Electronic Supplementary Information

A Triple-Color Fluorescent Probe for Multiple Nuclease Assays

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Experimental Section

Materials and Apparatus

All oligonucleotides (Table S2) were synthesized and HPLC purified by Sangon Biotechnology Co. Ltd. (Shanghai, China) and Takara Biotechnology Co., Ltd. (Dalian, China), with all modifications being characterized by mass spectrometry. SYBR Gold was purchased from Invitrogen (Carlsbad, CA, USA). Mirin and aurintricarboxylic acid (ATA) were obtained from Sigma (St. Louis, MO, USA). Exonuclease T (Exo T), exonuclease III (Exo III), T7 exonuclease (T7 Exo), T5 exonuclease (T5 Exo), exonuclease I (Exo I), exonuclease V (Exo V), lambda exonuclease (lambda Exo), restriction endonucleases including MseI, HaeIII and DdeI, 10 × NEBuffer 4 and the dilution buffer A (50 mM KCl, 10 mM Tris-HCl, 1 mM DTT, 0.1 mM EDTA, 50% Glycerol, 200 μg/ml BSA, pH 7.4) were obtained from New England Biolabs (Ipswich, MA, USA). Before use, Exo T, Exo III, T7 Exo, Exo I and lambda Exo were diluted to 1 U/μL with dilution buffer A. All fluorescence measurements were performed on an F-4600 spectrofluorometer (Hitachi, Japan) equipped with a circulating water bath for temperature control. The gel electrophoresis was carried out on a Bio-Rad apparatus (Hercules, CA, USA), and the images were obtained by the Image Station 4000MM (Rochester, NY, USA).

Gel Electrophoresis

To obtain the branched DNA probe, three ssDNAs were mixed at the molar ratio of 1:1:1 with a final concentration of 1.0 μM in 1× NEBuffer 4 (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 1 mM DTT, pH 7.9). The mixture was heated at 95°C for 5 min, followed by gradual cooling to 25°C for 1 h. The formation of the three-way DNA junction structure was characterized and confirmed by using 15% non-denaturing gel electrophoresis (Fig. S1). For the exonuclease assay, the branched DNA probe was prepared in the mixture of ssDNAs (I), (II) and (III). Different exonucleases (0.5 U) were added into 5.0 μL of solution containing 1.0 μM annealed probe, and then incubated at 30°C for 15 min. After the addition of 5.0 μL of formamide and 2.0 μL of 6× loading buffer, the reaction solution was denatured at 95°C for 15min, and then loaded onto 20% denaturing polyacrylamide gel (prepared with 8 M urea) followed by electrophoresis at 250 V in 1× TBE buffer (45 mM Tris-boric acid, 10 mM EDTA, pH 8.0) for 25 min. For the endonuclease assay, the branched DNA probe was prepared in the mixture of ssDNAs (IV), (V) and (VI). Different endonucleases (0.5 U) were added into 5.0 μL of solution containing 1.0 μM annealed probe, and then incubated at 37°C for 1.5 h. The reaction products were analyzed by 15% native polyacrylamide gel at 120 V in 1× TBE buffer.

Fluorescence Measurement of Nucleases

For the exonuclease assay, equal moles of ssDNAs (I), (II) and (III) were mixed and annealed to obtain the branched probe. The 5.0 μL of branched probe solution (1.0 μM) was mixed with different exonucleases (0.5 U) in 50 μL of 1× NEBuffer 4 at 30°C for 10 min. The fluorescence spectra were measured by the fluorometer at an appropriate excitation wavelength. For the real-time monitoring, the fluorescence signals were collected continuously at the excitation/emission wavelength of 310/365 nm, 350/450 nm and 496/520 nm for 2-Ap, P-dC, and FAM, respectively. For the endonuclease assay, the mixture of ssDNAs (IV), (V) and (VI) was used to prepare the branched probe. The 5.0 μL of branched probe solution (1.0 μM) was mixed with different endonucleases (0.5 U) in 50 μL of 1× NEBuffer 4 at 37°C for 1 h. For the exonuclease discrimination, the mixture of ssDNAs (VII), (VIII) and (IX) was used to prepare the branched probe. The 5.0 μL of branched probe solution (1.0 μM) was mixed with different exonucleases (2.0 U) in 50 μL of 1× NEBuffer 4 at 37°C for 30 min. The fluorescence intensity was measured by the fluorometer at the excitation/emission wavelength of

310/365 nm, 350/450 nm and 496/520 nm for 2-Ap, P-dC, and FAM, respectively. The PCA statistic analysis was performed with Matlab (The Mathworks Inc., USA).

Cell Extract Analysis and Inhibition Assay

For real sample analysis, the nuclear proteins were extracted from Hela cells, MCF-7 cells, A549 cells and 293T cells, respectively, using a commercial cell nucleoprotein extract kit (Shanghai Sangon, China). The protein concentrations were quantified by a BCA Protein Assay Kit (Merck Millipore, Germany). The cell extracts were mixed with 5.0 μL of annealed probes consisting of DNA Ib, II and IIIb (1.0 μM) in 50 μL of 1× NEBuffer 4 at 30°C for 30 min (the concentration of each protein is 126 μg/mL). The fluorescence intensity was measured by a fluorometer at the excitation/emission wavelength of 310/365 nm, 496/520 nm and 550/580 nm for 2-Ap, FAM, and TAMRA, respectively. For inhibition assays, two inhibitors (mirin and ATA) with various concentrations were mixed with 10.0 μL of annealed probes consisting of DNA Ib, II and IIIb (1.0 μM) in 100 μL of 1× NEBuffer 4, followed by the addition of Exo T, Exo III and T7 Exo. After incubation at 30°C for 30 min, the fluorescence intensity was measured by Gemini microplate reader (Molecular Devices, USA) at the excitation/emission wavelength of 310/365 nm, 496/520 nm and 550/580 nm for 2-Ap, FAM, and TAMRA, respectively.

Supplementary Results and Discussion

Specificity of the Designed DNA Probe for Three Exonucleases Assay

In the 2-Ap end, the hairpin ssDNA with a 3'-overhang can be degraded by Exo T but not by Exo III,[1] and its 5'-end modified by phosphorothioate can inhibit the hydrolysis of T7 Exo. In the P-dC end, the 5'-end modified by phosphorothioate can protect it from the cleavage of T7 Exo, and the dsDNA with the 3'-blunts can be degraded by dsDNA-specific Exo III but not by ssDNA-specific Exo T. In the FAM end, the phosphorothioate-modified dsDNA with the 3'-blunts cannot be digested by either Exo T or Exo III, but it can be degraded by T7 Exo.

To verify the specificity of the designed DNA probe, its hydrolysis products in the presence of different exonucleases are analyzed by denaturing gel electrophoresis. We designed a branched probe

containing a FAM-labeled ssDNA (I') (Table S2) instead of ssDNA (I) to facilitate the observation. In the absence of any specific exonuclease, the branched probe shows two distinct bands (Fig. 1a, lane 1) of FAM-labeled ssDNA (II) (60 nt) and FAM-labeled ssDNA (I') (37 nt). In the presence of T7 Exo, the band of ssDNA (I) shows no observable migration change due to the blocking of its 5'-end by phosphorothioate modification, while the hydrolysis of 5'-end of ssDNA (II) by T7 Exo (Fig. S-A) results in the appearance of FAM-dC band (Fig. 1a, lane 2). In contrast, no FAM-dC band is observed in the absence of T7 Exo (Fig. 1a, lanes 1, 3 and 4). In the presence of Exo T, a new rapid migration band derived from ssDNA (II) can be observed (Fig. 1a, lane 3) due to the digestion of ~5 nt from the 3'-end of ssDNA (II) by Exo T, but no such band is observed in the absence of Exo T (Fig. 1a, lanes 1 and 2). In the presence of Exo III, a new rapid migration band derived from ssDNA (I) can be observed (Fig. 1a, lane 4) due to the digestion of ssDNA (I) by Exo III, but no such band is observed in the absence of Exo III (Fig. 1a, lanes 1 and 2). In the presence of T7 Exo and Exo T, both the FAM-dC band and the new rapid migration band derived from ssDNA (II) can be simultaneously observed (Fig. 1a, lane 5). While in the presence of T7 Exo and Exo III, both the FAM-dC band and the new rapid migration band derived from ssDNA (I) can be simultaneously observed (Fig. 1a, lane 6). In the presence of Exo T and Exo III, both the new rapid migration bands derived from ssDNA (II) and ssDNA (I) can be simultaneously observed (Fig. 1a, lane 7). When three exonucleases are co-present, the FAM-dC band and the new rapid migration bands derived from ssDNA (II) and ssDNA (I) can be simultaneously observed (Fig. 1a, lane 8). These results clearly demonstrate the specificity of the designed DNA probe for multiplex detection of various nucleases.

In addition, the results of gel electrophoresis demonstrate that the exonucleases cannot degrade the branched DNA itself (Fig. 1a), consistent with the fact that exonucleases catalyze the DNA hydrolysis in a manner of direction dependence other than DNA structure dependence. It should be noted that some structure-specific nucleases may cleave the branched DNA,[2-4] and this problem may be solved by protecting the fragile phosphate linkage with a phosphorothioate modification.

Multiplex Detection of Various Restriction Endonucleases

We further demonstrated the feasibility of the proposed method for multiple endonucleases assay. As shown in Fig. S5a, the fluorescent nucleotides of 2-Ap and P-dC are used to substitute an adenosine in ssDNA (IV) and a cytidine in ssDNA (V), respectively, and the 3'-end of ssDNA (VI) is labeled by a FAM. The hybridization of ssDNAs (IV), (V) and (VI) results in the formation of a branched DNA probe containing three terminals of 2-Ap, P-dC, and FAM with specific recognition sequences for DdeI, MseI and HaeIII, respectively. In addition, four successive guanosine bases are used to efficiently quench FAM,[5] and two of them locates in the recognition sites of restriction endonuclease (HaeIII) to shorten the length of cleaved dsDNA fragments. In fact, one guanosine can efficiently quench FAM.[6,7] For the detection of restriction endonucleases without guanosine base in their recognition sites, we may simply employ one guanosine to quench FAM or design several guanosines away from the recognition sites of restriction endonuclease. To disassociate the cleaved dsDNA fragments (< 12 bp) to ssDNAs for the elimination of DNA-quenching, the distance between the cleavage site and the fluorophore label should be less than 12 bp. In the case of the generation of long dsDNA fragment (> 12bp), the DNA-quenching may be stopped through adding extra Exo III or lambda Exo to disassociate the long dsDNA fragments and simultaneously blocking the unwanted degradation by phosphorothioate modification.

In the absence of endonucleases, three fluorophores of 2-Ap, P-dC and FAM are quenched by the adjacent DNA bases through either base stacking or PIET. In the presence of DdeI restriction endonucleases, 2-Ap terminal is specifically cleaved, resulting in the disassociation of 2-Ap-labeled ssDNA from the branched probe and consequently the recovery of 2-Ap fluorescence signal. Similarly, the addition of MseI and HaeIII will result in the recovery of P-dC and FAM fluorescence, respectively. Therefore, each fluorescence signal indicates the presence of one specific restriction endonuclease, and the simultaneous detection of multiple restriction endonucleases can be achieved in a turn-on mode in a single assay.

We used native gel electrophoresis to confirm the specific hydrolysis of various endonucleases (Fig. S5b). The distinct band of FAM-labeled oligonucleotide fragment is observed in the presence of HaeIII (Fig. S5b, lanes 2, 5, 6 and 8, the bottom band) but not in the absence of HaeIII (Fig. S5b, lanes

1, 3, 4 and 7), suggesting that the FAM terminal can be specifically cleaved by HaeIII. The hydrolysis products of DNA probe cleaved by DdeI and MseI can be clearly resolved with long electrophoresis time of 4.5 h (Fig. S5c). The band of DNA probe moves rapidly with the addition of DdeI (Fig. S5c, lane 3) and MseI (Fig. S5c, lane 4), further confirming that DdeI and MseI can specifically cleave the 2-Ap and P-dC terminals of DNA probe, respectively. The more DNA bases are cleaved (14 bases for MseI and 7 bases for DdeI), the rapider the band of DNA probe moves (Fig. S5c, lane 4 *vs* lane 3). Moreover, the simultaneous addition of two or three endonucleases leads to more rapidly migrating bands of DNA probe (Fig. S5c, lanes 5, 6, 7 and 8).

We further measured the fluorescence signal of the triple-color DNA probe in response to various restriction endonucleases. As shown in Fig. S5d, only 2-Ap fluorescence enhancement is observed in presence of DdeI endonuclease. Similarly, P-dC and FAM fluorescence enhancements are observed in presence of MseI and HaeIII, respectively. In the presence of DdeI and MseI, the fluorescence enhancements of 2-Ap and P-dC are simultaneously observed, but no significant FAM fluorescence enhancement is observed. Similarly, the fluorescence enhancements of P-dC and FAM are simultaneously observed in the presence of MseI and HaeIII, while the fluorescence enhancements of 2-Ap and FAM are simultaneously observed in the presence of DdeI and HaeIII. When all three restriction endonucleases are co-present, the fluorescence enhancements of 2-Ap, P-dC and FAM are simultaneously observed. These results clearly demonstrate the feasibility of the triple-color DNA probe for multiplex detection of various restriction endonucleases.

References

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Table S1. Properties of Exonucleases

exonuclease	direction	ss-/ds-DNA	note
Exo T	3'→5'	ss-DNA	
Exo I	3'→5'	ss-DNA	DNA with an extension length of more than 8 nt is required for the initiation of hydrolysis.
Exo III	3'→5'	ds-DNA	DNA with an extension length of more than 4 nt at 3'-end is resistant to the cleavage.
Exo V	5'→3' 3'→5'	ds-DNA	The addition of ATP is required for the reaction buffer.
T5 Exo	5'→3'	ds-DNA ss-DNA	
T7 Exo	5'→3'	ds-DNA	
Lambda Exo	5'→3'	ds-DNA	DNA with 5'-phosphorylation is the preferred substrate.

Table S2. Sequences of Oligonucleotides[α]

note	sequence (5'-3')
ssDNA (I)	G*C*T* GAG GCT GAT TCG GTT CAT GCG GAT C*C*A* G**p-dC**A AGA T
ssDNA (I')	FAM-G*C*T* GAG GCT GAT TCG GTT CAT GCG GAT C*C*A* GCA AGA T
ssDNA (Ib)	G*C*T* GAG GCT GAT TCG GTT CAT GCG GAT C*C*A* GCA ACC C-TAMRA
ssDNA (II)	FAM-CCC CAC CCC* T*A*C GGC GAA TGA CCG AAT CAG CCT CAG CCA CGC ATG* T*C*T* ATG CGT GA**Ap** CTG
ssDNA (III)	A*T*C* TTG CTG GAT CCG CAT GAC ATT CGC CGT AGG GGT GG*G* G*
ssDNA (IIIb)	G*G*G* TTG CTG GAT CCG CAT GAC ATT CGC CGT AGG GGT GG*G* G*
ssDNA (IV)	CGG CTT TAA AGA GGC TGA TTC GGT TCA TGC GGA TCC AGC AAG ATA TCT **Ap**AG C
ssDNA (V)	GGG GCC CCT TAC GGC GAA TGA CCG AAT CAG CCT CTT TAA AG**p-dC** CG
ssDNA (VI)	GCT TAG ATA TCT TGC TGG ATC CGC ATG ACA TTC GCC GTA AGG GGC CCC-FAM
ssDNA (VII)	PO_4-GGG CCC ACT TAC GGC GAA TGA CCG AAT CAG CCT CA*G* C*TG **Ap**AA
ssDNA (VIIII)	ATC TTG CTG GAT CCG CAT GAC ATT CGC CGT AAG TGG GC*C* C*-FAM
ssDNA (IX)	GCT GAG GCT GAT TCG GTT CAT GCG GAT C*C*A* G**p-dC**A AGA T

[α]FAM and TAMRA indicate the fluorescein and carboxytetramethyl rhodamine dye modifications, respectively. The asterisk indicates the phosphorothioate modification. The bold Ap and p-dC indicate the 2-aminopurine and pyrrolo-dC substitutions, respectively.

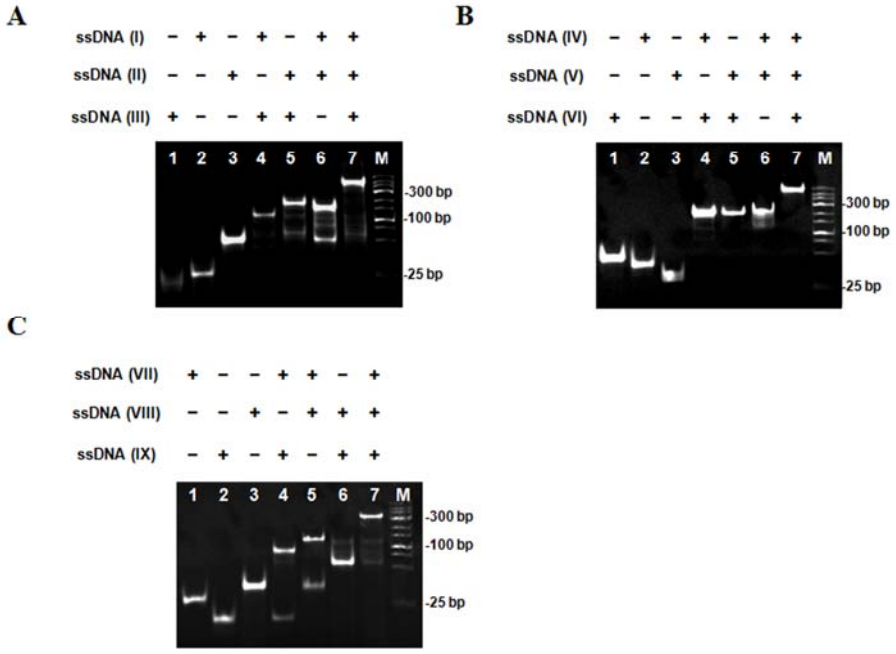

Fig. S1 Characterization of the three-way DNA junction structure by 15% non-denaturing gel electrophoresis. The three-way DNA junction structure is formed by the hybridization of ssDNA (I), ssDNA (II) and ssDNA (III) (A, lane 7), the hybridization of ssDNA (IV), ssDNA (V) and ssDNA (VI) (B, lane 7), and the hybridization of ssDNA (VII), ssDNA (VIII) and ssDNA (IX) (C, lane 7), respectively.

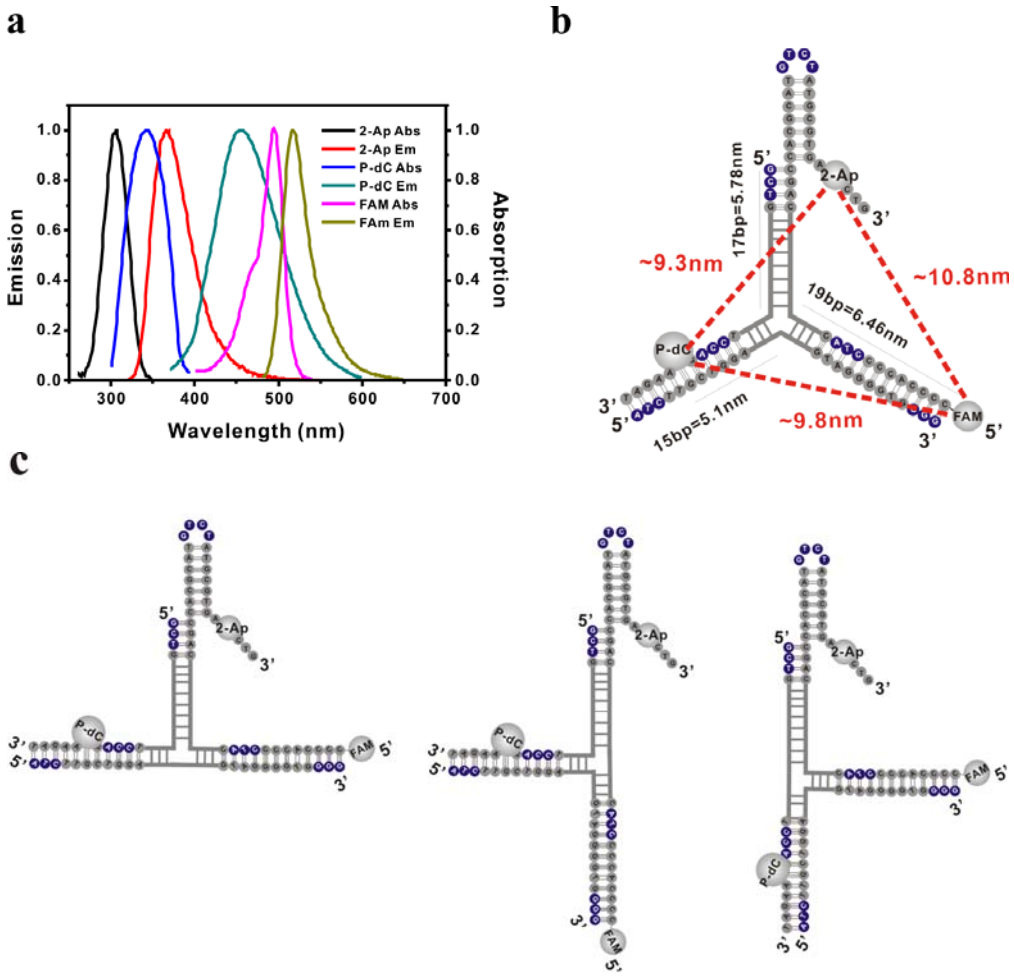

Fig. S2 (a) The normalized absorption and emission spectra of 2-Ap, P-dC, and FAM labels in oligonucleotides. Black line, absorption spectrum of 2-Ap; red line, emission spectrum of 2-Ap; blue line, absorption spectrum of P-dC; cyan line, emission spectrum of P-dC; magenta line, absorption spectrum of FAM; yellow line, emission spectrum of FAM. (b-c) Estimation of the distance among three fluorophores. The DNA three-way junction probe may exist in three different conformations (c) because two of the helices are stacked and continuous (coaxial), with the third helix outside in most cases. The cartoon drawing in (b) shows a case with the average distance.

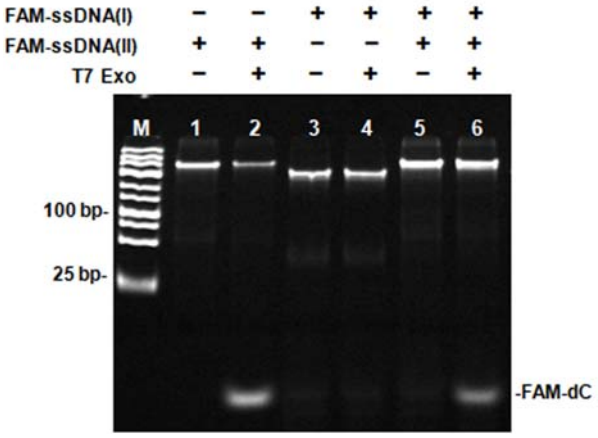

Fig. S-A Electrophoresis experiment confirms that FAM is released from ssDNA (II) instead of ssDNA (I) upon the cleavage by T7 Exo. "-" indicates the DNA without FAM label, and "+" indicates the DNA with FAM label.

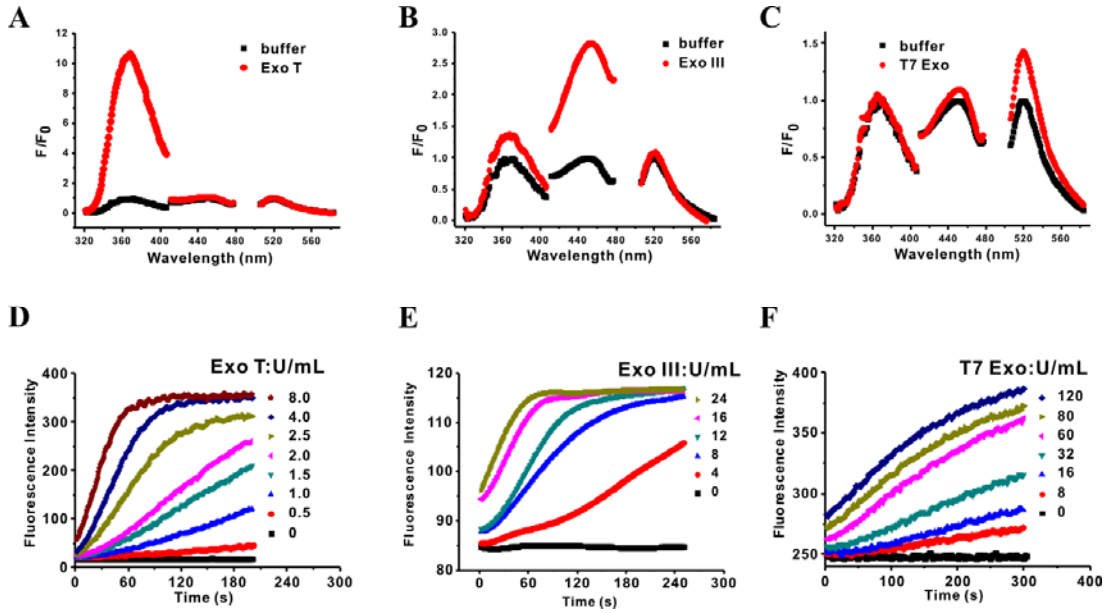

Fig. S3 Fluorescence spectra of DNA probe in the presence of exonucleases (A-C) and real-time monitoring of exonuclease activity (D-F). (A-C) Fluorescence spectra of DNA probe in response to Exo T (A), Exo III (B) and T7 Exo (C). (D-F) Time courses of 2-Ap (D), P-dC (E) and FAM (F) fluorescence in response to various concentrations of exonucleases. It should be noted that the enhancement of FAM fluorescence is only 1.4-fold (Fig. S3C) because of the poor PIET efficiency when FAM is at 5'-end in comparison with that at 3'-end (J. Am. Chem. Soc., 1995, 117, 6406-6407), but we do not employ the DNA probe with FAM at 3'-end because it exhibits high background signal in response to Exo III even if the phosphodiester linkage is modified by phosphorothioate replacement (Fig. S-B).

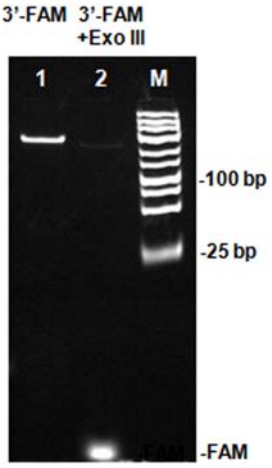

Fig. S-B Electrophoresis experiment indicates that the phosphorothioate modification cannot inhibit the Exo III-catalyzed cleavage of FAM label at 3'-end (lanes 1 and 2).

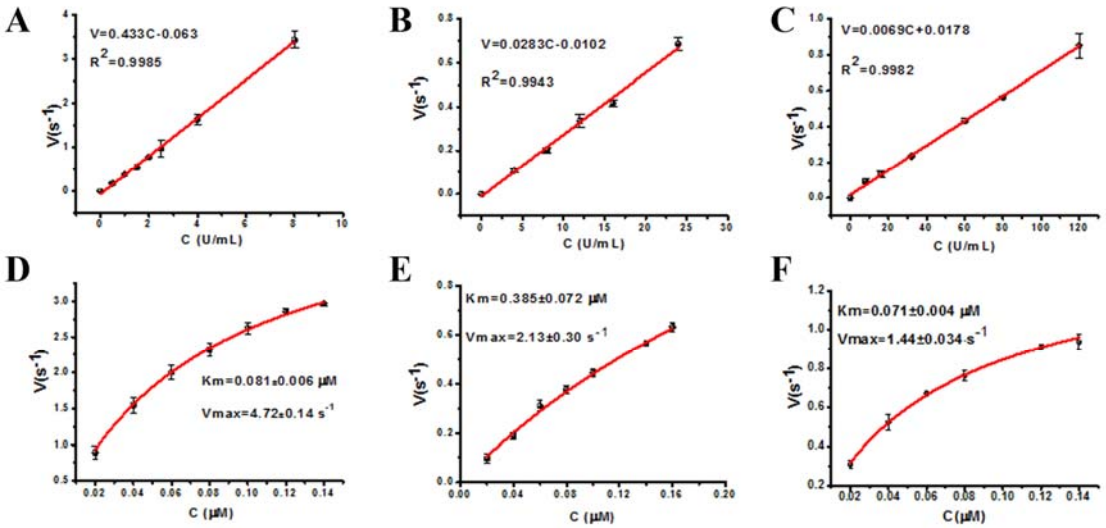

Fig. S4 Measurement of the concentration (A, B and C) and the steady-state kinetic parameters (D, E and F) of Exo T (A and D), Exo III (B and E), and T7 Exo (C and F) by real-time fluorescence monitoring. Error bars show the standard deviation of three experiments.

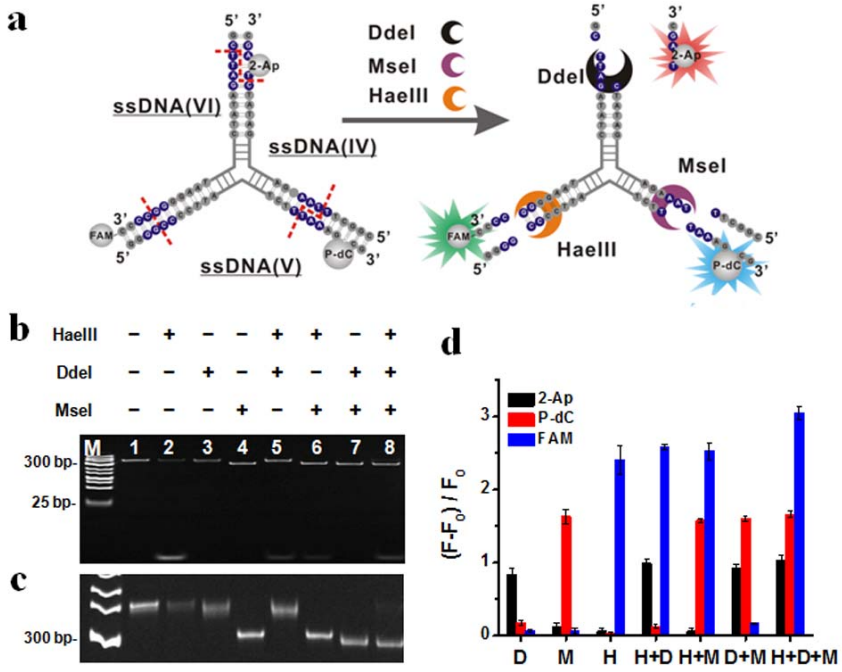

Fig. S5 Multiplex detection of various restriction endonucleases. (a) Design of self-quenched triple-color DNA probe for multiple endonucleases assay. The blue DNA bases indicate recognition sequence of restriction endonuclease. The red dash lines indicate the cleavage site of restriction endonuclease. (b and c) Native gel electrophoresis analysis of the cleavage of DNA probe by various endonucleases. The images were obtained by electrophoresis for 1 h (b) and 4.5 h (c), respectively, with FAM as the fluorescence indicator. (d) Enhanced fluorescence signals of 2-Ap (black color), P-dC (red color) and FAM (blue color) indicate the presence of HaeIII (H), DdeI (D) and MseI (M), respectively. Error bars show the standard deviation of three experiments.

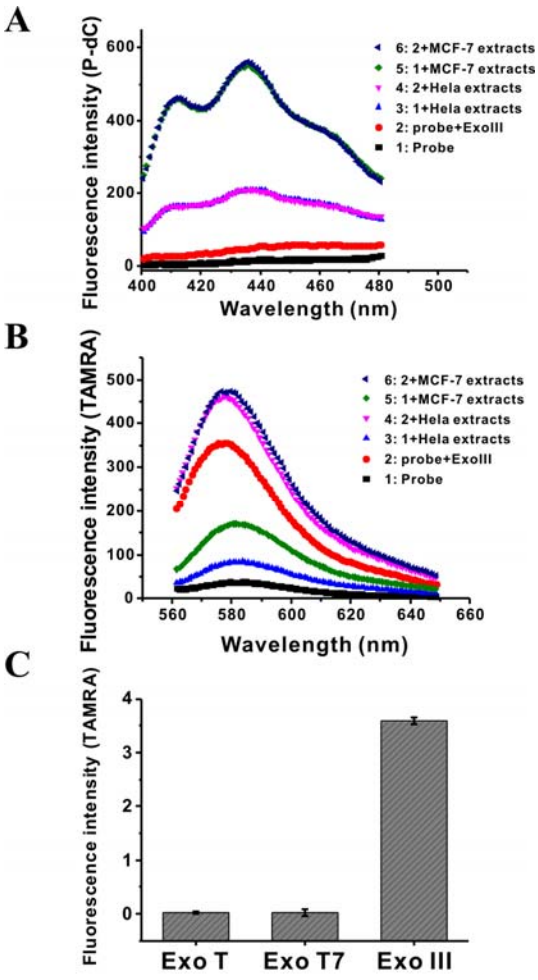

Fig. S6 Substitution of TAMRA fluorophore (B) for P-dC (A) can overcome the fluorescence interference of cell extracts without affecting the specificity of DNA probe (C). The fluorescence spectra of 2-Ap (A) and TAMRA (B) were obtained at the excitation wavelength of 310 nm and 550 nm, respectively. The fluorescence intensity of TAMRA (C) was measured at excitation/emission wavelength of 550 nm / 580 nm. Error bars show the standard deviation of three experiments.

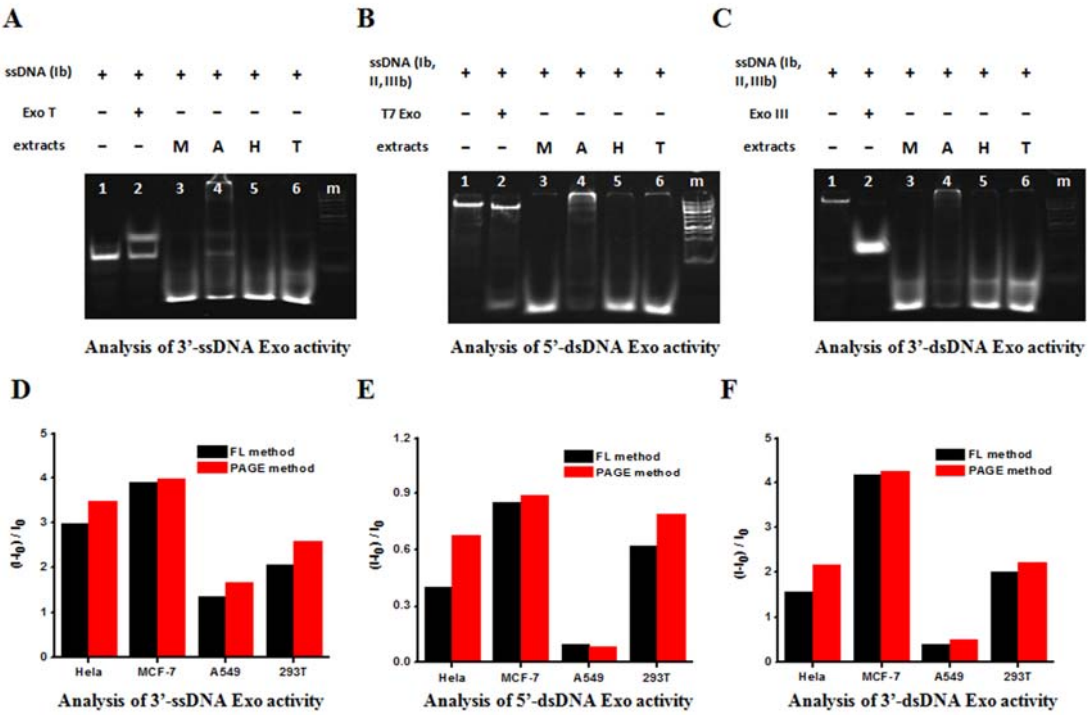

Fig. S7 Native PAGE (15%) analysis of 3'-ssDNA Exo activity (A), 5'-dsDNA Exo activity (B) and 3'-dsDNA Exo activity (C) in the nuclear extracts of different cells and the comparison of 3'-ssDNA Exo activity (D), 5'-dsDNA Exo activity (E) and 3'-dsDNA Exo activity (F) obtained by the PAGE method and the triple-color FL method. The abbreviations of M, A, H and T in the nuclear extracts in panels A-C indicate that the nuclear proteins obtained from MCF-7 cells (M), A549 cells (A), Hela cells (H) and 293T cells (T), respectively. I_0 and I represent the measured fluorescence intensity in the absence and in the presence of cell extracts, respectively. The fluorescence intensity was quantified by Quantity Onesofterware (Bio-Rad).

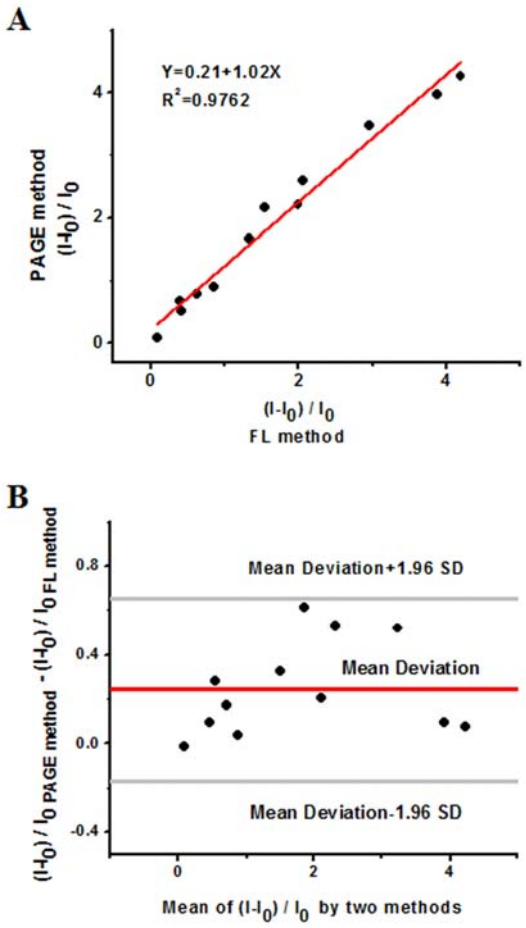

Fig. S8 Comparison of the proposed method (FL method) with the PAGE method. (A) Linear relationship between the FL method and the PAGE method. (B) Result of Bland-Altman analysis between the FL method and the PAGE method. I_0 and I represent the measured fluorescence intensity in the absence and in the presence of cell extracts, respectively.