Bright fluorescent nucleic acid detection with CRISPR-Cas12a and poly(thymine) templated copper nanoparticles.

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ABSTRACT

Fluorescence-based diagnostic tools are attractive and versatile tests with multiple advantages: ease of use, sensitivity and rapid results. The advent of CRISPR-Cas technology has created new avenues for the development of diagnostic testing tools. In the current study, by effectively combining the specific functions of two enzymes, CRISPR-Cas12a and terminal-deoxynucleotidyl-transferase (TdT), we developed a DNA detection assay that generates copper nanoparticles that are easily visible to the naked eye under UV-light; we named this detection assay CANTRIP: Cas12a Activated Nuclease poly-T Reporter Illuminating Particles. Upon specific target DNA recognition by Cas12a, single-stranded DNA (ssDNA) reporter oligos with blocked 3'-ends are cut into smaller ssDNA fragments, thereby generating neo 3'-OH moieties. TdT subsequently elongates these newly formed ssDNA fragments, incorporating only dTTP nucleotides, and these poly(thymine)-tails subsequently function as scaffolds for the formation of copper nanoparticles (CuNPs). These CuNPs produce a bright fluorescent signal upon UV excitation, and thus, this bright orange signal indicates the presence of target DNA, which in this proof-of-concept study consisted of anthrax lethal factor plasmid DNA. CANTRIP, which combines two detection platforms consisting of CRISPR-Cas12a and fluorescent copper-nanoparticles into a single reaction, appears to be a robust, low-cost and simple diagnostic tool.

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INTRODUCTION

Early identification of pathogens is important for the timely administration of appropriate countermeasures. New innovative diagnostic tools have been developed to detect nucleic acids using the programmable properties of CRISPR-Cas endonucleases (1). These tools exploit a convenient key characteristic of specific Cas-enzymes, i.e., the triggering of non-specific DNase/RNase enzyme activity upon foreign pathogenic DNA recognition.

CRISPR-Cas enzymes are components of the bacterial adaptive immune system and provide a robust mechanism for host defence. Cas endonucleases can first detect invasive nucleic acids (e.g., bacteriophage infection) using specific target recognition RNAs (gRNA) complementary to the invading DNA. This recognition complex, comprised of a Cas endonuclease and foreign DNA, then triggers indiscriminate single-stranded DNA or RNA cleavage activity of the Cas endonuclease, resulting in cell death/suicide of the infected bacteria and protection of the uninfected bacterial population (2–5). This "collateral cleavage activity" is harnessed for use in diagnostic testing by releasing a fluorescent signal after cutting ssDNA or ssRNA reporters, which indicates that the target sequence of the pathogen is present in the tested sample. A myriad of viral or bacterial pathogens can be detected using this CRISPR-Cas detection technology. Cas13a targets RNA and has been adapted for the detection of RNA viruses such as ZIKA, Dengue, RSV and Ebola (3, 6, 7), whilst Cas12a targets DNA and has been applied for the detection of DNA viruses, like HPV and ASFV, and bacteria, such as Mycobacterium (4, 8–10).

These diagnostic CRISPR-Cas assays, named DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter), SHERLOCK (Specific High Sensitive Enzymatic Reporter) or HOLMES (one-HOur Low-cost Multipurpose highly Efficient System), rely on synthetic RNA/DNA oligonucleotides containing a reporter fluorophore (donor) on one end and a quencher (acceptor) in proximity on the other end (3, 4, 11). Intact, these fluorescence resonance energy transfer (FRET) based reporters show no fluorescence, but upon DNA or RNA cleavage by the activated Cas enzyme, the released donor fluorophore is no longer quenched, and a fluorescent signal is detectable. Ideally, there should be extensive overlap in the FRET pair between the donor emission-spectrum and the acceptor absorption-spectrum, but no overlap in the excitation spectra. Distinguishing these fluorescent signals, however, requires a narrow set of filters in a fluorometer which discards portions of the spectra, thereby affecting the sensitivity. Moreover, directly measuring FRET fluorescence in biological samples may add interference sources, thus causing absorbance, autofluorescence or light scattering, and (12) ultimately affecting the signal. Non-fluorescent SHERLOCK and DETECTR assays have also been designed based on the destruction of a FAM-biotin reporter, allowing for detection on lateral flow strips (13).

To overcome the limitations of FRET reporters, we coupled the CRISPR-Cas detection to an alternative reporter using fluorescent copper nanoparticles (CuNPs). CuNPs exhibit great potential for fluorescent bioassays as a result of their high fluorescence yield, good photostability and a visible emission (orange/red) when excited with UV-light (14–16). For CuNPs to form, DNA has been used as a template to support the binding of copper ions and the subsequent chemical reduction. Qing *et al*. reported that single stranded poly(thymine) could specifically act as an efficient template for the formation of copper nanoparticles, whilst random ssDNA, poly(adenine), poly(cytosine), or poly(guanine) failed to synthesize CuNPs (17). CuNPs are formed along poly-T homopolymers through the addition of ascorbate and Cu^{2+}, which takes only a few minutes (18). Fluorescence spectra of CuNPs have an emission peak at 625 nm when excited at 340 nm, which makes CuNPs well-suited for detection in

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complex biological matrices, as the large Stokes shift (i.e., the difference in wavelength between the absorption- and emission-spectra maxima) reduces strong background signals.

A wide range of applications for CuNPs has been explored, e.g., measuring disease related enzymes and biomarkers in fluids, such as serum, plasma, urine and cell lysates (reviewed in (16)). In a publication by Luo and colleagues (19), the formation of CuNPs was suggested as a method for detecting deoxyribonuclease I (DNAse I) activity in blood plasma of cancer patients. Through the clever use of the special function of the enzyme terminal deoxynucleotidyl transferase (TdT), which produces long poly(thymidine)-tails, a sensitive assay for DNAse I was developed. Other biomarker tests that use CuNPs as an indicator include biological thiol levels (cysteine, homocysteine, and glutathione) in metabolic disorders (20), the microRNA-155 biomarker in cancer (21) or alkaline phosphatase levels used to detect liver or bone disorders (22).

In the current study, we developed a bright fluorescent nucleic acid detection test, CANTRIP (Cas12a Activated Nuclease poly-T Reporter Illuminating Particles). CANTRIP consists of CRISPR-Cas12a and TdT dependent poly(thymine)-templated copper nanoparticles functional in a single reaction tube. As a proof-of-concept, purified DNA containing the anthrax lethal factor gene as a representative target was used to demonstrate the potential of CANTRIP.

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MATERIALS & METHODS

Chemicals/reagents

Sodium L-ascorbate (#A7631) and copper(II) sulfate pentahydrate (Sigma-Aldrich, #203165) from Sigma-Aldrich (Zwijndrecht, The Netherlands). Engen LbCas12a (#M0653T), and terminal deoxynucleotidyl transferase (#M0315L) were obtained from New England Biolabs(Ipswich, USA) as were the matching $CoCl_2$ (2.5 mM stock) plus TdT buffer (final concentration: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate; pH 7.9). dTTP (ThermoFisher #R0171, Bleiswijk, The Netherlands).

Apparatus

HTX Synergy multi-mode plate reader (Biotek Instruments, Inc.).

Plasmids

The plasmid encoding the partial gene of anthrax toxin lethal factor, amino acids 253-380, GCTTTTGCATATTATATCGAGCCACAGCATCGTGATGTTTTACAGCTTTATGCACCGGAAGCTTTTAATTACATG GATAAATTTAACGAACAAGAAATAAATCTATCCTTGGAAGAACTTAAAGATCAACGGATGCTGGCAAGATATG AAAAATGGGAAAAGATAAAACAGCACTATCAACACTGGAGCGATTCTTTATCTGAAGAAGGAAGAGGACTTT TAAAAAAGCTGCAGATTCCTATTGAGCCAAAGAAAGATGACATAATTCATTCTTTATCTCAAGAAGAAAAAGA GCTTCTAAAAAGAATACAAATTGATAGTAGTGATTTTTTATCTACTGAGGAAAAAGAGTTTTTAAAAAAGCTAC AAATTGATATTCGTGATTC, was synthesized and cloned into pTwist Amp High Copy plasmid by Twist Biosciences (San Francisco).

Oligonucleotides

Reporter ssDNA oligonucleotides according to (19) were ordered from Isogen Life Sciences (Utrecht, The Netherlands): 5'-AAC TAT GCA ACC TAC TAC CTC T-OH-3' (RepU) and 5'-AAC TAT GCA ACC TAC TAC CTC T-PO_3-3' (RepB). The crRNAs designed to recognize the anthrax toxin lethal factor were ordered from Isogen Life Sciences included the following ssRNA sequences: 5'-UAA UUU CUA CUA AGU GUA GAU CAU AUU AUA UCG AGC CAC AGC-OH-3' (crRNA1); 5'-UAA UUU CUA CUA AGU GUA GAU UGC ACC GGA AGC UUU UAA UUA C-OH-3' (crRNA2); 5'-UAA UUU CUA CUA AGU GUA GAU GCU CAA UAG GAA UCU GCA GC-OH-3' (crRNA3); and 5' UAA UUU CUA CUA AGU GUA GAU ACA TAT AGT GAA CCG CCA CA-OH-3' (not targeting crRNA).

In vitro Cas12a activation

Presence of the gene sequence was evaluated *in vitro* through the addition of the plasmid carrying the partial *lef* gene to a reaction vessel. The final solution contained: 50 nM LbCas12a, 62.5 nM crRNA2 (unless results were labelled with 'crRNA1' or 'crRNA3'), 100 nM reporter-3'PO_3, 2 nM plasmid (unless otherwise stated) and 1x TdT buffer (New England Biolabs). The total reaction volume used was 50 µl. The samples were incubated at 37 °C for 60 min, after which heat inactivation of the LbCas12a was performed by incubation at 70 °C for 15 min.

Elongation of 3'OH ends into poly-T tails by TdT

Elongation of the 3'OH ends was started by the addition of TdT to the products generated in the Cas12a activation step. The 50 µl of added volume contained 1x TdT buffer (New England Biolabs), 0.5 mM $CoCl_2$ (New England Biolabs, provided with TdT), 0.8 U/µl TdT and 8 mM dTTP. The final concentrations in the 100 µl total volume of all the relevant components were therefore 1x TdT buffer,

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0.25 mM $CoCl_2$, 0.4 U/µl TdT, and 4 mM dTTP. The samples were subsequently incubated at 37 °C for 3 hours.

Synthesis of poly-thymine and CuNPs

Copper nanoparticle synthesis was initiated by the addition of 4 mM ascorbate and 200 µM $CuSO_4$ with immediate mixing. Samples were transferred to a black 96-well plate without a clear bottom. Fluorescence measurements were performed at room temperature in the HTX synergy plate reader on fluorescence mode with the following filters: excitation 360/40, emission 590/35. Each bar represents the mean of two technical duplicates of an independent experiment and the corresponding differences between these two experiments. Background subtracted graphs show the absolute fluorescence value minus the value of an empty well. Samples were transferred to Eppendorf tubes or a clear 96-well plate and placed on a UV-light source for visual assessment.

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RESULTS

Strategy for specific DNA detection using Cas12a dependent poly(thymine) formation

Figure 1 shows the strategy of the CANTRIP assay, which can be divided into three steps, starting after DNA recognition and activation of the trans-cleavage activity of Cas12a with a specific CRISPR RNA (crRNA). cRNAs consist of a 20-nucleotide sequence complementary to the target DNA and serve as a binding and activation scaffold for the Cas nuclease.

Step 1: After activation, Cas12a cleaves the 3' end blocked reporter oligomers. The reporter oligomers have been synthesized with a 3'-phosphate end, so no nucleotides can be added to the primer terminus in the next step if no cleavage has occurred.

Step 2: After a short heat treatment (15 min, 70 °C) which inactivates the cleavage activity of Cas12a, terminal deoxynucleotidyl transferase (TdT) and dTTP nucleotides are added to the sample. TdT is a specialized DNA polymerase that adds nucleotides to the 3' end of an ssDNA strand without requiring a template. When there is Cas12a activation and trans-cleavage, the 3' end blocked oligo will be cut at multiple sites, and TdT will be able to add nucleotides at all the new 3' hydroxyl (3'OH) ends that have been created by the Cas12a cuts. Only dTTP is added to the mixture, so only Ts will be incorporated, forming a poly-T tail.

Step 3: Cu^{2+} and sodium ascorbate are added to the sample. Sodium ascorbate catalyses the conversion from Cu^{2+} to Cu^{o}. In this Cu^{2+}reduction process, the long poly-T tails provide scaffold nucleation sites for the formation of metallic copper-nanoparticles (CuNPs). Poly-T templated CuNPs can be excited with UV-light (340 nm) and emit a strong red fluorescence (emission 615 nm) visible to the naked eye.

Thus, in summary, if there is Cas12a mediated trans-cleavage of a 3' blocked oligo, it will be extended by TdT, forming poly-T tails and CuNPs, along with a fluorescent signal. If there is no Cas12a trans-cleavage activity, as in the absence of the target pathogen, no signal will be observed.

Feasibility of CANTRIP

To achieve Cas12a trans-cleavage activation, we employed *Lachnospiraceae* bacterium Cas12a (NEB), a plasmid containing a partial *lef* gene of *Bacillus anthracis* (*ALF*; aa 253-380) and three corresponding crRNAs with a 20 nucleotide sequence complementary to the partial *lef* gene (see M & M). Activation of Cas12a was first confirmed for each crRNA using a fluorophore-quenched DNase Alert system (IDT) (data not shown). To combine the three-step CANTRIP into a one reaction tube system, a single reaction buffer needed to be established. Fortuitously, all three key components in the reaction, Cas12a, TdT and CuNPs, function optimally in weakly alkaline conditions. TdT is inhibited by the high concentrations of chloride ions present in the recommended Cas12a buffers. Therefore, the reactions were performed in an acetate-containing buffer (potassium acetate, TRIS acetate and magnesium acetate; pH 7.9; see M & M). As illustrated in Figure 2, a strong fluorescent signal is emitted in the presence of the complete reaction mixture. All three crRNAs we developed caused Cas12a activation, and strong fluorescent signals. Next, omission of any of the components in the reaction (crRNA, the *lef* gene, the Cas12a enzyme or the blocked reporter) did not result in any observable signal above background. Clearly, both the Cas12a and TdT enzymes were active and performed well within these buffer conditions. The presence of 0.25 mM $CoCl_2$, an essential cofactor for TdT added in step 2, was also compatible with downstream copper nanoparticle formation.

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Sensitivity and reaction time of CANTRIP

After establishing that the three-step CANTRIP assay can be performed in a single reaction tube, the sensitivity of the detection method was tested using serially diluted plasmid DNA (Figure 3).

Based on both the fluorescence measurement and visual assessment, the detection limit of CANTRIP was evaluated at 10 picomolar of the *lef* gene. At concentrations above 10 nM, we observed a slight but reproducible decrease in signal. In the reactions described above, we allowed the maximum time for Cas12a activation (1 hr) and TdT elongation (3 hrs). To investigate the effect of the reaction time on the signal intensity, identical reaction mixtures were incubated over varying time intervals.

Cas12a was added to each of the tubes and incubated for time intervals of 15, 30 and 60 minutes. Subsequently, TdT was added, and the reaction tubes were incubated for time intervals of 1, 2 and 3 hours. As depicted in Figure 4, the results showed that the maximal incubation times (1 hr for Cas12a, 3 hrs for TdT) generated the highest signal intensity for both enzymes. Incubation times of 15 min for Cas12a and 1 hr for TdT allowed detection above the background signal using a fluorescence plate reader, although this signal was hard to discriminate using the naked eye (Figure 4, inset).

Two step isothermal CANTRIP

After step-1, a heat inactivation step is added to inactivate Cas12a as continuous collateral cleavage by Cas12a will cleave the poly-T tails produced. Conversely, extra 3'OH ends within the poly-T tails will be created by Cas12a cleavage providing extra substrate for TdT to initiate new extra poly-T additions. To test this, we left out the heat inactivation after step 1 in the most optimal CANTRIP condition (60 min CaS12a, 3 hrs TdT). Both reactions, with and without inactivation, show a high fluorescent signal which shows that, despite continuous cleavage activity of Cas12a during the TdT reaction, heat inactivation is not necessary, and an isothermal reaction at 37°C is possible (*Fig.5, left*). In addition, CaS12a and TdT can be added simultaneous (*Fig.5, right*) at the start of the reaction thereby creating a two-step isothermal CANTRIP reaction. A one-step isothermal CANTRIP reaction, i.e., including copper nanoparticle formation during Cas12a cleavage and TdT elongation, is not possible as both enzymes are inhibited (data not shown).

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DISCUSSION

In addition to their remarkable gene editing abilities, CRISPR-Cas systems have been used in a variety of detection applications (1, 23, 24). In this research, we combined the activity of programmable CRISPR-Cas12a with poly(thymine) templated copper nanoparticles and established a proof-of-concept for a novel fluorescent specific nucleic acid detection assay, i.e., CANTRIP. The read-out under UV-light is an unmistakable bright orange/red fluorescence visible to the naked eye when the targeted DNA sequence is present in the sample. In addition, the colour intensity can be quantified by measuring the emittance using a fluorescence plate-reader.

There are several advantages to the CANTRIP test. First, the large Stokes shift of the CuNPs avoids self-quenching and light scattering, thus allowing for performing the assays in biological fluids. Second, performing the CANTRIP assay does not require a well-equipped laboratory and may therefore have the potential to be used in point-of-care. The assay only requires isothermal incubation at 37 °C. Third, the formation of fluorescent CuNPs is efficient, since it requires only a small amount of ssDNA. Finally, in contrast to ssDNA FRET-reporters connected by a fluorophore and a quencher, where multiple cuts do not result in an increased signal, multiple cuts in the blocked reporter oligomers before the TdT addition result in more poly-T tails and therefore an increase in CuNP formation and a higher signal. CANTRIP fully utilizes this amplification phenomenon.

Cas-dependent assays, such as DETECTR, SHERLOCK and HOLMES are combined with a preceding isothermal or PCR multiplication step to amplify the targeted pathogen DNA or RNA before adding the Cas enzymes for detection. This combination substantially lowers the detection limit, as more Cas-enzymes become activated by the increased number of amplified targets. These DNA/RNA amplification assays depend on sequence specific primers that contain 3'OH ends for the DNA polymerases and are therefore not suitable for CANTRIP, since the TdT would initiate poly-T formation from these primers rather than from the cleaved reporters. This inherent limitation lowers the sensitivity of CANTRIP, even though the Cas12 cleavage and TdT step amplify the detection signal.

Published data of various CRISPR-Cas FRET based detection methods without pre-amplification show detection limits between 10 pM and 2 nM (4, 13, 25) which places CANTRIP, with 10 pM detection limit, in the higher sensitivity range.

Cas13a used in the SHERLOCK assay binds to RNA and cleaves the surrounding RNA rather than the DNA substrates. In this assay, target-specific RNA is produced with T7 transcription to convert amplified DNA to RNA for subsequent recognition by Cas13a and activation of its promiscuous RNA cleavage (3). As TdT is unable to use RNA 3'OH ends to initiate poly-T formation, Cas13a, and thus RNA pathogen recognition, cannot be used for the current CANTRIP assay. Human Polθ terminal transferase can modify the 3' terminal ends of both DNA and RNA and might therefore be used in the formation of Cas13a dependent poly-T CuNPs (26, 27). This reaction would also require that the added crRNA is blocked at its 3' end to prevent it from being elongated by Polθ terminal transferase.

An intrinsic limitation of the fluorescent poly-T CuNPs based systems is a lack of multiplex ability in a single reaction (25), which permits the simultaneous testing of multiple targets. However, since immobilization of the 3' blocked ssDNA reporter using adsorption or covalent binding on a surface is possible, multiple (micro-)compartmentalization with subsequent fluorescence read-outs are feasible (28).

Developments are rapidly advancing in both CRISPR-Cas and copper nanoparticle fluorescent biosensors (14, 15, 29). Interestingly, in addition to enzymes and nucleic acids, these applications are beginning to include the detection of small molecules, such as uric acid (30), metals (31–33), iodide

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(34), biothiols (20) and dopamine (35). Here, we show the first promising example of a combination of both techniques.

The CANTRIP assay provides a method for rapid initial screening for pathogens in non-laboratory settings without requiring specialized equipment. Subsequently, more comprehensive centralized laboratory testing could be used to confirm the initial screening results and provide more detailed information. Both the performance sensitivity (high picomolar) and incubation times (hours) of the CANTRIP assay require further improvement, but this method clearly has the potential to serve as a simple and easily visible pathogen detection assay for biofluids.

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FIGURE LEGENDS

Figure 1. Schematic representation of the CANTRIP (Cas12a Activated Nuclease poly-T Reporter Illuminating Particle) DNA detection assay. See text for further details.

Figure 2. Copper nanoparticle fluorescence emission intensity of the CANTRIP assay on anthrax lethal factor template gene recognition. To verify the correct coupling of the Cas12a target activation via poly-T scaffolded CuNPs formation, a synthetic ALF gene was detected. Three different crRNA targeting ALF gene were compared, and the negative controls consisted of leaving out individual components of the assay and a non-targeting crRNA (nt crRNA). Bars represent the mean of two technical duplicates of an experiment and the corresponding differences between these two experiments. RepB = blocked reporter. Inset: Fluorescence emission image upon illumination with UV light corresponding to bar chart (excluding nt crRNA).

Figure 3. Sensitivity of the fluorescent CANTRIP assay using serial dilution of anthrax lethal factor plasmid as the target dsDNA in combination with crRNA2. The negative control contained no ALF target plasmid (0 pM). Bars represent the mean of two technical duplicates of an experiment and the corresponding differences between these two experiments. Inset: Corresponding fluorescence emission image upon illumination with UV light. Image was rearranged to match the bar graph.

Figure 4. Variation of incubation times for the complete CANTRIP assay reaction using crRNA2. Incubation time with the Cas12a (Cas) enzyme was 15, 30 or 60 min. Incubation time with TdT was one, two or three hours. Bars represent the mean of two technical duplicates of an experiment and the corresponding differences between these two experiments. Inset: Corresponding fluorescence emission image upon illumination with UV light.

Figure 5. CANTRIP can be performed in a two-step reaction without Cas12a heat inactivation. Pre-incubation time with the Cas12a enzyme, before TdT addition, was 60 min or 0 min after which the Cas12a reaction mix was heat inactivated at 70 °C or not. Subsequent incubation time with TdT was three hours. Bars represent the mean of two technical duplicates of an experiment and the corresponding differences between these two experiments.

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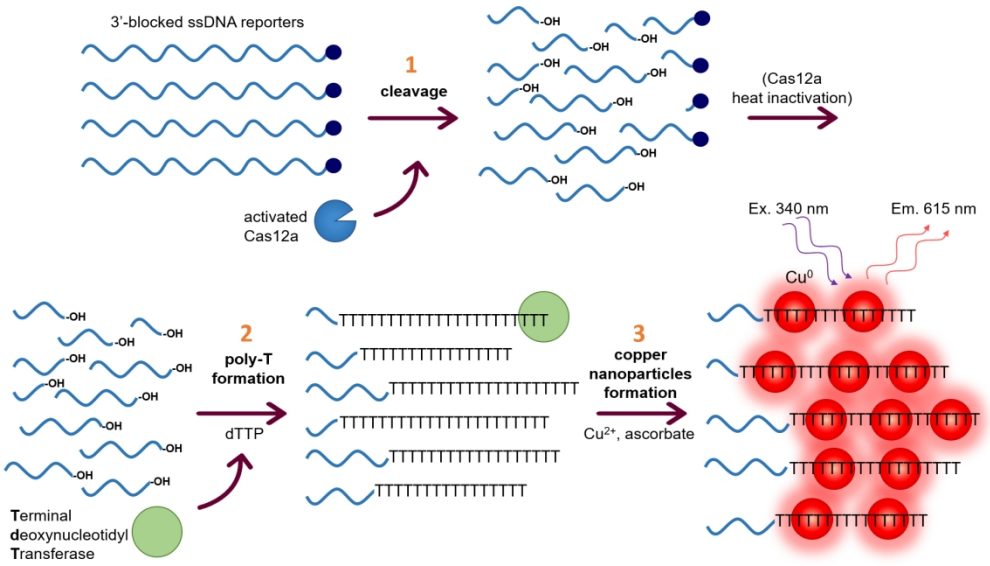


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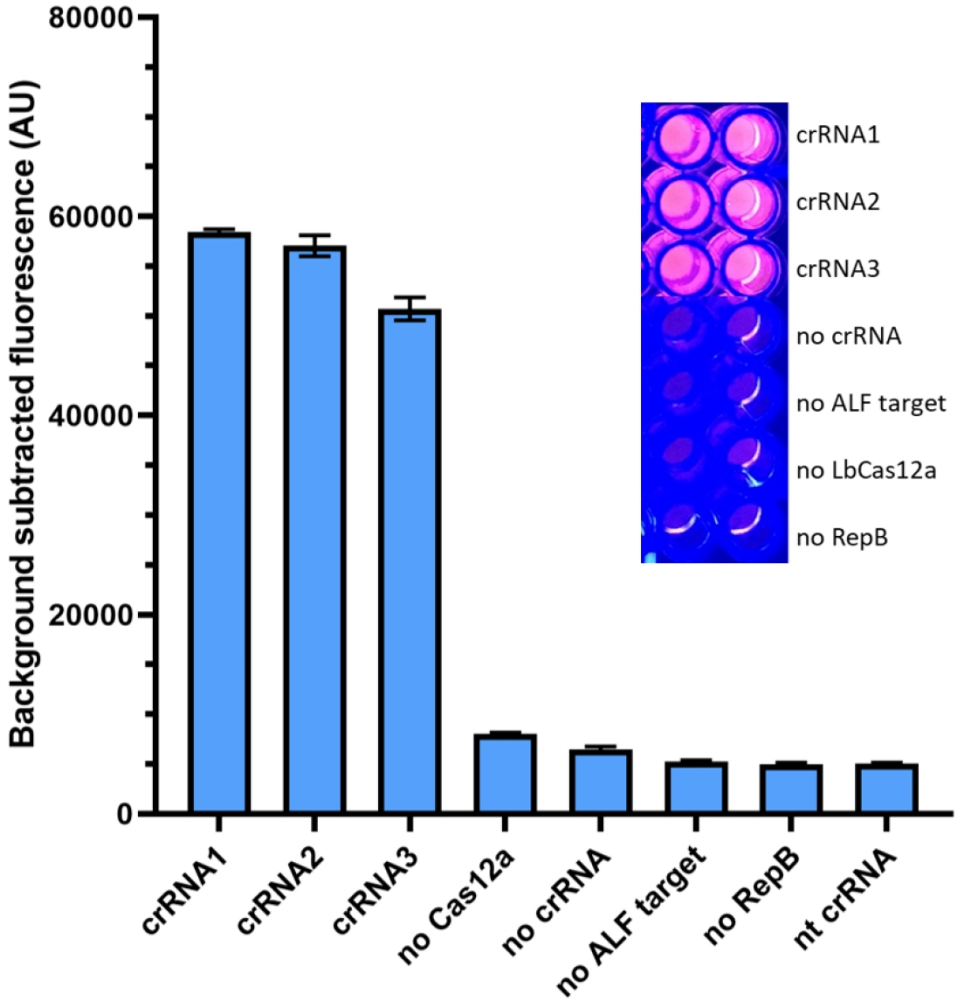


Figure 2. Copper nanoparticle fluorescence emission intensity of the CANTRIP assay on anthrax lethal factor template gene recognition. To verify the correct coupling of the Cas12a target activation via poly-T scaffolded CuNPs formation, a synthetic ALF gene was detected. Three different crRNA targeting ALF gene were compared, and the negative controls consisted of leaving out individual components of the assay and a non-targeting crRNA (nt crRNA). Bars represent the mean of two technical duplicates of an experiment and the corresponding differences between these two experiments. RepB = blocked reporter. Inset: Fluorescence emission image upon illumination with UV light corresponding to bar chart (excluding nt crRNA).

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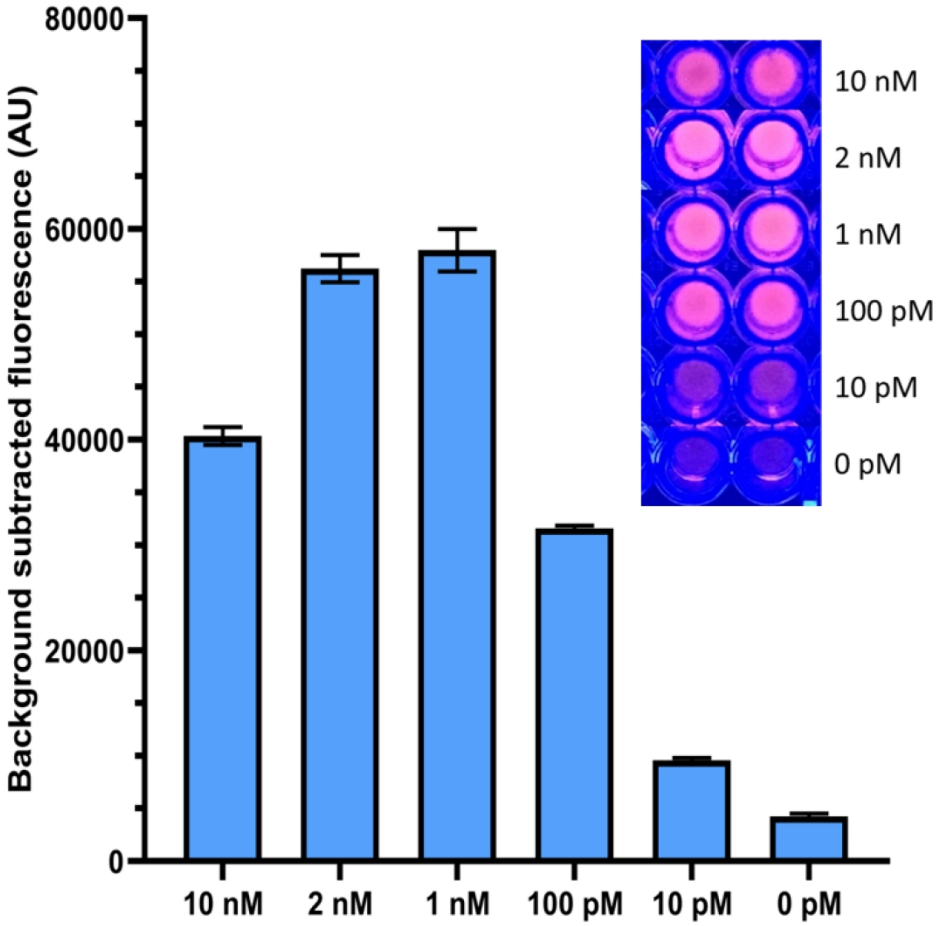


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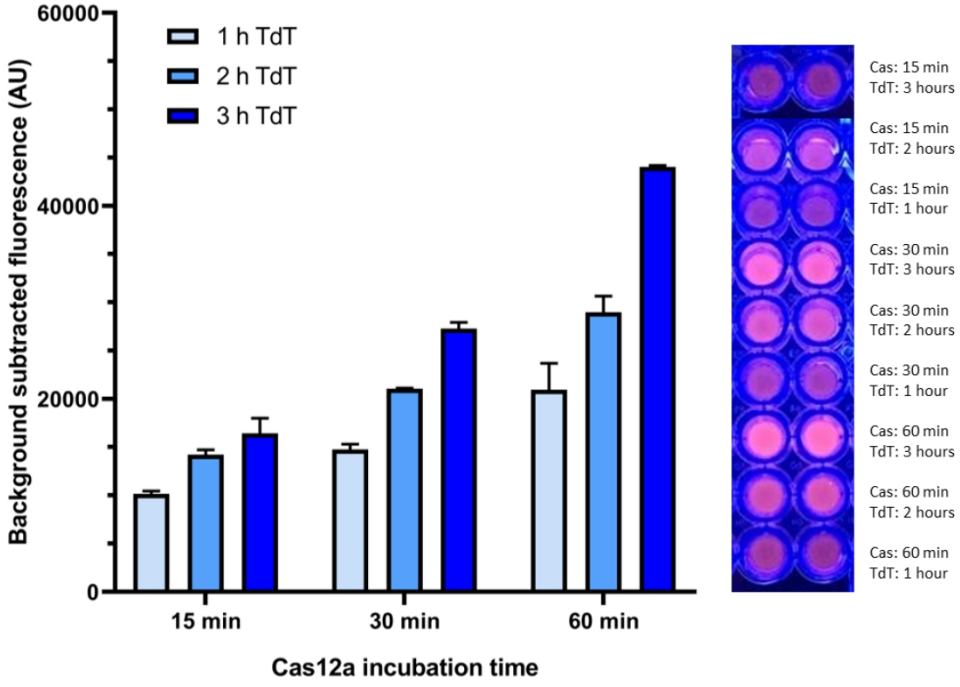


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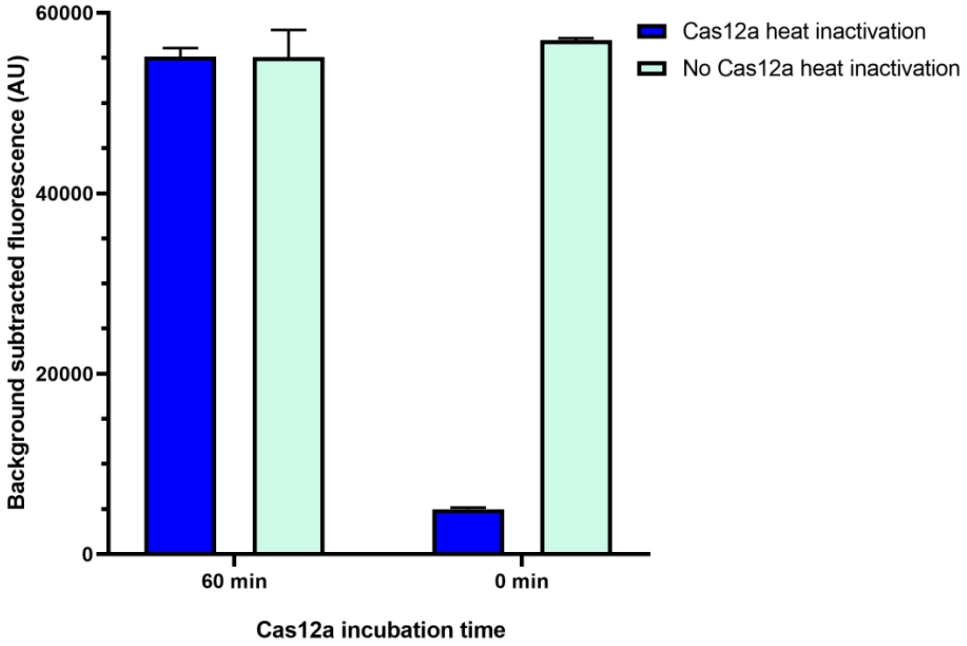


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