Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

DNAzyme Based Visual Detection of DNA Methylation

Mei Li[a], Lei Zhang[b], Gangyi Chen[a], Jing Zhou[b], Yi Yuan[a], Jiawei Zou[a], Mingming Yuan[b], Rong Chen[a], Feng Du[a], Xin Cui[a], Xin Huang[a], Juan Dong[a], and Zhuo Tang[a]*

[a]Natural Products Research Center, Chengdu Institute of Biology of the Chinese Academy of Sciences, Chengdu, Sichuan, China; [b]Sichuan Academy of Chinese Medicine Sciences, Chengdu, Sichuan, China.

*To whom correspondence should be addressed: Chengdu Institute of Biology of the Chinese Academy of Sciences, No.9 Section 4, Renmin Nan Road, Chengdu, Sichuan, China. Fax: +86-28-82890648; E-mail: tangzhuo@cib.ac.cn.

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Oligonucleotides used in the experiment

Table S1. oligonucleotides information

Identity	Sequences[a]
T-1	TATTTAGTTAATCGGCGGGTTTTCGACGGGAATGGGGAGC
T-2	TATTTAGTTAATTGGTGGGTTTTTGATGGGAATGGGGAGC
T-3	TATTTAGTTAATAGGAGGGTTTTAGAAGGGAATGGGGAGC
T-4	TATTTAGTTAATAGGAGGGTTTTCGACGGGAATGGGGAGC
T-5	TATTTAGTTAATAGGCGGGTTTTCGACGGGAATGGGGAGC
probe-1	ATTCCCGTCGAAAACCCGCCGTTCCCAACCCGCCAAGGGTAGGGCGGGTTGGGAAAAAA
P-T-M	TTATATGTCGGTTACGTGCGTTTATATTTAGTTAATCGGCGGGTTTTCGACGGGAATGGGGAGCGTTTTGGTTCGTATTT
P-T-U	TTATATGTCGGTTATGTGTGTTTATATTTAGTTAATTGGTGGGTTTTTGATGGGAATGGGGAGTGTTTTGGTTTGTATTT
P-1-M-F	TTATATGTCGGTTACGTGCGTTTATAT
P-1-M-R	AAATACGAACCAAAACGCTCCCC
MT	CCACATGTCGGTCACGTGCGCCCACACCCAGCCAATCGGCGGGCTCCCGACGGGAATGGGGAGCGCCCTGGTCCGCATCC
UT	CCACATGTCGGTCACGTGCGCCCACACCCAGCCAATCGGCGGGCTCCCGACGGGAATGGGGAGCGCCCTGGTCCGCATCC
APC-F	CCACATGTCGGTCACGTGCGCCCACAC
APC-R	GGATGCGGACCAGGGCGCTCCCC
CatG4	TGGGTAGGGCGGGTTGGGAAA
probe-APC-1	ATTCCCGTCGAAAACCCGCCGTTCCCAACCCGCCAAGGGTAGGGCGGGTTGGGAAAAAA
probe-APC-2	CCCGTCGAAAACCCGCCGTTCCCAACCCGCCAAGGGTAGGGCGGGTTGGGAAAAAA
probe-APC-3	GTCGAAAACCCGCCGTTCCCAACCCGCCAAGGGTAGGGCGGGTTGGGAAAAAA
probe-APC-4	GAAAACCCGCCGTTCCCAACCCGCCAAGGGTAGGGCGGGTTGGGAAAAAA
probe-APC-5	ATTCCCGTCGAAAACCTTCCCAACCCGCCAAGGGTAGGGCGGGTTGGGAAAAAA
S-1	ATTCCCGTCGAAAACCCGCCGTTCCCAACCCGCCAAGG
S-2	GTAGGGCGGGTTGGGAAAAAA
S-splint	AACCCGCCCTACCCTTGGCGGGTT
M-26	CAAGGGTAGGGCGGGTTGGGAAAAAA
M-28	GCCAAGGGTAGGGCGGGTTGGGAAAAAA
M-30	CCGCCAAGGGTAGGGCGGGTTGGGAAAAAA
M-32	ACCCGCCAAGGGTAGGGCGGGTTGGGAAAAAA

[a] The oligonucleotides **MT** and **UT** are sequences of the fragment chose for the detection with different methylation pattern. The cytosine labeled with red in **MT** is synthesized methylation sites. And the red cytosine in **P-T-M** represents the corresponding methylation site. The nucleotides marked in red in **T-2** to **5** are mutated sites.

Experimental procedures

General information

Taq DNA polymerase and dNTPs mixture were purchased from Beijing TransGen Biotech Co., Ltd. (Beijing, China). 10 × Taq buffer was prepared according to the instruction of Taq DNA polymerase. Lambda exonuclease was purchased from Fermentas. Hemin was purchased from Alfa Aesar. ABTS was purchased from Wolsen (Xi'an, China). H_2O_2 was purchased from Bodi Chemical Holding Co., Ltd. (Tianjin, China). T4 polynucleotide kinase (PNK) and T4 DNA ligase were purchased from Thermo Scientific. [γ-^{32}p] ATP was purchased from Furui Biological Engineering (Beijing, China). ATP was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The EZ DNA Methylation-Gold™ Kit was purchased from THE Epigenetics COMPANY™. (Shanghai, China). The DNA extraction kit was purchased from Bioteke Corporation. (Beijing, China). All of the oligonucleotides were polyacrylamide gel electrophoresis (PAGE) purified by Sangon Biotech Co., Ltd. (Shanghai, China) and are shown in Table S1. The cell lines (L-O2 and HepG2) were received from State Key Laboratory of Biotherapy. The pEASY®-T1 Cloning Vector was purchased from Beijing TransGen Biotech Co., Ltd. (Beijing, China). The PCR reaction was manipulated in a C1000 thermal cycler (Bio-Rad). The detection of the absorbance produced by oxidized ABTS was carried out in a microplate reader (Thermo Scientific). The absorbance wavelength was 414 nm. About 40 readings with a 30 s interval were recorded.

Labeling reaction

Oligonucleotides **M-26**, **M-28**, **M-30** and **M-32** were incubated with 10 units of Polynucleotide kinase (PNK) at 37°C for 1 h for DNA phosphorylation in a reaction mixture containing 50 mM Tris-HCl (pH 7.8, 25°C), 40 mM NaCl, 10 mM $MgCl_2$, 1 mg/ml BSA and 10 μCi [γ-^{32}p] ATP. The labeled product was purified by 10% denaturing polyacrylamide gel and used as DNA marker.

Ligation reaction

5 μM ^{32}p-labeled **S-2** and 5 μM 5'-phosphorylated **S-1** were incubated with 5 units of T4 DNA ligase for 2 h at 37°C in condition as follows: 400 mM Tris-HCl (pH 7.8, 25°C), 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP and 5 μM **S-splint**. Afterwards, the ligation product was loaded on 10% denaturing polyacrylamide gel for purification.

DNA extraction and bisulfite modification

The genomic DNA was extracted from HepG2 (a human hepatoma cell line, ATCC No., HB-8064) and L-O2 (normal human hepatic cell) according to the standard protocol of DNA extraction kit respectively. Then, the DNA sample was converted by bisulfite and purified under the instruction of the EZ DNA Methylation-Gold™ Kit before PCR amplification, followed by storage at -20°C until future use. The DNA concentration was calculated according to the OD value at 260 nm.

Extraction validation of the genomic DNA of HepG2 and L-O2 cell lines

The PCR reaction was conducted in a volume of 25 μl with 50 ng genomic DNA, 0.3 μM **APC-F**, 0.3 μM **APC-R**, 0.2 mM dNTPs, 1 × Taq buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$),

2.5 units of Taq DNA polymerase. The PCR procedure was as follows: 94°C for 3 min followed by 10 cycles of 94°C for 30 s, 65°C (-0.5°C /cycle) for 30 s and 72°C for 30 s, then, 18 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s.

PCR amplification for colorimetric detection

The PCR reaction was carried out in a 50 µl mixture containing 5 µl DNA template, 0.3 µM forward primer (phosphorylated at 5'-end), 0.3 µM reverse primer, 0.2 mM dNTPs, 1 × Taq buffer, 5 units of Taq DNA polymerase. Following was the optimal touchdown PCR program: 94°C for 3 min followed by 10 cycles of 94°C for 30 s, 63°C (-0.5°C /cycle) for 30 s and 72°C for 30 s, then, 27 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s.

DNAzyme based colorimetric detection

1 × Taq buffer, 1 µM probe (phosphorylated at 5'-end), 110 mM NaCl (the dosage can be adjusted according to the actual situation) and sterilized water were added to the PCR products to make a total volume of 50 µl. Then Lambda exonuclease with the activity of 5'-3' exonuclease was added into the mixture to digest the 5'-phosphorylated labeled strand from double-strand by incubation at 37°C for 1 h 50 min. Finally, hemin (1 µM), ABTS (2 mM) and H_2O_2 (1 mM) were added, followed by absorbance detection of $ABTS^{\bullet+}$ at 414 nm immediately.

Results and discussion

The specificity analysis of the probe (Fig. S1)

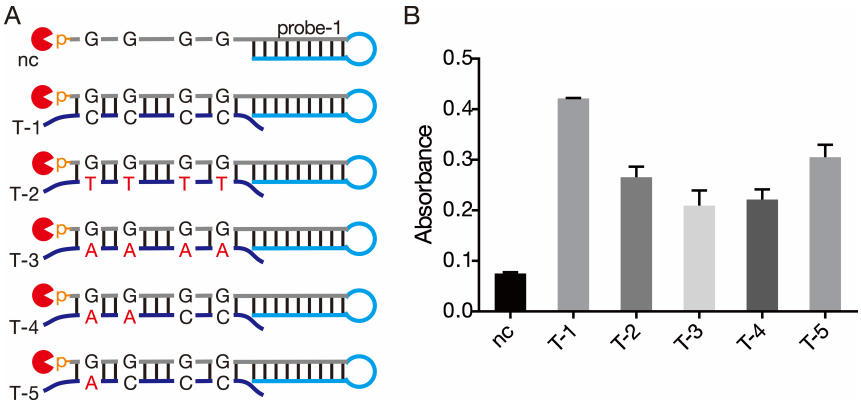

Fig. S1 Specificity analysis of the probe. (A) Schematic diagram of the reaction. The mutation sites in **T-2** to **5** are marked in red. (B) nc: negative control which use sterile water as sample. 0.3 μM **T-1** to **5** were added to the reaction buffer which contained 0.8 μM **probe-1** and 110 mM NaCl, respectively. The 50 μl reaction mixture was treated with 10 units of Lambda exonuclease at 37°C for 50 min. The absorbance was recorded in triplicate at 414 nm.

As shown in Fig. S1A, four mutated target sequences **T-2**, **T-3**, **T-4**, **T-5** which have four or two or only one mutations comparing to **T-1** were designed to investigate the specificity of the probe. The colorimetric reaction was carried out in reaction buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$) with 110 mM NaCl. The adding of NaCl is very important to decrease the background caused by the low activity on single-stranded DNA of Lambda exonuclease. 0.3 μM **T-1** to **5** were added to the reaction buffer which contained 0.8 μM **probe-1**, respectively. And there was a negative control which use sterile water as sample. Then, the 50 μl reaction mixture was treated with 10 units of Lambda exonuclease at 37°C for 50 min. After the addition of Hemin, ABTS and H_2O_2, the absorbance was recorded at 414 nm (Fig. S1B). Low absorbance was obtained in the negative control which utilizes sterile water as sample. While, the absorbance of the tube containing perfectly matched template **T-1** could be easily distinguished from the mutated targets, even though just one base is mutated in **T-5**. And lower absorbance was obtained in the presence of two or more mutations. It indicates that the probe behaved with good specificity.

The selection and evaluation of different methylation specific probes (Fig. S2)

In the pilot experiment, five probes with different length (**probe-APC-1**, **probe-APC-2**, **probe-APC-3**, **probe-APC-4** and **probe-APC-5**) were designed against the different regions of the *APC* gene (Fig. S2). **Probe-APC-1** is a sequence of 59 nt which contains 21 nt complementary to the target, differently, **probe-APC-2** and **probe-APC-3** have a complementary sequence of 18 nt, 15 nt respectively. Four methylation sites are included in both three probes. There are three methylation sites in **probe-APC-4** while **probe-APC-5** have only two. The synthesized **P-T-M** was used as the target. The colorimetric reaction was carried out in reaction buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$) with 90 mM NaCl. And 5 units of Lambda exonuclease was added into the 20 µl reaction mixture which containing 1 µM probe. There's a negative control performed without template for each probe. After 50 min of digestion at 37°C, hemin, ABTS and H_2O_2 were added to report the detection result. As illustrated in Fig. S2, different colorimetric results by using those five probes were turned out. It was demonstrated that **probe-APC-1** exhibited the best colorimetric result comparative to that of other probes. Therefore, the **probe-APC-1** was proved to be the best fitted probe for DNA methylation detection based on our strategy.

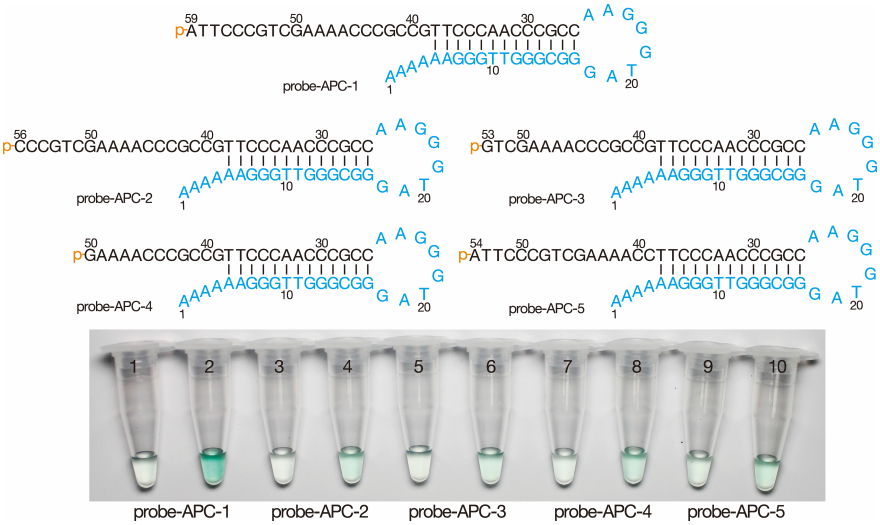

Fig. S2 Sequence information of the five probes with different length and the colorimetric detection results with five different probes. Tube 1, 2: **probe-APC-1**; Tube 3, 4: **probe-APC-2**; Tube 5, 6: **probe-APC-3**; Tube 7, 8: **probe-APC-4**; Tube 9, 10: **probe-APC-5**; Tube 1, 3, 5, 7, 9: the colorimetric reaction containing no target DNA; Tube 2, 4, 6, 8, 10: the colorimetric reaction containing 0.3 µM methylated DNA (**P-T-M**).

Assay sensitivity (Fig. S3)

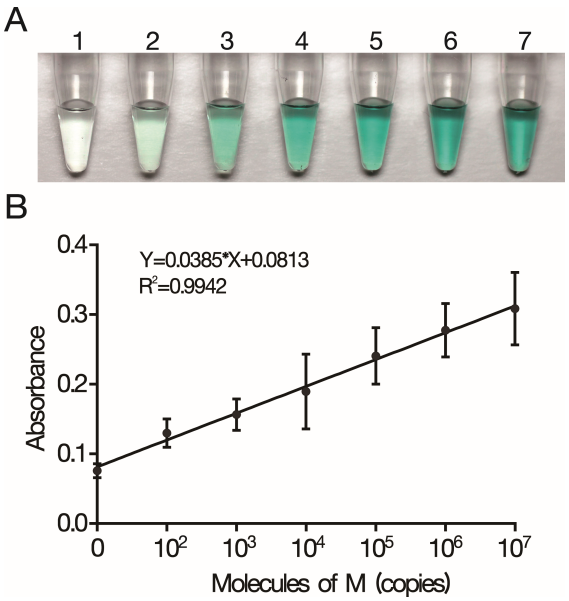

Fig. S3 Assay sensitivity. (A) Tube 1: negative control without template; Tubes 2 to 7 containing 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 copies of **P-T-M**, respectively. (B) The calibrated curve plotted against the concentration of methylated DNA. 'M': methylated DNA.

Extraction validation of the genomic DNA of HepG2 and L-O2 cells (Fig. S4)

To investigate whether the DNAzyme based colorimetric method can be applied to real-world samples, a human hepatoma cell line named HepG2 that has been widely used in the field of methylation research and verified to be hypermethylated at the *APC* gene (data not shown) was utilized here. And the normal hepatic cell line named L-O2 was used as a control. At first, the genomic DNA of the two cell lines was extracted according to the standard protocol of DNA extraction kit respectively. Then, a pair of universal primers (**APC-F**: 5′-CCACATGTCGGTCACGTGCGCCCACAC-3′, **APC-R**: 5′-GGATGCGGACCAGGGCGCTCCCC-3′) for *APC* gene were designed for the amplification of the genomic DNA, which is carried out as control for DNA extraction. The PCR reaction was conducted in a volume of 25 μl with 50 ng genomic DNA, 0.3 μM **APC-F**, 0.3 μM **APC-R**, 0.2 mM dNTPs, 1 × Taq buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$), 2.5 units of Taq DNA polymerase. And the PCR procedure was as follows: 94°C for 3 min followed by 10 cycles of 94°C for 30 s, 65°C (-0.5°C /cycle) for 30 s and 72°C for 30 s, then, 18 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. As shown in Fig. S4, Obvious objective strap was obtained from both L-O2 (lane 2) and HepG2 (lane 3) cells, and, the target band from each template was cloned into pEASY®-T1 Cloning Vector followed by automatic DNA sequencing provided by Sangon Biotech Co., Ltd. (Shanghai, China) (Fig. S7). The results indicate that the genomic DNA of both HepG2 and L-O2 cells could be extracted successfully.

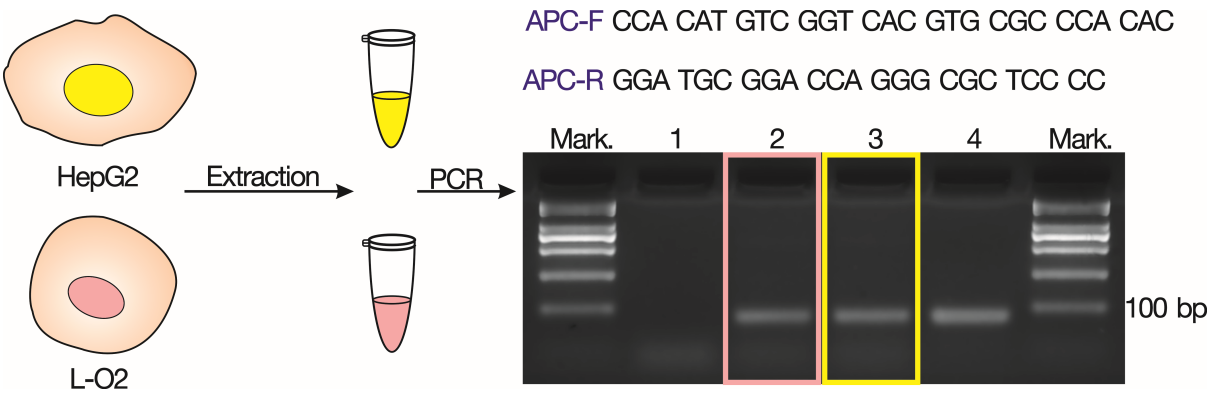

Fig. S4 The extraction validation of the genomic DNA of HepG2 and L-O2 cell lines. **APC-F** and **APC-R** are primers used in this experiment. Lane 1: negative control which use sterile water as sample; Lane 2: the amplification with the genomic DNA of L-O2; Lane 3: the amplification with the genomic DNA of HepG2; Lane 4: positive control.

Repeatability of three batches of converted HepG2 and L-O2 DNA (Fig. S5)

As shown in Fig. S5, the genomic DNA of both HepG2 and L-O2 were firstly extracted according to the standard protocol of DNA extraction kit respectively. And then, the extracted DNA was converted by bisulfite and purified under the instruction of the EZ DNA Methylation-Gold™ Kit before PCR amplification. To study the reproducibility of the bisulfite modification, three batches of converted HepG2 and L-O2 DNA were used for PCR (the sequencing result of the amplification product of the bisulfite converted HepG2 DNA was provided in Fig. S8) and colorimetric detection under the optimized conditions. Finally, the absorbance was recorded at 414 nm with the addition of hemin, ABTS and H_2O_2 and the result indicates that high repeatability of the bisulfite modification could be obtained.

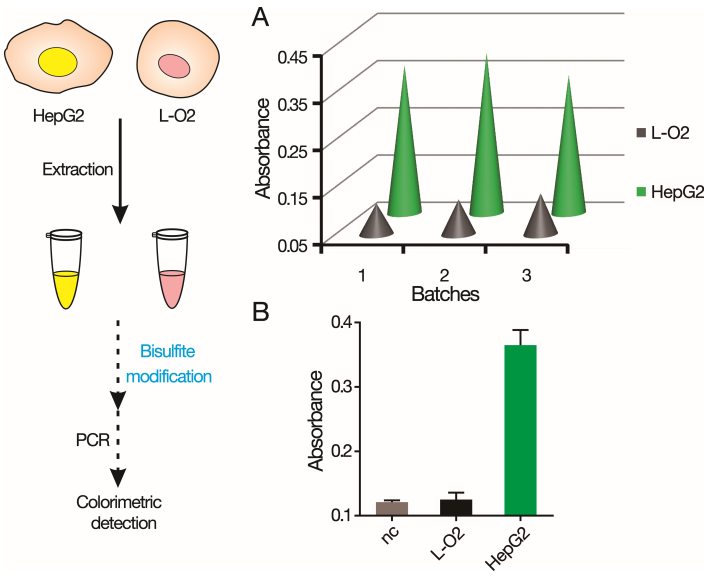

Fig. S5 Reproducibility of the bisulfite modification. (A) The optical absorption of different batches of converted HepG2 and L-O2 DNA which were recorded at 414 nm. 35 ng converted HepG2 and L-O2 DNA were used for the colorimetric detection respectively. (B) The average absorbance (414 nm) obtained from the three batches of converted HepG2 and L-O2 DNA. The error bars were determined by standard deviation (SD) of the data of three batches of sample.

Sequencing results obtained in the experiment

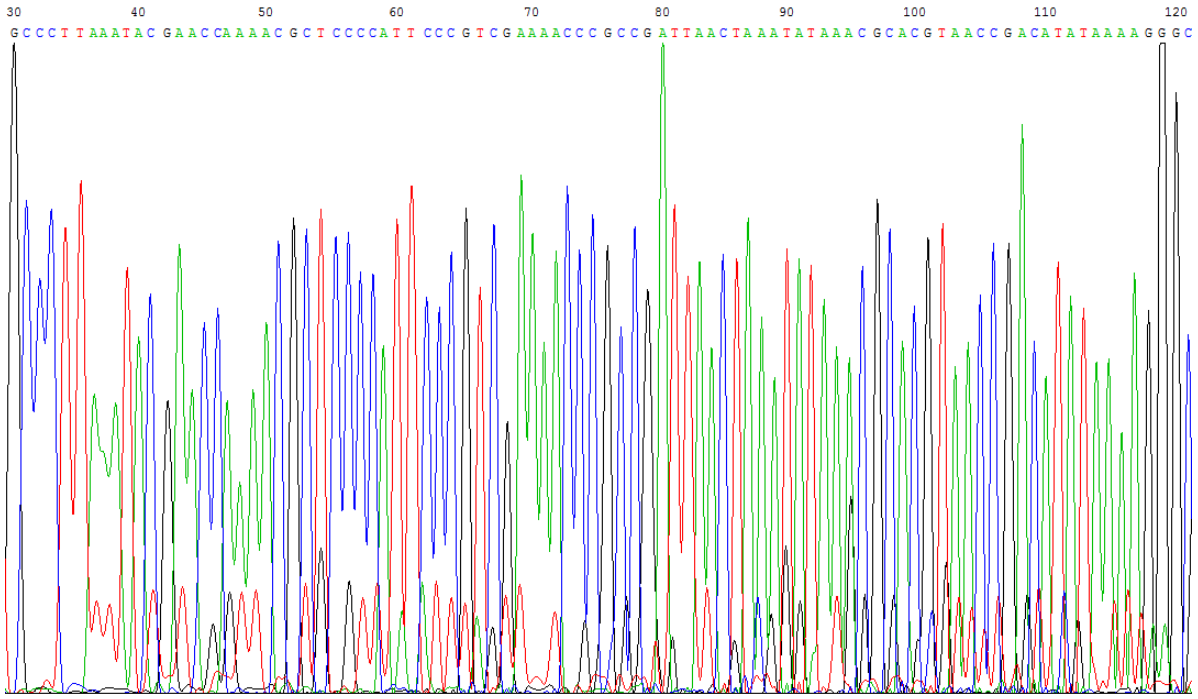

Fig. S6 Sequencing result of the amplification product of **P-T-M.** Primers that were used for the amplification are **P-1-M-F** and **P-1-M-R**.

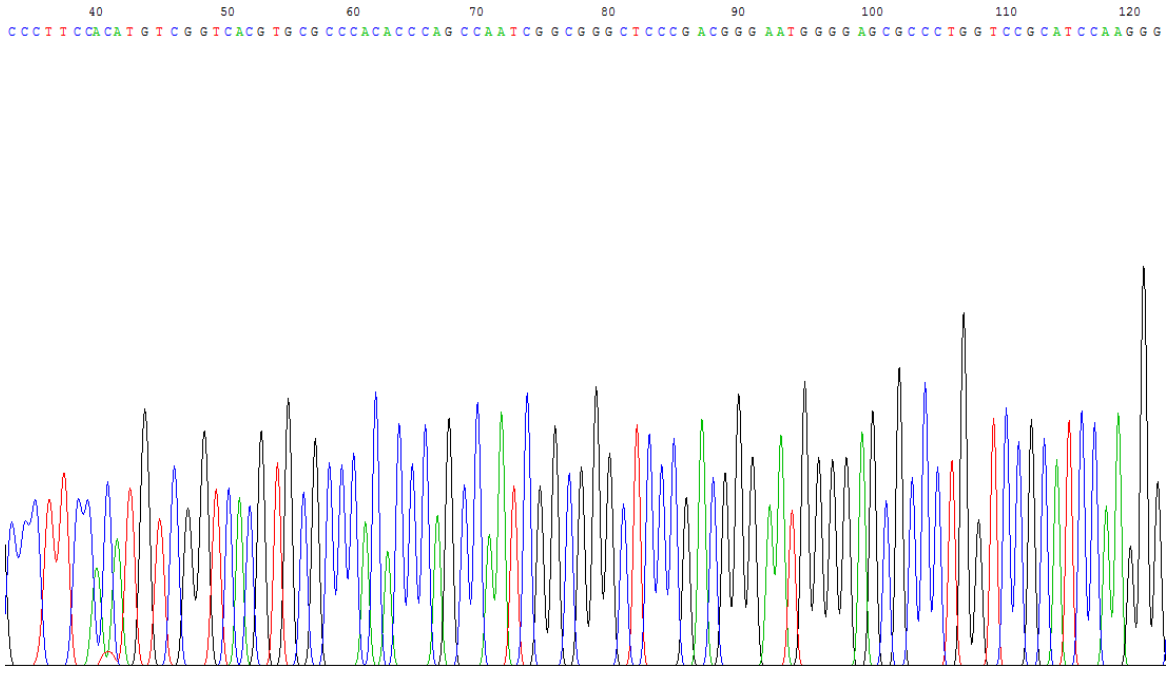

Fig. S7 Sequencing result of the amplification product of HepG2 DNA. Primers that were used for the amplification are **APC-F** and **APC-R**.

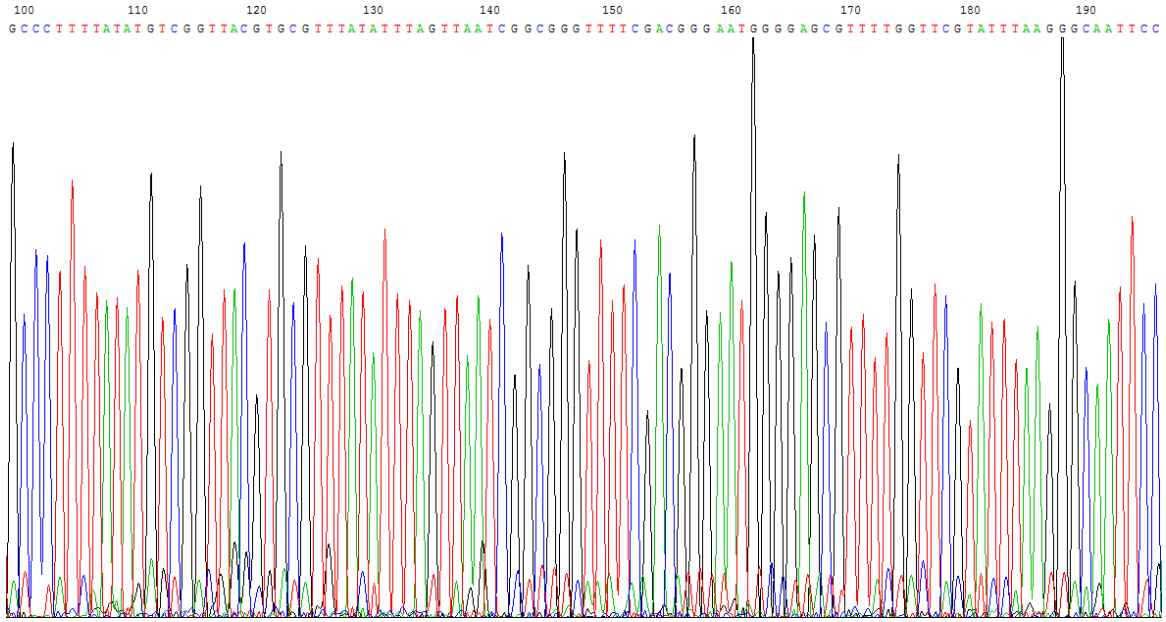

Fig. S8 Sequencing result of the amplification product of the bisulfite converted HepG2 DNA. Primers that were used for the amplification are **P-1-M-F** and **P-1-M-R**.

Characterization of the modified oligonucleotides by ESI-MS

Name:	P-1-M-R		
Sequence (5’ to 3’):	AAA TAC GAA CCA AAA CGC TCC CC		
Primer Length:	23	Tm:	58.07
MW (µg/µmole):	6939.58	%GC:	47.83
Purification:	HPLC	Modification:	5`P
Modification MW:	79.90	Aggregate MW:	7019.48

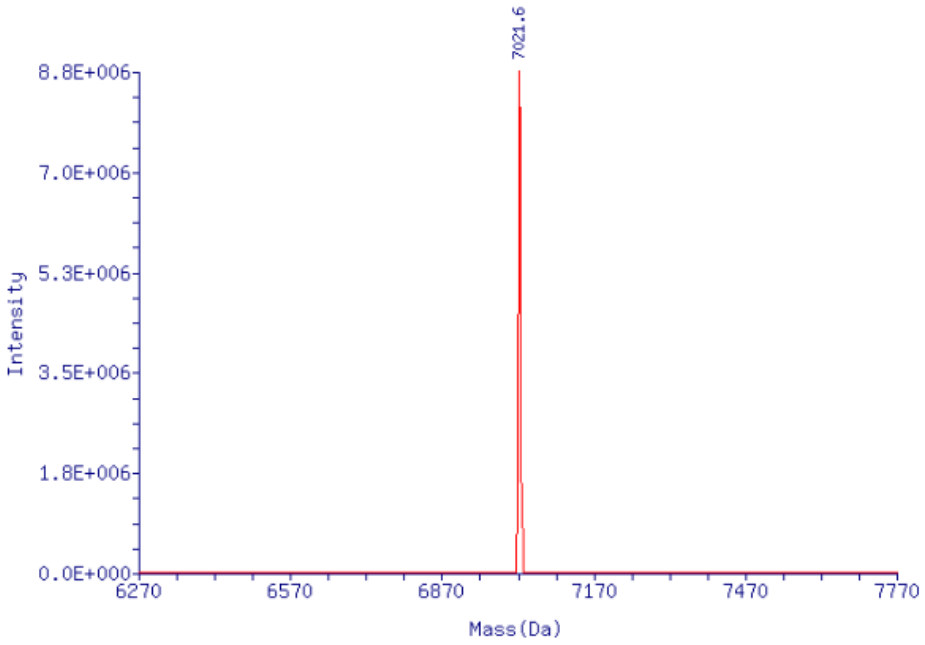

Fig. S9 Characterization of the 5’-phosphorylated **P-1-M-R** by ESI-MS.

Name:	probe-1		
Sequence (5’ to 3’):	ATT CCC GTC GAA AAC CCG CCG TTC CCA ACC CGC CAA GGG TAG GGC GGG TTG GGA AAA AA		
Probe Length:	59	Tm:	80.03
MW (µg/µmole):	18184.75	%GC:	59.32
Purification:	HPLC	Modification:	5`P
Modification MW:	79.90	Aggregate MW:	18264.65

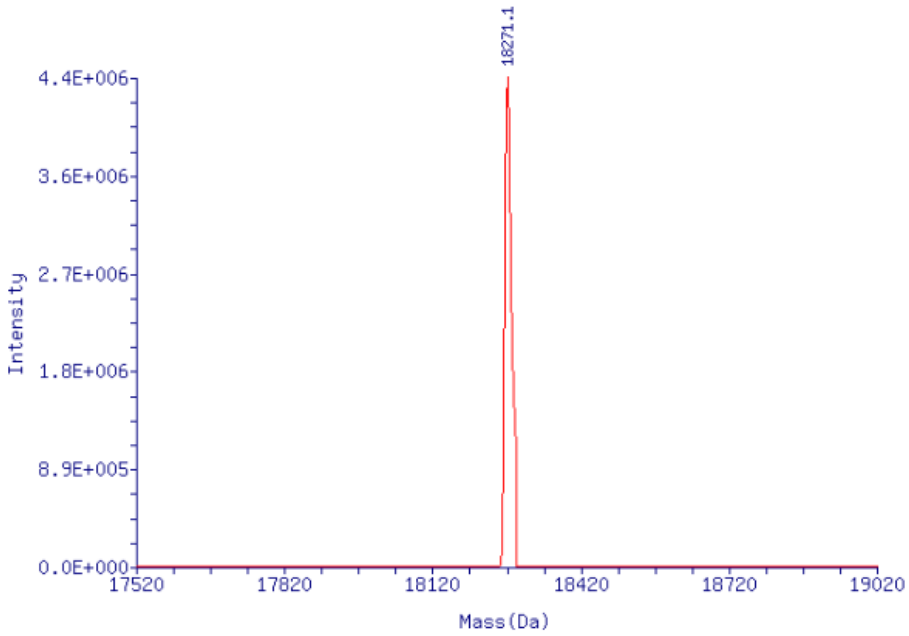

Fig. S10 Characterization of the 5’-phosphorylated **probe-1** by ESI-MS.