Supporting Information

Detecting 5-Methylcytosine Using an Enzyme-free DNA Strand Exchange Reaction Without Pretreatment Under Physiological Conditions

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Methods

Nucleic acids. The labeled oligonucleotides (5mC and fluorophores) were purchased from TaKaRa (Dalian) and the label free oligonucleotides were purchased from Invitrogen (Shanghai).

Preparation of SERs samples. Every oligonucleotide was quantified by measuring the UV absorbance at 260 nm. In all the double-stranded DNAs, the 5mC containing strands and homologous non-methylated strands were excess than the complementary strands (1.2N or 1.5N). DNAs were annealed at 95℃ for 5 min, and then slowly cooled to room temperature.

SER processes. In the buffer of 40 mM KCl, 20 mM Na_2HPO_4/NaH_2PO_4 (pH 7.4), the incubation was carried out at 37℃(except for temperature gradient experiments) with variety time. After that, the samples were added bromophenol blue as loading buffer. The final concentration of target dsDNA is 1 μM. Native PAGE gel was employed to electrophoresed the samples. The SERs products were scanned and integrated by Pharos FX Molecular Imager (Bio-Rad). All those gels were scanned with fluorescent detector. And all the SERs yields were calculated based on the concentration ratio of the reacted labeled ssDNAs and un-reacted labeled ssDNAs.

SER yield. The SERs products were scanned and integrated by Pharos FX Molecular Imager. This precise instrument can help us calculate the fluorescence intensity of pointed area of the gel. Assume that the quantity of the target DNA is *x* than the quantity of the labelled probe DNA is *nx (n>0),* the fluorescence intensity of the SER product band is *a* and the fluorescence intensity of unreacted labelled probe DNA band is *b,* Than the yield of the SER will be:

$$y = \frac{a}{\frac{a+b}{n}} \times 100\%$$

Additional details

ODNs	Sequences
48nt	5′-TTGCGCGGCTCTCCTCAGCTCCTTCCCGCCGCCCAGTCTGGATCCTGG-3′
48nt-com	5′-CCAGGATCCAGACTGGGCGGCGGGAAGGAGCTGAGGAGAGCCGCGCAA-3′
48nt-5mC	5′-TTGCGCGGCTCTCCTCAGCTCCTTCCCGC(5mC)GCCCAGTCTGGATCCTGG-3′
7nt(48nt)-FAM	5'-(FAM)CGCCGCC-3'
13nt(48nt)-FAM	5′-(FAM)TCCCGCCGCCCAG-3′
15nt(48nt)-FAM	5′-(FAM)TTCCCGCCGCCCAGT-3′
17nt(48nt)-FAM	5′-(FAM)CTTCCCGCCGCCCAGTC-3′
19nt(48nt)-FAM	5′-(FAM)CCTTCCCGCCGCCCAGTCT-3′
21nt(48nt)-FAM	5′-(FAM)TCCTTCCCGCCGCCCAGTCTG-3′

Table S1: Sequences of 48bp sets.

ODNs	Sequences
21nt	5′-TCCTTCCCGCCGCCCAGTCTG-3′
21nt-5mC	5′-TCCTTCCCGC(5mC)GCCCAGTCTG-3′
21nt-com	5′-CAGACTGGGCGGCGGGAAGGA-3′
21nt-com-Cy5	5′-CAGACTGGGCGGCGGGAAGGA(Cy5)-3′
21nt(21nt)-Cy3	5′-(Cy3)TCCTTCCCGCGCCCAGTCTG-3′
21nt (21nt)-FAM	5′-(FAM)TCCTTCCCGCGCCCAGTCTG-3′

Table S2: Sequences of FRET experiments.

ODNs	Sequences
47nt	5'-TCCCTGCACCCAGGTTTCCATTGGCGGCTCTCCTCAGCTCCTTCCC-3'
47nt-mC	5'-TCCCTGCACCCAGGTTTCCATTG(5mC)GCGGCTCTCCTCAGCTCCTTCCC-3'
47nt-2mC	5'-TCCCTGCACCCAGGTTTCCATTG(5mC)G(5mC)GGCTCTCCTCAGCTCCTTCCC-3'
47nt-com	5'-GGGAAGGAGCTGAGGAGAGCCGCGCAATGGAAACCTGGGTGCAGGGA-3'
21nt(47nt)-FAM	5’-(FAM)GTTTCCATTGCGCGGCTCTCC-3'

Table S3: Sequences of the single 5mC/two adjacent 5mC experiments.

ODNs	Sequences
70nt	5’-TTCCATTGCGCGGCTCTCCTCAGCTCCTTCCCGCCGCCCAGTCTGGATCCTGGGGGAGGCGCTGAAGTCG-3’
70nt-3mC	5’-
70nt-com	5’-CGACTTCAGCGCCTCCCCCAGGATCCAGACTGGGCGGCGGGAAGGAGCTGAGGAGAGCCGCGCAATGGAA-3’
15nt(70nt)-FAM	5’-(FAM)CATTGCGCGGCTCTC-3’
15nt(70nt)-Cy5	5’-(Cy5)TCCTTCCCGCCGCCC-3’
15nt(70nt)-Cy3	5’-(Cy3)GGGGAGGCGCTGAAG-3’

Table S4: Sequences of multi-sites methylation 70bp sets.

ODNs	Sequences
24nt	5'-ACGAGGAGGGCTCGGAGGAGAAGG-3'
24nt-mC	5'-ACGAGGAGGGCT(5mC)GGAGGAGAAGG-3'
24nt-com	5'-CCTTCTCCTCCGAGCCCTCCTCGT-3'
9nt(24nt)-FAM	5'-(FAM)GGCTCGGAG-5'
13nt(24nt)-FAM	5'-(FAM)AGGGCTCGGAGGA-3'
17nt(24nt)-FAM	5'-(FAM)GGAGGGCTCGGAGGAGA-3'

Table S5: Sequences of 24bp sets.

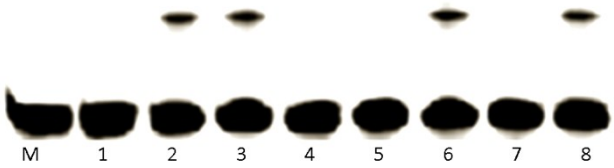

Figure S1. Study of incubation temperature. In the buffer of 40mM KCl, 20mM phosphate buffer (pH 7.4) and 20vol% PEG200. 20% native PAGE. Sequences see in Table S1. Incubation time is 1h. Probe is 21nt(48nt)-FAM. Template : Probe = 1 : 2. Lane M is probe. Lane 1, 4, 5 and 7 are unmethylated samples, lane 2, 3, 6 and 8 are methylated samples. Incubation temperature of lane 1 and 2 is 37℃; lane 3 and 4, 55°C; lane 5 and 6, 70°C; lane 7 and 8, 85°C. SER yields of each sample are y_1 = 0%; y_2 = 13.2%; y_3 = 12.7%; y_4 = 0%; y_5 = 0%; y_6 = 13%; y_7 = 0%; y_8 = 11.9%.

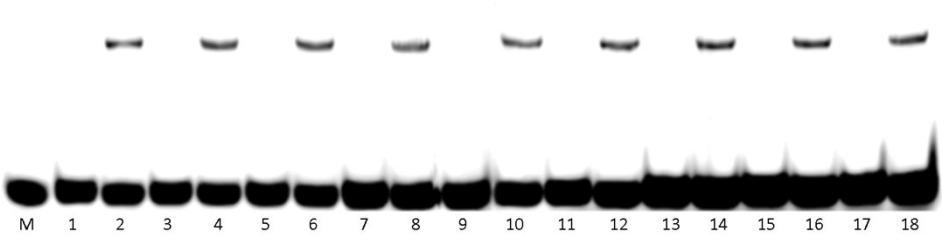

Figure S2. Study of incubation time and probe density. In the buffer of 40mM KCl, 20mM phosphate buffer (pH 7.4) and 20vol% PEG200. 20% native PAGE. Sequences see in Table S1. Probe is 21nt(48nt)-FAM. Lane M is probe. Lane 1, 3, 5, 7, 9, 11, 13, 15 and 17 are unmethylated samples, lane 2, 4, 6, 8, 10, 12, 14, 16 and 18 are methylated samples. The probe density (template : probe) of lane 1-6 is 1 : 1; lane 7-12 is 1 : 2; lane 13-18, 1 : 5. Incubation time of lane 1, 2, 7, 8, 13 and 14 is 5min; lane 3, 4, 9, 10, 15 and 16, 30min; lane 5, 6, 11, 12, 17 and 18, 60min. SER yields of each sample are y_1 = 0%; y_2 = 13.0%; y_3 = 0%; y_4 = 13.0%; y_5 = 0%; y_6 = 12.5%; y_7 = 0%; y_8 = 15.3%; y_9 = 0%; y_{10} = 16.0%; y_{11} = 0%; y_{12} = 15.7%; y_{13} = 0%; y_{14} = 22.0%; y_{15} = 0%; y_{16} = 21.1%; y_{17} = 0%; y_{18} = 22.2%.

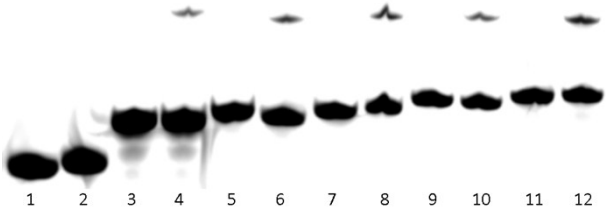

Figure S3. Study of probe length. Sequences see in Table S1. In the buffer of 40mM KCl, 20mM phosphate buffer (pH 7.4) and 20vol% PEG200. 20% native PAGE. Incubation time is 2h. Incubation temperature is 37℃. Template : Probe = 1 : 5. Lane 1, 3, 5, 7, 9 and 11 are unmethylated samples, lane 2, 4, 6, 8, 10 and 12 are methylated samples. Probe of lane 1 and 2 is 7nt(48nt)-FAM; lane 3 and 4, 13nt(48nt)-FAM; lane 5 and 6, 15nt(48nt)-FAM; lane 7 and 8, 17nt(48nt)-FAM; lane 9 and 10, 19nt(48nt)-FAM; lane 11 and 12, 21nt(48nt)-FAM. SER yields of each sample are y_1 = 0%; y_2 = 0.5%; y_3 = 0%; y_4 = 26.0%; y_5 = 0%; y_6 = 44.3%; y_7 = 0%; y_8 = 71.1%; y_9 = 0%; y_{10} = 75.9%; y_{11} = 0%; y_{12} = 77.6%.

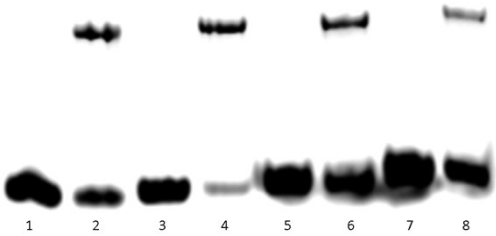

Figure S4. Study of PEG200 vol%. Sequences see in Table S1. In the buffer of 40mM KCl, 20mM phosphate buffer (pH 7.4). 20% native PAGE. Incubation time is 3h. Incubation temperature is 37℃. Probe is 21nt(48nt)-FAM. Template : Probe = 1 : 1. Lane 1, 3, 5 and 7 are unmethylated samples, lane 2, 4, 6 and 8 are methylated samples. PEG200 volume fraction of lane 1 and 2 is 0%; lane 3 and 4, 20%; lane 5 and 6, 40%; lane 7 and 8, 60%. SER yields of each sample are y_1 = 0%; y_2 = 62.0%; y_3 = 0%; y_4 = 83.7%; y_5 = 1.7%; y_6 = 44.1%; y_7 = 3.1%; y_8 = 32.0%.

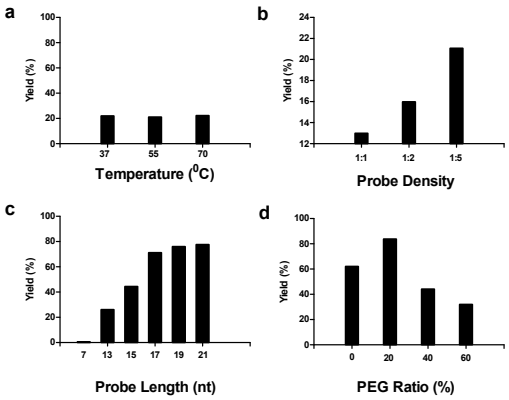

Figure S5. The histograms of the SER yields performed in various conditions. (a) Incubation Temperature (37℃, 55℃, 70℃); (b) Probe Density (the ratio of template to probe from 1:1 to 1:5); (c) Probe Length (7nt, 13nt, 15nt, 17nt, 19nt and 21nt); (d) Volume fraction of PEG200 from 0% to 60%.

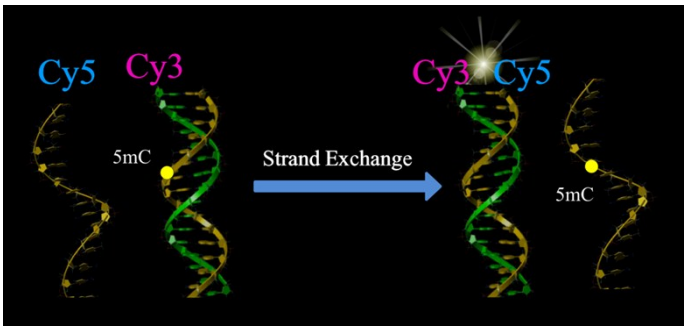

Figure S6. FRET assay of strand exchange kinetics. After the hybridization of Cy3-labelled single strand with the Cy5-labelled methylated template, the adjacencies of two fluorophores result in the excitation of Cy5.

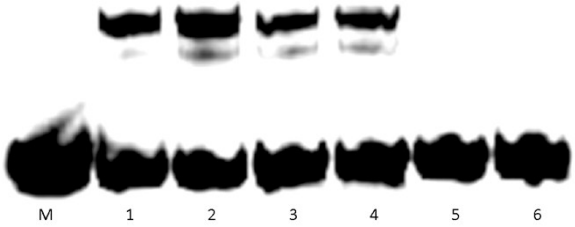

Figure S7. Study of single methylation site and double adjacent methylation sites. Sequences see in Table S3. In the buffer of 40mM KCl, 20mM phosphate buffer (pH 7.4) and 20vol% PEG200. 20% native PAGE. Incubation time is 30min. Incubation temperature of lane 1, 3 and 5 is 0℃; lane 2, 4 and 6, 37℃. Template : Probe = 1 : 1. Probe is 21nt(47nt)-FAM. Lane M is probe. Lane 1 and 2 are samples containing double methylation sites. Lane 3 and 4 are samples containing single methylation site. Lane 5 and 6 are non-methylated samples. SER yields of each sample are $y_1 = 24.0\%$; $y_2 = 30.0\%$; $y_3 = 11.0\%$; $y_4 = 12.0\%$; $y_5 = 0\%$; $y_6 = 0\%$.

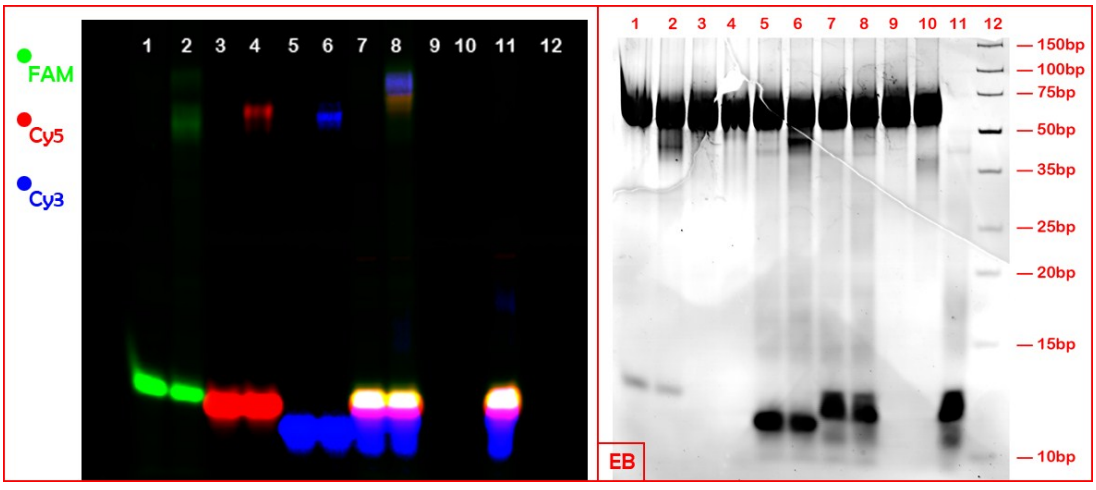

Figure S8. Study of multi-sites methylation detection. Sequences see in Table S4. In the buffer of 40mM KCl, 20mM phosphate buffer (pH 7.4) and 20vol% PEG200. 20% native PAGE. Incubation time is 2 hours. Incubation temperature is 37℃. Template : Probe = 1 : 2. Lane 1, 3, 5, 7 and 9 are unmethylated samples, lane 2, 4, 6, 8 and 10 are methylated samples. Lane 9 is unmethylated template. Lane 10 is methylated template. Lane 11 is three

probes. Lane 12 is DNA ladder. Probe of lane 1 and lane 2 is 15nt(70nt)-FAM; lane 3 and lane 4, 15nt(70nt)-Cy5; lane 5 and 6, 15nt(70nt)-Cy3; lane 7 and 8, 15nt(70nt)-FAM、15nt(70nt)-Cy5 and 15nt(70nt)-Cy3. Colors in the left figure are generated and integrated by Pharos FX Molecular Imager. The right is the same gel dyed with EB.

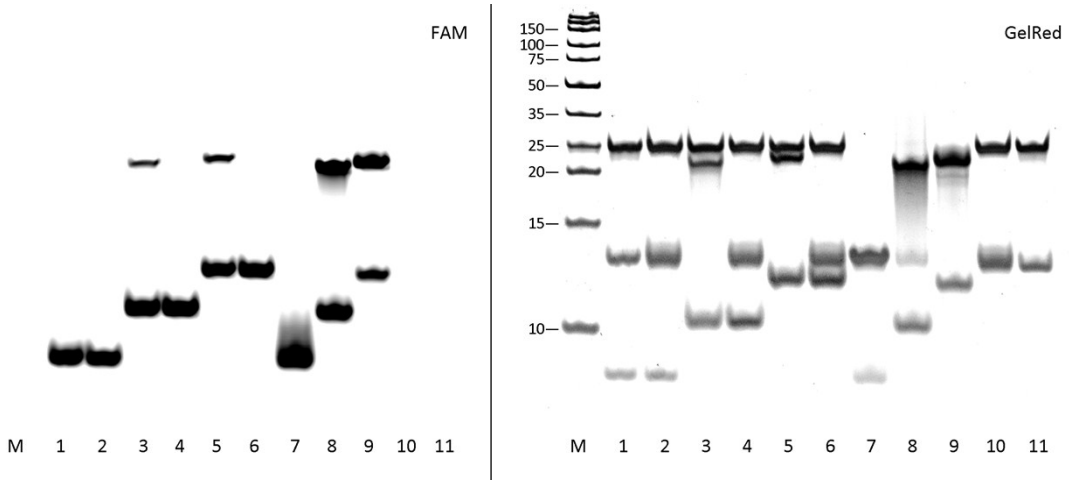

Figure S9. Study of SER product structure (24bp sets). In the buffer of 40mM KCl, 20mM phosphate buffer (pH 7.4) and 20vol% PEG200. 30% native PAGE. Sequences see in Table S5. Incubation time is 2 hours. Incubation temperature is 37℃. Template : Probe = 1 : 3. Lane M is DNA ladder. Lane 7 is the annealing product of 9nt(24nt)-FAM and 24nt-com. Lane 8 is the annealing product of 13nt(24nt)-FAM and 24nt-com. Lane 9 is the annealing product of 17nt(24nt)-FAM and 24nt-com. Lane 1, 3 and 5 are methylated samples, lane 2, 4 and 6 are unmethylated samples. The right is the left dyed with GelRed.

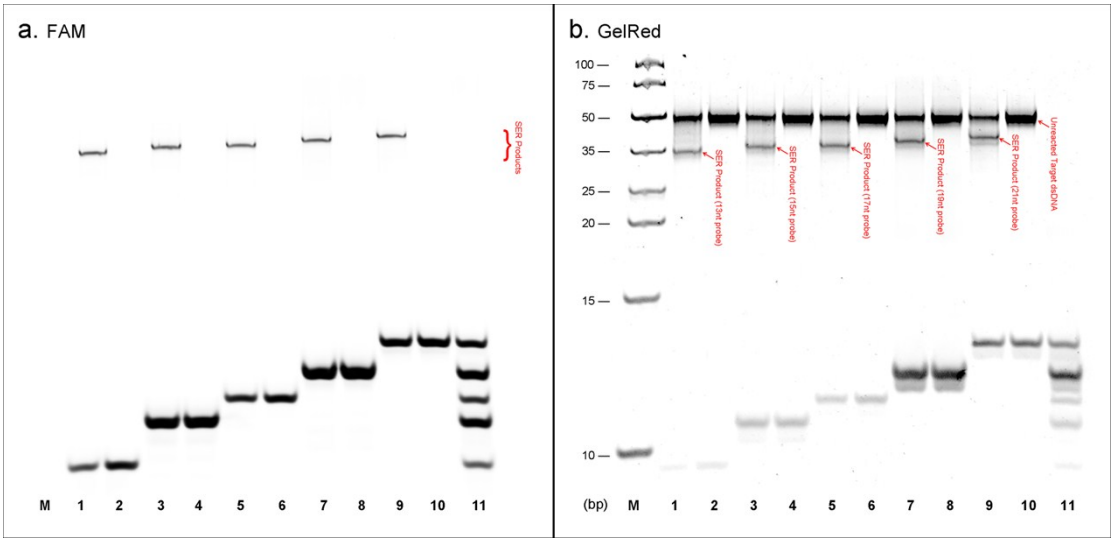

Figure S10. Study of SER product structure (48bp sets). Sequences see in Table S1. 30% native PAGE. Incubation time is 2 hours. Incubation temperature is 37℃. Template : Probe = 1 : 3. Lane M is DNA ladder. Lane 11 is five probes. Lane 1, 3, 5, 7 and 9 are methylated samples, lane 2, 4, 6, 8 and 10 are unmethylated samples. The right is the left dyed with GelRed.

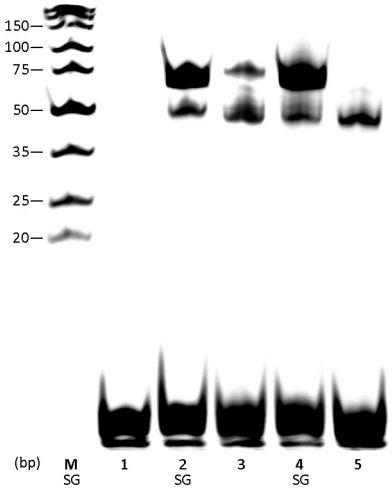

Figure S11. Study of SER product structure (70bp sets, 15nt(70nt)-FAM). Sequences see in Table S4. 30% native PAGE. Incubation time is 2 hours. Incubation temperature is 37℃. Probe is 15nt(70nt)-FAM. Template : Probe = 1 : 3. Lane M is DNA ladder. Lane 5 is the annealing product of 15nt(70nt)-FAM and 70nt-com. Lane 1 and 2 are the same unmethylated sample, but lane 2 is dyed with Sybr Green. lane 3 and 4 are the same methylated samples, but lane 4 is dyed with Sybr Green. Both FAM and Sybr Green were scaned with the same wavelength. Only the methylated sample exchanged with the probe and generated two product bands. The upper one was above the template band. The nether one was under the template band and migrated the same speed with the upper band in lane 5.

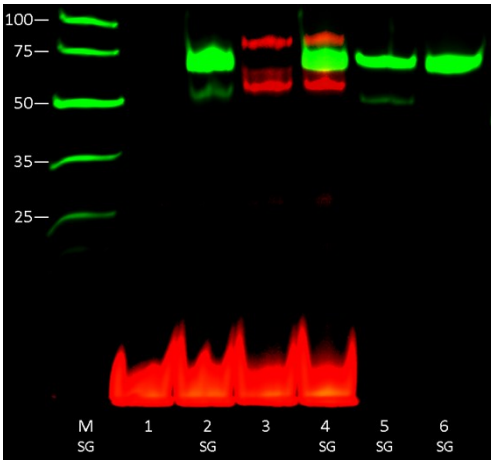

Figure S12. Study of SER product structure (70bp sets, 15nt(70nt)-Cy3). Sequences see in Table S4. 30% native PAGE. Incubation time is 2 hours. Incubation temperature is 37℃. Probe is 15nt(70nt)-Cy3. Template : Probe = 1 : 3. Lane M is DNA ladder. Lane 5 is the unmethylated template dyed with Sybr Green. Lane 6 is the methylated template dyed with Sybr Green. Lane 1 and 2 are the same unmethylated sample, but lane 2 is dyed with Sybr Green. lane 3 and 4 are the same methylated samples, but lane 4 is dyed with Sybr Green. Cy3 and Sybr Green were scaned with different wavelength and then integrated by Pharos FX Molecular Imager. Only the methylated sample exchanged with the probe and generated two product bands. The upper one was above the template band. The nether one was under the template band.

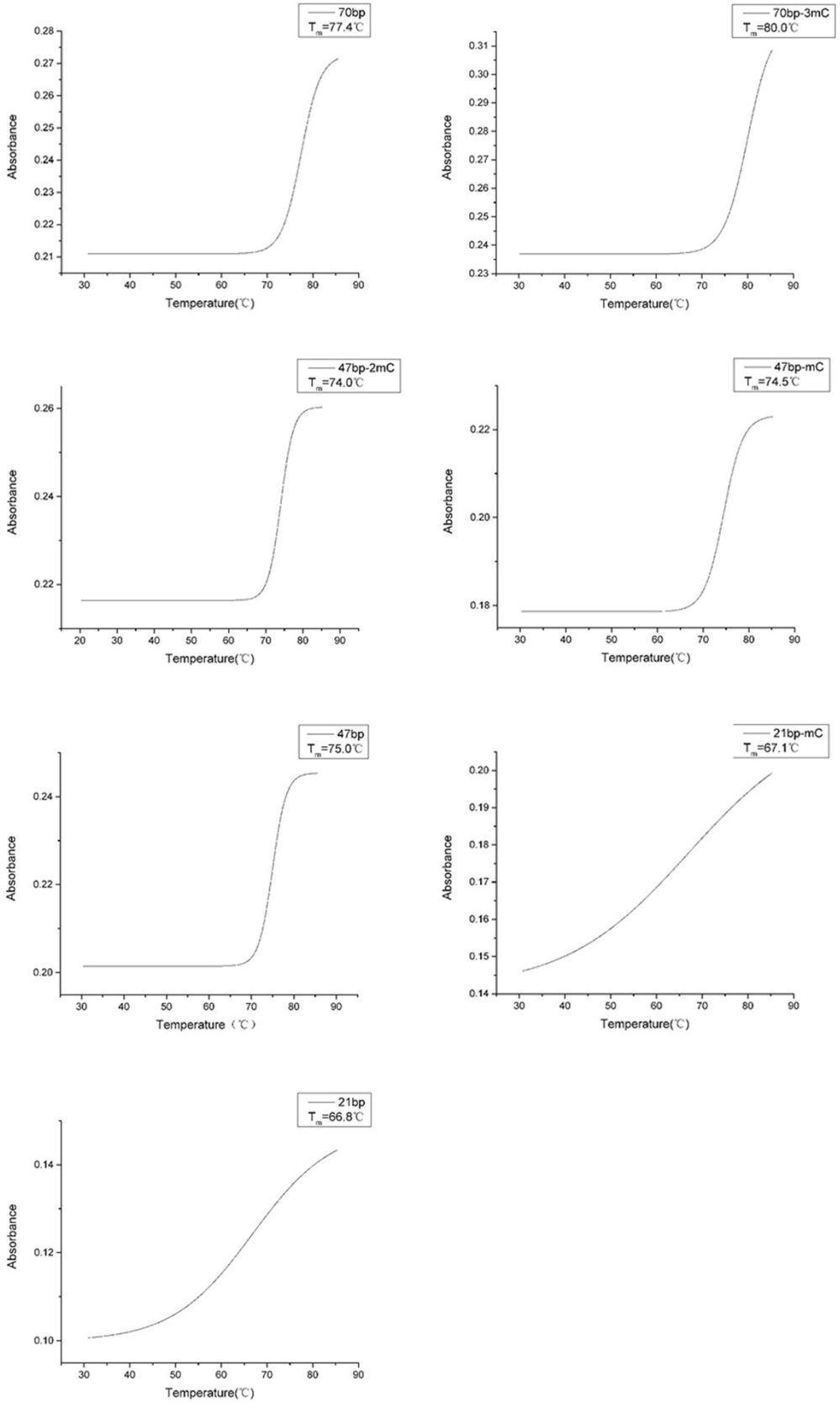

Figure S13. CD melting curves of double stranded DNAs recorded in a quartz cuvette of 0.1cm path length, at a scan rate of 0.5 ℃/min.