Electronic Supplementary Information

Label-free and amplified detection of apoptosis-associated caspase using branched rolling circle amplification

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EXPERIMENTAL SECTION

Materials. All oligonucleotides (Table S1) were HPLC-purified and synthesized by TaKaRa Bio Inc. (Dalian, China). The peptide was obtained from Chinese Peptide Company (Hangzhou, China), and conjugated to DNA by TaKaRa Bio Inc. (Dalian, China). The caspase-8 (human, recombinant, active), caspase-3 (human, recombinant, active) and caspase-9 (human, recombinant, active) were obtained from Enzo Biochem, Inc. (Farmingdale, NY, USA), and one unit enzyme is defined as the amount of caspase-8 that cleaves 1 nmol of substrate IETD-pNA per hour at 37 °C in a reaction solution containing 50 mM HEPES (pH 7.2), 50 mM sodium chloride, 0.1% CHAPS,

10 mM EDTA, 5% glycerol and 10 mM DTT. The phi29 DNA polymerase and its reaction buffer, molecular biology grade bovine serum albumin (BSA) and deoxynucleotide (dNTP) solution mix were purchased from New England Biolabs (Ipswich, MA, USA). The streptavidin-coated magnetic beads (Dynabeads^{TM} M-280 Streptavidin), 1× phosphate buffered saline (1× PBS, pH 7.4), SYBR Gold was bought from Thermo Fisher Scientific (Waltham, MA, USA). The caspase-8 inhibitor benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (Z-IETD-FMK) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Mitomycin C (MMC) was purchased from Millipore Corporation (Billerica, MA, USA). The 20-bp DNA ladder (dye plus) and the DL10,000 DNA marker were purchased from TaKaRa Bio Inc. (Dalian, China). Human cervical cancer cell line (HeLa cells) and human breast cancer cell line (MCF-7 cells) were obtained from cell bank of Chinese academy of sciences (Shanghai, China). All other reagents were of analytical grade and used as received without further purification. Ultrapure water obtained from a Millipore filtration system (Temecula, CA, USA) was used throughout all experiments.

Table S1. Sequences of the DNA oligonucleotides and peptide-DNAs [a]

note	sequence (5'−3')
detection probe	biotin-(COOH)-Lys-Ser-His-Ser-His-Gly-Asp-Thr-Glu-Ile-Cys-(NH_2)-CAT TCG GTA GTA GGT TGT ATA GTT G
cleaved peptide-DNA	Asp-Thr-Glu-Ile-Cys-CAT TCG GTA GTA GGT TGT ATA GTT G
circular template	CAA CTA TAC AAC CTA CTA CCG AAT GAA TAT GAA CAC ATT CTA AGT CTC TAT GTC TGG CAA CAG TGT
secondary primer	CAT TCT AAG TCT CTA TGT CTG

[a] The closed circular DNA template is synthesized by TaKaRa Bio Inc. (Dalian, China) using ligase-mediated cyclization reaction, and it can be directly applied for RCA reaction.

Assembly of the detection probes with the streptavidin-coated magnetic beads. The assembly of detection probes onto the magnetic beads (MBs) was carried out according to the protocol of manufacturer. The 100 μL of 10 mg/mL streptavidin-coated MBs solution was washed twice using 1× PBS. After resuspending with 190 μL of 1× PBS, 10 μL of 10 μM detection probes was added to form the detection probe-MB nanostructure through biotin–streptavidin interaction at room temperature for 30 min. The detection probe-MBs were then washed five times using 1× PBS to remove the uncoupled probes by magnetic separation and resuspended in 50 μL of 1× PBS.

Detection of caspase-8. The caspase-8 assay includes two steps: (1) caspase-8 digestion, and (2) branched RCA reaction. The caspase-8 digestion was performed in a volume of 20 μL. The 13 μL of 1× PBS, 4 μL of detection probe-functionalized MBs and 3 μL of different-concentration recombinant caspase-8 were mixed and incubated at 37 ℃ for 30 min. After magnetic separation, the supernatant solution containing digestion products was used for the next step. For branched RCA reaction, mixs A and B were prepared separately. Mix A contained 10 nM circular template, 10 nM secondary primer, 1× phi29 DNA polymerase reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM DTT, pH 7.5), and 2 μL of the supernatant solution mentioned before. Mix B contained 600 μM dNTPs, 200 μg/ml BSA, and 2 U of phi29 DNA polymerase. Mix A was first denatured at 95 ℃ for 5 min, followed by cooling to room temperature. After mixs A and B were mixed, the branched RCA reaction was performed at 30 ℃ for 1 h. The reaction was terminated by heating at 65 ℃ for 20 min.

Fluorescence measurement. The 40 μL of amplification reaction products was diluted to a final volume of 60 μL with ultrapure water and 1× SYBR Gold. The fluorescence spectra were measured using a trace quartz cuvette on a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan) with an excitation wavelength of 495 nm. The emission spectra were recorded over the wavelength range of 510 nm – 610 nm with a slit width of 5 nm for both excitation and emission. The fluorescence intensity at 524 nm was used for data analysis. The real-time fluorescence measurements were performed in a CFX connect Real-Time System (Bio-Rad, Hercules, CA, USA), and the fluorescence intensity was monitored at intervals of 30 s. To determine the sensitivity of the proposed assay, the limit of detection is calculated by evaluating the average signal of blank plus three times standard deviation.

Gel electrophoresis. The caspase-8 digestion products were analyzed by 12% nondenaturing polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer (9 mM Tris-HCl, 9 mM boric acid, 0.2 mM ethylenediaminetetraacetic acid, EDTA, pH 7.9) at a 110 V constant voltage at room temperature for 50 min. The amplification products of branched RCA reaction were analyzed by 1% agarose gel electrophoresis in 1× TAE buffer (40 mM Tris-acetic acid, 2 mM EDTA, pH 8.0) at a 120 V constant voltage at room temperature for 60 min. The gels were stained by SYBR Gold and analyzed by a Bio-Rad ChemiDoc MP Imaging System (Hercules, CA, USA).

Kinetic analysis. To evaluate the enzyme kinetic parameters of caspase-8, we measured the initial velocity in the presence of 15 U/mL caspase-8 and different-concentration detection probe-functionalized MBs at 37 ℃ for 5 min. The kinetic parameter is fitted to the Michaelis−Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]} \qquad (1)$$

Where V_{max} is the maximum initial velocity, [S] is the concentration of detection probe, and K_m is the Michaelis−Menten constant.

Inhibition assay. To evaluate the effect of Z-IETD-FMK upon the activity of caspase-8, different concentrations of Z-IETD-FMK were preincubated with 15 U/mL caspase-8 at room temperature for 10 min, respectively. Then 4 μL of detection probe-functionalized MBs and 6 μL of 1× PBS were added into the mixture. The following detection process was same as the procedure of caspase-8 digestion-induced branched RCA reaction. The relative activity (RA) of caspase-8 was measured according to equation 2:

$$RA = \frac{F_i - F_0}{F_t - F_0} \qquad (2)$$

where F_0, F_t, F_i represent the fluorescence intensity in the absence of caspase-8, in the presence of 15 U/mL caspase-8, and in the presence of 15 U/mL caspase-8 and different-concentration Z-IETD-FMK, respectively. The IC_{50} value was calculated from the curve of RA versus the Z-IETD-FMK concentration.

Recovery assay. A total volume of 20 μL of reaction mixture containing 1% fetal bovine serum (FBS) spiked with varying-concentration caspase-8, 4 μL of detection probe-functionalized MBs and 1× PBS were incubated at 37 ℃ for 30 min. The following detection process was same as the procedure of caspase-8 assay described above.

Cell culture and preparation of cell extracts. HeLa cells and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, USA) with 10% FBS (Life Technologies, USA) and 1% penicillin-streptomycin (Gibco, USA). The cells were cultured at 37 ℃ in a humidified atmosphere containing 5% CO_2. For real sample analysis, cells in the exponential phase of growth were collected with trypsinization and counted using Countstar BioTech

Automated Cell Counter IC1000 (Shanghai, China), washed twice with ice-cold 1× PBS, and centrifuged at 800 rpm for 5 min. Then the cells were suspended in 100 μL of lysis buffer (150 mM NaCl, 1% NP-40, 0.25 mM sodium deoxycholate, 1.0% glycerol, and 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 10 mM Tris-HCl, pH 8.0), incubated on ice for 30 min with simultaneously vortexing for 30 s every 5 min, and then centrifuged at 12,000 g for 20 min at 4 ℃. For MMC-induced apoptosis analysis, cells were incubated in 5 mL of DMEM containing 100 μg/mL MMC for 2 h prior to the cell lysis procedure. The supernatant was transferred into a fresh tube and used immediately. The protein concentration was measured using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

SUPPLEMENTARY RESULTS

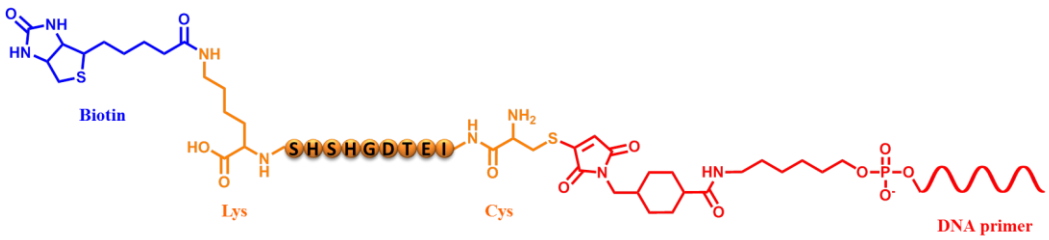

Fig. S1 Chemical structure of the peptide-DNA detection probe.

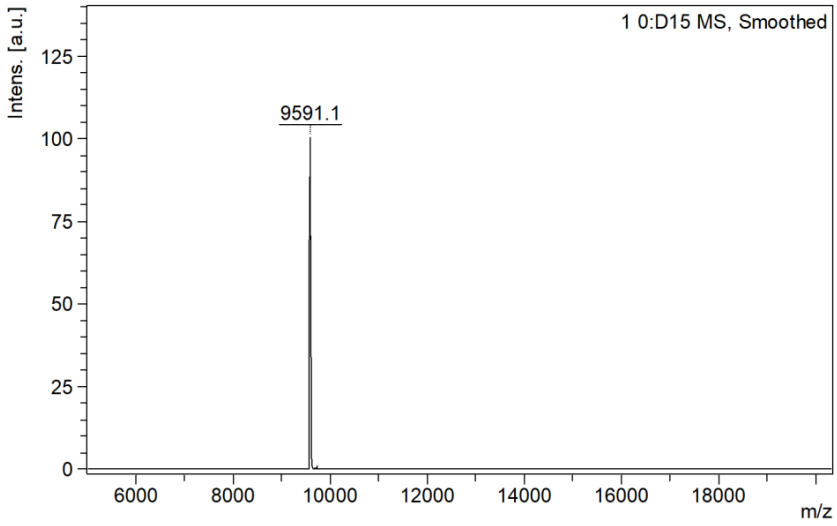

Fig. S2 MS spectrum of the synthetic peptide-DNA detection probe. The calculated MS value of the detection probe is 9596.1.

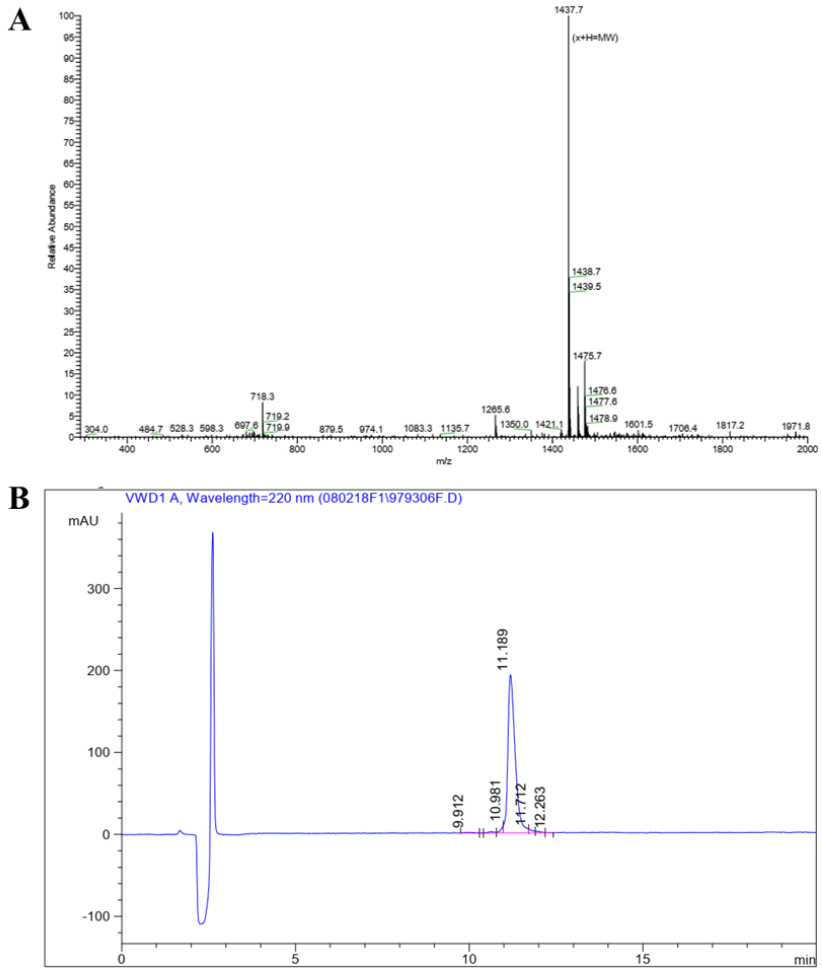

Fig. S3 MS spectrum (A) and HPLC chromatogram (B) of the peptide substrate. The calculated MS value of the peptide substrate is 1439.6.

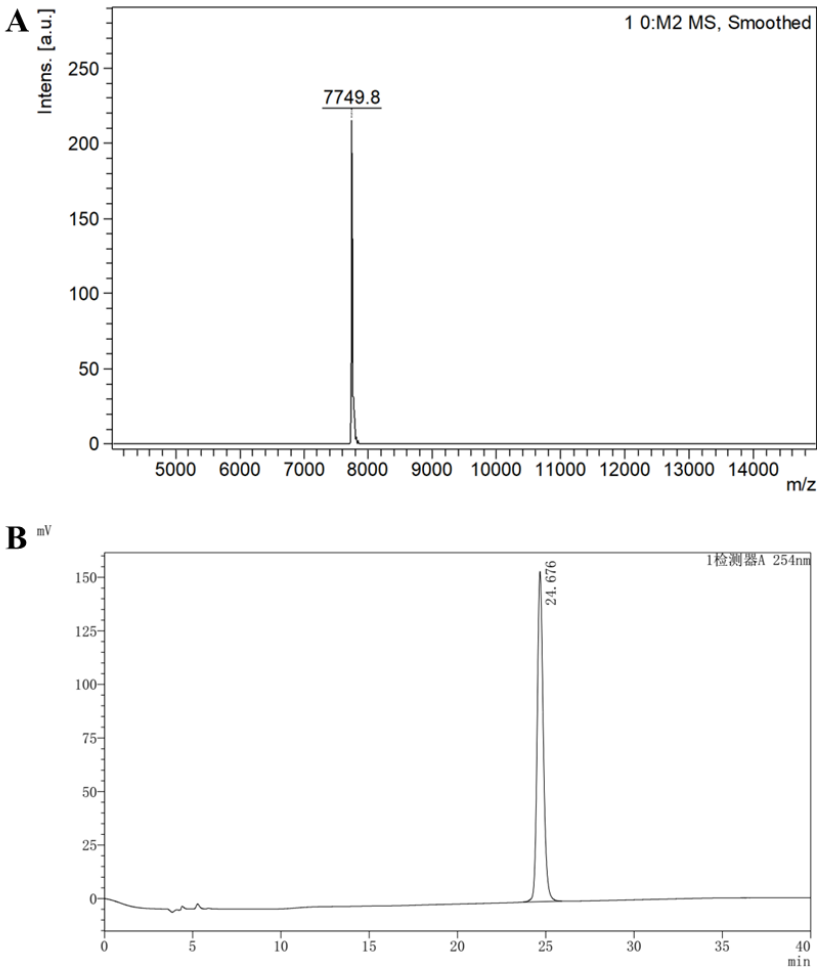

Fig. S4 MS spectrum (A) and HPLC chromatogram (B) of the DNA primer. The calculated MS value of the DNA primer is 7758.1.

Optimization of experimental conditions. To achieve the best assay performance, we optimized caspase-8 reaction temperature (Fig. S5), circular template concentration (Fig. S6A), secondary primer concentration (Fig. S6B), dNTP concentration (Fig. S7), phi29 DNA polymerase amount (Fig. S8), RCA reaction time (Fig. S9A), and RCA reaction temperature (Fig. S9B).

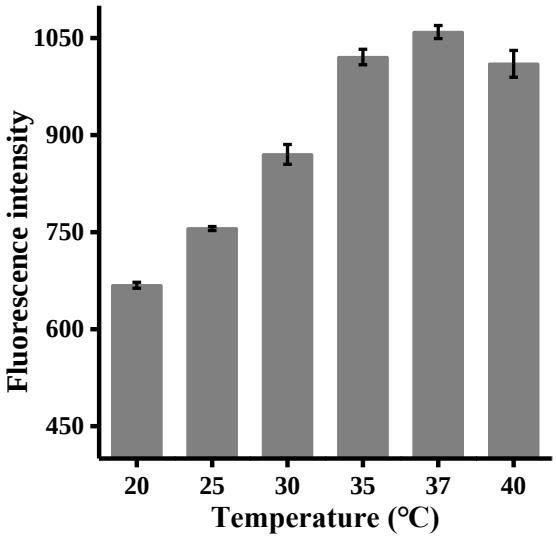

Fig. S5 Effect of caspase-8 reaction temperature upon the fluorescence intensity. Error bars represent the standard deviation of three experiments.

We first investigated the effect of caspase-8 reaction temperature upon the caspase-8 digestion. As shown in Fig. S5, the fluorescence intensity enhances with the increasing temperature from 20 ℃ to 37 ℃, and levels off beyond the temperature of 37 ℃. Therefore, the reaction temperature of 37 ℃ is used in the subsequent researches.

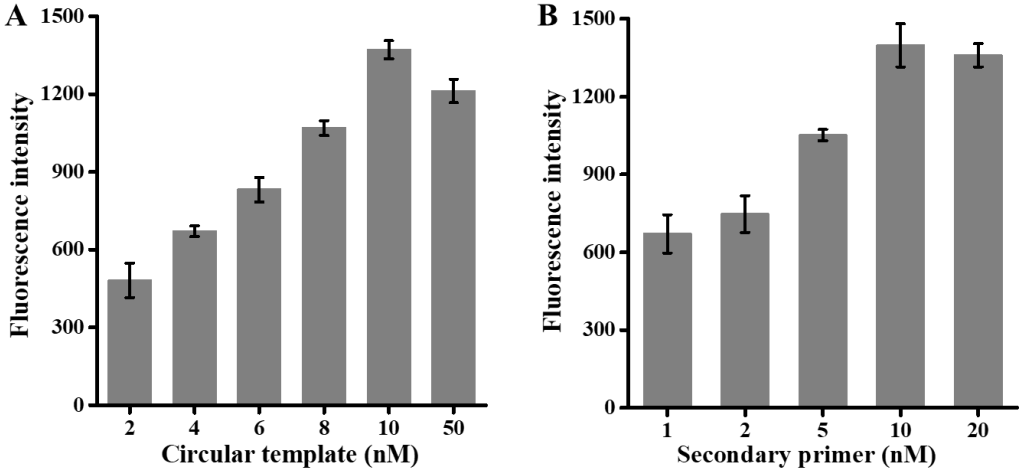

Fig. S6 Variance of fluorescence intensity with the concentrations of circular template (A) and secondary primer (B). Error bars show the standard deviation of three experiments.

The concentration of circular template and secondary primer may influence the amplification efficiency of branched RCA reaction.[1] As shown in Fig. S6A, the fluorescence intensity enhances with the increasing concentration of circular template and reaches the highest value at 10 nM, followed by the decrease beyond 10 nM. Therefore, the optimal concentration of circular template is 10 nM. Moreover, the fluorescence intensity enhances with an increasing concentration of secondary primer due to the increase of branched RCA products, followed by slightly decrease beyond 10 nM (Fig. S6B). Therefore, 10 nM secondary primer is used in the subsequent researches.

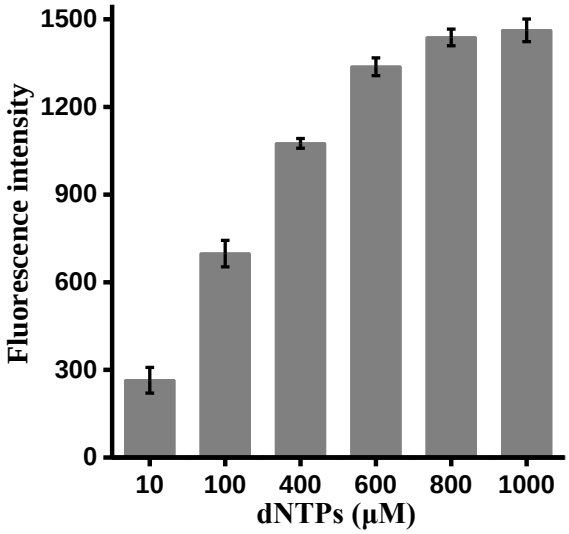

Fig. S7 Variance of fluorescence intensity with the concentration of dNTPs. Error bars show the standard deviation of three experiments.

Deoxynucleotide triphosphates (dNTPs) are building blocks of the RCA product and they can influence the amplification effect. We studied the influence of the concentration of dNTPs upon the branched RCA reaction. As shown in Fig. S7, the fluorescence intensity enhances when the concentration of dNTPs increases from 10 to 600 μM, followed by leveling off beyond the concentration of 600 μM. Thus, 600 μM is selected as the optimal concentration of dNTPs.

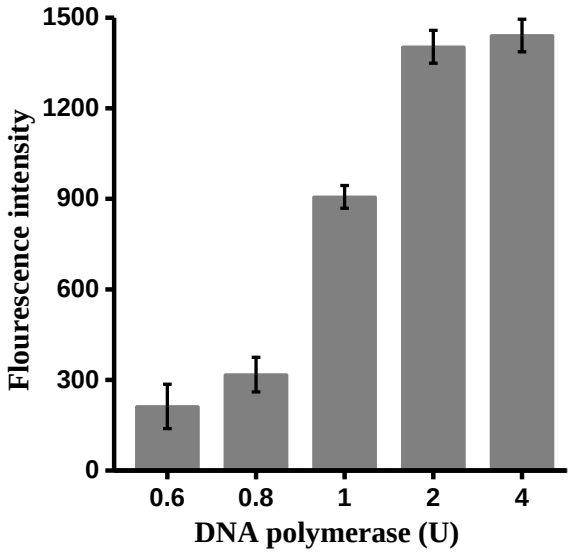

Fig. S8 Variance of fluorescence intensity with the amount of phi29 DNA polymerase. Error bars show the standard deviation of three experiments.

Phi29 DNA polymerase has been most frequently used due to its exceptional processivity and strand displacement ability.[2] We investigated the influence of phi29 DNA polymerase upon the branched RCA reaction. As shown in Fig. S8, the fluorescence intensity enhances with the increasing amount of phi29 DNA polymerase from 0.6 to 2 U, and levels off beyond the amount of 2 U. Therefore, 2 U is selected as the optimal concentration of phi29 DNA polymerase.

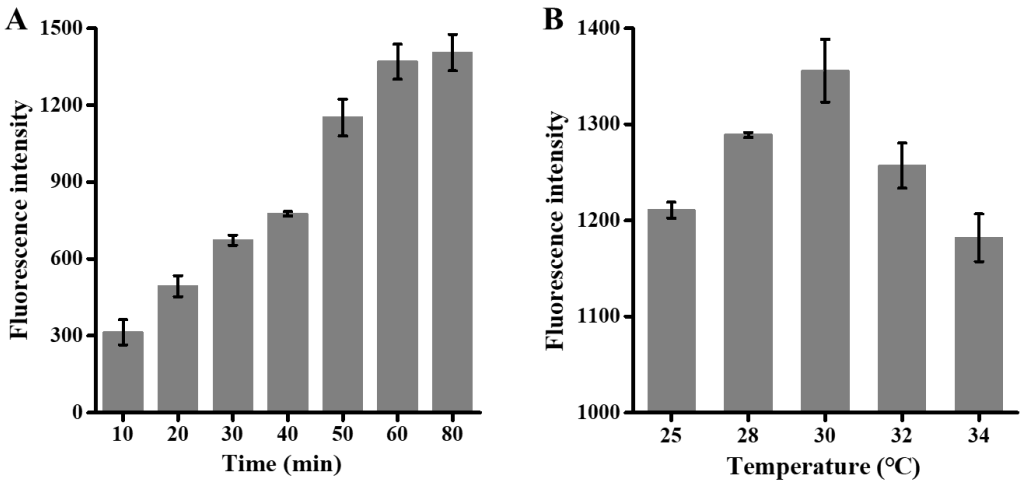

Fig. S9 Variance of fluorescence intensity with reaction time (A) and temperature (B) of the branched RCA. Error bars represent the standard deviations of three experiments.

The reaction time and temperature are critical factors of isothermal amplification.[3] We investigated the effect of reaction time and temperature upon the fluorescence signal (Fig. S9). As shown in Fig. S9A, the fluorescence intensity enhances with the reaction time of the branched RCA, and reaches the maximum at 60 min. Thus, 60 min is used in the subsequent experiments. As shown in Fig. S9B, the fluorescence intensity enhances with the increase of the branched RCA reaction temperature from 20 to 30 ℃, followed by the decrease beyond the temperature of 30 ℃. Thus, 30 ℃ is used as the reaction temperature of branched RCA reaction in the subsequent researches.

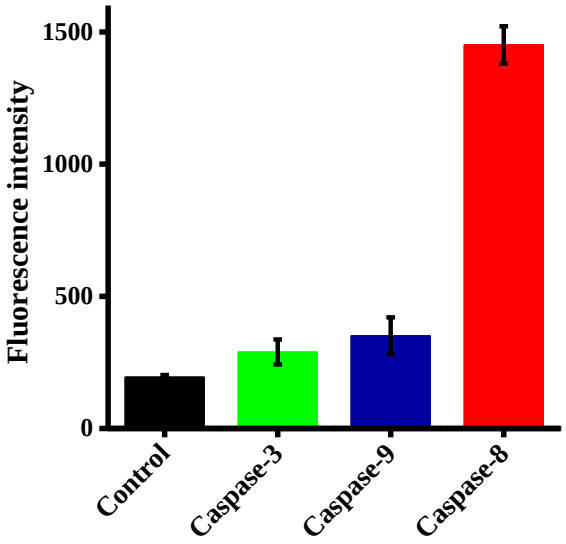

Fig. S10 Measurement of fluorescence intensity in response to the control group (black column), caspase-3 (green column), caspase-9 (blue column) and caspase-8 (red column). The 25 U/mL caspase-3, 25 U/mL caspase-9 and 25 U/mL caspase-8 were used in this experiment. Error bars represent the standard deviation of three experiments.

Detection selectivity. To evaluate the selectivity of this assay, we used caspase-3 and caspase-9 as the nonspecific interferences.[4] As shown in Fig. S10, no signal enhancement is observed in the presence of caspase-3 (Fig. S10, green column) and caspase-9 (Fig. S10, blue column) compared with the control group with only reaction buffer (Fig. S10, black column). While in the presence of caspase-8, a significantly enhanced fluorescence signal is detected (Fig. S10, red column). The fluorescence intensity of target caspase-8 is 7.5-, 5.0- and 4.1-fold higher than that of control group, caspase-3, and caspase-9, respectively. These results demonstrated that the proposed method possesses high selectivity and it can be used to discriminate different caspase family members.

Recovery study. We performed recovery study by adding varying-concentration caspase-8 (5, 10, 15, 25, and 5 U/mL) to the caspase-free fetal bovine serum samples. As shown in Table S2, a quantitative recovery rate ranging from 95.2% to 101.5% with a relative standard deviation (RSD) of 0.7% – 2.1% is obtained. These results demonstrated that the proposed method can be used for accurate detection of target caspase-8 in complex biological matrix.

Table S2. Recovery of caspase-8 spiked in fetal bovine serum (1%) samples

added (U/mL)	measured (U/mL)	recovery (%)	RSD (%)
5	5.2	101.5	1.2
10	8.7	97.1	0.7
15	16.9	98.7	2.1
25	25.8	95.2	0.7
50	49.5	100.3	2.1

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4. O. Julien and J. A. Wells, *Cell Death & Differentiation*, 2017, **24**, 1380-1389.