# Bifunctional circular DNA amplifier transforms a classic CRISPR/Cas sensor into an ultrasensitive autocatalytic sensor

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**Article**

# Abstract

The CRISPR/Cas sensors represent a new approach in molecular diagnostics. Based on the programmable *trans*-cleavage of Type V & VI Cas proteins to degrade single-strand DNA/RNA reporters they detect nucleic acids with single-nucleotide specificity. However, their clinical usefulness is limited by their pM level sensitivity, which is insufficient unless integrated with preamplification. To overcome this limitation, we designed a bifunctional circular DNA amplifier to report nucleic acid detection events and simultaneously facilitate an autocatalytic reaction network. This minimalistic design converts a classic CRISPR/Cas sensor into a DNA amplifier-enhanced CRISPR/Cas autocatalytic sensor (DANCER), without the need for additional amplification reaction or device. DANCER achieved 1 aM level (< 1 copy/uL) DNA detection ($10^6$ times improvement), within 15 mins, at room temperature, and with 11 orders of magnitude detection range. When combined with a colorimetric lateral flow assay, DANCER realized point-of-care testing of circulating tumor DNA in plasma of a colorectal cancer-mouse.

# Introduction

Nucleic acid detection is widely used in a range of fields, including medical diagnostics, food safety testing, and environmental monitoring.[1] The ability to program the Cas ribonucleoprotein (RNP) in CRISPR/Cas systems - by aligning the sequence of guide RNA (gRNA) with specific molecular targets - offers an effective approach for detecting nucleic acids with ultrahigh selectivity.[2,3] Such CRISPR/Cas RNP based biosensors represent a flexible platform that only needs gRNA replacement to target specific sequences.[2] When activated by a nucleic acid target, type V & VI Cas RNPs switch on their sequence-nonspecific *trans*-cleavage utilised in a classic CRISPR/Cas sensor scheme,[2,4] which catalytically cleave the supplied single strand DNA/RNA reporters. A range of optimised reporters have been reported with increased signal intensity and/or reduced background, with designs such as hairpin,[5] G4[6] and G3[7], achieving pM sensitivity without additional amplification.[5,8] However, thus far there were no efforts to endow the DNA/RNA reporters with novel additional functions which could lead to a quantum leap in sensor performance, and eliminate the need for pre-amplification. Stripped-down designs that do not introduce additional system complexity would be particularly valuable.

In this work, specially designed circular DNA amplifiers (Cir-amplifiers) were introduced to act both as a highly efficient reporter for signal readout and, simultaneously, to establish an autocatalytic reaction network with Cas12a RNPs (Fig. 1). This DNA Cir-amplifier comprises a circular single strand DNA (ssDNA) and a slightly shorter linear complementary DNA strand (cDNA) labelled at both ends with a fluorophore and a matching quencher. These two sequences together create a hybrid circular structure with a dsDNA section joined by a very short ssDNA linker. The dsDNA sequence is the same as the target sequence. When the linker is cleaved by the *trans*-ssDNA activity of activated Cas12a RNP,[2] the dsDNA becomes linearised which restores the fluorescence signal. Furthermore, upon this cleavage, these now linearized Cir-amplifiers become "fake targets", due to sequence identity with the real targets. This identity then drives the autocatalysis reaction. In this way, the Cir-amplifier plays a dual role in our system: of a

catalytic substrate for *trans*-cleavage by activated Cas RNP, exactly like the reporter in a classic CRISPR sensor design,[2,4] and an autocatalytic substrate for the yet-to-be activated Cas RNPs.

We stress that our DANCER sensor only contains two ingredients, Cas RNPs and circular DNA amplifiers, in precise correspondence to classical CRISPR sensors with their Cas RNPs and their DNA/RNA reporters – but its performance is dramatically improved in terms of sensitivity, reaction speed and detection range. Since in DANCER sensor under suitable conditions one target is able to activate all Cas12a RNPs available, the signal amplification is theoretically unlimited. As a result, we were able to show that without the need for any additional amplification reaction or device, the DANCER is capable of detecting DNA at a remarkable 1 aM level (< 1 copy/uL) sensitivity under 15 mins at isothermal condition (room temperature), and with uncompromised specificity compared to classical CRISPR/Cas sensors.

# Results

1. Synthesis and characterisation of circular ssDNA.

We first synthesised a single ring circular-ssDNA (Cir-ssDNA). Streptavidin-modified magnetic beads were utilized to immobilize the biotinylated (linear) ssDNA (Fig. 2a). Subsequently, the click chemistry approach was applied to form the Cir-ssDNA by bonding of azide and alkyne (CHCH) functional groups.[9,10] The remaining linear ssDNA was degraded by exonuclease, and Cir-ssDNA was released from the streptavidin beads by heating to 95 °C.[11] Single ring Cir-ssDNA thus synthesised was characterised by using denaturing polyacrylamide gel (dPAGE) electrophoresis assay (Fig. 2b), where the band of Cir-ssDNA (column 3) was found to move more slowly than the band of linear ssDNA (column 2), confirming the formation of Cir-ssDNA.[12] In addition, only a single band of Cir-ssDNA (column 3) was observed, consistent with the formation of single ring Cir-ssDNA. We optimized the synthesis efficiency of Cir-ssDNA by varying the concentration of magnetic beads, and over 90% synthesis efficiency was achieved (Fig. 2c & Fig. S1). The Cir-ssDNA synthesis method was highly reproducible (Fig. 2d, coefficient of variation of 1.06%).

2. Performance of Cir-amplifiers as reporters in a classic CRISPR/Cas12a biosensing system.

The Cir-amplifiers are designed to be a catalytic substrate for activated Cas12a RNPs and produce a fluorescent reporter signal once linearized (Fig. 3a). This requires a significant fluorescence signal difference between Cir-amplifier and linearized Cir-amplifier shown in Fig. 3b. Limited fluorescent background was observed in the case of Cir-amplifiers since the fluorophore-quencher distance (equivalent to the ssDNA linker length) was within the FRET distance of the Texas/BHQ2 pair (5nt) [13]. After cleavage, the fluorescence signal of a linearized Cir-amplifier was restored since the distance of fluorophore and quencher (18nt, estimated to be 6.12 nm[14,15]) exceeded the FRET distance of Texas/BHQ2 (over 10nt)[13]. The fluorescence signal of the Cir-amplifier increased over 16.5 times upon linearisation, confirming that it can act as a reporter in classical CRISPR/Cas12a biosensing systems.

Optimisation of the reporter function in our DNA amplifier was carried out by changing the linker length (Fig. 3c). Higher fluorescent background was observed for longer linker lengths, which was due to increased fluorophore-quencher distance. We then exposed the Cir-amplifiers with different ssDNA linker lengths to activated Cas12a RNPs (Fig. 3d). The fluorescence signal was found to increase for ssDNA linker lengths from 0 to 3, since longer ssDNA linkers could be more easily cleaved by Cas12 RNP.[16] With further increase of ssDNA linker length from 3 nt to 7nt, the fluorescence signal remained similar. Thus, 3nt ssDNA linker length was found to be optimal. The optimised Cir-amplifier with 18nt dsDNA and 3nt ssDNA linker was also compared with a classic linear ssDNA reporter (TTATT, with identical fluorophore-quencher pairs) in a classic CRISPR/Cas12a biosensing system, and higher fluorescence signal by a factor of 3 was observed for the Cir-amplifier (Fig. S2). Additionally, the limit of detection (LOD) of Cir-amplifier-assisted CRISPR/Cas12a biosensing system (0.1 pM) was found to be 10 times lower than that of linear ssDNA reporter-assisted CRISPR/Cas12a biosensing system (1 pM) (Fig. 3e). Therefore, our Cir-amplifier represents a high-performance reporter for CRISPR/Cas12a biosensors.

3. RNP activation efficiency of Cir-amplifier and linearised Cir-amplifier in a CRISPR/Cas12a biosensing system

We assessed the RNP activation efficiency of Cir-amplifiers in a CRISPR/Cas12a biosensing system (Fig. 4a). Since the Cir-amplifier contains two key regions, the dsDNA section and ssDNA linker, we systematically investigated the lengths of each. As shown in Fig. 4b, a certain amount of RNP activation by Cir-amplifiers giving rise to a background signal was observed when the dsDNA length was higher than 18nt, and RNP activation was significantly reduced when the dsDNA length was lower than 18nt. Since minimal RNP activation by Cir-amplifiers is desirable, 18nt dsDNA length was selected for following studies.[17] Afterwards, we investigated the ssDNA linker length in Cir-amplifiers. As shown in Fig. 4c, the RNP activation (background signal) slightly increased from 0–3 nt, and then grew sharply from 5–10 nt, thus the ssDNA linker length below 3 nt was found to be optimal for background control. Additionally, since longer ssDNA linkers are easier to be cleaved than shorter ones (Fig. S3), a 3nt ssDNA linker was selected for the following work.[16]

Furthermore, the RNP activation efficiency by linearized Cir-amplifier in a CRISPR/Cas12a biosensing system was investigated (Fig. 4d). A linearized Cir-amplifier with 18nt dsDNA and 3nt ssDNA was applied to activate a CRISPR/Cas12a biosensing system. Excellent activation efficiency with over 20 times fluorescence increase was observed (Fig. 4e), which was comparable to that of linear dsDNA (18nt) (Fig. 4f), indicating that the ability of Cir-amplifier to activate RNPs was fully recovered upon its linearisation. This means that a Cir-amplifier with 18nt dsDNA and 3nt ssDNA only minimally activates the available Cas12a RNPs but linearized Cir-amplifiers with 18nt dsDNA and 3nt ssDNA are able to activate these RNPs just like the original molecular targets.

4. Establishment of the DNA amplifier-enhanced CRISPR/Cas autocatalytic sensor (DANCER)

The CRISPR/Cas autocatalytic sensor (DANCER) is established by using two components, Cas12a RNPs and Cir-amplifiers. This seemingly minor modification of replacing linear ssDNA reporters in a classic CRISPR/Cas sensor system with Cir-amplifiers has a profound impact on the reaction network within the sensor. As schematically shown in Fig. 1a, with the introduction of target DNA, the DANCER autocatalysis system is initiated. The target activated RNP linearises a number of Cir-amplifiers which then continue to generate additional activated Cas12a RNPs, and these create an avalanche of additional linearised Cir-amplifiers – each of which reports the detection. This avalanche continues producing an ever-increasing signal as long as the Cir-amplifier substrate and Cas12a RNPs are not depleted. In comparison with a classical CRISPR/Cas12a sensor (Fig. 1b), whose signal linearly increases with time (Fig. 5a, control), DANCER provides an exponentially increasing signal (Fig. 5a). Additionally, in DANCER for the same amount of Cas12a RNP (20nM), with increasing supply of Cir-amplifiers, an increasing fluorescence signal was observed (Fig. 5a). Correspondingly, with the same amount of Cir-amplifiers (200 nM), with increasing Cas12a RNP levels, an increasing fluorescence signal was observed as well (Fig. 5b), which is indicative of higher total levels of RNP activation in DANCER compared with a classic CRISPR/Cas sensor. Finally, the biosensing performance of DANCER was investigated (Fig. 5c), and 1 aM of limit of detection (LOD) was achieved, with more than 11 orders of magnitude detection range. This is 6 orders of magnitude higher than the LOD of a classic CRISPR/Cas12a biosensing system (1 pM) (Fig. S4). Therefore, DANCER is an ultrasensitive biosensing system capable of detection of single nucleic acid targets per microlitre.

To further expand the biosensing applications of DANCER, we combined a classic CRISPR/Cas12a biosensing system with DANCER to establish a versatile DANCER-2 system (Fig. S5), in which the DANCER system functions as an additional signal amplification loop for a classical CRISPR/Cas12a biosensing system. A comparable performance of DANCER-2 (with LOD of 1 aM) was observed with DANCER system (Fig. S5) confirming its versatility.

The autocatalysis-driven biosensing performance of DANCER was further interpreted by a model system of chemical kinetics rate equations[18] (Supplementary Note) which introduces the autocatalysis loop for Cas12a. It allows to establish that the experimentally observed increase is approximately exponential when the RNPs and Cir-amplifier are sufficiently abundant and do not get noticeably depleted, which has been confirmed by an exponential fit in Fig. 5a.

5. Application of DANCER for the point-of-care quantification of ctDNA in plasma of mice with human colorectal cancer xenografts

The biosensing performance of DANCER in future clinical settings was evaluated via the detection of circulating tumor DNA (ctDNA, PIK3CA E542KM[19]) in mice with orthotopic human colorectal cancer xenografts. Three groups of mice were prepared, including normal mice, mice bearing human colorectal cancer (CRC-mouse), and X-ray treated CRC-mouse. Blood samples were collected from all animal groups for the analyses of ctDNA in blood plasma.[20] Prior to the analysis of ctDNA from animal models, the biosensing performance of DANCER in a synthesized ctDNA (PIK3CA E542KM[19]) spiked plasma sample

was first evaluated. As shown in Fig. 6a, 1 aM sensitivity was achieved in a non-diluted plasma sample although higher background signal was observed than in plasma-free controls. A DANCER calibration curve extending over 4 orders of magnitude was obtained by testing of different concentrations of ctDNA spiked into prepared plasma samples (Fig. 6b). The DANCER system was then used for the detection of ctDNA from mouse plasma in a procedure shown in Fig. 6c, in which 10 μL plasma sample was sufficient for a test. The analysis results confirm that the DANCER system was able to distinguish ctDNA from normal and diseased mice (Fig. 6d), while a classic CRISPR biosensing system was not able to realize such detection, confirming superiority of the DANCER system in future clinical settings. In addition, lower ctDNA concentration was observed in X-ray treated CRC-mouse (Fig. 6d), confirming the feasibility to monitor cancer treatment effect by using DANCER. Furthermore, based on the standard calibration curve (Fig. 6b), the ctDNA concentrations in CRC and X-ray treatment groups were estimated to be 74.5 aM and 19.9 aM, respectively, while no ctDNA was found in the normal mouse group.[20]

To further expand the applicability of a fluorescent DANCER system to a point-of-care setting, a new colorimetric Cir-amplifier was established (Fig. 6e).[21] The core structure of colorimetric Cir-amplifier was identical to the fluorescent Cir-amplifier discussed earlier, the only difference was the extension of 18nt cDNA with 5nt CCCCC with a biotin on 3' end. After the DANCER reaction was deemed to be sufficiently executed, a lateral flow assay (LFA) was applied for the colorimetric signal readout of Cir-amplifiers (Fig. 6f). An anti-FAM antibody with gold nanoparticle on the conjugation pad was applied to recognize the fluorophore FAM on the 5' end of colorimetric Cir-amplifier (Fig. 6e). As the reaction product flowed through the LFA, the antibody & Cir-amplifier complex was captured by the streptavidin on the control line through the binding of biotin on the 3' end of colorimetric Cir-amplifier (Fig. 6e), and red colour appeared on the control line (Fig. 6g). With further flow of the sample, the secondary antibody on the test line captured the anti-FAM antibody for colorimetric signal readout. In terms of the whole DANCER & LFA assay, in the presence of a genuine target, the DANCER system was activated, and colorimetric Cir-amplifiers were cleaved to yield linearized Cir-amplifiers (Fig. 6e), which were further captured by the secondary antibody on the test line for signal readout due to the loss of their 3'-5C biotin tails (positive test). Conversely, without a genuine target, the DANCER system was not activated, and colorimetric Cir-amplifier remained intact (Fig. 6e), and was further captured by the streptavidin on the control line for signal readout (negative test). Finally, the established DANCER & LFA assay was applied to test the plasma ctDNA from normal and CRC-mice (Fig. 6g), and an obvious colour intensity difference was observed between the CRC-mouse (74.5 aM of target) and normal mouse (0 aM of target) samples (Fig. 6f), confirming the potential to apply the DANCER system in a point-of-care setting.

# Discussion

In this study, we developed a novel DNA amplifier-enhanced CRISPR/Cas autocatalytic sensor (DANCER). DANCER is capable of ultrasensitive detection of a single copy of nucleic acids without amplification at room temperature (Fig. 5). Additionally, DANCER is able to quantify ctDNA from clinical samples and reveal the presence of abnormal status at a point-of-care setting, demonstrated here in blood plasma of

mice with human CRC xenografts using both fluorescent and LFA based colorimetric readout (Fig. 6). DANCER is an elegant autocatalysis system, which only contains two molecular components, Cas12a RNPs and Cir-amplifiers. The Cir-amplifier as the key component of DANCER system, has the ability of both activating downstream Cas12a RNPs to drive the autocatalysis reaction system (Fig. 5) and acting as a fluorescent reporter for real-time signal readout (Fig. 3). The formation of Cir-amplifiers is based on intramolecular linkage to form single ring. Herein, we introduced a novel magnetic beads-based click chemistry method for the synthesis of Cir-ssDNA, which shows high efficiency (> 90%), excellent reproducibility, and it yields only a single ring circular ssDNA (Fig. 2).[12]

Being a single pot reaction, DANCER is compatible with point-of-care ultrasensitive quantification of nucleic acids. In contrast to conventional nucleic acid amplification technologies[22], such as PCR, RPA, and LAMP, DANCER provides a comparable sensitivity (1 aM) but it is free from their disadvantages such as the requirement for temperature cycling, or the potential for primer polymerisation. Additionally, in comparison with the established CRISPR biosensing system, such as DETECTR system[2] (RPA with Cas12a), SHERLOCK system[4] (RT-RPA with Cas13), HOLMES[2] (PCR with Cas12a), which have high sensitivity and specificity, but still require nucleic acid amplification step, our DANCER provides a rapid amplification free approach with compatible sensitivity and specificity, but without amplicon contamination. Furthermore, in comparison with other signal amplification technologies assisted CRISPR biosensors, such as SERS[23], metal-enhanced fluorescence[24], nanoenzyme[25], and field-effect transistors[26,27], DANCER does not require any sophisticated instrumentation and it can be performed at room temperature with a point-of-care setting. In comparison with the recently established Cas tandem[21,28,29] and the Cas feedback circuit [30], which require more than one hour turnover time due to a complex system design with multiple components, our DANCER system is capable of rapid detection of 1 aM nucleic acids within 15 min (Fig. 5c), with the possibility of further reduction.

DANCER provides a versatile approach for signal amplification for future biosensing solutions. Through the programmability of Cas nucleases, it is capable of ultrasensitive detection of a broad range of nucleic acid analytes. Additionally, it can be directly integrated into all the type V based CRISPR/Cas biosensing system (based on Cas12 & Cas14) as an additional signal amplifier to enhance their sensitivity, without any additional changes of the original reagents or setup (Fig. S5).[28] In conclusion, DANCER offers a breakthrough approach for rapid, point-of-care, and ultrasensitive quantification of nucleic acids.

# Declarations

## Acknowledgements:

contributed ideas design and data analysis. R.S and W.D. contributed the animal model. E.G contributed ideas design and the theoretical model. All authors contributed to and approved the final manuscript.

# Online Methods

Acronyms related to specific molecules are explained in the Supplementary Material.

## 1. Synthesis and characterisation of circular ssDNA

To synthesize Cir-ssDNA, 0.4 mL of 0.5% w/v streptavidin modified magnetic beads (0.74 μm) were first blocked with 1% BSA solution for 1 h to eliminate non-specific binding. Afterwards, 1 mL of 0.5 μM biotinylated linear-ssDNA was incubated with the beads for 1 h following a PBS wash to remove the residual free linear-ssDNA. Subsequently, 1 mL of the click chemistry reaction solution (1.0 mM $CuSO_4$, 2.0 mM TCEP, and 100 μM TBTA) was added and incubated with the beads for 12 h at room temperature. After synthesis, the magnetic beads were collected and washed with PBS buffer to remove excess chemicals. Subsequently, 100 μL of 100 units/mL Exonuclease VII solution was added and incubated at 37 °C for 30 min to remove the linear ssDNA. After washing with PBS buffer, the synthesized Cir-ssDNA was released from the streptavidin-modified magnetic beads by heat treatment at 95 °C for 30 min,[11] and the supernatant was collected for further use. All the Cir-ssDNA used in this research are synthesized based on this approach. The sequence listed in Table S1 is a demonstration example. Nanodrop was utilized to test the concentration of synthesized Cir-ssDNA.

The formation of Cir-ssDNA was verified by using denaturing polyacrylamide gel (dPAGE) electrophoresis assay. 10 μL of Cir-ssDNA aliquoted with 2 μL 6X DNA gel loading dye was loaded into the gel for electrophoresis, which was carried out for 40 min at a constant voltage of 100V. 5 μL of 10 bp DNA ladder was used for molecular weight reference. Gel images were visualized by using Gel Doc + XR image system (Bio-Rad Laboratories Inc., USA).

## 2. Investigation of the reporter performance of Cir-amplifiers in a classic CRISPR/Cas12a biosensing system

The Cir-amplifier was assembled by mixing Cir-ssDNA with fluorophore labelled cDNA (Texas-cDNA-BHQ2). The Cir-amplifier based CRISPR/Cas12a reaction mixture was prepared as follows: 1 μL 100 μM (100 pmol) of Cas12a protein was gently mixed with 5 μL 20 μM (100 pmol) of gRNA-C in 3.6 mL 1X NEB 2.1 buffer. Then, 120 μL of 5 μM (0.6 nmol) of Cir-amplifier with different linker length (0−7 nt) were added and well mixed to form the standard Cir-amplifier involved reaction mixture. For comparison, linear ssDNA reporter assisted CRISPR/Cas12a reaction mixture was prepared: 1 μL 100 μM (100 pmol) of Cas12a protein was gently mixed with 5 μL 20 μM (100 pmol) of gRNA-C in 3.6 mL 1X NEB 2.1 buffer. Then, 6 μL of 100 μM (0.6 nmol) of pre-synthesized fluorescent quenched ssDNA reporters (Texas red-TTATT-BHQ2) were added and well mixed to form the standard linear ssDNA reaction mixture.

Afterwards, 10 µL of different concentrations (0, 0.1, 1, 10, 100, 1000 pM) of target-C ssDNA were added to 90 µL of the prepared reaction mixture containing either Cir-amplifiers or ssDNA reporters and incubated for 120 min. A SpectraMax iD5 multi-Mode Microplate Reader (Molecular Devices) was used for the detection of fluorescence readout. The Ex/Em of Texas-Cir-amplifier-BHQ2 was 570/615 nm. All the DNA and RNA oligos used in this experiment are listed in Table S2.

### 3. Investigation of the RNP activation ability of Cir-amplifier in a classic CRISPR/Cas12a biosensing system

In this experiment, the CRISPR/Cas12a reaction mixture was prepared as follows: 1 µL 100 µM (100 pmol) of Cas12a protein was gently mixed with 5 µL 20 µM (100 pmol) of gRNA-D in 3.6 mL 1X NEB 2.1 buffer. Then, 6 µL of 100 µM (0.6 nmol) of pre-synthesized fluorescent quenched ssDNA reporters (Texas red-TTATT-BHQ2) were added and well mixed to form the standard reaction mixture.

Afterwards, 10 µL 0.25 µM of a range of Cir-amplifiers with different dsDNA length and different ssDNA linker lengths were added to 90 µL of the prepared reaction mixture and incubated for 120min. A SpectraMax iD5 multi-Mode Microplate Reader (Molecular Devices) was applied for the detection of fluorescence readout. The Ex/Em of Texas red-TTATT-BHQ2 reporter was 570/615 nm. For comparison, linear dsDNA was also applied to activate the CRISPR/Cas12a reaction mixture under the same conditions. All the DNA and RNA oligos used in this experiment are listed in Table S3 & S4.

### 4. Investigation of the RNP activation efficiency of linearized Cir-amplifiers in a classic CRISPR/Cas12a biosensing system

The RNP activation efficiency of linearized Cir-amplifiers was evaluated using the CRISPR/Cas12a reaction mixture prepared by Method 3. Afterwards, 10 µL 0.25 µM of linearized Cir-amplifiers was added to 90 µL of the prepared reaction mixture and incubated for 120min. A SpectraMax iD5 multi-Mode Microplate Reader (Molecular Devices) was applied for the detection of fluorescence readout. For comparison, linear dsDNA (18nt) was also applied to activate the CRISPR/Cas12a reaction mixture under the same conditions. All the DNA and RNA oligos used in this experiment are listed in Table S4.

### 5. Evaluation and biosensing application of DANCER

The DANCER reaction mixture was prepared as follows: 1 µL 100 µM (100 pmol) of Cas12a protein was gently mixed with 5 µL 20 µM (100 pmol) of gRNA-D to form the Cas12a RNP in 5 mL 1X NEB 2.1 buffer. Subsequently, 200 µL of 5 µM (1 nmol) of Cir-amplifier solution was added and well mixed to form the reaction mixture.

Afterwards, 10 µL of target-D ssDNA at different concentrations were added to 90 µL of the prepared reaction mixture for activating trans-cleavage of Cas12a and enabling the CRISPR/Cas autocatalysis biosensing reaction. A SpectraMax iD5 multi-Mode Microplate Reader (Molecular Devices) was applied for the detection of fluorescence readout. The Ex/Em of Texas red-TTATT-BHQ2 reporter was 570/615 nm. All the DNA and RNA oligos used in this experiment are listed in Table S5.

## 6. Establishment of orthotropic CRC mouse model

All animal experiments were approved by the UNSW Animal Care and Ethics Committee (project approval 20/95B, 21/39B, and 21/77B). NOD/SCID (6-8-week-old) mice were provided by Animal Services from the Animal Resources Centre (ARC, Perth, WA). Mice were housed in specific pathogen free conditions at 22°C with a light/dark cycle of 12 h. Mice were kept in standard ventilated cages and acclimated for one-week following arrival into the UNSW animal facility. Mice were provided food and water ad libitum and their wellbeing was monitored regularly.

An orthotropic CRC mouse model was established by using intra-rectal tumour cell injection method with minor modifications of the previously reported work.[31] In brief, 6-8-week-old female NOD/SCID mice were fasted of food for 6 h prior to cancer cell injections, followed by rapid anesthesia induction with 2−4% isoflurane and maintenance at 1−3% with 1 L/min oxygen. Lubricated blunt-tip forceps were used to dilate the anal canal, exposing the distal anal and rectal mucosa. Subsequently, $4 \times 10^5$ HCT-116-Luc2 cells suspended in 10 μL PBS and 10 μL Matrigel were orthotopically inoculated into the distal posterior rectal submucosa, 1−2 mm above the anal canal using a 30-gauge needle (Terumo, Tokyo, Japan). Mice were closely monitored for 1 to 72 h post-injection for early detection of adverse events, with subsequent monitoring occurring at least bi-weekly.

Tumour formation and growth over time were monitored once a week by using the IVIS Spectrum CT imaging system (Perkin Elmer, Waltham, US). Typically, mice were intraperitoneally injected with 150 mg/kg of D-Luciferin. Mice were then anesthetized with isoflurane, with anaesthesia maintained throughout imaging using the IVIS spectrum imaging system for bioluminescence detection via Living Image® 4.5.2 software. When tumour reached the 100 mm$^3$ volume (equivalent to approximately 4−$6 \times 10^{10}$ photons/s of bioluminescence signal in this study), one group of mice were treated with X-ray radiation. At 27 days post treatment, the terminal blood collection (500 ~ 750 μL per mouse) was performed by the cardiac puncture technique with 25-gauge needles. K3 EDTA tubes were used for blood samples collection, allowing the isolation of blood plasma through centrifugation (1000 × g, 10 min). The isolated mice blood plasma was stored at -80°C for further use.

## 7. Application of DANCER for the detection of ctDNA in mouse plasma

The DANCER reaction mixture for ctDNA (PIK3CA E542KM[19]) detection was prepared as follows: 1 μL 100 μM (100 pmol) of Cas12a protein was gently mixed with 5 μL 20 μM (100 pmol) of gRNA-ct to form the Cas12a RNP in 5 mL 1X NEB 2.1 buffer. Subsequently, 200 μL of 5 μM (1 nmol) of Cir-amplifier solution was added and well mixed to form the reaction mixture.

Afterwards, 10 μL of spiked in sample or collected mouse plasma was added to 90 μL of the prepared reaction mixture for activating *trans*-cleavage of Cas12a and enabling the CRISPR/Cas biosensing reaction. A SpectraMax iD5 multi-Mode Microplate Reader (Molecular Devices) was applied for the

detection of fluorescence readout. The Ex/Em of Tex-Cir-reporter-BHQ2 was 570/615 nm. All the DNA and RNA oligos used in this experiment are listed in Table S6.

## 8. Application of colorimetric Cir-amplifier based DANCER for ctDNA detection in mouse plasma using lateral flow assay

The colorimetric Cir-amplifier based DANCER reaction mixture was prepared as follows: 1 μL 100 μM (100 pmol) of Cas12a protein was gently mixed with 5 μL 20 μM (100 pmol) of gRNA-ct to form the Cas12a RNP in 5 mL 1X NEB 2.1 buffer. Subsequently, 200 μL of 5 μM (1 nmol) of colorimetric Cir-amplifier were added and well mixed to form the reaction mixture.

Afterwards, 10 μL of collected mouse plasma was added to 90 μL of the prepared reaction mixture for activating *trans*-cleavage of Cas12a and enabling the CRISPR/Cas biosensing reaction. After 15min reaction, 5 μL of the reaction mixture was added to 95 μL of HybriDetect assay buffer (Milenia) and run on HybriDetect lateral flow strips (Milenia). All the DNA and RNA oligos used in this experiment are listed in Table S6.

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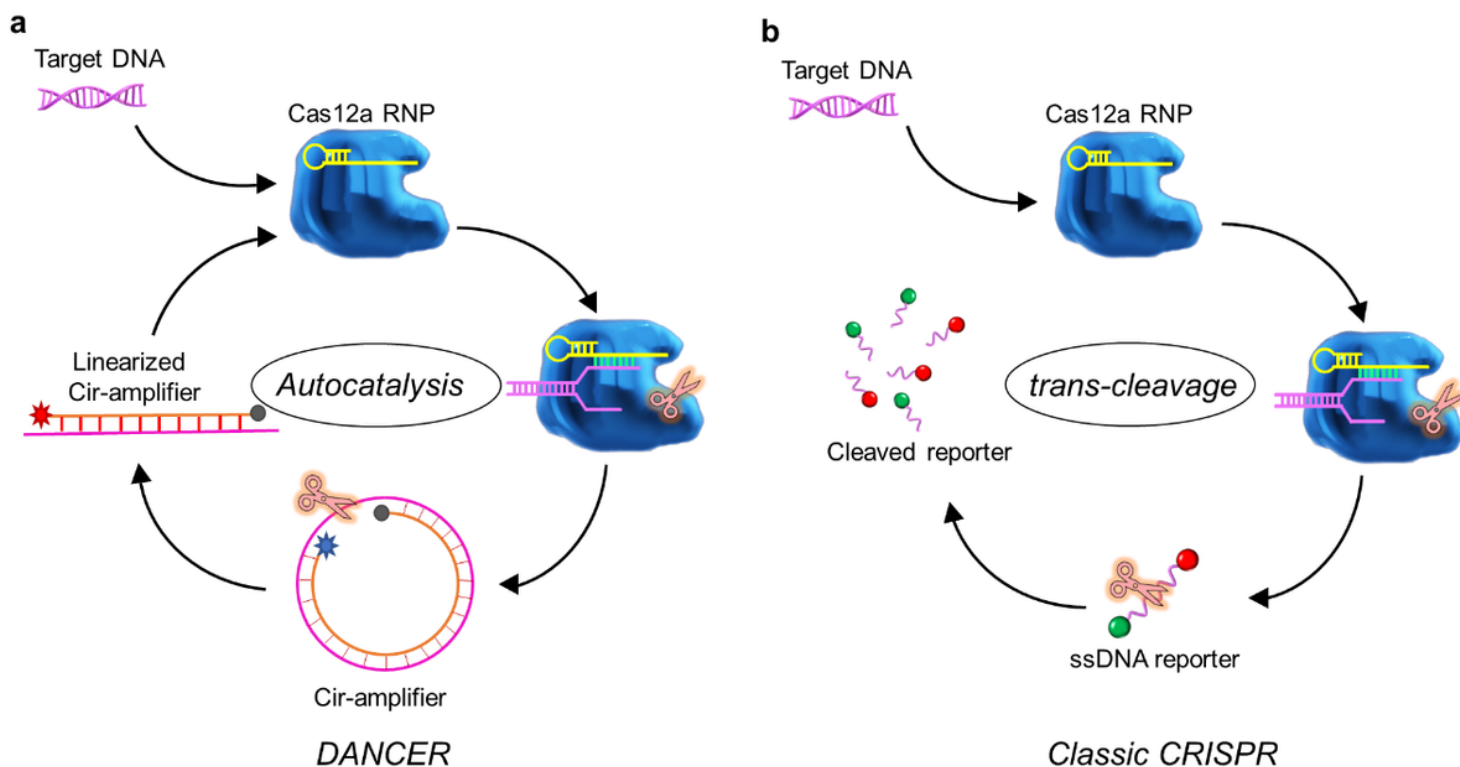
# Figures



## Figure 1

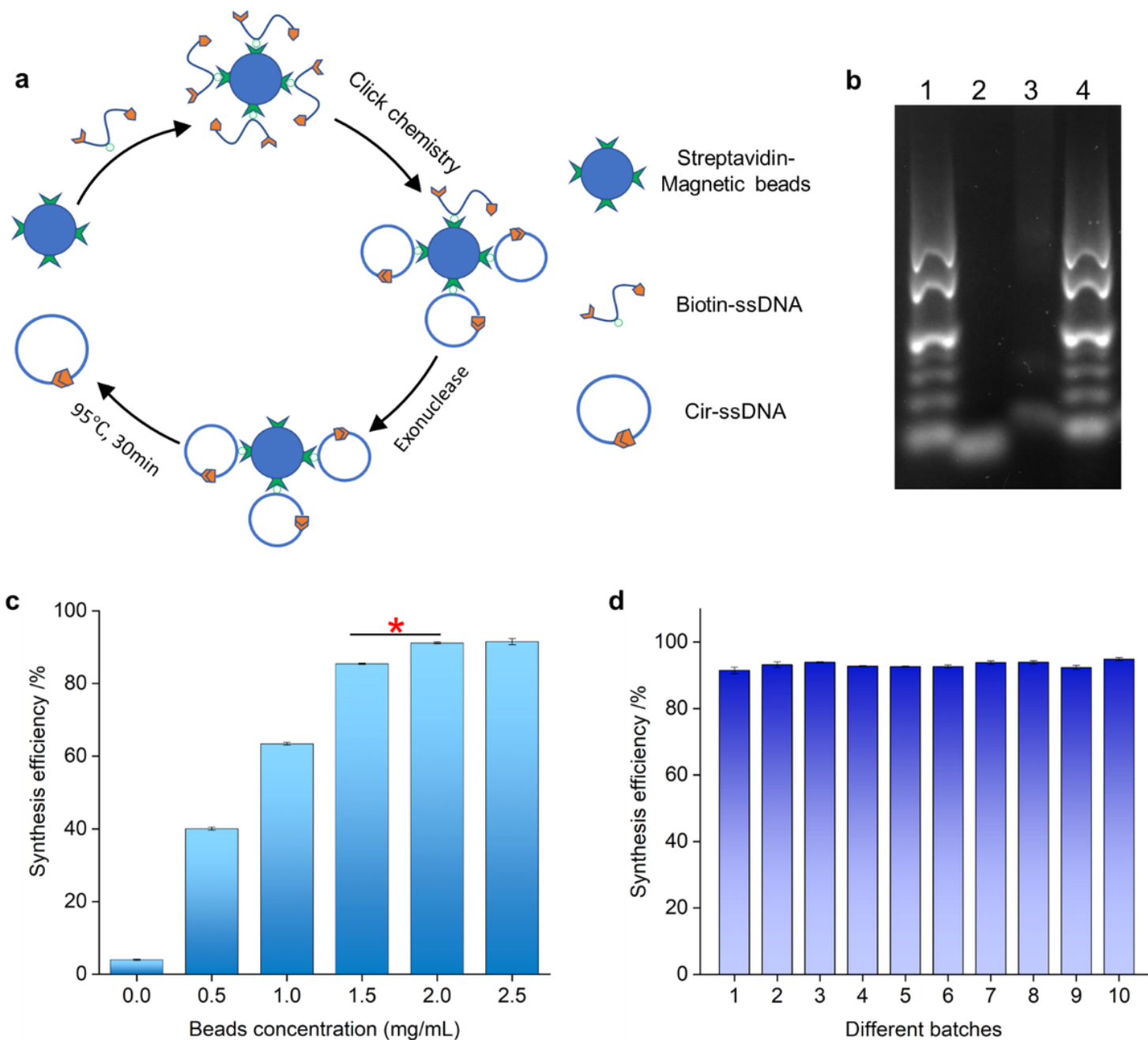The schematics of DANCER system (a) and classic CRISPR/Cas12a sensors (b).

Figure 2

Synthesis and characterization of Cir-ssDNA (Method 1). (a) Schematic for the synthesis of Cir-ssDNA using a bead-based click chemistry method. Biotin-ssDNA with specific modifications (5'-Azide (N3); 3'-CHCH; internal-Biotin); (b) Demonstration of the formation of Cir-ssDNA using denaturing polyacrylamide gel (dPAGE) electrophoresis ( from left to right :1. 10 bp ladder; 2. 19nt linear ssDNA; 3. 19nt Cir-ssDNA; 4. 10 bp ladder.); (c) Optimization of Cir-ssDNA synthesis efficiency using a bead-based click chemistry method; (d) Reproducibility of Cir-ssDNA synthesis using a bead-based click chemistry method.
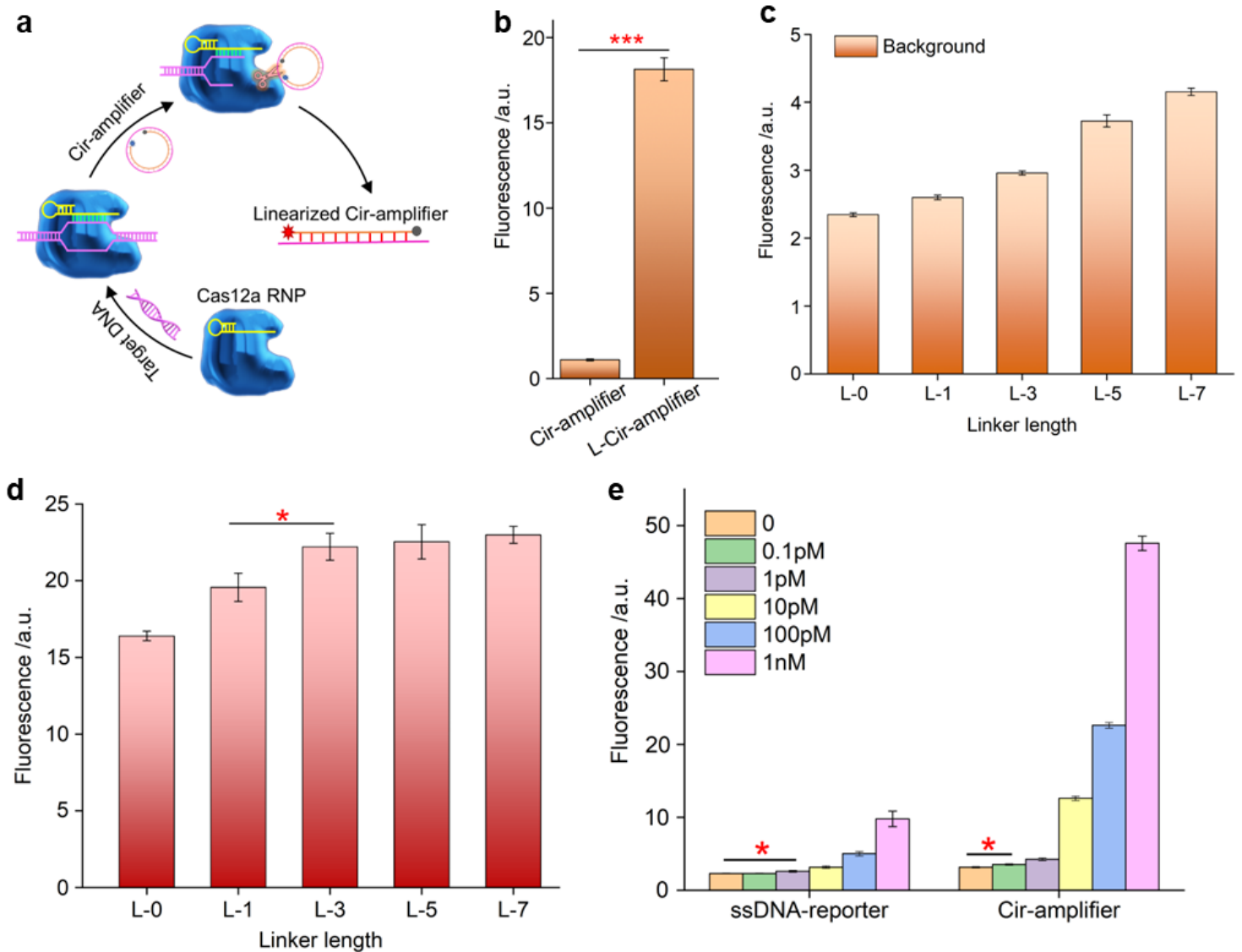
**Figure 3**

Performance of Cir-amplifiers as reporters in a classic CRISPR/Cas12a biosensing system (Method 2). (a) Schematics for the investigation of reporter performance of Cir-amplifier; (b) Comparison of the fluorescence signals of a Cir-amplifier and linearized Cir-amplifier (L-Cir-amplifier) (18nt dsDNA with 3nt ssDNA); (c) Background signals of Cir-amplifiers with different linker lengths (18nt dsDNA). Here L-x represents the linker length is x nt; (d) Investigation of the Cir-amplifier linker length in a classic CRISPR/Cas12a biosensing system (18nt dsDNA, 100pM target DNA). Here L-x represents the linker length is x nt; (e) Comparison of the detection limits of classic CRISPR/Cas12a biosensors with Cir-amplifiers (18nt dsDNA with 3nt ssDNA) and with linear ssDNA reporters (TTATT) with identical fluorophore-quencher pairs.
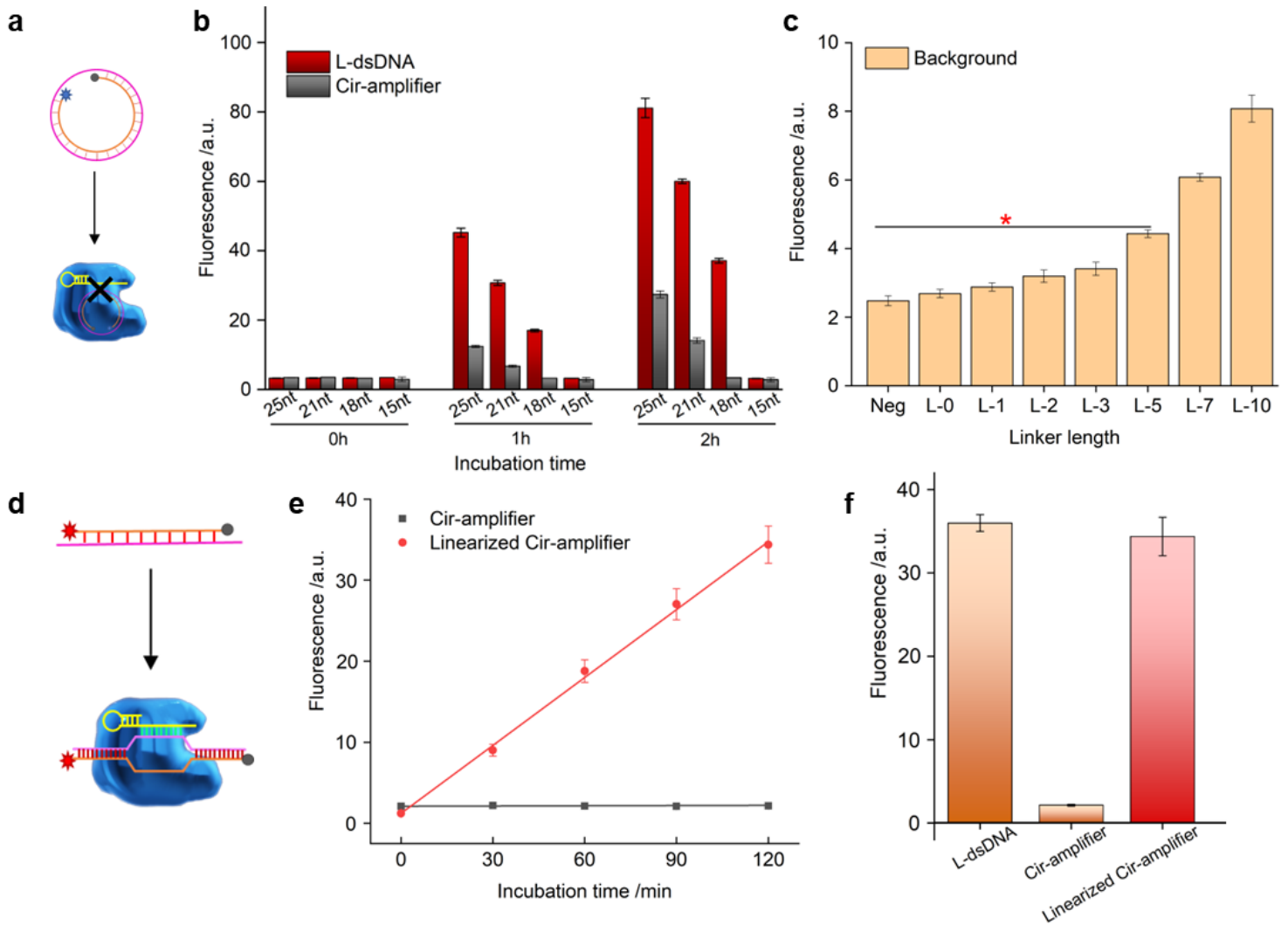
**Figure 4**

RNP activation efficiency of Cir-amplifiers and linearised Cir-amplifiers in a CRISPR/Cas12a biosensing system (Method 3&4). (a) Schematics for the application of Cir-reporters as activators for Cas12a RNP; (b) Evaluation of the dsDNA length in Cir-amplifier for the activation of a classic CRISPR/Cas12a biosensing system (with a 3nt ssDNA linker); (c) Evaluation of the linker length in Cir-amplifier for the activation of a classic CRISPR/Cas12a biosensing system (with 18nt dsDNA "fake target"). Here L-x represents the linker length is x nt; (d) Schematic for the application of linearized Cir-amplifier as activators for Cas12a RNP; (e) Evaluation of the RNP activation efficiency of linearized Cir-amplifiers; (f) Comparison of the RNP activation efficiency by linearized Cir-amplifiers and by corresponding linear dsDNA.
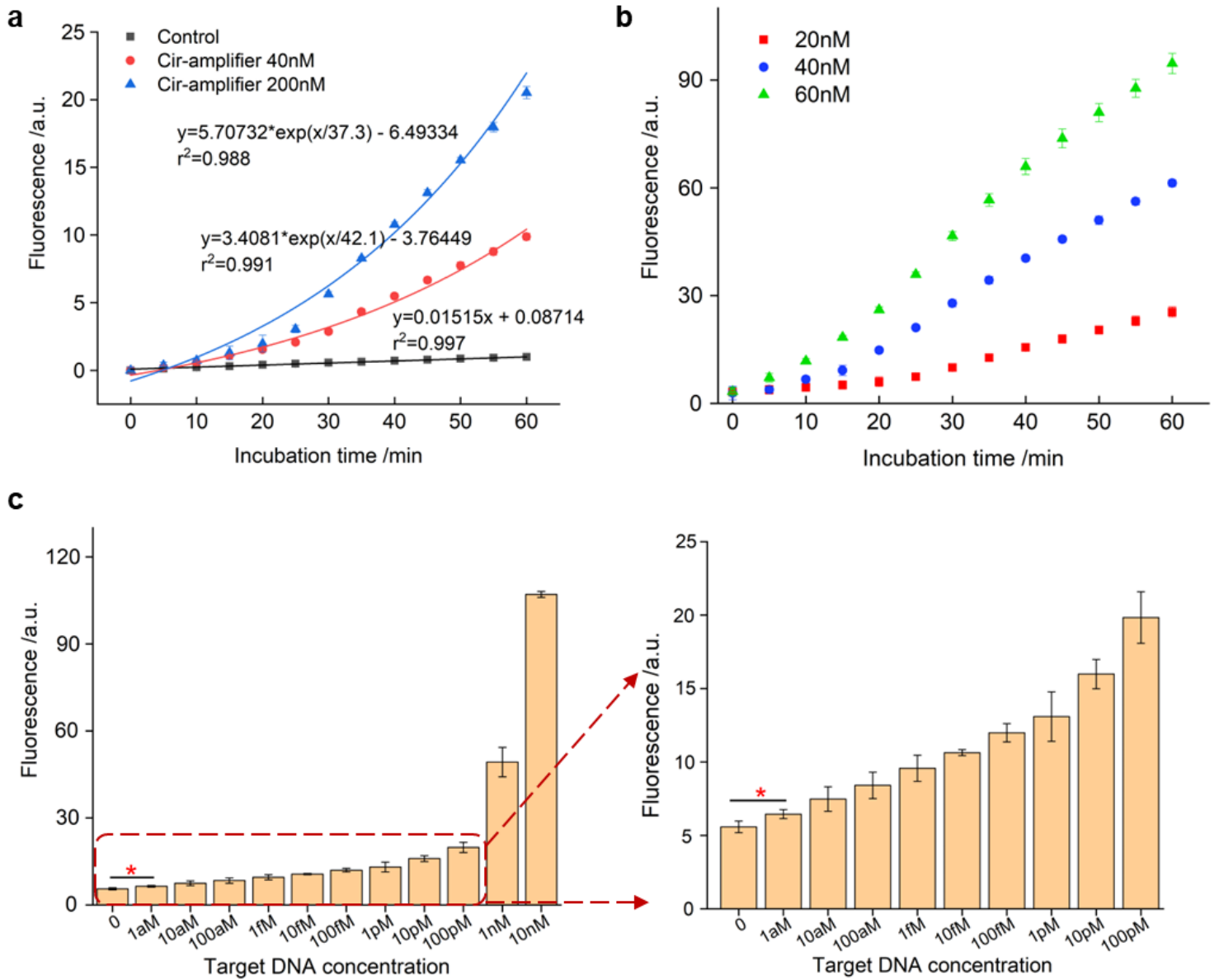
**Figure 5**

Characterisation of the DANCER sensor (Method 5). (a) The DANCER fluorescent signal as a function of Cir-amplifier concentration (20 nM of Cas12a RNP, and 1 pM of target DNA); (b) The DANCER fluorescent signal as a function of Cas12a RNP concentration (200 nM of Cir-amplifier, and 1 pM of target DNA); (c) The calibration curve of DANCER (60 nM of Cas12a RNPs, and 200 nM of Cir-amplifiers).
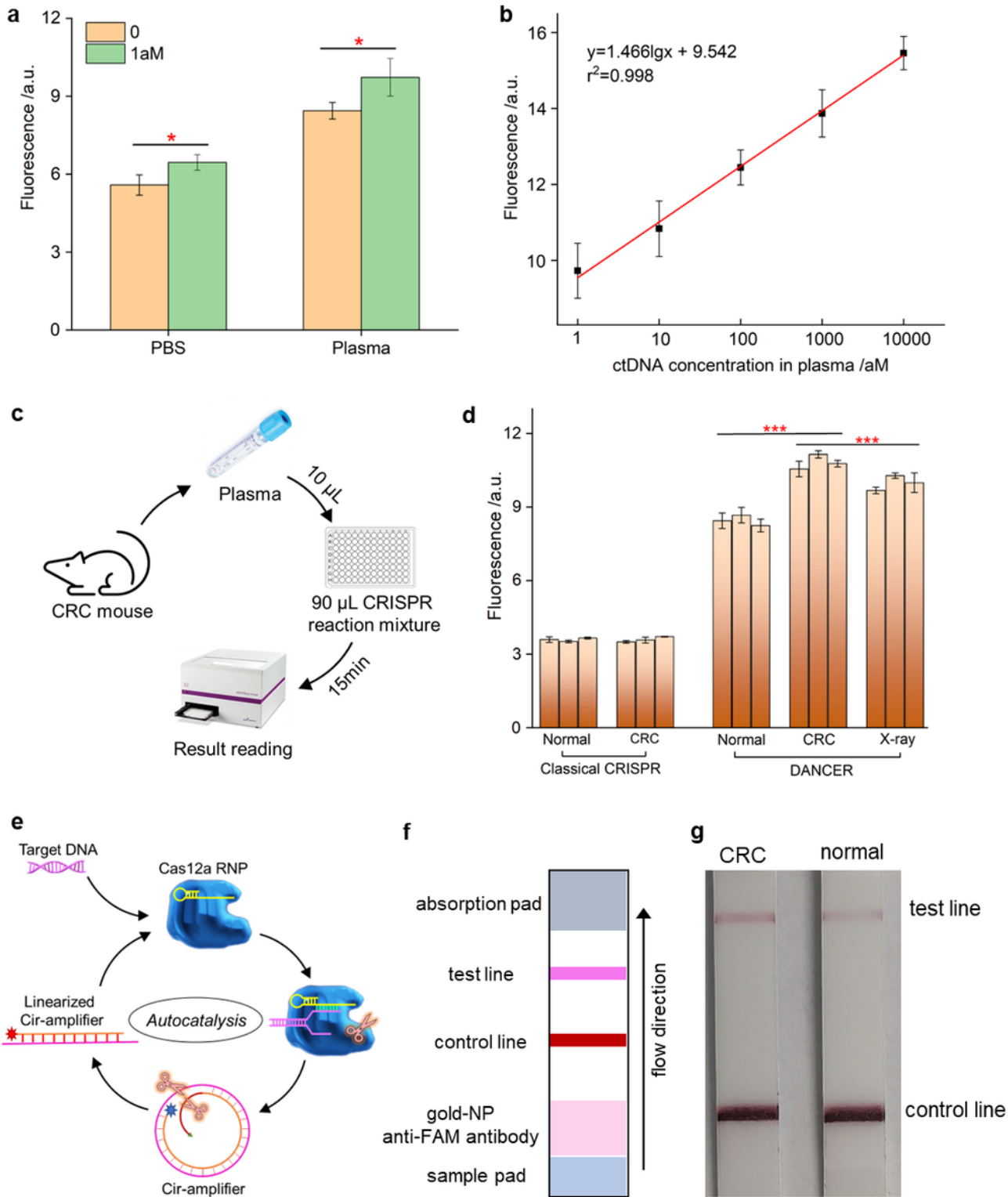
## Figure 6

The application of DANCER for the point-of-care detection of ctDNA from mouse plasma (Method 7&8). (a) Biosensing performance of DANCER in PBS and mouse plasma; (b) The calibration curve of DANCER in mouse plasma; (c) The procedure of DANCER for ctDNA detection from mouse plasma; (d) The application of DANCER for ctDNA detection in mouse plasma (n=3); (e) The establishment of a colorimetric Cir-amplifier through extending of the cDNA with 5nt CCCCC and a biotin on 3' end; (f) The

schematic of the colorimetric lateral flow assay for the detection of biotin-DNA-FAM reporter (control line: streptavidin; test line: secondary antibody); (g) The application of colorimetric Cir-amplifier-based DANCER for ctDNA detection in mouse plasma with a lateral flow assay.

## Supplementary Files

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- SupplementaryInformation.docx