Electronic Supplementary Information (ESI)

Amplification-free detection of telomerase activity at the single-cell level via Cas12a-lighting-up single microbead (Cas12a-LSMB)

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1. Experimental section

1.1 Materials

The Streptavidin Mag Sepharose microbeads (STV-MBs), which are highly cross-linked spherical agarose (Sepharose) containing magnetite and surface-coupled with STV through NHS, were obtained from Cytiva (Uppsala, Sweden). The CHAPS lysis buffer was bought from Millipore Co., Ltd (USA). The Triton X-100, JumpStart DNA polymerase, and 10 × PCR Buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, pH 8.3) were bought from Sigam-Aldrich (Shanghai, China). Recombinant CRISPR-Cas12a protein (LbCas12a) was supplied by Editgene Co., Ltd, (Guangzhou, China). Recombinant RNase Inhibitor (RRI), dNTPs, and RNase-free water were supplied by TaKaRa Biotechnology Co. Ltd. (Dalian, China). 1 × PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4, pH 7.4 at 25 ℃) and 1 × PBST (1 × PBS with 0.05% tween-20) flowed from Sangon Biotech Co. Ltd. (Shanghai, China). 10 × NEBuffer 2.1 (100 mmol/L Tris-HCl, 500 mmol/L NaCl, 100 mmol/L $MgCl_2$, 100 μg/mL BSA, pH 7.9 at 25°C) was purchased from New England BioLabs Inc. (Bejing, China). Telomerase substrate (TS or TSP), ACX primer, crRNA, off-target crRNA, TSE-9 DNA, off-target DNA, and Biotin-ssDNA reporter were custom synthesized and purified by Sangon Biotech (Shanghai, China). 20 × SYBR Green I (20 ng/μL stock solution in DMSO) was purchased from Xiamen Zhishan Biological Technology Co., Ltd. (Xiamen, China). All the reagents were of analytical grade and were used as received without further purification. The sequences of the oligonucleotides were given in Table S1. The HeLa, HL-60, NB4, MCF-7, A549, and MRC-5 cell lines were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China).

Table S1. Sequences of the oligonucleotides used in this work.

Name	Sequence（5′-3′ direction）
Telomerase substrate (TS)	AATCCGTCGAGCAGAGTT
TSE-DNA crRNA	UAAUUUCUACUAAGUGUAGAUCCCUAACCCUAACCCUAACC
Off-target crRNA	UAAUUUCUACUAAGUGUAGAUUGAAGUAGAUAUGGCAGCAC
TSE-9	AATCCGTCGAGCAGAGTTTTAGGGTTAGGGTTAGGGTTAGGGTT AGGGTTAGGGTTAGGGTAAGGGTTAGGG
Off-target ssDNA	CGTAAATGCACTTGCTTCAGGACCATATTTCTCTACACCTTTTTT AGGATGCTTTGTTTCAGGTGTATCAACCAATAATAGTCTGAATGT CATTGGTTGACC
TS primer (TSP)	AATCCGTCGAGCAGAGTT
ACX	GCGCGGCTTACCCTTACCCTTACCCTAACC
Biotin-ssDNA reporter	6-FAM-/IBI ODT/TATTATATT-BHQ1

Notes: The letter /IBI ODT/ indicates the Biotin modification at the T.

1.2 Cell culture and extraction of telomerase

The MRC-5 (Human embryonic lung fibroblasts cell), NB4 (Human acute promyelocytic leukemia cell), A549 (Human non-small cell lung cancer cell line), HL-60 (Human acute promyelocytic leukemia cell) cell lines were cultivated in 5 mL RPMI 1640 Medium (Sangon Biotech) contains 10% (v/v) fetal bovine serum, 1.5 g/L $NaHCO_3$, 2.5 g/L glucose, 0.11 g/L Sodium Pyruvate. HeLa (Human cervical cancer cell) and MCF-7 cells (Human breast ductal carcinoma cell line) were cultured in the DMEM Medium (Sangon Biotech), and it includes 10% (v/v) fetal bovine serum (Sangon Biotech), 1% $NaHCO_3$, 100 U/mL penicillin, 100 mg/mL streptomycin, and 3 mmol/L L-glutamine. All of the cell lines were cultured at 37 °C and the CO_2 content in humid air was 5%.

After digesting the cultured Hela cells or other cells with trypsin (0.2% trypsin, 1 mmol/L EDTA, Invitrogen), all cells were centrifuged at 1000 rpm for 5 minutes and then removed medium. The cells were washed with cold D-PBS buffer (10 mmol/L sodium phosphate buffer, 0.1 mol NaCl, pH 7.4 at 25 °C), accurately counted by a blood counting chamber, and then centrifuged to obtain aggregated cells. Each cell pellet was resuspended in an appropriate volume of CHAPS cell lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.1 mmol/L PMSF, 5 mmol/L mercaptoethanols, 0.5% CHAPS, 10% glycerol), in which ensures the cell concentration is 10^4 cells/μL, placed on ice for 30 minutes, and then centrifuged at 12,000 rpm at 4 °C for 20 minutes. The upper layer cell extracts

containing telomerase were divided and stored in an ultra-low temperature refrigerator at -80 °C. The telomerase extracts are appropriately diluted with CHAPS lysis buffer as a sample for telomerase activity detection.

1.3 Preparation of single-cell lysates

To obtain a single cell, we continuously diluted the above cell suspension to 1 cell/μL. 1 μL of the diluted cell suspension was transferred to the hydrophobic 96-well plate cover, and then one cell was accurately aspirated utilizing a micromanipulation system equipped with a monitor. Finally, 1 cell was dropped into 1 μL of cold Triton X-100 (0.2%) solution to lysate for 5 min, and its telomerase activity was immediately detected as the sample.

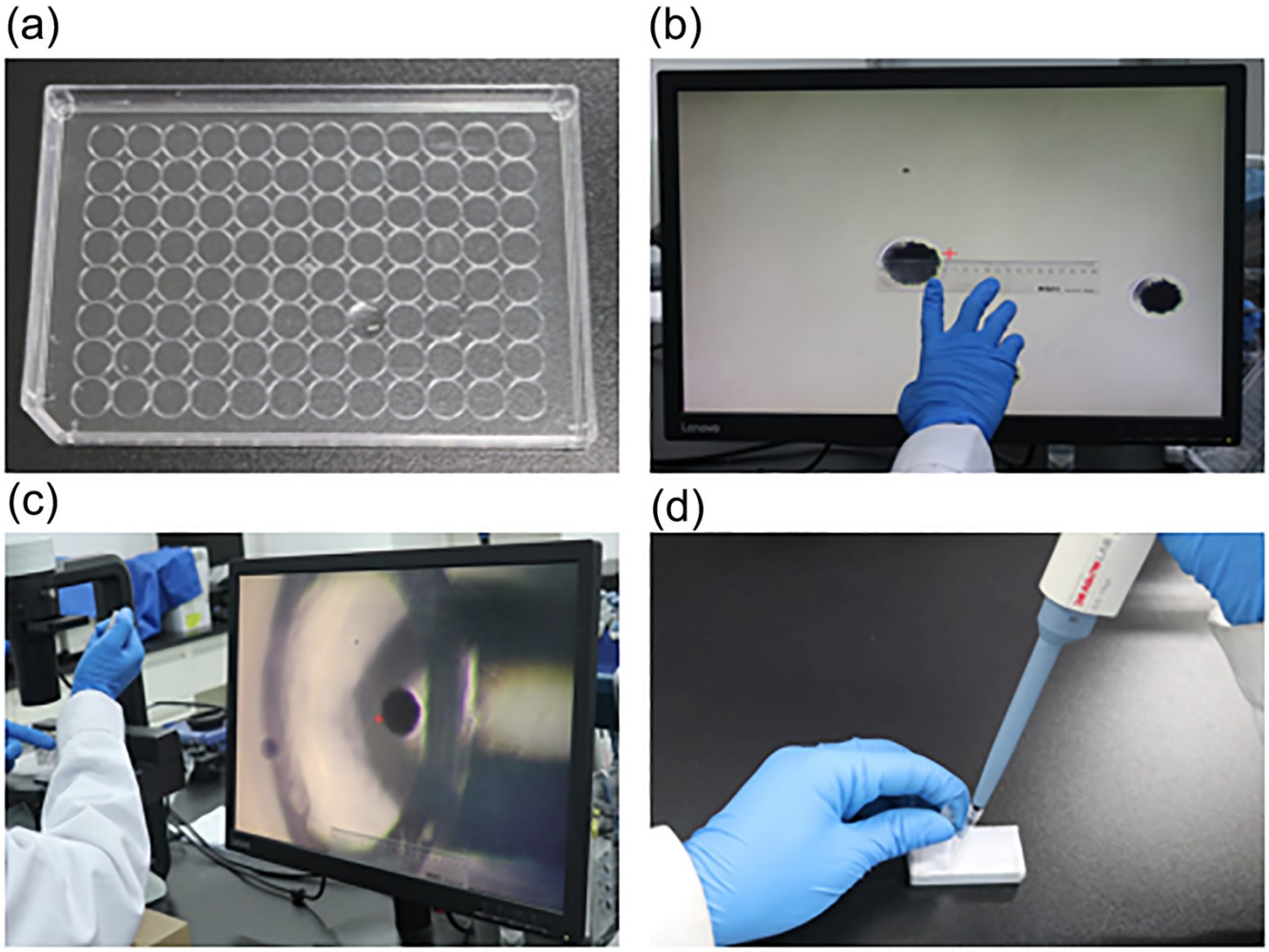

Fig. S1. Illustration of the capturing process of a single STV-MB.

1.4 Preparation of the single microbeads (SMBs)

Typically, 50 μL of PBST solution suspended with hundreds of STV-MBs was dropped onto a transparent hydrophobic 96-well plate cover to assist and capture individual MB (Fig. S1a). A DIY micromanipulation system, an Olympus IX53 inverted microscope equipped with a monitor was used to operate individual STV-MBs. With the help of the monitor, we can search the suitable MBs on the monitor with the naked eye. A ruler was employed to help us select the beads with the desired diameter size (a diameter of ~6 cm on the monitor and the actual diameter is ~ 80 μm). Once the desired SMB was selected, we only needed a pipette to capture and transfer it to a PCR tube (Fig. S1d). To ensure the reproducibility of the Cas12a-LSMB-based telomerase assay, the size of the selected STV-MBs was strictly controlled at 80 ± 5 μm.

1.5 Functionalization of SMBs

Place a single SMB in a PCR tube containing 9.5 μL PBS, add 0.5 μL Biotin-ssDNA reporter (50 μmol/L), and oscillate for one hour at room temperature. Then, the SMB was washed three times by magnetic separation to remove the excess probes for use.

1.6 Homogeneous Cas12a assay

Real-time fluorescence detection manner: 10 μL of the homogeneous Cas12a reaction mixture was prepared with 100 nM-1600 nM Cas12a, 50 nM-800 nM TSE DNA-crRNA, 500 nM Biotin-FQ-ssDNA reporter, 1× NEBuffer 2.1, and 1 nM TSE-9. Before adding TSE-9 DNA, all reaction mixtures were incubated at 37°C for 10 min to form the crRNA/Cas12a complex. The fluorescence of the bulk Cas12a assay was real-time monitored by Step-One Real-Time Thermal Cycler (Thermal Fisher Scientific) at 20 °C-55 °C for 60 min.

Endpoint fluorescence detection manner: 100 μL of the homogeneous Cas12a reaction mixture was prepared with 400 nM Cas12a, 200 nM TSE DNA-crRNA, 500 nM Biotin-FQ-ssDNA reporter, 1× NEBuffer 2.1, and cell extracts. Before adding cell extracts, all reaction mixtures were incubated at 37°C for 10 min to form the crRNA/Cas12a complex. The mixture solution was incubated at 37 °C for 60 min to perform the *trans*-cleavage reaction of Cas12a. Then, 100 μL 1 × PBS was added to the reaction solution, and the fluorescence spectra were measured on an F-7000 fluorescence spectrophotometer (Hitachi, Japan).

1.7 Cas12a-LSMB-based telomerase activity assay

(1) Telomerase reverse transcriptional extension reaction

5 μL reaction mixture containing 1 × telomerase reverse transcriptional extension buffer (20 mM Tris HCl, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.05% Tween-20, pH 8.3), 4 U RNase inhibitor (RRI), 500 μM dNTPs，10 nM telomerase substrate (TS), and various cell samples (including cell extracts, or heating-treated cell extracts, or single cell lysates) were incubated at 37 °C for 60 min (30 min for RQ-TRAP assay) to obtain the telomerase extension product and then incubated at 95 °C for 5 min to inactive the telomerase.

(2) *Trans*-cleavage reaction of the CRISPR/Cas12a system

5 μL reaction mixture (containing 400 nM LbCas12a protein, 200 nM TSE-DNA crRNA, and 2 × NEBuffer 2.1 (20 mM Tris-HCl，100 mM NaCl，20 mM $MgCl_2$，2 μg/mL BSA), and Functionalized-SMB) was added into the telomerase reverse transcription extension mixture (5 μL). Then the reaction mixture (10 μL) was incubated at 37 °C for 60 min under gentle oscillation to complete the trans-cleavage reaction of the CRISPR/Cas12a system.

(3) Fluorescence imaging

After the reaction, the reaction solution was removed. Then, the Functionalized-SMB was washed twice with 200 μL PBST and resuspended in 5 μL of PBS. Subsequently, fluorescence imaging of the individual Functionalized-SMB was obtained on TCS-SP8 confocal fluorescence microscopy (Leica) by collecting the fluorescence at the wavelength range from 500 nm to 570 nm under the excitation of a 488 laser.

1.8 RQ-TRAP-based telomerase activity assay

5 μL of the telomerase reverse transcription extension mixture was added to 15 μL of the TRAP assay solution, which contains 1 × PCR Buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, pH 8.3), 1 × SYBR Green I, 1 U JumpStart Taq DNA polymerase, 250 nM TS primer, 250 nM ACX primer, and 500 μM dNTPs. The PCR was performed using a Step-One Real-Time Thermal Cycler (Thermal Fisher Scientific) with the following procedure: 94°C for 2 min; 40 cycles at 94°C for 20 s, 50°C for 30 s, and 72°C for 90 s.

2. Additional data

2.1 Optimization of experimental conditions for *trans*-cleavage of Cas12a assay

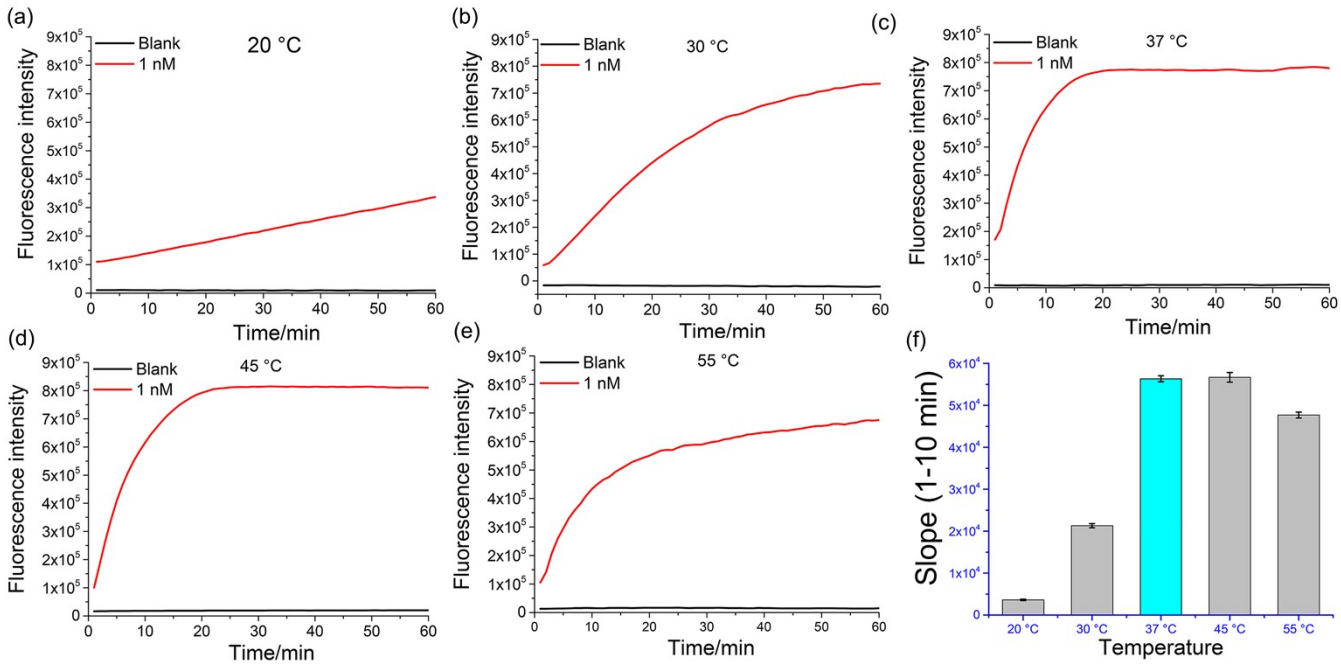

Fig. S2 Effect of the reaction temperature on *trans*-cleavage efficiency of Cas12a. (a-e) Real-time fluorescence curves of Cas12a *trans*-cleavage initiated by 1 nM TSE-9 DNA at different temperatures, including 20 °C (a), 30 °C (b), 37 °C (c), 45 °C (d), and 55 °C (e). (f) The slope of real-time fluorescence curves (1-10 min) produced by 1 nM TSE-9 DNA. Other conditions: TSE-DNA crRNA, 200 nM; Cas12a protein, 400 nM. Error bars show the standard deviations of three repeated experiments.

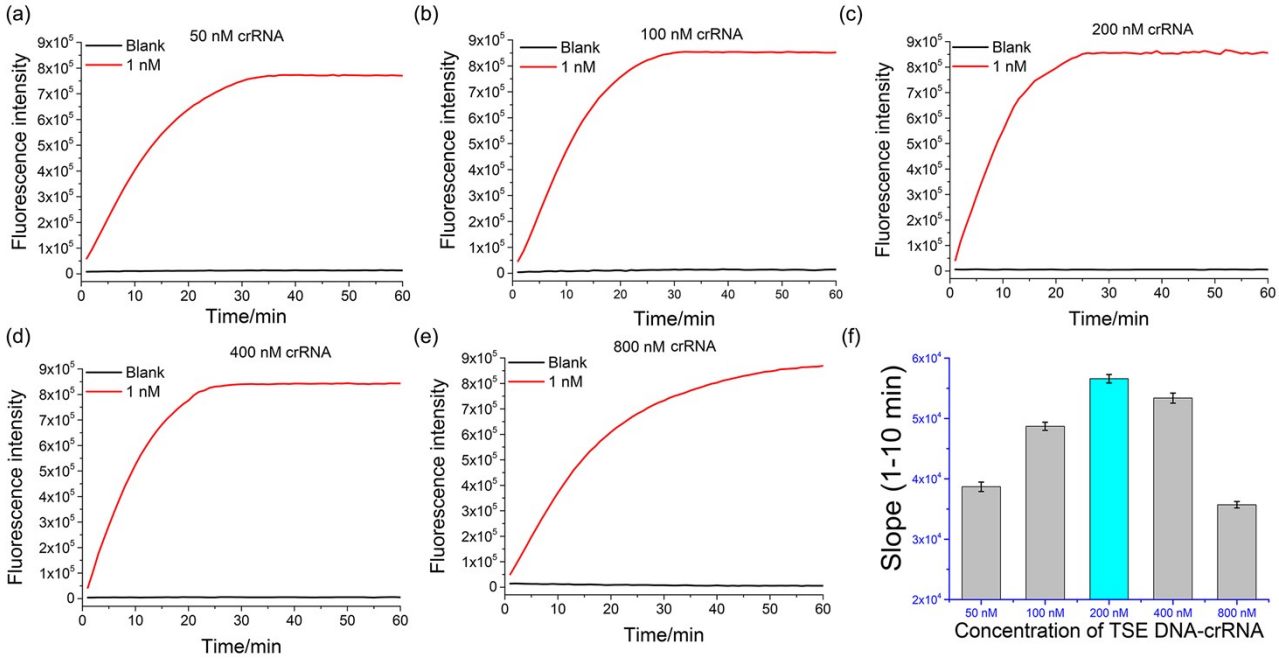

Fig. S3 Influence of the TSE-DNA crRNA concentration on *trans*-cleavage efficiency of Cas12a. (a-e) Real-time fluorescence curves of Cas12a *trans*-cleavage initiated by 1 nM TSE-9 DNA at different concentrations of TSE-DNA crRNA, including 50 nM (a), 100 nM (b), 200 nM (c), 400 nM (d), and 800 nM (e). (f) The slope of real-time fluorescence curves (1-10 min) produced by 1 nM TSE-9 DNA. Other conditions: Cas12a protein, 400 nM; reaction temperature, 37 °C. Error bars show the standard deviations of three repeated experiments.

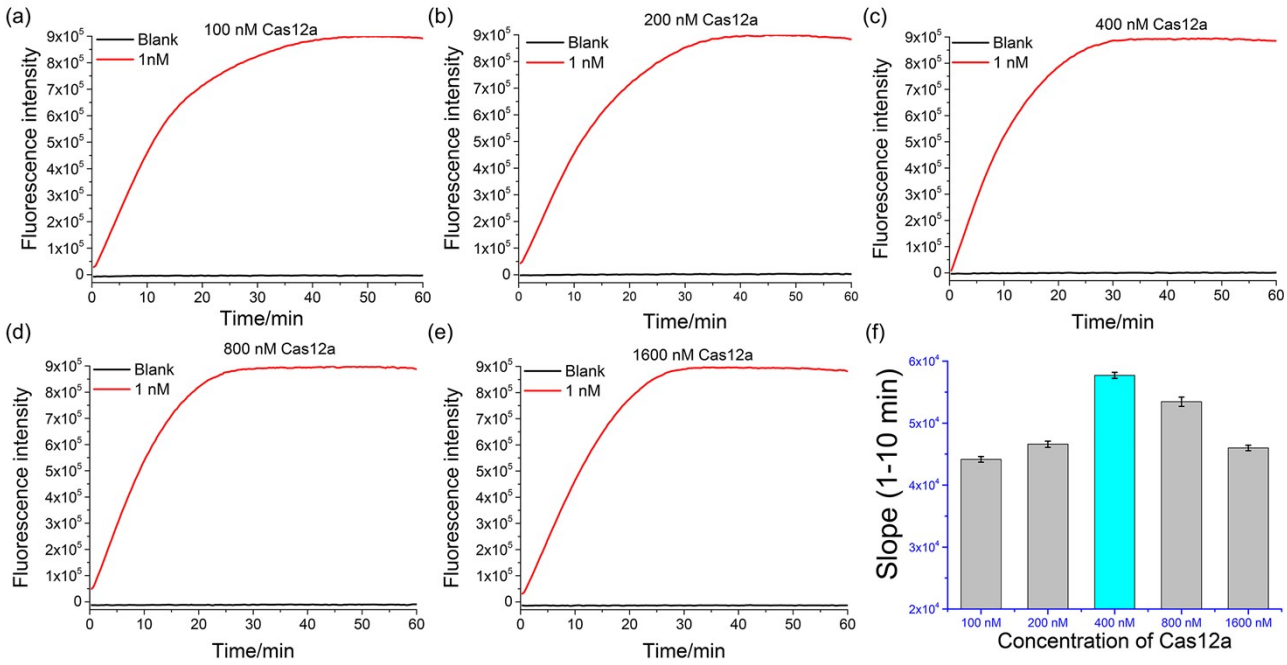

Fig. S4 Effect of the Cas12a protein concentration on *trans*-cleavage efficiency of Cas12a. (a-e) Real-time fluorescence curves of Cas12a *trans*-cleavage initiated by 1 nM TSE-9 DNA at different concentrations of Cas12a protein, including 100 nM (a), 200 nM (b), 400 nM (c), 800 nM (d), and 1600 nM (e). (f) The slope of real-time fluorescence curves (1-10 min) produced by 1 nM TSE-9 DNA. Other conditions: TSE-DNA crRNA, 200 nM; reaction temperature, 37 °C. Error bars show the standard deviations of three repeated experiments.

2.2 Evaluation of telomerase activity by using homogeneous Cas12a assay

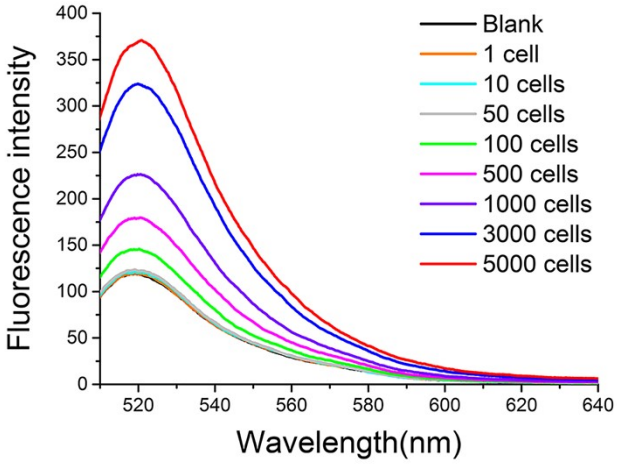

Fig. S5 Evaluating the telomerase activity in different numbers of HeLa cell extracts by using the endpoint fluorescence homogeneous Cas12a assay. Experimental conditions: TSE DNA-crRNA, 200 nM; Cas12a protein, 400 nM; Telomerase self-extension reaction time, 60 min; *Trans*-cleavage reaction time, 60 min; Telomerase self-extension and *Trans*-cleavage reaction temperature, 37°C.

2.3 Detection of telomerase activities in the cell extracts

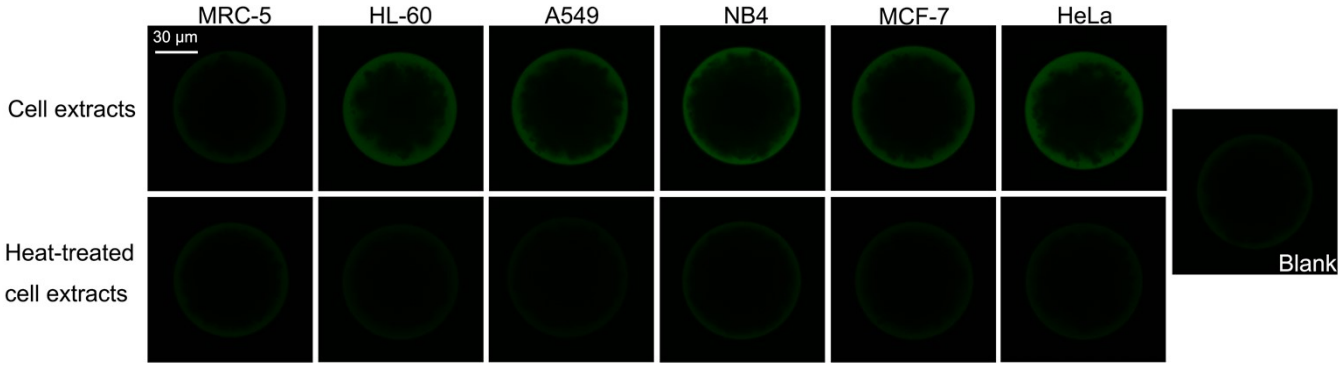

Fig. S6 Detection of telomerase activity in cell extracts and heat-treated cell extracts (10 cells) from different cell lines, including MRC-5, HL-60, A549, NB4, MCF-7, and HeLa.

2.4 Evaluation of telomerase activities in the cell extracts with RQ-TRAP assay

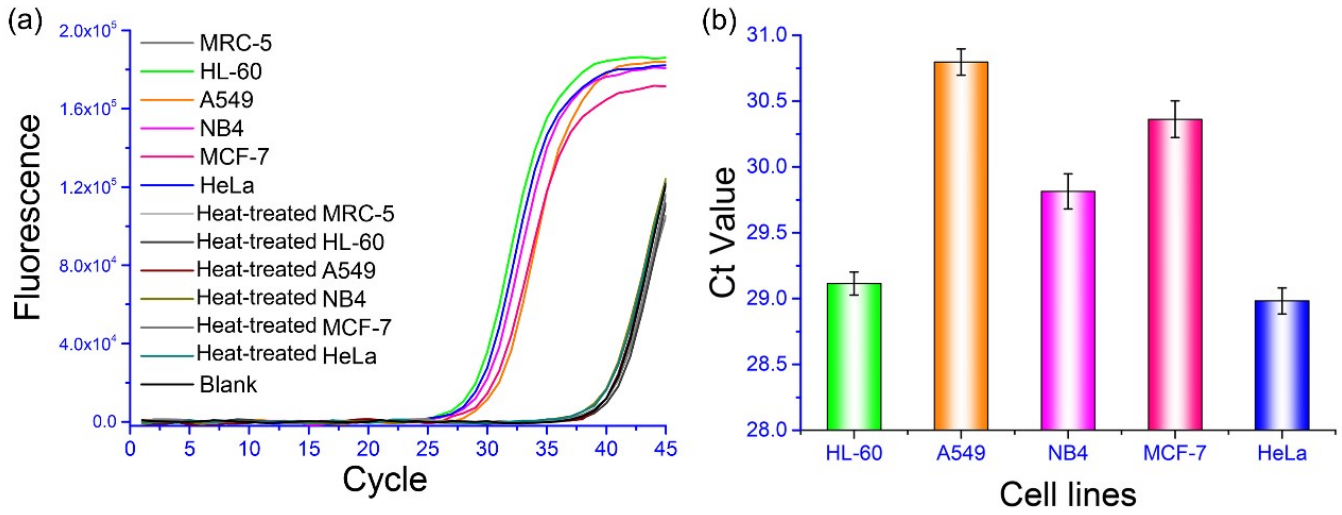

Fig. S7 Evaluation of telomerase activities in the cell extracts with RQ-TRAP assay. (a) Real-time fluorescence curves produced by 10 cell equivalent extracts and heat-treated extracts from different cell lines. (b) Ct value of real-time fluorescence curves generated by 10 cell equivalent extracts from HL-60, A549, NB4, MCF-7, and HeLa.

2.5 Telomerase activities analysis in human serum

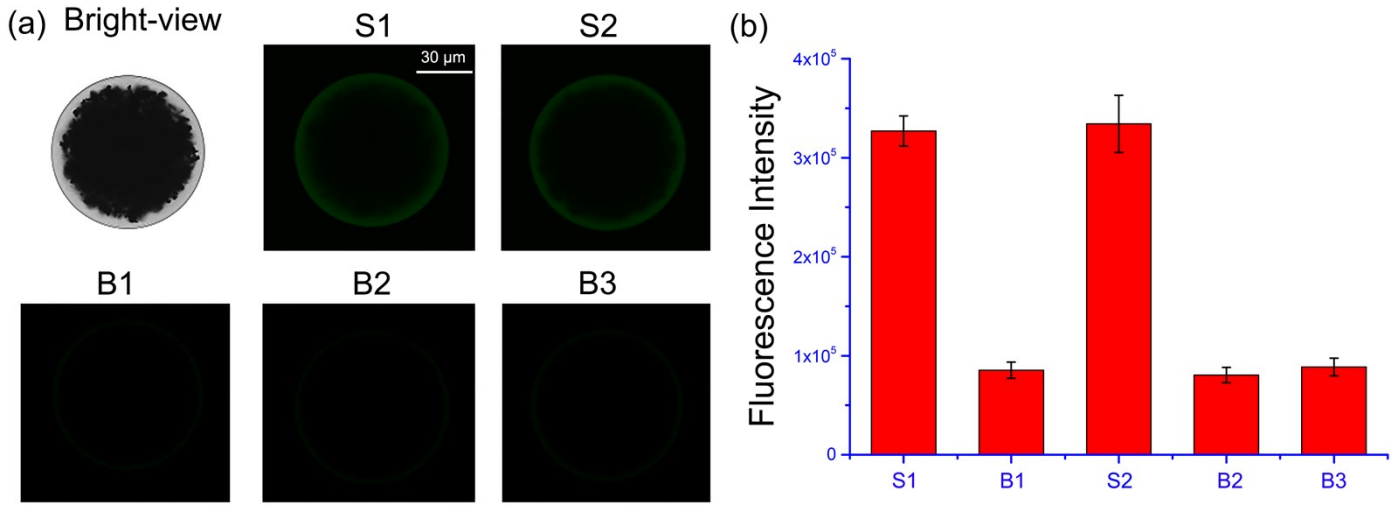

Fig. S8 Telomerase activity analysis in Human Serum by using Cas12a-LSMB. (a) Fluorescence images of SMBs produced by different samples. (B) The adopted fluorescence intensity of the SMB images. The left-top image in (a) is the bright-view image of the individual SMB. S1: 10 HeLa cell equivalent extracts; S2: 10 HeLa cell equivalent extracts-spiked serum (0.2 μL) sample; B1: CHAPS buffer; B2: Serum; B3: 10 HeLa cell equivalent heat-treated extracts-spiked serum (0.2 μL) sample. Error bars show the standard deviations of three repeated experiments from the same samples.

2.6 Detection of telomerase activities in individual cells

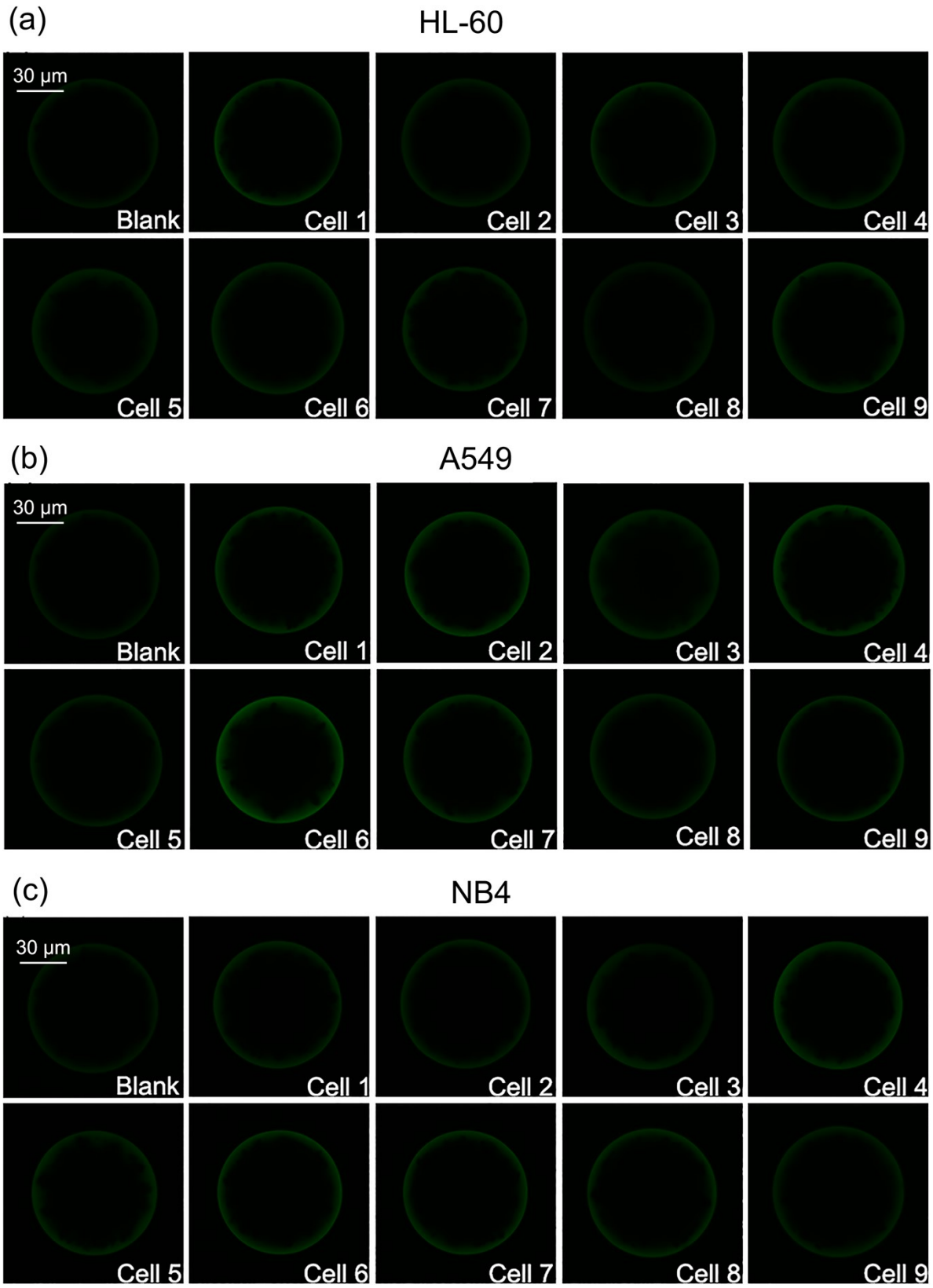

Fig. S9 Telomerase activity assay in individual cells. (a) HL-60, (b) A549, and (c) NB4.

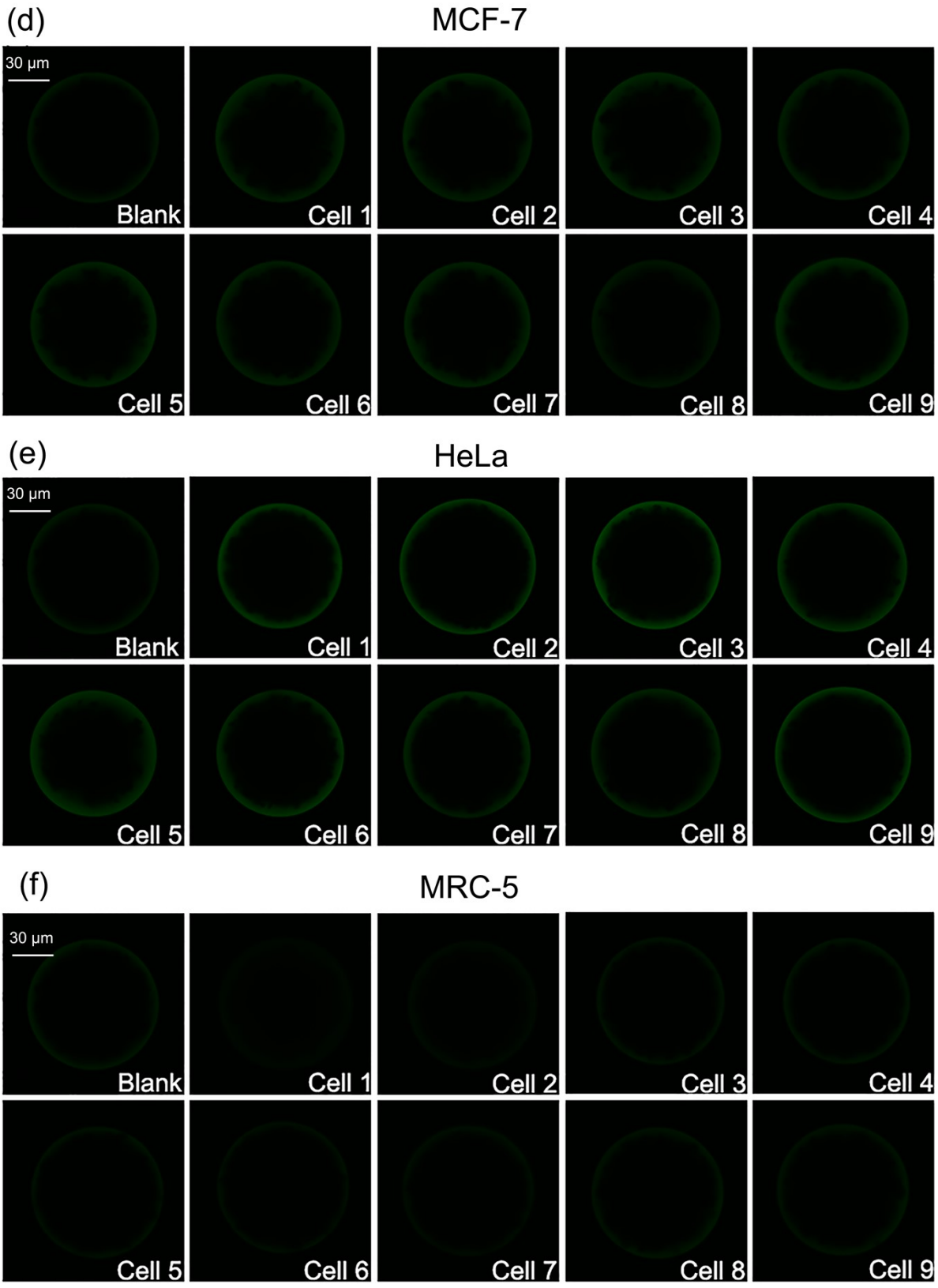

Fig. S9 Telomerase activity assay in individual cells. (d) MCF-7, (e) HeLa, and (f) MRC-5.

2.7 Comparison of the performance of Cas12a-LSMB with other methods for telomerase analysis

Table S2. Comparison of the performance of Cas12a-LSMB with other methods for telomerase analysis.

Method	Probe design and numbers	Amplification	Preparation process and time of sensors	Processes and temperature of basic measurement	Analysis time	Sensitivity	Signal output	Ref
Real-time TRAP	Simple, 2	PCR	No need	Self-extension, 25°C; Thermal cycles	150 min	1 cell	Real-time fluorescence	[1, 2]
RCA	Complex, 3	RCA	No need	Pre-Hybridization, 95°C and 37°C; Self-extension, 37°C; ligation, 16°C; RCA 30 °C	7 h	1 cell	Fluorescence	[3]
3WJ-EXPAR	Complex, 3	EXPAR	No need	Self-extension 30°C; Hybridization, 37°C; EXPAR 55 °C	180 min	3 cells	Fluorescence	[4]
EXPAR	Complex, 2	EXPAR	No need	Self-extension 37°C; EXPAR 55 °C	30-60 min	1 cell	Fluorescence	[5,6]
SPEA	Complex, 4	LAMP	No need	Self-extension 37°C; LAMP 65 °C	100 min	1 cell	Real-time fluorescence	[7]
HCR	Complex, 4	HCR	Complex, > 24 h	Self-extension 37°C; Hybridization, room temperature; HCR, room temperature	4 h	2 cells	Electrochemical	[8]
Electrochemistry sensor	Complex, 3	CHA	Complex, ~ 60 h	Self-extension 37°C; incubation and CHA, 37°C	~250 min	100 cells	Electrochemical	[9]
Smart DNA Tweezer	Complex, 4	SDA	Simple, < 10 min	Self-extension 37°C; Telomerase inactivated 95°C; SDA 37°C	~ 12 h	280 cells	Fluorescence	[10]

PCR-free fluorescence assay	Complex, 4	No need	Simple, < 10min	Self-extension 30°C; Telomerase inactivated 95°C; SDA 37°C	110 min	5 cells	Fluorescence	[11]
Nanoparticles-based assay	Simple, 2	No need	Complex, ~ 24 h	Self-extension 30°C; Telomerase inactivated 95°C; Hybridization	~ 70 min	100 cells	Color or UV-Vis spectra	[12]
SPR assay	Simple, 2	No need	Complex, > 24 h	Self-extension 38°C	~65 min	18 cells	SPR spectra	[13]
One-pot Cas12a assay	Simple, 2	No need	No need	One step 37°C	60 min	100 cells	Fluorescence	[14]
Two-step Cas12a assay	Simple, 2	No need	No need	Self-extension 37°C; Telomerase inactivated 95°C; Trans-cleave reaction 37°C	155 min	5 cells	Fluorescence	[15]
pcDNA Cas12a assay	Complex, 3	No need	No need	Self-extension 37°C; Trans-cleave reaction 37°C	180 min	10 cells (*in vitro*) or 1 cell (*in vivo*)	Fluorescence or Fluorescence imaging	[16]
Cas12a-LSMB	Simple, 2	No need	Simple, ~ 60 min	Self-extension 37°C; Telomerase inactivated 95°C; Trans-cleave reaction 37°C	125 min	1 cell	Fluorescence imaging	This work

2.8 Experimental data analysis of Cas12a-LSMB-based telomerase assay

Table S3 Experimental data analysis of Cas12a-LSMB-based telomerase assay

Cell Number	Cell Line	Average	SD	SD/Average	Fig
1 cell	HeLa	183266	11093	0.061	Fig. 4b
10 cells	HeLa	354244	15166	0.043	Fig. 4b
100 cells	HeLa	557706	17935	0.032	Fig. 4b
500 cells	HeLa	655312	30920	0.047	Fig. 4b
1000 cells	HeLa	804679	28458	0.035	Fig. 4b
10 cells	MRC-5	107520	8856	0.082	Fig. 4c
10 cells	HL-60	328149	12626	0.038	Fig. 4c
10 cells	A549	276787	8282	0.030	Fig. 4c
10 cells	NB4	321356	9368	0.029	Fig. 4c
10 cells	MCF-7	296750	10243	0.035	Fig. 4c
10 cells	Hela	333775	8926	0.027	Fig. 4c
10 cells	MRC-5	99857	8795	0.088	Fig. 4c
10 cells	HL-60	101694	10599	0.104	Fig. 4c
10 cells	A549	90287	9821	0.109	Fig. 4c
10 cells	NB4	103102	10484	0.102	Fig. 4c
10 cells	MCF-7	102000	9829	0.096	Fig. 4c
10 cells	Hela	95851	9980	0.104	Fig. 4c
	Blank	88157	8625	0.098	Fig. 4c
1 cell	MRC-5	83438	8657	0.104	Fig. 5
1 cell	HL-60	131169	17879	**0.136**	Fig. 5
1 cell	A549	152613	23545	**0.154**	Fig. 5
1 cell	NB4	135977	22178	**0.163**	Fig. 5
1 cell	MCF-7	158614	23171	**0.146**	Fig. 5
1 cell	HeLa	178342	26847	**0.151**	Fig. 5

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