

Electronic Supplementary Information

Highly Sensitive DNA Methylation Analysis by Surface-enhanced Raman Scattering (SERS) via Ligase Chain Reaction

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Experimental Section:

Reagents. 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), 4-mercaptobenzoic acid (MBA), HAuCl₄, sodium citrate dehydrate, sodium borohydride and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma/Aldrich/Fluka. DNA oligonucleotides were purchased from IDT. 4-Mercapto-3-nitro benzoic acid (MNBA) was prepared by breaking the disulfide bond in DTNB with NaBH₄ as reducing agent before usage.

Preparation of SERS nanotags. Gold nanoparticles (AuNPs) with the diameter of 60 nm were synthesized by citrate reduction of HAuCl₄.¹ SERS nanotags were made by modifying AuNPs with Raman reporters and DNA oligonucleotides according to previous reports with slight modification.²⁻³ Briefly, 1.5 mL as prepared AuNPs were concentrated into 1 mL and mixed with 10 μL 50 μM thiolated DNA oligonucleotides (IDT) at RT for 12 hours. Thiolated DNA was first activated with TCEP prior to use as recommended by the manufacturer. Next, 100 μL 1 mM Raman reporters (MNBA and MBA) were then added the gold nanoparticles and incubated at RT for several more hours. Then 0.6 M NaCl in 1 mM PBST was used to age the SERS nanotags at RT for 12 hours. After the salt aging step, stable oligonucleotide-modified SERS nanotags can be obtained and stored at 4 degrees for several weeks. Finally, SERS nanotags were centrifuged and re-dispersed into 10 mM PBS solution prior to use on the gold assay.

Ligase chain reaction (LCR) protocol. LCR was carried out as described previously.⁴⁻⁶ Briefly, 20 μL reaction mix (DNA Tag ligase kit, NEB) consisting of 2.0 μL 10x reaction

buffer, 100 nM of each DNA probes, 2.0 units of DNA Ligase, 50 ng salmon sperm DNA as blocking agent and 1 μ L of initial DNA target was used for each LCR reaction. All the sequences of DNA oligonucleotides used are listed in Table S1. The thermocycling used for LCR reactions was 95°C for 3min, 12 cycles of 95°C for 30s followed by 60°C for 2 min. 5 μ L of the LCR reaction was then analyzed by standard ethidium bromide gel electrophoresis to verify amplification (Figure S1).

Table S1. Sequences of DNA oligonucleotides used in 5 to 3 orientation. DNA base being interrogated is highlighted in underlined font. Modifications are as indicated. HS-: Thiol, PO4-: 5'Phosphate.

Oligos	5'-Sequence-3'
Capture probe	HS-AGTTGTGCAGGTGGT
SERS detection probe 1	HS-CAGATCGTCATGTTC
SERS detection probe 2	HS-TCTGCACCAATGTAC
P1	CCCCTCGACGCACCTAACC <u>G</u>
P2	PO4-CCCTACCCGCCTACCTAACCACCACCTGCACAAC
P3	PO4-GGTTAGGTGCGTCGAGGGGGAACATGACGATCTG
P4	GGTTAGGTAGGCGGGTAGGG <u>C</u>
P5	AAACACCCCTCAACACACCTAACC <u>A</u>
P6	PO4-CCCTACCCACCTACCTAACCACCACCTGCACAAC
P7	PO4- GGTTAGGTGTGTTGAGGGGTGTTtGTACATTGGTGCAGA
P8	GGTTAGGTAGGTGGGTAGGG <u>T</u>
Methylated target	GGGATTCGGTTAGGTAGGCGGGTAGGG <u>C</u> GGTTAGGTGCG TCGAGGGGCGTTTTTA
Un-methylated target	GGGATTCGGTTAGGTAGGCGGGTAGGG <u>T</u> GGTTAGGTGCG TCGAGGGGCGTTTTTA

Gold Assay. Capture DNA oligonucleotides were activated by TCEP at RT for 30 min. Then 70 μ L 2.5 μ M DNA oligonucleotides were spotted onto the gold surface for the binding of the capture DNA on the gold surface by Au-S bond at RT for 6 hours in a humid environment. The surface was then blocked by 6-mercaptohexanol overnight. The surface was washed by nanopore water and hybridized with the LCR product at RT for 2 hours. The surface was

washed again and hybridized with the corresponding SERS nanotags at RT for another 2 hours. After the reaction, the gold surface was washed for several times prior to SERS detection.

Bisulfite PCR and sequencing. Genomic DNA was extracted from MDA-MB-231, MDA-MB-468 and HCC1937 cell lines (ATCC) using the DNeasy Blood and Tissue DNA extraction kit (Qiagen) following the manufacturer's protocol. Bisulfite conversion was done using the MethylEasy™ Exceed kit (Human Genetic Signatures) as recommended by the manufacturer. Finally the miR200b P2 promoter sequence was PCR amplified from all three cell lines using protocols previously described.⁷ Briefly, PCR was performed in a total volume of 20 µl containing: 1x Buffer, 4 mM MgCl, 200 µM of each dNTP, 200 nM of each primer, 0.5U HotStarTaq polymerase (Qiagen) and 2ng of bisulphite modified template. The amplification conditions consisted of 3 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 15 sec at 60°C and 30 sec at 72°C. Primer sequences (PCR Fwd and PCR Rev) are given in Table S1. The derived 262bp PCR products were then outsourced to the Australian Genome Research Facility (AGRF, Brisbane Node) for NGS on the 454-platform. For LCR experiments, PCR reactions were first diluted 1 in 200 dilution in water and 1 µL was then use as input in LCR.

Table S2. PCR primer sequences

PCR Fwd	GTCGGGCGTTTTATTTTATTTTAGTT
PCR Rev	AAAACCGCCCCAACAAAAA

Methylation Data Analysis: The methylation calibration plot was generated by using synthetic targets of various known methylation levels in a duplexed LCR reaction which simultaneously amplified both methylated and unmethylated DNA targets. For breast cancer cell line experiments, methylation events (CpG12 of the miR200b P2 promoter) were amplified by LCR using a 1 in 100 dilution of bisulfite PCR samples generated as described above. To estimate the degree of methylation, the general formula below was used:

$$\% \text{ Methylation} = \text{Methylated DNA} / \text{Total DNA}$$

$$= \text{CpG}_M / (\text{CpG}_M + \text{CpG}_U)$$

$$= I_C / (I_C + I_T)$$

$$= I_{MNBA} / (I_{MNBA} + I_{MBA})$$

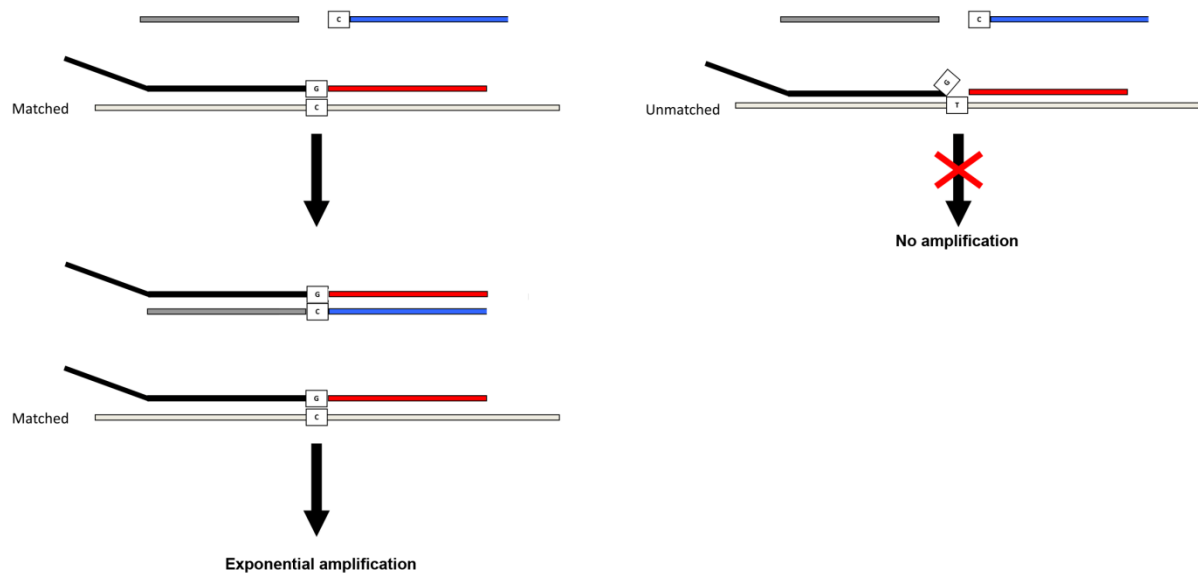
where M and U are methylated and unmethylated respectively; I is the Raman signal intensity; C and T are the respective LCR reactions; MNBA and MBA the respective SERS labels.

Instruments. Gold nanoparticles (AuNPs) were characterized by TEM (JEOL-2100) and UV-vis extinction spectroscopy (Nanodrop 1000, Thermo Scientific). Scanning electron microscopy (SEM) (Philips XL30) was utilized for characterization of the binding of SERS nanotags on the gold surface. SERS spectra were recorded with a portable IM-52 Raman Microscopy. The 785 nm line was used for excitation of Raman scattering. SERS spectra were obtained at 0.1 sec integration with laser power (70 mW) by with a long distance objective (40 x, NA=0.60).

References

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Supporting Figures



Scheme S1. Ligase chain reaction. Perfectly matched sequences allowed for the black and red probes to anneal and ligate whereas no ligation can occur in the presence of an unmatched sequence. Ligated black/red DNA can then serve as a template for the blue and grey probes, and ligated blue/grey serve as templates for subsequent black and red probe reaction, resulting in exponential amplification.



Figure S1. Image of DNA agarose gel electrophoresis of LCR reaction with a no template control (NoT), 5 pM of unmatched template (UM), 5 pM of matched template (M) and 50 pM of unmatched template (UM) stained with ethidium bromide.

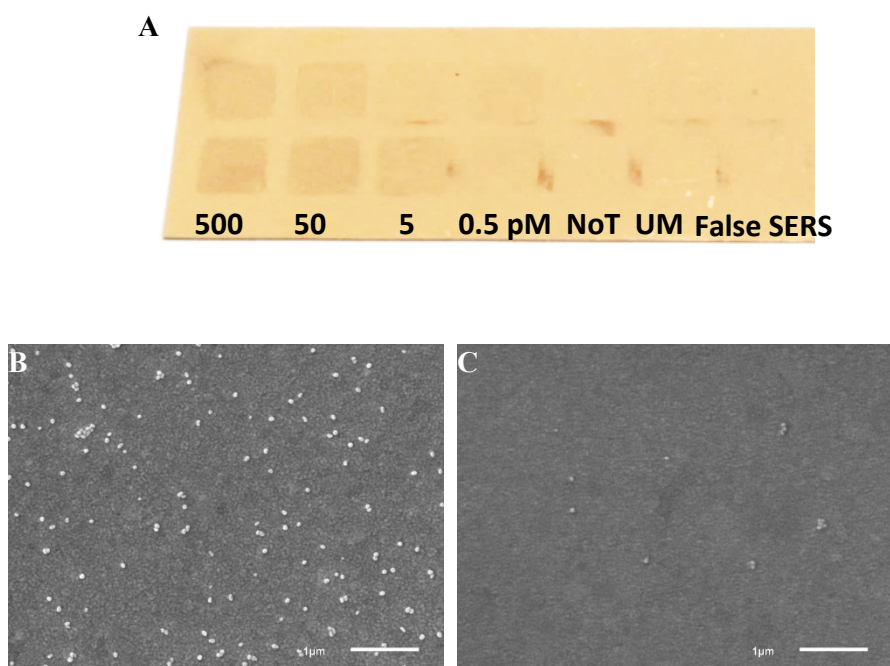


Figure S2. Optical image of the gold assay with different concentration of LCR target and the negative control including no-template (NoT), un-matched primer (UM) and false SERS nanotags (A), SEM images of gold surface with LCR target at 5 pM (B) and with NoT as control (C).

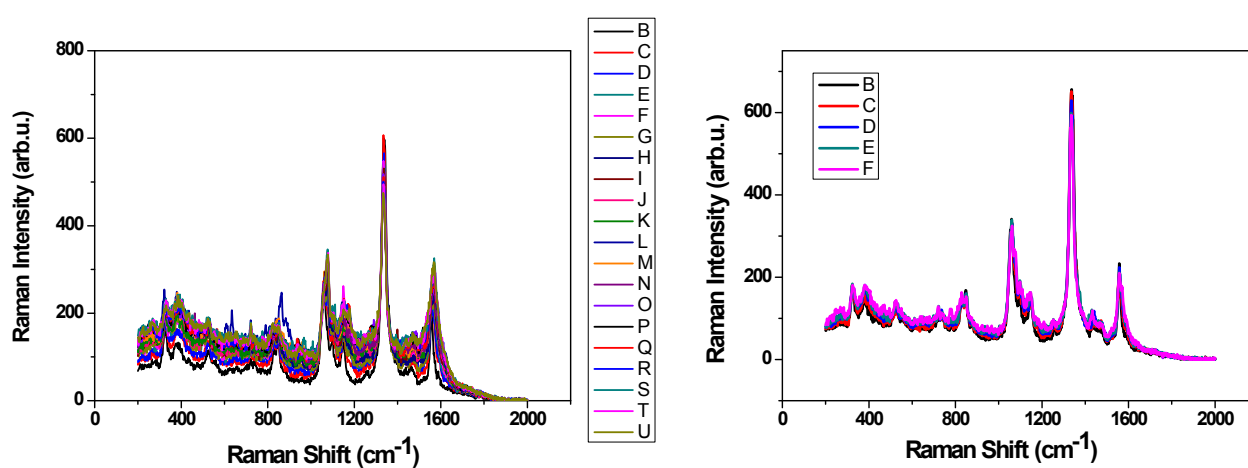


Figure S3. SERS spectra collected from 20 different points (A) with the average SERS spectra (B) in 5 independent tests at each sample (0.5 pM in this case).

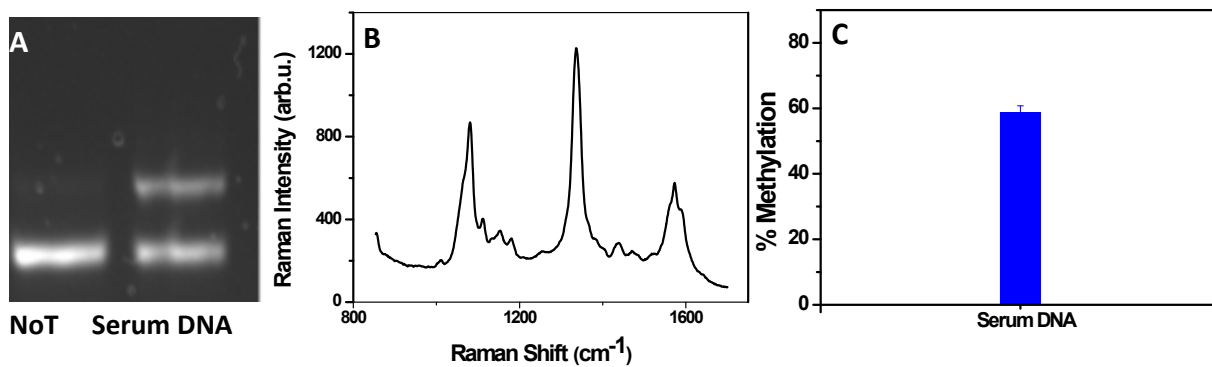


Figure S4. Image of DNA agarose gel electrophoresis of LCR reaction with a no template control (NoT) and a serum DNA sample (A), corresponding SERS spectra (B) and estimated methylation level (C).