Supporting Information

A fuel-limited isothermal DNA machine for sensitive detection of cellular deoxyribonucleoside triphosphates

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Experimental Section

Materials

Vent (exo-) polymerase (2,000 U/mL), Nt.AlwI (10,000 U/mL), endonuclease IV (Endo IV) (10,000 U/mL), NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9 @ 25 °C), ThermoPol Reaction Buffer (20 mM Tris-HCl, 10 mM KCl，10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 0.1% Triton X-100, pH 8.8 @ 25 °C), CutSmart Buffer (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 100 μg/ml BSA, pH 7.9 @ 25 °C), NEBuffer 3 (100mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$, 1mM DTT, pH 7.9 @ 25 °C) were purchased from New England Biolabs (MA, USA). dNTPs (100 mM) and rNTPs (100 mM) were purchased from Sangon Biotech Co. (Shanghai, China). DNase/RNase free deionized water was purchased from Tiangen Biotech Co. (Beijing, China). All DNA strands listed in Table S1 and Table S2 were purchased from Sangon Biotech Co. (Shanghai, China, purified by HPLC). The human alveolar epithelial cell line A549 was a kind gift from Prof. Xiangdong Li at the State Key laboratory of Agro-Biotechnology at China Agricultural University (Beijing, China). Ham's F-12K (Kaighn's) Medium (Gibco, Rockville, MD), fetal bovine serum (FBS) (Invitrogen, Rockville, MD), penicillin-streptomycin (10,000 U/mL) (Gibco, Rockville, MD) and phosphate-buffered saline (PBS) were purchased from Thermo Fisher Scientific Inc. (MA, USA). 5-Fluoro-2'-deoxyuridine (FUdR) and 5-fluorouracil (5-FU) were purchased from D&B Biological Science and Technology Co.Ltd (Shanghai, China).

Table S1. DNA sequences used in this work.

Name	Abbr.	Sequence (5' to 3')	T_m (°C)[a]
Short Primer	SP	CCGCCTCCACCGCC	64.1
Long Primer-dA	LP-A	CCGCCTCCACCGCCTTTA	66.2
Long Primer-dC	LP-C	CCGCCTCCACCGCCAAAC	67.6
Long Primer-dT	LP-T	CCGCCTCCACCGCCAAAT	67.2
Long Primer-dG	LP-G	CCGCCTCCACCGCCAAATAAAG	67.4
Template-dA[b]	T-A	*ACACAACACACACA*CAAAGA**T**CCAAA**T**AAAGGCGGTGGAGGCGG	76.7
Template-dC[b]	T-C	*ACACAACACACACA*CAAA**G**ATCCTTT**G**TTTGGCGGTGGAGGCGG	77.6
Template-dT/G[b]	T-T/G	*TGTGTTTTGTGGTT*TGGTGA**T**CCTTTA**T**TTGGCGGTGGAGGCGG	77 .0
AP Probe-dA/C[c]	P-A/C	**FAM**/ACACAA**X**ACACACA/**BHQ1**	47.2
AP Probe-dT/G[c]	P-T/G	**FAM**/TGTGTT**X**TGTGGTT/**BHQ1**	47.1
Template-dAO[b, d]	T-AO	*AGCAGAGACAGACAAACA*GAAAGA**T**CC**T**AAAGGCGGTGGAGGCGG	77 .0
AP Probe-dAO[c, d]	P-AO	**FAM**/AGCAGAG**X**CAGACAAACA/**BHQ1**	58.8

[a] T_m was calculated in the presence of 25 mM Na^+, 6 mM Mg^{2+}, 0.1 mM dNTPs and 0.1 mM oligonucleotides by Integrated DNA Technologies (IDT) OligoAnalyzer 3.1.

[b] Bases in bold denote the measuring points where the limiting dNTP binds opposite. The underlined bases represent the recognition sequence of Nt.AlwI. Bases in italics refer to the sequence excluding any measuring point which has the responsibility to produce a single stranded reporter (SSR).

[c] Letters in bold represent the location and type of probe modifications and "X" denotes dSpacer.

[d] Template-dAO and AP Probe-dAO are only used for the experimental optimization of the concentration of non-limiting dNTPs.

Detection of the limiting dNTP

To a 200 μL PCR tube, 11.5 μL (for the positive group) or 12.5 μL (for the blank) of water, 1.25 μL of 10×NEBuffer 2 Reaction Buffer, 1.25 μL of 10×ThermolPol Reaction Buffer, 1 μL of primer (2.5 μM) and 1 μL of template (2.5 μM) were added and mixed well. The solution was heated to 85°C for 5 min, followed by gradually cooling down to 37°C. Then 1 μL of AP probe (2.5 μM), 1 μL of non-limiting dNTP mixture (2.5 mM), 2 μL of Nt.AlwI (1,250 U/mL), 1 μL of Endo IV (250 U/mL), 3 μL of Vent(exo-) polymerase (250 U/mL) and 1 μL of the limiting dNTP (various concentrations for the positive group) were added and then detected at 37°C on a Rotor-Gene Q 5plex HRM Instrument (QIAGEN, Hilden, Germany). The excitation and emission wavelengths were set to 470 nm and 510 nm, respectively. The gain level was

set to 8 for dATP and dCTP detection while 9.67 for dTTP and dGTP detection. Fluorescence intensity was measured once a cycle (5 s per cycles). When all curves went into the phase of fluorescence rise, the instrument could be stopped to determine the time of fluorescence threshold. The total detection time varies in the range from 5 to 60 min according to different targets, primers and templates.

Cell culture and extraction of intracellular dNTPs

A549 cells were maintained in Ham's F-12K (Kaighn's) medium supplemented with 10% FBS and 1% penicillin-streptomycin in a Thermo Scientific 3111 incubator (Thermo Fisher Scientific Inc., MA, USA) at 37°C with 5% CO_2. Cells were counted on the TC 10 automated cell counter (Bio-Rad Co., CA, USA) with a trypan blue dye method. Before extraction, cells were plated in 10-cm tissue culture dishes (Corning, NY, USA) at 1×10^6 and incubated for 48 h to enter log-phase growth. Then the medium was replaced by normal, FUdR (1 μM) or 5-FU (5 μM)-contained medium and cells were incubated for 5 h. The steps of extraction were referred to the literature.[1] Briefly, the medium was first aspirated and cells were rinsed with PBS. Then digested by trypsin, the adherent cells were detached and suspended in 10 mL of ice-cold PBS. After counting, the cell suspension was centrifuged for 5 min at 3000*g* at 4°C in 5424R Refrigerated Centrifuge (Eppendorf, AG, Germany). Subsequently the supernatant was discarded and the cell deposits were resuspended in 500 μL of ice-cold 60% methanol. After heating at 95°C for 3 min, the solution is sonicated for 30 s in a Sonics Ultrasonic Cell Disrupter system (Sonics & Materials, Inc. Newtown, CT, USA). The cell extracts were centrifuged for 5 min at 16,000*g* at 4°C and further purified by ultrafiltration through pre-equilibrated Amicon Ultra-0.5 ml-3 kDa-centrifugal filters (Millipore, MT, USA) for 2 h at 14000*g* at 4°C. At last, the filtrate was evaporated at 80°C under centrifugal vacuum and the dry solute was dissolved in 50 μL DNase/RNase free deionized water. The solution was stored at -80°C until use.

Detection of intracellular dNTPs

The dNTP sample was first diluted into a certain concentration so that it fell within the linear range of a calibration curve. Then the sample was detected in a 200 μL PCR tube at 37°C in the same manner as described above.

Supplementary results

Optimization of experimental conditions

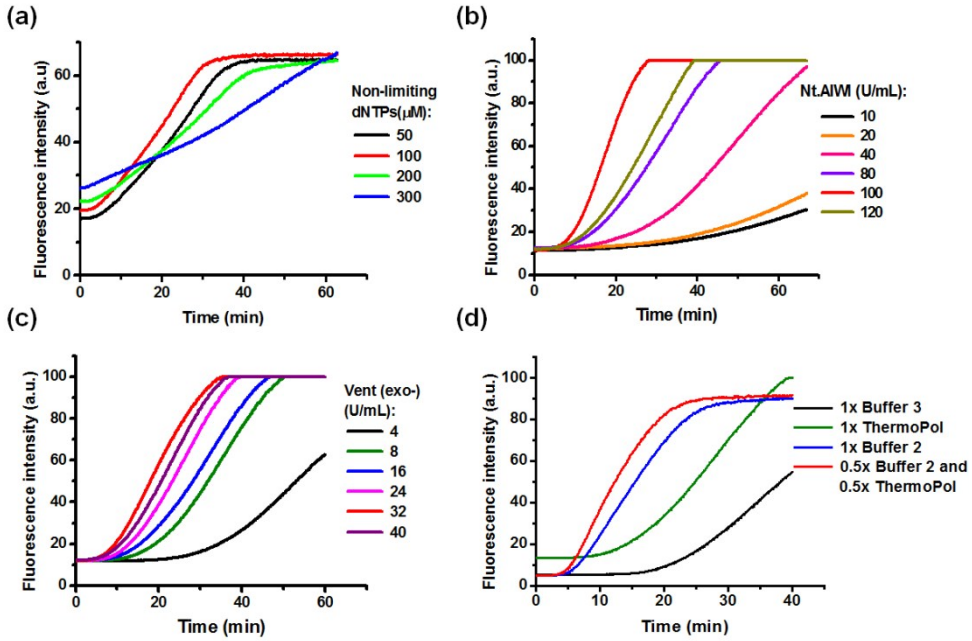

Fig. S1 Optimization of the experimental conditions by utilizing SP-dATP-2 detection system: (a) the concentration of non-limiting dNTPs, (b) the amount of Nt.AlwI, (c) the amount of Vent (exo-) polymerase, (d) the composition of the reaction buffer. Template-dAO and AP Probe-dAO were used in the optimization of the concentration of non-limiting dNTPs while Template-dA and AP Probe-dA were used in other optimization experiments. The amounts of all DNA sequences and the limiting dATP were 2.5 pmol and 5 pmol, respectively. The concentration of Endo IV was 10 U/mL, which had been optimal.[2] Before the optimization of buffer, all the experiments were performed in 1×ThermoPol Reaction Buffer.

To achieve the optimal performance of the fuel-limited isothermal DNA machine, the concentrations of non-limiting dNTPs, Nt.AlwI and Vent (exo-) polymerase and the composition of the reaction buffer were all examined by utilizing SP-dATP-2 detection system. Both the time required to reach the fluorescence threshold (F_T, defined as 5SD above the mean baseline fluorescence intensity) and the slope of the initial fluorescence curve were investigated as the parameters of the fluorescence signals to be correlated to the initial concentration of the dNTPs. Calibration curves with better linear relationship were obtained by plotting the reciprocal of t (t^{-1}) vs. $[dATP]_0$. The optimal concentration of non-limiting

dNTPs was found to be 100 μM (Fig. S1a). When the concentrations of dNTPs increase to higher than 100 μM, the DNA machine showed a poor performance, probably due to the inhibition of the enzyme activity by the high salt concentration. The concentrations of Nt.AlwI and Vent (exo-) polymerase were determined to be 100 U/mL and 32 U/mL, respectively (Fig. S1b, c). Because the buffer suitable for Vent (exo-) polymerase, Nt.AlwI and Endo IV were ThermoPol Reaction Buffer, NEBuffer 2 and NEBuffer 3, respectively, we used 1×buffer and a mixture of 0.5×ThermoPol Reaction Buffer and 0.5×NEBuffer 2 to test the performance of the machine. According to Fig. S1d, the machine showed the best performance in the mixed buffer.

Detection of dTTP and dGTP

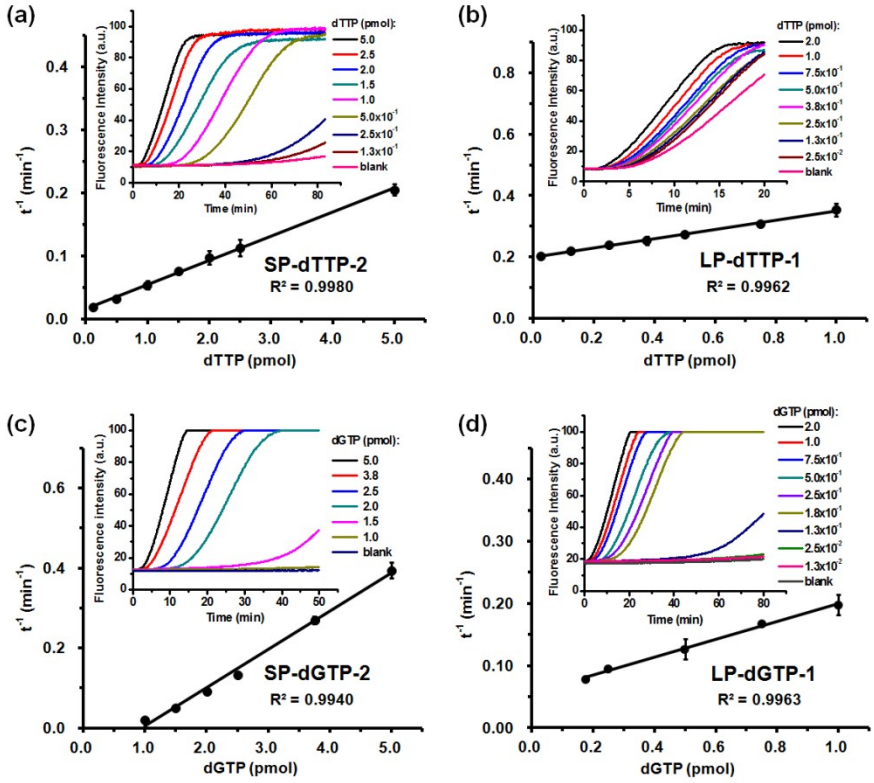

Fig. S2 Calibration curves by plotting t^{-1} *vs.* $[dNTP]_0$ for the limiting dNTP detection: (a) SP-dTTP-2, (b) LP-dTTP-1, (c) SP-dGTP-2, (d) LP-dGTP-1. Error bars, SD, n=3. Inset: Real-time fluorescence curves of the DNA machine upon the input of various amounts of the limiting dNTP.

Table S2 The linear range, accuracy and coefficient of variation (CV) for dNTP-2 and dNTP-1.

dNTP	dATP-		dCTP-		dTTP-		dGTP-	
	1	2	1	2	1	2	1	2
Linear Range (pmol)	0.13–1.00	1.00–5.00	0.025–1.00	0.25–5.00	0.025–1.00	0.13–5.00	0.25–1.00	1.00–5.00
Accuracy (%)	98.7±5.8	101.4±8.4	108.1±14.3	98.7±11.4	101.1±5.2	97.9±7.0	99.3±6.2	101.2±7.6
CV(%)	2.1±1.8	4.7±3.7	5.7±3.0	2.6±1.9	2.3±2.2	7.5±3.7	5.6±5.3	3.1±1.6

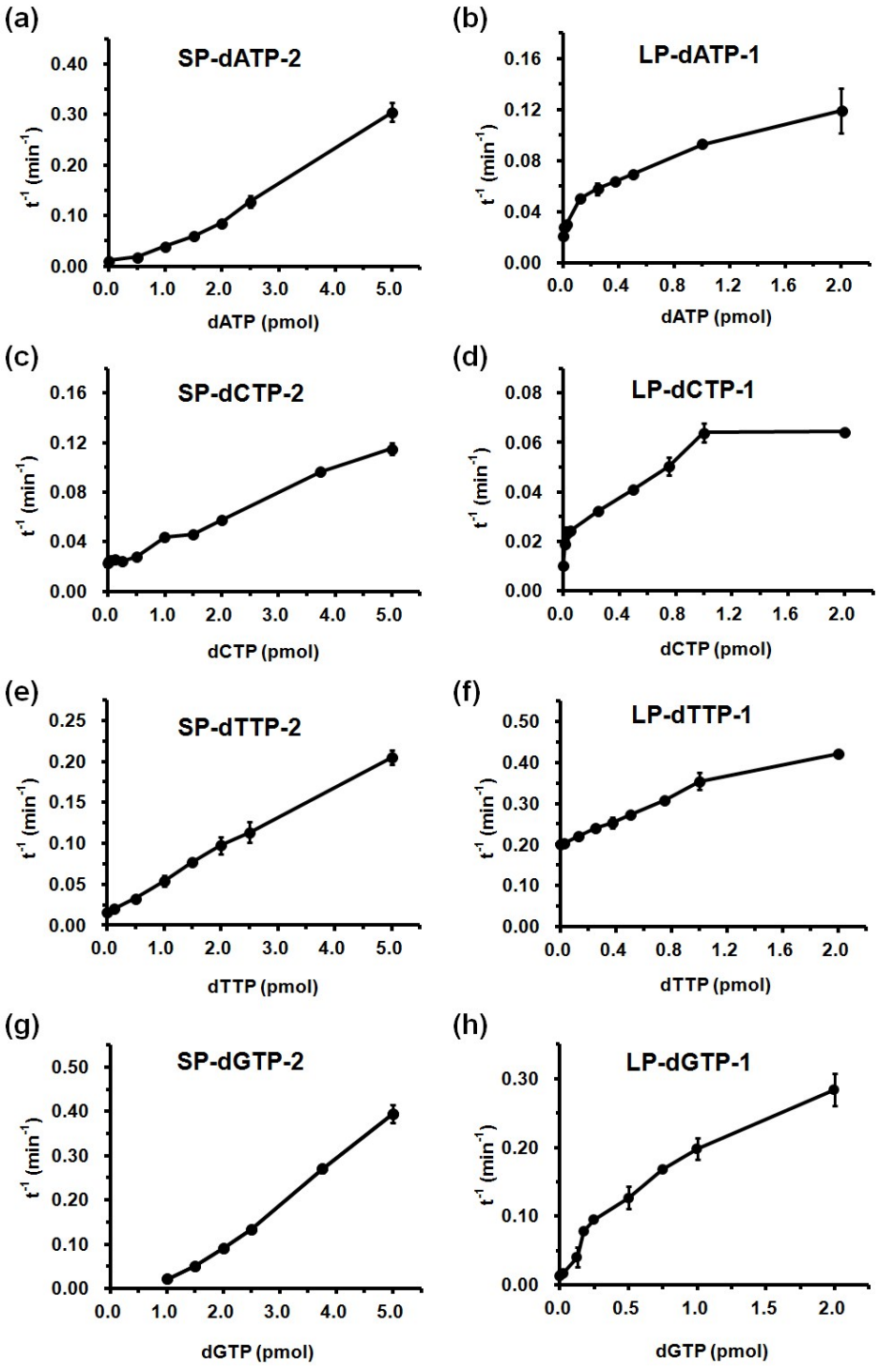

Fig. S3 The plots of t^{-1} *vs.* $[dNTP]_0$ corresponding to all the curves involved in dNTP-2 and dNTP-1 detection: (a) SP-dATP-2, (b) LP-dATP-1, (c) SP-dCTP-2, (d) LP-dCTP-1, (e) SP-dTTP-2, (f) LP-dTTP-1, (g) SP-dGTP-2, (h) LP-dGTP-1. Error bars, SD, n=3.

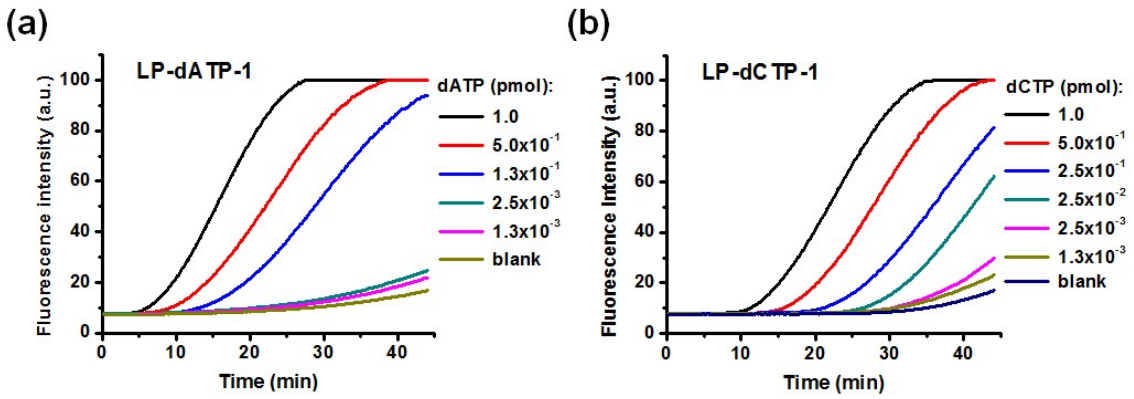

Fig. S4 The initial amount of limiting dNTPs was further reduced to verify the sensitivity of the machine for dATP and dCTP detection.

References

1 P. M. Wilson, M. J. Labonte, J. Russell, S. Louie, A. A. Ghobrial and R. D. Ladner, *Nucleic Acids Res.*, 2011, **39**, e112.

2 X. Xiao, C. Song, C. Zhang, X. Su and M. Zhao, *Chem. Commun.*, 2012, **48**, 1964-1966.