Electronic Supplementary Information

Deamination-triggered exponential signal amplification for chemiluminescent detection of cytosine deaminase at single-cell level

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EXPERIMENTAL SECTION

Chemicals and reagents

All oligonucleotides (Table S1) were synthesized and HPLC purified by Accurate Biotechnology (Changsha, China). Recombinant APOBEC3A protein was purchased from Active Motif (Carlsbad, CA, USA). SwaI and 10 × H buffer (500 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 1 M NaCl) were obtained from TaKaRa Biotechnology Co., Lid. (Dalian, China). Bst DNA polymerase (large fragment), Nb.BsmI nicking enzyme, deoxynucleotide solution mixture (dNTPs), 10× ThermoPol® Reaction Buffer (200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1% Triton X-100, pH 8.8), and 10 × NEBuffer™ r3.1 (1 M NaCl, 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 mg/mL Recombinant Albumin, pH 7.9) were obtained from New England Biolabs (Ipswich, MA, USA). Aurintricarboxylic acid (ATA), hemin, luminol, H_2O_2, 4-(2-hydroxyethy) piperazine1-ethanesulfonic acid sodium salt (HEPES), $MgCl_2$ and NaCl were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). SYBR Gold was obtained from Invitrogen Co. (Carlsbad, CA, USA). Human breast adenocarcinoma cell line (MCF-7 cells), human lung adenocarcinoma cell line (A549 cells), human cervical carcinoma cell line (HeLa cells) and human embryonic kidney cell line (HEK-293T cells) were bought from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The ultrapure water was prepared by a Millipore filtration system (Millipore, Milford, MA, USA).

Table S1. Sequences of the oligonucleotides

Oligonucleotides	**Sequences (5′-3′)**
Substrate probe	TAG TTG TAG GTG GTG AGG TAT TCA AAT GAA TGG TTG-NH_2
Complementary probe	CAA CCA TTC ATT TGA ATA CC-NH_2
Circular template	CCA CCT ACA ACT AAT CGA ATG CAA CCC AAC CCG CCC TAC CCG AAT GCA ACC CAA CCC GCC CTA CCC AAA TAC CTC A

APOBEC3A assay

50 nM DNA substrate probe was incubated with various concentrations of APOBEC3A in 1× HEPES reaction buffer (pH 6.5) at 37 ℃ for 120 min. The resultant deaminated substrate probe was incubated with 50 nM complementary probe for 5 min at 95 ℃, followed by slowly cooling to room temperature to obtain the duplex probe. SwaI-mediated cleavage reaction was carried out in 10 μL of solution containing 5 μL of above duplex probe, 3 U of SwaI, 1× H buffer at 30 ℃ for 150 min. Then, 2.5 μL of cleavage products was mixed with 5 nM circular template, 300 μM dNTP, 0.1 U/μL Nb.BsmI, 0.1 U/μL Bst DNA Polymerase, 1× ThermoPol® reaction buffer, and 1× NEBuffer™ r3.1buffer, followed by incubation at 65 ℃ for 60 min.

Gel electrophoresis

SwaI-mediated cleavage products were analyzed by 12% native polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer (9 mM boric acid, 0.2 mM EDTA, 9 mM Tris-HCl, pH 7.9) at 110 V for 55 min. The RCA products were analyzed by 12% native polyacrylamide gel electrophoresis (PAGE)

in 1 × TBE buffer (9 mM boric acid, 0.2 mM EDTA, 9 mM Tris-HCl, pH 7.9) at 120 V for 50 min. The gel was stained with SYBR Gold and imaged by using a Bio-Rad ChemiDoc MP Imaging System (Hercules, CA, USA).

Real-time fluorescence measurement

The real-time fluorescence measurement of RCA reaction was performed in a Bio-Rad CFX Connect TM Real-Time System (Hercules, CA, USA) with 1× SYBR Gold as the fluorescent indicator, and the fluorescence intensity was monitored at a time interval of 20 s.

Chemiluminescence measurement

The freshly prepared luminol solution (40 μM) and hemin solution (600 nM) were added into the mixture containing 5 μL of RCA reaction products and 3 μL of incubation buffer (40 mM HEPES, 20 mM KCl, 300 mM NaCl, pH 8.0), and incubated at room temperature for 30 min to make the G-rich polymerization products fold into four-stranded G-quadruplex structures. Upon the addition of 30 μL of H_2O_2 (50 mM) into the mixture, the chemiluminescence signals were measured by using a GloMax 96 Microplate Luminometer (Promega, Madison, WI, USA) with a time interval of 1.5 s.

Inhibition assay

For inhibition assay, various concentrations of ATA were incubated with 1 nM APOBEC3A, 50 nM substrate probe at 37 ℃ for 120 min. The relative activity (RA) of APOBEC3A was calculated according to equation 1.

$$\mathrm{RA}\ (\%) = \frac{C_i}{C_t} \times 100\% = 10^{(F_i - F_t)/1459900} \times 100\% \qquad (1)$$

where F_i represents the fluorescence intensity in response to APOBEC3A + ATA, and F_t represents the fluorescence intensity in response to APOBEC3A. C_i and C_t were obtained according to the linear correlation equation in Fig. 2B. The RA was plotted versus the logarithm of the ATA

concentration, and the IC_{50} value of ATA was calculated from the fitting curve.

Cell culture and preparation of cell extracts

Human breast adenocarcinoma cell line (MCF-7 cells), human lung adenocarcinoma cell line (A549 cells), human cervical carcinoma cell line (HeLa cells) and human embryonic kidney cell line (HEK-293T cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10 % fetal bovine serum (FBS) and 50 U/mL penicillin plus 50 μg/mL streptomycin in a 100 % humidified chamber containing 5% CO_2 at 37 °C. Protein extracts were prepared by using a nuclear extract kit (Active Motif, Carlsbad, CA) according to the manufacturer′s protocol. Prior to the extraction, the numbers of cells were counted by a Countstar automated cell counter (IC 1000, Inno-Alliance Biotech Inc., Wilmington, DE, USA). Then cells were digested and collected using trypsin, followed by washing with ice-cold PBS twice and centrifuging at 800 rpm for 5 min. The obtained cell extract was immediately subjected to APOBEC3A assay.

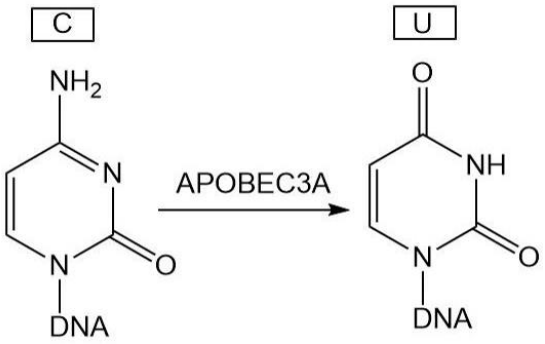

Fig. S1 Schematic illustration of APOBEC3A-catalyzed cytosine (C) to uracil (U).

Optimization of experimental conditions

To achieve the best assay performance, we optimized a series of experimental parameters including the concentration of substrate probe, the concentration of SwaI, the concentration of circular

template, the amount of dNTP, the concentrations of Bst DNA polymerase and Nb.BsmI nicking endonuclease. We investigated the influence of experimental parameters upon the value of $I_i - I_0$, where I_i and I_0 are the chemiluminescence intensity in the presence and absence of target APOBEC3A, respectively.

The concentration of substrate probe is the essential element that directly determines the yield of primer for PG-RCA reaction. Therefore, we optimized the concentration of substrate probe. As shown in Fig. S2, the $I_i - I_0$ value enhances with the increasing concentration from 20 to 50 nM, and levels off beyond 50 nM. Thus, 50 nM substrate probe is selected for APOBEC3A assay in the following researches.

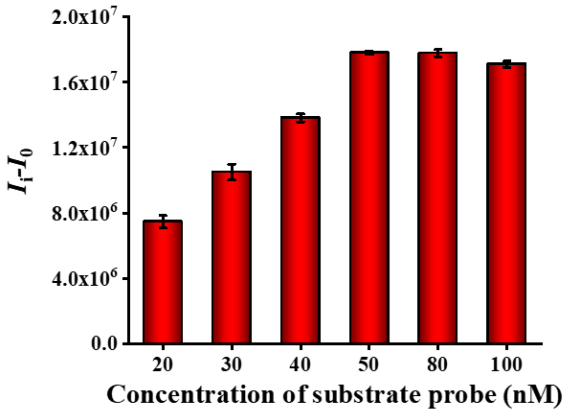

Fig. S2 Variance of the $I_i - I_0$ value with different concentrations of substrate probe. Error bars show the standard deviation of the three experiments.

SwaI-mediated cleavage of deaminated dsDNA substrate probe produces large numbers of primers to initiate PG-RCA reaction. Therefore, the concentration of SwaI should be carefully optimized. As shown in Fig. S3, the $I_i - I_0$ value enhances with the increasing concentration of SwaI from 0.05 to 0.3 U/μL, followed by the decrease beyond the concentration of 0.3 U/μL. Thus, 0.3 U/μL SwaI is selected for APOBEC3A assay in the following researches.

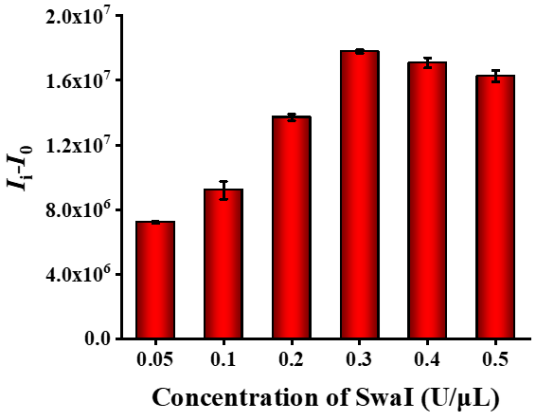

Fig. S3 Variance of the $I_i - I_0$ value with different concentrations of SwaI. Error bars show the standard deviation of the three experiments.

The concentration of circular template is a curial element that affects the amplification efficiency of PG-RCA. On one hand, the high-concentration circular template can induce high amplification efficiency, but it might increase the amplification efficiency of background as well. On the other hand, the low-concentration circular template can decrease the background, but it may induce low amplification efficiency. Therefore, we monitored different $I_i - I_0$ value in response to different concentrations of circular template. As shown in Fig. S4, a maximum value of $I_i - I_0$ is obtained at the circular template concentration of 3 nM. Thus, 3 nM circular template is selected for APOBEC3A assay in the following researches.

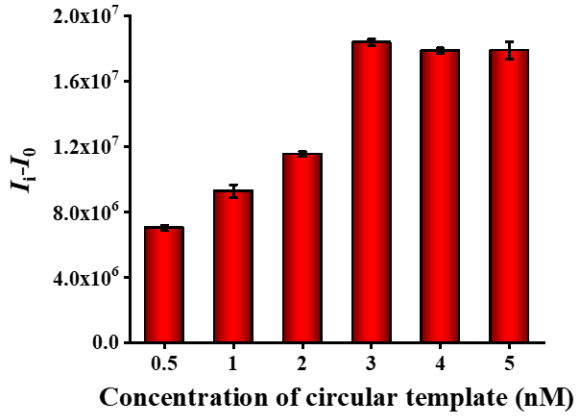

Fig. S4 Variance of the $I_i - I_0$ value in response to the concentration of circular template. Error bars represent the standard deviation of three experiments.

We further explored the effect of dNTP upon the amplification efficiency of PG-RCA. As shown in Fig. S5, the $I_i - I_0$ value enhances with increasing amount of dNTP from 50 to 300 μM, follow by the decrease beyond the amount of 300 μM. Thus, 300 μM dNTP is selected for APOBEC3A assay in the following researches.

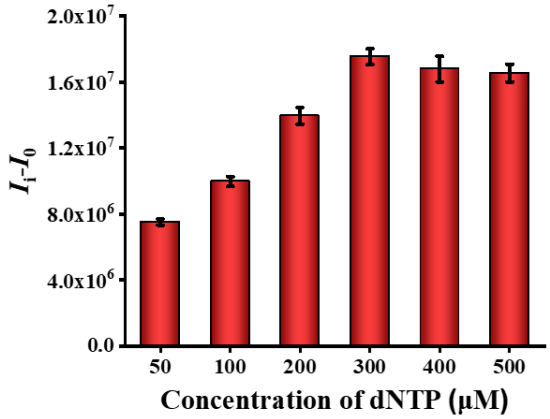

Fig. S5 Variance of the $I_i - I_0$ value in response to different amounts of dNTP. Error bars represent the standard deviation of three experiments.

In this assay, the amplification efficiency of PG-RCA relies on the cooperation of Bst DNA

polymerase and Nb.BsmI nicking endonuclease. Therefore, the concentrations of Bst DNA polymerase and Nb.BsmI should be carefully optimized. We explored the influence of Bst DNA polymerase upon the chemiluminescence signal. A shown in Fig. S6, the $I_i - I_0$ value enhances with the increasing concentration of Bst DNA polymerase from 0.02 to 0.1 U/μL, and reaches a plateau beyond the concentration of 0.1 U/μL. Thus, 0.1 U/μL Bst DNA polymerase is selected for APOBEC3A assay in the following researches.

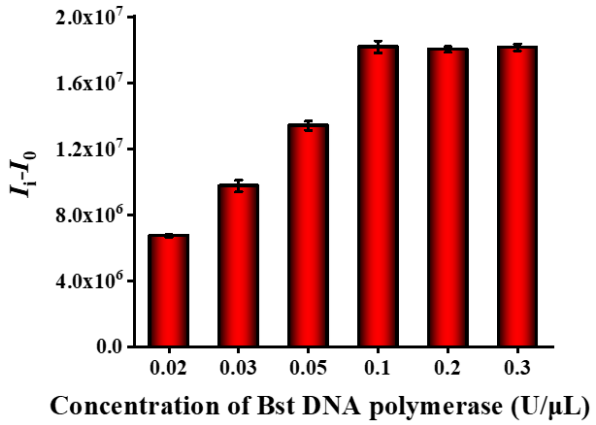

Fig. S6 Variance of the $I_i - I_0$ value in response to different concentrations of Bst DNA polymerase. Error bars represent the standard deviation of three experiments.

We further explored the influence of Nb.BsmI upon the chemiluminescence signal. As shown in Fig. S7, the $I_i - I_0$ value enhances with the increasing concentration of Nb.BsmI from 0.01 to 0.1 U/μL, followed by a plateau beyond the concentration of 0.1 U/μL. Thus, 0.1 U/μL U Nb.BsmI is selected for APOBEC3A assay in the following researches.

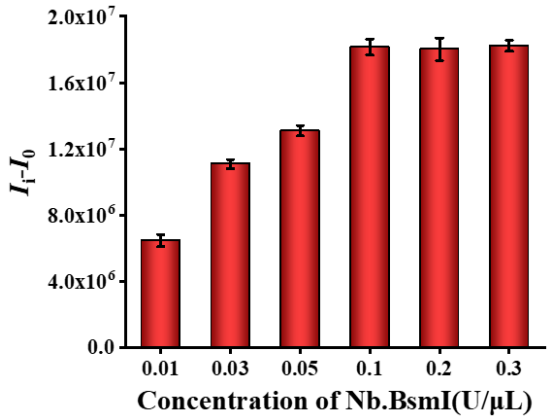

Fig. S7 (A) Variance of the $I_i - I_0$ value in response to different concentrations of Bst DNA polymerase. (B) Variance of the $I_i - I_0$ value in response to different concentrations of Nb.BsmI. Error bars represent the standard deviation of three experiments.

Table S2. Recovery studies in spiked human serum samples.

Sample	Added (nM)	Measured (nM)	Recovery (%)	RSD (%, n=3)
1	5.0×10^{-2}	5.0×10^{-2}	100.23	3.61
2	2.0×10^{-2}	1.9×10^{-2}	97.26	1.87
3	1.0×10^{-2}	9.9×10^{-3}	99.98	2.19
4	5.0×10^{-3}	5.1×10^{-3}	103.36	3.36
5	1.0×10^{-3}	1.0×10^{-3}	102.28	3.26

To investigate the performance of the proposed biosensor in complex sample analysis, we assessed the recovery of APOBEC3A by spiking varying amounts of APOBEC3A (1.0×10^{-3} to 5.0×10^{-2} nM) into 10% human serum samples. As shown in Table S2, a quantitative recovery ranging from 97.26% to 103.36% is obtained, suggesting high accuracy of the proposed method in

complex biological matrix.