Supporting information for:

One-pot isothermal amplification permits recycled strand elongation and recycled activation of CRISPR/Cas12a for sensing terminal deoxynucleotidyl transferase activity

Experimental section

Materials and reagents. The reporter ssDNA (5′-/FAM/-TTATT/BHQ/TATCTGATTT-3′) is purified by the High-Performance Liquid Chromatography (HPLC) and acquired from Sangon Biotechnology Co., Ltd. (Shanghai, China). The crRNA (5′ -UAAUUUCUACUAAGUGUAGAUUUUUUUUUUUUUUUUUUUU U-3′) was synthesized by Tsingke Biotechnology Co., Ltd. The Terminal deoxynucleotidyl Transferase (20 U/μL), 1×Terminal Transferase Reaction Buffer (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, pH=7.9), Lba Cas12a (Cpf1), Klenow polymerase, Phi29 DNA polymerase, 1×NEBuffer 1(10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH=7.0), 1×NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH=7.9), 1×NEBuffer r2.1 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 μg/ml recombinant albumin, pH=7.9), 1×Cutsmart (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 100 μg/mL BSA, pH=7.9), phi29 DNA polymerase, and 10 × Phi29 buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 40 mM dithiothreitol) were purchased from New England Biolabs (USA) Ltd. (Beijing, China). Taq DNA polymerase, Bst DNA polymerase, dATP (10 mM), 25–500 bp DNA Marker, and Double distilled water (dd-H_2O) were acquired from Sangon Biotechnology Co., Ltd. (Shanghai, China).

Apparatus. To conduct the fluorescent measurement, the FAM fluorophore was excited at a wavelength of 490 nm using a F97 Pro fluorescence spectrophotometer (Shanghai Cold 116 Light Technology Co., Ltd.), and the emission spectra were recorded in the range of 500 to 650 nm. Additionally, both the excitation and emission slits were configured with a width of 10 nm, and the PMT voltage was set to 650 V. To perform the isothermal reaction, the temperature was controlled on a T100 thermal cycler (Bio-Rad Laboratories, Inc. To characterize the reaction mechanism, the gel was visualized on a Tanon MINI Space 1000 gel imager (Shanghai Tanon Life Sciences Co., Ltd, China).

TdT-triggered one-pot isothermal CRISPR/Cas12a amplification. We used one-pot isothermal CRISPR/Cas12a amplification to ultrasensitive Analysis of TdT Activity. A total volume of 20 μL reaction mixture contains 10.5 μL of dd-H_2O, 2 μL of 10×Terminal Transferase Reaction Buffer, 2 μL of 10×NEBuffer 1, 2 μL of 2.5 μM reporter ssDNA, 2 μL of 2.5 μM crRNA, 1 μL of 1 μM Cas12a protein and 0.5 μL of a series of TdT with different concentrations. The reaction was maintained at 37℃ for 2 h and then terminated at 80 ℃ for 20 min. After that, we supplemented the mixture with 80 μL of dd-H_2O and proceeded to conduct fluorescence measurements.

Gel electrophoresis characterization. The 12% native-PAGE was prepared using a mixture of 5.9 mL dd-H_2O, 6 mL of 30% acrylamide: bis-acrylamide (29: 1), 3 mL of 5×TBE, 110 μL of ammonium persulfate, and 10 μL of N,N,N',N'-Tetramethylethylenediamine (TEMED). For electrophoresis, 10 μL of the reaction product was combined with 2 μL of 10× 4S Green Plus and 2 μL of 6× DNA loading buffer. Electrophoresis was carried out in a constant voltage mode at 80 V using 1× TBE buffer for 60 min in an electrophoresis apparatus (Bio-Rad Laboratories, Inc.). The resulting gel images were analyzed for the separation and characterization of reaction products.

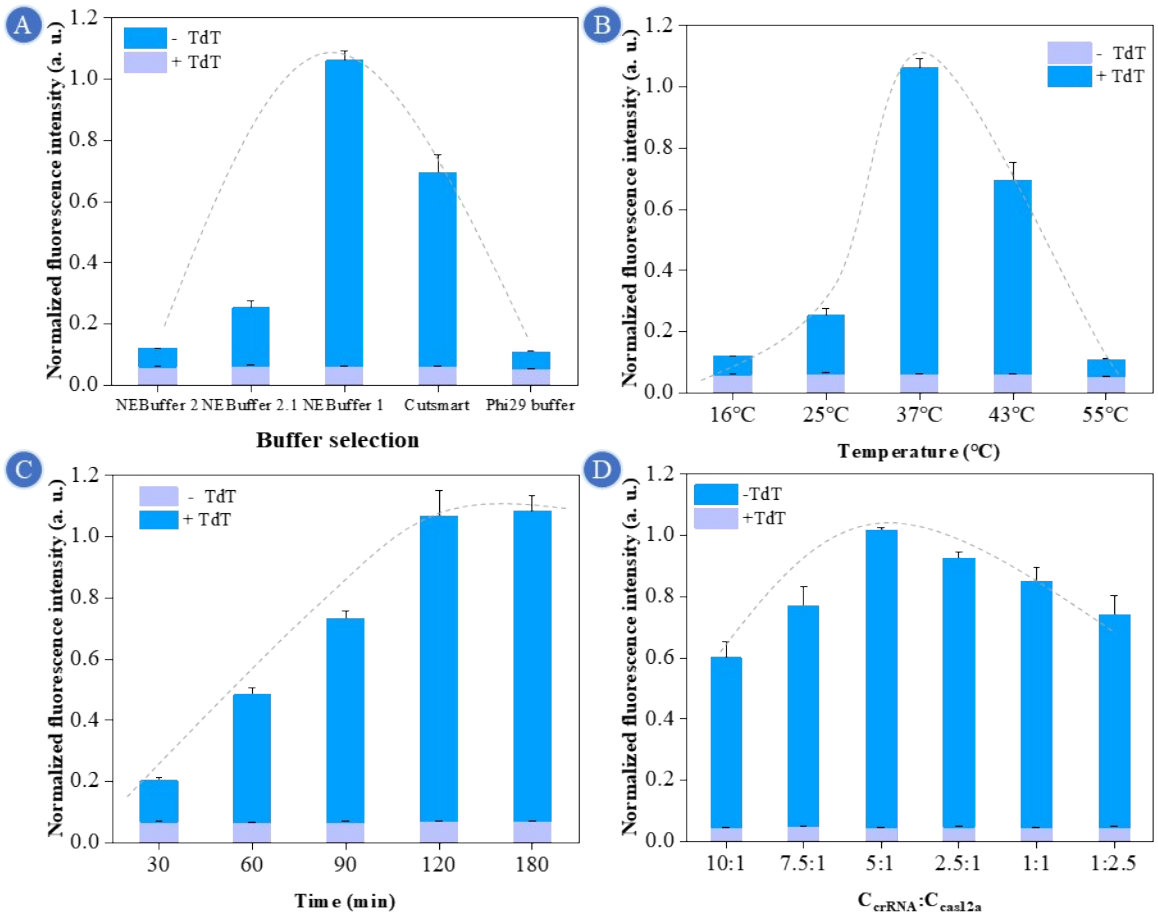

Figure S1. Evaluation of different reaction buffers (A), variation of reaction temperature (B), different reaction times (C), and the concentration ratio between crRNA and cas12a protein (D) on the assay performance. Error bars are from three repetitive tests. (A) Experimental conditions: [reporter ssDNA] = 250 nM; [crRNA] = 250 nM; [cas12a protein] = 50 nM; [TdT] = 250 U/mL; (B) Experimental conditions: [reporter ssDNA] = 250 nM; [crRNA] = 250 nM; [cas12a protein] = 50 nM; [TdT] = 250 U/mL; (C) Experimental conditions: [reporter ssDNA] = 250 nM; [crRNA] = 250 nM; [cas12a protein] = 50 nM; [TdT] = 250 U/mL; (D) Experimental conditions: [reporter ssDNA] = 250 nM; [crRNA] =500, 375, 250, 125, 50, and 20 nM, respectively; [cas12a protein] = 50 nM; [TdT] =250 U/mL.

Optimization of experimental conditions

To achieve optimal experimental conditions, we systematically optimized various parameters, including reaction buffers, reaction temperatures, and overall reaction time (**Figure S1**). Initially, we evaluated the performance of NEBuffer 2, NEBuffer r2.1, NEBuffer 1, Cutsmart, and Phi29 buffer on assay performance. The results in **Figure S1A** illustrate that different buffers generated distinct target signals due to their varying compositions, while background signals remained relatively constant. NEBuffer 1 exhibited the highest target signal compared to reactions in other buffers, making it the optimal choice. Subsequently, the reaction temperature emerged as a critical factor influencing enzyme activity. Systematic variations in temperature (16, 25, 37, 43, and 55°C) were examined. As shown in **Figure S1B**, both excessively high temperatures (43, 55°C) and excessively low temperatures (16, 25°C) resulted in decreased enzyme activity. The target signal was significantly enhanced at 37°C, and considering that the background signal remained relatively stable at this temperature, we selected 37°C as the optimal reaction condition. Thereafter, we optimized the reaction time in **Figure S1C**. According to the results, the fluorescence signal gradually intensified within the first 120 min, reaching near equilibrium by that point. Similarly, the background signal remained nearly unchanged. Consequently, we determined 120 min as the optimal reaction time. Finally, we assessed the concentration ratio between crRNA and cas12a protein. To ensure comparability, we maintained the final concentration of cas12a at 50 nM while adjusting the concentration of crRNA. **Figure S1D** demonstrated that employing a concentration ratio of 5:1 between crRNA and cas12a protein yielded maximal signal output in the amplification system. Consequently, we adopted this ratio for evaluating assay performance.

Table S1. Comparison of the current method with previously reported methods for TdT detection.

Methods	Linear range	Reaction time	LOD	Ref.
C-rich DNA nanotail-templated silver nanoclusters	0.4-90 U/mL	182 min	0.08 U/mL	1
Strand-elongation initiated DNAzyme walker	0.2-20 U/mL	240 min	0.09 U/mL	2
In-situ growth G4-nanowire-driven electrochemical biosensor	0.002-1 U/mL	180 min	0.081U/mL	3
Iridium (III)-based i-motif probe	0-8 U/mL	120 min	0.25 U/mL	4
TdT-yielded DNA-CuNCs	0.5-160 U/mL	152 min	0.1 U/mL	5
Long poly-T-templated copper nanoparticles	0-500 U/mL	125 min	3.75 U/mL	6
TdT-triggered one-pot isothermal CRISPR/Cas12a amplification	0.01-100 U/mL	120 min	0.01 U/mL	This work

Table S2. Recovery rates of TdT detection in human serum samples using the proposed biosensor.

Samples	Added (U/mL)	Found (U/mL)	Recovery (%)	RSD (%)
1	1	1.043	104.3	2.58
2	10	9.405	94.05	4.69
3	80	72.48	90.6	4.06

As mentioned in the literature, TdT is a potential important biomarker. Therefore, the practicality of the one-pot isothermal CRISPR/Cas12a amplification was verified by measuring TdT in human serum samples. It should be noted that human serum was chosen as the matrix due to the presence of abundant nucleases, which poses a significant challenge for nucleic acid sensing systems. Simulated biological samples were prepared by spiking standard concentrations of TdT into human serum. The recovery rates were determined and are presented in **Table S2**. From the data in the table, it can be observed that at indicated concentrations of 1 U mL^{-1}, 10 U mL^{-1}, and 80 U mL^{-1}, the recovery rates of TdT were 104.3%, 94.05%, and 90.6%, respectively, indicating good recovery rates. Moreover, all the relative standard deviation (RSD) values were below 5%, indicating good reproducibility of the current method. Therefore, these data demonstrate the promising practicability of the proposed method.

References

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