Supporting Information

Triple-Helical Binding of Peptide Nucleic Acid Inhibits Maturation of Endogenous MicroRNA-197

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Synthesis and Purification of PNA

All PNA sequences were synthesized on an automated Expedite 8909 DNA synthesizer at 2 µmol scale using NovaSyn TG Sieber resin. Commercial PNA-T-monomer and AEEA spacer were purchased from Link Technologies, HiLyte Fluor 488 carboxylic acid was purchased from Anaspec. M and E monomers were synthesized using our previously developed procedures.[1] HiLyte Fluor 488 was coupled as the last step in PNA synthesis on solid support using our previously developed procedures.[2] PNA sequences were cleaved from the solid support following standard cleavage conditions using 1.0 mL of 20 % m-cresol in TFA for 2.0 h using two-syringe pull-push method. The cocktail was collected in several Eppendorf tubes (300 µL in each tube) and the resin again washed with an additional 1.0 mL of the fresh cleaving cocktail. Crude PNA (300 µL in each tube) was precipitated by the addition of chilled diethyl ether (~1.0 mL) followed by centrifugation. The crude PNA was dissolved in distilled water and analyzed by RP-HPLC or LC-MS (see Figures S1-S6 below). All the PNA sequences were RP-HPLC purified using a linear gradient of acetonitrile in water containing either 0.1% TFA or 0.1% HCOOH. The purity and identity of the PNA sequences were confirmed by LC-MS (ESI) or MALDI-TOF analysis (Table S1).

[1] Hnedzko, D.; Cheruiyot Samwel, K.; Rozners, E. Using triple-helix-forming Peptide nucleic acids for sequence-selective recognition of double-stranded RNA. *Current Protocols in Nucleic Acid Chemistry* **2014**, *58*, 4.60.1-4.60.23.
[2] Hnedzko, D.; McGee, D.W.; Rozners, E. Synthesis and properties of peptide nucleic acid labeled at the N-terminus with HiLyte Fluor 488 fluorescent dye. *Bioorg. Med. Chem*. **2016**, *24*, 4199-4205.

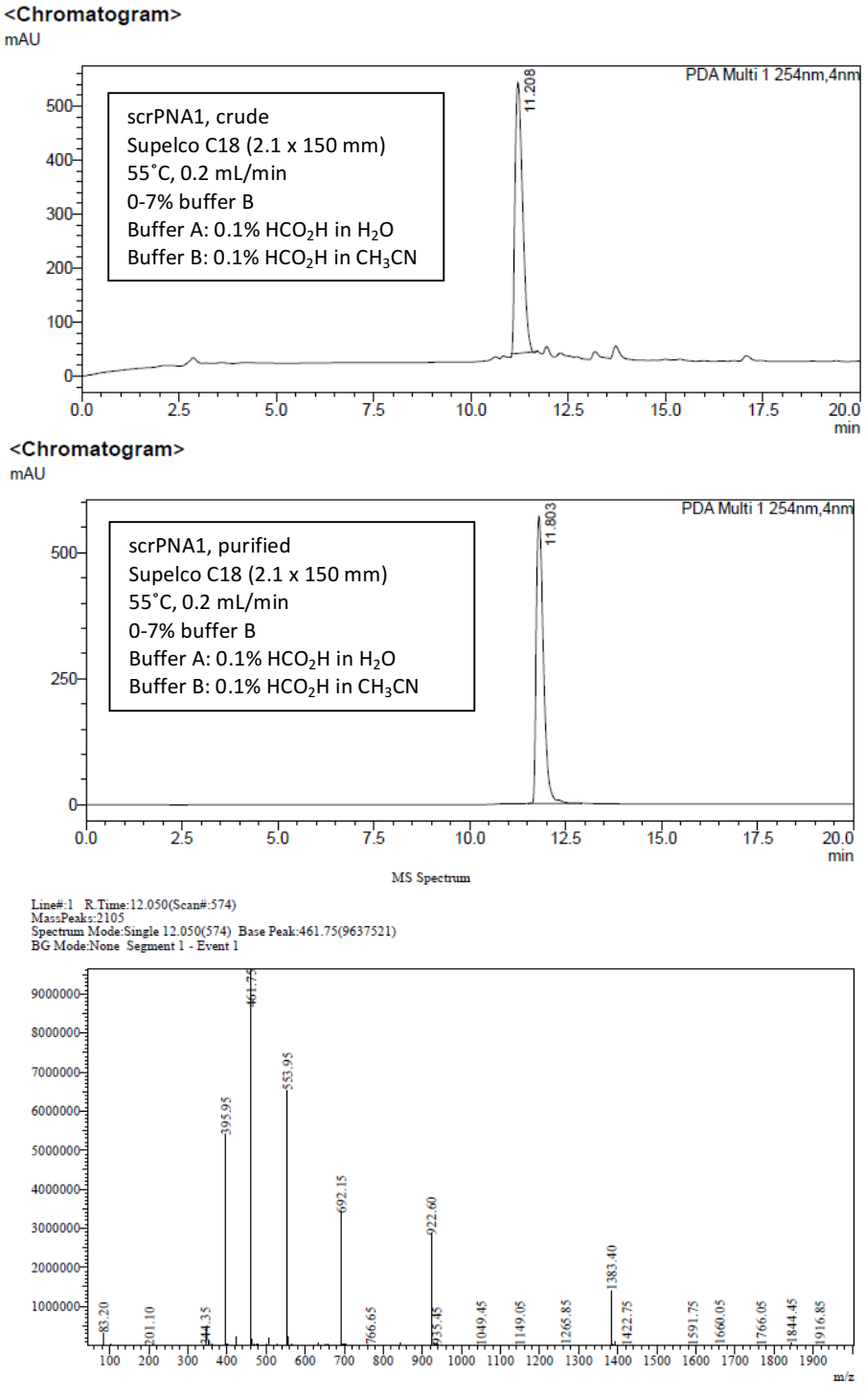

Figure S1. LC-MS analysis of scrPNA1.

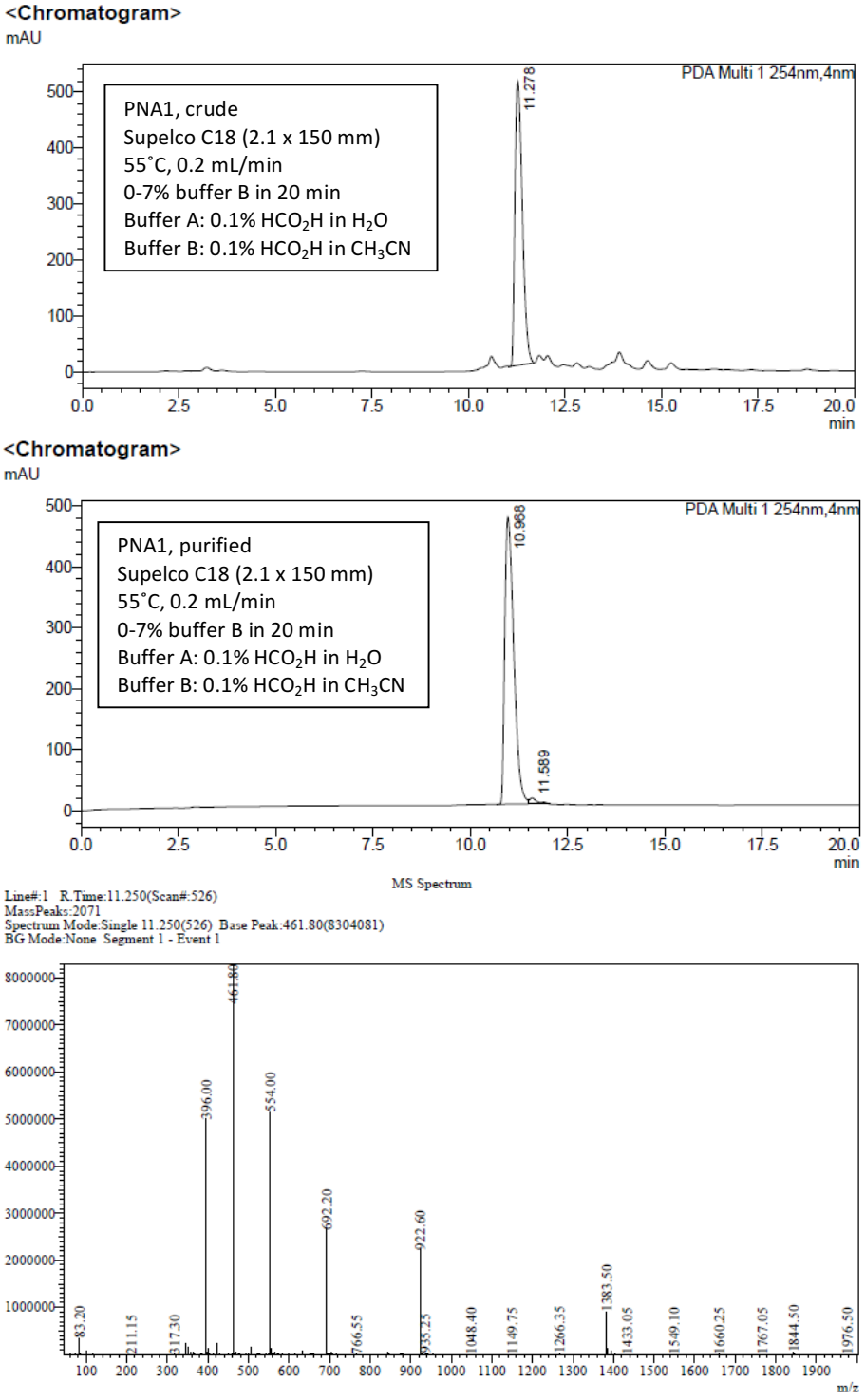

Figure S2. LC-MS analysis of PNA1.

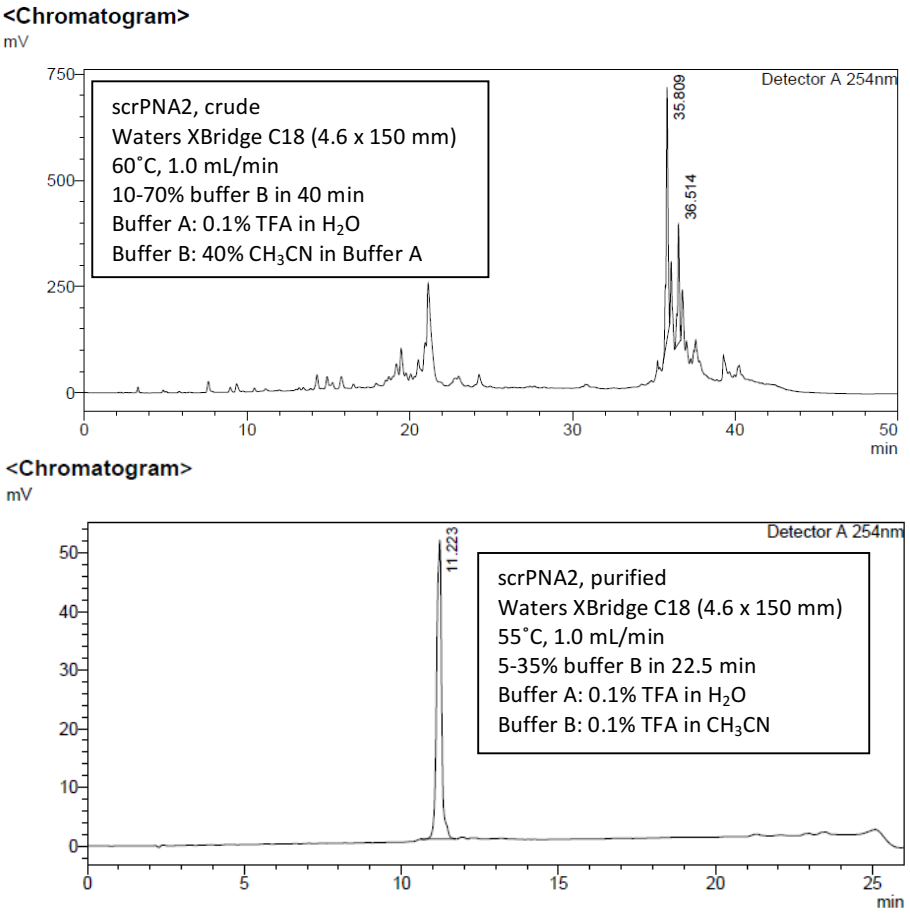

Figure S3. HPLC analysis of scrPNA2.

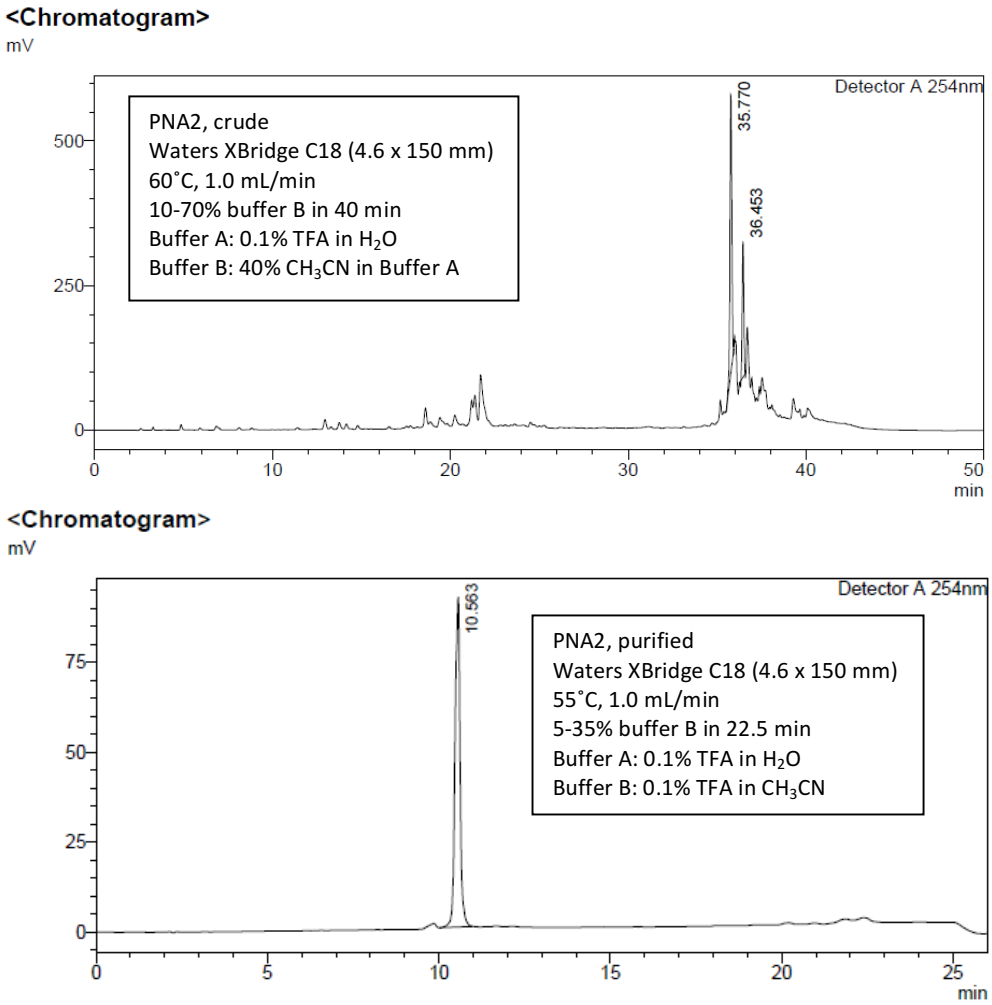

Figure S4. HPLC analysis of PNA2.

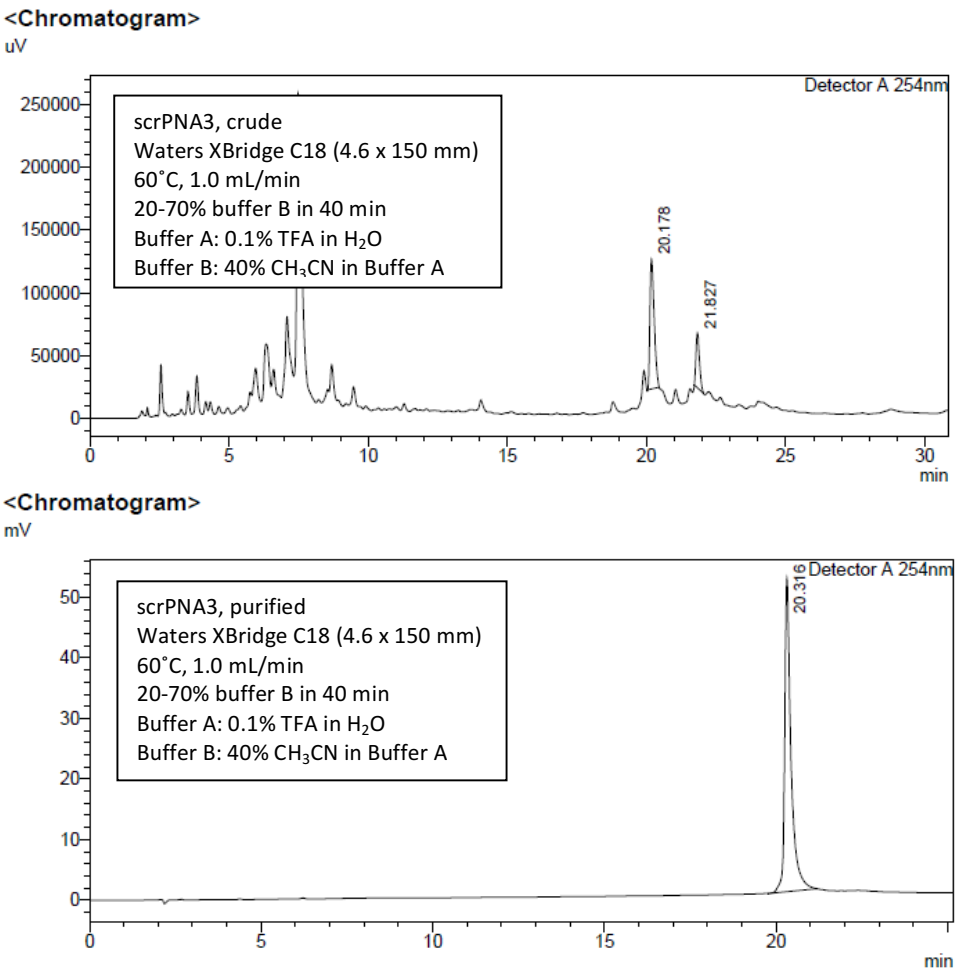

Figure S5. HPLC analysis of scrPNA3.

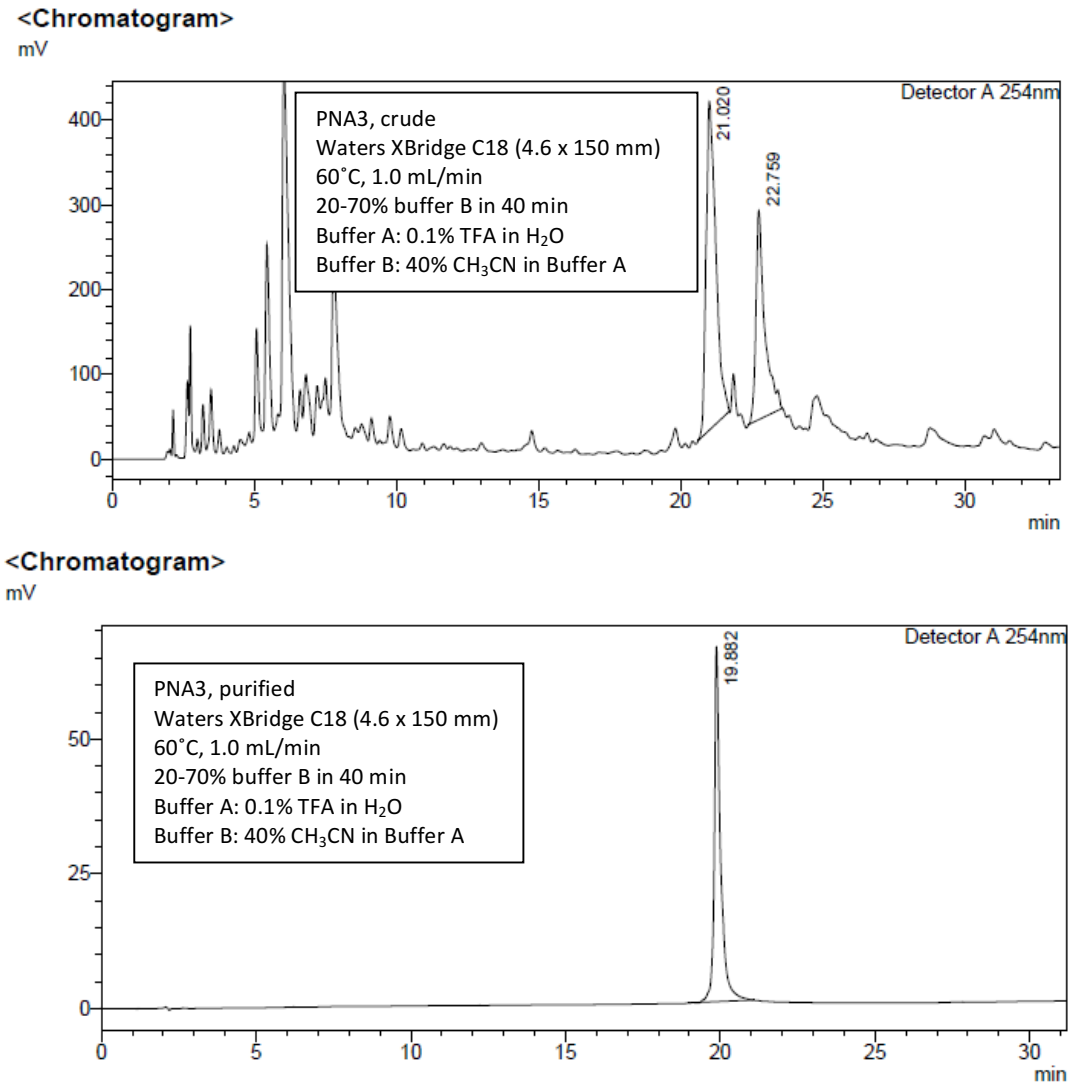

Figure S6. HPLC analysis of PNA3.

Table S1. PNA sequences and MS analysis results.

PNA	***Sequence***	***Mass calc.***	***Mass found (LCMS) $(M+2H)^{2+}$, $(M+3H)^{3+}$, $(M+4H)^{4+}$, $(M+5H)^{5+}$***
PNA1	H_2N-K-MMMETMTMT-KKK-$CONH_2$	2763.4	1383, 922, 692, 554
scrPNA1	H_2N-K-EMTMMTMTM-KKK-$CONH_2$	2763.4	1383, 922, 692, 554

PNA	***Sequence***	***Mass calc.***	***Mass found (MALDI-TOF)***
PNA2	HF-AEEA-AEEA-K-MMMETMTMT-$CONH_2$	3138.8	3139.1
scrPNA2	HF-AEEA-AEEA-K-EMTMMTMTM-$CONH_2$	3138.8	3139.2
PNA3	HF-AEEA-AEEA-K-MMMETMTMT-KKK-$CONH_2$	3523.0	3523.2
scrPNA3	HF-AEEA-AEEA-K-EMTMMTMTM-KKK-$CONH_2$	3523.0	3523.3

HF – HiLyte Fluor 488; AEEA - 2-(2-aminoethoxy)ethoxy acetic acid linker

Fluorescence quenching assay

HPLC-purified BHQ1-modified HRP-197 (Figure 1) was purchased from Japan Bio Services Co. Ltd. (Saitama, Japan) and refolded by cooling from 95 ˚C to 25 ˚C at 5 ˚C min^{-1} in a buffer containing 30 mM HEPES-KOH (pH 7), 100 mM KCl, and 0.01% CHAPS before addition of PNA. HiLyte Fluor 488 (HF)-labeled PNAs, **PNA2**, **scrPNA2**, **PNA3**, or scr**PNA3**, at 500 pM were mixed with various concentrations of HRP-197 in a buffer containing 30 mM HEPES-KOH (pH 7), 100 mM KCl, 10 ng/μL Yeast tRNA (Thermo Fisher Scientific, Waltham, MA, USA), and 0.01% CHAPS. The mixtures were incubated at 37 ˚C for 60 min, and the fluorescence signals were measured using a microwell plate reader (Infinite 200 Pro; Tecan) with 490 nm excitation and 530 nm emission. Equilibrium association constants at 37 ˚C ($K_{A\,37}$) between PNAs and HRP-197 were calculated from fluorescence signal as a function of the concentration of HRP-197. The fluorescence signals were fitted by Equation 1 assuming a 1:1 binding reaction:

Equation 1:

$$F = F_{\text{initial}} + \left(\frac{F_{\text{final}} - F_{\text{initial}}}{2 \times [\text{PNA}]} \right) \times \left\{ \left([\text{PNA}] + [\text{HRP}] + \frac{1}{K_{A\,37}} \right) - \sqrt{\left([\text{PNA}] + [\text{HRP}] + \frac{1}{K_{A\,37}} \right)^2 - 4 \times [\text{PNA}] \times [\text{HRP}]} \right\}$$

where F is the fluorescence signal of HF-labeled PNA at each concentration of HRP-197, F_{initial} is the initial fluorescence signal without HRP-197, F_{final} is the fluorescence signal after fluorescence decrease is saturated, [PNA] is the total concentration of the HF-labeled PNA, and [HRP] is the concentration of HRP-197.

Preparation of miRNA

pri-miR-197 hairpin (Figure 1) was transcribed in vitro from DNA template, which was prepared by primer extension using sense and antisense DNA strands: 5′-CTTAATACGACTCACTATAGGCTGTGCCGGGTAGAGAGGGCAGTGGGAGGTAAGAGCTCTTCACCCTTC-3′ (sense) and 5′-GGCCATGCTGGGTGGAGAAGGTGGTGAAGGGTGAAGAGCTCTTACCTC-3′ (antisense), by using ScriptMAX Thermo T7 Transcription Kit (Toyobo, Osaka, Japan). Transcribed RNA was purified by denaturing polyacrylamide gel electrophoresis. RNA concentration was determined by measuring the absorbance at 260 nm at 90 °C using a UV/Vis spectrophotometer (Shimadzu UV-1700, Shimadzu, Kyoto, Japan) connected to a thermo programmer. Single-strand extinction coefficients were calculated from mononucleotide and dinucleotide data using the nearest neighbour approximation.

Labeling of miRNA with Cy3 fluorophore

The 3′ terminus of pri-miR-197 hairpin was labeled with Cy3 fluorophore using an enzymatic reaction. Purified pri-miR-197 hairpin (2 μM) was mixed with pCp-Cy3 (Jena Bioscience, Jena, Germany) (4 μM) and T4 RNA Ligase 1 (New England BioLabs Ipswich, MA, USA) (0.4 U/μL) in a buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 10 % DMSO. The reaction mixture was incubated at 16 ˚C overnight. The reaction product was purified by using RNeasy MinElute Cleanup Kit (Qiagen, Venlo, Netherlands) according to manufacturer's protocol, except that 3.8-fold more (compared to the standard protocol) of ethanol was added before the sample was transferred to

MinElute spin column. After elution of the Cy3-labeled pri-miR-197 hairpin, the concentration was adjusted based on absorbance at 260 nm, which was measured by NanoDrop 1000 (Thermo Fisher Scientific), based on that of non-labeled pri-miR-197 hairpin.

Dicer inhibition assay in vitro

Cy3-labeled pri-miR-197 hairpin (250 nM) was mixed with PNA (500 nM) in a buffer of Recombinant Turbo Dicer Enzyme Kit (Genlantis, San Diego, CA, USA), in which the amount of additional BSA was reduced to 50%. Recombinant Turbo Dicer enzyme was added to the sample at indicated concentration. The reaction mixture was incubated at 37 °C for 3 hours. The reaction was terminated by addition of equivalent volume of 8 M urea. Reaction product was separated on an 10% denaturing polyacrylamide gel at 70 °C. Fluorescence signals of Cy3 in the gel were analysed using FLA5100 fluorescence image scanner (Fuji Film, Tokyo, Japan) with 532 nm excitation laser and 575 nm emission. The gel was subsequently stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific) and imaged using FLA5100 with 473 nm excitation and 510 nm emission. Efficiency of RNA cleavage was calculated by Cy3 intensities of intact and cleaved RNAs using ImageQuant TL software (GE Healthcare, Chicago, IL, USA).

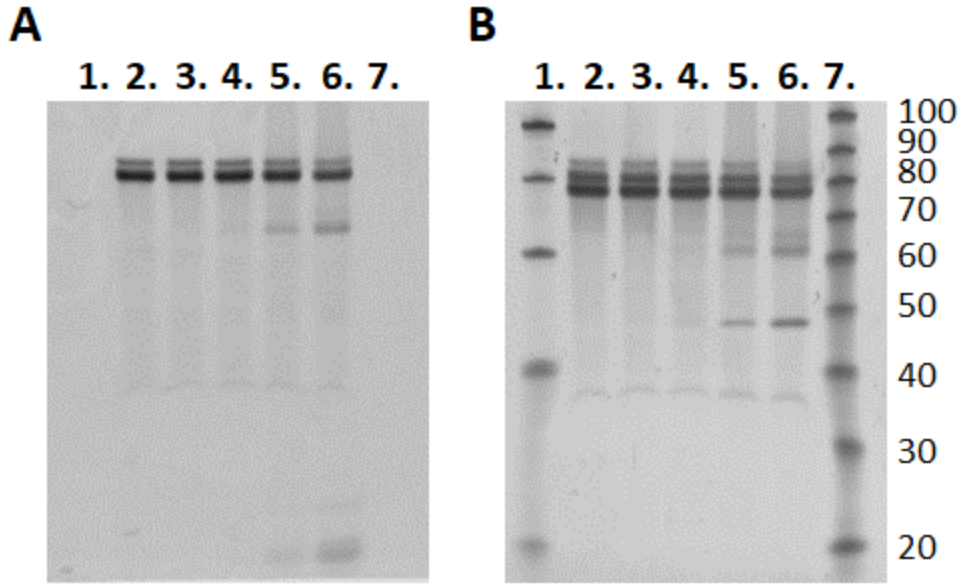

Figure S7. Processing of Cy3-labeled pri-miR-197 hairpin dependent on concentration of human Turbo Dicer enzyme. Products of Dicer cleavage reaction were separated by denaturing PAGE and imaged by (A) Cy3 fluorescence and (B) SYBR Gold staining. Samples are 20 base pair ladder (lane 1), reaction mixtures with 0 (lane 2), 0.0125 (lane 3), 0.025 (lane 4), 0.05 (lane 5), and 0.1 (lane 6) U/µL Dicer enzyme, and 10 base pair ladder (lane 7).

Electroporation of PNA into cells

Human neuroblastoma (SH-SY5Y) and cervix epithelioid carcinoma (HeLa) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and maintained at 37 °C under 5% CO_2. Cells were trypsinized and counted. PNA without fluorophore labeling (400 pmol) or PNA labeled with HF (360 pmol) was mixed with SH-SY5Y (5×10^5 cells) or HeLa (2×10^5 cells) in a solution of 4D-Nucleofector X Kit S (Lonza, Basel, Switzerland) and introduced into the cells using 4D-nucleofector (Lonza). Optimal electroporation program by manufacturer was used with SF and SE Cell Line Kit for SH-SY5Y and HeLa cells, respectively. To analyze mature miRNA levels, cells were re-plated on 2-wells of a collagen-coated 24-well plate. To analyze cellular proliferations, cells were re-plated on 3-wells of a collagen-coated 96-well plate, in which each well contains 1/10 cells used for the electroporation. The cells were continuously cultured at 37 °C under 5% CO_2.

Reverse transcription of miRNA

After 48 or 72 hours from the electroporation, short RNA fragments were purified from the cells cultured on 24-well plate using miRNeasy Mini Kit (Qiagen) and robotic workstation for automated purification (QIAcube, Qiagen) according to the standard program without optional DNase treatment. Concentration of the purified RNA fragments was measured by NanoDrop 1000. RNA concentrations of the samples and values of absorbance at 260 nm relative to 280 nm (260/280) for each experimental replicate are listed in Tables S2, S3 and S4. Multiplex reverse transcription (RT) of miRNAs was performed using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, purified short RNA fragments (50 ng) were mixed with the premixed RT-primers for miRNAs to be analysed (TaqMan MicroRNA Assays, Thermo Fisher Scientific) in total reaction volume of 7.5 μL. RT reaction was done with MultiScribe Reverse Transcriptase (25 U, Thermo Fisher Scientific) according to the manufacturer's protocol, which incubates reaction mixtures at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. Pre-mixed RT-primers for miR-197 (miR-197-3p), miR-27a (miR-27a-3p), and miR-155 (miR-155-5p) were used for samples obtained after 72 hours with electroporation of PNA without fluorophore labeling. Pre-mixed RT-primers for miR-197 and miR-27a were used for samples obtained after 48 hours with electroporation of PNA labeled with HF.

Table S2. Concentrations (ng/μL) and 260/280 values of purified RNAs from SH-SY5Y cells after electroporation of non-labeled PNAs [a]

	Replicate 1 [b]	**Replicate 2** [b]	**Replicate 3** [b]	**Replicate 4** [b]
PNA1	56.8 (1.74)	65.4 (1.91)	67.0 (1.76)	61.0 (1.93)
scrPNA1	46.2 (1.58)	38.1 (1.77)	36.0 (1.64)	35.4 (1.82)
without PNA	101 (1.79)	124 (1.8)	73.9 (1.79)	80.9 (1.85)

a) Values of absorbance at 260 nm relative to 280 nm (260/280) are shown in parentheses.

b) Values obtained from individually performed experimental replicates are shown.

Table S3. Concentrations (ng/μL) and 260/280 values of purified RNAs from SH-SY5Y cells after electroporation of HF-labeled PNAs [a]

	Replicate 1 [b]	Replicate 2 [b]	Replicate 3 [b]
PNA2	44.2 (1.84)	49.0 (1.74)	38.4 (1.66)
scrPNA2	58.7 (1.69)	42.6 (1.71)	33.9 (1.65)
without PNA	65.2 (1.68)	53.6 (1.78)	44.0 (1.85)

c) Values of absorbance at 260 nm relative to 280 nm (260/280) are shown in parentheses.

d) Values obtained from individually performed experimental replicates are shown.

Table S4. Concentrations (ng/μL) and 260/280 values of purified RNAs from HeLa cells after electroporation of HF-labeled PNAs [a]

	Replicate 1 [b]	Replicate 2 [b]	Replicate 3 [b]
PNA2	67.4 (1.90)	52.5 (1.73)	54.7 (1.81)
scrPNA2	53.6 (1.91)	45.5 (1.70)	51.0 (1.82)
without PNA	68.7 (1.99)	64.6 (1.60)	54.3 (1.85)

e) Values of absorbance at 260 nm relative to 280 nm (260/280) are shown in parentheses.

f) Values obtained from individually performed experimental replicates are shown.

Quantitative RT-PCR assay of mature miRNA

Amplification profile of mature miRNAs was monitored by Mx3000P qPCR System (Agilent, Santa Clara, CA, USA) using PCR primers and TaqMan probes for miRNAs to be analysed (TaqMan MicroRNA Assays, Thermo Fisher Scientific) and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific). Specifically, four samples in the 3-fold dilution series (5-, 15-, 45-, and 135-fold dilutions) were prepared as standard using an RT product obtained from cells, in which electroporation was performed in the absence of PNA. RT products from samples to be analysed were diluted 15-fold. The diluted samples (7 μL) were mixed with specific PCR primers and TaqMan probe for each miRNA to be analysed, which are provided as premixed solution (1.75 μL) in TaqMan MicroRNA Assays. The mixtures were subsequently mixed with TaqMan Universal PCR Mater Mix (17.5 μL), and the volume was adjusted to 35 μL by H_2O. The reaction mixtures were divided to 3-wells (each 10 μL) in a 96-well plate and quantitative real time PCR was performed according to manufacturer's protocol, which involves preincubation at 95 °C for 10 min followed by two-step PCR reaction by cycling 95 °C for 15 sec and 60 °C for 1 min for 50 cycles. Signals of TaqMan probe at 492 nm excitation and 516 nm emission and reference baseline signals at 440 nm excitation and 610 nm emission were monitored by Mx3000P qPCR System. For analysis of the amplification profiles, signals of baseline-corrected normalized fluorescence were plotted against the number of PCR cycles (Figure S8a, S8b, and S8c) and threshold cycle (Ct) values were determined by using MxPro QPCR Software (Mx3005P v4.10, Agilent). The baseline correction and normalization of the signals were proceeded according to the default settings of the software. Slope and y-intercept of the standard samples, amplification efficiencies, and r2 values calculated from the linearity of the standard curves (Figure S8d) are shown in Table S5, S6, and S7. The expression levels of mature miRNAs were evaluated as relative levels to the standard. Of the replicates of standard dilution series prepared in 3 wells, if there was a point that deviates significantly from the standard straight line, the well was omitted.

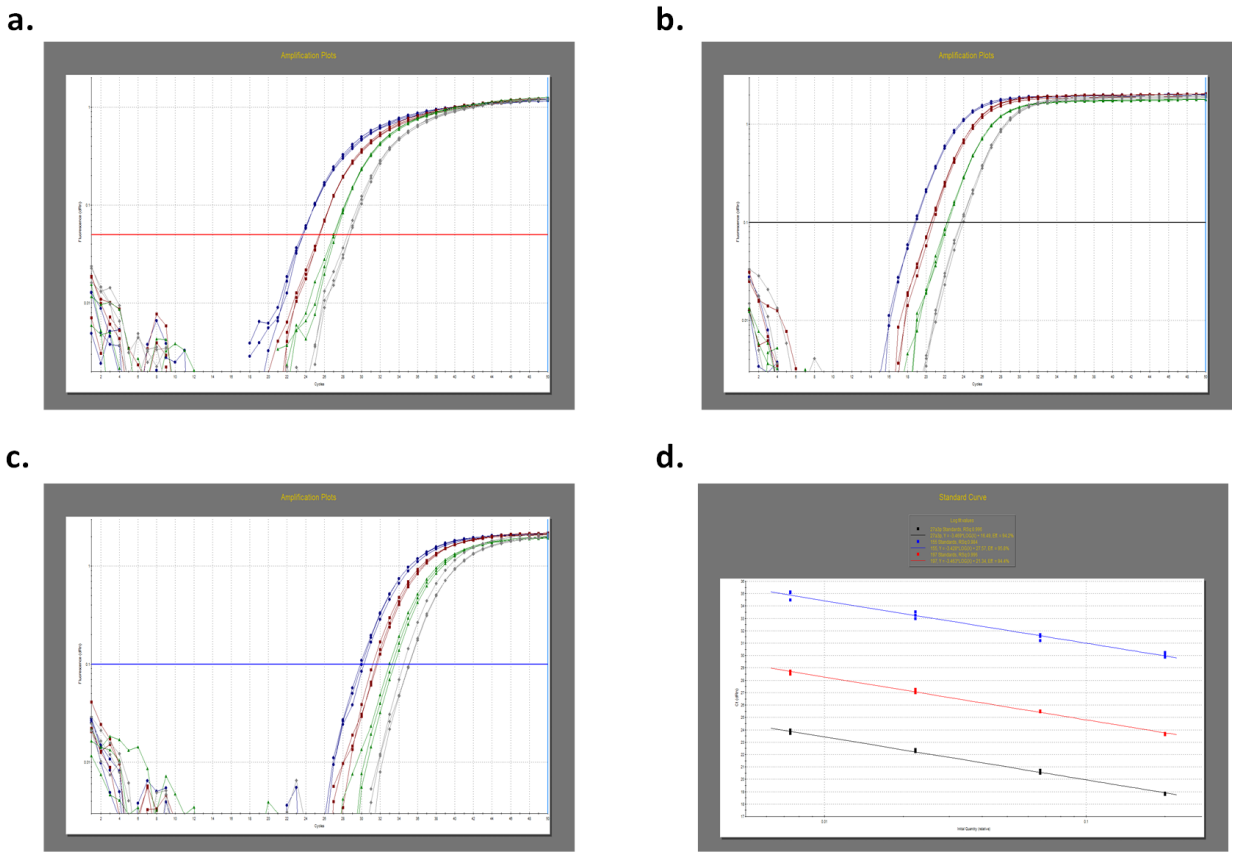

Figure S8. Typical signal amplification profiles of standard dilutions series analyzed with single experimental replicate. (a-c) Exported amplification profiles of standard samples for (a) miR-197, (b) miR-27a, and (c) miR-155. Threshold values of the baseline-corrected normalized fluorescence signals to calculate the Ct values were 0.05, 0.1, and 0.1 for miR-197, miR-27a, and miR-155, respectively, which are shown as colored lines in the graphs. (d) standard curves obtained from Ct values from the amplification profiles for miR-197 (red), miR-27a (black), and miR-155 (blue). Values of slope, y-intercept, amplification efficiency, and r square for each miRNA, which were obtained from replicated experiments, are serialized in Tables S5, S6, and S7.

Table S5. Validity of standard curves in qRT-PCR of samples obtained from SH-SY5Y cells after electroporation of non-labeled PNAs.

		slope	y-intercept	amplification efficiency (%)	r^2
Replicate 1	miR-197	-3.39	22.0	97.1	0.996
	miR-27a	-3.57	16.4	90.5	0.997
	miR-155	-3.45	28.2	95.0	0.957
Replicate 2	miR-197	-3.33	22.2	99.6	0.997
	miR-27a	-3.49	16.7	93.4	0.997
	miR-155	-3.67	28.0	87.2	0.992
Replicate 3	miR-197	-3.46	21.3	94.4	0.996
	miR-27a	-3.47	16.5	94.2	0.996
	miR-155	-3.43	27.6	95.8	0.984
Replicate 4	miR-197	-3.48	21.0	94.0	0.988
	miR-27a	-3.20	17.0	105.5	0.995
	miR-155	-2.93	28.9	119.5	0.98

Table S6. Validity of standard curves in qRT-PCR of samples obtained from SH-SY5Y cells after electroporation of HF-labeled PNAs

		slope	y-intercept	amplification efficiency (%)	r^2
Replicate 1	miR-197	-3.45	23.3	93.9	0.991
	miR-27a	-3.29	27.1	101.4	0.972
Replicate 3	miR-197	-3.72	23.4	85.8	0.996
	miR-27a	-3.94	26.9	79.4	0.985
	miR-197	-3.67	24.6	87.2	0.986
	miR-27a	-3.50	26.9	93.1	0.977

Table S7. Validity of standard curves in qRT-PCR of samples obtained from HeLa cells after electroporation of HF-labeled PNAs

		slope	y-intercept	amplification efficiency (%)	r^2
Replicate 1	miR-197	-3.77	24.3	84.3	0.991
	miR-27a	-3.52	24.0	92.2	0.993
Replicate 2	miR-197	-3.65	25.3	87.9	0.986
	miR-27a	-3.57	25.0	90.6	0.991
Replicate 3	miR-197	-3.55	24.4	91.2	0.987
	miR-27a	-3.61	23.7	89.4	0.997

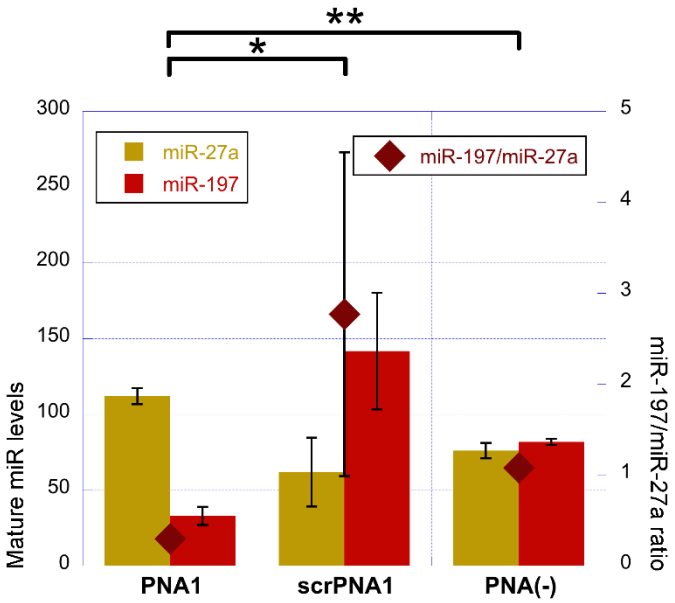

Figure S9. Levels of mature miRNA in cells after 72 hours treated with PNAs. Levels of miR-27a (dark yellow) and miR-197 (red) are represented on left axis, and their relative values (dark red) are plotted on right axis. Values are means ± standard deviations of experiments replicated four times. Asterisk and double asterisks indicate two-tailed *P* values of less than 0.1 and 0.02, respectively, as calculated using Student's *t*-test.

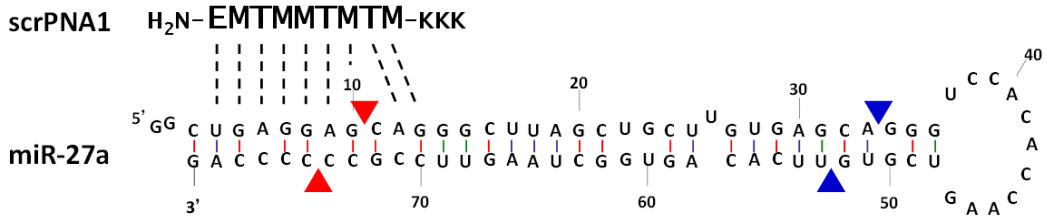

Figure S10. Sequence alignment of **scrPNA1** and pri-miR-27a hairpin. The Dicer and Drosha cleavage sites are shown with blue and red arrows, respectively, and the partial complementarity is highlighted with dashed lines.

Cell proliferation assay

After 72 hours from the electroporation, the number of cells in the 96-well plate was evaluated by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, the cellular media was replaced to that containing 10 µL/well substrate and incubated at 37 °C. The 450 nm absorbance of each well was measured after 60-min incubation by using Infinite 200 Pro, and the values were normalized by subtracting those of the wells without cells. Cellular proliferation was evaluated by comparing the values with cells that were electroporated without PNA, for which the value was set as 100%.

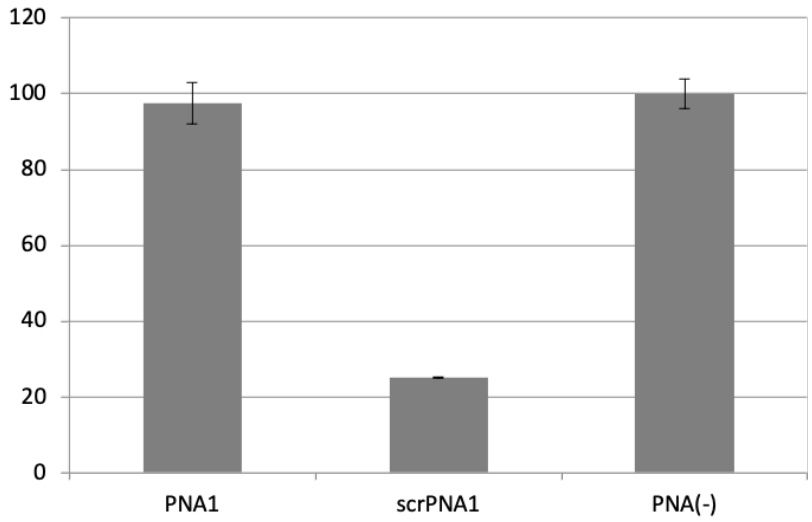

Figure S11. Cell proliferation activity after PNA electroporation.

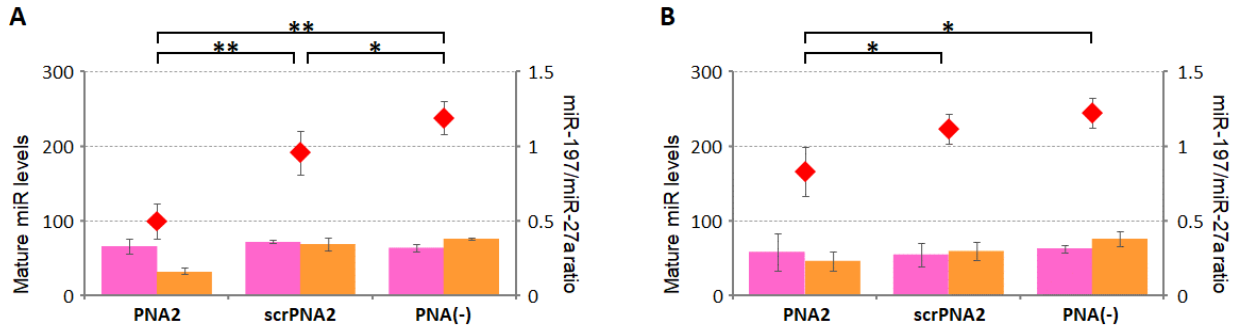

Figure S12. Levels of mature miRNAs in (A) SH-SY5Y and (B) HeLa cells after treatment with PNAs for 48 hours. Levels of miRNA-27a (pink) and miRNA-197 (orange) are represented on left axis, and their relative values (green) are plotted on right axis. Values are means ± standard deviations of triplicated experiments. Asterisk and double asterisks indicate two-tailed *P* values of less than 0.1 and 0.02, respectively, as calculated using Student's *t*-test.

Table S8. Bioinformatic survey of possible targets among *H. Sapiens* miRNAs in the miRBase assuming that PNAs may form triplex with dsRNA region in a minimum of 7-mer length (underlined in miRNA sequences). The search included A-C non-canonical base pairs.

Potential target miRNA		miRNA sequence
PNA1	hsa-mir-197	GGCUGUGCCGGGUAGAGAGGGCAGUGGGAGGUAAGAGCUCUUCACCCUUCACCACCU UCUCCACCCAGCAUGGCC
	hsa-mir-1285-1	UGUAGAGAUAGGAUCUCACUUUGUUGCCCAGGCUGGUCUCAAACUCCUGGUCUGGG CAACAAAGUGAGACCUUAUCUCUACAAG
	hsa-mir-3138	CCCUCCUCGGCACUUCCCCCACCUCACUGCCCGGGUGCCCACAAGACUGUGGACAGUG AGGUAGAGGGAGUGCCGAGGAGGG
	hsa-mir-3129	GUACUUGGGCAGUAGUGUAGAGAUUGGUUUGCCUGUUAAUGAAUUCAAACUAAUCU CUACACUGCUGCCCAAGAGC
	hsa-mir-3609	GUAACAGUAACUUUUAUUCUCAUUUUCCUUUUCUCUACCUUGUAGAGAAGCAAAGU GAUGAGUAAUACUGGCUGGAGCCC
scrPNA 1	hsa-mir-27a	CUGAGGAGCAGGGCUUAGCUGCUUGUGAGCAGGGUCCACACCAAGUCGUGUUCACAG UGGCUAAGUUCCGCCCCCCAG
	hsa-mir-657	GUGUAGUAGAGCUAGGAGGAGAGGGUCCUGGAGAAGCGUGGACCGGUCCGGGUGGG UUCCGGCAGGUUCUCACCCUCUCUAGGCCCCAUUCUCCUCUG
	hsa-mir-877	GUAGAGGAGAUGGCGCAGGGGACACGGGCAAAGACUUGGGGGUUCCUGGGACCCUCA GACGUGUGUCCUCUUCUCCCUCCUCCCAG
	hsa-mir-1249	GGGAGGAGGGAGGAGAUGGGCCAAGUUCCCUCUGGCUGGAACGCCCUUCCCCCCCUU CUUCACCUG
	hsa-mir-3150b	GAGGGAAAGCAGGCCAACCUCGAGGAUCUCCCCAGCCUUGGCGUUCAGGUGCUGAGG AGAUCGUCGAGGUUGGCCUGCUUCCCCUC
	hsa-mir-3160-1	GGACCUGCCCUGGGCUUUCUAGUCUCAGCUCUCCUCCAGCUCAGCUGGUCAGGAGAG CUGAGACUAGAAAGCCCAGGGCAGGUUC
	hsa-mir-3160-2	ACCUGCCCUGGGCUUUCUAGUCUCAGCUCUCCUGACCAGCUGAGCUGGAGGAGAGCU GAGACUAGAAAGCCCAGGGCAGGU
	hsa-mir-4769	GAGGAGAGGUGGGAUGGAGAGAAGGUAUGAGCUAAAAAUCCCCAAGCUCUGCCAUCC UCCCUCCCCUACUUCUCCCCC
	hsa-mir-6076	AGCAUGACAGAGGAGAGGUGGAGGUAGGCGAGAGUAAUAUAAUUUCUCCAGGAGAA CAUCUGAGAGGGGAAGUUGCUUUCCUGCCCUGGCCCUUUCACCCUCCUGAGUUUGGG
	hsa-mir-6740	GAAAGAGUUUGGGAUGGAGAGAGGAGAAACUUGAGGUCUCUGGGAGUUGCUUAAAC CAGUUGACCGUAACCUGGCCAGAGAAUUCUGAUAGUGUCUUCUCUCCUCCCAAACAG