Supplementary Material

Leveraging Cas13a's trans-cleavage on RNA G-quadruplexes for amplification-free RNA detection

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Experimental

Nucleic acids preparation

Sequences of various RNA sequences including RNA G-quadruplexes (rG4s), ssRNA, RNA targets (microRNA-20a, microRNA-20b, SARS-CoV-2 E-gene, and SARS-CoV-2 N-gene), as well as the corresponding crRNAs were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The design of crRNAs targeting SARS-CoV-2 N-gene and E-gene referred to previous reports (*Cell*, 2021, 184(2), 323; *Nat. Biomed. Eng.*, 2020, 4(12), 1140). To ensure RNA integrity, a murine RNAse inhibitor capable of effectively inhibiting various RNases (RNase A, B, C) was obtained from Vazyme Co., Ltd. (Nanjing, China). To prepare rG4 stock solutions, the oligonucleotides were dissolved in DEPC-water at a concentration of 100 μM, heated at 95°C for 10 min, followed by cooling to room temperature. Before usage, the stock solutions were appropriately diluted with a cleavage buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 6 mM $MgCl_2$, unless mentioned otherwise) to achieve the desired concentrations.

Typical LwaCas13a-based cleavage assay

The *Leptotrichia wadei* Cas13a (LwaCas13a) enzyme used in this study was obtained from Magigen company (Guangzhou, China). Unless mentioned otherwise, Cas13a-mediated cleavage assays were carried out using the cleavage buffer (20 mM HEPES, 50 mM NaCl, 6 mM $MgCl_2$, pH 7.5). The experimental procedure involved the following steps. Initially, LwaCas13a (1 μL, 1 μM), crRNA (1 μL, 1 μM), RNAse inhibitor (1 μL, 40 U/μL) and 17 μL of the cleavage buffer were mixed and pre-incubated at 37°C for 10 min. After the formation of the LwaCas13a/crRNA complex, synthesized target RNA or SARS-CoV-2 RNA standards (obtained from the China National Institute of Metrology with the code GBW(E)091089) at various concentrations (10 μL), or cleavage buffer (10 μL), along with rG4 or ssRNA (20 μL, 1 μM) were added to create a 50-μL reaction solution. The cleavage reaction was conducted at 37°C in a mini-metal bath obtained from Yeasen Biotechnology (Shanghai, China) for 60 min, followed by measuring the corresponding signal.

To investigate the *trans*-cleavage potential of the rG4 sequences by other Cas13a variants, the *trans*-cleavage activity of *Leptotrichia buccalis* Cas13a (LbuCas13a) on TERRA G4 was also examined. The LbuCas13a enzyme (Addgene #: 115267) was expressed and purified in accordance with a previously published protocol (Chen et al. 2019). The LbuCas13a-based cleavage assay was performed following the same protocol as the LwaCas13a-based assay.

Fluorescence measurement

For the fluorescence-based assay, the 5'-FAM/3'-TAMRA (FRET) or 5'-FAM/3'-BHQ1 (FQ) labelled rG4 reporters in the 50-μL cleavage reaction solution were diluted to a final concentration of 66.7 nM. 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.

The time-course cleavage assays were carried out on a QuantStudio real-time PCR instrument (Thermo Fisher Scientific Inc, USA). The *trans*-cleavage rates of Cas13a on rG4 and ssRNA were examined. First, the pre-incubated LwaCas13/crRNA (1 μL, 1 μM) complex along with RNAse inhibitor (1 μL, 40 U/μL) in 20 μL of cleavage buffer was mixed with the FQ-labelled rG4 (telomeric-repeat containing RNA, TERRA) or ssRNA (20 μL, 5 μM) in the PCR tubes. The measurement was conducted after adding the target (SARS-CoV-2 E-gene, 10 μL, varied concentration) into the tube. Measurements were taken at a 30-s interval over a duration of 60 min.

Circular dichroism (CD) experiments

CD experiments were conducted using a Chriascan instrument (Applied Photophysics, UK). Measurements were performed in a cuvette with a path length of 0.1 cm. Data were collected by averaging three scans recorded from 220 to 320 nm at a scanning rate of 1 nm/s. The experiments were carried out at room temperature (~25°C). To prepare the samples, LwaCas13a (1 μL, 10 μM) was pre-incubated with crRNA (1 μL, 10 μM) and RNAse inhibitor (1 μL, 40 U/μL) in a total volume of 20 μL buffer containing either

50/100 mM Na^+/K^+. Next, the target (SARS-CoV-2 E-gene, 10 μL, 2 μM) or buffer (as control) and rG4 oligonucleotides (10 μL, 100 μM) were added into the reaction solution. The mixtures were then incubated at 37°C for 2 h. After the incubation period, the mixture was diluted to a total volume of 200 μL for the CD measurements. All spectra were corrected with the relevant buffer blanks.

For the time-course CD measurement, the cuvette holder was pre-heated and maintained at a temperature of 37℃. The temperature of the testing solution was monitored using a sensor throughout the experiment. To prepare the reaction mixture, 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 TERRA G4 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. The solution was rapidly mixed, and the CD measurement was started immediately. The measurements were taken at intervals of 5-10 minutes for a duration of up to 2 h.

Analysis of Cas13a-digested products by urea PAGE

Urea polyacrylamide gel electrophoresis (PAGE) was employed to analyze the fragments of rG4 reporters (the sequences were listed in Table S2) after the *trans*-cleavage. LwaCas13a (2 μL, 1 μM), crRNA (2 μL, 1 μM) and RNAse inhibitor (1 μL, 40 U/μL) were pre-incubated in a total volume of 20-μL buffer for 10 min at 37°C. Then the FRET-labeled TERRA G4 oligonucleotide (20 μL, 10 μM) and the target (10 μL, 10 nM) or buffer (10 μL, as a control) were added into the reaction solution. The mixtures were then incubated at 37°C for an appropriate time. To inactivate the LwaCas13a, the reaction system was incubated at 65℃ for 15 min. Subsequently, the cleaved solution (8 μL) was mixed with the loading buffer (2 μL) and loaded into 30% denaturing PAGE containing 8 M urea. Electrophoresis was carried out at 180 V (about 40 V/cm) for about 120 min using a Mini-PROTEAN Tetra Cell system from Bio-Rad in 1×TBE buffer. Finally, the gel was scanned using a Bio-rad ChemiDoc MP (170-8280) (BioRad Company, Shanghai, China) under the “fluorescein” mode.

NMR experiments

^{1}H NMR was measured on a Bruker AVANCE 700 MHz spectrometer. The spectra were recorded at a temperature of 37°C. To prepare the non-cleaved sample (without target), a solution containing LwaCas13a/crRNA complex (0.5 μM) and RNAse inhibitor (1 μL, 40 U/μL) in 400 μL buffer was utilized. The buffer composition was 90% H_2O/10% D_2O, 20 mM HEPES, 50 mM NaCl, 6 mM $MgCl_2$, pH 7.5. The TERRA G4 oligonucleotide was dissolved in this solution with a final concentration of 0.3 mM. The NMR experiment was initially performed on this non-cleaved sample. Then the target RNA (SARS-CoV-2 E-gene, 15 nM, final concentration) was added to the sample, and the mixture was incubated at 37°C for overnight cleavage. After the cleavage reaction, the cleaved samples were subjected to ^{1}H NMR measurement using the same experimental settings as the non-cleaved sample.

Single-Molecule FRET (smFRET) analysis

Prior to the smFRET experiments, three samples were prepared to investigate different cleavage time durations. LwaCas13a (2 μL, 1 μM), crRNA (2 μL, 1 μM) and RNAse inhibitor (1 μL, 40 U/μL) were preincubated in a total of 17 μL buffer for 10 min at 37°C, followed by adding the FRET-labeled TERRA G4 oligonucleotide (3 μL, 100 μM). The first sample was prepared by mixing 20 μL of the reaction solution and 10 μL buffer (cleavage time=0). The second and third sample was prepared by mixing 20 μL of the reaction solution and 10 μL target (10 μM) and incubated at 37°C for 5 min and 120 min, respectively. To inactivate the LwaCas13a, the reaction system was incubated at 65°C for 15 min.

Subsequently, the smFRET experiments were conducted using the laser confocal fluorescence microscope MicroTime 200 (PicoQuant, Berlin, Germany). The experiment setup, data collection and analysis were performed following previously described protocols (Guan et al. 2023; Pei et al. 2021).

Michaelis-Menten enzyme kinetic measurements and data analysis

To compare the *trans*-cleavage activity of activated Cas13a system on rG4 and ssRNA, the *cis*-cleavage activity was activated in advance, followed by adding the reporters to assess the *trans*-cleavage activity (Avaro and Santiago 2022; Huyke et al. 2022). Briefly, the pre-incubated LwaCas13/crRNA (1 μL, 1 μM) complex with RNAse inhibitor (1 μL, 40 U/μL) in 20 μL cleavage buffer was mixed with the target (SARS-CoV-2 E-gene, 10 μl, 0.5 nM), and incubated at 37°C for 30 min. After the *cis*-cleavage activation, the FQ-labelled TERRA G4 or ssRNA (5 μL, different concentrations) and 15 μL cleavage buffer (pre-heated at 37°C) were added into the relevant tubes. The final concentrations for reporter TERRA G4 or ssRNA were 62.5, 125, 250, 500, 1000, 2000, and 4000 nM. Then the measurement was immediately initiated with a 30-s interval between readings.

We determined the initial reaction velocities (V_0) for each concentration of reporters by fitting the data collected during the initial 300 seconds to a linear regression model. The calculated reaction velocities were then converted from arbitrary units per second (a.u./s) to nanomoles per second (nM/s), assuming a positive correlation between measured fluorescence and cleaved reporters. To establish fluorescence signals corresponding to fully cleaved reporters at the reported concentrations, we incubated the reporters at 37°C for up to 5 hours under the same experimental conditions as previously mentioned, assuming completion of the *trans*-cleavage reaction. The calculated reaction velocities (nM/s) were graphed against the concentration of FQ-labelled TERRA G4 or ssRNA reporters (substrates) and fitted to the Michaelis−Menten equation using GraphPad Prism software (GraphPad, CA, USA) to derive V_{max} and K_m values. The Michaelis−Menten equation is as follows: $V_0 = (V_{max} \times [S])/(K_m + [S])$, where [S] represents the substrate concentration. Lastly, the turnover number (K_{cat}) was calculated using the formula: $K_{cat} = V_{max}/[E]$, where [E] denotes the concentration of the active Cas13a/crRNA/target ternary complex, which was set to 0.1 nM.

Molecular docking analysis of Cas13a with TERRA G4 or ssRNA reporter

The docking experiments were conducted through utilization of the HDOCK server (http://hdock.phys.hust.edu.cn/) (Remmert et al. 2012; Yan et al. 2020; Yan et al. 2017). The server is grounded in a hybrid algorithm combining template-based modeling and *ab* initio free docking. To elaborate, HDOCK's scoring function was enhanced by integrating a long-range shape-based scoring (LSC) function with the well-established Fast-Fourier Transform (FFT)-based algorithm. During the sampling phase, the scoring of a ligand grid considers contributions not only from the nearest receptor grids but also from distant ones, determined by the distance parameter r (in the form of $\sim e^{-1/r\hat{}2}$). The ligand undergoes rotation and translation: the ten best translations for each rotation are refined by an iterative knowledge-based scoring function capable of predicting the reference state, enabling extraction of realistic interaction potentials. This produces a distinct binding mode for each rotation. For each model, the interface information encompasses residue pairs within a 5.0 Å range between the receptor and the corresponding ligand.

The HDOCK server received the Cas13a/crRNA/target ternary complex PDB structures (PDB: 5XWP) as the receptor input. Additionally, either the TERRA G4 structure (PDB: 3IBK) or the scrambled TERRA G4 or the ssRNA sequence (AAUGGCA) was submitted as the ligand input to the HDOCK server. This was carried out under specific conditions: the binding site residue (s) of the receptor were confined to the range of 1 to 1153 and located in chain A. After the docking calculations, the result files with the docking scores and the docked structures were retrieved from the server. The docked structures, in PDB format, were directly loaded on PyMOL 2.5 software (Schrödinger Inc., USA) for structure inspection.

ThT-based label-free measurement with TERRA G4

LwaCas13a (1 μL, 1 μM), crRNA (1 μL, 1 μM) and RNAse inhibitor (1 μL, 40 U/μL) were incubated in a total of 20 μL cleavage buffer for 10 min at 37°C. Next, the target (SARS-CoV-2 E-gene) with varying concentrations (10 μL) and TERRA G4/ThT complex (20 μL) were added to form a 50-μL reaction solution. The TERRA G4/ThT complex was made of TERRA G4 (3 μL, 10 μM), ThT (6 μL, 100 μM) and buffer (11

μL). The cleavage reaction was performed at 37°C for 60 min, followed by measuring the fluorescence signal. To measure ThT fluorescence, the excitation and emission slits were set as 5 nm. Excitation wavelength was set at 420 nm, and emission was collected from 450 to 600 nm. The fluorescence intensity at 490 nm was extracted for further analysis.

rG4 based electrochemical measurement

The electrochemical-based detection was performed on a wireless USB-like electrochemical platform from Refresh Biosensing Technology Co., Ltd. (Shenzhen, China). Screen-printed electrodes (SPEs) purchased from the above company were used for electrochemical analysis. TERRA G4 was used as the label-free reporter, and SARS-COV-2 E-gene was tested with various concentrations. LwaCas13a (1 μL, 1 μM), crRNA (1 μL, 1 μM) and RNAse inhibitor (1 μL, 40 U/μL) were pre-incubated in a total of 20 μL cleavage buffer. Next, the target with various concentrations (10 μL) and TERRA G4 (5 μL, 10 μM) were added to form a 50-μL reaction solution, followed by the *trans*-cleavage at 37°C for 30 min. Then, the cleavage reaction (50 μL) was mixed with hemin (25 μL, 10 μM) and incubated at room temperature for 15 min. H_2O_2 (2.5 μL, 50 mM) and HQ (2.5 μL, 50 mM) were added into the above solution to a total of 100 μL electrochemical detection solution and incubated at room temperature for 15 min. The electrochemical measurement was performed by placing the detection solution on the electrodes and collecting the data under the differential pulse voltammetry (DPV) mode. The parameters were set as: potential increment, 5 mV; modulation amplitude, 100 mV; and potential range, -0.6–0.1 V. Each sample was measured in three biological replicates.

SARS-CoV-2 clinical sample collection and detection

De-identified patient samples (nasopharyngeal swabs) were collected from Maternal and Child Health Hospital (MCHH) of Hubei Province, Huazhong University of Science and Technology. The study was approved by the MCHH Committee on Human Research (2023IEC055). Prior to our assay, these samples were screened for SARS-

CoV-2 infection in the clinical laboratory based on RT-qPCR (Maccura Biotechnology Co., Ltd., Chengdu, China) by testing the RNA extracted (Weimi Bio-tech, Guangzhou, China) from the swab samples. The clinical sample information was listed in Table S3. To test the clinical samples, FQ-labelled TERRA G4 was used as the reporter in the LwaCas13a-based assay. The extracted RNA sample (10 μL) was used as the target. The cleavage assays were performed at 37°C for 60 min, followed by measuring the fluorescence signal.

Table S1. Overview of the limit of detection (LOD) of CRISPR-Cas13a based detection platforms.

Platforms	Target Amplification	Detection Strategy	LOD	Reference
SHERLOCK	None	Fluorescence	50 pM	*Science*, **2017**, 356, 438.
E-CRISPR	None	Electrochemical	10 pM	*Adv. Mater.,* **2019**, 31, 1905311.
FL-CRISPR	None	Fluorescence	91 fM	*ACS Sens.,* **2019,** 4, 1048.
FL-CRISPR	None	Fluorescence	16 fM	*Cell***, 2021***,* 184, 323
SATORI	None	Fluorescence (microwell-based confinement)	10 fM	*Commun. Biol.*, **2021**, 4, 476.
HRP-CRISPR	None	Fluorescence (transfer the reporter to HRP)	10 fM	*J. Am. Chem. Soc.*, **2022**, 144, 16310.
rG4-CRISPR	None	Fluorescence/ Electrochemical	10 fM/ 2 fM	This work

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

Name	Sequence (from 5' to 3')
SARS-CoV-2 E-gene RNA	AGUUACACUAGCCAUCCUUACUGCGCUUCGAUUG
SARS-CoV-2 E-gene crRNA (LwaCas13a)	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACUCGAAGCGCAGUAAGGAUGGCUAGUGUA
SARS-CoV-2 E-gene crRNA (LbuCas13a)	GACCACCCCAAAAAUGAAGGGGACUAAAACAGCGCAGUAAGGAUGGCUAG
SARS-CoV-2 N-gene RNA	UGGCAAUGGCGGUGAUGCUGCUCUUGCUUUGCUG
SARS-CoV-2 N-gene crRNA (LwaCas13a)	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACCAAAGCAAGAGCAGCAUCACCGCCAUUG
miRNA-20a crRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACACACUACCUGCACUAUAAGCACUUUAGUGC
miRNA-20a RNA	GUAGCACUAAAGUGCUUAUAGUGCAGGUAGUGUUUA
miRNA-20b crRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACAAACUACCUGCACUAUGAGCACUUUGGUAC
miRNA-20b RNA	GUAGUACCAAAGUGCUCAUAGUGCAGGUAGUUUUGG
Fluo TERRA G4	FAM-UUAGGGUUAGGGUUAGGGUUAGGG-TAMRA
TERRA G4	UUAGGGUUAGGGUUAGGGUUAGGG
FQ TERRA G4	FAM-UUAGGGUUAGGGUUAGGGUUAGGG-BHQ1
BCL2 RNA G4	GGGGGCCGUGGGGUGGGAGCUGGGG
KRAS RNA G4	GGCGGCGGCAGUGGCGGCGG
ORN-N RNA G4	AGGGUUAGGGUUAGGGUUAGGG
Fluo BCL2 RNA G4	FAM-GGGGGCCGUGGGGUGGGAGCUGGGG-TAMRA
FQ ssRNA	FAM-mAAUGGCmA-BHQ1

Note: The underlined sequence indicates the pairing region between crRNA and target.

Table S3. Patient sample detection results of SARS-CoV-2 from the clinical lab and our Cas13a-based assay. Samples with Ct value>38.0 was identified as Negative in the clinical test.

Sample	Gender	Clinical Results (qRT-PCR)	Ct	Our Cas13a-based results
1	Female	+	17.24	+
2	Female	+	16.19	+
3	Female	+	24.19	−
4	Female	+	18.01	+
5	Male	+	14.98	+
6	Female	+	15.99	+
7	Male	+	21.69	+
8	Female	−	>38.0	−
9	Male	−	>38.0	−
10	Male	+	18.44	+
11	Female	−	>38.0	−
12	Male	+	18.9	+
13	Male	−	>38.0	−
14	Female	−	>38.0	−
15	Female	+	21.74	+
16	Female	−	>38.0	−
17	Male	+	18.22	+
18	Female	+	20	+
19	Female	−	>38.0	−
20	Male	−	>38.0	−
21	Female	+	22.98	+
22	Female	+	25.51	+
23	Female	+	16.99	+
24	Male	−	>38.0	−
25	Female	+	20.39	+

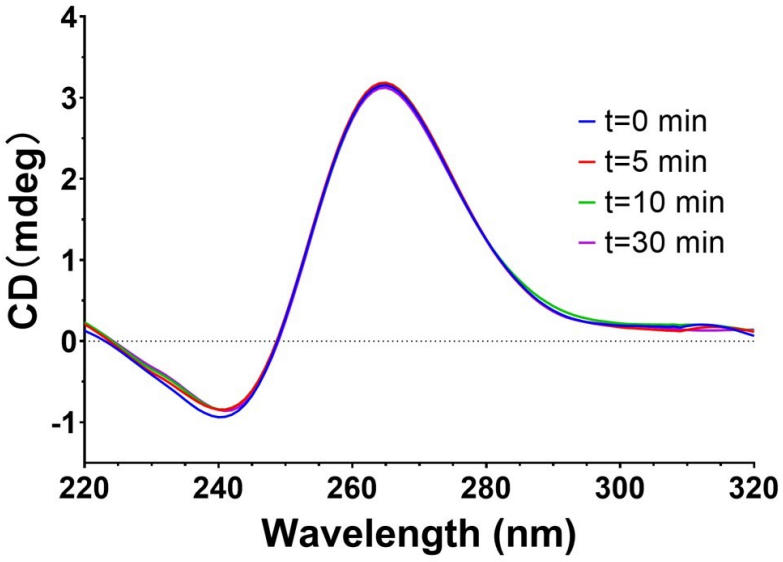


Figure S1 Time-course CD spectra to check the degradation of TERRA G4 in the absence of target (as the control sample) in the Cas13a-based cleavage assay (50 mM NaCl). No obvious degradation was observed.

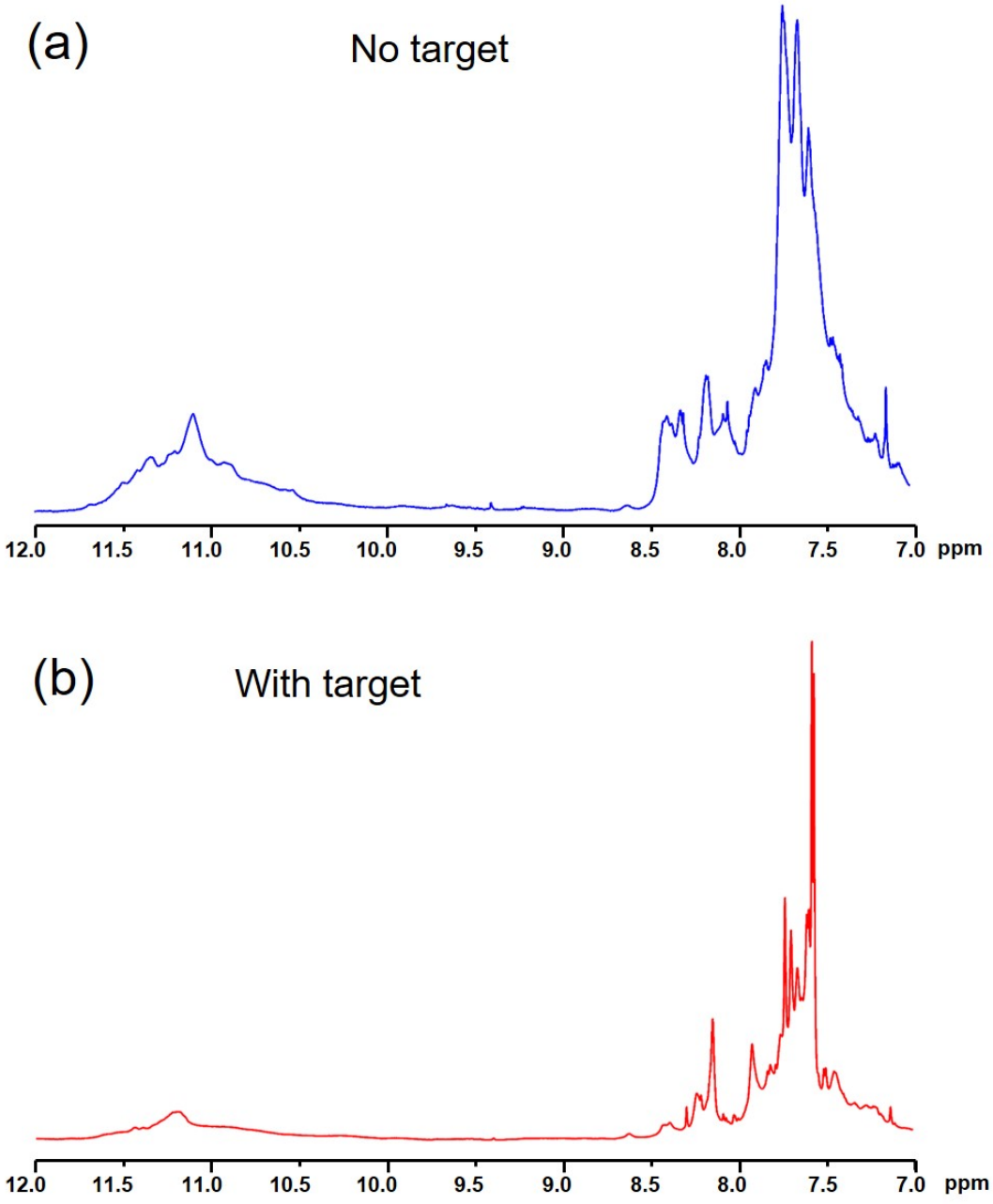


Figure S2 The ^{1}H NMR spectrum of TERRA G4 at 7-12 ppm without (a) or with (b) the target to activate the Cas13a-based cleavage assay (50 mM NaCl).

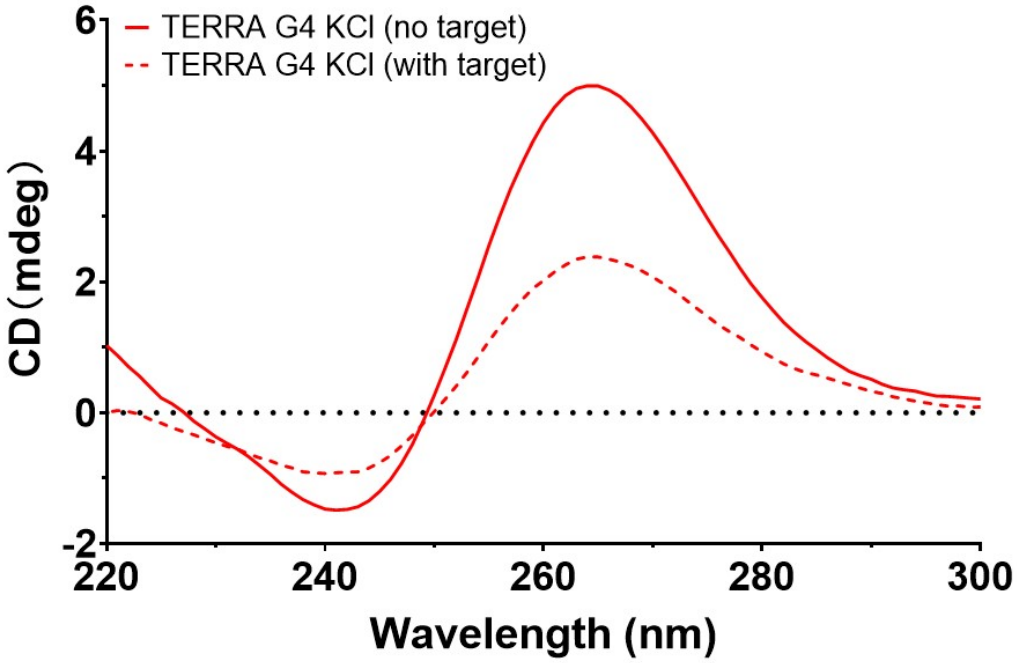


Figure S3 Investigation of the *trans*-cleavage activity of Cas13a on TERRA G4 in 50 mM KCl solution by CD.

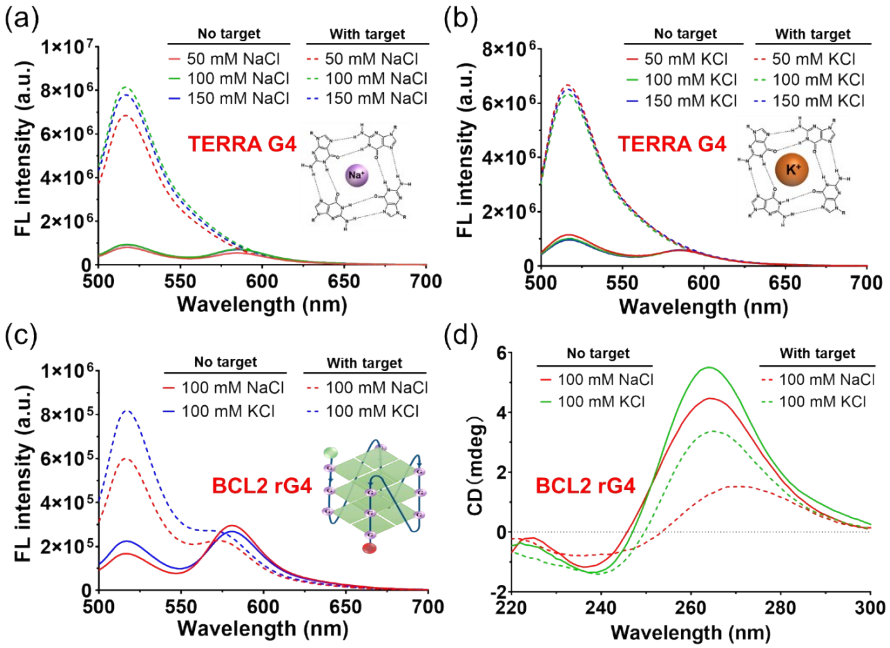


Figure S4 The universal trans-cleavage ability of Cas13a on different rG4s. (a-b) FRET measurements show that LwaCas13a trans-cleaves TERRA G4 under NaCl (a) and KCl (b) conditions, respectively. (c-d) LbuCas13a shows trans-cleavage efficiency on BCL2 rG4 under NaCl or KCl conditions, as evidenced by FRET measurements (c) and CD spectra (d).

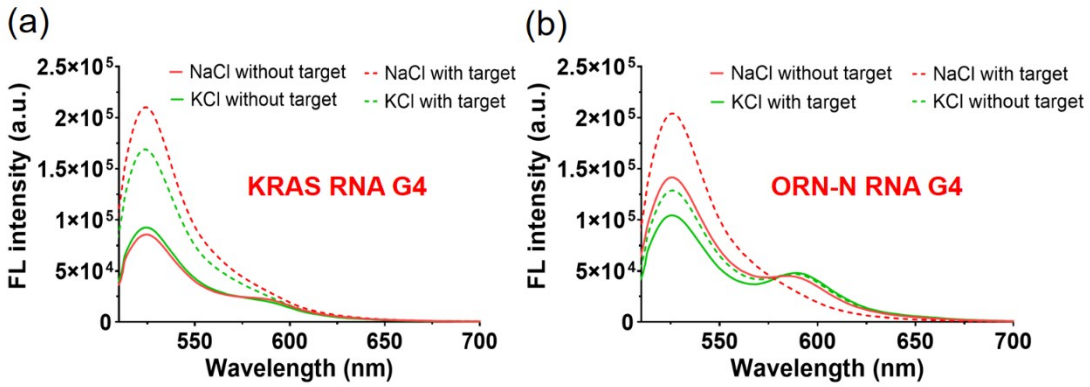


Figure S5 Using fluorescence spectroscopy to characterize the *trans*-cleavage activity of Cas13a on KRAS rG4 (a) and ORN-N rG4 (b) under NaCl and KCl conditions.

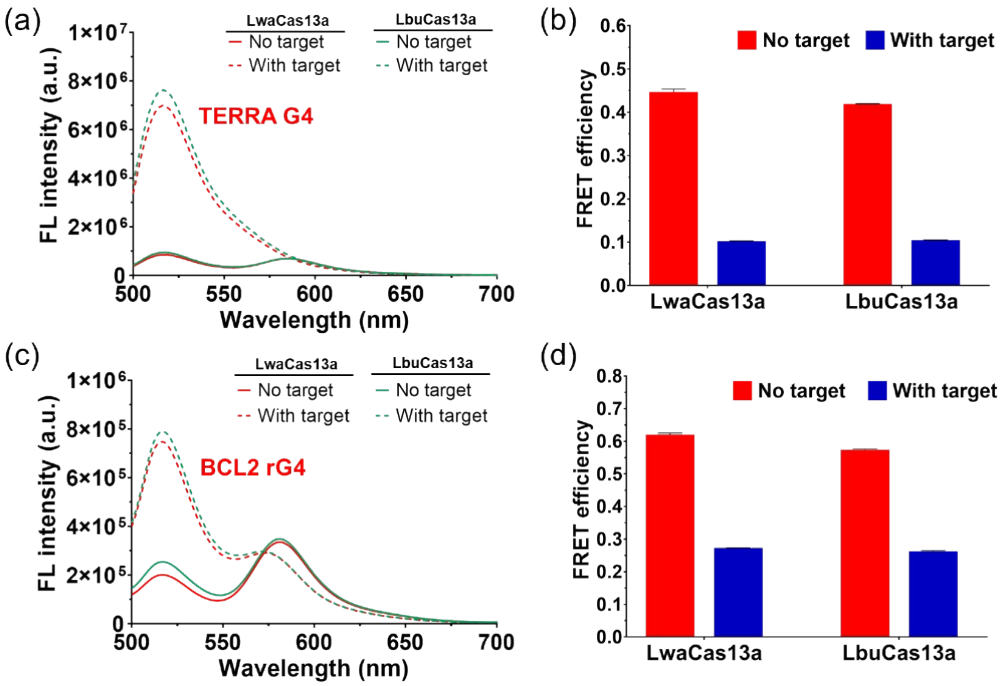


Figure S6 LwaCas13a and LbuCas13a *trans*-cleave rG4. (a-b) FRET measurements show that LwaCas13a and LbuCas13a *trans*-cleave TERRA G4. (c-d) FRET measurements show that LwaCas13a and LbuCas13a *trans*-cleave BCL2 rG4. Error bars represent the standard derivations (SDs) of three measurements.

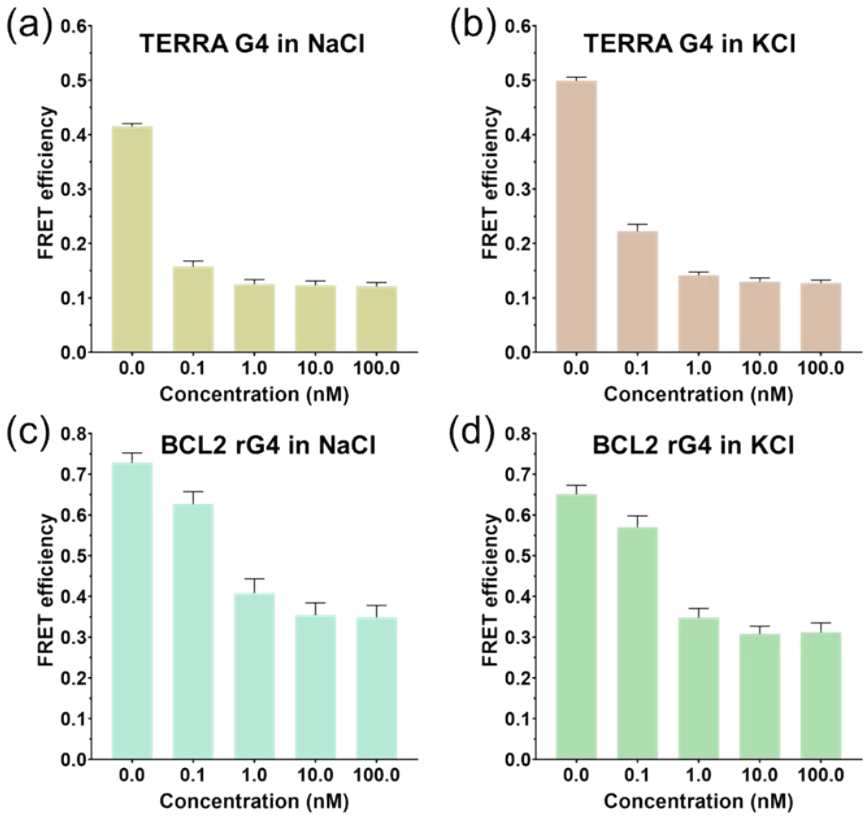
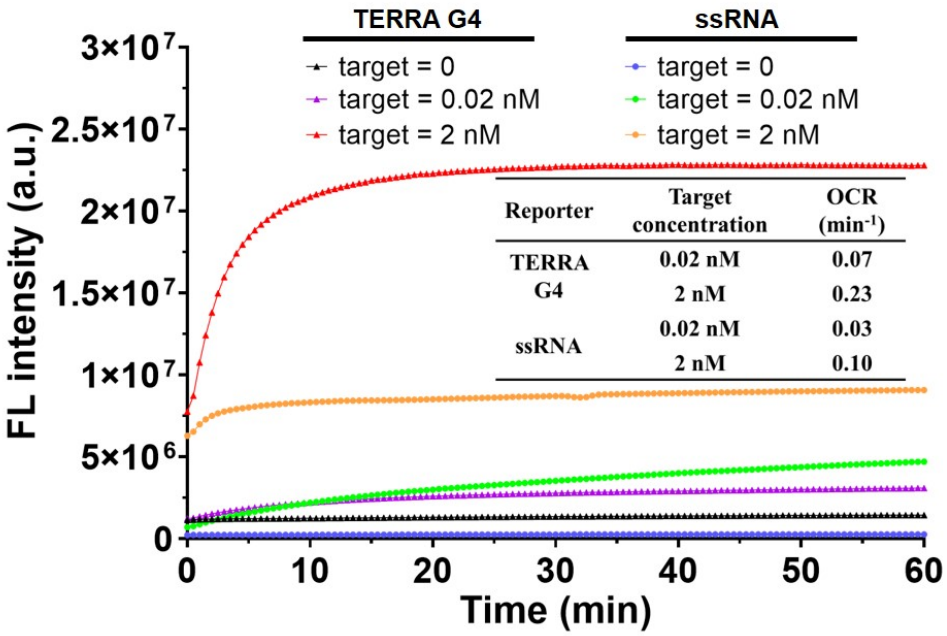


Figure S7 Cas13a *trans*-cleaves TERRA and BCL2 rG4s under different target concentrations. (a-b) FRET efficiency of TERRA G4 under different conditions in NaCl (a) and KCl (b) conditions. (c-d) FRET efficiency of BCL2 rG4 under different conditions in NaCl (c) and KCl (d) conditions.



Reporter	Target concentration	OCR (min^{-1})
TERRA G4	0.02 nM	0.07
	2 nM	0.23
ssRNA	0.02 nM	0.03
	2 nM	0.10

Figure S8 Analysis of the OCRs of Cas13a on FQ-TERRA-G4 and FQ-ssRNA reporter.

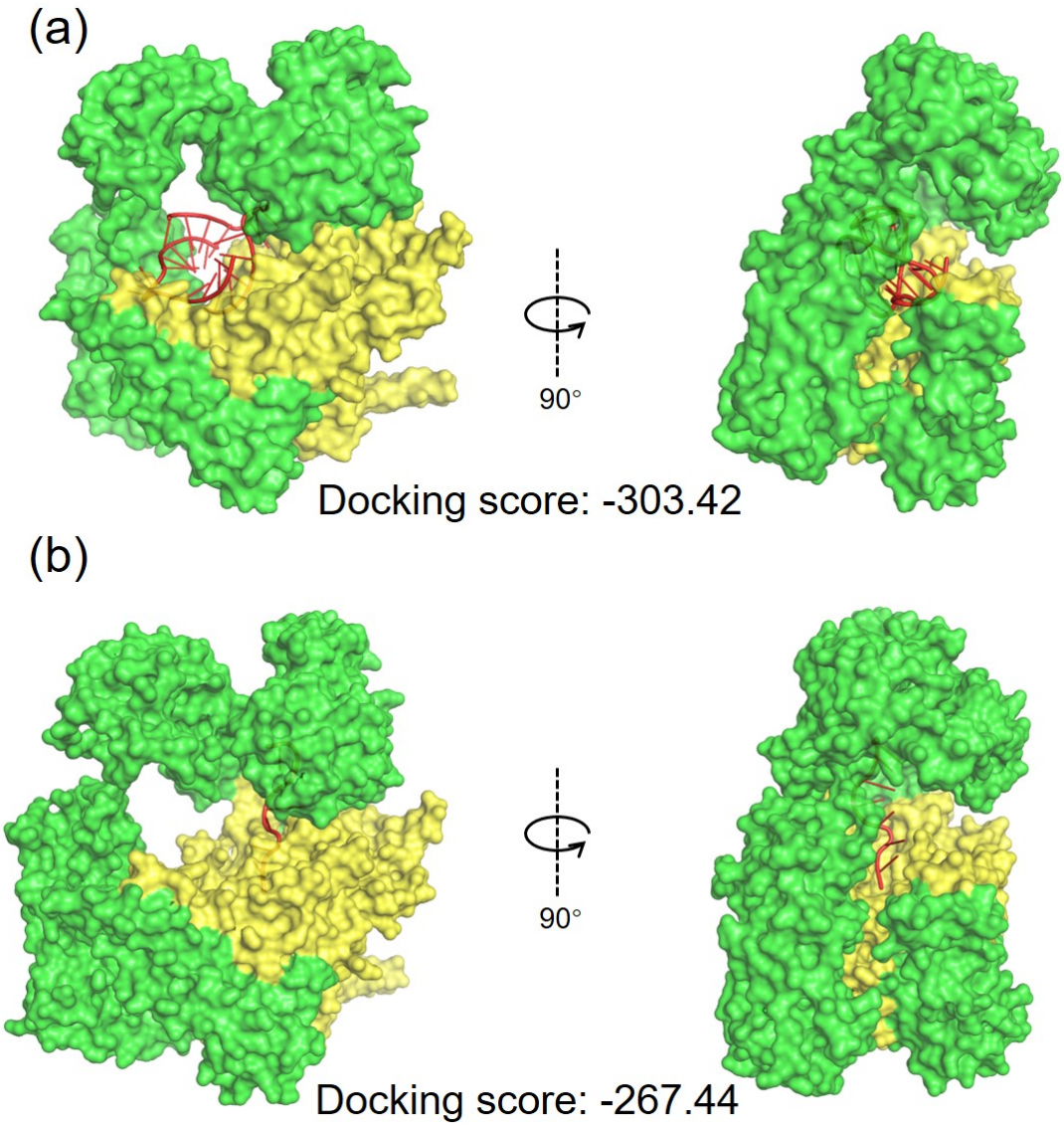
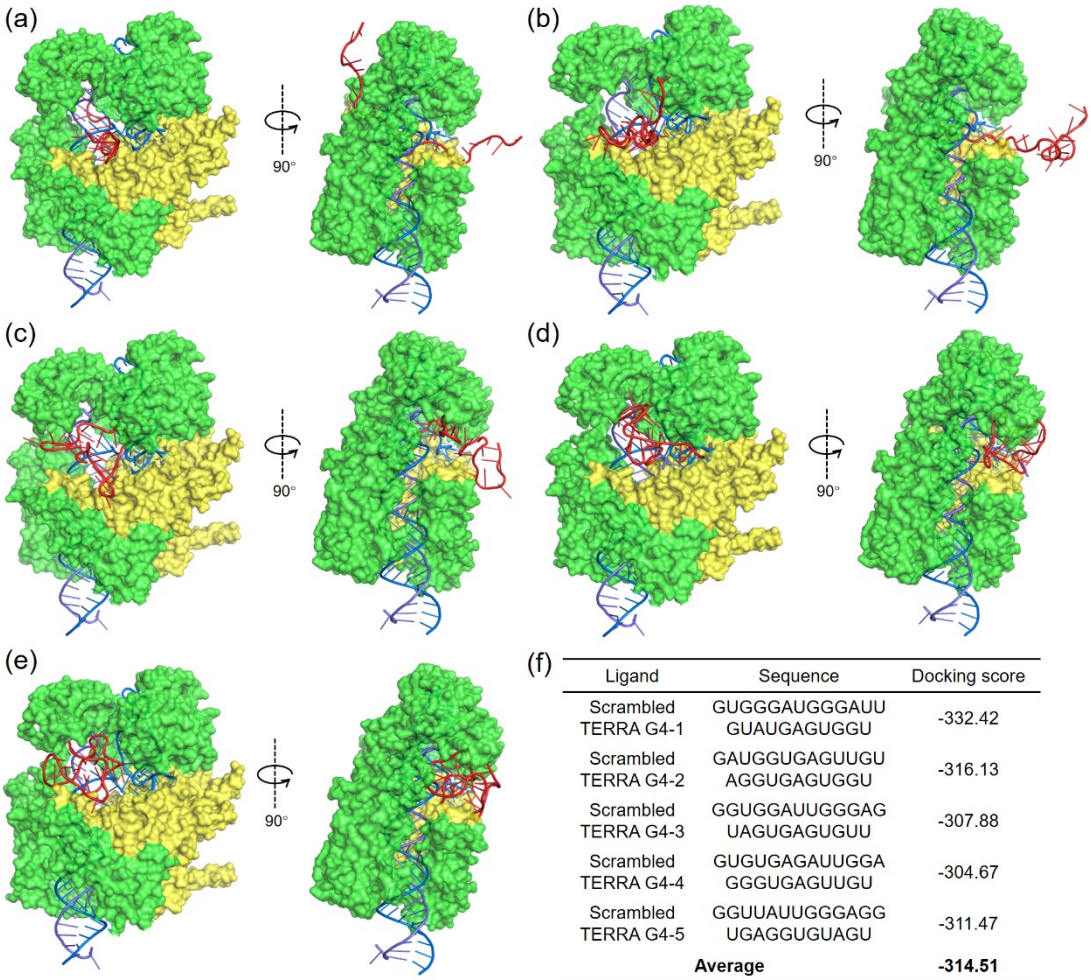


Figure S9 Molecular docking analysis of the Cas13a with TERRA G4 (a) or ssRNA (b) substrate. The Cas13a was retrieved from the Cas13a/crRNA/target ternary complex (PDB ID: 5XWP).



Ligand	Sequence	Docking score
Scrambled TERRA G4-1	GUGGGAUGGGAUU GUAUGAGUGGU	-332.42
Scrambled TERRA G4-2	GAUGGUGAGUUGU AGGUGAGUGGU	-316.13
Scrambled TERRA G4-3	GGUGGAUUGGGAG UAGUGAGUGUU	-307.88
Scrambled TERRA G4-4	GUGUGAGAUUGGA GGGUGAGUUGU	-304.67
Scrambled TERRA G4-5	GGUUAUUGGGAGG UGAGGUGUAGU	-311.47
Average		**-314.51**

Figure S10 Molecular docking analysis on Cas13a/crRNA/target ternary complex (PDB ID: 5XWP) in conjunction with five different scrambled TERRA G4s (a-e). The sequence along with its corresponding docking score is presented in (f).

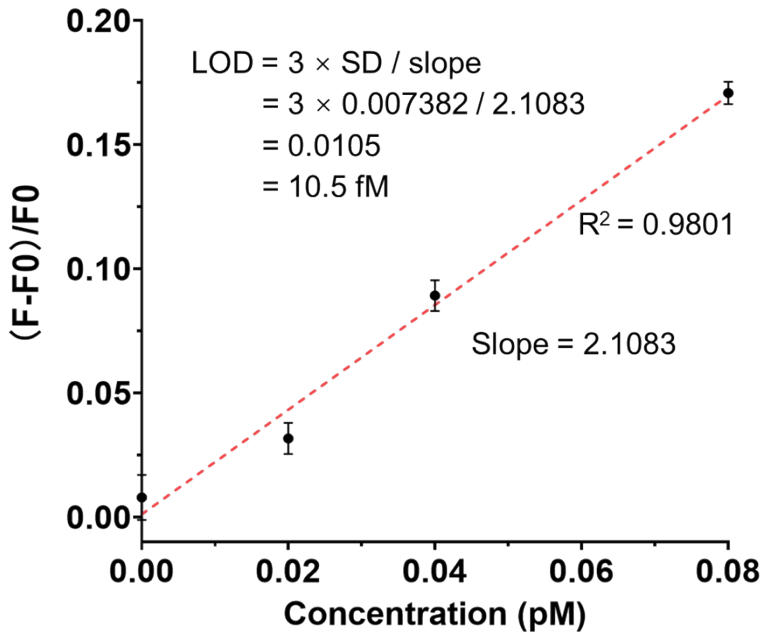


Figure S11 Limit of detection (LOD) calculation of the FQ-TERRA-G4 based Cas13a assay (corresponding to Figure 4a in Maintext). Error bars represent the standard derivations (SDs) of three measurements.

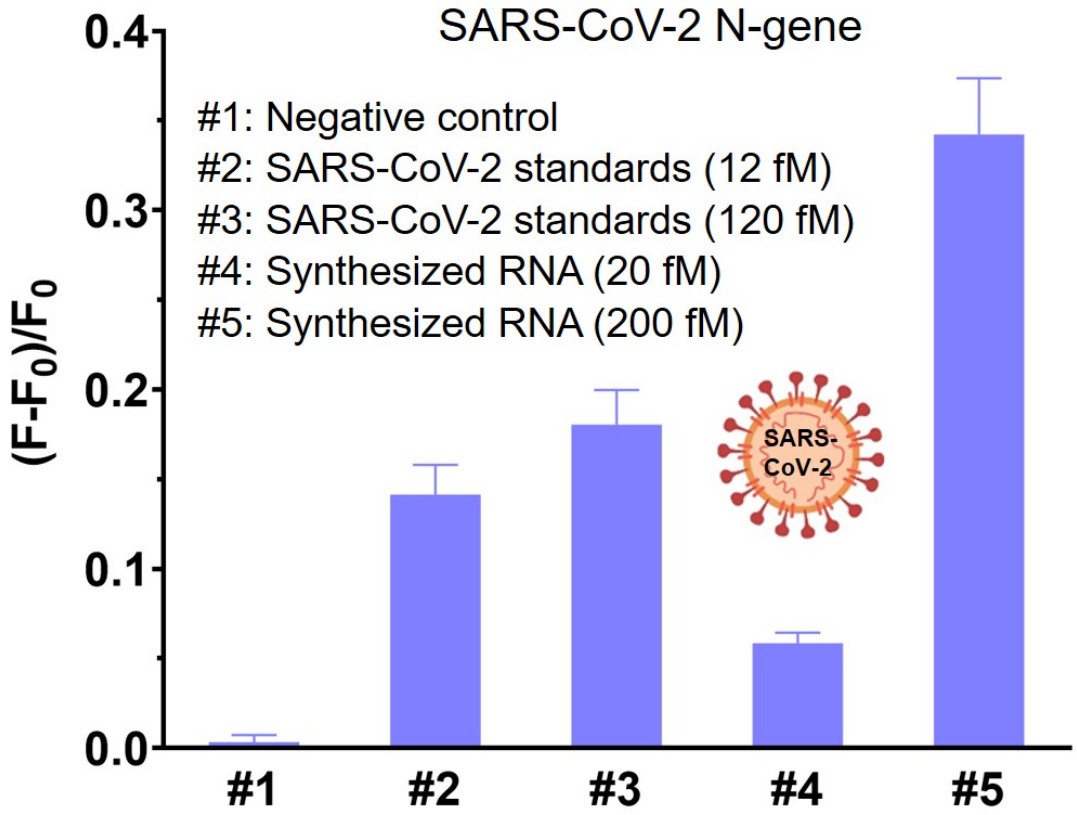


Figure S12 Detection of SARS-CoV-2 N-gene RNA in the standards. Error bars represent the standard derivations of three measurements.

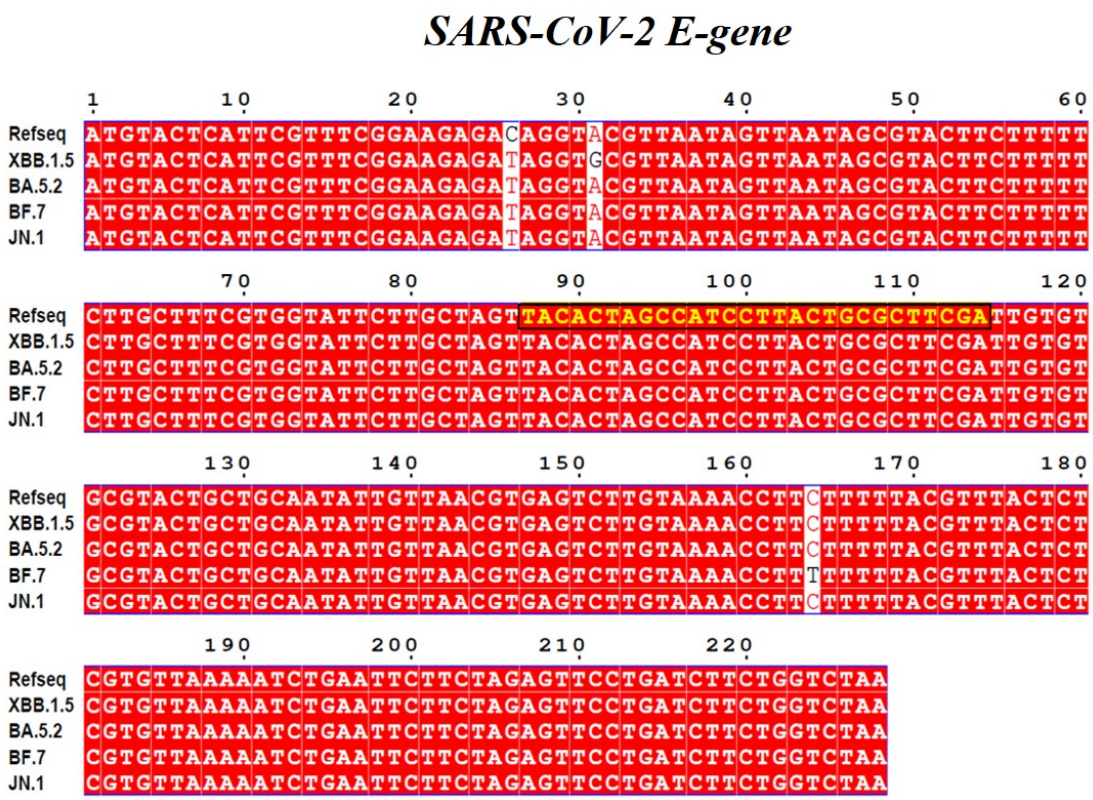


Figure S13 The sequence alignment results of SARS-CoV-2 E-gene from current popular variants. The black rectangle with the yellow color indicates the region targeted by the crRNA.

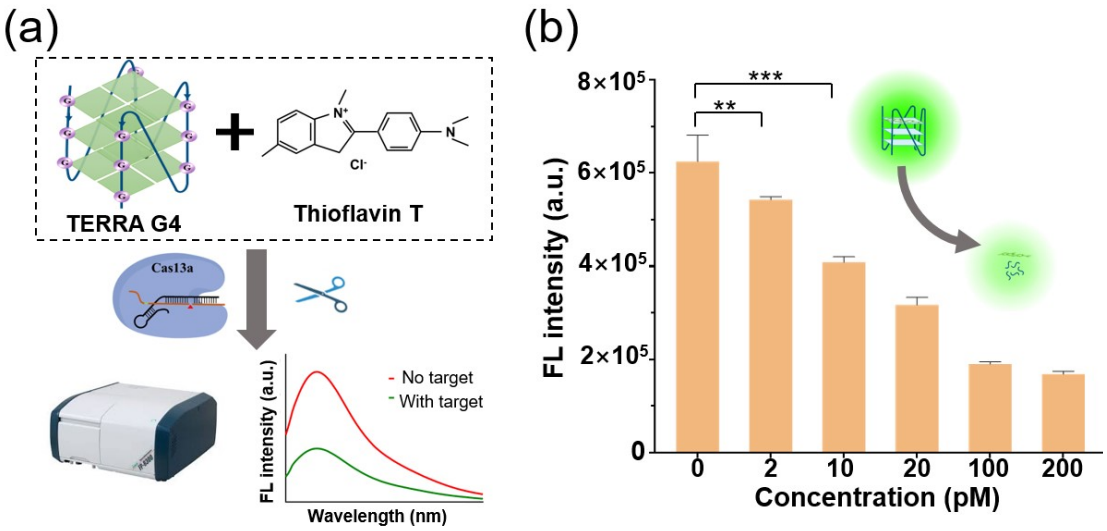


Figure S14 Cas13a-based label-free detection assay using unlabeled TERRA G4 and ThT as the reporter, with (a) showing the scheme, and (b) showing the detection of SARS-CoV-2 E-gene RNA.

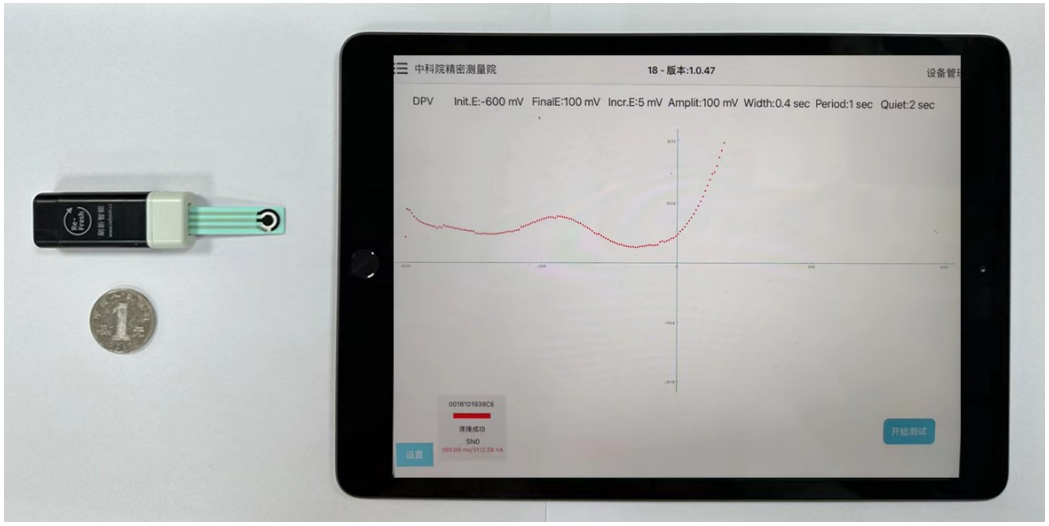


Figure S15 The portable electrochemical instrument containing the USB-like electrochemical device, the electrodes and the iPad for readout.