Protocol 1. Co-transfecting plasmid DNA and PNAs™ miRNA inhibitor into mammalian cell lines

The amounts below are given for a 24-well plate format.

Cell plating

1. One day before transfection, seed 5.0 X 10^4 cells per well in 0.5 ml of the appropriate complete growth medium without antibiotics.
2. Incubate cells at 37ºC with 5% CO_2 overnight.

Transfection with Lipofectamine™ 2000 (Invitrogen, Cat# 11668-027)

1. Prepare the stock solution of PNAs™ miRNA inhibitors of 100 μM in RNase-free water. Store at - 20 ºC until used.
   √**Notice!**
   Before use, incubate PNA for 10 min at 70 ºC in a water bath or heating block.
2. Dilute 200 ng target DNA, 20 ng control DNA and PNAs™ miRNA inhibitor in 50 μl Opti-MEM® I Medium. PNAs™ miRNA inhibitor is added into culture medium at a final concentration of 500 ~ 2,000 nM.
   Mix gently [suspension 1].
3. Dilute 1 μl of Lipofectamine™ 2000 in 50 μl Opti-MEM® I Medium and mix gently [suspension 2].
4. Incubate each tube for 15 minutes at room temperature.
5. Mix [suspension 1] and [suspension 2] gently by pipetting and incubate for 15 minutes at room temperature [suspension 3].
6. Add [suspension 3] onto each well containing cells and medium and mix gently by rocking the plate back and forth.
7. Incubate the cells at 37ºC in 5% CO_2 incubator for 48 hours.

Luciferase assay (Promega, Cat# E1910)

1. Remove growth medium from the cultured cells, and gently apply a PBS to rinse the bottom of the culture vessel.
2. Prepare 1 X Passive Lysis Buffer (PLB) just before use.
3. Apply 100 μl of 1 X PLB into each well and lyse the cell.
4. Transfer the lysate to a new tube or vial.
5. For firefly luciferase assay, carefully transfer 10 μl of cell lysate and 10 μl of Lucifearase Assay Reagent II (LAR II) into luminometer tube. Mix by pipetting two times.
6. Place the tube in the luminometer and initiate reading.
7. For Renilla luciferase assay, remove the sample tube from the luminometer, add 10 μl of Stop & Glo® Reagent and mix briefly. Replace the sample in the luminometer and initiate reading.
8. Normalize the firefly luciferase activity to the Renilla luciferease activity.

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Protocol 2. Transfection and real-time PCR methods using PNAs™ miRNA inhibitors

The amounts below are given for a 6-well plate format.

Cell plating

1. One day before transfection, seed 1.5 X 10^5 cells per well in 1.5 ml of the appropriate complete growth medium without antibiotics.
2. Incubate cells at 37 ºC with 5% CO_2 overnight.

Transfection with PNAs miRNA inhibitors

1. Prepare the stock solution of PNAs™ miRNA inhibitors of 100 μM in RNase-free water. Store at - 20 ºC until used.

   √**Notice!**

   Before use, incubate PNA for 10 min at 70 ºC in a water bath or heating block.
2. Dilute PNAs™ miRNA inhibitor solution of appropriate concentration in 150 μl Opti-MEM® I Medium and mix gently.

   √**Notice!**

   We recommend a final PNAs miRNA inhibitors concentration range of 1 μM ~ 2 μM after adding the solutions to cells. Exact concentration depends on cell lines, culture conditions and target genes.
3. Incubate the solutions for 15 minutes at room temperature.
4. Add the solutions onto each well containing cells and medium and mix gently by rocking the plate back and forth.
5. Incubate the cells at 37 ºC in 5% CO_2 for 48 hours.

Extraction of total RNA or miRNAs

1. Remove growth medium from the cultured cells.
2. Extract total RNA or miRNAs from the samples using PureLink™ RNA kit or miRNA Isolation kit (Invitrogen Co.), respectively, according to the manufacturer's instruction.

   √**Notice!**

   Do not use Trizol for total RNA extraction because PNA-miRNA complexes are dissociated during Trizol purification.

Real-time PCR

1. Perform reverse transcription reaction using 10 ng of total RNA or miRNA by using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) and then perform real-time PCR by using TaqMan® Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instruction.
2. Analyze data.

   √**Notice!**

   This protocol has been optimized for use with HeLa, A549 or MCF7 cells in a 6-well plate format. Please optimize the protocol according to your experiment purpose.

References

1. Fabani MM, Gait MJ. 2008. miR-122 targeting with LNA/2'-O-methyl oligonucleotide mixmers, peptide nucleic acids (PNA), and PNA-peptide conjugates. *RNA*. 14(2):336-46.
2. Oh SY, Ju Y, Park H. 2009. A highly effective and long-lasting inhibition of miRNAs with PNA-based antisense oligonucleotides. *Mol. Cells*. 28:341-345.