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Supporting Information

Multi-STEM MePCR: a bisulfite-free, multiplex, high-sensitive and high-specific assay to measure DNA methylation

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Experimental Procedures

Main materials

All the primers, modified oligonucleotides, Taqman probe and plasmids were obtained from Shanghai Sangon Biological Corporation (Shanghai, Chain) with the sequence information in Supplementary Table 1. The Jurkat genomic DNA, CpG Methylated Jurkat Genomic DNA and CpG Methyltransferase (M.SssI) were obtained from ThermoFisher Scientific. The methylation-dependent restriction endonuclease (MDRE) Gla I was purchased from SibEnzyme (Russian). Champagne TaqTM DNA polymerase and Phanta UC Super-Fidelity DNA Polymerase for BS-sequencing were purchased from Vazyme Biotech Co. Ltd. Nucleic acid dye and DNA marker (20bp, DL2000, DL10000) were purchased from Tanon and Takara Biomedical Technology (Beijing) Co., Ltd respectively. The QIAamp Fast Tissue Kits, QIAquick PCR Purification Kit and EpiTect DNA Bisulfite Kit were all obtained from Qiagen.

Instruments

The Real-time amplification process of multi-STEM-MePCR was performed on a LightCycler instrument 480 II (ROCHE). The gel was electrophoresed at a voltage of 150 mV for 30 min and then imaged on an UVP ChemStudio^{TM} (Analytlk Jena). The capillary electrophoresis of PCR products was performed on a Qsep100 Fully Automated Nucleic Acid and Protein Analysis System (Bioptic, Taipei, TW, China).

Preparation of methylated plasmid

The C5 position on the base moiety of all cytosine nucleotides in a 5'-CpG-3' context on plasmids constructed different target genes (Septin9, RASSF1 and SDC2) were methylated by Methyltransferase (M.SssI) respectively. The 20 µL reaction included 1 µg of plasmid, 1 x M.SssI Buffer, 1 x S-adenosylmethionine and 1 x M.SssI was incubated at 37^{o}C for 30 min then inactivated by heating at 65^{o}C for 20 min, finally purified by QIAquick PCR Purification Kit and quantified by digital PCR. The methylated plasmids were treated with the methylation-sensitive restriction endonuclease (HpaII) and the isoschizomer (MspI) respectively and further validated by the gel electrophoresis.

The methylation-dependent restriction endonuclease (MDRE) digestion

The MDRE digestion process was carried out in a total volume of 10 µL including 5 U Gla I, 1 x buffer and templates with certain concentration and incubated with following program: 37^{o}C for 30 min and inactivated at 65^{o}C for 20 min, finally stored at 4^{o}C.

Real-time amplification

The Real-time amplification process of multi-STEM-MePCR was performed in a total of 20 µL volume containing 10 nM TFP (tailored-foldable primer), 0.2 µM TSP (terminal specific primer), 0.2 µM UP (universal primer), and 0.2 µM Taqman probe, 0.3 mM of dNTP (containing dUTP), 1 × PCR buffer and 1 U Champagne Taq DNA Polymerase, 1 U UDG and 10 µL MDRE-digested product. The mixture was heated at 95 ^{o}C for 5 min, 10 cycles of 95^{o}C for 10 s, 66^{o}C for 90 s, and followed by second stage of 40 cycles of 95^{o}C for 10 s, 65^{o}C for 30 s. The signal acquisition was carried out during the annealing step of stage 2 by monitoring the fluorescence signal.

Agarose gel electrophoresis and Capillary electrophoresis of PCR products

5 µL of PCR product was mixed with 1 µL of 6 x loading buffer, and electrophoretic analysis was carried out in 3% agarose gel stained with Tanon™ Nucleic Acid Dye (10000 ×). The gel was electrophoresed at a voltage of 150 mV for 30 min and then imaged on an UVP ChemStudio^{TM} (Analytlk Jena). The PCR product of multiplex system was analyzed on a Qsep100 Fully Automated Nucleic Acid and Protein Analysis System (Bioptic, Taipei, TW, China) following the manufacturer's protocol. Digital data, gel-image format and capillary electropherograms can be exported by the Q-Analyzer software (Bioptic).

DNA extraction and bisulfite sequencing

The tissue samples were obtained from patients undergoing surgery at Renji hospital (Shanghai, China) with consent having been obtained prior to surgery-Human Research Ethics Committee approval RGH 09/04. The genomic DNA of tissue samples were extracted by using QIAamp Fast DNA Tissue Kit, then the quantity and purity of genomic DNA were assessed by Nanodrop spectrophotometer (ThermoFisher). 10 ng genomic DNA of each sample was analyzed by multi-STEM MePCR. Then 1 µg genomic DNA of different samples were converted and purified via EpiTect DNA Bisulfite Kit respectively. Then 2 µL of converted products were amplified by Bisulfite sequencing PCR (BSP) primers and the PCR products were analyzed by high-throughput sequencing.

Table S1. Sequence of primers used in the study.

name	sequence 5′-3′	describe
UP1	CGGCGTCAGATGTGGCACTGACAA	
UP2	GCCTGTCAGCCAACGGTATTCATC	
TSP-septin9	TGCCAACGGTATTCATCGTTGACC	
probe-septin9	VIC-CCATCATGTCGGACCC-MGB	
TFP-septin9	TGCCAACGGTATTCATCGTTGACCGCGGGGTCCG/ISP18/AGATGTGGCACTGACAAGCGACCAGCTGCCCACCAGC	0/17 (Methyl/total)
	TGCCAACGGTATTCATCGTTGACCGCG**GGGTCCG**/ISP18/AGATGTGGCACTGACAAGCGACCAGCTGCCCACCAGC	7/17
	TGCCAACGGTATTCATCGTTGACC**GCGGGGTCCG**/ISP18/AGATGTGGCACTGACAAGCGACCAGCTGCCCACCAGC	10/17
	TGCCAACGGTATTCATCGTTGA**CCGCGGGGTCCG**/ISP18/AGATGTGGCACTGACAAGCGACCAGCTGCCCACCAGC	12/17
	TGCCAACGGTATTCATCGTT**GACCGCGGGGTCCG**/ISP18/AGATGTGGCACTGACAAGCGACCAGCTGCCCACCAGC	14/17 & FR-17nt
	TGCCAACGGTATTCATC**GTTGACCGCGGGGTCCG**/ISP18/AGATGTGGCACTGACAAGCGACCAGCTGCCCACCAGC	17/17
	TGCCAACGGTATTCATCGTTGACC**GCGGGGTC**/ISP18/AGATGTGGCACTGACAAGCGACCAGCTGCCCACCA	FR-15nt
	TGCCAACGGTATTCATCGTTGACC**GCGGG**/ISP18/AGATGTGGCACTGACAAGCGACCAGCTGCCCACCAGC	FR-12nt
	TGCCAACGGTATTCATCGTTGACC**GC**/ISP18/AGATGTGGCACTGACAAGCGACCAGCTGCCCACCAGC	FR-9nt
	TGCCAACGGTATTCATCGTTGACC/ISP18/AGATGTGGCACTGACAAGCGACCAGCTGCCCACCAGC	FR-7nt
TSP-RASSF1	AGCCAACGGTATTCATCGCGGCTC	
probe-RASSF1	FAM-CTTCCCGCCGCCCAG-MGB	
TFP-RASSF1	AGCCAACGGTATTCATCGCGGCTC**TCCTCAGCTC**/ISP18/AGATGTGGCACTGACAAGCCTCCCCCAGGATCCAGACT	
TSP-SDC2	GCCAACGGTATTCATCGCGATCT	CpG 3
probe-SDC2	CY5-CAAGCTCCGCCTCCC-MGB	
TFP-SDC2	GCCAACGGTATTCATCGCGATCT**CGGCCCACTG**/ISP18/AGATGTGGCACTGACAAGAGGCAGGAGAATGACGTGAACCT	
TSP-SDC2-CpG1	GCCAACGGTATTCATCGCACACG	CpG 1
probe-SDC2-CpG1	VIC-CAGAGTACCGCAGCGA-MGB	
TFP-SDC2-CpG1	GCCAACGGTATTCATCGCACACG**AATCCGGAGC**/ISP18/AGATGTGGCaCTGACAACCGAGTCCCCGAGCCTGAGCC	
TSP-SDC2-CpG2	CCAACGGTATTCATCGCCGCAT	CpG 2
probe-SDC2-CpG2	FAM-ACCGGCTGCTCCCAG-MGB	
TFP-SDC2-CpG2	GCCAACGGTATTCATCGCCGCAT**ATTCCCCAGG**/ISP18/AGATGTGGCaCTGACAACTGAGCAGGAGGCTTCGTTTTGCC	
target-75bp	GTTGACCGCGGGGTCCGACATTTTTTTTTTTGATTTTTTTTTTTGGCTTTTTTTTTTTGGTGGGCAGCGGGTCGC	Templates With different size
target-65bp	GTTGACCGCGGGGTCCGACATTTTTTTTTTTGATGGCTTTTTTTTTTTGGTGGGCAGCGGGTCGC	
target-55bp	GTTGACCGCGGGGTCCGACATTTTTTTTTTTGATGGCTGGTGGGCAGCGGGTCGC	
target-45bp	GTTGACCGCGGGGTCCGACATGATGGCTGGTGGGCAGCGGGTCGC	
target-35bp	GTTGACCGCGGGGTCCGTGGTGGGCAGCGGGTCGC	

target-28bp	GTTGACCGCGGGGTGGTGGGCAGCGGGT	
BSP-FP	GTTAGTGTTGTATTGTAGGAG	bisulfite sequencing
BSP-RP	CAACCCAACACCCACCTT	

Note. Underlined and bold letters are 2'-O-mehthyl (2'Ome) bases, /ISP18/ is polyethylene glycol 18 used as extension blocker.

Table S2. Thermodynamic parameters of P1 with different FR length.

Name of TFP	Length of FR (nt)	Tm of folding structure (°C)	ΔG (kcal.mole^{-1}) at 65°C
TFP-FR-17nt	17	79.9	-6.16
TFP-FR-14nt	14	76	-4
TFP-FR-12nt	12	71.5	-1.86
TFP-FR-9nt	9	58.2	/
TFP-FR-7nt	7	43.7	/

The Tm and ΔG was calculated using the IDT OligoAnalyzer Tool available online.

Table S3. The Ct values measured by multi-STEM-PCR and ratio of methylation from NGS in 14 CRC samples. Septin 9 gene was used as target.

Sample	repeat			AVE	SD	CV	Ratio of methylation (%)
1	28.52	28.34	28.36	28.41	0.10	0.35%	21.40
2	27.47	27.37	27.62	27.49	0.13	0.46%	25.24
3	31.27	30.51	29.84	30.54	0.72	2.34%	9.99
4	27.66	26.93	27.73	27.44	0.44	1.61%	16.18
5	/	/	/	/	/	/	2.28
6	28.43	28.38	27.8	28.20	0.35	1.24%	17.05
7	34.31	33.94	31.8	33.35	1.36	4.06%	8.08
8	28.95	28.81	28.26	28.67	0.36	1.27%	17.04
9	25.53	25.53	24.79	25.28	0.43	1.69%	35.69
10	28.71	28.54	28.46	28.57	0.13	0.45%	7.21
11	29.9	30.26	30.9	30.35	0.51	1.67%	5.28
12	36.29	36.73	33.86	35.63	1.55	4.34%	0.22
13	26.9	27.39	26.98	27.09	0.26	0.97%	20.19
14	26.44	26.45	27.39	26.76	0.55	2.04%	22.53

Table S4. A comprehensive list of abbreviations and their corresponding full terms used in the main text.

Abbreviation	**Full terms**
multi-STEM MePCR	multiple Specific Terminal Mediated Methylation PCR
MSP	methylation-specific PCR
QuARTS	quantitative allele-specific real-time target and signal amplification
QM-MSP	quantitative multiplex methylation-specific PCR
MSRE	methylation-sensitive restriction enzyme
MDRE	methylation-dependent restriction endonucleases
TFP	tailored-foldable primer
UP	universal primer
TSP	terminal specific primers
HP	hairpin structure
UR	universal region
CR	capture region
FR	folding region
CRC	colorectal cancer
2'Ome	2'-O-methyl

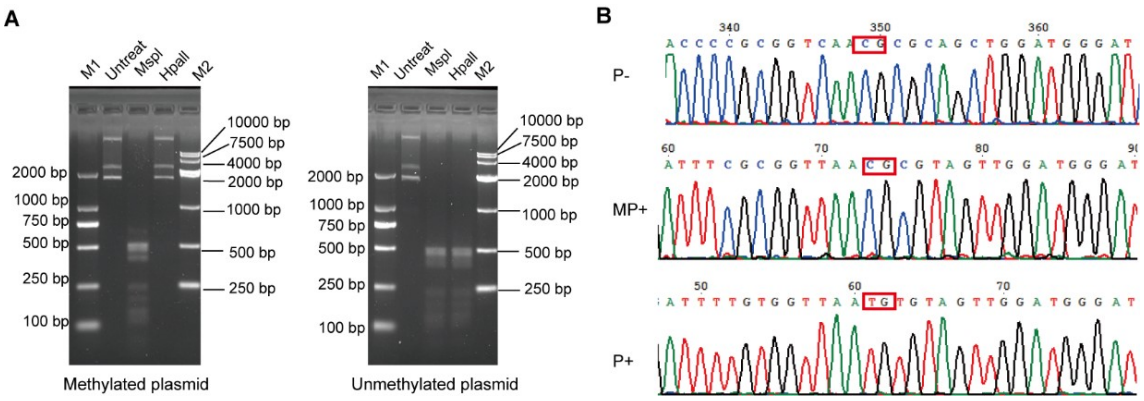

Fig. S1 (A) 1 µg of plasmid DNA (pUC57) were methylated using M.SssI. 100ng methylated plasmid DNA and un-methylated plasmid DNA were digested in parallel with methylation sensitive HpaII restriction enzyme and methylation insensitive isoschizomer MspI. Agarose gel electrophoresis map of methylated plasmid (left) and un-methylated plasmid (right). (B) The methylated and unmethylated plasmid DNA were test by bisulfite sequencing. The sequencing result of original plasmid with no treatment (P-), methylated plasmid treated by bisulfite (MP+) and un-methylated plasmid treated by bisulfite (P+).

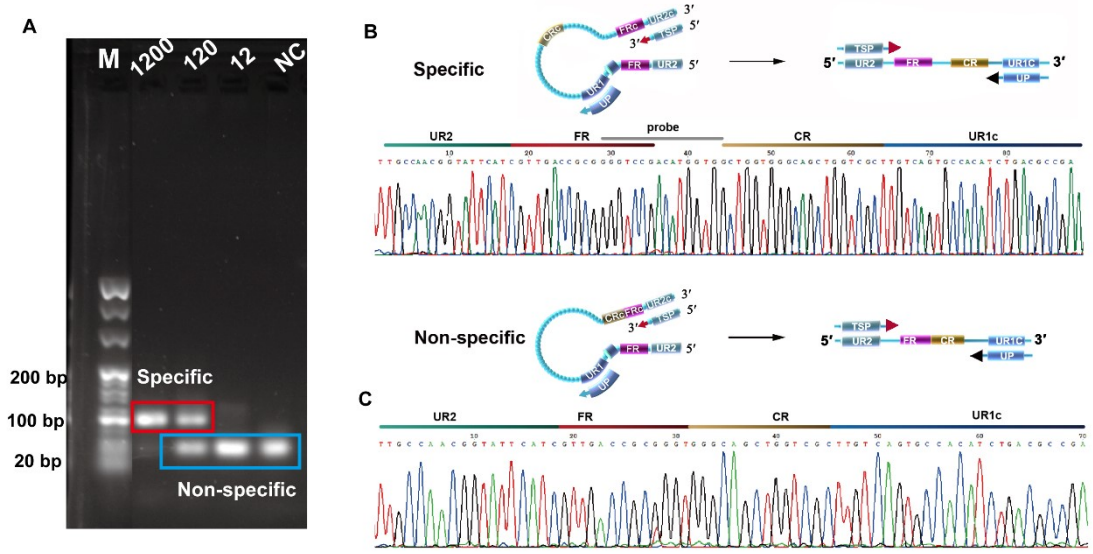

Fig. S2 (A) Agarose gel electrophoresis map of amplification product from 1200, 120 and 12 copies/reaction of methylated genomic DNA. (B) Monoclonal sequencing of specific and (C) non-specific amplification products from methylated site of Septin9 gene.

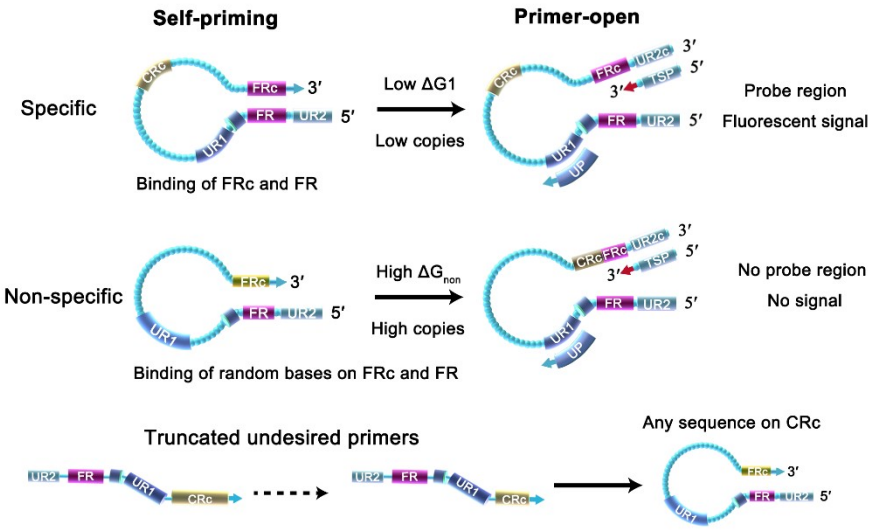

Fig. S3 In specific process, the FR and FRc of extension product formed a strong intramolecular binding to generate a partial stem-loop structure. In non-specific process, the partial region of CRc bound with FR randomly with low affinity to mediate a similar amplification. Any sequence on CR is at risk of causing non-specific amplification due to the truncated primers.

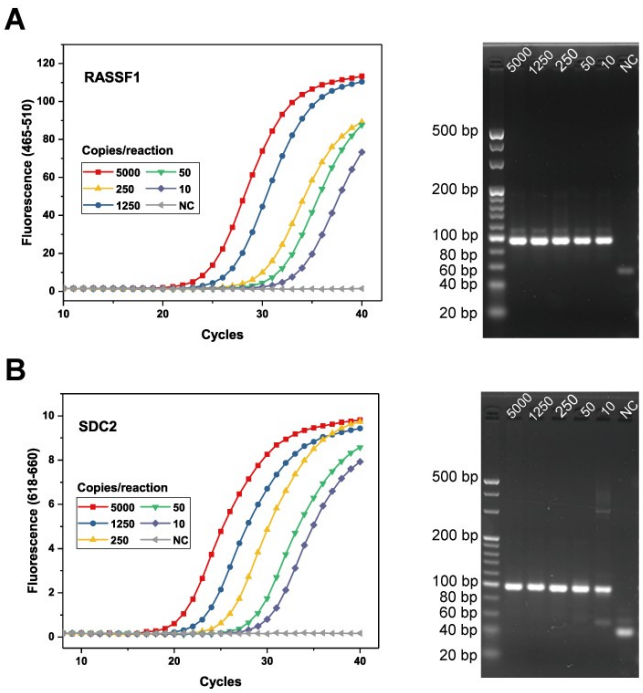

Fig. S4 (A) Real-time amplification curves (left) of multi-STEM MePCR with serially diluted methylated Jurkat genomic DNA 5000, 1250, 250, 50, 10 (copies/reaction), free water as a negative control (NC) on RASSF1 gene and Agarose gel electrophoresis map (right) of PCR product from different concentrations of methylated DNA and NC. (B) Real-time amplification curves (left) on SDC2 gene and Agarose gel electrophoresis map (right) of corresponding PCR product.

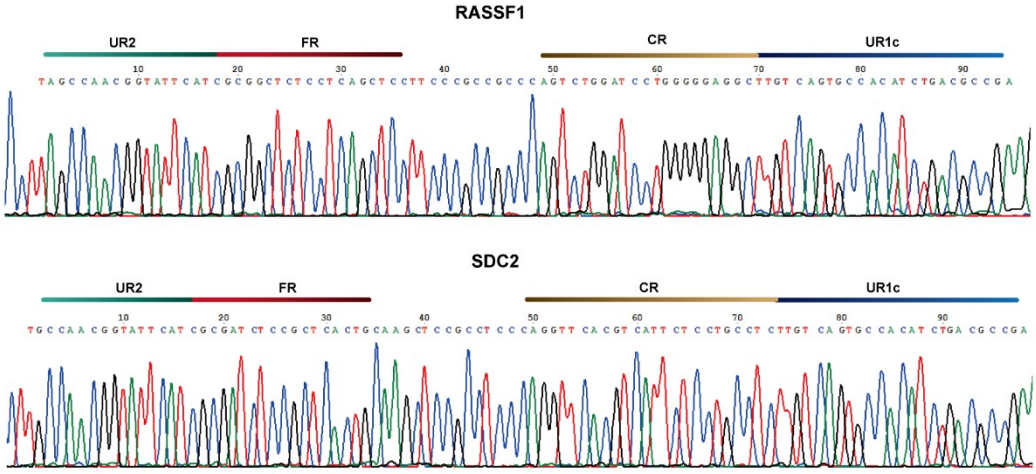

Fig. S5 Monoclonal sequencing of PCR products from methylated site of RASSF1 gene and SDC2 gene.