Remove the reaction from the magnetic stand and allow it to air-dry for 5 min at room temperature.

39 Add 50 µL of UltraPure water to the dry beads and incubate for 5 min at room temperature.

40 Place the reaction on a magnetic stand and wait 5 min to allow for separation of the beads from eluted crRNA sample.

41 Transfer the clear supernatant to a new nuclease-free tube and determine the concentration with a spectrophotometer. To ensure the correct size, run an aliquot on a 10% TBE-urea gel and stain with SYBR Gold to visualize nucleic acid species.

▲ **CRITICAL STEP** Try not to transfer any magnetic beads to the final eluant, because this can inhibit downstream reactions. Using this protocol, ~50–100 µg of RNA is made. See the Troubleshooting section if the result substantially deviates from this.

? TROUBLESHOOTING

Sample extraction ● Timing 5 min to 1 h

▲ **CRITICAL** SHERLOCK detection is compatible with both pure and crude sample extractions, as long as contaminating nucleases are inactivated. For pure extraction of genomic DNA or RNA, we recommend using the QIAamp DNA Blood Mini Kit (DNA) for isolating genomic DNA from human serum, saliva, or blood, as well as genomic DNA from bacterial cultures. For viral RNA detection from

urine and tissue culture, we recommend using the QIAamp Viral RNA Mini Kit or RNeasy Plus Mini Kit. The manufacturer's instructions for the above-mentioned extractions are sufficient.

42 Follow the procedures for crude DNA extraction from human saliva (option A) or plant material (option B).

(A) **Crude DNA extraction from human saliva** ● **Timing 15 min**

(i) Collect 2 mL of human saliva from patients or volunteers after appropriate institutional review board (IRB) approval is in place.
! CAUTION IRB approval and written informed consent must be obtained for all experiments involving materials from human subjects.

(ii) Add 400 µL of PBS to 100 µL of saliva, and centrifuge for 5 min at 1,800*g* at 22 °C (or room temperature).

(iii) Carefully remove the supernatant and resuspend the pellet in 30 µL of PBS supplemented with 0.2% Triton X-100. Denature the sample for 5 min at 95 °C.

(iv) Centrifuge for 5 min at 1,800g at 22 °C and use 1 µL of the supernatant as sample input for RPA.

(B) **Crude extraction from plant material** ● **Timing 5 min**

(i) To 20 mg of crushed seeds, leaf, or root tissue, add 200 µL of plant extraction buffer (500 mM NaOH, 10 mM EDTA). Vortex the sample for 30 s and incubate at room temperature for 1 min. The raw alkaline extraction can be stored at −20 °C for up to 2 weeks.

(ii) Transfer a 5-µL aliquot to a new tube and dilute 1:20 with 95 µL of UltraPure water. Only 1 µL of sample is needed for RPA. The remaining dilution can be stored at −20 °C for up to 2 weeks.
▲ CRITICAL STEP Reaction inhibitors might still be present after dilution, and we recommend that the sample volume in the reaction does not exceed 10% of the final volume.

RPA of nucleic acids ● Timing 30 min to 1 h

▲ CRITICAL The recommended procedures for RNA and DNA pre-amplification are nearly identical. However, important distinctions are summarized in the 'Experimental design' section. Here, we provide a protocol for single target identification using two-step SHERLOCK. For one-pot SHERLOCK detection, follow the instructions provided in Box 2. If multiplexed nucleic acid detection or quantification are desired, the reader should follow the guidance provided in the 'Experimental design' section. We also recommend performing positive and negative controls alongside tested samples. A negative control could include a water-only input sample for RPA or a sample in which the target molecule is known to be absent. Positive controls include synthetic RNA or DNA targets or purified DNA/RNA in which the target is known to be present.

43 Prepare the pre-amplification area by wiping down the work surface and pipettors with RNase Away.
▲ CRITICAL STEP RPA is an isothermal amplification technology that produces large quantities of amplified product within minutes. This can lead to sample contamination of reagents and the work area, generating a false-positive signal. It is therefore highly recommended to set up separate pre- and post-amplification work areas. Laminar flow hoods used for standard tissue culture provide an optimal pre-amplification work environment, and primer aliquots, rather than stocks, are recommended as another measure to prevent contamination spread.

44 On ice, prepare the following master mix (sufficient for four replicates) in a 1.5-mL Eppendorf or PCR tube:

Component	Volume (µL)
Forward primer 10 µM	2.4
Reverse primer 10 µM	2.4
TwistAmp Rehydration Buffer (from the TwistAmp Basic kit)	29.5
UltraPure water	8.65

45 Add 40 µL of master mix to a single pellet aliquot and carefully resuspend the mixture on ice. Then transfer the entire reconstituted reaction back to the initial Eppendorf or PCR master mix tube.
▲ CRITICAL STEP One pellet yields approximately four individual RPA reactions. If a larger number of reactions are needed, scale up the master mix volume and pellets accordingly. When doing so, it is critical not to change the pipette between transfers of resuspended pellets, as the

stickiness of the RPA reaction components could result in a substantial loss of reaction enzymes when using multiple pipette tips.

46 Add 2.5 µL of 280 mM TwistAmp magnesium acetate (from the TwistAmp Basic kit) to the reaction, and then briefly vortex and quickly spin down (using a minifuge for 2–3 s at room temperature).

▲ **CRITICAL STEP** Addition of magnesium acetate to the reconstituted RPA pellet activates the enzymes and may lead to strong primer–dimer formation. It is therefore important to quickly proceed to sample addition.

47 Prepare aliquots of 10 µL of the reconstituted RPA reaction in PCR strip tubes or PCR plates, and then add 1 µL of extracted sample to each reaction. Mix by carefully pipetting up and down. Include an UltraPure water–only negative control.

48 Run the reaction at 37° in a pre-heated PCR thermocycler or incubator for 10–30 min. If performing RT-RPA, run the reaction at 42 °C. The reaction time can be extended to 1 h for highly sensitive reactions.

■ **PAUSE POINT** After the run is completed, you can either immediately proceed to Cas13 SHERLOCK detection or store the reaction at 4 °C for 2–3 d. Alternatively, reactions can be stored at −20 °C for up to several weeks.

Cas13-SHERLOCK nucleic acid detection ● Timing 1–4 h

▲ **CRITICAL** Here, we provide details for two-step detection. For details of one-pot detection, follow the instructions in Box 2.

▲ **CRITICAL** SHERLOCK is a highly sensitive RNA-based detection assay and requires RNase-free post-amplification. As with RPA, we recommend that you wipe down the surface and pipettors with RNase Away. We also recommend performing positive controls and negative controls alongside the experiment. Suitable negative controls are water-only RPA reactions or RPA reactions in which RPA is absent. Suitable positive controls include synthetic RNA or DNA targets or purified DNA/RNA in which the target is known to be present.

49 Thaw sufficient amounts of normalized crRNA (300 ng/µL), RNaseAlert Reporter (2 µM, from RNaseAlert Lab Test Kit v2), and a Cas13 protein aliquot (2 mg/mL, 14.44 µM) on ice, covered with aluminum foil to protect from light exposure. Sufficient amounts are calculated on the basis of the number of desired reactions, with a minimum 25% excess.

50 Dilute LwaCas13a to a 10× concentration (63.3 µg/mL) by adding 153 µL of protein SB to 5 µL of LwaCas13a at 2 mg/mL. Reactions are performed in 60 mM NaCl by adding 2 µL of diluted protein per 20 µL of SHERLOCK reaction mixture. If multiplexed SHERLOCK is performed, dilute the proteins in SB accordingly. For example, for dual-target multiplexing, dilute each CRISPR enzyme to 20× and add 1 µL of each per 20 µL of SHERLOCK reaction. For triplex detection, dilute to 30× and add 0.67 µL of each enzyme. For quadruplex detection, dilute to 40× and add 0.5 µL of each enzyme to the reaction.

▲ **CRITICAL STEP** It is critical that the volume of Cas13 enzyme used per reaction is the same for single-plex and multiplex experiments. When doing multiplex detections, the sum of the individual Cas13 enzyme volumes must equal the volume used for a single Cas13 enzyme in a single-plex reaction.

51 Dilute the crRNA to 10 ng/µL by adding 145 µL of UltraPure water to 5 µL of crRNA (300 ng/µL). Add 1 µL of crRNA for each Cas12/Cas13 ortholog to the reaction at 10 ng/µL. Then add 1.25 µL of each ortholog's sequence-specific reporter to the reaction at a 2 µM concentration. See below for information regarding which reporters to use for single-plexing and multiplexing applications.

▲ **CRITICAL STEP** For multiplexing applications, the reporters are specific to each ortholog and are as follows: LwaCas13a (/5TEX615/T*A*rArUG*C*/3IAbRQSp/), CcaCas13b (/5Cy5/T*A*rUrAG*C*/3IAbRQSp/), PsmCas13b (/56-FAM/rArArArArA/3IABkFQ/), and AsCas12a (DNaseAlert Kit). In cases of single-plexing, RNase Alert can be used for LwaCas13a, CcaCas13b, and PsmCas13b. For single-plexing with AsCas12a, DNase alert can be used.

52 Perform a fluorescence-based detection assay using a fluorescence plate reader (option A) or a colorimetric lateral flow detection assay (option B).

(A) **Fluorescence-based detection assay**

(i) Pre-heat the fluorescence plate reader to 37 °C.

(ii) Prepare the Cas13-SHERLOCK master mix by adding the following components to an Eppendorf tube in the order they are listed, starting with water. This master mix is for a single-plex reaction. Modifications for multiplexing are described above.

Component	Volume per reaction (μL)	Volume for four replicates (μL)
UltraPure water	11.27	56.35
HEPES, pH 6.8, 1 M	0.4	2
$MgCl_2$, 1 M	0.18	0.9
rNTP solution mix, 25 mM each	0.8	4
LwaCas13a in SB, 63.3 μg/mL	2	10
Murine RNase inhibitor, 40 U/μL	1	5
T7 RNA polymerase, 5 U/μL	0.5	2.5
crRNA (10 ng/μL)	1	5
RNaseAlert v2 (2 μM)	1.25	6.25
Total	19	95

▲ **CRITICAL STEP** We recommend routinely performing four technical replicates per SHERLOCK condition. When working with multiple conditions (e.g., if adding positive and negative controls or additional samples), scale up the reaction accordingly and adjust for pipetting errors by adding a 15% excess volume. For example, the following calculation would be required for an experiment with one sample, a positive control, and a negative control: amount per four technical replicates × 3 × 1.15.

(iii) For one condition and four technical replicates, transfer 87.4 μL of master mix to a PCR-strip tube or 96-well PCR plate. Scale up according to total number of conditions and replicates.

(iv) Spin down the RPA pre-amplification reaction sample (using a minifuge or plate spinner for 5 s at room temperature), carefully open the Eppendorf tube, and transfer 4.6 μL of RPA reaction mix to 87.4 μL of aliquoted master mix on ice. Briefly vortex and spin down (using a minifuge or plate spinner for 5 s at room temperature) in a 96-well plate to collect the entire reaction mix in the well.

(v) Carefully open each Eppendorf tube and transfer 20 μL per technical replicate and condition to a 384-well, round black-well, clear-bottom plate on ice. To group technical replicates, we recommend to routinely pipette the remaining technical replicates beside and below, to form a square of 2 × 2 grouped reactions. When transferring the reaction from a 96-well plate to a 384-well plate, we recommend using an 8-well or 12-well multichannel pipette for quicker setup, which will also load every second well of a 384-well plate. We therefore recommend a 2 × 2 setup to avoid pipetting mistakes and provide better grouping of replicates.

(vi) Briefly spin down the plate (500*g*, 22 °C, 15 s) to remove potential bubbles and place it into a pre-heated BioTek plate reader, or equivalent.
▲ **CRITICAL STEP** After plate preparation, the samples should be placed into the fluorescence plate reader quickly because SHERLOCK Cas13 reactions may begin as soon as they are removed from ice.

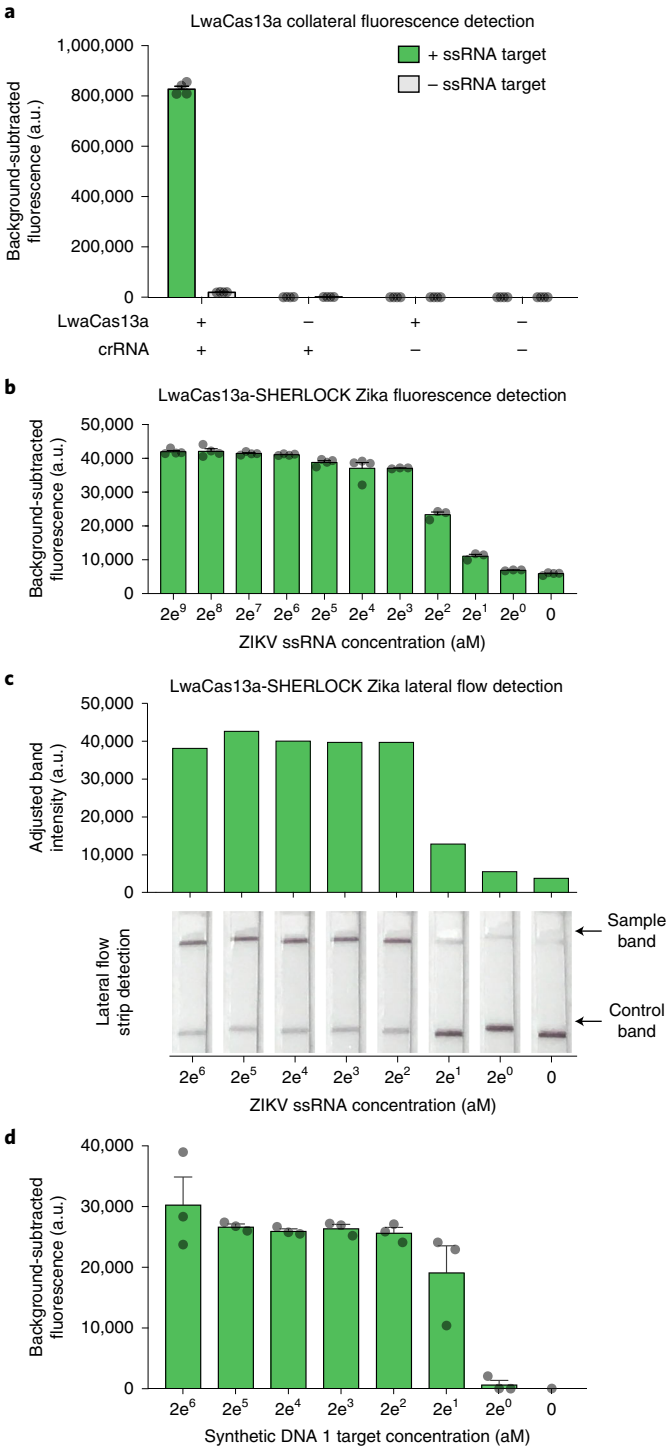
(vii) Start data acquisition by monitoring fluorescence over 3 h at 37 °C with a 5-min interval between well-data acquisitions. See Anticipated results and Fig. 5a,b for representative results.
? TROUBLESHOOTING

(B) **Colorimetric-based lateral-flow detection assay**

(i) Prepare the Cas13-SHERLOCK master mix by adding the following components to an Eppendorf tube in the order they are listed, starting with water.

Component	Volume per reaction (μL)	Volume for four replicates (μL)
UltraPure water	12.32	61.6
HEPES, pH 6.8, 1 M	0.4	2
$MgCl_2$, 1 M	0.18	0.9
rNTP solution mix, 25 mM each	0.8	4
LwaCas13a in SB (63.3 μg/mL)	2	10
Murine RNase inhibitor, 40 U/μL	1	5

Table continued



(continued)

Component	Volume per reaction (μL)	Volume for four replicates (μL)
T7 RNA polymerase, 5 U/μL	0.5	2.5
crRNA (10 ng/μL)	1	5
LF-RNA reporter 1 (100 μM)	0.2	1
Total	19	95

◀ **Fig. 5 | Anticipated fluorescence and lateral flow results. a**, LwaCas13a detection of ssRNA 1 is dependent on the formation of a protein–crRNA–target complex. Reactions contain LwaCas13a and crRNA, LwaCas13a or crRNA alone, or neither protein nor crRNA, in the presence or absence of a single ssRNA 1 target. Bar indicate mean ± s.e.m. of background-subtracted fluorescence measured from four technical replicates; each individual replicate is represented as a dot. **b**, Fluorescence detection for a synthetic RNA version of Zika virus with decreasing input concentrations with a two-step SHERLOCK reaction. Each bar represents the detected collateral cleavage activity for a given input concentration. Bars indicate mean ± s.e.m. of background-subtracted fluorescence measured from either three or four technical replicates; each individual replicate is represented as a dot. **c**, Lateral flow detection for a synthetic RNA version of Zika virus with decreasing input concentrations with a two-step SHERLOCK reaction. Samples were detected with a 30-min RT-RPA incubation followed by a 1-h LwaCas13a reaction before lateral flow strip detection. Adjusted band intensities (determined from lateral flow strips) are shown, as well as individual lateral flow strips, with positive and control sample lines indicated with arrows[16]. **d**, Fluorescence detection of synthetic DNA 1 target with decreasing input concentrations using a one-pot SHERLOCK reaction combining the RPA, T7, and LwaCas13a detection steps. Bars indicate mean ± s.e.m. of background-subtracted fluorescence measured from either three technical replicates; each individual replicate is represented as a dot[14]. **c**,**d**, adapted with permission from refs. [16] and [14], respectively, American Association for the Advancement of Science. aM, attomolar; a.u., arbitrary units.

(ii) For one condition and four technical replicates, transfer 87.4 µL of master mix to a PCR-strip tube or 96-well PCR plate. Scale up according to total number of conditions and replicates.

(iii) Spin down (in a minifuge or plate spinner for 5 s at room temperature) the RPA pre-amplification reaction mix, carefully open the Eppendorf tube, and transfer 4.6 µL of RPA reaction mix to 87.4 µL of master mix on ice. Briefly vortex and spin down for 15 s (in a minifuge or plate spinner for 5 s at room temperature) in a 96-well plate to collect the entire reaction mix in the well.

(iv) Carefully open the Eppendorf tube for each reaction mix and transfer 20 µL per technical replicate and condition to a 96-well plate. Incubate the plate for 1 h at 37 °C in a PCR thermocycler or incubator.

(v) Transfer the 20 µL of reaction mix to a 2-mL Eppendorf tube and add 100 µL of HybriDetect 1 assay buffer (from the Milenia HybriDetect 1 kit). A nuclease-free 2-mL 96-well block can be used instead of Eppendorf tubes.

(vi) Perform lateral flow detection by placing a HybriDetect 1 lateral flow strip (from the Milenia HybriDetect 1 kit) into diluted reactions and waiting for 1–2 min for the development of the colored readout.

(vii) After completion, remove the lateral flow strip and place it on a white background for visual inspection. See Anticipated results and Fig. 5c for positive and negative reactions. Alternatively, for quantitative assessment, remove the lateral flow strip and place it on a white background for image acquisition with conventional smart-phone cameras or a gel imaging system. Use a gel image or image-processing software to measure band intensity and determine positive detection against a positive-control dilution series.

▲ **CRITICAL STEP** It is important to perform positive and negative controls alongside tested samples to verify a functional reaction and rule out false-positive and false-negative results.

? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 3.

Table 3 | Troubleshooting table

Step	Problem	Possible reason	Solution
3	No colonies	Wrong antibiotic	Check if the correct antibiotic has been used (ampicillin for LwaCas13a plasmid)
		Inefficient heat shock	Repeat transformation procedure
8	No visible difference between induced and uninduced cells	Poor expression	Optimize temperature and IPTG induction conditions

Table continued

Table 3 (continued)

Step	Problem	Possible reason	Solution
18	No specific protein band in final eluate	Protein did not bind to beads or it precipitated	Check flow-through for recombinant protein, try re-batch binding of flow-through with more resin
	Two protein bands or wrong-size band	Insufficient SUMO protease cleavage	Add more SUMO protease, incubate for 4–12 h, and monitor cleavage by SDS-PAGE
22	No protein peak	Sample did not bind to the column	Salt too high; check FPLC waste for protein and change the initial buffer A/B mix ratio
23, 27	Slow spin-filter sample concentration	Glycerol sedimentation or protein precipitation	Carefully pipette up and down and centrifuge again
26	No protein peak	Sample lost during application	Check FPLC waste or spin-filter flow-through
41	Low crRNA concentration	Incorrect crRNA design	Check crRNA design for T7 promoter sequence
		Ethanol concentration in washing solution was too low	Prepare fresh 85% EtOH solution
52A(vii), 52B(vii), Box 2,step 12	No signal	No amplification during RPA because of incorrect primer design	Redesign primers
		No amplification during RPA because of contaminants in sample	Re-purify or check for presence of nucleic acid by other technologies, such as PCR
	Signal in all samples, including negative control	RNase contamination	Incubate the reaction without the sample and remove suspected components to determine whether the signal is reduced
		DNA template contamination in the reagent	Use new reagents and primer aliquots

Timing

Steps 1–28, recombinant expression and purification of LwaCas13a: 5 d
Steps 29–41, IVT and purification of crRNA: 5 h or overnight
Step 42A, sample extraction from human saliva: 15 min
Step 42B, crude extraction from plant material: 5 min
Steps 43–48, RPA pre-amplification: 30 min to 1 h
Steps 49–52, Cas13-SHERLOCK detection: 1–4 h
Box 2, one-pot Cas13-SHERLOCK nucleic acid detection: 1–4 h

Anticipated results

The presented protocol for expression and purification of LwaCas13a yields ~0.5–1 mg of pure protein. A recommended culture volume of 4 L therefore yields enough protein to perform thousands of individual Cas13-SHERLOCK reactions from the same batch. Moreover, the enzyme is free of contaminating nucleases because water input SHERLOCK controls do not result in background fluorescence (Fig. 5a,b).

Nucleic acid detection as described above can detect DNA and RNA with attomolar sensitivity. Figure 5b shows the result from a Cas13-SHERLOCK fluorescence detection of a synthetic RNA dilution series down to 2 aM. The same dilution series was used to detect RNA with a Cas13-SHERLOCK lateral flow colorimetric readout with similar sensitivity (Fig. 5c). Combining the RPA, T7, and Cas13 steps in a single one-pot reaction can detect synthetic DNA 1 with a sensitivity of 20 aM (Fig. 5d).

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data and materials availability

Reagents from the Zhang lab are widely available to the academic community through Addgene, and additional information and resources can be found on the Zhang lab website (https://zlab.bio/) and GitHub (github.com/fengzhanglab).

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Author contributions

M.J.K., J.G.K., J.S.G., O.O.A., and F.Z. designed and conducted all experiments and wrote the manuscript.

Competing interests

M.J.K., J.S.G., O.O.A., and F.Z. are co-inventors on patent applications filed by the Broad Institute relating to work in this article. J.S.G., O.O.A., and F.Z. are co-founders of Sherlock Biosciences. F.Z. is a co-founder and advisor of Beam Therapeutics, Editas Medicine, Pairwise Plants, and Arbor Biotechnologies. O.O.A. and J.S.G. are advisers for Beam Therapeutics. J.S.G. is a campus advisor for Benchling, Inc.

Additional information

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