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Supplemental Information

Base-modified aptamers obtained by cell-internalization SELEX facilitate cellular uptake of an antisense oligonucleotide

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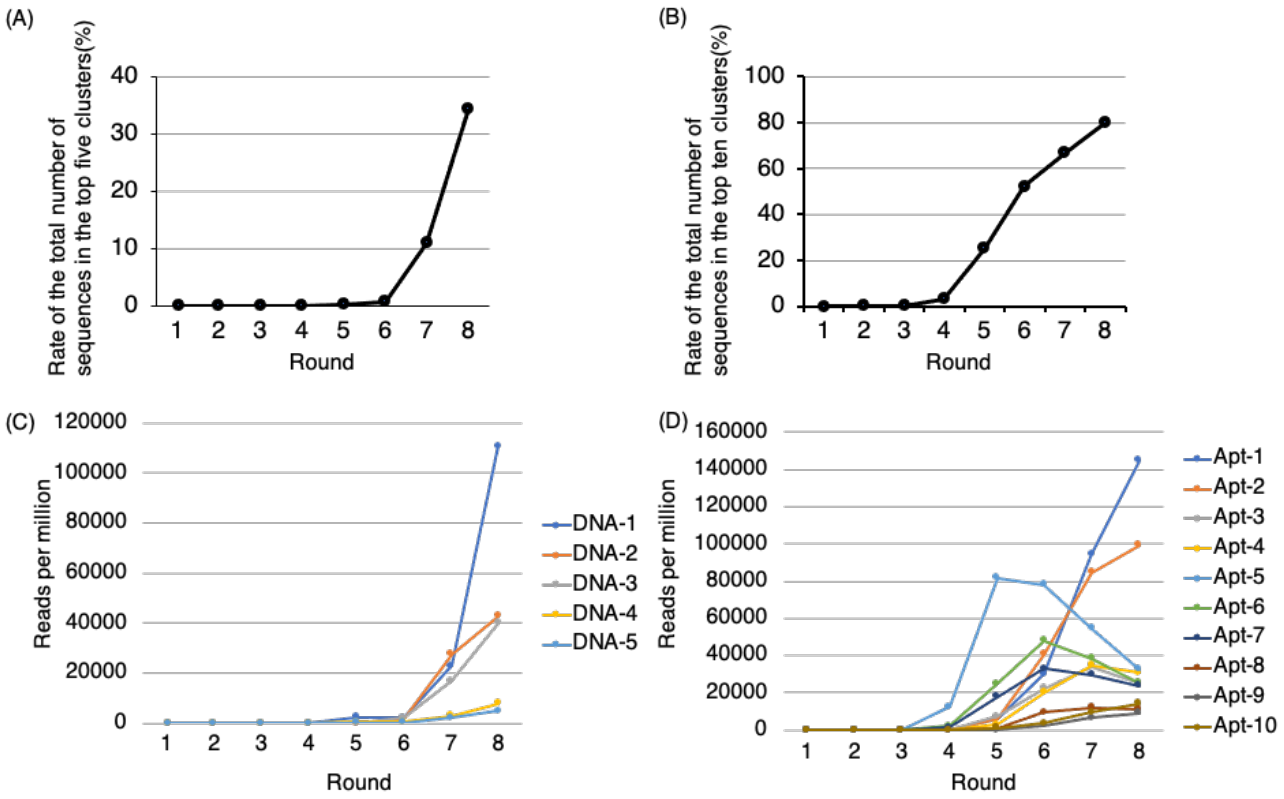


Fig.S1 The enrichment of aptamers

The total number of sequences in the top five or ten clusters were divided by the total number of read sequences in each library (A) natural DNA library (B) artificial DNA library. Reads per million of each sequence in each round library are shown in (C) (DNA-1 to -5) and (D) (Apt-1 to -10).

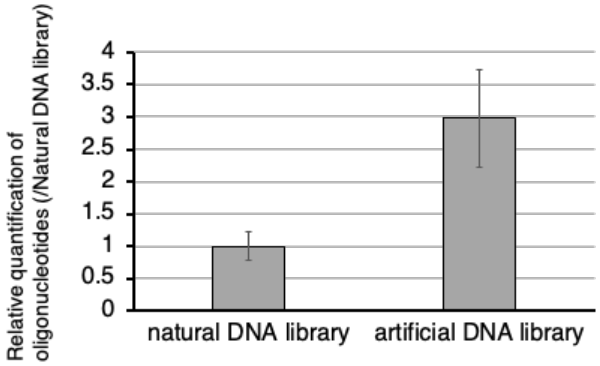


Fig. S2 Comparison of natural and artificial DNA library

The natural DNA library and artificial DNA library were incubated with A549 cells for 30 min at 37°C. The amounts of internalized aptamers were evaluated by real-time PCR and were normalized with the amount of internalized natural DNA library. Error bars show the mean ± SD values of three cell culture wells. See also Fig. S4(B).

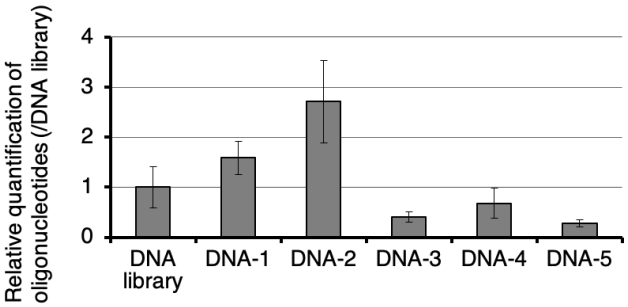


Fig. S3 Quantification of the aptamers that internalized into cells.

DNA library and DNA-1 to -5 were incubated with A549 cells for 30 min at 37°C. The amounts of internalized aptamers were evaluated by real-time PCR and were normalized with the amount of internalized natural DNA library. Amplification efficiencies of DNA-2 to -5 were significantly lower than DNA library, because sequences of DNA-2 to -5 are guanosine rich. So, on this experiment, we used standard curves of each sequence. Error bars show the mean ± SD values of four cell culture wells.

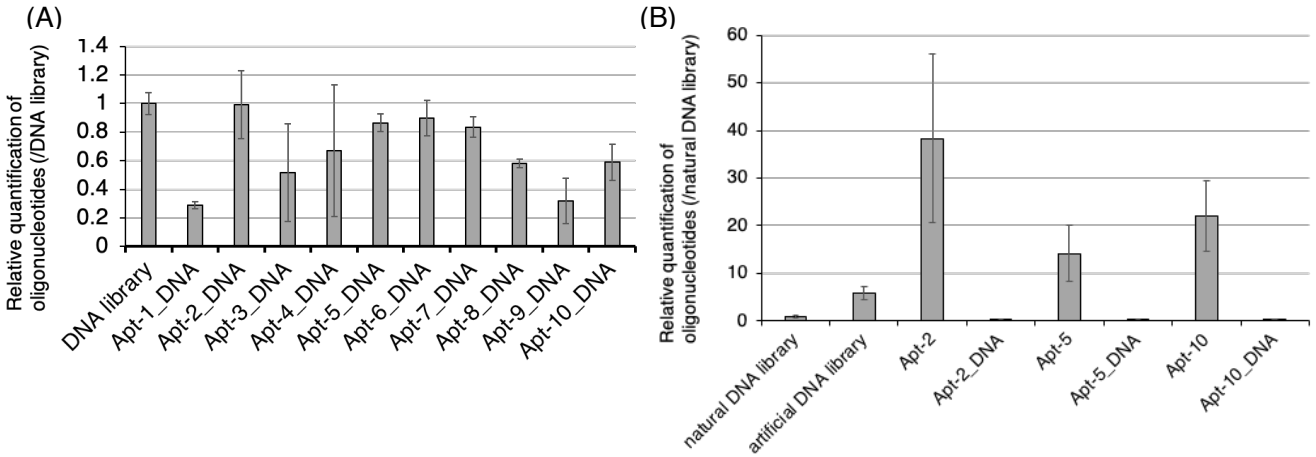


Fig. S4 Quantification of the aptamers that internalized into cells.

(A) Natural DNA library and Apt-1_DNA to -10_DNA were incubated with A549 cells for 30 min at 37°C. Apt-1_DNA to -10_DNA are sequences that U^{trp}s in Apt-1 to -10 are substituted by deoxythymidine. The amounts of internalized aptamers were evaluated by real-time PCR and were normalized with the amount of internalized natural DNA library. Apt-1_DNA to -10_DNA did not internalize into the cells more than natural DNA library. Error bars show the mean ± SD values of three cell culture wells.

(B) Natural DNA library, artificial DNA library, Apt-2, Apt-2_DNA, Apt-5, Apt-5_DNA, Apt-10, and Apt-10_DNA were incubated with A549 cells for 30 min at 37°C. The amounts of internalized aptamers were evaluated by real-time PCR and were normalized with the amount of internalized natural DNA library. The substitution of U^{trp}s in the aptamers reduced their internalization ability. Error bars show the mean ± SD values of three cell culture wells.

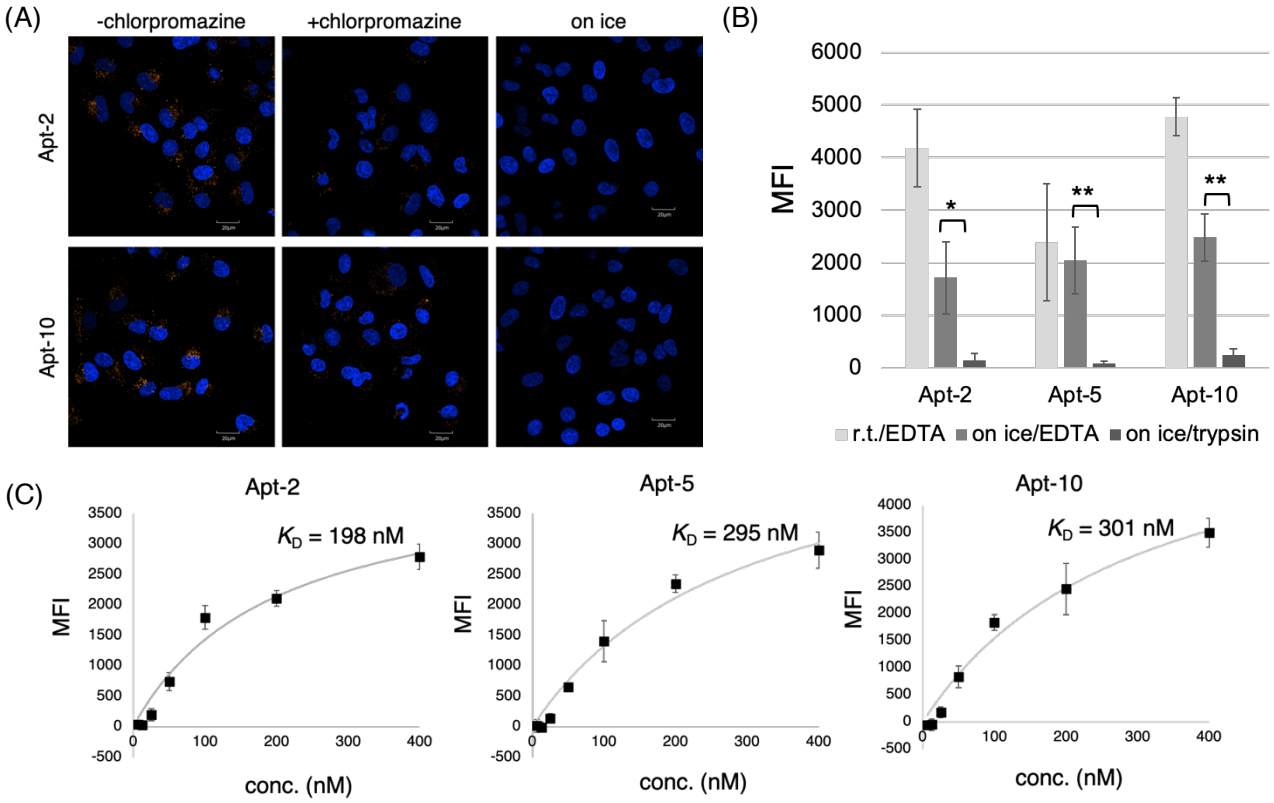


Fig. S5 Investigation of the internalization pathway of Apt-2 and -10

(A) The cells were incubated in the presence or absence of 10 μg/mL chlorpromazine for 1 h. Alexa594-labeled Apt-2 and -10 were added to the cells and incubated for 2 h at 37°C (w/ or w/o chlorpromazine) or on ice (w/o chlorpromazine). The concentration of aptamers was 100 nM. After incubation, the cells were washed with PBS. Cell nuclei were stained with Hoechst33342. The images were taken using CV7000. Scale bars represent 20 μm. The experiment of incubation on ice was not carried out at the same time as the incubation with or without chlorpromazine experiment, but the laser power and the contrast of the red channel were adjusted to the same levels for both experiments.

(B) The cells were detached by 5 mM EDTA or 2.5 g/L trypsin/1 mM EDTA. FAM-labeled Apt-2, -5, and -10 (200 nM) were incubated with the cells for 30 min on ice or at room temperature (r.t.). After incubation, the cells were washed and fixed. The mean fluorescence intensity (MFI) of the cells was measured using LSRFortessa. MFI of non-treated cells was subtracted from each MFI of aptamer treated cells. Error bars show the mean ± SD values of three independent experiments. Student's *t*-test, $*P = 0.052$, $**P < 0.05$.

(C) The cells were detached by 5 mM EDTA. FAM-labeled Apt-2, -5, and -10 (6.25–400 nM) were incubated with the cells for 30 min on ice. After incubation, the cells were washed and fixed. The mean fluorescence intensity (MFI) of the cells was measured using LSRFortessa. MFI of non-treated cells was subtracted from each MFI of the aptamer treated cells. Error bars show the mean ± SD values of three independent experiments. The points were fitted according to the following formula: $Y = B_{MAX}X/(K_D + X)$, where X was the aptamer concentration, Y was the mean fluorescence intensity.

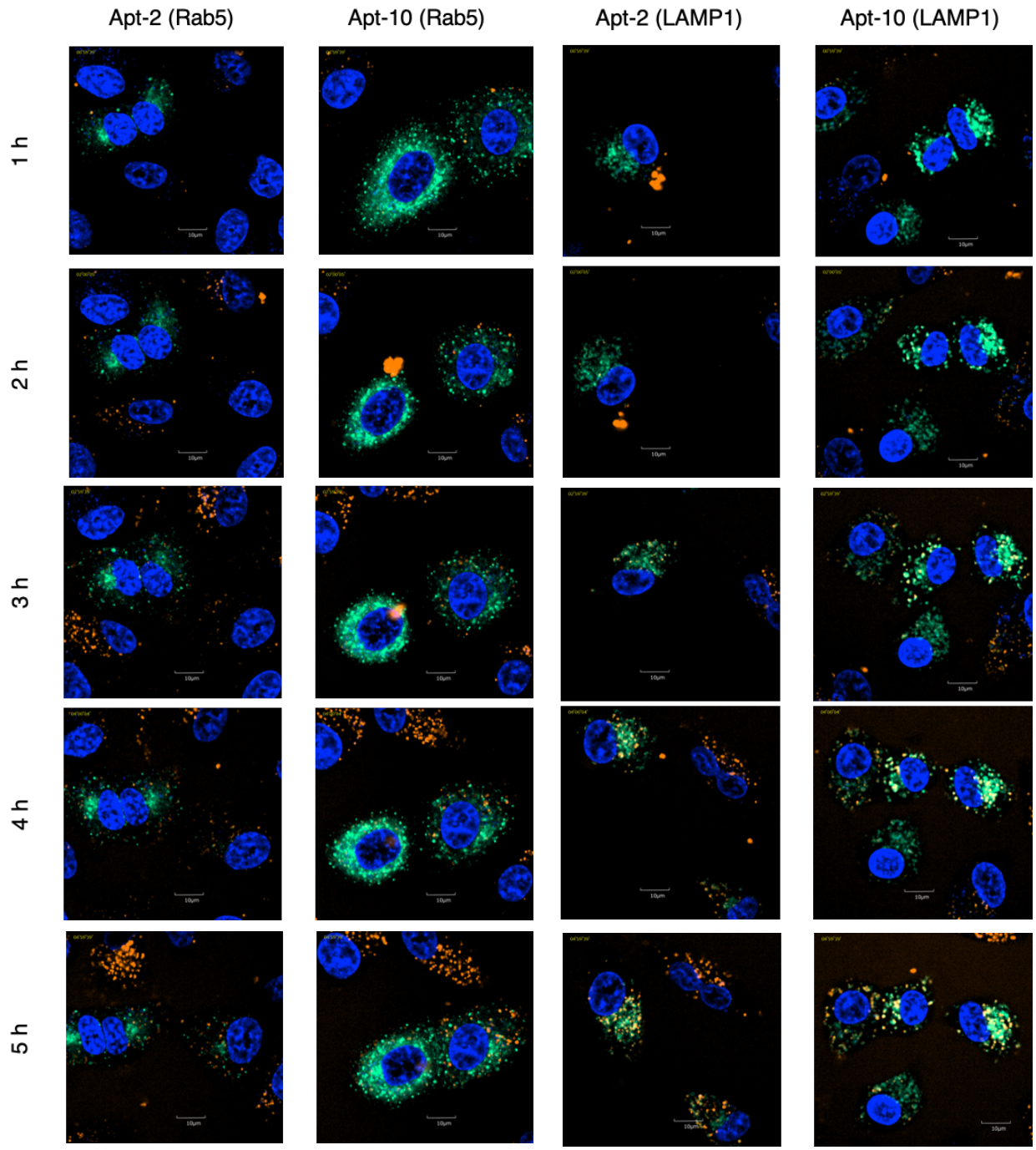


Fig. S6 Localization study of Apt-2 and -10

Fluorescence images were taken every 20 min, while Alexa594-labeled Apt-2 and -10 were incubated with GFP-Rab5- or GFP-LAMP1-expressing A549 cells. Images taken after 1, 2, 3, 4, and 5 h of incubation are shown. Cell nuclei were stained with Hoechst33342. Scale bars represent 10 μm. The contrast of green channels is different in GFP-Rab5 and GFP-LAMP1. The contrast of the images was adjusted to the levels so that the fluorescence of the medium was not visible using CellPathfinder.

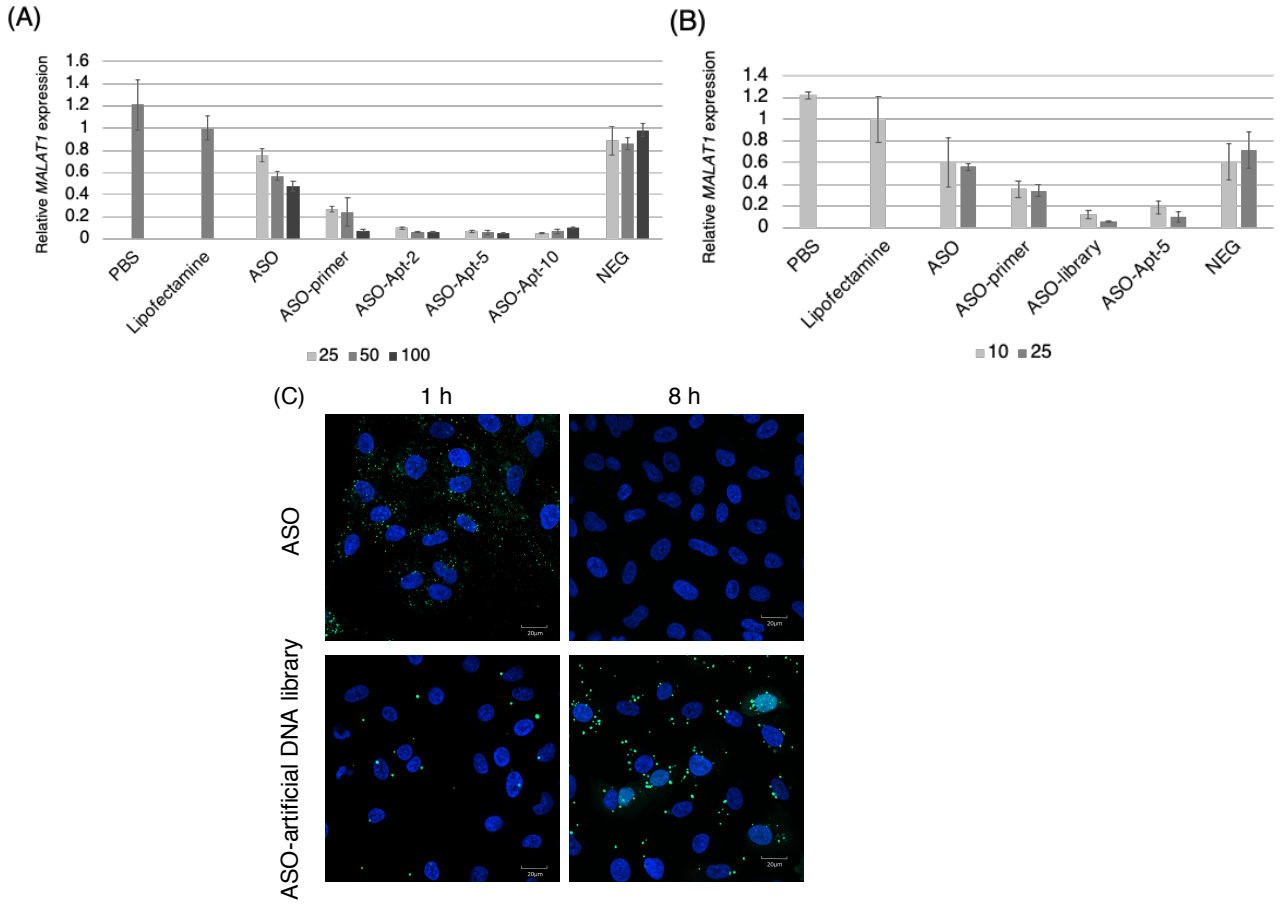


Fig.S7 Confirmation of ASO efficiency with Lipofectamine 3000

(A) ASO and ASO-aptamer conjugates were incubated with A549 cells in the presence of Lipofectamine 3000 for 24 h. The final concentrations of ASOs were 25, 50, or 100 nM. *MALAT1* expression was evaluated by qRT-PCR. *GAPDH* expression was used as a control, and *MALAT1* expression was normalized with Lipofectamine control. ASO-primer was an ASO with the forward primer sequence of aptamers. NEG was a non-targeting antisense oligonucleotide. Error bars show the mean ± SD values of three cell culture wells.

(B) RNA degradation activity of ASO-natural DNA library conjugate with Lipofectamine 3000 was investigated. The final concentrations of ASOs were 10 or 25 nM. *MALAT1* expression was evaluated by qRT-PCR. *GAPDH* expression was used as a control, and *MALAT1* expression was normalized with Lipofectamine control. ASO-primer was an ASO with the forward primer sequence of aptamers. NEG was a non-targeting antisense oligonucleotide. Error bars show the mean ± SD values of three cell culture wells.

(C) Transfection efficiency of ASO and ASO-artificial DNA library conjugate. FAM-labeled ASO and ASO -artificial DNA library conjugate (25 nM, each) was transfected into the cells with Lipofectamine 3000. After 1 h or 8 h incubation, the cells were fixed and permeabilized. Cell nuclei were stained with Hoechst33342. The images were taken using CV7000. Scale bars represent 20 μm. The contrast of the green channel is different between 1 h and 8 h images.

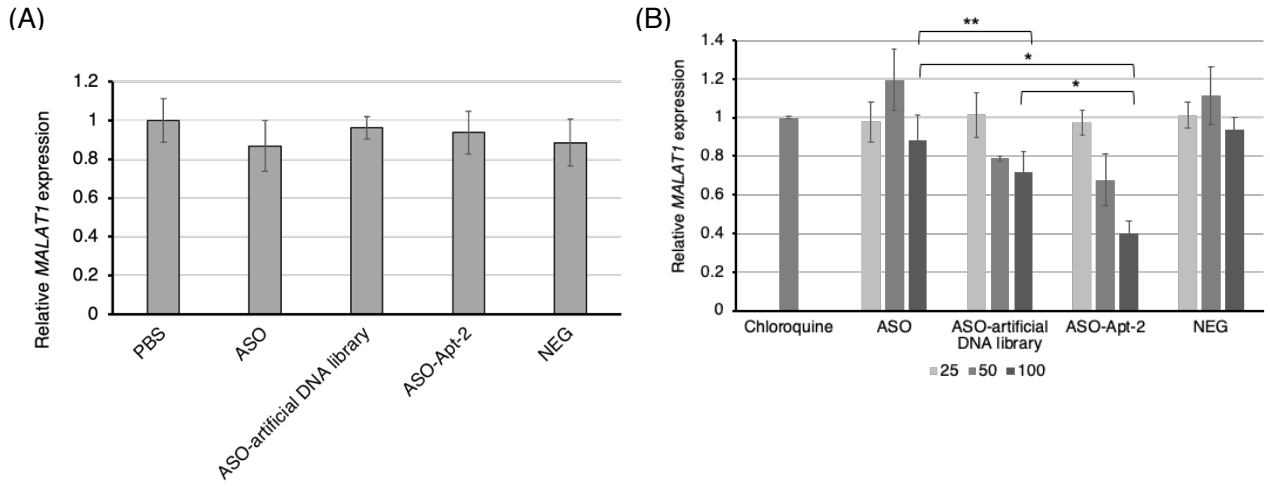


Fig. S8 RNA degradation activity of ASO-artificial DNA library conjugate

ASO, ASO-artificial DNA library conjugate, and ASO-Apt-2 were incubated with A549 cells in the absence (A) or presence (B) of 100 μM chloroquine for 8 h, after which the oligonucleotide and chloroquine were removed. The concentrations of oligonucleotides were 400 nM (A) and 25, 50, and 100 nM (B). The cells were incubated without oligonucleotide and chloroquine for 16 h. *MALAT1* expression was evaluated by qRT-PCR. *GAPDH* expression was used as a control, and *MALAT1* expression was normalized to that of the control cells treated with chloroquine alone. Error bars show the mean ± SD values of three independent experiments. Student's *t*-test, $*P < 0.02$ (ASO-Apt-2 (100 nM) *v.s.* ASO (100 nM) or ASO-artificial DNA library (100 nM)), $**P > 0.15$ (ASO (100 nM) *v.s.* ASO-artificial DNA library (100 nM)).

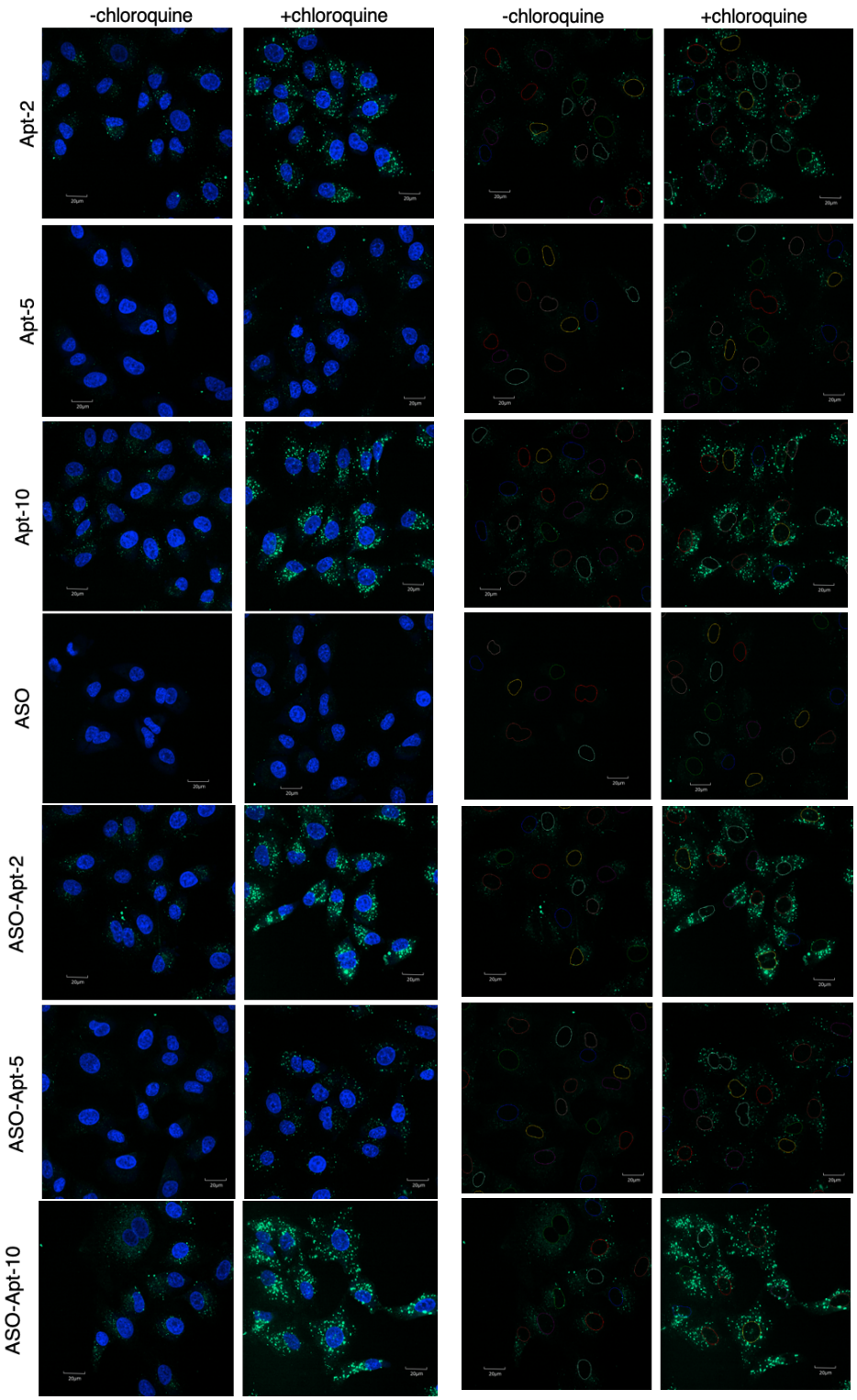
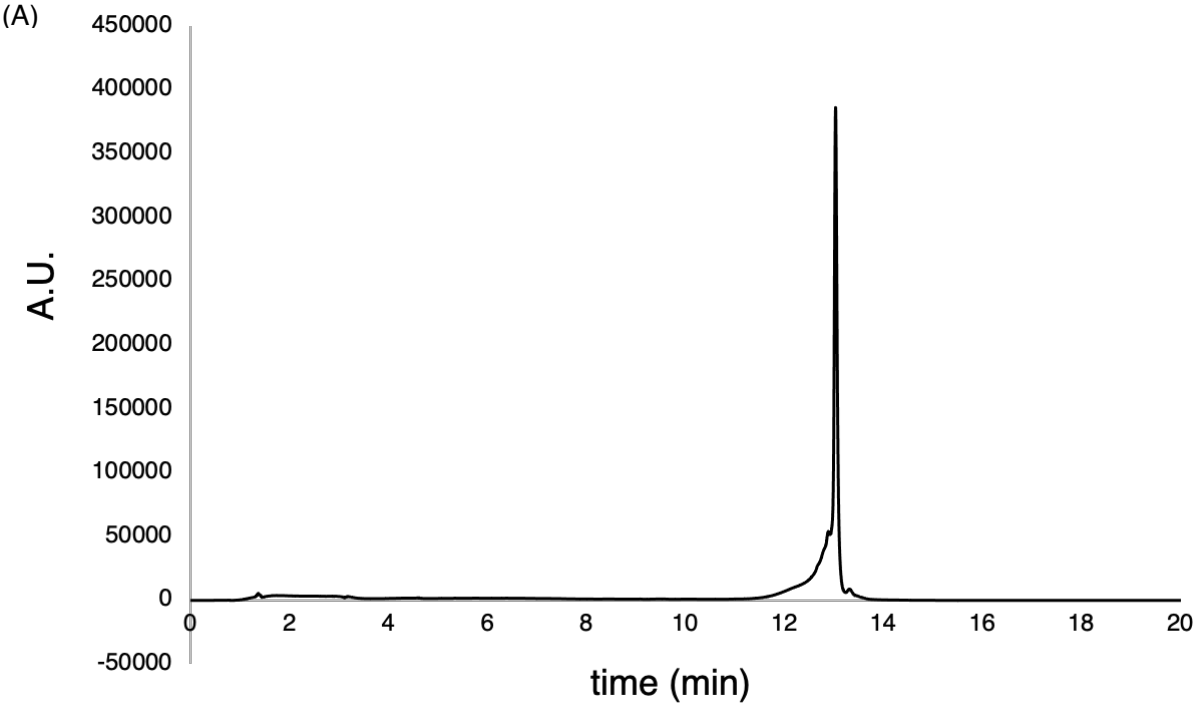
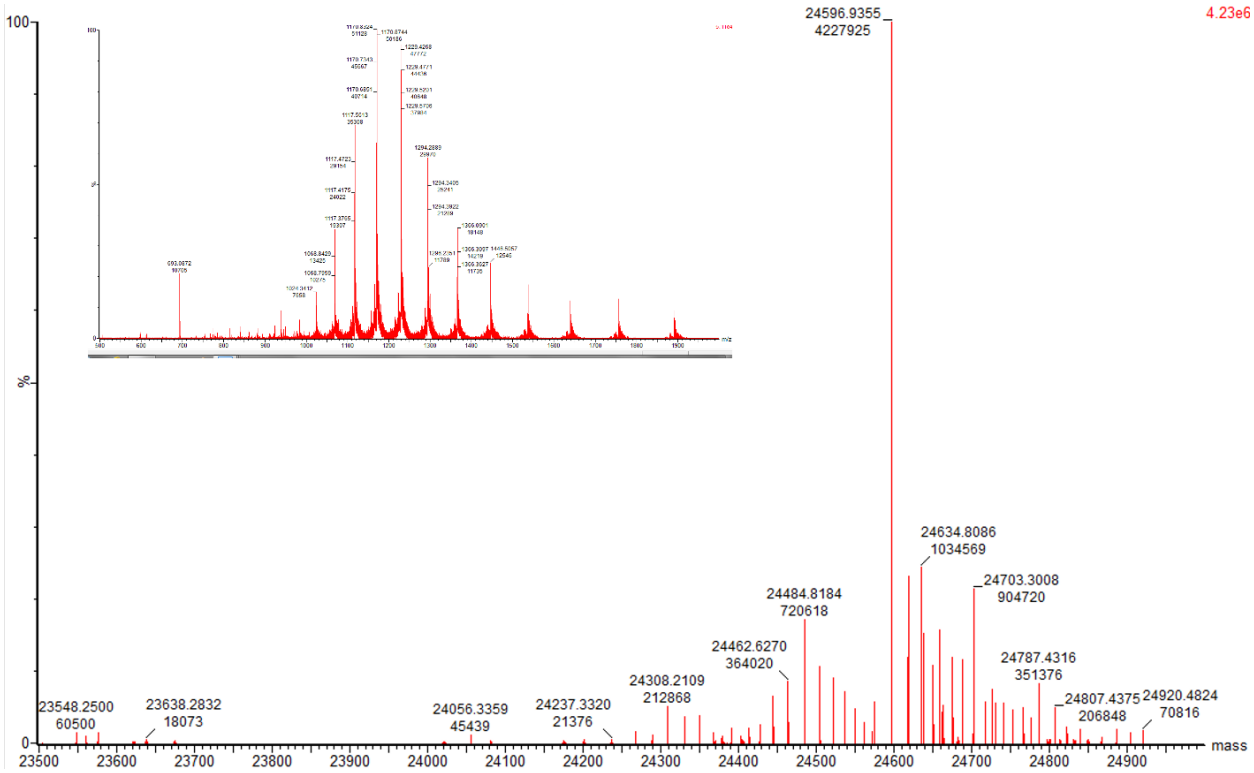


Fig.S9 Internalization and nuclear translocation of aptamers in the presence of chloroquine

Fluorescence images were taken after 1 h incubation with FAM-labeled ASO, Apt-2, -5, -10, ASO-Apt-2, -5, and -10. Cell nuclei were stained with Hoechst33342. The boundary of the nucleus was detected using CellPathfinder. Scale bars represent 20 μm.

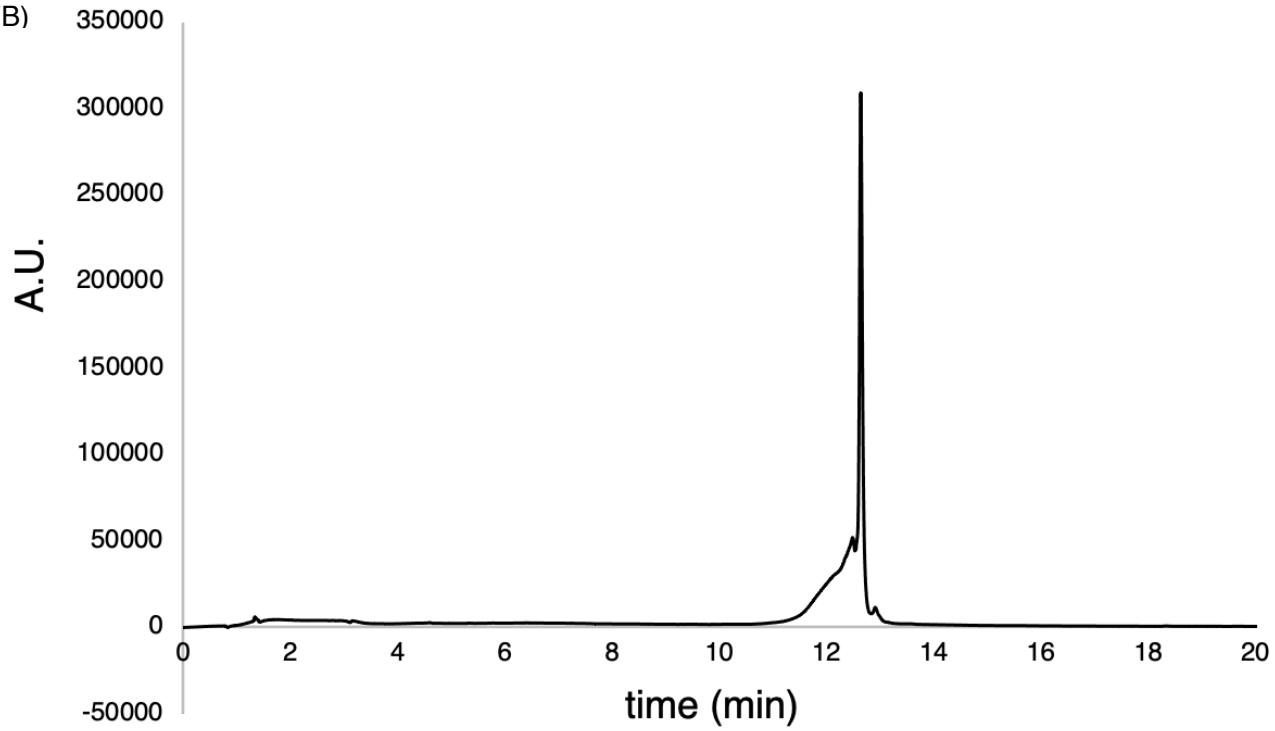
(A)

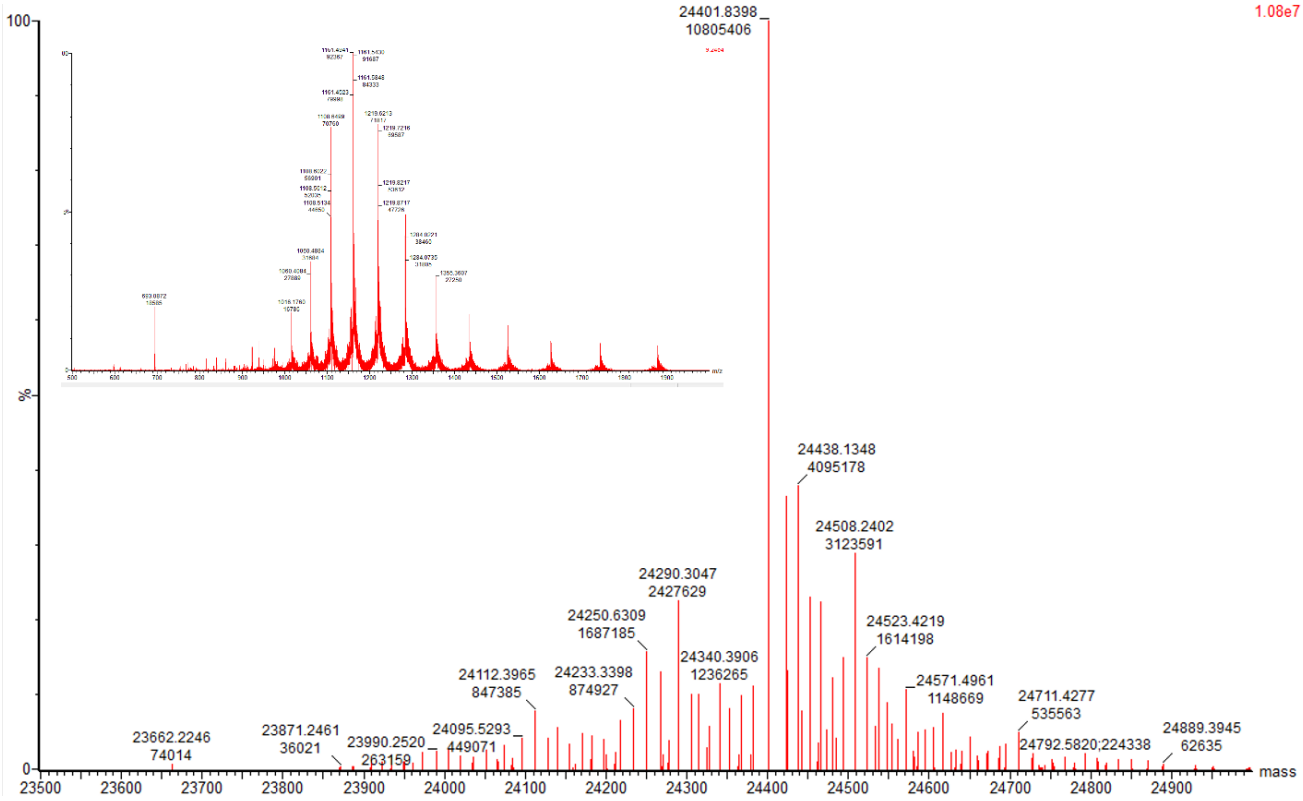




	Calcd.	Found.
$[M-H]^-$	24597	24597

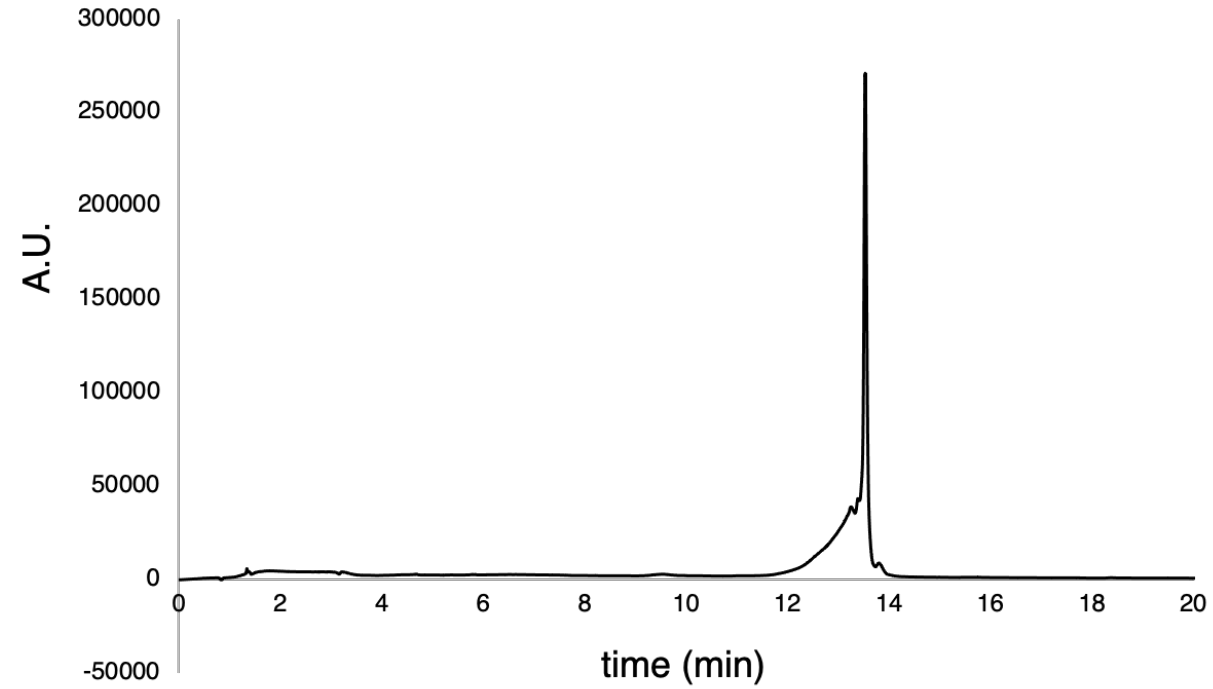
(B)

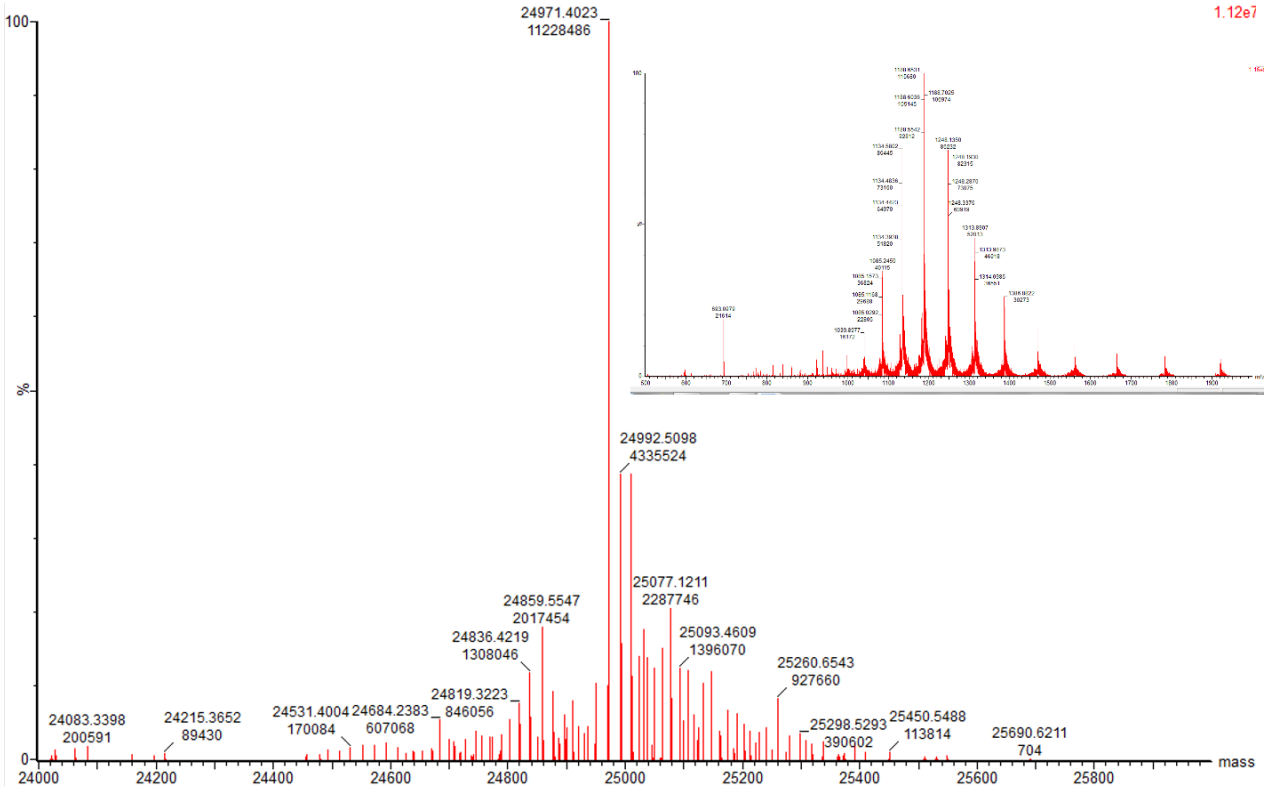




	Calcd.	Found.
$[M-H]^-$	24402	24402

(C)





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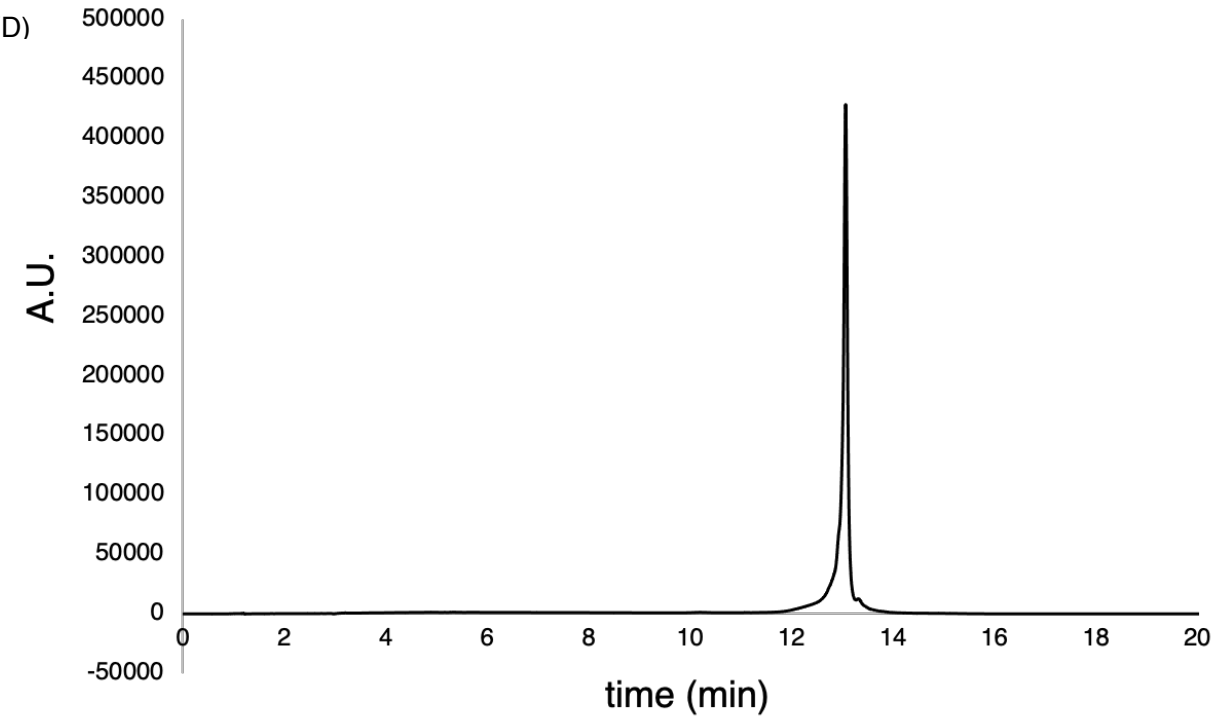
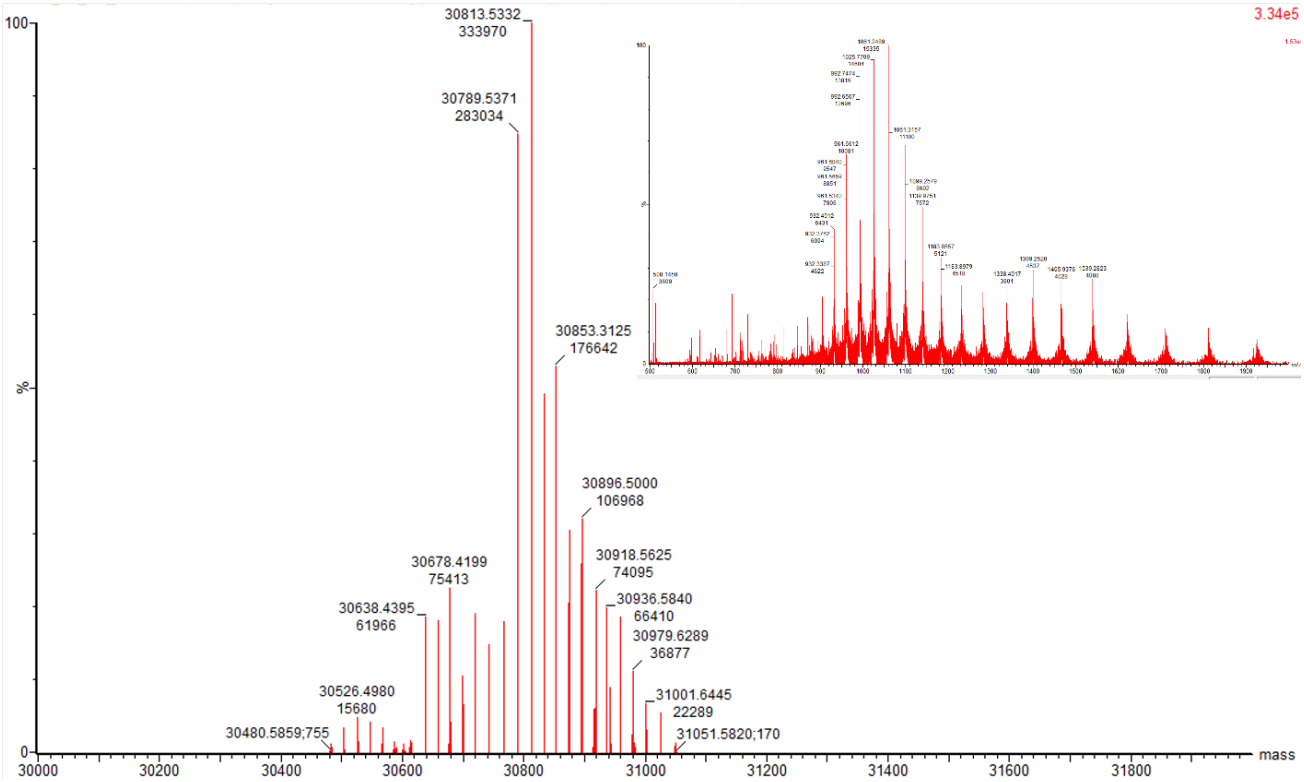
$[M-H]^-$	24971	24971

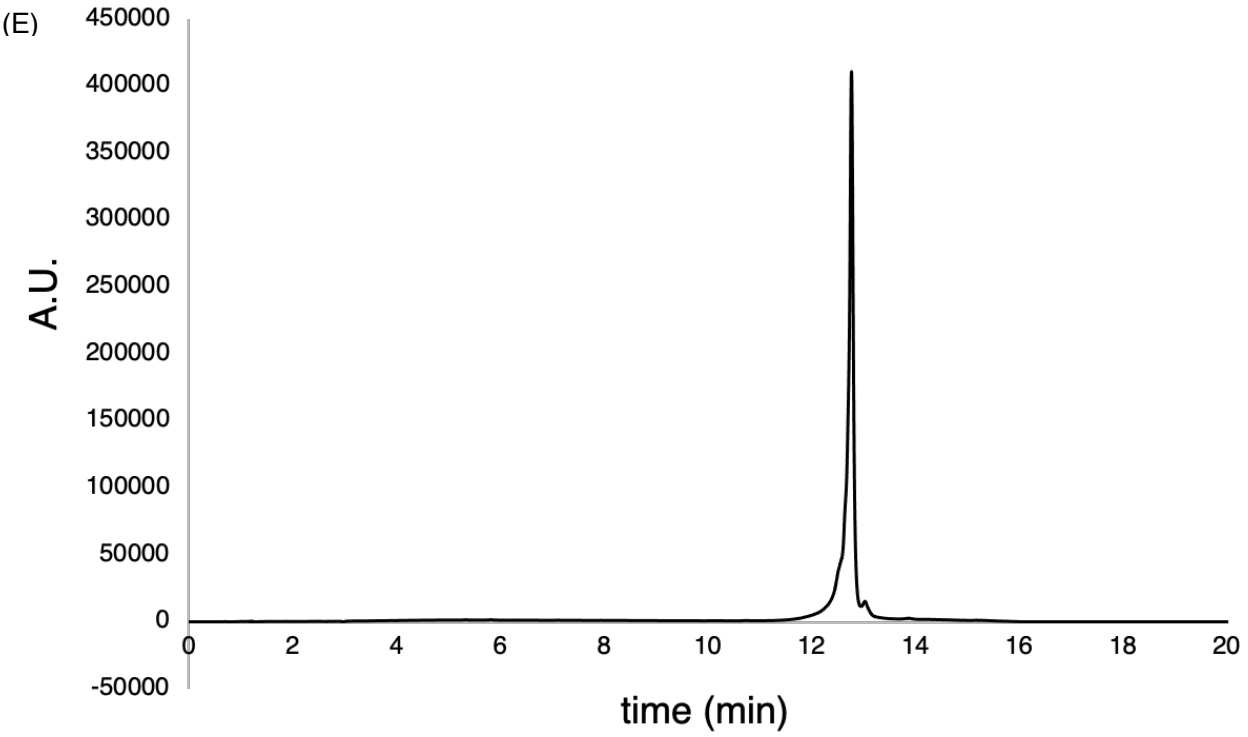
(D)

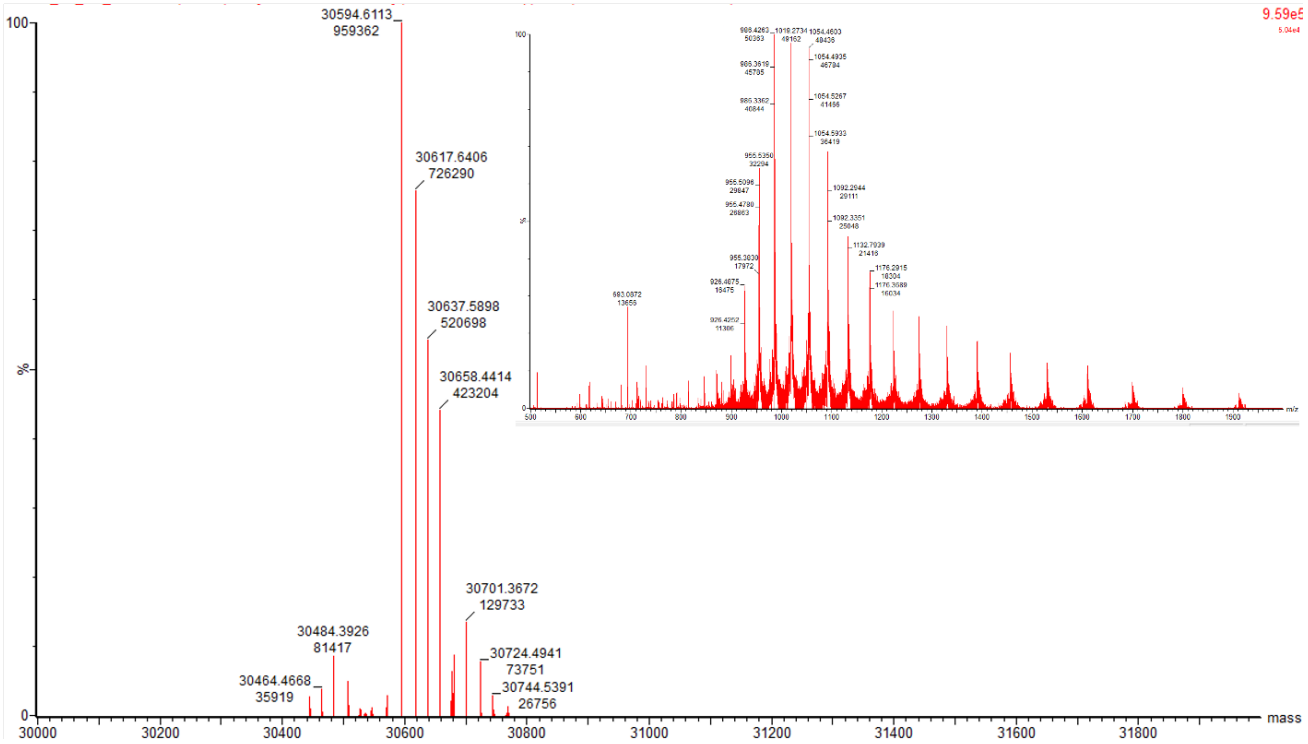




	Calcd.	Found.
$[M-H]^-$	30790	30790
$[M-2H+Na]^-$	30812	30814

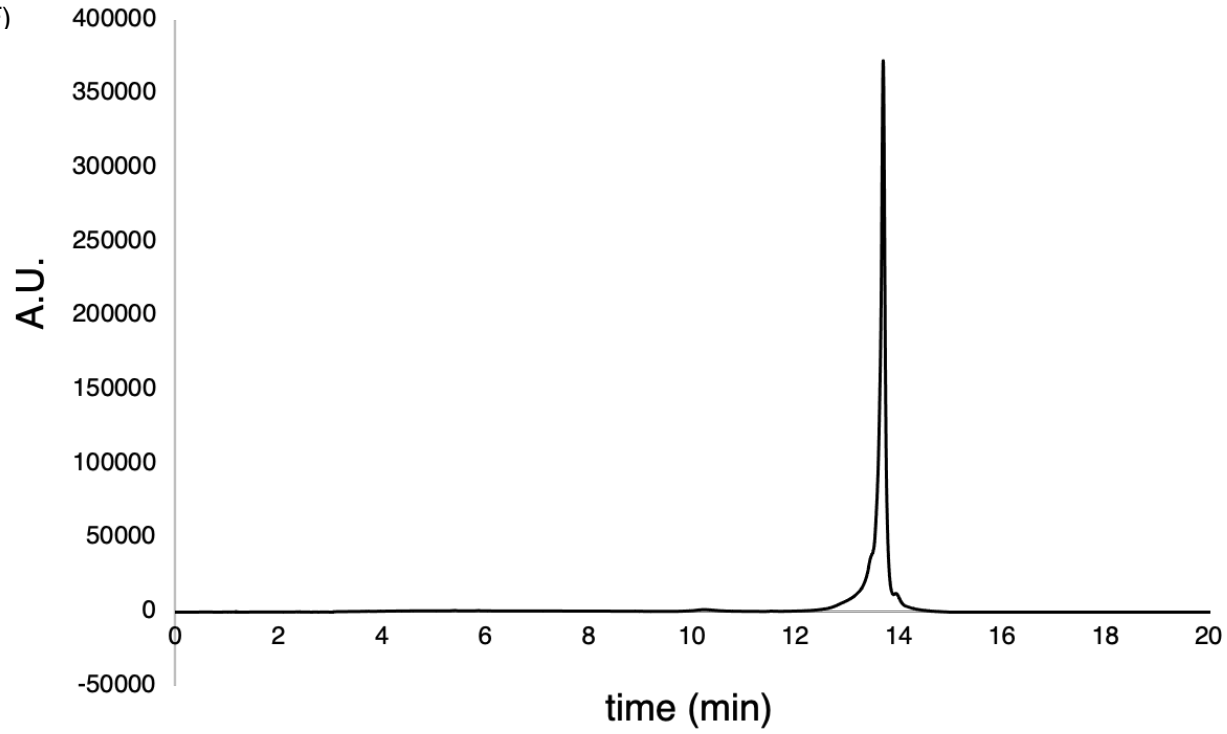
(E)

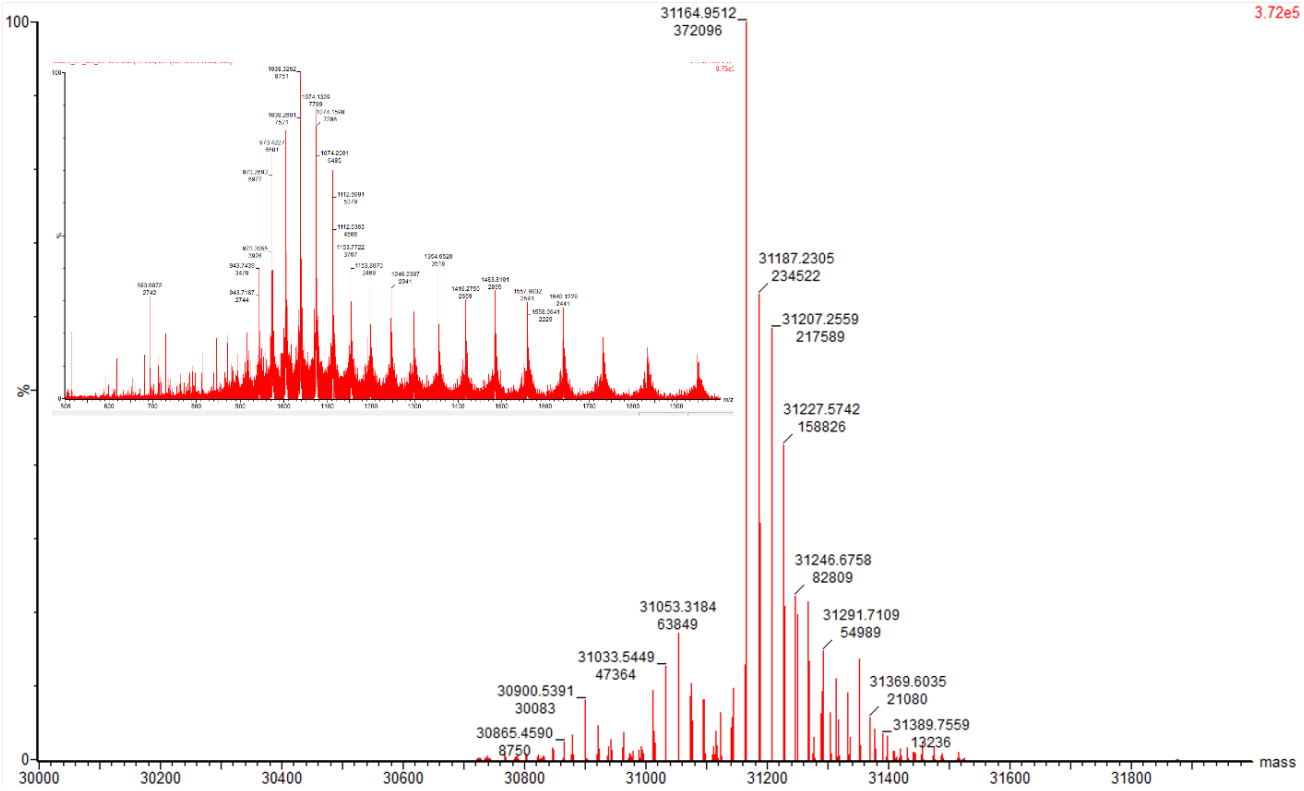




	Calcd.	Found.
$[M-H]^-$	30594	30595
$[M-2H+Na]^-$	30616	30618

(F)





	Calcd.	Found.
$[M-H]^-$	31164	31165
$[M-2H+Na]^-$	31186	31187

Fig. S10 Verify the purification and MS of the aptamers and the ASO-aptamer conjugates

HPLC analysis was performed using the ACQUITY UPLC H-Class system (Waters) with ACQUITY UPLC Oligonucleotide BEH C18 Column (1.7 μm, 2.1 mm × 100 mm, 50 °C) determined by 260 nm absorbance. The sample was eluted (0.3 mL/min) with buffer A (15 mM triethylamine, 400 mM hexafluoro-2-propanol prepared in water) and buffer B (15 mM triethylamine, 400 mM hexafluoro-2-propanol prepared in methanol) with the following: 0 min 20% B, 20 min 50% B.

MS analysis was performed using Xevo G2-S QTof (Waters) (negative mode). The MS results were deconvoluted using the MaxEnt3 program. The results obtained before deconvolution are shown above the results obtained after deconvolution.

(A) FAM-labeled Apt-2, (B) FAM-labeled Apt-5, (C) FAM-labeled Apt-10, (D) ASO-Apt-2 conjugates, (E) ASO-Apt-5 conjugates, (F) ASO-Apt-10 conjugates.

TableS1 Sequences of libraries, primers, and templates that were used in this study.

Capital and small letters indicate DNA and LNA respectively. 6FAM, PHO, and HEX indicate 6-carboxyfluorescein, phosphate, and hexachloro-fluorescein modification respectively. ^ indicate phosphorothioate linkage. C3 indicate C3 spacer. ^{m}c indicate 5-Methylcytidine LNA. N indicates mix of A, G, C, and T.

Name	Sequence
Forward primer	TCGCCTTGCCGGATCGCAGA
Forward primer_FAM	6FAM_TCGCCTTGCCGGATCGCAGA
Forward primer_PHO	PHO_TCGCCTTGCCGGATCGCAGA
Forward primer_Alexa	Alexa594_TCGCCTTGCCGGATCGCAGA
ASO_forward primer	t^t^^{m}c^T^A^T^C^T^T^C^T^A^t^a^CTTTTTTTCGCCTTGCCGGATCGCAGA
ASO_forward primer_FAM	6FAM_t^t^^{m}c^T^A^T^C^T^T^C^T^A^t^a^CTTTTTTTCGCCTTGCCGGATCGCAGA
Reverse primer	GGTGTCAGGCTCACGGACCA
Reverse primer_PHO	PHO_GGTGTCAGGCTCACGGACCA
Reverse primer_HEX	HEX_AAAAAAAAAAAAAAA_C3_GGTGTCAGGCTCACGGACCA
DNA library (sense)	6FAM_TCGCCTTGCCGGATCGCAGANNNNNNNNNNNNNNNNNNNNNNNNNNNNTGGTCCGTGAGCCTGACACC
DNA library (antisensen)	HEX_GGTGTCAGGCTCACGGACCANNNNNNNNNNNNNNNNNNNNNNNNNNNNTCTGCGATCCGGCAAGGCGA
ASO	t^t^^{m}c^T^A^T^C^T^T^C^T^A^t^a^C
NEG	t^g^a^A^C^A^A^A^A^T^A^A^t^a^C
R_Apt-1	GGTGTCAGGCTCACGGACCATCGACATCCCCCGATTCAGAACTGTCGATGTCTGCGATCCAA
R_Apt-2	GGTGTCAGGCTCACGGACCAATAGAGGAGACTCGCTTCTCCCAGGAGACCTCTGCGATCCAA
R_Apt-3	GGTGTCAGGCTCACGGACCAAGCACGGTACACCGACTCGAGGGCGTACCATCTGCGATCCAA
R_Apt-4	GGTGTCAGGCTCACGGACCAGTGGACGACTTCGAGTAACGCTCCGGTTACTCTGCGATCCAA
R_Apt-5	GGTGTCAGGCTCACGGACCAACGTCGGGGATCGCGGGCTCCACAAACCCATCTGCGATCCAA
R_Apt-6	GGTGTCAGGCTCACGGACCAATAGGCGGATTACGCGAGGGCTCCCAAACCTCTGCGATCCAA
R_Apt-7	GGTGTCAGGCTCACGGACCACTGTGCCTGCTCGAGGGCACCACAAACCCTTCTGCGATCCAA
R_Apt-8	GGTGTCAGGCTCACGGACCAACCCGCCGGTAGGAGGAACGCGCTGCTTGCTC

	TGCGATCCAA
R_Apt-9	GGTGTCAGGCTCACGGACCATACCCTCCGTACGAACGTGTCGGTACTCCGTCT GCGATCCAA
R_Apt-10	GGTGTCAGGCTCACGGACCAGATAGTGGACTCCCGCAGACTTCAGAGAAATC TGCGATCCAA
R_Apt-2_PHO	GGTGTCAGGCTCACGGACCAATAGAGGAGACTCGCTTCTCCCAGGAGACCTC TGCGATCCGGCAA_PHO
R_Apt-5_PHO	GGTGTCAGGCTCACGGACCAACGTCGGGGATCGCGGGCTCCACAAACCCATC TGCGATCCGGCAA_PHO
R_Apt-10_PHO	GGTGTCAGGCTCACGGACCAGATAGTGGACTCCCGCAGACTTCAGAGAAATC TGCGATCCGGCAA_PHO
Apt-1_DNA	TCGCCTTGCCGGATCGCAGACATCGACAGTTCTGAATCGGGGGATGTCGATGG TCCGTGAGCCTGACACC
Apt-2_DNA	TCGCCTTGCCGGATCGCAGAGGTCTCCTGGGAGAAGCGAGTCTCCTCTATTGG TCCGTGAGCCTGACACC
Apt-3_DNA	TCGCCTTGCCGGATCGCAGATGGTACGCCCTCGAGTCGGTGTACCGTGCTTGG TCCGTGAGCCTGACACC
Apt-4_DNA	TCGCCTTGCCGGATCGCAGAGTAACCGGAGCGTTACTCGAAGTCGTCCACTG GTCCGTGAGCCTGACACC
Apt-5_DNA	TCGCCTTGCCGGATCGCAGATGGGTTTGTGGAGCCCGCGATCCCCGACGTTGG TCCGTGAGCCTGACACC
Apt-6_DNA	TCGCCTTGCCGGATCGCAGAGGTTTGGGAGCCCTCGCGTAATCCGCCTATTGG TCCGTGAGCCTGACACC
Apt-7_DNA	TCGCCTTGCCGGATCGCAGAAGGGTTTGTGGTGCCCTCGAGCAGGCACAGTG GTCCGTGAGCCTGACACC
Apt-8_DNA	TCGCCTTGCCGGATCGCAGAGCAAGCAGCGCGTTCCTCCTACCGGCGGGTTG GTCCGTGAGCCTGACACC
Apt-9_DNA	TCGCCTTGCCGGATCGCAGACGGAGTACCGACACGTTCGTACGGAGGGTATG GTCCGTGAGCCTGACACC
Apt-10_DNA	TCGCCTTGCCGGATCGCAGATTTCTCTGAAGTCTGCGGGAGTCCACTATCTGG TCCGTGAGCCTGACACC
MALAT1_F-primer	GGTCTCCCCACAAGCAACTT
MALAT1_R-primer	AACCCACCAAAGACCTCGAC
GAPDH_F-primer	GAGTCAACGGATTTGGTCGT
GAPDH_R-primer	GACAAGCTTCCCGTTCTCAG

TableS2 Sequences of aptamers

The positions of U^{trp} are shown as X.

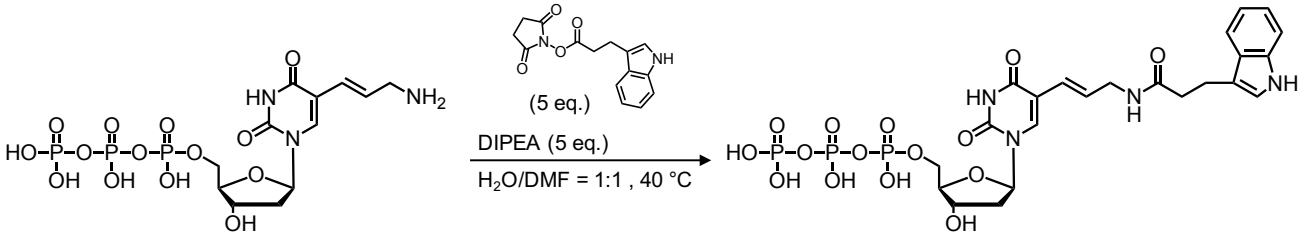
Name of aptamers	Sequence	Number of U^{trp} in random region (30 base)
DNA-1	TCGCCTTGCCGGATCGCAGAGGTCCTCGACTCGTCCTACTGCACTTCTACTGGTCCGTGAGCCTGACACC	0
DNA-2	TCGCCTTGCCGGATCGCAGAATGGGTGGGAGTGGGGTGGGGGCGGGTGGGTGGTCCGTGAGCCTGACACC	0
DNA-3	TCGCCTTGCCGGATCGCAGAGGTGGGGGGTTGGGGGCGGTGGTGGGTCCATGGTCCGTGAGCCTGACACC	0
DNA-4	TCGCCTTGCCGGATCGCAGACTTGGGTGGGAGGGGGTTTTGGGGGGGTGGTGGTCCGTGAGCCTGACACC	0
DNA-5	TCGCCTTGCCGGATCGCAGAGGTTGGGTGGGGGTCTTTCTGGGGGCGGGTTGGTCCGTGAGCCTGACACC	0
Apt-1	TCGCCTTGCCGGATCGCAGACAXCGACAGXXCXGAAXCGGGGGAXGXCGAXGGXCCGXGAGCCXGACACC	7
Apt-2	TCGCCTTGCCGGATCGCAGAGGXCXCCXGGGAGAAGCGAGXCXCCXCXAXXGGXCCGXGAGCCXGACACC	8
Apt-3	TCGCCTTGCCGGATCGCAGAXGGXACGCCCXCGAGXCGGXGXACCGXGCXXGGXCCGXGAGCCXGACACC	8
Apt-4	TCGCCTTGCCGGATCGCAGAGXAACCGGAGCGXXACXCGAAGXCGXCCACXGGXCCGXGAGCCXGACACC	6
Apt-5	TCGCCTTGCCGGATCGCAGAXGGGXXXGXGGAGCCCGCGAXCCCCGACGXXGGXCCGXGAGCCXGACACC	7
Apt-6	TCGCCTTGCCGGATCGCAGAGGXXXGGGAGCCCXCGCGXAAXCCGCCXAXXGGXCCGXGAGCCXGACACC	8
Apt-7	TCGCCTTGCCGGATCGCAGAAGGGXXXGXGGXGCCCXCGAGCAGGCACAGXGGXCCGXGAGCCXGACACC	6
Apt-8	TCGCCTTGCCGGATCGCAGAGCAAGCAGCGCGXXCCXCCXACCGGCGGGXXGGXCCGXGAGCCXGACACC	5
Apt-9	TCGCCTTGCCGGATCGCAGACGGAGXACCGACACGXXCGXACGGAGGGXAXGGXCCGXGAGCCXGACACC	5
Apt-10	TCGCCTTGCCGGATCGCAGAXXXCXCXGAAGXCXGCGGGAGXCCACXAXCXGGXCCGXGAGCCXGACACC	10

Table S3 The washing condition of each SELEX round

After incubation with the libraries, cells were washed once with PBS and several times with 0.5 M NaCl and incubated with 0.5 M NaCl for several minutes. The number of times of 0.5 m NaCl wash and the length of time of 0.5 M NaCl incubation are listed below.

Round	1	2	3	4	5	6	7	8
0.5 M NaCl wash (times)	2	2	2	2	4	5	6	6
0.5 M NaCl incubation time (min)	5	5	5	7	10	10	10	10

Scheme S1 Synthesis of $U^{trp}TP$



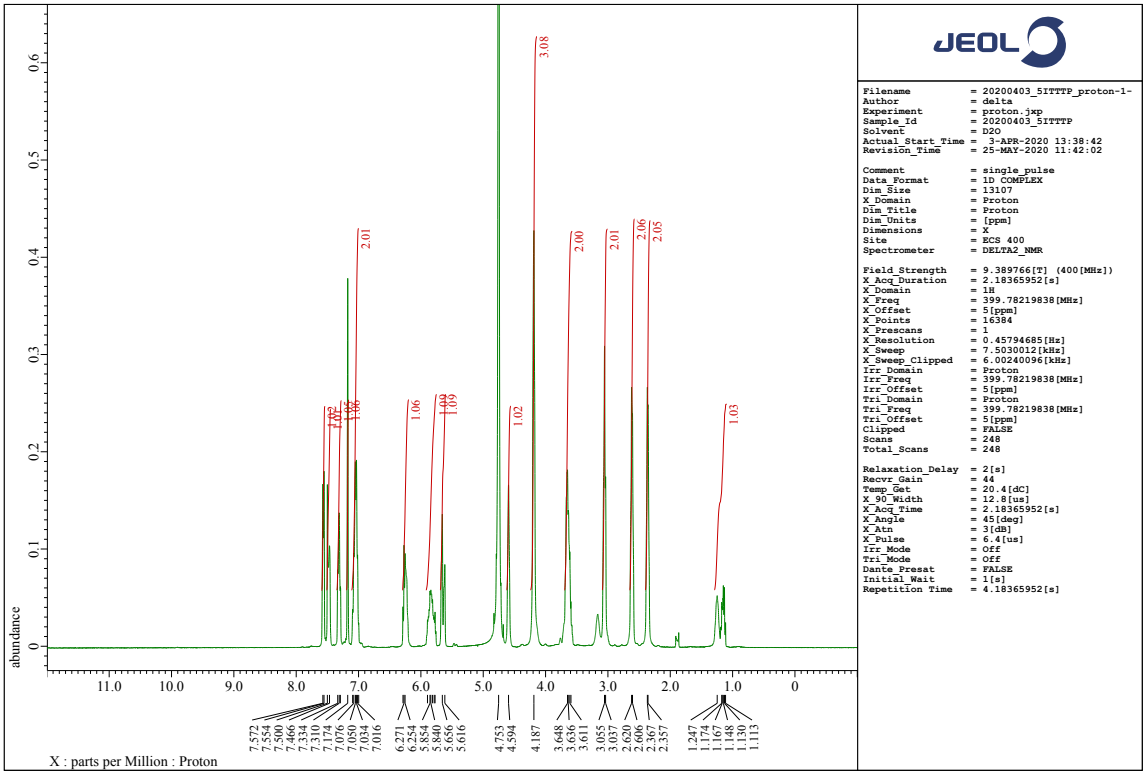
5-(3-aminoprop-1-en-1-yl)-2'-deoxyuridine triphosphate (N-1062, TriLink Bio Technologies (CA, USA)) (10μmol) was dissolved in 100μL distilled water. *N*-Succinimidyl-3-indolepropanoate (50μmol in 100μL DMF), DIPEA (50μmol, 8.3μmol) was added and the reaction mixture was stirred for 3 h at 40 °C. The triphosphate was purified by ethanol precipitation and HPLC (linear gradient: A conc.: B conc. = 99:1 to 60:40, A = 0.1 M TEAA, B = MeCN over 30 min, pH = 7.0, rt, Det.: 260 nm). NMR spectra were recorded on a JEOL JNM-ECS400. FAB mass spectra was measured using a JEOL JMS-S3000 mass spectrometer.

^{1}H NMR (400 MHz, D_2O): δ 7.56 (d, J = 7.2 Hz, 1H), 7.48 (d, J = 13.6 Hz, 1H), 7.31 (d, J = 9.6 Hz, 1H), 7.17 (s, 1H), 7.04 (dd, J = 13.6 Hz, 7.2 Hz, 2H), 6.26 (m, 1H), 5.85 (m 1H), 5.63 (d, J = 16 Hz, 1H), 4.59 (br, 1H), 4.19 (br, 3H), 3.64 (m 2H), 3.04 (t, J = 7.2 Hz, 2H), 2.61 (t, J = 7.2 Hz, 2H), 2.36 (d, J = 4.0 Hz, 2H)

^{31}P NMR (120 MHz, D_2O): δ -5.56, -10.91, -20.88

HRMS (MALDI) calcd for $C_{23}H_{28}N_4O_{15}P_3$ (M^-) 693.0770, found 693.0756.

U^{trp}TP ^{1}H NMR



U^{trp}TP ^{31}P NMR