



Combining CRISPR–Cas12a with terminal deoxynucleotidyl transferase dependent reporter elongation for pathogen detection using lateral flow test strips

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Abstract

CRISPR–Cas (CC)-based detection technologies have some exceptional features, which hold the promise of developing into the next-generation diagnostic platforms. One of these features is the ability to trigger non-specific single-stranded DNA/RNA cleavage activity after specific target recognition and Cas enzyme activation. This cleavage activity can be visualized either by single-stranded DNA/RNA fluorescence resonance energy transfer quenching reporters or via lateral flow strips, which separate and detect the cleaved reporters. In a previous study, we reported coupling CC-cleavage activity with the enzyme terminal deoxynucleotidyl transferase (TdT) that elongates cleaved ssDNA reporter fragments with dTTP nucleotides. These elongated poly(thymine) tails then act as scaffolds for the formation of copper nanoparticles which generate a bright fluorescent signal upon UV excitation. In the current study, we visualize the poly(thymine) tails on lateral flow strips, using different combinations of biotinylated or fluorescein-labeled nucleotides, various reporters, and capture oligos. One particular approach, using a fluorescein reporter, reached a target sensitivity of <1 pM and was named Cas activity assay on a strip and was tested using *Bacillus anthracis* genomic DNA.

Keywords: CRISPR–CAS; DNA detection; lateral flow assay; point of care; pathogen; FRET

Introduction

DNA/RNA-polymerase enzymes are commonly used in pathogen identification approaches. These enzymes specifically amplify the target DNA/RNA in repetitive (thermocycling) steps, which can then be detected with fluorescent dyes [1] or probes [2]. With the advent of programmable CRISPR–Cas (CC) enzymes, which recognize specific DNA/RNA sequences and subsequently activate inbuilt specific and non-specific DNA/RNA cleavage, another strategy for pathogen identification is evolving [3–6]. The non-specific DNA/RNA cleavage activity (also called trans-cleavage) of CC enzymes can be utilized to generate a measurable and visible signal. A commonly applied read-out of these CC-tests can be achieved using Fluorescence Resonance Energy Transfer (FRET) technique as a reporter method [7, 8]. Here, DNA oligonucleotides are labeled at one end with a fluorophore and a quencher at the other end. After CC activation and cleavage, the oligonucleotide reporter starts to fluoresce, which can be measured in spectrofluorometers [9].

A drawback is that the CC-tests must still be used in combination with DNA/RNA-polymerase target pre-amplification in order to have clinically relevant detection limits. Current limits of

pathogen detection using this pre-amplified CC-test combination are reported to be in the femto- to even attomolar range [10, 11]. Depending on the DNA/RNA pre-amplification technique and CC enzymes used, the three main techniques are named as SHERLOCK [3], DETECTR [5], and HOLMES [12]. Such pre-amplification steps, however, reduce the simplicity and increase the duration of analysis of Cas diagnostic tests. Without pre-amplification CC-FRET-based detection methods show detection limits in nano- or even picomolar range [5, 13, 14].

Next to FRET reporters, lateral flow assays (LFAs) provide an easy-to-use read-out of CC detection assays. LFA is a paper-based technology offering a quick, easy, and inexpensive detection test that requires no instruments or electricity. Here, the DNA-reporter oligonucleotides are labeled with a biotin group on one end and a fluorophore on the other end. On the paper strip, CC-cleaved oligonucleotides can be captured with immobilized streptavidin and visualized using conjugated antibodies that recognize the fluorophore [15]. These CC-LFA combinations might become powerful tools in point-of-care diagnostics [16].

Instead of using pre-amplification of the DNA/RNA target template to increase the substrate activation of CC enzymes, several techniques amplify the signal during or after template recognition

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by CC [17, 18]. We previously reported a detection assay using a combination of CRISPR-Cas12a with terminal deoxynucleotidyl transferase (TdT). TdT is a specialized DNA polymerase that adds nucleotides to the 3'-end of a ssDNA strand without requiring a template. When specific Cas12a activation and subsequent non-specific trans-cleavage of a ssDNA reporter has taken place, the newly formed 3'-end can then be extended with deoxythymidine triphosphate (dTTP) nucleotides and the poly-T tails thus formed visualized using Copper nanoparticles [19].

TdT in conjunction with Cas12a can also be used to detect activity of DNA modifying enzymes such as polynucleotide kinase, DNA glycosylase, or DNA methylase [20–22]. These DNA modifying enzymes trigger TdT elongation by generating a free 3'-hydroxyl (3'-OH) end from specifically designed DNA sequences that are used as indicators of the enzymatic activity.

Here, we report the use of lateral flow test strips to detect poly-T tails formed by TdT after pathogen recognition by CRISPR-Cas12a. In total, we examined six variations recognizing the poly-T signal for LFA read-out.

Materials and methods

Chemicals/reagents

Engen LbCas12a (#M0653T) and TdT (#M0315L) were obtained from New England Biolabs (Ipswich, MA, USA) as were the matching CoCl₂ (2.5 mM stock) plus TdT buffer (final concentration: 50 mM potassium acetate, 20 mM Tris-acetate, and 10 mM magnesium acetate; pH 7.9). dTTP (ThermoFisher #R0171, Bleiswijk, The Netherlands). Fluorescein-12-dUTP (Jena Bioscience, Germany) (#NU-803-FAMX-L). Biotin-16-dUTP (Jena Bioscience, Germany) (#NU-803-BIO16-S). HybriDetect, Universal LFA Kit (Milenia Biotec, Gießen Germany). G-25 Sephadex Columns (Roche, #11273949001). Dideoxycytidine (GE Healthcare, USA) (#27-2061-01).

Apparatus

SimpliAmp Thermal Cycler (Applied Biosystems). Thermo Mixer C, ThermoTop, Smartblock PCR 96 (Eppendorf).

Plasmids

The plasmid encoding the partial gene of anthrax lethal factor [19].

Oligonucleotides

Reporter ssDNA oligonucleotides were ordered from Isogen Life Sciences (Utrecht, The Netherlands) or from IDT (Leuven, Belgium).

Name	Type	Sequence
10C blocked reporter	Reporter	5'-C = C = CCCCCCCC-PO ₃ -3'
Biotin 10C blocked reporter	Reporter	5'-Biotin-C = C = CCCCCCCC-PO ₃ -3'
F6C	Reporter	5'-Fluorescein-C = C = CCCC-PO ₃ -3'
F8C	Reporter	5'-Fluorescein-C = C = CCCCC-PO ₃ -3'
F10C	Reporter	5'-Fluorescein-C = C = CCCCCCCC-PO ₃ -3'
F12C	Reporter	5'-Fluorescein-C = C = CCCCCCCCCC-PO ₃ -3'

(continued)

Name	Type	Sequence
F20C	Reporter	5'-Fluorescein-C = C = CCCCCCCCCCCCCCCC-CC-PO ₃ -3'
Biotin-Poly-A	Capture	5'-Biotin-CCC CCA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA A-3'
FAM-Poly-A crRNA 2	Visualization crRNA	5'-AAAAAAAAA-3' fluorescein 5'-UAA UUU CUA CUA AGU GUA GAU UGC ACC GGA AGC UUU UAA UUA C-3' [19]

'=' indicates phosphorothioate backbone and PO₃ indicates a phosphate group.

Blocking of reporters

Blocking of the reporter was performed in a buffer containing 0.4 U/μl TdT, 4 mM ddCTP (GE healthcare), 1× TdT buffer (New England Biolabs), 0.25 mM CoCl₂ (New England Biolabs, provided with TdT), nuclease-free water (New England Biolabs), and 10 μM reporter. The total reaction volume used was 50 μl. The mixtures were subsequently incubated at 37°C for 3 h and heat inactivated at 70°C for 15 min. Afterward the reactions were filtered with G25 columns (Roche) according to the manufacturer's instructions. The total end concentration of the reporter was calculated via the dilution factor (end concentration = (original reaction volume (50 μl) × original concentration (20 μM))/end volume). Afterward the blocked reporters were checked for remaining 3'-OH ends by performing an elongation reaction with TdT and dTTP nucleotides. This reaction contained 1× TdT buffer (New England Biolabs), 0.25 mM CoCl₂ (New England Biolabs, provided with TdT), 0.4 U/μl TdT, 4 mM dTTP (ThermoFisher), and 300 nM reporter. The final end volume was 50 μl. The samples were subsequently incubated at 37°C for 1 h and heat inactivated 15 min at 70°C.

In vitro Cas12a activation and elongation of 3'-OH ends into poly-T tails by TdT

Cas12a activation and TdT elongation were performed in one step containing 50 nM LbCas12a (New England Biolabs), 62.5 nM crRNA2, 300 nM blocked reporter (as indicated in text, e.g. F20C), plasmid (concentration indicated in figures), 1× TdT buffer, 0.25 mM CoCl₂ (New England Biolabs), 0.4 U/μl TdT (New England Biolabs), 4 mM dTTP, and F-dUTP (1 μM) or B-dUTP (1 μM) (as indicated). The final volume was 100 μl. The samples were subsequently incubated at 25° or 37°C for 30 min and heat inactivated by incubation at 70°C for 15 min or via addition of 20 mM EDTA final concentration before adding to the lateral flow stick.

Lateral flow detection reactions

The biotin- and fluorescein-labeled reporter samples (10 μl) were applied to the sample pad of the HybriDetect lateral flow stick (Milenia Biotec). The HybriDetect lateral flow stick was inserted directly into the HybriDetect assay buffer (Milenia Biotec, 80 μl) in an upright position and incubated for 15 min at room temperature (RT) before inspection. The sticks were then taken out of the buffer and photographed.

To EDTA-inactivated samples, biotin-polyA-oligo (300 nM end concentration) was added simultaneously and samples were incubated for 5 min on the bench. The pad of the HybriDetect lateral flow stick (Milenia Biotec) was inserted directly into the tube

containing the complete reaction. The dipstick was incubated for 15 min at RT before inspection. The strips were then removed and photographed.

Bacillus anthracis bacterial cells and DNA isolation

Bacillus anthracis (str. Vollum) was cultured on blood agar (BA) plates and incubated for 2 days at 35°C and 5% CO₂. Ten colonies were suspended in 1 ml demi water and by plating 1/10th the number of colony forming units was determined. Bacteria were heat inactivated for 1 h at 95°C. DNA was isolated using the Qiaamp DNA Micro Kit (Qiagen, cat no: 56304). The inactivation of *B. anthracis* was checked and confirmed by plating 1/10th of the isolated DNA on BA plates and incubation for 14 days at 35°C and 5% CO₂. DNA quantification was performed by absorbance measurements at 260 nm using a NanoDrop instrument (Westburg). For mass-to-mole conversion, a genome length of 5.5 million basepairs was used (Genbank, NZ_CP007664.1, NZ_CP007665.1, and NZ_CP007666.1).

Results

Design and testing of lateral flow strips for poly-T capture

The principle underpinning the Cas Activity Assay on a STRIP (CANSTRIP) CC assay is that upon Cas activation (i.e. after target pathogen DNA recognition), CRISPR-Cas12a trans-cleavage of a blocked reporter-oligonucleotide creates free 3'-OH ends, which are subsequently elongated into poly-thymine tails by TdT using only dTTP nucleotides supplied (Fig. 1a and Supplementary Fig. S1). To detect and visualize these poly-T tails on lateral flow strips, we use the capillary flow of strips coated with a streptavidin biotin-ligand line and an antibody capture line. Therefore, the newly formed T-tails must be labeled, directly or indirectly, with fluorescein as well as biotin to capture and visualize the T-tails at the streptavidin test line (see example in Fig. 1c). The 3'-blocked reporter oligonucleotides can be labeled at the 5'-end with either a fluorescein or a biotin group (Fig. 1b). Adding the second label can be achieved by including labeled nucleotides containing fluorescein or biotin, such as fluorescein-12-dUTP or biotin-16-dUTP, which are additionally incorporated by TdT during poly-T tail synthesis (Fig. 1b). As an alternative to biotin coupled to the reporter or biotin addition via TdT-incorporated nucleotides, the poly-T tails can be captured via hybridization to a biotinylated adenine polymer (see Fig. 2, methods 5 and 6). Similarly, the fluorescein label can be added via hybridization using a fluorescein-labeled adenine decamer (see Fig. 2, methods 3 and 4). Overall, six different approaches, numbered 1–6, were designed (Fig. 2, top) and subsequently tested (see Fig. 2, bottom) to select the most promising option which was then chosen for more in-depth development. As target DNA, purified *Bacillus anthracis* plasmid DNA containing the *lef* gene (lethal factor) was used [19].

As a first approach, in addition to the dTTP nucleotide, a fluorescein-12 labeled deoxyuridine triphosphate analog (TF) is included as a substrate for TdT (method 1, Fig. 2). This labeled analog can be directly recognized by the anti-FITC antibody. Thus, the formed poly-T tails contain intermittent fluorescein groups, depending on the dTTP:F-dUTP molar ratio. Capture is done with a biotin coupled to the 5'-end of the reporter. Unexpectedly, when the poly(T/TF) reaction was run on an LFA strip, no signal was observed at the test-line (Supplementary Fig. S2). The amount of F-dUTP used in this 100 µl reaction is 0.1 nmol (ratio dTTP:F-dUTP = 4000:1), which is in excess compared with

the available anti-FITC antibody (<1 pmol binding). Free, non-incorporated F-dUTP will bind the anti-FITC antibody and compete for antibody binding with F-dUTP incorporated in the poly-T tails. We therefore applied a Sephadex G-25 spin-column designed to remove free nucleotides from DNA labeling reactions (see M&M). Removal of these free nucleotides indeed resulted in a clear signal at the test-line (Supplementary Fig. S2). Using this approach, titration of the target plasmid, containing the anthrax *lef* gene showed that the detection limit of this target template is close to 10 pM (method 1, Fig. 2).

In the second approach, when in addition to the dTTP nucleotide, biotinylated-dUTP (TB) nucleotides are used and the fluorescein label has to be coupled to the reporter (method 2, Fig. 2). As for the previous method, a Sephadex spin-column has to be applied to remove unincorporated nucleotides (data not shown). This approach with biotinylated dTTPs demonstrated low sensitivity of approximately 100 pM (method 2, Fig. 2).

Since the previous strategy showed that free unincorporated F-dUTP nucleotides interfere with the signal, we opted for a third approach using 5'-fluorescein labeled adenine decamer oligonucleotides which anneals directly to the biotinylated reporter poly-T tail (method 3, Fig. 2). Fluorescein adenine decamers can be used at the threshold of fluorescein antibodies, thereby obtaining a maximal signal. The results show that the detection limit for this approach is 10 pM (method 3, Fig. 2).

As the fourth approach, we used the 5'-fluorescein-labeled adenine decamer oligonucleotides to anneal to the reporter poly-T tails but by the use of biotinylated nucleotides plus spin-column, a low detection limit of 100 pM is observed (method 4, Fig. 2).

The fifth and sixth approaches use immobilization at the streptavidin-line by annealing the poly-T tail to a complementary 5'-biotinylated poly-adenine oligonucleotide. The annealed duplex strand can thus be captured at the test-line. Visualization requires addition of fluorescein nucleotides plus Sephadex spin column (method 5, Fig. 2) or use of fluoresceinated reporter (method 6, Fig. 2). Method 5 showed a threshold of detection of 10 pM but the combination of fluorescein-coupled reporter and biotin-poly-A capture (method 6, Fig. 2) achieved the highest sensitivity of 1 pM and was therefore further explored. This last method was termed CANSTRIP (Cas activity Assay on a STRIP). Of note, the total binding capacity of fluorescein antibody on a LFA strip, according to the manufacturer, is approximately 1 pmol and biotin binding capacity of immobilized streptavidin is 5 pmol. We use 3 pmol fluorescein reporter, biotinylated reporter, or fluorescein-adenine decamers which is at its capacity to occupy all fluorescein with a gold-labeled antibody and below biotin binding capacity to ensure complete capture.

Reporter composition and length

The reporter used in the CANSTRIP assay described above consists of 10 consecutive cytidine nucleotides with a 5'-fluorescein, a 3'-PO₃, and a phosphorothioate backbone at positions 2 and 3. Poly-C was selected as previously was shown that Cas12a cleaved a poly-C reporter with the highest efficiency [23]. The presence of phosphorothioate groups in oligodeoxynucleotides stabilizes such compounds against degradation by nucleases including Cas12a cleavage [24]. Cas12a cleavage between position C1–C2 or C2–C3 is unwanted because the minimum primer length for efficient initiation and elongation by TdT is a trioxynucleotide [25]. In addition, trans-cleavage by Cas12a typically leaves 2-nt from the 3'-end [6]. Therefore, the 10-nt cytidine reporter only contains 6 inter-nt linkages available for Cas12a cleavage and subsequent elongation. We therefore tested how, shorter (6- and

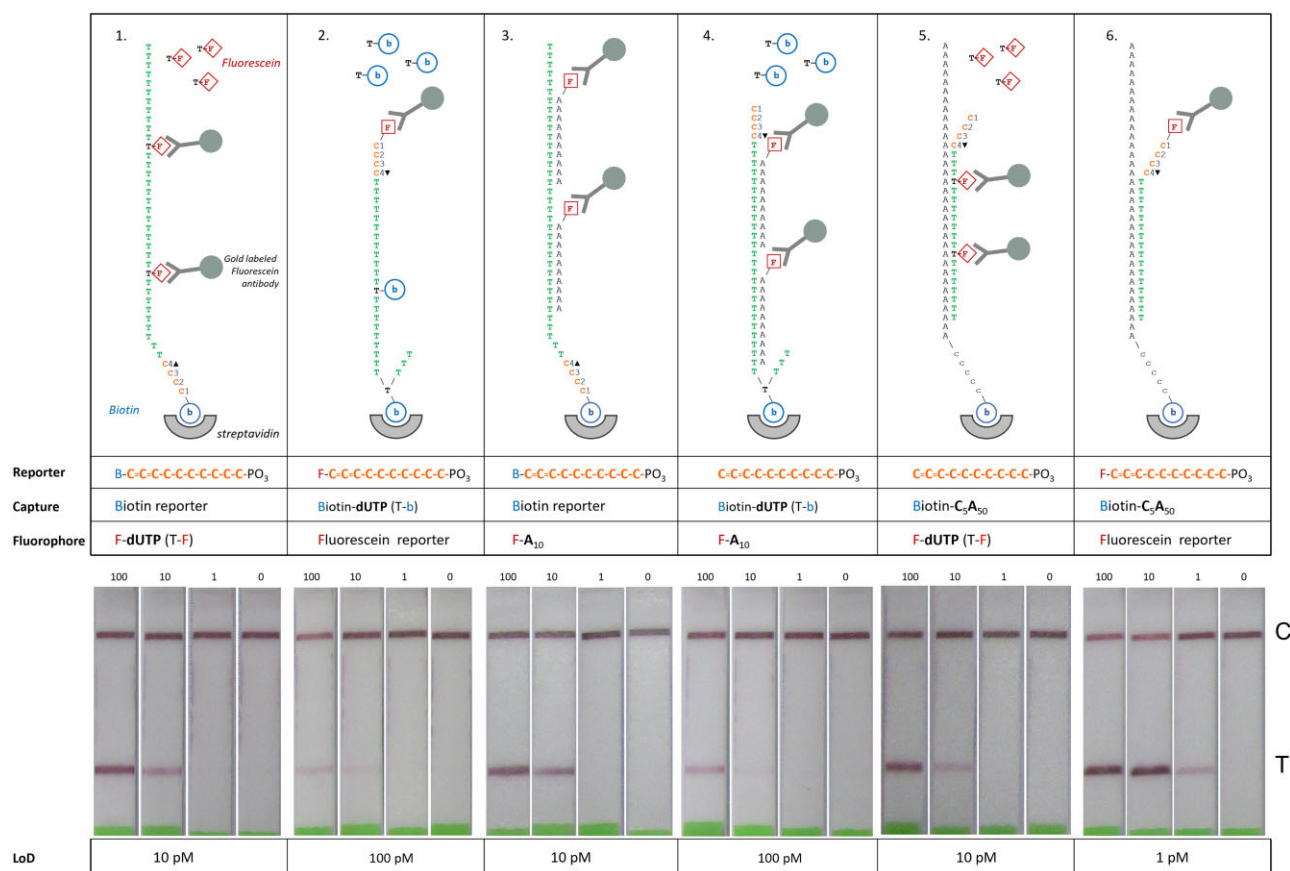


Figure 2: Six different LFA strategies tested to detect poly-T tails formed by activated Cas12a and TdT. (Top) Schematic of reporter-poly-T capture at streptavidin test-line. b, biotin (blue); -PO₃, blocked 3'-end; F, fluorescein (red); T-F, fluorescein-dUTP; T-b, biotin-dUTP; A, adenine; T, thymine; C, cytidine. Reporter oligo in orange. (Bottom) Results of poly-T capture lateral flow strip assay corresponding to above schematic. Indicated above each strip the amount in picomolar target ALF plasmid DNA. C, control line; T, test-line; LoD, limit of detection. (A color version of this figure appears in the online version of this article.)

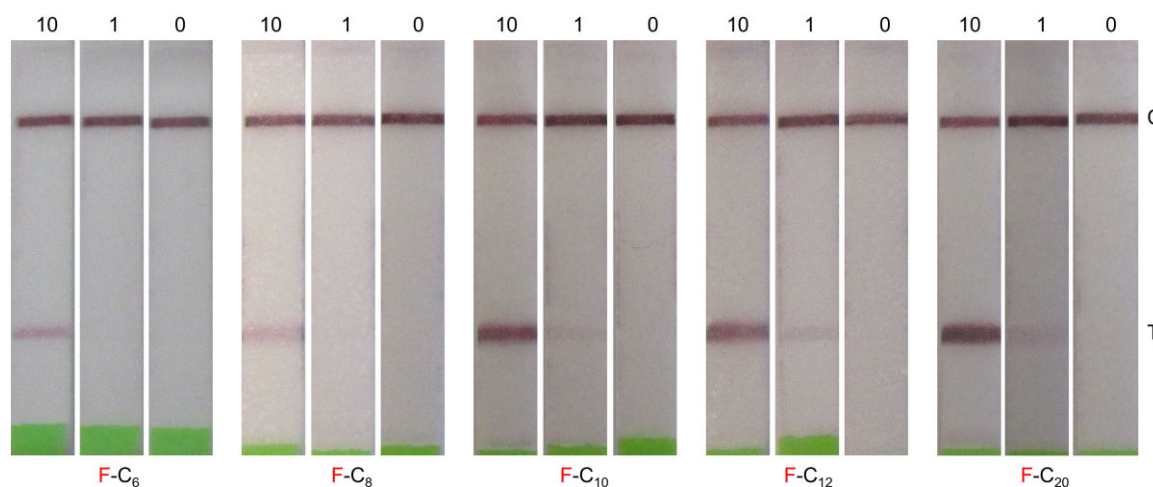


Figure 3: Effect of poly-cytidine reporter length on DNA target pathogen detection sensitivity measured on CANSTRIP LFA. Indicated above each strip is the amount in picomolar target ALF plasmid DNA. F, fluorescein. Indicated below strips is the number of consecutive cytidines. C, control line; T, test-line.

8-nt) and longer (12- and 20-nt) reporters would perform in the CANSTRIP assay (Fig. 3). This experiment shows that reporters smaller than 8 nt exhibit reduced sensitivity in the CANSTRIP assay. Increasing reporter length from 10- to 12-nt showed no increased sensitivity. However, the signal intensity of the 20-nt poly-C reporter is slightly higher and therefore used in subsequent experiments. This 20-nt reporter was further tested at

concentration below 1 pM, which showed an even lower detection limit of 0.5–0.25 pM (Supplementary Fig. S3).

Reaction time and temperature

In the CANSTRIP reaction with enzymes TdT and Cas12a, there is a continuous formation and cleavage of poly-T tails. The elongated reporter oligo carries a 5'-fluorescein enabling antibody

binding and visualization. Unfortunately, a second Cas12a cleavage of the generated poly-T tail will result in loss of this fluorescein group in the cleaved off product which will still bind to the capture poly-A50 anchor and compete for binding with the fluorescein containing reporter. We aimed to reduce cleavage of the poly-T by shortening the incubation time and reducing the

incubation temperature (Fig. 4, top). This experiment showed that incubation time can be reduced to 30 min at 37°C with a clear detectable signal at 1 pM (Fig. 4, middle). Incubation at RT did lead to some loss of signal intensity compared with 37°C resulting in somewhat lower sensitivity and a weak ambiguous signal at 1 pM (Fig. 4, bottom).

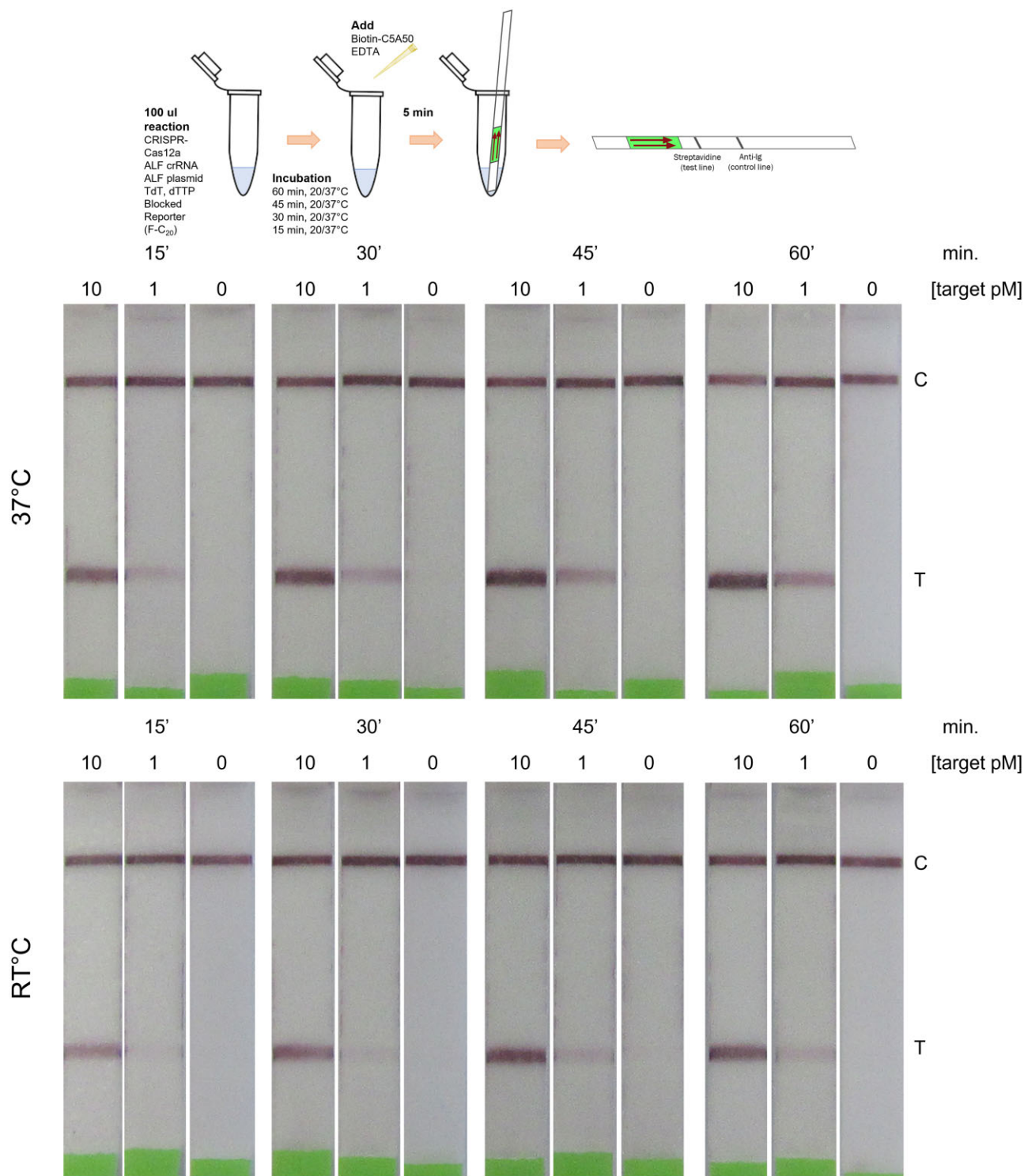


Figure 4: Effect of incubation time and temperature on DNA target detection sensitivity measured using CANSTRIP LFA. (Top) Schematic of experiment. (Bottom) Results of poly-T capture LFA. Indicated above each strip is the amount in picomolar target ALF plasmid DNA. The incubation time is indicated above each panel of three strips. C, control line; T, test-line.

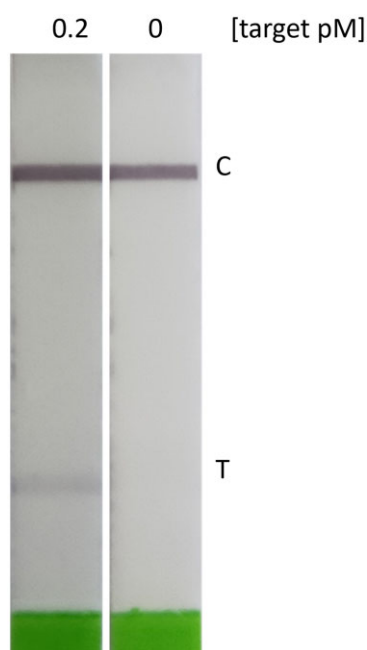


Figure 5: CANSTRIP LFA testing isolated genomic DNA from *Bacillus anthracis* cells. (Left) 0.2 pM genomic DNA corresponding to 10×10^6 genome copies. (Right) 0 is no cells control.

DNA detection of genomic DNA isolated from *B. anthracis* vegetative cells

The optimal CANSTRIP reaction (method 6) was tested on complete genomic DNA isolated from cultured *B. anthracis* strain Vollum cells. The concentration tested, 0.2 pM, corresponding to 10×10^6 cells, resulted in weak but clear signal (Fig. 5), showing that this quantity is suitable for detection using the CANSTRIP method.

Discussion

In this proof-of-principle study, we have demonstrated that sensitive pathogen DNA detection on a lateral-flow strip using CRISPR-Cas12a in combination with TdT-generated poly-T tails is feasible. Six different approaches have been designed and compared, which resulted in selection and optimization of the most sensitive method. This optimal procedure, named CANSTRIP, can be performed in 30 min reaction time and a 5-min lateral flow assay reaching a 0.5-pM sensitivity.

This CANSTRIP method (method 6), using biotin-poly-adenine capturing of the poly-T tails, outperformed the other tested approaches, and has several additional advantages. Methods 1, 2, 4 and 5 (Fig. 2) make use of labeled nucleotides which first need to be removed to avoid competition in binding to the fluorescein antibody or streptavidin test line. Having to remove nucleotides using centrifuge-, or even gravity-, spin columns is neither practical nor feasible for a point-of-care test. In a laboratory setting, the use of labeled nucleotides could potentially be useful, provided that much higher concentrations of fluoresceinated- or biotinylated nucleotides will boost the current signal intensity and sensitivity.

Reporters with a 5'-biotin or a 5'-fluorescein label (methods 1, 2, 3 and 6, Fig. 2) have the advantage that these are not hindered by a background signal. Background noise can be caused by endogenous free 3'-OH ends in the biological sample giving rise to unwanted poly-T tails as might occur with unlabeled reporter

capture approaches (methods 4 and 5, Fig. 2). In contrast, the 5'-labeled reporters are hampered by a second cut by Cas12a of the poly-T tail which will result in the loss of the fluorescent or biotin signal in the fragment cleaved off. The rate of TdT-oligo elongation is variable depending on initiator, nucleotides, concentrations, buffer conditions, and temperature but typically 2–3 nt are being incorporated per second [26]. Cas12a trans-cleavage kinetic parameters are calculated at 0.1–0.4 cleavages per second [23, 27], which suggests that poly-T tail synthesis rate is faster than cleavage. Moreover, the pool of reporter molecules of 300 nM used in this approach will exceed the number of T-tails at the start of the reaction suggesting that these will be initially cleaved more often than the poly-T tails formed later on in the reaction.

The six approaches tested in this study could be optimized even further by combining methods provided that these do not compete with each other, e.g. adding fluorescein-dUTP to fluorescein reporter (method 2) or adding biotin-dUTP to biotinylated reporter (method 1). Combined approaches will be subject of further studies.

Lateral flow immune assays using antibody-antigen recognition are a well-established platform for point-of-care testing due to its low cost and user friendliness [28]. The cost of the materials and reagents used in a CANSTRIP reaction, at one-time purchase, is around 7 euros, and probably much less if this is done on a large scale. A major advantage of CC-LFA-based tests is their short development time eliminating the need for producing immuno-LFA-specific monoclonal antibodies.

CC-dependent LFA tests could become a great addition to the assortment point-of-care testing, especially because target design using pathogen-specific crRNAs will only take days compared with months creating specific antibodies.

CC-LFA assays hold a promising prospect for quick production and execution as point-of-care tests. The CANSTRIP CC-LFA does not use pre-amplification of the target DNA and consequently does not match the extreme high sensitivity of approaches that do, but further improvements in sensitivity can make this a useful addition to the arsenal of CC tests.

Supplementary data

Supplementary data is available at *Biology Methods and Protocols* online.

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Author contributions

N.F.B. and H.C.v.L. conceived and designed the experiments. N.F.B. and R.M.-G. performed the experiments. H.C.v.L. wrote the article, with input from R.W.B. and A.P.

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Conflict of interest statement

H.C.v.L. reports a patent 'Nucleic acid fluorescence detection' (patent application number WO2021177833A1).

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