

Electronic Supplementary Information for Chemical Communications

Bioimaging of multiple piRNAs in a single breast cancer cell using molecular beacons

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Experimental procedures

Cell culture and transfection

Normal cell line, CHO and breast cancer cell lines, MCF-7, BT474, MDA-MB-231, and MDA-MB-453 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All about cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Gibco, Gland Island, NY), 50 Units/mL penicillin, 50 µg/mL streptomycin (Gibco) in a 5% CO₂-humidified 37 °C incubator. These cell lines were cultured according to ATCC protocols. The cells were transfected with piR-36026 MB, piR-36743 MB or exogenous piRNA using Lipofectamine 2000 (Invitrogen, Grand Island, NY).

Design of piRNA MB

Both piR-36026 MB and piR-36743 MB, and piR-126541 MB used as a negative control were designed to form a partially double stranded oligonucleotide. The reporter oligo containing the piR-36026 or piR-36743 or piR-126541 binding sequence, which is a reverse complimentary sequence against mature piR-36026 or piR-36743, was phosphorylated at 3' end and was as follows: piR-36026 MB, 5'-Cy5.5-CCGCGAGAGTGCTGACCATTACACC ATGGGGCC-3'; piR-36743 MB, 5'-Cy3-CCGCGAGGCGAACGTGATGACCACTACACT ACGGAAAC-3'; piR-126541 MB, 5'-Cy3-CCGCGAATCTATCACTCCTGAACTGAGAT CAC-3' and the quencher oligo are as follows: piR-36026 MB, 5'-GCACTCTCGCGG-BHQ2-3'; and piR-36743 MB, 5'-TTCGCCTCGCGG-BHQ1-3'; piR-126541 MB, 5'-ATAGATTCGCGG-BHQ1-3' These oligonucleotides were purchased from Bioneer (Bioneer Corporation, Daejeon, Republic of Korea).

Quenching efficiency of piR-36026 MB and piR-36743 MB were determined by 30 pmol of reporter oligo was reacted with different concentrations(0, 15, 30, 60 and 100 pmol) of its quencher oligo in black tube contained annealing buffer (100 mM Tris-HCl, 1 mM MgCl₂) for 1hr at room temperature. To analysis specificity if piR-36026 MB and piR-36743 MB, 50 pmol of piR-36026 MB or piR-36743 MB was reacted with different concentrations (0, 10, 20, 50, 100 and 200 pmol) of its exogenous piRNA in black tube for 1hr at room temperature. To evaluate the stability of piR-36026 MB and piR-36743 MB, 100 pmol of each piR30626-MB, piR-36743 MB were incubated in PBS, FBS, and DMEM culture media at 37 °C for 2 days, then each exogenous piRNA added into piRNA MB incubated PBS, FBS, or DMEM media for additional 1 hr at 37 °C. Cy5.5 fluorescence signal of piR-36026 reporter oligo was measured at excitation of 694 nm and emission of 705 nm and Cy3 fluorescence signal of piR-36743 reporter oligo was measured at excitation of 550 nm and emission of 570 nm using a Varioskan Flash Fluorescencet Microplate Fluoremeter (Thermo Fisher Scientific Ind., Waltham, MA).

RNA Purification, Real-time quantitative RT-PCR

Small RNA was extracted from normal cells (CHO) and breast cancer cells (MCF-7, BT474, MDA-MB-231, MDA-MB-453) using mirVana™ isolation kit (Ambion, Grand Island, NY). 100 ng of small RNA was reverse transcribed using NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen) for poly(a) tailing and cDNA synthesis. cDNAs were diluted to a final concentration of 20 ng per reaction. Real time quantitative RT-PCR was performed using a SYBR Master Mixes (Applied Biosystems, Odessa, Texas, USA) on Step One Plus™ Real-Time PCR Systems (Applied Biosystems) and expression values were

normalized against 5srRNA. The sequence of the primer for piR-36743 amplification was 5'-GTTTCCGTAGTGTAGTGGTCATCACGTTTCGCC-3' (Forward) and 5'-GTTTCCGTAGTGTAGTGGTCATCACGTTTCGCC (Reverse). The sequence of the primer for piR-36026 amplification was 5'-GGCCCCATGGTGTAATGGTCAGCACTC-3' (Forward) and 5'-GGC CCCATGGTGTAATGGTCAGCACTC-3' (Reverse).

Fluorescence measurement of dual signal of piR-36026 MB and piR 36743 MB

1 x 10⁴ cells of normal and breast cancer cell lines were seeded onto a 24 well plate in each well containing 25-mm-diameter cover slip and cultured for 24 hrs at 37 °C. To analysis piR-36026 MB or piR-36743 MB in CHO cells, 50 pmol of piR-36206 MB or piR-36743 MB was co-transfected with various concentration of 0, 0.05, 0.1, and 1 nmol of exogenous piR-36026 or piR-36743 by Lipofectamine 2000 and incubated in a 5% CO₂-humidified incubator for 6 hrs. Exogenous piR-126541 (1 nmol) was used as a negative control. And then, breast cancer cell lines (MCF-7, BT474, MDA-MB-231, and MDA-MB-453 cells) were transfected with (0, 5, 10, 20 and 50 pmol) piR-36260 MB or piR-36743 MB or combined with piR-36026MB and piR-36743 MB using Lipofectamin 2000 and incubated in 37 °C incubator for 6 hrs. Exogenous piR-126541 MB (50 pmol) was used as a negative control. After incubation, cells were washed with PBS twice for 10 min. The cells were fixed with 3.7% formaldehyde (Sigma Aldrich, St. Louis, MO), washed three times with PBS and cover slip was mounting with mounting solution contained 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, CA). Fluorescence signal of piR-36026 MB (Cy5.5; excitation/emission, 694/705 nm) and piR-36743 MB (Cy3; excitation/emission, 550/570 nm), DAPI (excitation/emission, 305/406 nm) were detected using confocal laser scanning microscopy (Carl Zeiss, Weimer, Germany).

The quantitative fluorescence intensities were measured using Varioskan Flash Fluorescent Micrometer. These data were presented as mean \pm SD calculated from triplicate samples and significant differences between samples were assessed using a Student's t-test.

In vivo fluorescence imaging analysis

All experimental animals were housed under specific pathogen free conditions and handled in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of Catholic Kwandong University International St. Mary's Hospital. All the experimental protocols included in this *in vivo* study were reviewed and approved by the Institutional Animal Ethics Committee (Ref No. CKU 03-2017-005). To visualize dual imaging of piR-36026 or piR-36743 *in vivo*, MCF-7 cells were co-treated with piR-36026 MB and piR-36743 MB. MCF-7 cells (1×10^7) transfected with and without piR-36026 MB and piR-36743 were injected into the right thigh and left thigh of male BALB/c nude mice (7 weeks old; n=3 of each group), respectively. The mice were scanned for 4 days using the IVIS spectrum imaging system (Perkin Elmer, Waltham, MA). For the fluorescence images, the mice were anesthetized in an IVIS imaging chamber equipped with a small holder connected to an isoflurane and oxygen tank. Anesthetic gas was administered with 2% isoflurane in O₂ gas at a flow rate 1 L/min. *In vivo* fluorescence imaging was conducted for piR-36026 (excitation/emission, 694/705 nm) and piR-36743 (excitation/emission, 550/570 nm).

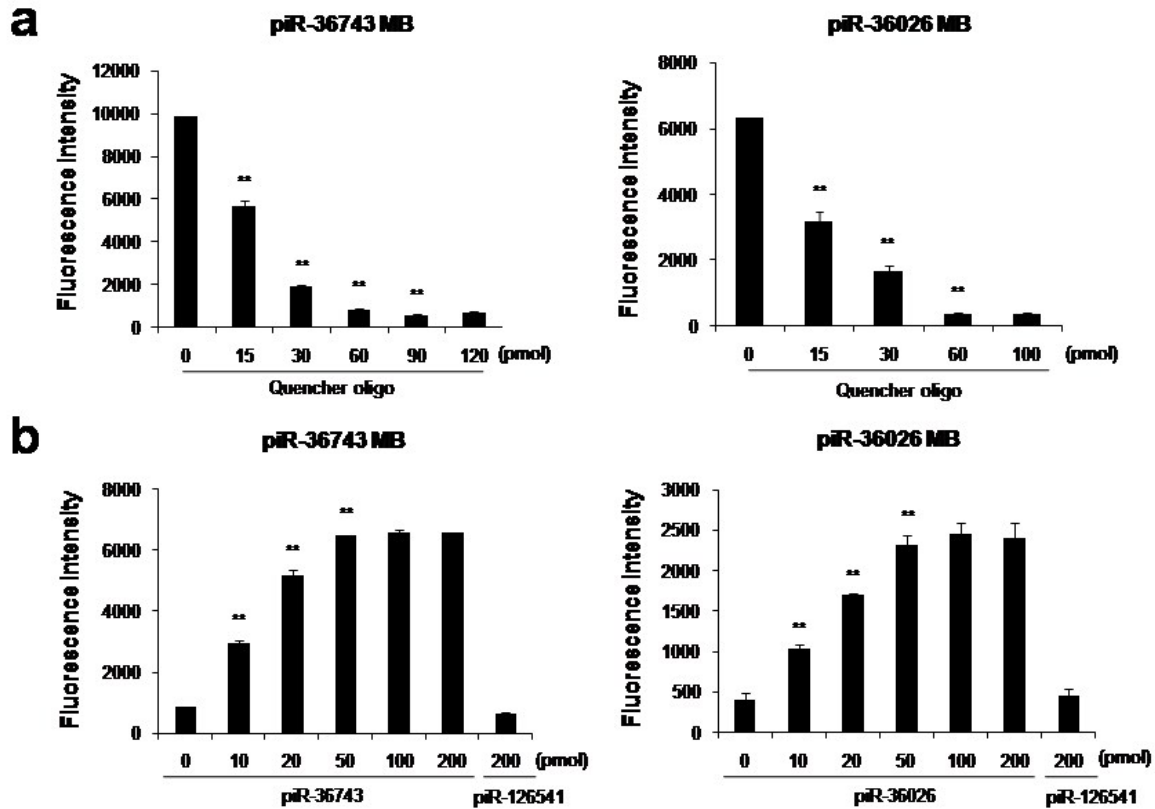


Figure S1. Measuring efficiencies of piR-36743 MB and piR-36026 MB. (a) To see optimal ratio of reporter oligo to quencher oligo for generating piR-36743 MB and piR-36026 MB, different concentrations (0, 15, 30, 60 and 100 or 120 pmol) of the quencher oligo were annealed with each reporter oligo (30 pmol) in a tube. Quantitative fluorescence intensities of Cy3 in piR-36743 reporter oligo and of Cy5.5 in the piR-36026 reporter oligo were measured. (b) The specificity of piR-36743 MB and piR-36026 MB to detect piR-36743 and piR-36026, respectively. Various concentrations (0, 10, 20, 50, 100 and 200 pmol) of exogenous piR-36743 and piR-36026 were incubated in the microtube with 30 pmol of piR-36743 MB and piR-36026 MB. There was no significant change of fluorescence intensity with negative control (exogenous piR-126541, 200 pmol). The data are displayed as means \pm standard deviation of triplicate samples (** $p < 0.005$)

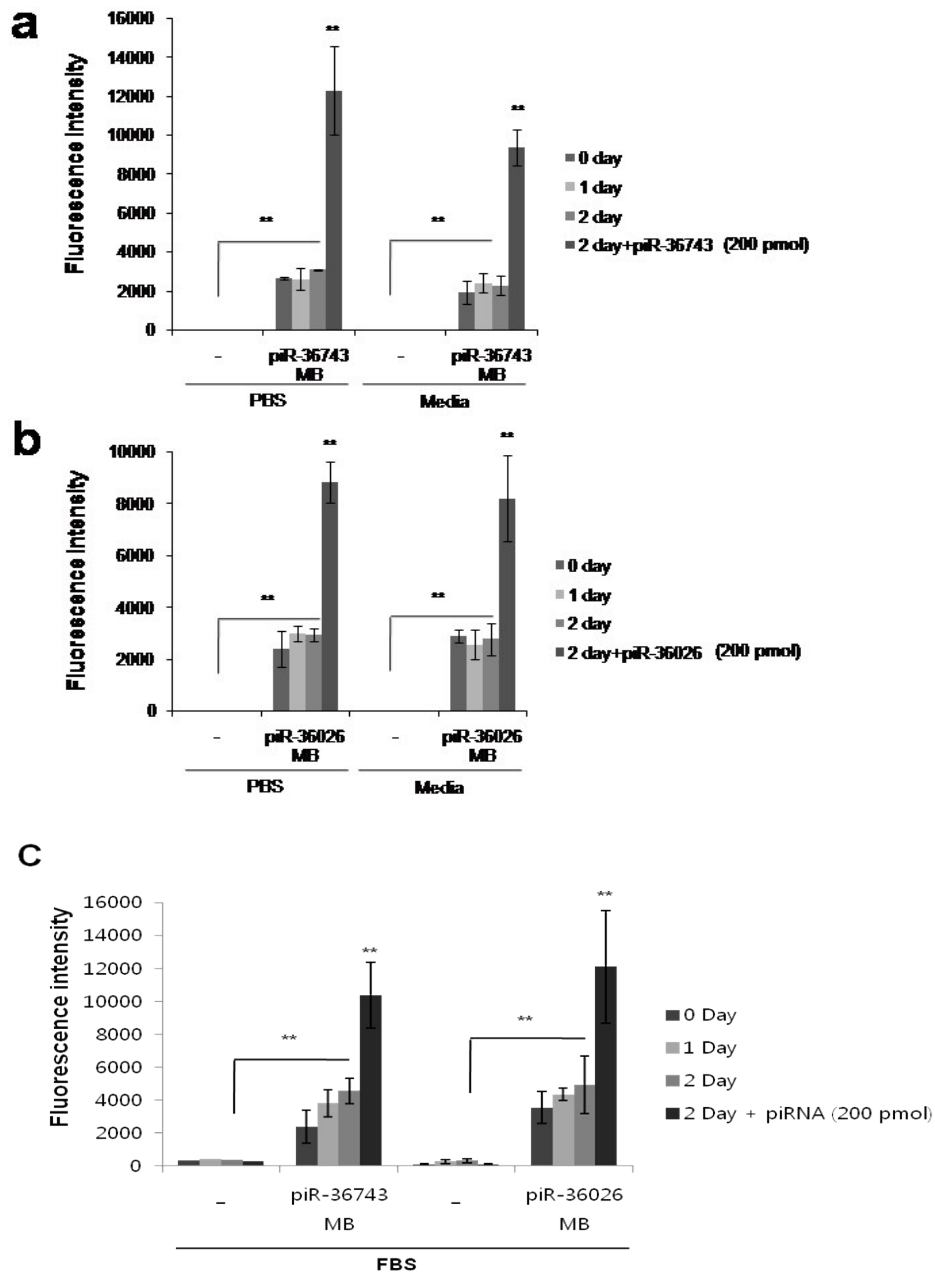


Figure S2. The chemical and functional stabilities of piR-36743 MB and piR-36026 MB. (a) 100 pmol of piR-36743 MB was incubated in PBS or culture media for 2 days with or without exogenous piR-36743 (200 pmol) and fluorescence intensity was measured. (b) 100 pmol of the piR-36026 MB was incubated in PBS or culture media for 2 days with or without exogenous piR-36026 (200 pmol) and fluorescence intensity was measured. (c) 100 pmol of piR-36743 MB or piR-36026 MB was incubated in FBS for 2 days with or without its corresponding exogenous piRNA (200 pmol) and fluorescence intensity was measured. The data are displayed as means \pm standard deviation (** $p < 0.005$).

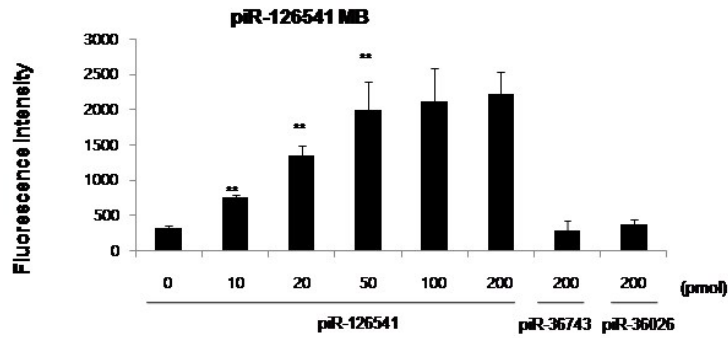


Figure S3. The specificity of piR-126541 MB to detect piR-126541. MCF-7 cell transfected with the piR-126541 MB was co-transfected with various concentrations (0, 10, 20, 50, 100 and 200 pmol) of exogenous piR-126541 or 200 pmol of exogenous piR-36743 or piR-36026 (used as negative control). The data are displayed as means \pm standard deviation of triplicate samples (** $p < 0.01$)

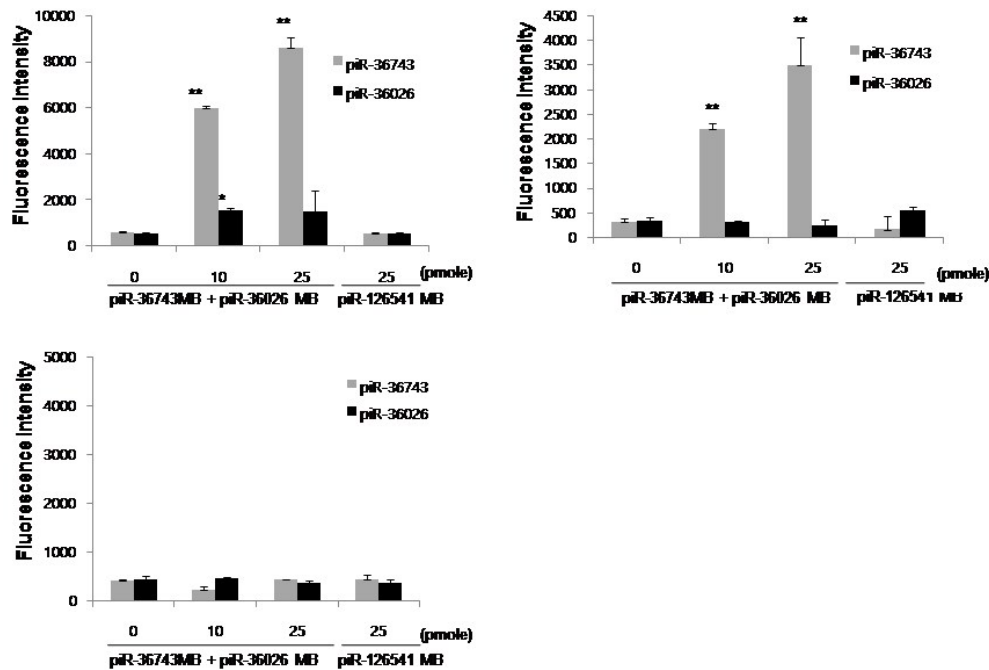


Figure S4. Simultaneous detection of endogenous piR-36743 and piR-36026 in different human breast cancer cells. Both piR-36743 MB and piR-36026 MB were co-transfected into BT-474 cells (Upper left), MDA-MB-231 cells (left upper), and MDA-MB-453 cells (left lower). piR-126541 MB was used as a negative control. The data are displayed as means \pm standard deviation of triplicate samples (** $p < 0.05$, ** $p < 0.01$)

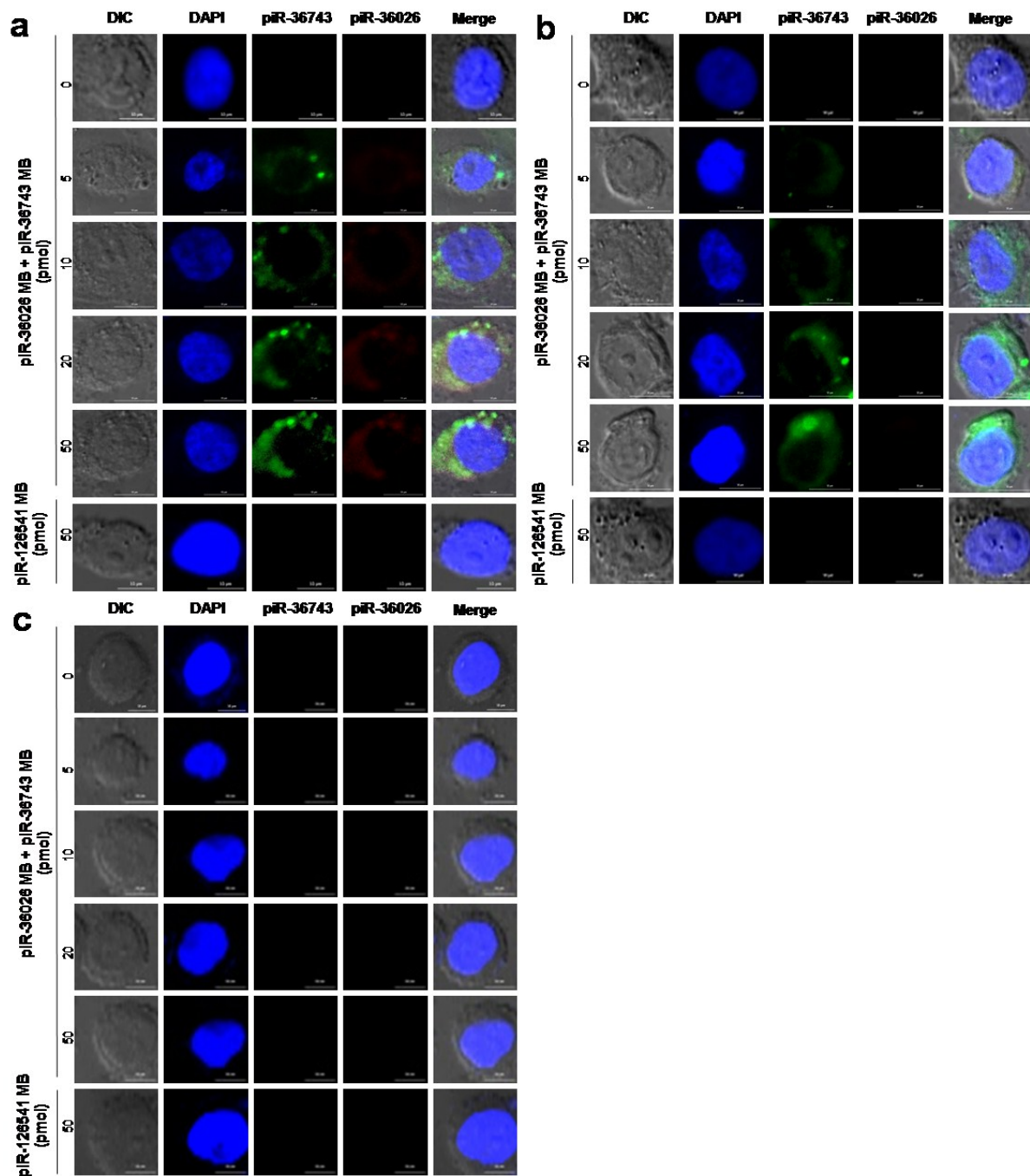


Figure S5. Simultaneous imaging of piR-36743 and piR-36026 in (a) BT-474 cells (b) MDA-MB-231 cells (c) MDA-MB-453 cells by piR-36026 MB and piR 36743 MB. The fifth column showed merged images and cellular morphology. Scale bars, 10 μ m.