Electronic Supplementary Information for

Endo IV-assisted cascade amplification for ultrasensitive TdT detection in acute lymphoblastic leukemia

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1.Materials and methods

1.1 Oligonucleotides and Reagents

All DNA oligonucleotides were synthesized by Sangon Biotech (Shanghai, China), with their sequences provided in the Supplementary Information (Table S1). TdT, UDG, T7 exonuclease, hAAG, Buffer 1, Buffer 2.1, Buffer 3.1, Buffer 4, and other reagents were obtained from New England Biolabs (NEB, USA).

1.2 Sample Collection

All experiments involving human subjects were conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. This study was reviewed and approved by the Institutional Ethics Committee of the General Hospital of Ningxia Medical University (Approval No.: KYLL-20241013). Written informed consent was obtained from all participants—including healthy volunteers (HC, n=20) and newly diagnosed acute lymphoblastic leukemia (ALL, n=10) patients—prior to sample collection. Peripheral blood samples from ALL patients were confirmed by clinical pathology. All procedures followed the institutional guidelines for human subject research, ensuring confidentiality and voluntary participation.

1.3 Condition Optimization

The reaction system utilized a 1000 nM PolyA chain (S-001) as the template. Endo IV concentrations varied from 100 to 800 U/mL, while the fluorescent probe concentrations ranged from 100 to 300 nM. The reactions were conducted at 37°C, with fluorescence detection occurring every 30 seconds for a total of 120 cycles.

1.4. Cell Lysis

Peripheral blood was collected and processed within 2 hours. Peripheral blood mononuclear cells (PBMC) were separated using density gradient centrifugation, and the cells were subsequently treated with lysis buffer to obtain the samples for analysis.

1.5. TdT Activity Assay Reaction System and Procedure

The TdT activity assay employs a two-stage reaction system: In the first stage, the TdT sample to be tested is reacted with 100 nM L-001 template strand and 1 mM dATP in TdT

buffer at 37°C for 30 minutes to complete the poly(dA) extension. In the second stage, 100 U/mL Endo IV and 200 nM fluorescent probe are added, and the mixture is further incubated at 37°C while fluorescent signals are detected every 30 seconds using a plate reader (Ex/Em = 495/520 nm), monitoring a total of 120 cycles (60 minutes).

1.6. TdT Quantification ELISA (Enzyme-Linked Immunosorbent Assay)

The procedure was strictly performed according to the manufacturer's instructions of the commercial kit (Ruifan Biotechnology Co., Ltd., Cat. No. RF5043).

1.7. Statistical Analysis.

All parameters were expressed as mean ± standard deviation (SD), with statistical significance defined as $P \leq 0.05$. The comparison of TdT enzyme reaction rates between the acute lymphoblastic leukemia (ALL) group and the healthy control group was conducted using an unpaired Student's t-test. Data analysis was performed using SPSS software version 25.0. For comparisons across multiple groups, one-way analysis of variance (ANOVA) was used, followed by Tukey’s post hoc test to evaluate significant differences.

Table S1. Sequences of the oligonucleotide probes in the biosensor

Name	Sequence (5'→ 3')
L-001	GCTCTAGCTAGCTAAATCGCAGTGCAGGTCCAAAAATTTAAAAAGGACC
	TGCACTGCGATTTAGCTAGCTAGAGCGCC
S-001	AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA
Probe	TTTTTTT/i6FAMdT/TTTTT/idSp/TTTTT-BHQ-1

Table S2. Comparative Analysis of Endonuclease IV-Assisted Cascade Amplification System and Conventional Methods for TdT Detection

Readout	Method	Linear range	LOD	Time	Ref.
Fluorescent readout	OPT-Cas	1×10^{-4} - 1×10^{-1} U μL^{-1}	6.16×10^{-5} U μL^{-1}	120 min	1
Fluorescent readout	Single quantum dot	0.001-1 U mL^{-1}	0.001 U mL^{-1}	170 min	2
Fluorescent readout	Silver nanoclusters	0.05-10 U mL^{-1}	0.042 U mL^{-1}	120 min	3
Fluorescent readout	Versatile biosensor	0-300 U mL^{-1}	3.18 U mL^{-1}	130 min	4
Fluorescent readout	Endonuclease IV-Assisted Cascade Amplification System	0-0.3 U μL -1	0.96 mU μL^{-1}	90 min	This work

References

[1] M. Yi, Y. Gong, Q. Zhan, Y. Dai, T. Yang, X. Cheng, S. Ding, B. Gu, W. Cheng, D. Zhang, A one-pot CRISPR-Cas12a-based toolbox enables determination of terminal deoxynucleotidyl transferase activity for acute leukemia screening, Analytica Chimica Acta, 1254 (2023) 341115.

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[3] X. Wang, J. Xu, P. Qin, C. Yan, G. Liu, W. Chen, Self-assembly of a polythymine embedded activatable molecular beacon for one-step quantification of terminal deoxynucleotidyl transferase activity, Anal. Chim. Acta. 1141 (2021) 127-135.

[4] Y. Yuan, W. Li, Z. Liu, Z. Nie, Y. Huang, S. Yao, A versatile biosensing system for DNA-related enzyme activity assay via the synthesis of silver nanoclusters using enzymatically-generated DNA as template, Biosens. Bioelectron. 61 (2014) 321-327.

Table S3. Cost analysis of the TdT-Endo IV Biosensors [a].

Reagents	Unit price	Usage	Cost
Endo IV	$150/50 μL	0.1 μL	$0.3
Probe	$200/100 μL	0.002 μL	$0.04
dATP	$30/1500 μL	0.01 μL	$0.001
	Total cost per reaction		$0.341

[a] Non-critical reagents (e.g., buffers, ssDNA) were omitted from cost analysis as they constituted <1% of total expenditure

Table S4. Recovery experiments of TdT in human serum samples.

TdT	Spiked (U μL^{-1})	Found (U μL^{-1})	Recovery %	RSD (n=3, %)
	0.01	0.0096	96.00	2.20
	0.1	0.098	98.00	1.29
	0.2	0.205	102.5	1.81
	0.4	0.397	99.25	3.80

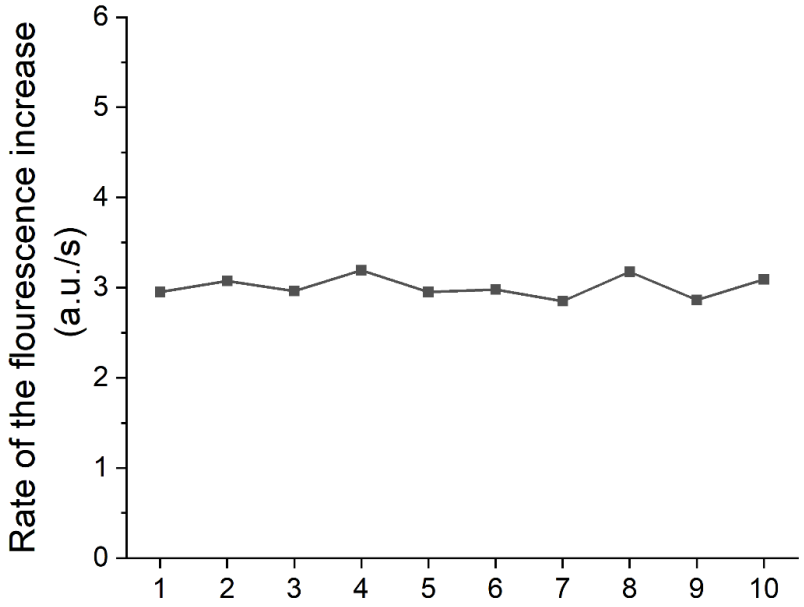

Figure. S1 Reproducibility assessment of the TdT activity detection system

Under optimized reaction conditions (100 nM L-001 template strand, 1 mM dATP, 100 U/mL Endo IV, and 200 nM fluorescent probe in TdT-specific buffer), ten replicate measurements were conducted with 0.1 U/μL TdT to evaluate the assay reproducibility.

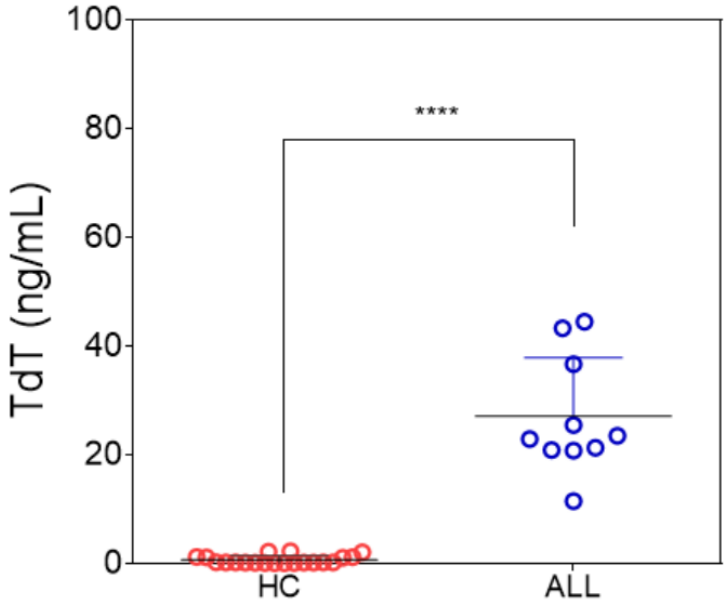

Figure. S2 Comparison of TdT Levels (ng/mL) Between Healthy Controls（n=20） and ALL Patients（n=10）

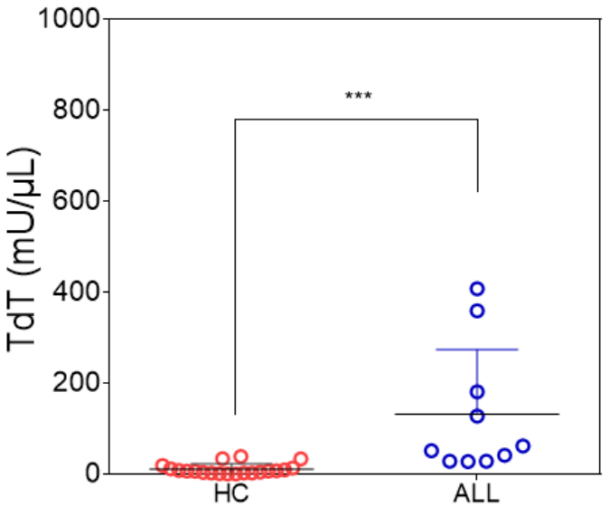

Figure. S3 Comparison of TdT Levels (mU/μL) Between Healthy Controls（n=20） and ALL Patients（n=10）