Supporting Information

Label-free real-time detection of DNA methylation based on quartz crystal microbalance measurement

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Materials and Methods:

QCM chips (AT-cut, 5 MHz) with gold coating were purchased from Dongwei Biotechnology Co., Ltd. (Hangzhou, China). Before probe immobilization, each QCM chip was cleaned for 30 minutes in UV/Ozone Cleaner (BioForce Nanosciences, Inc., Aspen, USA), then rinsed with water and ethanol and dried under nitrogen airflow. A home-built QCM with commercial control software (Resonant Probes GmbH, Goslar, Germany) was used, operating at the third overtone, i.e., at 15 MHz.

The primers for nested PCR and probe sequences used for the *p16* and *GALR2* genes were as follows: for *P16*, outer-forward, 5′-AGC CCA GTC CTC CTT CCT T-3′; outer-reverse, 5'-CTG CCT GCT CTA CCC CTC TC-3'; inner-forward, 5'-TCC TTC CTT GCC AAC GCT-3';

inner-reverse, 5'-GCC CCT CCT CTT TCT TCC TC-3'; and probe for *p16*, sulfhydryl-5'-TTT TTT TTT TTT TTT GCC CCT CCT CTT TCT TCC TC-3'; and primers for *GALR2*, outer-forward, 5'-CCG ATA TCA TGC CAC TAC ACT CCA-3'; outer-reverse, 5'-CGA GGT TGT CCC CAG CAG C-3'; inner-forward, 5'-ATC TCC CAG GGG TCC TCT TTT G-3'; inner-reverse, 5'-GCT TGT ACC TGC TTC CGA GCT-3'; and probe for *GALR2*, sulfhydryl-5'-TTT TTT TTT TTT TAT CTC CCA GGG GTC CTC TTT TG-3'. The synthetic probe DNA sequences complementary to the *GALR2* sequence were as follows: sense, 5'-GAG GGG CTG GGG TGC CCT CAA AAG AGG ACC CCT GGG AGA T-3'; antisense, 5'-ATC TCC CAG GGG TCC TCT TTT GAG GGC ACC CCA GCC CCT C-3'.

Genome extraction, digestion, and PCR. The HT29 and HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum plus 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. At 80% confluence, genomic DNA was extracted with a TIANamp genomic DNA kit (including approximately 1×10^6 cells). Electrophoresis was used to analyze the extracted DNA on a 1% (wt/vol) agarose gel.

To prepare the digestion sample, we mixed 2 μL HpaII enzyme, 2 μL 10× HpaII enzyme buffer, 2 μL genome DNA solution, and 14 μL sterilized water. To prepare the mock sample, we mixed 2 μL 10× HpaII enzyme buffer, 2 μL genome DNA solution, and 16 μL sterilized water. These samples were then incubated at 37°C for 5 minutes to digest genome DNA. Samples were then incubated at 65°C for 5 minutes to inactivate HpaII enzyme. The HpaII-digested, MspI-digested and mock sample were amplified by nested PCR, with the first round product diluted 10-fold with sterilized H_2O to serve as DNA template for the second-round PCR.

Primer design for nested PCR. For HpaII-digested DNA sample, a primer pair was designed to amplify the sequence that covers more than two 5′-CCGG-3′ sites. The primers contain no 5′-CCGG-3′ site, with lengths between 18 and 25 mers. Primer pair sequences were designed online

with Primer3, checking for secondary structures. The 35-mer oligonucleotide probe, functionalized with a thiol group at its 5′ end, hybridized with the PCR end-product.

With H_2O as a negative control and mock sample as a positive control to verify DNA integrity, we mixed 1 μL 25× dNTP mixture, 1 μL sense primer (10 μM), 1 μL antisense primer (10 μM), 12.5 μL 2× GC buffer I, 0.5 μL Fermentas Hot Start Taq DNA polymerase (5 UμL−1), 1 μL digested DNA sample (5 ng), and 8 μL high-purity H_2O for nested PCR solution. The nested PCR conditions were as follows: one step at 95 °C for 3 minutes, 32 cycles at 95 °C for 15 seconds, and 55 °C to 62 °C for 1 minute.

The experimental results of methylation detection and inhibitor evaluation were included below. Typically, data from four samples were collected, namely HpaII or inhibitor, Mock, negative control and Msp samples. The HpaII group represented that cell genome was firstly digested by HpaII enzyme and PCR amplified. Then, the PCR product flowed through QCM. The inhibitor group represented that cells were first treated inhibitors before underwent HpaII processing. The mock group represented that genome DNAs were not digested by HpaII enzyme before PCR. The negative control represented that the addition of equivalent reagent mixtures of PCR. The Msp-PCR represented that genome DNAs were digested by MspI enzyme before PCR.

Methylation assay.

We applied QCM to detect methylation statues of *P16* gene in HepG2 cell and *GALR2* gene in HT29 cell.

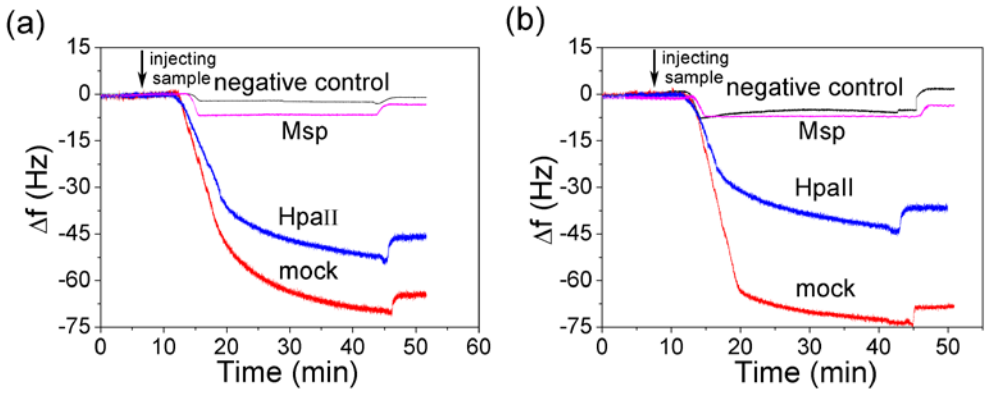

Figure S1. The representative QCM curves that denote methylation status of *GALR2* gene in HT29 cell (a) and *P16* gene in HepG2 cell (b). (a) *GALR2* gene in HT29 cell. The frequency change of HpaII, Mock, negative control and MspI samples were $\Delta f = 46 \pm 4$ Hz, 65 ± 2 Hz, 4 ± 1 Hz, and 3 ± 1 Hz, respectively. Methylation index = 69.4% ± 6.8%. (b) *P16* gene in HepG2 cell. The frequency changes of HpaII, Mock, negative control and MspI groups were $\Delta f = 38 \pm 2$ Hz, 69 ± 4 Hz, 2 ± 1 Hz, and 4 ± 1 Hz, respectively. Methylation index = 52.3% ± 4.5%.

Evaluation of inhibitor.

The cells are cultured with inhibitor 5-Aza-2'-deoxycytidine for 48 hours, then Mehtylation statues of *P16* gene in HepG2 cell and *GALR2* gene in HT29 cell was assayed.

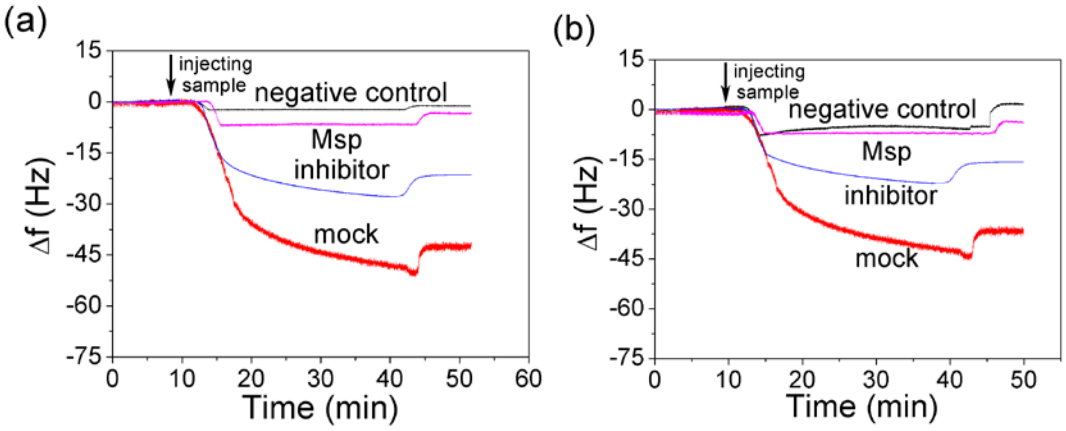

Figure S2. The representative QCM curves that evaluated methyltransferase inhibitor. (a) *GALR2* gene in HT29 cell. HT29 cell was cultured with 5-Aza-2' deoxycytidine for 48 hours. The frequency change of inhibitor, Mock, negative control and MspI groups were $\Delta f = 22 \pm 2$ Hz, 46 ± 3 Hz, 2 ± 1 Hz, and 3 ± 1 Hz, respectively; Inhibition effect index = 55.8% ± 5.7%. (b)

p16 gene in HepG2 cell. HepG2 cells were cultured with 5-Aza-2' deoxycytidine for 48 hours. The frequency changes of inhibitor, Mock, negative control and MspI groups were $\Delta f = 16 \pm 2$ Hz, 38 ± 3 Hz, 2 ± 1 Hz, and 4 ± 1 Hz, respectively. Inhibition effect index = 64.7% ± 6.9%.

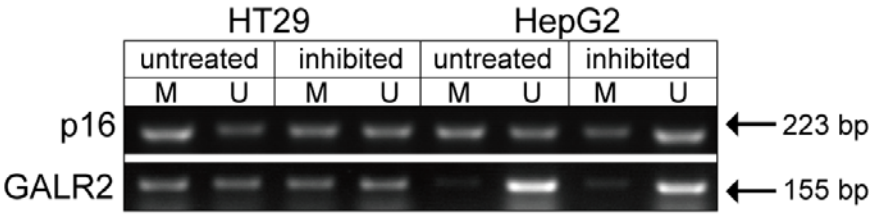

Figure S3. MSP electrophoresis analysis for DNA methylation in 2% (wt/vol) agarose gel. The result has shown the methylation of *p16* and *GALR2* genes in HT29 and HepG2 cell lines and the inhibition effect of Decitabine. For each sample, the left lane labeled with M is the specifically-designed primers for methylated target DNA, and the right lane labeled with U is the specifically-designed primers for unmethylated target DNA. Untreated sample means samples without inhibition from inhibitors. Inhibited sample means inhibitor-treated sample. The MSP result confirmed the result of QCM. *p16* is methylated in HT29 and HepG2 cell lines. *GALR2* is almost unmethylated in HepG2 cells. After being inhibited by Decitabine, methylation of these genes is inhibited in both HT29 and HepG2 cell lines.

We further performed bisulfite pyrosequencing for *GALR2* gene in HT29 and HepG2 cell lines. Because the target DNA sequence is over 250 bp, we relay the detection in two runs covering the four CCGG loci of *GALR2* gene. The average methylation level (of the ten loci in the figures) of *GALR2* in HT29 before and after inhibition was 0.7 and 0.07, respectively. The average methylation level of *GALR2* in HepG2 before and after inhibition was 0.08 and 0.06, respectively. These results supported the QCM detection results.

Before

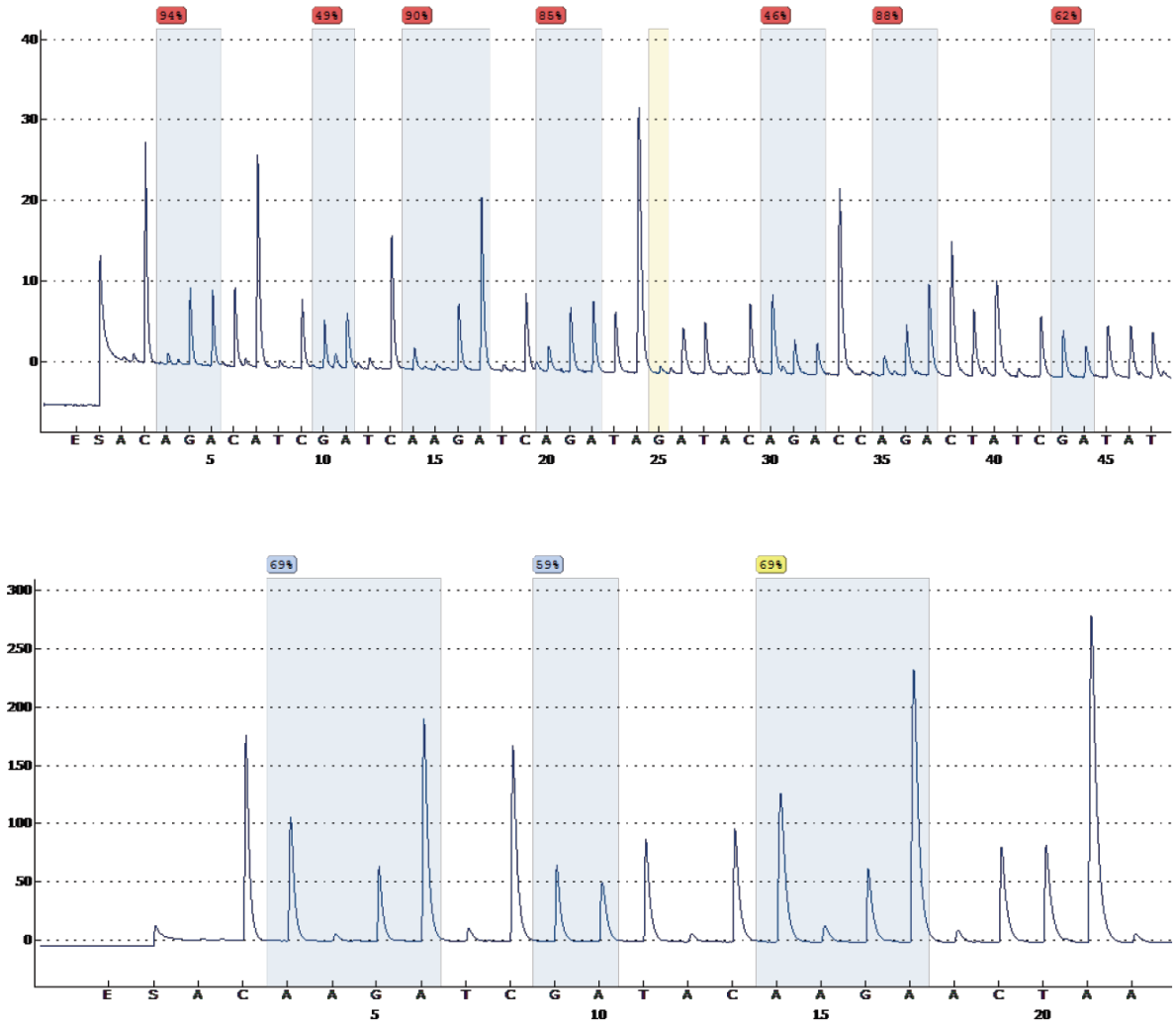

Figure S4 (1) GALR2 in HT29 cell line before inhibition.

After

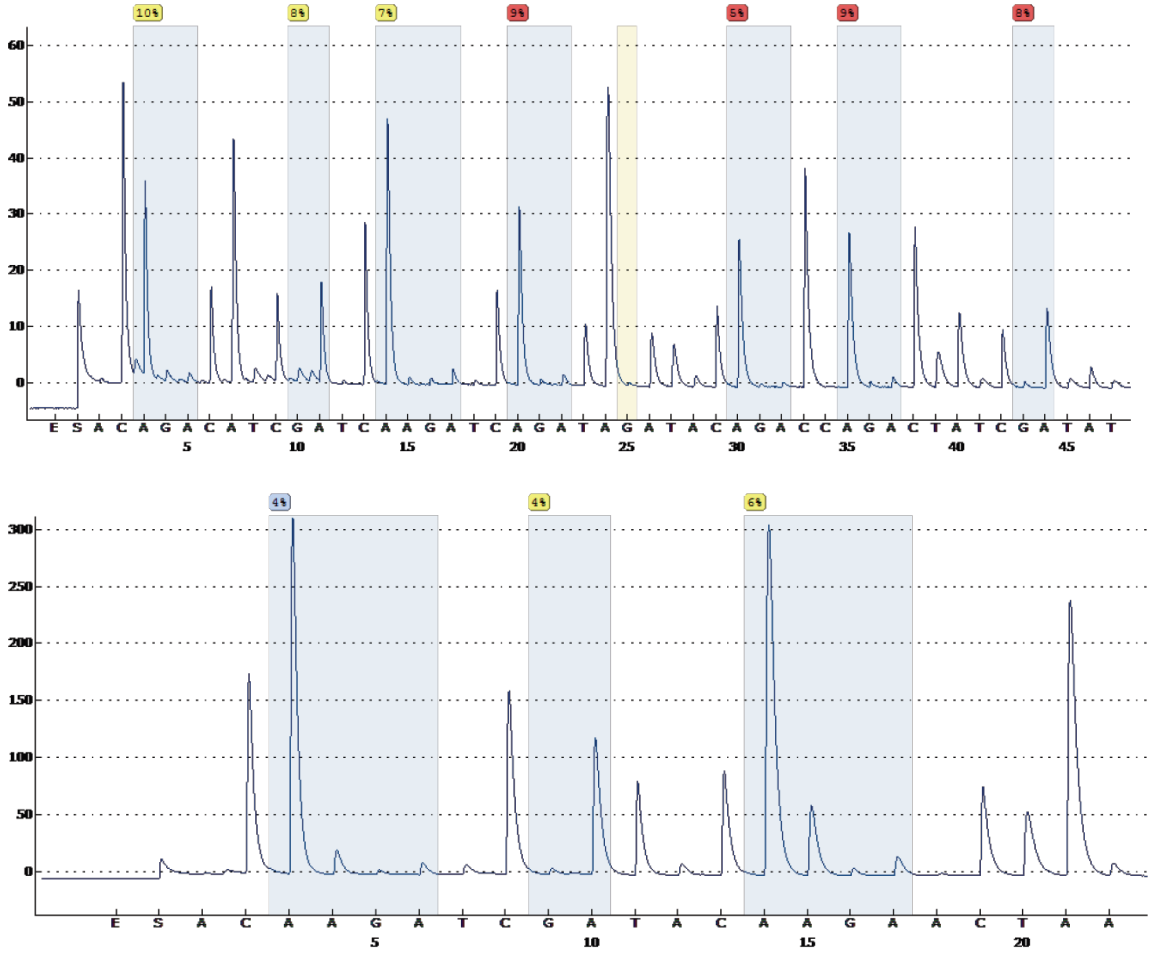

Figure S4 (2) *GALR2* gene in HT29 cell line after inhibition.

Before

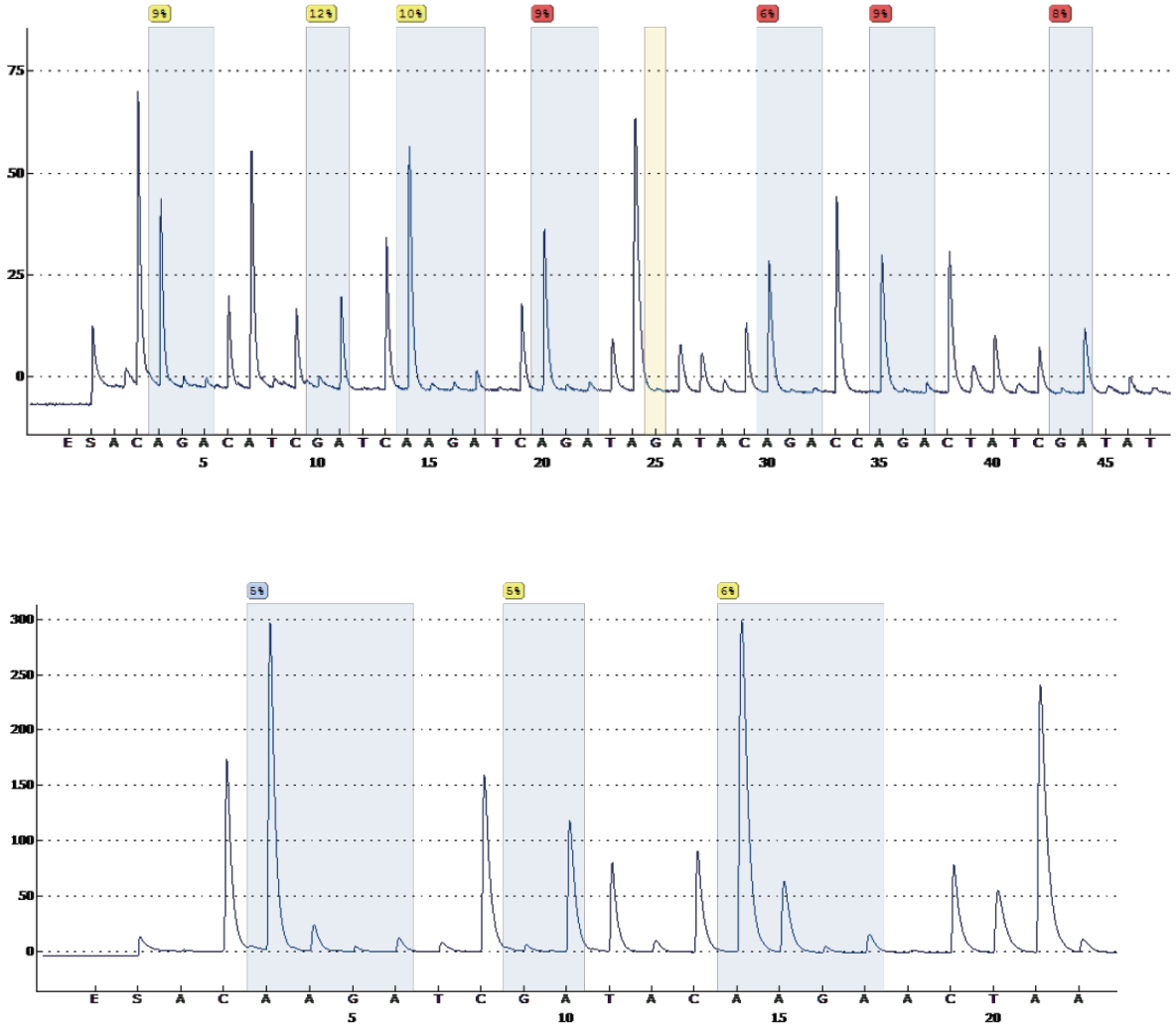

Figure S4 (3) *GALR2* gene in HepG2 cell line before inhibition.

After

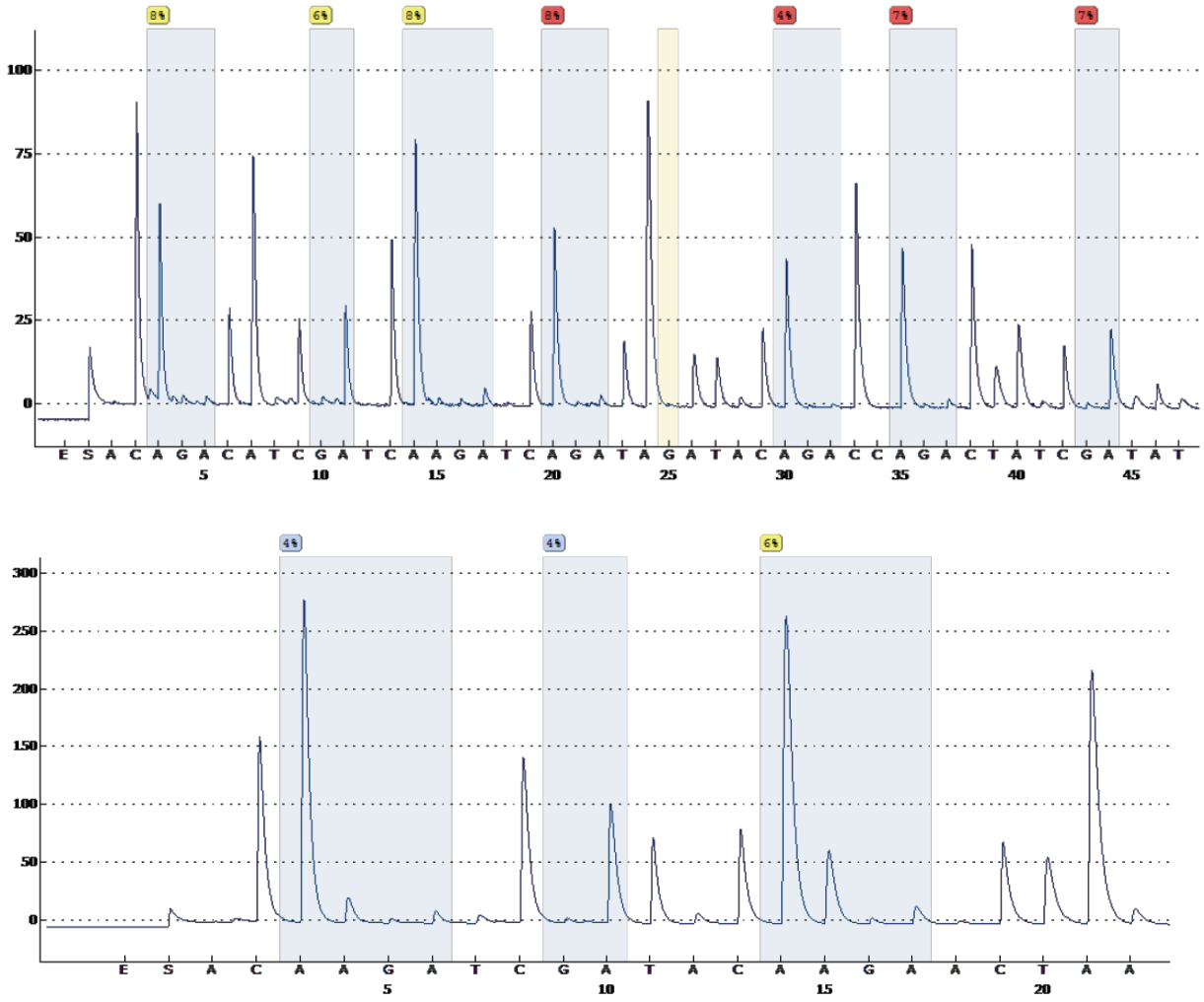

Figure 4S (4) *GALR2* gene in HepG2 cell line after inhibition.

Table S1 MSP primers sequence.

primers	Primer sequence(5'----3')
P16-M	TTTTTTTTCGGTGTTGGC
	CCAACGCTAACTCTAACGAA
P16-U	TTTTTTTTTTTTGGTGTTGGT
	TTACCAACACTAACTCTAACAAA
GALR2-M	TTAGGTTGTAGGGAAGCGTC
	CCCGCAATCGAAAATTCT
GALR2-U	TTGTTAGGTTGTAGGGAAGTGTT
	ACCCCCACAATCAAAAATTCT

Table S2. MSP PCR parameters for the first-round PCR:

	temperature	time
1	95 ℃	10 min
35	95 ℃	30 s
	58 ℃	30 s
	72 ℃	1 min
1	72 ℃	5 min

Table S3. MSP PCR parameters for the second-round PCR：

	temperature	time
1	95 ℃	10 min
35	95 ℃	30 s
	55 ℃	30 s
	72 ℃	1 min
1	72 ℃	5 min