Unveiling a Novel RNA G-Triplex Structure: Its Function and Potential in CRISPR-based Diagnostics

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1. Experimental

1.1 Materials

Details of the nucleotide sequences used in this study are provided in Table S2. The RNA G-triplex sequences, including T-TERRA, BCL2-△5 rG3 and BCL2-△3 rG3 (derived from TERRA G4 and BCL2 rG4, respectively), were provided by *General BioSystem Co., Ltd.* (Anhui, China) as HPLC grade lyophiles. The S-TERRA sequence, derived from T-TERRA, has the same nucleotide composition but was rearranged to prevent G3 structure formation. The *Leptotrichia wadei* Cas13a (LwaCas13a) enzyme was supplied by *Magigen Company* (Guangzhou, China). The crRNA and target design were based on our previous study (*Chemical Communications*, 2024, 60, 3166). The Cas13a cleavage reporters including T-TERRA-FQ and T-TERRA-FRET were synthesized by *Sangon Biotech Co., Ltd.* (Shanghai, China). To prevent RNA degradation, a murine RNAse inhibitor was purchased from *Vazyme Co., Ltd.* (Nanjing, China).

1.2 Sample Preparation

Before use, all RNA stock solutions were dissolved in DEPC-treated water, heated at 95°C for 10 minutes, cooled to room temperature, and diluted to the desired concentrations in Tris buffer (10 mM Tris-HCl, 1 mM EGTA, pH 7.5) or cleavage buffer (20 mM HEPES, 6 mM $MgCl_2$, pH 7.5), which also contained either 50 or 100 mM KCl or NaCl, as needed.

1.3 Circular Dichroism (CD) analysis

CD measurements were conducted using a Chirascan spectropolarimeter (Applied Photophysics, UK) with a 0.1 cm path-length cuvette. Spectra were obtained by averaging three scans collected from 220 to 320 nm at a scanning rate of 1 nm/s, at room temperature (~25°C). For sample preparation, 10 μL of either rG3 or S-TERRA oligonucleotides (100 μM) was added to 190 μL of Tris buffer containing 50 mM NaCl or KCl, and the mixture was vortexed at room temperature. All spectra were recorded in triplicate and baseline-corrected using corresponding buffer blanks.

For T_m measurement experiments based on CD spectra, we measured the absorbance of the sample at 265 nm at different temperatures and calculated the T_m value through curve fitting using a specific formula. To prepare the samples, 20 μL of either T-TERRA or S-TERRA oligonucleotides (100 μM) was added to 180 μL of Tris buffer containing varying concentrations of NaCl or KCl. The well-mixed sample was then placed in a cuvette, and the temperature was gradually increased from 10°C to 90°C. Spectra were collected at 2°C intervals, and the measurement was repeated three times. The resulting curve of CD 265 nm absorbance as a function of temperature is fitted using the Boltzmann formula: $Y= (Y_{min}+(Y_{max}-Y_{min})/(1+\exp((X-X_{mid})/HillSlope)))$

1.4 NMR spectroscopy

^{1}H NMR data were acquired using a Bruker Ascend 700 MHz NMR spectrometer (*Bruker Corporation*, Billerica, MA, USA) at temperature of 25℃ and 37℃. Oligonucleotides samples were prepared in KP buffer (10 mM KH_2PO_4/K_2HPO_4, 100 mM KCl, 90%/10% H_2O/D_2O) to a final concentration of 0.5 mM. The excitation sculpting with gradients was applied for water suppression, with DSS (4, 4-dimethyl-4-silapentane-1-sulfonic acid) serving as the reference for chemical shift calibration. Data processing and analysis were carried out using TopSpin 4.4.1 (Bruker).

1.5 ThT/NMM/Hemin binding to rG3 and functional analysis

To verify that G3 forms higher-order structures with properties similar to G4, we tested its interactions with ThT, NMM, and Hemin, all of which were obtained from *Macklin Biochemical Technology Co., Ltd.* (Shanghai, China). For analyzing fluorescence intensity changes upon ThT binding to rG3, we combined 5 μL of rG3 (10 μM) and 1 μL of ThT (1 mM) in an eppendorf tube containing 194 μL of Tris buffer with 100 mM KCl or NaCl. The solution was vortexed at room temperature for 30 minutes in the dark, then transferred to a microplate for fluorescence measurement using a SpectraMax i3 microplate reader with an excitation at 420 nm, emission from 450 to 600 nm (2 nm intervals), and a set temperature of 25°C. Each experiment was

conducted in triplicate.

For NMM binding studies, rG3 (5 μL, 10 μM) and NMM (1 μL, 1 mM) were similarly combined with Tris buffer containing 100 mM KCl or NaCl, vortexed under the same conditions, and measured on the microplate reader with an excitation at 400 nm, emission from 550 to 700 nm, and 2 nm increments. Each trial was performed three times.

To assess hemin binding, 5 μL of rG3 (10 μM) and 1 μL of hemin (0.1 mM) were added to an Eppendorf tube with 194 μL of Tris buffer containing either KCl or NaCl (100 mM). After vortexing for 30 minutes in the dark, 100 μL of TMB substrate solution (containing TMB and H_2O_2, purchased from *Macklin Biochemical Technology Co., Ltd.*) was added, and time-course absorbance measurements at 370 nm were recorded every 2 min over a 0–30 min period.

1.6 Cas13a-based cleavage assays

1.6.1 Fluorescence measurement of the Cas13a-based cleavage assay results

LwaCas13a (1 μL, 1 μM) was first pre-incubated with crRNA (1 μL, 1 μM) and RNAse inhibitor (1 μL, 40 U/μL) in a total volume of 20 μL HEPES cleavage buffer (20 mM HEPES, 50 mM Na^+, 6 mM MgCl2, pH 7.4) at 37°C for 10 min. Next, the target (SARS-CoV-2 E-gene, 10 μL, 10 nM) or buffer (as control) and T-TERRA-FRET reporter (20 μL, 10 μM) were added into the reaction solution. The mixtures were then incubated at 37°C for a cleavage period of 30 minutes, ensuring sufficient time for the Cas13a enzyme to cleave the reporter and generate a detectable fluorescence signal. After the incubation period, the mixture was diluted to a total volume of 300 μL for the fluorescence measurements. Fluorescence measurements were conducted using a FluoroMax-4 spectrofluorometer (Horiba, Japan) at room temperature (~25°C). The excitation and emission slits were both set to 5 nm. Excitation was set at 488 nm, and emission was collected within the range of 500 to 750 nm. For sensitivity analysis of T-TERRA-FQ as a Cas13a cleavage reporter, target concentrations were adjusted to a final gradient of 0, 0.02, 0.04, 0.08, 0.16, 0.20, and 1.00 pM, while all other experimental conditions were maintained as described above.

1.6.2 CD measurement of the Cas13a-based cleavage assay results

In preparation, LwaCas13a (1 μL, 10 μM) was incubated with crRNA (1 μL, 10 μM) and RNAse inhibitor (1 μL, 40 U/μL) in a total volume of 20 μL HEPES cleavage buffer. The target (SARS-CoV-2 E-gene, 10 μL, 2 μM) or buffer (as control) and T-TERRA oligonucleotides (10 μL, 100 μM) were then added, followed by incubation at 37°C for 2 h. After the incubation period, the mixture was diluted to a total volume of 200 μL for the CD analysis.

For the time-course CD measurement, the cuvette holder was pre-heated to 37°C and maintained at this temperature with continuous monitoring throughout the experiment. LwaCas13a (1 μL, 10 μM), crRNA (1 μL, 10 μM), and RNAse inhibitor (1 μL, 40 U/μL) were pre-incubated in a total volume of 20 μL cleavage buffer at 37°C for 10 min. Following the pre-incubation step, the T-TERRA sequence (10 μL, 100 μM) was added to the reaction mixture. Subsequently, the target (SARS-CoV-2 E-gene, 10 μL, 2 μM) and 160 μL buffer were added. After rapid mixing, CD measurements commenced immediately, with readings taken every 5–10 minutes over 2 h.

1.6.3 Urea-PAGE analysis of Cas13a-based cleavage assay results

Urea polyacrylamide gel electrophoresis (PAGE) was used to evaluate the *trans*-cleavage activity of Cas13a on T-TERRA. LwaCas13a (2 μL, 1 μM), crRNA (2 μL, 1 μM) and RNAse inhibitor (1 μL, 40 U/μL) were pre-incubated in a 20-μL HEPES cleavage buffer at 37°C for 10 min. Next, FRET-labeled T-TERRA rG3/G4 oligonucleotides (20 μL, 10 μM) and the target (10 μL, 10 nM) or buffer control (10 μL) were added to the reaction mixture, followed by incubation at 37°C for a cleavage period. The reaction was then heated to 65°C for 15 min to inactivate LwaCas13a. A portion of the cleaved sample (8 μL) was combined with 2 μL of loading buffer and loaded onto a 30% denaturing PAGE with 8 M urea. Electrophoresis was performed at 180 V (approximately 40 V/cm) for 120 minutes using a Mini-PROTEAN Tetra Cell system (Bio-Rad) in 1×TBE buffer. The gel was subsequently scanned under "fluorescein" mode using a Bio-Rad ChemiDoc MP system (170-8280) from Bio-Rad (Shanghai, China).

1.7 Molecular modelling

To construct the RNA G-triplex (rG3) model, we started with a truncated TERRA sequence $[UUAGGG]_3$. Using the parallel DNA G-quadruplex structure (PDB ID: 2KQG) as a template, we manually adjusted the base sequence to form a three-layer G-triad model, in which each guanine was arranged to allow Hoogsteen hydrogen bonding within each plane. We utilized Discovery Studio Visualizer v24.1.0.23298 to convert DNA bases to RNA, adjusting for the necessary structural and torsional differences. The final RNA G-triplex model was then optimized to minimize steric clashes and refined to improve hydrogen bonding interactions between layers. For stabilization, potassium ions were introduced into the central cavity between each G-triad plane, positioned to form six coordination bonds with adjacent guanine O6 atoms, further reinforcing the structure.

2. Supplementary Tables

Table S1 T_m values of T-TERRA and S-TERRA under different conditions.

Name	Conditions	T_m (℃)
T-TERRA	Without NaCl/KCl	38.05
	50 mM NaCl	47.44
	100 mM NaCl	51.32
	50 mM KCl	84.17
	100 mM KCl	88.97
S-TERRA	Without NaCl/KCl	NA
	50 mM NaCl	39.21
	100 mM NaCl	39.52

Table S2 Information of the nucleotide sequences used in this study.

Name	Sequence (5' → 3')
TERRA G4	UUAGGGUUAGGGUUAGGGUUAGGG
T-TERRA	UUAGGGUUAGGGUUAGGG
T-TERRA-FRET	FAM-UUAGGGUUAGGGUUAGGG-TAMRA
T-TERRA-FQ	FAM-UUAGGGUUAGGGUUAGGG-BHQ1
BCL2 rG4	GGGGGCCGUGGGGUGGGAGCUGGGG
BCL2-△5 rG3	CCGUGGGGUGGGAGCUGGGG
BCL2-△3 rG3	GGGGGCCGUGGGGUGGGAGCU
S-TERRA	GGUGGAUUGAUGGAGUGU
crRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAA ACUCGAAGCGCAGUAAGGAUGGCUAGUGUA
Target	AGUUACACUAGCCAUCCUUACUGCGCUUCGAUUG

Table S3 Comparative responses of T-TERRA and TERRA G4 to various small molecules under different ionic conditions

Conditions	Fluorescence intensity in response to small molecules		
	ThT	**NMM**	**Hemin**
100 mM NaCl	T-TERRA＞TERRA G4	T-TERRA≈TERRA G4	T-TERRA≈TERRA G4
100 mM KCl	T-TERRA＞TERRA G4	T-TERRA＜TERRA G4	T-TERRA＜TERRA G4

Note: The symbols represent relative fluorescence intensity: ">" indicates a higher intensity, "<" indicates a lower intensity, and "≈" indicates similar intensity.

3. Supplementary Figures

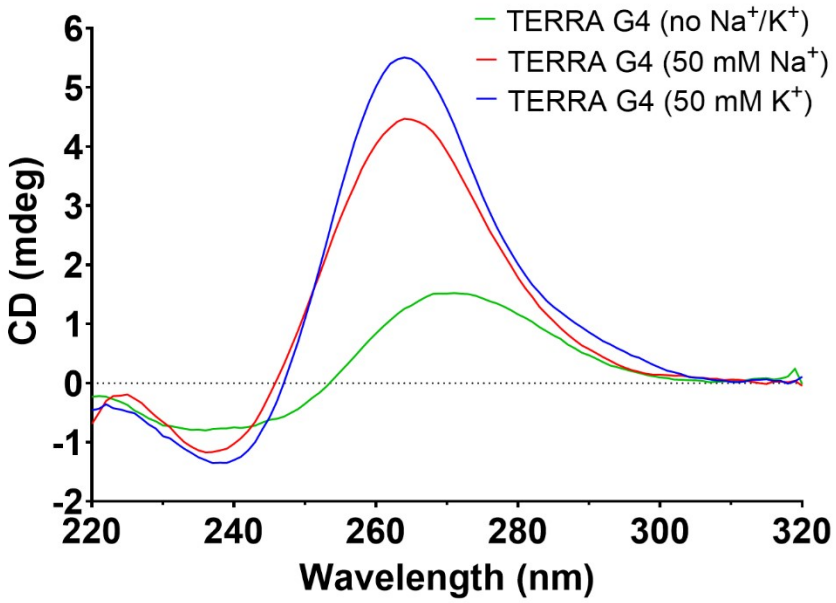

Figure S1. CD spectra of TERRA G4 under different Na^+ and K^+ conditions.

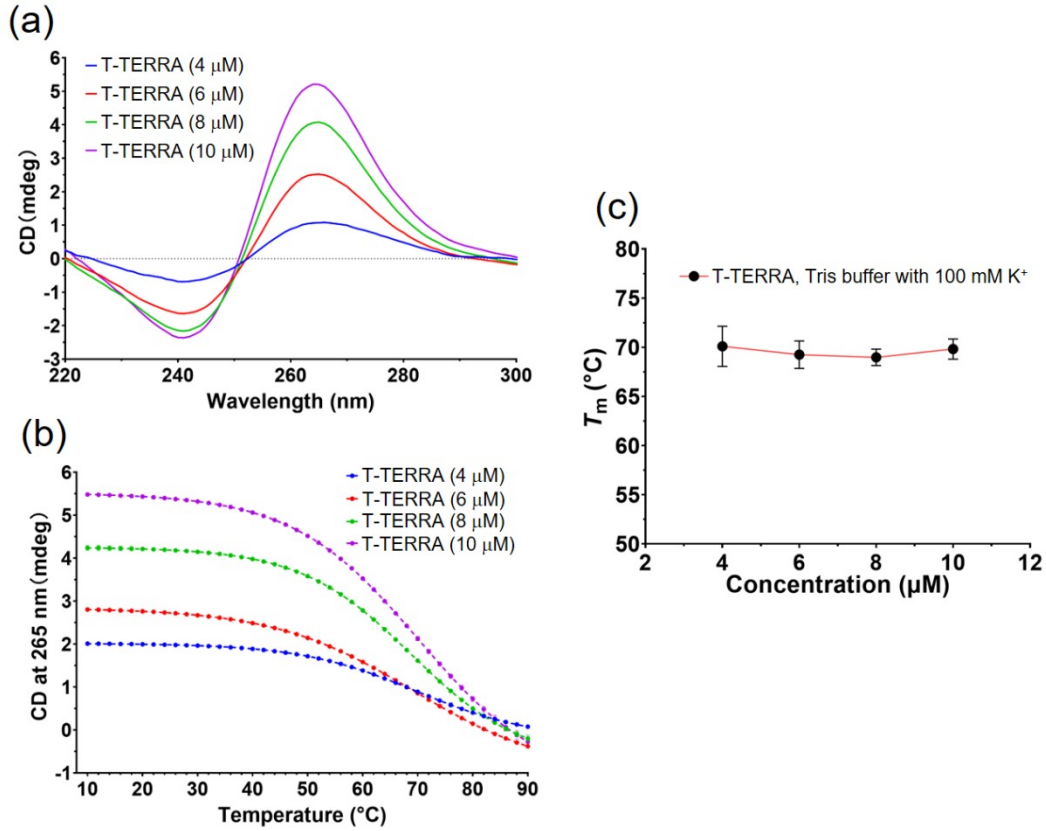

Figure S2. Plots of melting temperature (T_m) versus concentration of T-TERRA. (a) CD spectra of T-TERRA at different concentrations. (b) Thermal melting curves of T-TERRA at different concentrations. (c) Relationship between *T*m and concentration for T-TERRA.

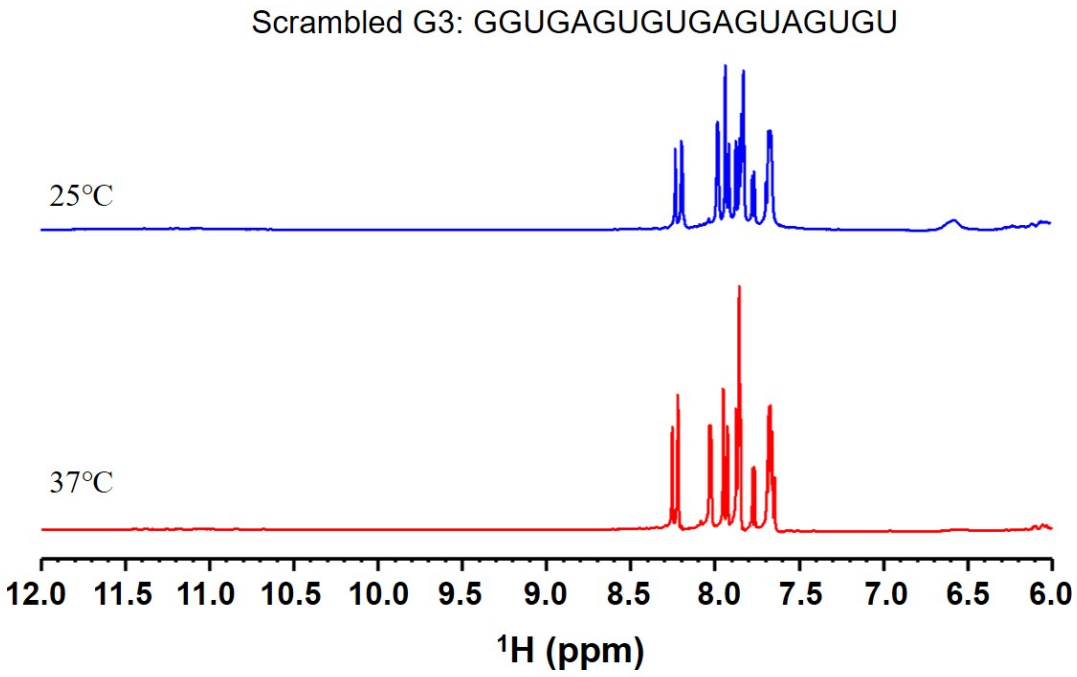

Figure S3 The ^{1}H NMR spectrum of S-TERRA at 6-12 ppm.

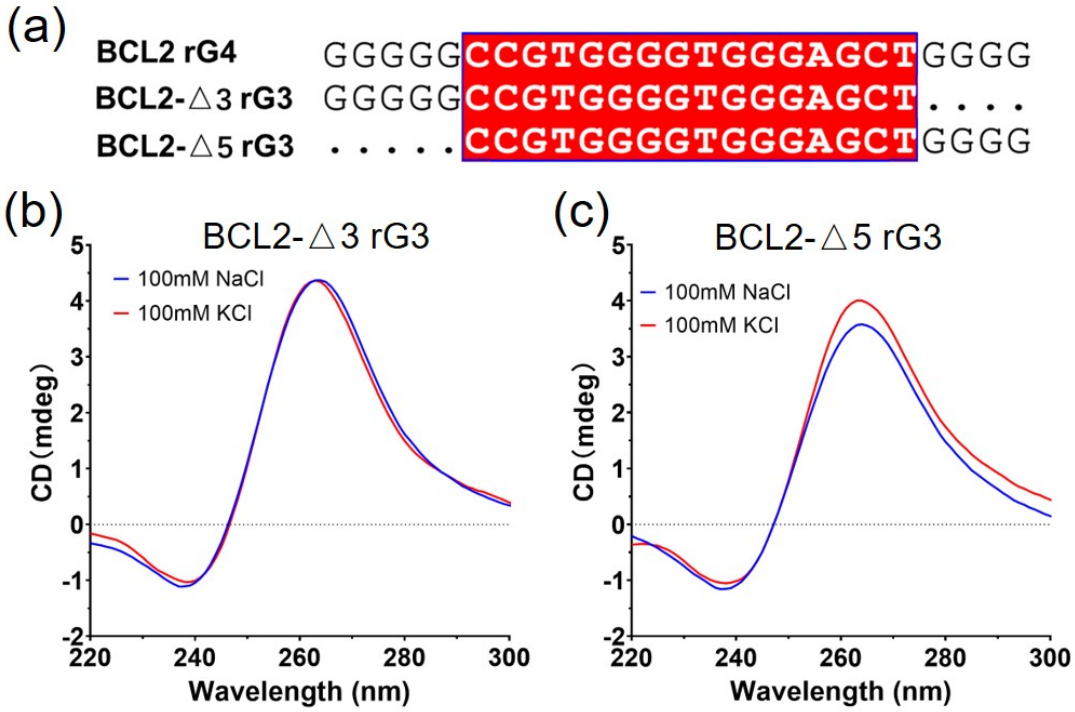

Figure S4 The CD spectrum of BCL2 rG3. (a) Sequence alignment results of BCL2 rG4, BCL2-△3 rG3 and BCL2-△5 rG3. (b-c) The CD spectrum of BCL2-△3 rG3 (b) and BCL2-△5 rG3 (c), indicating the formation of a parallel structure.

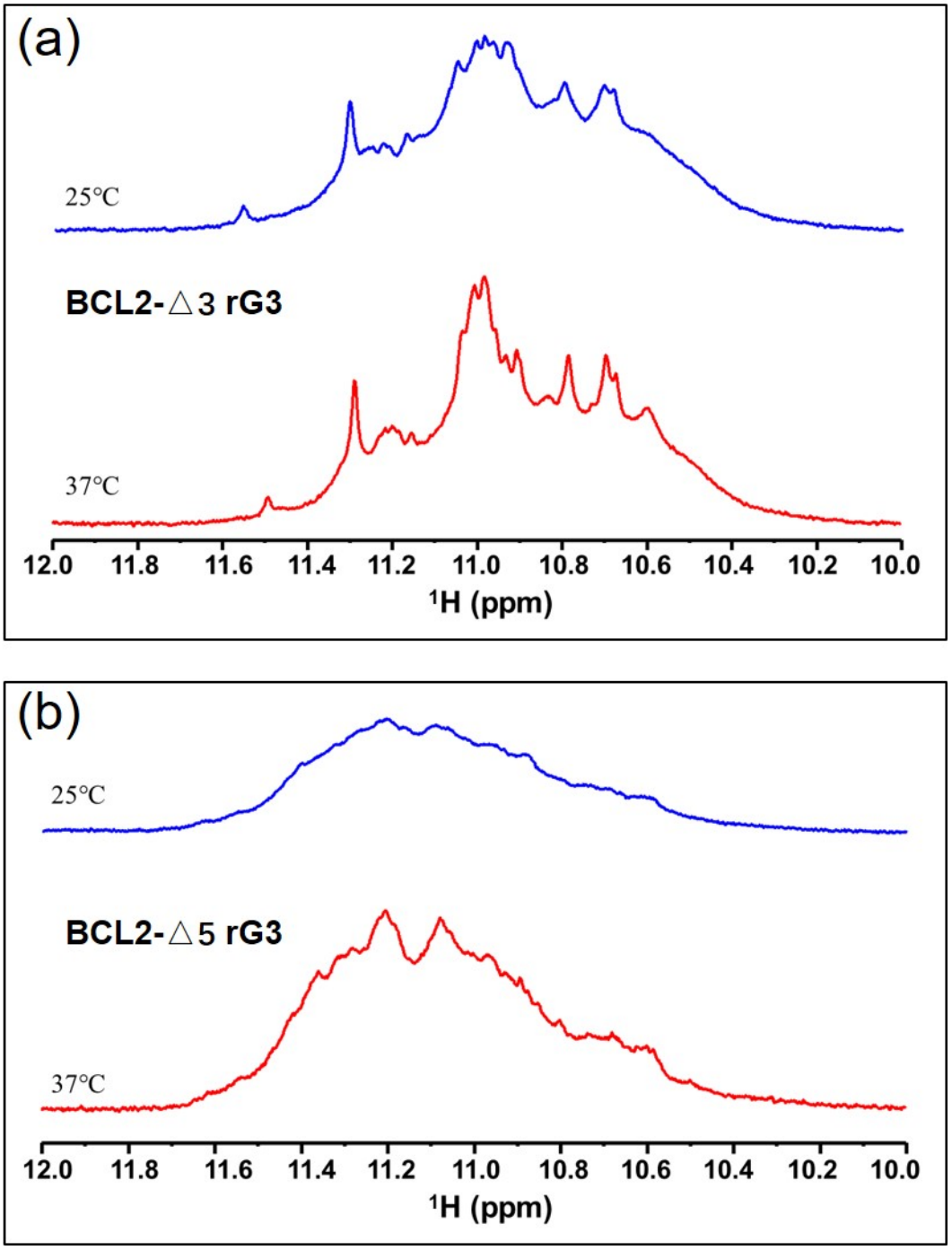

Figure S5 The ^{1}H NMR spectrum of BCL2 rG3. (a-b) The ^{1}H NMR spectrum of BCL2-△3 rG3 (a) and BCL2-△5 rG3 (b) at imino proton regions of 10-12 ppm.

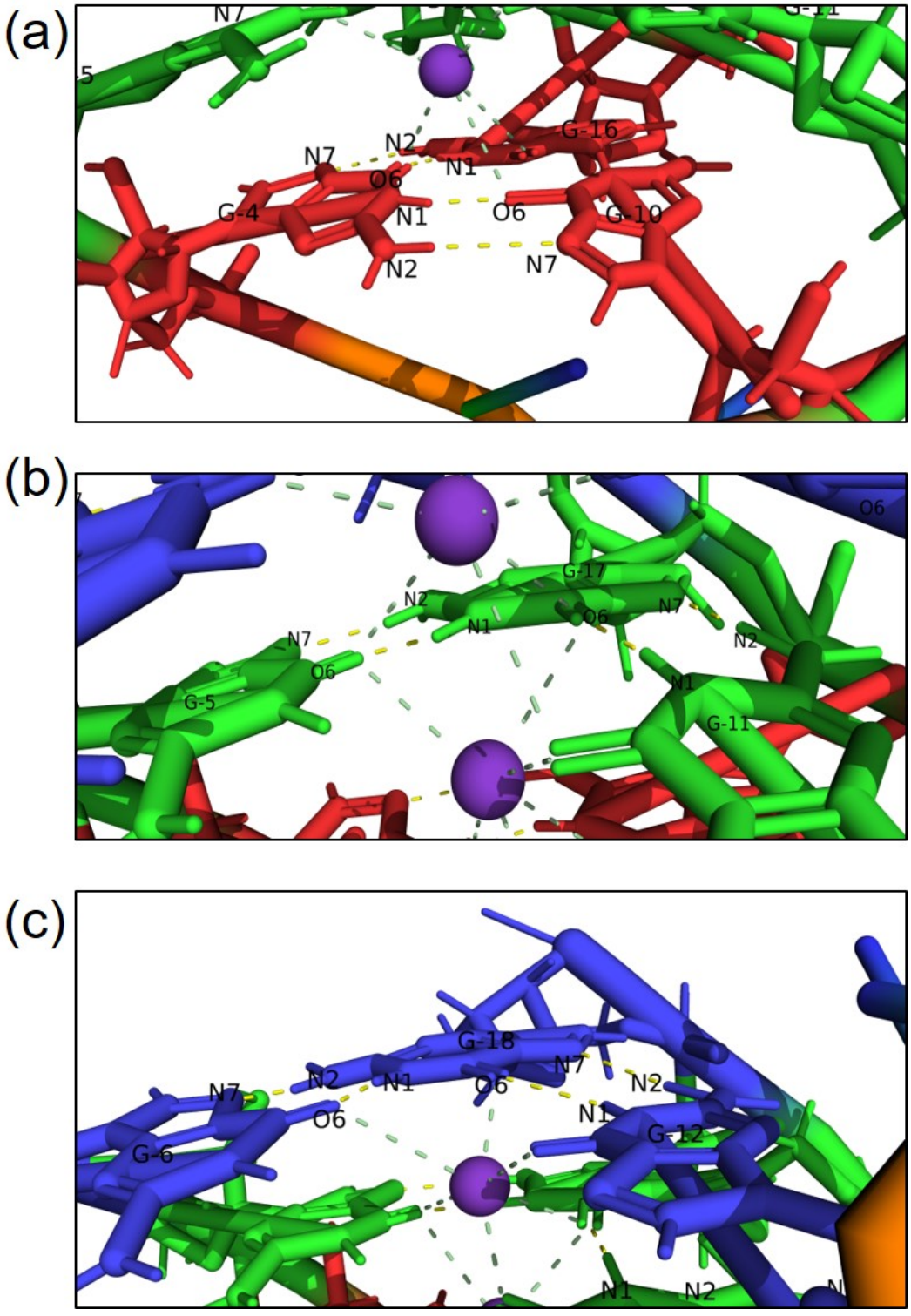

Figure S6 The illustration of three G-triads of T-TERRA G-triplex. (a-c) The G4-G10-G16 (a), G5-G11-G17 (b) and G6-G12-G18 (c) G-triad of T-TERRA G-triplex. The yellow dashed lines represent hydrogen bonding interactions, while the cyan dashed lines indicate K^+ ion coordination interactions.

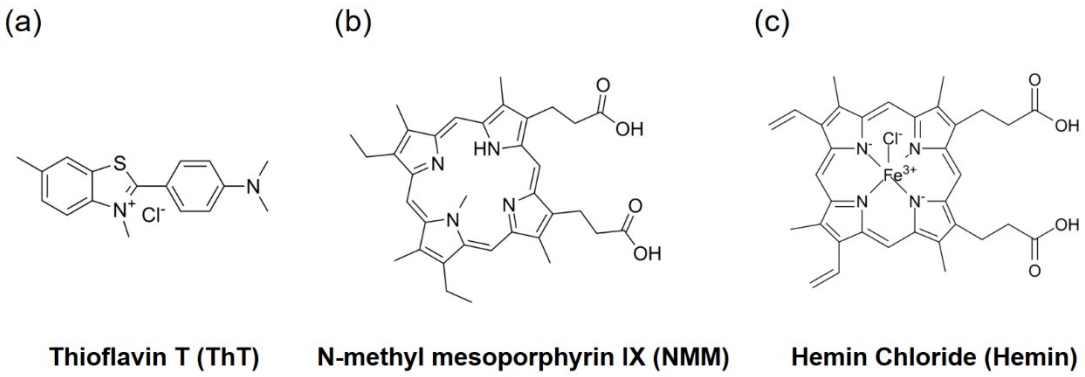

Figure S7. Chemical structure of rG3 functional small molecule ThT (a), NMM (b) and hemin (c).

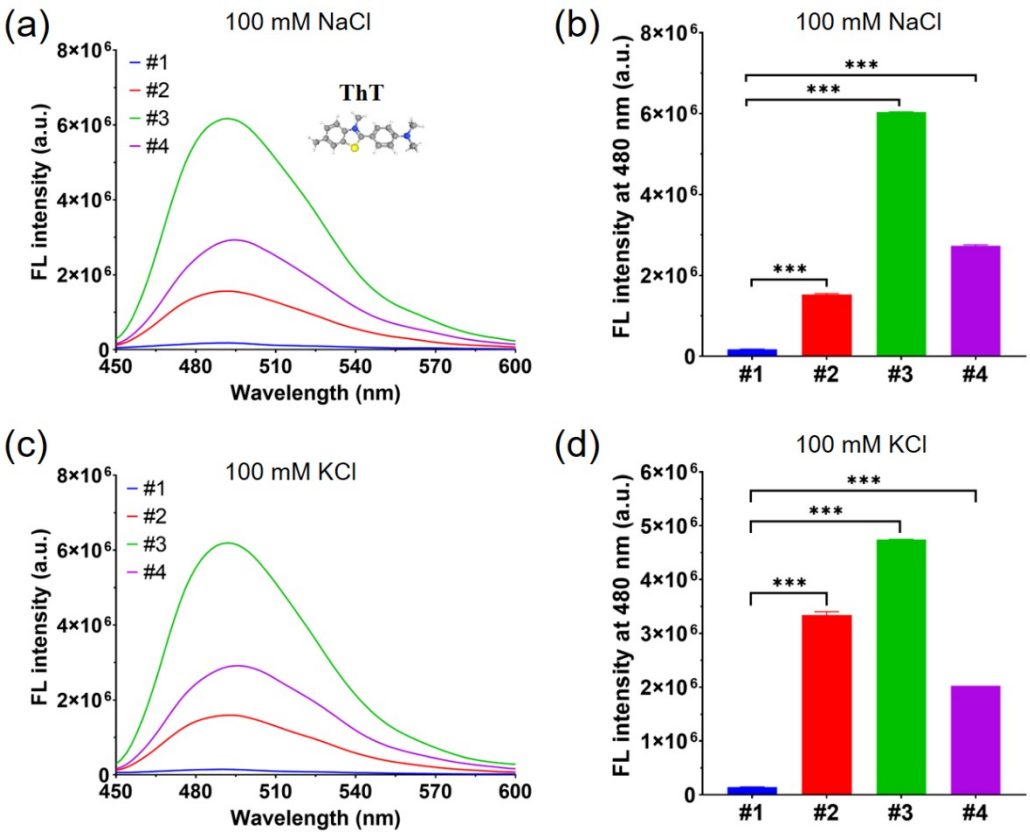

Figure S8 The fluorescence spectra of ThT with BCL2 rG3. (a) The fluorescence spectrum under 100 mM NaCl. (b) The fluorescence intensity at 480 nm under 100 mM NaCl. (c) The fluorescence spectrum under 100 mM KCl. (d) The fluorescence intensity at 480 nm under 100 mM KCl. #1—#4 represent ThT binding with buffer, BCL2 rG4, BCL2-△3 rG3 and BCL2-△5 rG3, respectively.

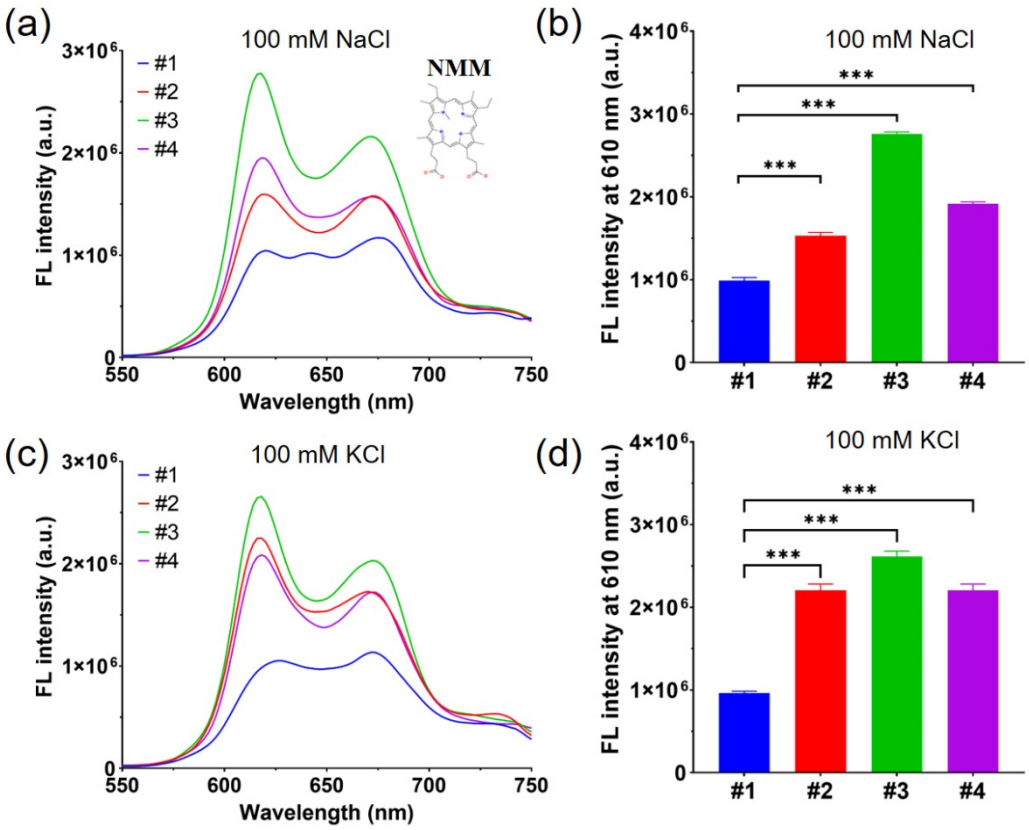

Figure S9 The fluorescence spectra of NMM with BCL2 rG3. (a) The fluorescence spectrum under 100 mM NaCl. (b) The fluorescence intensity at 680 nm under 100 mM NaCl. (c) The fluorescence spectrum under 100 mM KCl. (d) The fluorescence intensity at 680 nm under 100 mM KCl. #1－#4 represent ThT binding with buffer (as control), BCL2 rG4, BCL2-△3 rG3 and BCL2-△5 rG3, respectively.

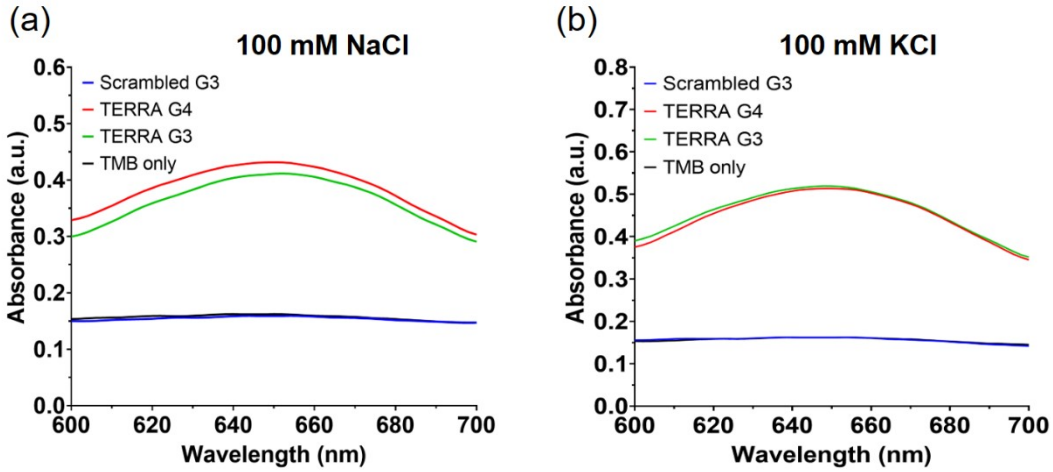

Figure S10 The absorbance spectrum of TMB oxidation products based on T-TERRA rG3/hemin peroxidase-like activity.

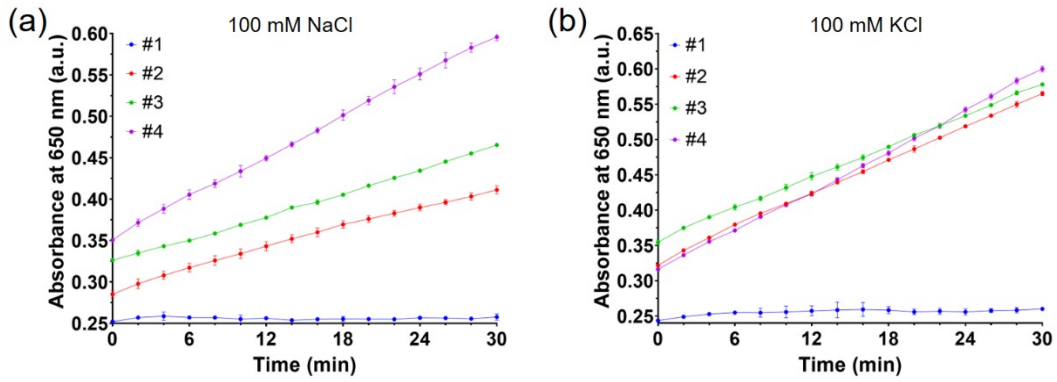

Figure S11 Time-course fluorescence intensity at 650 nm of TMB oxidation products catalyzed by the peroxidase-like activity of BCL2 rG3/hemin. #1－#4 represent hemin binding with buffer (as control), BCL2 rG4, BCL2-△3 rG3 and BCL2-△5 rG3, respectively.

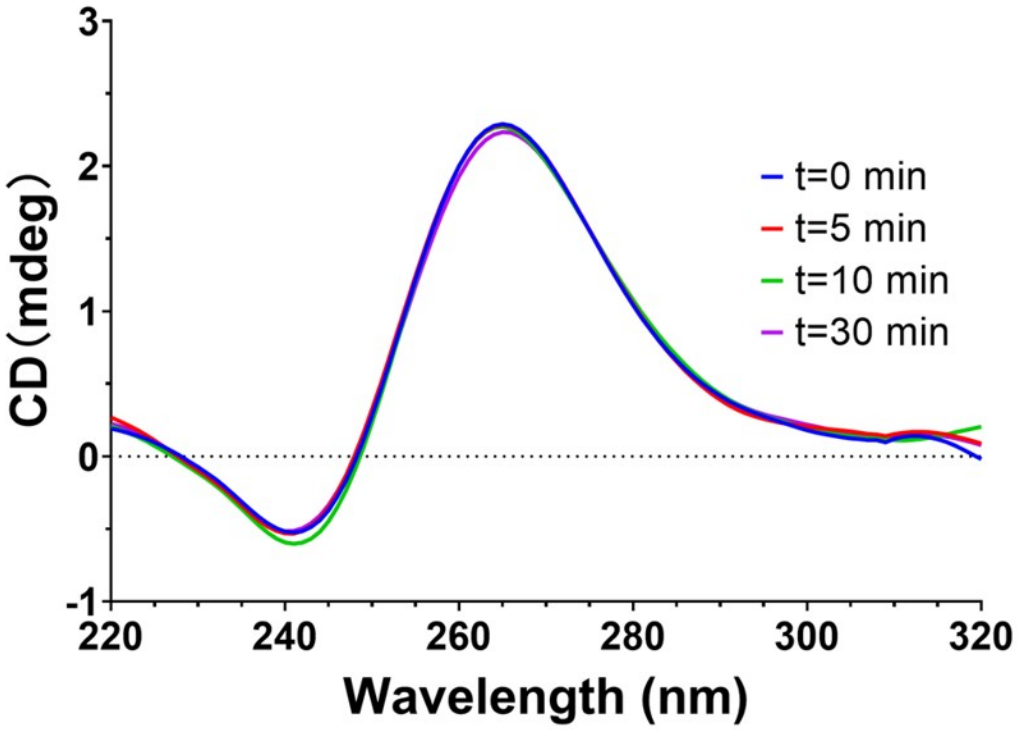

Figure S12 Time-course CD spectra of T-TERRA in the absence of target.

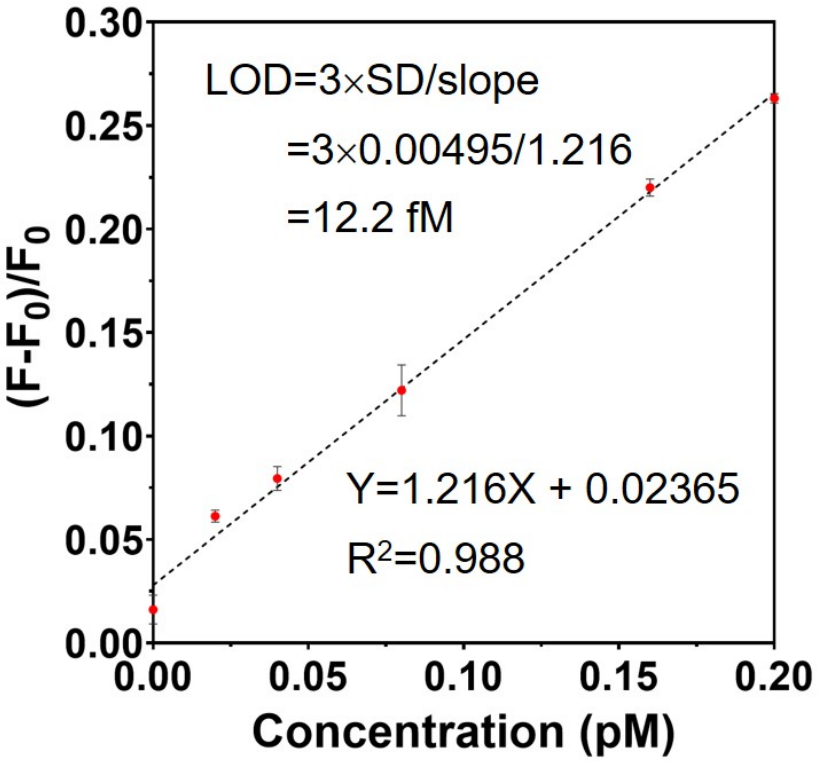

Figure S13 Limit of detection (LOD) calculation of the T-TERRA-FQ based Cas13a assay (corresponding to Figure 3d in Maintext). Error bars represent the standard derivations (SDs) of three measurements.

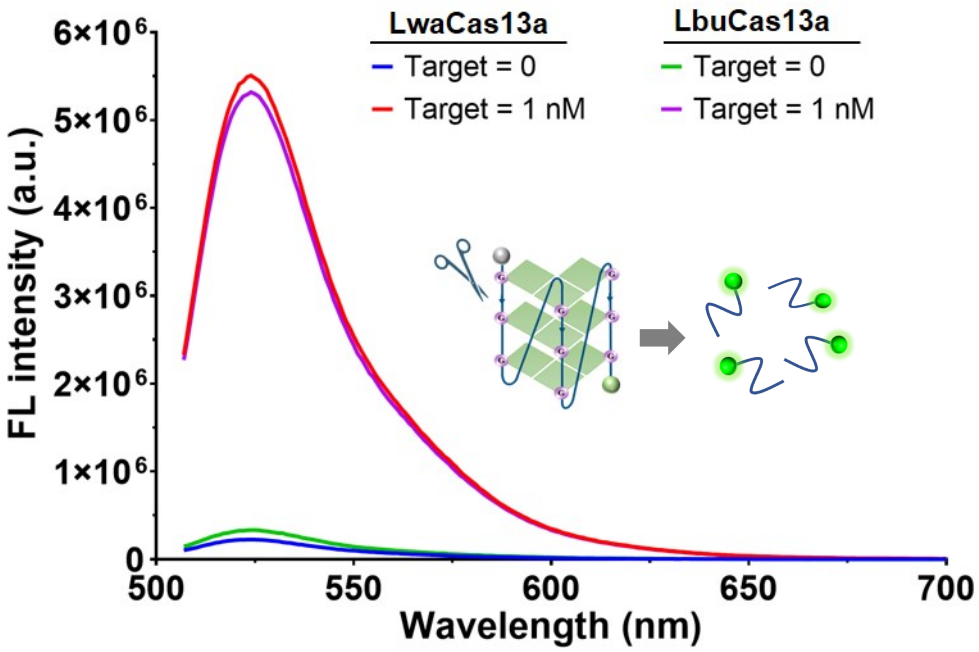

Figure S14 Results showing the capability of LwaCas13a and LbuCas13a to *trans*-cleave the T-TERRA-FQ reporter.