Supplementary Information

Ultrasensitive Gastric Cancer Circulating Tumor Cellular CLDN18.2 RNA Detection Based on a Molecular Beacon

(short title: Detect *CLDN 18.2* RNA in GC CTCs by Molecular Beacon)

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Materials and Methods

Design of *CLDN 18.2* molecular beacon

CLDN 18.2 MB is an oligomeric deoxyribonucleotide labeled with Cy3 reporter dye (exciting: 550nm and emitting: 570nm) at the 5' end and a BHQ2 quencher at the 3' end: 5' Cy3-mCmGmUmA*mU*mG*mC*mC*mC*mG*mC*mA*mA*mU*mC*mC*mC*mA*mA*mU*mC*mA*mG*mU*mUm AmCmG-BHQ2 3'. Italic sequence is the interactive (loop) region. Underline sequence is stem region. M represents 2'-O-methyl RNA modification. * represents phosphorothioate (PS) linkage modification. MB hybridization was analyzed by BLAST in NCBI oligoblast. The molecular folding was analyzed by Srna program (http://sfold.wadsworth.org/cgi-bin/srna.pl) and viewed minimum free energy (MFE) structure diagram for original sequence. All oligonucleotide probes and targets were synthesized and purified from GENEWIZ Technologies, Inc. (Suzhou, China). *CLDN 18.2* plasmid was synthesized by Sangon Biotech Co(Shanghai).

Target oligonucleotide synthesis

CLDN18.1 RNA target: AAGUGGUGGCGUUCCUCCUGUCC.

CLDN 18.2 RNA target: GUUUC*ACUGAUUGGGAUUGCGGGCA*UCA.

CLDN 18.2 ssDNA target: GGTTTC*ACTGATTGGGATTGCGGGCA*TCATTG.

CLDN 18.2 primer F: CAGGACGATGCCTACGA.

CLDN 18.2 primer R: TTTCACTGATTGGGATTGC.

CLDN 18.2 plasmid: PET-GFP plasmid with *CLDN 18.2* whole open reading frame region.

Performance test for molecular beacon hybridized to target oligonucleotide

To evaluate the kinetics of MB and the targeting oligonucleotide hybridization, MB and binding buffer were mixed in cuvette and measured fluorescence intensity immediately. Fluorescence intensity measurement required 550nm exciting light and 570nm emitting light for 600 s. Hybridization kinetics measurements were performed on Spectro fluorophotometer (shimadzu RF-5301PC) at room temperature.

To evaluate the selectivity of MB hybridize to target. MB was incubated in 0-80nM *CDLN 18.2* RNA/*CLDN 18.2* single-stain-DNA/ plasmid/ CLDN 18.1 RNA and fluorescence intensity was measured by PerkinElmer Enspire Multiscan Spectrum. Next, thermodynamics was analyzed when MB bind to the target. MB and CLDN18.1RNA/18.2 RNA /binding buffer mixed and melting curve program ran (95 ˚C 15 s, 30 ˚C 60 s, 32-80 ˚C ramp rates 1% and collect data on-ramp) directly in Mastercycler ep realplex (Eppendorf). Furthermore,

CLDN18.1/18.2 RNA and MB were incubated in 25/30/35/40/45/50 ˚C and fluorescence intensity was measured.

Cell lines and Cell Culture

SNU-5, MKN-45, KATO-Ⅲ, 293T, GES-1 and AGS cells were provided by Peking University Cancer Hospital (Beijing, China). MKN-45 cells were cultured in RPMI Medium supplemented with 20% FBS (Hyclone) at 37 ˚C, 5% CO2, and 90% relative humidity. Other cells were cultured in Roswell Park Memorial Institute Medium (RPMI, Gibco) supplemented with 10% FBS.

PMA (Beyotime Biotechnology, China) was diluted by RPMI medium supplemented with 10 % FBS at a final concentration of 100 ng/mL and both cells were incubated in PMA. Total RNA was extracted by TRIzol reagent kit (Tiangen, China) and *CLDN 18.2* RNA expression was measured by RT-PCR (Takara, Japan) on Arktik Thermal Cycler (Thermo Scientific, USA) set program(Pre-heat for 95˚C 15s, 35 cycles amplified for 95˚C 15s, 54˚C 20s, 72˚C 20s, and for a final extension at 72˚C 10min).

***CLDN 18.2* MB evaluations at cellular level**

Claudin 18.2 protein was measured by western blot which was performed on Mini-Protean Tetra Electrophoresis System (Bio-Rad, USA). Total protein was extracted by RIPA reagent (Beyotime Biotechnology) and 10% polyacrylamide gel electrophoresis (PAGE) gel, anti-Claudin 18.2 antibody (Abcam, clone ID: EPR19202) and anti-GAPDH (Abcam) were incubated. ECL detection were analyzed on ChemiDoc XRS system (Bio-Rad). Cellular level analysis using Zeiss LSM 710 confocal microscope equipped with a plan-apochromat 63×/1.40 oil M27 objectives. Cells were pre-treated by 100 ng/mL PMA/PBS and strained by Hoechst 33342. We used PBS to wash cells for 3 times and anti-*CLDN 18.2* antibody pre-labeled by Alexa Fluor 647 NHS Ester (Thermo Fisher, USA) for 2 hours. After washed 3 times, cells were detected by confocal laser scanning microscopy (CLSM) with different laser transmitters (Cy5 and Hoechst33342 channels).

Cellular delivery method, using Streptolysin O (SLO) reagent, 2 U/mL of SLO was incubated with 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) for 30 min. Then MB and 0.5 U/mL SLO incubated cells for 30 min. At last cells were washed by PBS. SNU-5 and MKN-45 cells pre-treated by PMA, then MB was transfected into cells by SLO delivery method and imaged by confocal microscope. *CLDN 18.2* RNA was transfected into MKN-45 cells by Lipofectamine 2000 (Invitrogen) standard method. In MB and nucleus colocalization analysis, Z-stack with step size of 1 μm was measured by confocal microscope.

FCM experiments were performed on BD FACSAria II Cytometer. Cells were treated by PMA for 0, 4, 8h

for MB performance test. Then MB was transfected into cells by SLO deliver for 20 min. Cells were washed twice with PBS to remove the extracellular beacon and incubated in growth medium for at least 1h. Cells were dissociated with trypsin-EDTA solution (Gibco) and stained in 1 μg/mL Propidium Iodide (Tiangen, China) for 10 min.

***CLDN 18.2* expression measurement in CTCs**

15 patients with metastatic late-stage gastric carcinoma from the department of gastrointestinal, Peking University Cancer Hospital were included in this study. Peripheral blood (2 mL) was obtained in the collection tube containing EDTA buffer (Nanopep Biotech, China). Paraffin embedded Informed consent was obtained from all patients in this study. Investigations were performed after approval of the Peking University Cancer Hospital ethical committee. Standard Pep@MNPs kit was operated and MB was applied to this system. 10 μL of Pep@MNPs were added into 2.0 ml of patient's peripheral blood, incubated at 37 ˚C in table concentrator at 100 rpm for 1 h. The Pep@MNPs captured CTCs and fixed. Cells were isolated under a magnetic field for 30 min and were labeled with fluorescent antibodies/MB which were specific for CD45, pan-cytokeratin and *CLDN 18.2* MB and then stained with Hoechst 33342. Fluorescence microscope experiments were performed on an Olympus IX 71 motorized inverted fluorescence microscope equipped with a 20× and 40×LUCPlanFLN objective lens. Images were acquired using Micro-Manager 1.4 software set DAPI/GFP/PE/RFP channel.

At last, we collected 8 patients of 63 CTCs and data analyzed, 5 outlier data were deleted and 58 CTCs' *CLDN 18.2* expression data were input to cluster using *Agglomerative Clustering* by Python 3.7 tool software. According to the distance between clusters, the parameter was set linkage as "ward" for a string and n clusters as 3 for clustering numbers. All 15 patients were enrolled for CLDN 18.2 detection and 10 patients' tissues were obtained and analyzed by immunohistochemistry(IHC).

Data analysis and statistics

Fluorescence intensity was analyzed by ImageJ software. Fluorescence intensity analysis and statistics in flow cytometry were processed by FlowJo_V10 software. Data were analyzed by SPSS 19.0 for significant difference evaluation and OriginPro 9.5 for drawing and signal to noise ratio calculation. One-way ANOVA was used for statistics. $P<0.05$ were regarded as statistically significant.

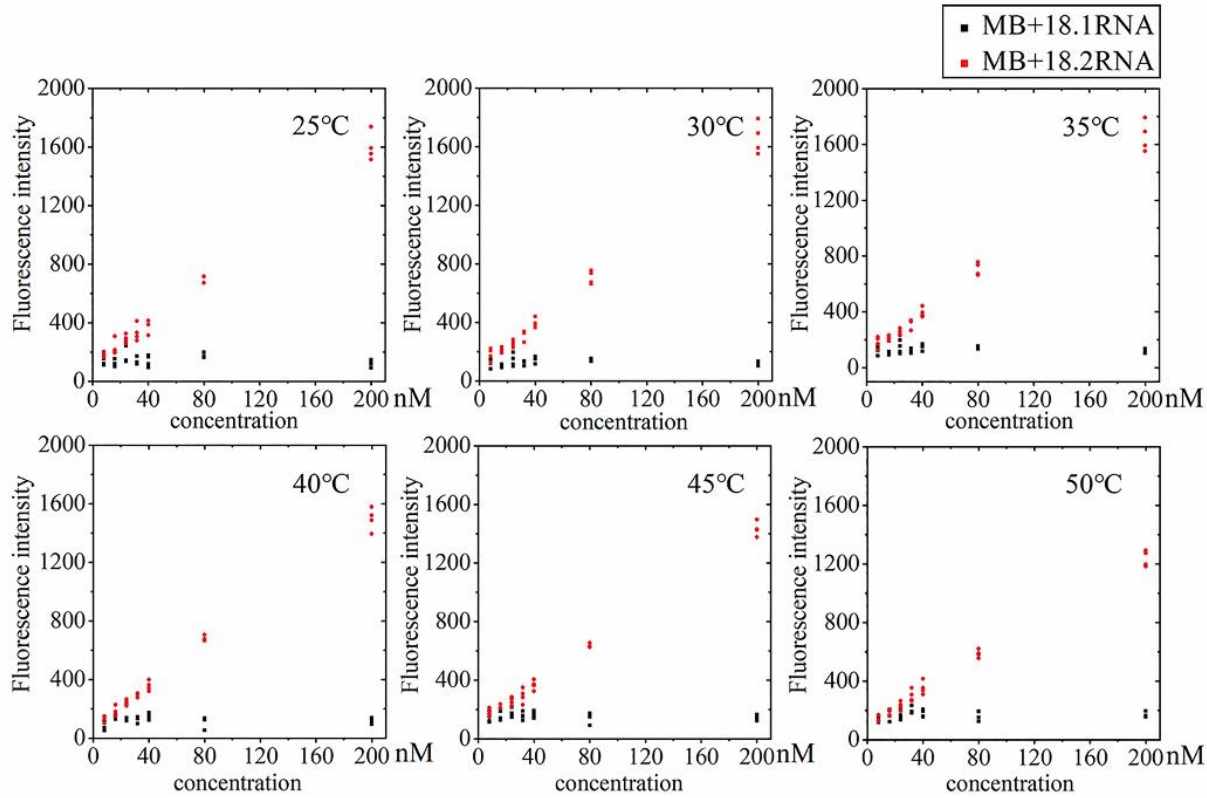

Figure S1 fluorescence intensity analyze Hybridize to CLDN 18.1/18.2 RNA in 25-50℃. Analyze MB fluorescence activated by different concentration of CLDN 18.2 and CLDN 18.1 RNA in 25-50℃ 100nM MB and the concentration of RNA range from 5 to 200 nM incubated for 1 hour. The fluorescence was measured by PerkinElmer Enspire Multiscan Spectrum with exciting light 550 nm and emitting light 570 nm. High temperature would partly decrease the fluorescence generated by MB + CLDN18.2, however the MB+ CDLN 18.2 fluorescence intensity were still higher than MB+CLDn18.1 RNA.

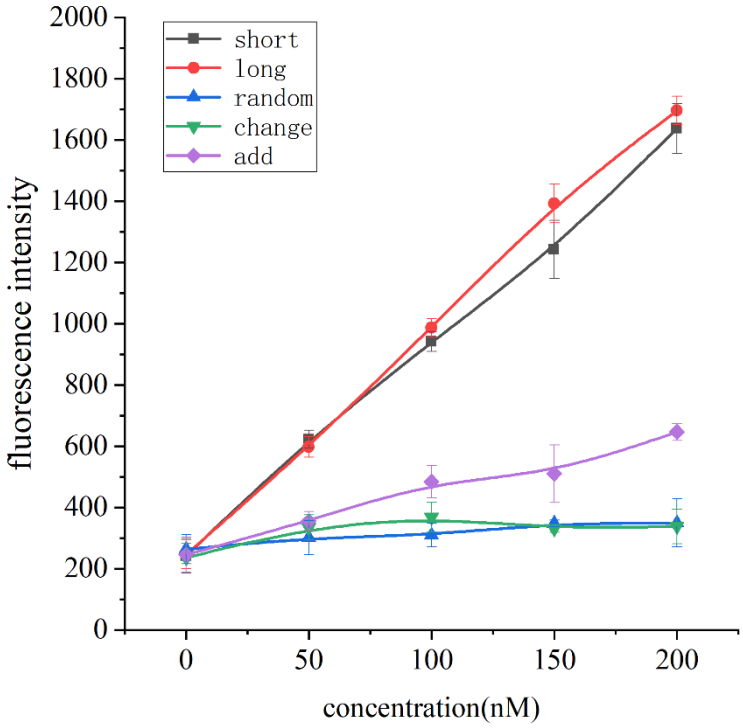

Figure S2 Specificity test for CLDN 18.2 MB

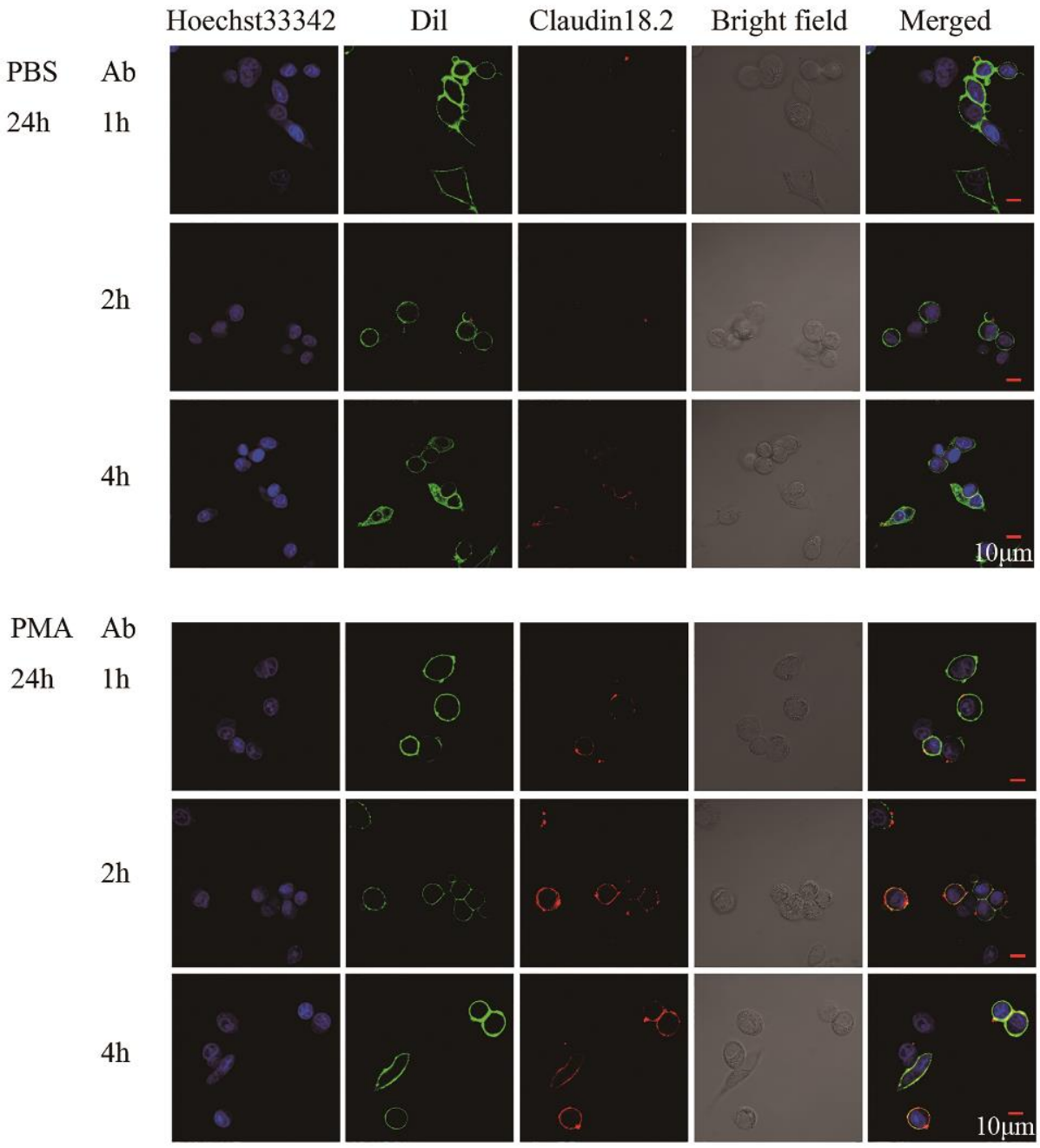

Figure S3 PBS/PMA induced MKN-45 cells and Claudin18.2 protein was measured. PBS/ PMA treated MKN-45 cells for 24 hours, then Dil stain cells for 15 min in room temperature, PBS wash out for 3 times and Claudin 18.2-647(1:100) incubated for 1,2,4 hour. At last Hoechst33342 stained cells for 5 min and DAPI/DIL/Cy5 channels were analyzed by Zeiss LSM 710 confocal microscope equipped with a plan-apochromat 63×/1.40 oil M27 objectives.

The result in FigS3 showed that the CLDN18.2 antibody did not interact with PBS/PMA induced MKN-45 cell in first 1hour, indicated that this antibody could be a little inconvenient applied for CTCs assay because

of this time-wasted and high cost (100 dollars/reaction).

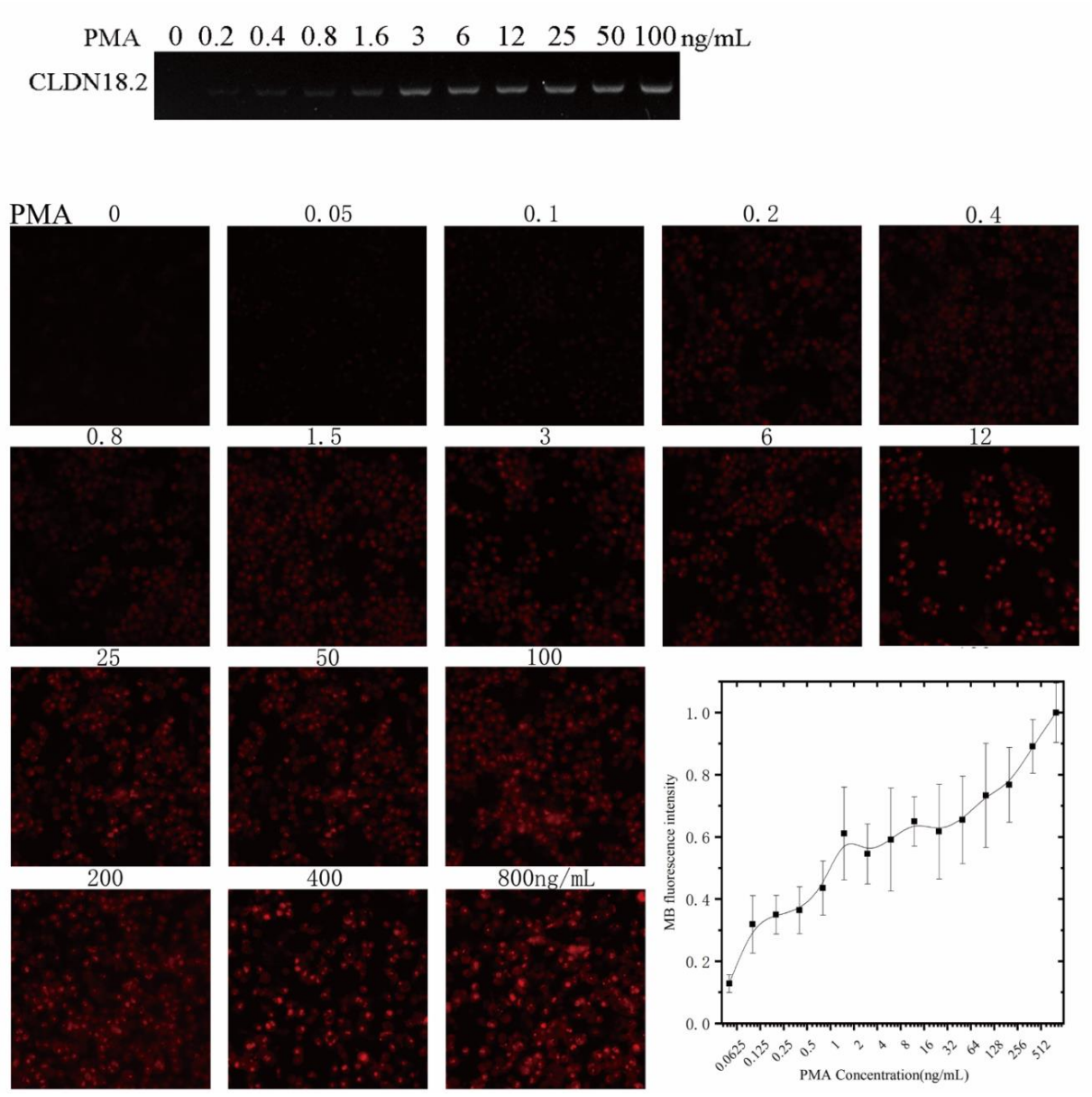

Figure S4 To investigate limit of detection (LOD) of MB in fluorescence microscope in which Pep@MNPs system was operated and analyzed, 14 times half dilution of PMA from 0.05-800 ng/mL were treated MKN-45 cells for 24 hours and only RFP channel was detected and 20 random cells were analyzed with fluorescence intensity in highest concentration of PMA was normalized as unit 1. The trend of fluorescence intensity was decrease with PMA concentration descend, indicating that MB had advantage in classifying

different level of *CLDN18.2* RNA expression in cellular level detection and even 0.2 ng/mL PMA induced MKN-45 cellular CLDN 18.2 could be detected by MB.

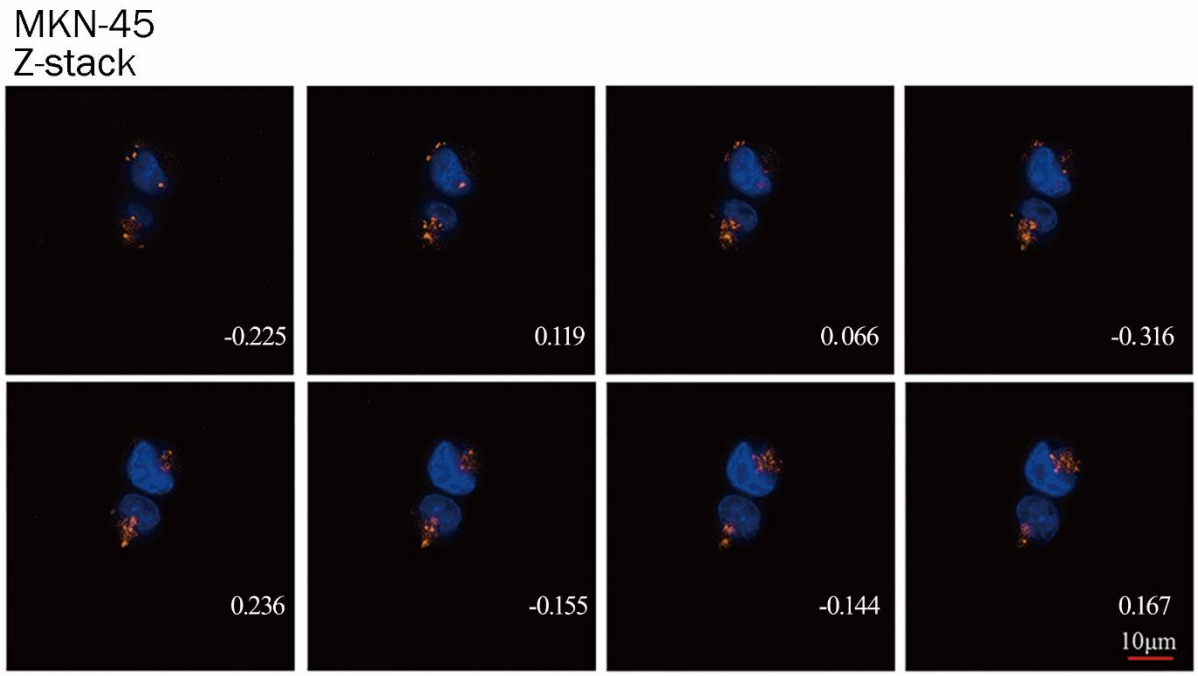

Figure S5 Pearson correlation coefficient analyze in cell nucleus and Molecular beacon. Z-stack analyze MKN-45 cells by Confocal with 1 μm/step, Pearson correlation coefficient analyze PMA induced MKN-45 cells, Pearson correlation coefficient was lower than 0.4 showed that MB and nucleus localization at different region.

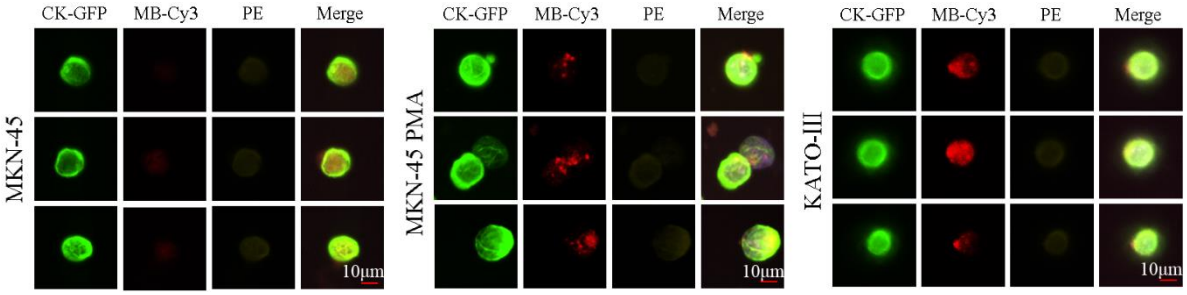

Figure S6 MB was detected in tumor by CTC standard method

Molecular beacon detect assay was applied to CTCs assay in fluorescence microscope, we select MKN-45, PMA induced MKN-45 and KATO-Ⅲ cells for fluorescence microscope detection. With Pep@MNPs standard procedure, MKN-45 cells MB showed negative while MKN-45 PMA and KATO-Ⅲ showed positive.

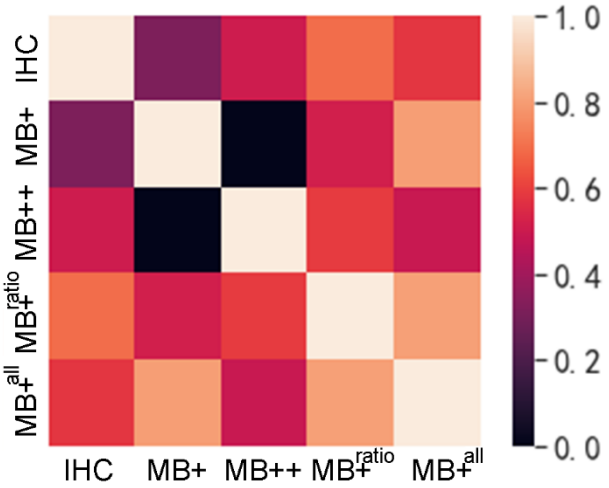

Figure S7 Correlation analysis between IHC and MB in 10 patients

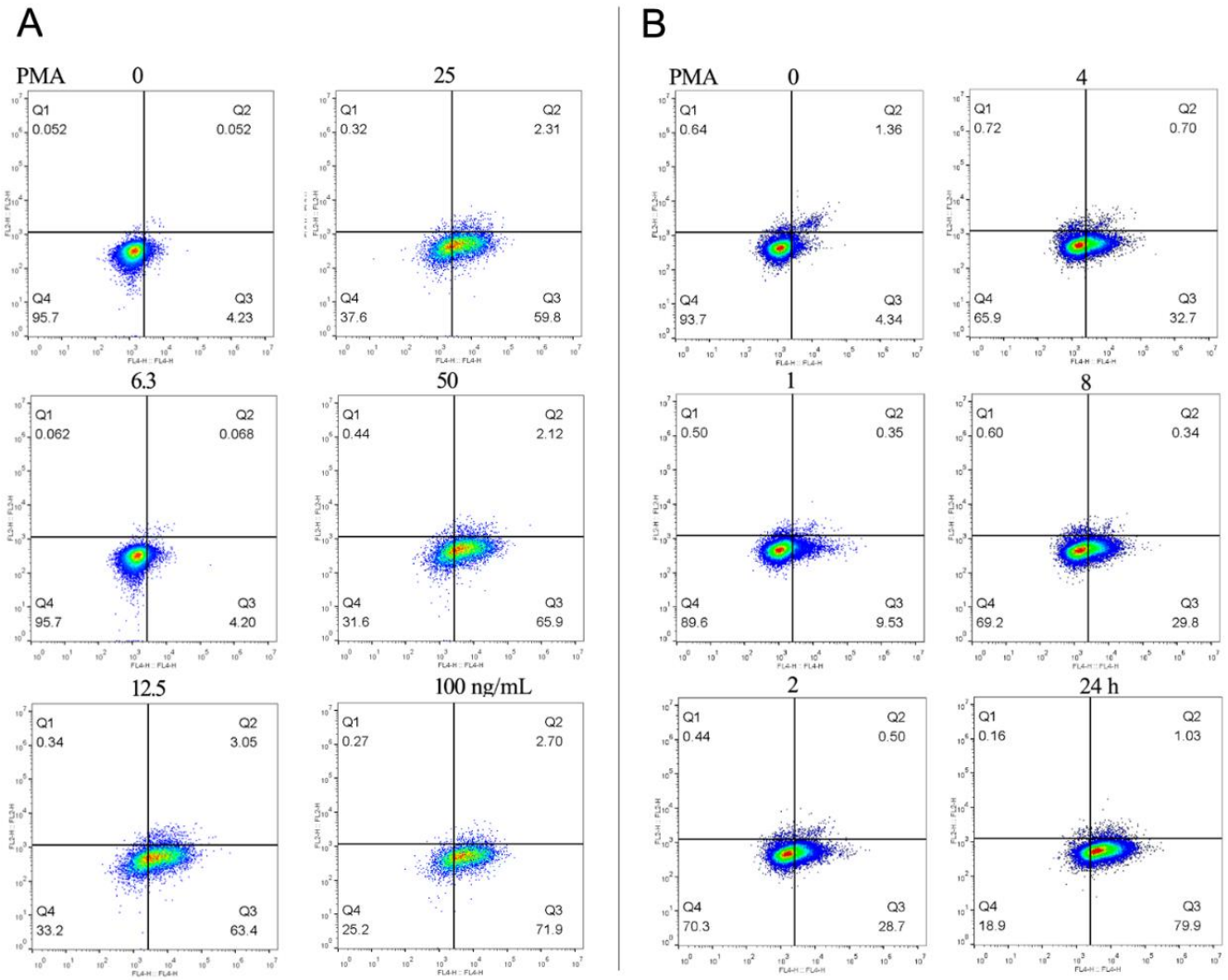

Figure S8 FCM experiments in PMA/PBS induced living cells. We labelled the bare MB for Cy5 and BHQ3, considering Cy3 has cross color to PI and Cy5 has not cross color to PI, Firstly, MB was transfected into cells, then PI treated for cells for 15min. the results showed that with PMA concentration(A) or treating time(B)'s increase, MB's fluorescence intensity is increase but PI invariant.to a low level., indicating that MB could detected target RNA in living cells.

Table S1 fluorescence intensity analysis for CTCs

Name	FITC	$FITC_b$	ΔFITC	PE	PE_b	ΔPE	MB	MB_b	ΔMB
MB-	825	126	699	226	114	112	385	139	246
MB+	697	120	577	417	113	304	1005	130	875
MB++	679	127	552	489	117	372	1245	143	1103

Statistics data and MB-, MB+ and MB++ have Significance differentiation between each other.

The fluorescence of CTC MB was analyzed by Image J. As a result, the fluorescence in CTCs MB could be classified as MB-, MB+ and MB++ with cutoff value 450 and 900. However, the fact of ΔPE is a little higher than MB- need to special considerations about cross color in different fluorescein.

At last, correlation analyze between IHC, MB+ number, MB++ number, MB + total number and MB+ ratio in 10 patients.

Table S2 Classification of molecular beacon level in 15 patients

Patients	IHC	*CLDN18.2*$^{MB-}$	*CLDN18.2*$^{MB+}$	*CLDN18.2*$^{MB++}$	CTCs Sum	MB+ ratio
01	/	1	2	2	5	80%
02	-	2	0	0	2	0%
03	++	0	2	5	7	100%
04	+	0	10	0	10	100%
05	-	4	0	0	4	0%
06	/	0	9	0	9	100%
07	++	11	3	0	14	21%
08	/	0	7	7	14	100%
09	++	4	1	0	5	20%
10	+++	0	23	2	25	100%
11	0	3	0	0	3	0%
12	++	7	1	0	8	14%
13	++	0	6	2	8	100%
14	+++	0	1	4	5	100%
15	+++	2	2	1	5	60%