Electronic Supplementary Information

Tetrahedral DNAzymes for enhanced intracellular gene-silencing activity

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Materials

Chloroform, isopropanol, magnesium chloride, xylene cyanol, bromophenolblue, formadehyde were purchased from Sigma-Aldrich (USA) Complete Protease inhibitor cocktail tablet was purchased from Roche (Switzerland). 20 × TBST was purchased from Translab (Korea). Methanol was purchased from Samchun Medicals (Korea). Absolute ethanol was purchased from Merck (Germany). Unless stated otherwise, all buffers were supplied by Biosesang (Korea). Bradford protein solution was purchased from Bio-Rad (USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) were purchased from Welgene (Korea). Antibiotics (penicillin and streptomycin) were purchased from Gibco (USA). Cell culture plates were purchased from LPS solution (Korea). Primers for reverse-transcriptase PCR of GFP and β-actin forward and reverse primers were purchased from Bioneer (Korea). 10 × RIPA buffer, anti-β-actin and anti-GFP antibody for Western Blot were purchased from Cell Signaling Technology (USA). Horseradish peroxidase (HRP)-conjugated anti-IgG secondary antibody was obtained from Santa Cruz Biotechnology (USA).

Oligonucleotides

DNA oligonucleotides for DNAzymes and 3′-fluorescein-labeled RNA substrate (54-mer) was synthesized on a controlled pore glass (CPG) resin (1 μmol, Bioautomation, USA) using Mermaid 4 DNA synthesizer (Bioautomation, USA). The CPG was deprotected in 30% aqueous NH_3 at 55 ˚C for 16 h. The crude residue was then purified by denaturing (7 M urea) polyacrylamide gel electrophoresis (PAGE), followed by extraction of DNA from gel with 0.1 × TBE. An equal amount of n-butanol was added to the DNA solution and mixed well by gentle shakes, and centrifuged at 3000 rpm for 3 min. The upper phase (n-butanol phase) was decanted. This

extraction step was repeated until the volume of the aqueous phase was approximately 500 µL. DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.2) and 3 volume of absolute ethanol, followed by storing the solution at - 80 ˚C for 2 h. DNA pellet was collected after a centrifugation 13,000 rpm for 20 min and washed with 70% ethanol (200 µL). The pellet was then dried and resuspended in autoclaved double distilled water. All purified oligonucleotides were quantified based on the absorbance at 260 nm measured by Libra S22 UV/Vis Spectrophotometer (Biochrom, UK).

Tetrahedral DNAzyme

Tetrahedral DNAzymes (TDzs) were prepared by self-assembly of three single stranded DNA as shown in Table S1. The oligonucleotides (600 nM) were incubated in TM buffer (10 mM Tris-HCl, 20 mM $MgCl_2$, pH 8) at 95 ˚C for 5 min and then slowly cooled down to 4 ˚C overnight. The assembled constructs were analyzed on 8% native PAGE and visualized based on the fluorescently labeled S1 or S6 in a gel documentation instrument (Chemidoc, Bio-rad, USA). The hydrodynamic sizes of TDzs were measured in TM buffer at room temperature by Zetasizer Nano ZS (Malvern, UK).

Kinetic analysis of RNA cleavage reactions

The RNA cleavage reaction was performed by incubating the fluorescein-labeled RNA substrate (100 nM) and a DNAzyme (100 nM) in reaction buffer (50 mM Tris HCl, 10 mM $MgCl_2$, pH 8). The reaction was quenched at various time points by adding the gel loading buffer (20 µL, 98% (*v/v*) formaldehyde, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). The reaction mixtures were analyzed on a 20% denaturing PAGE (7 M urea) and then visualized by

Chemidoc (Bio-rad, USA). The amounts of the remaining RNA substrate (S) was estimated using ImageJ. The cleaved RNA product (Pt) was estimated by 100 – S (%). The kinetic parameter k_{obs} was determined by fitting the time-course curve of the cleaved RNA product (P_t) based on the peudo-first order kinetics formula $P_t = P_{max} \cdot [1 - \exp(-k_{obs} \cdot t)]$, where P_{max} and t denote the maximum cleaved RNA product and the reaction time, respectively. Curve-fitting was performed using the Origin™ software (OriginLab, USA).

Cell culture

NIH3T3 cells used for cellular uptake experiments were kindly provided by Dr. Eun Gyeong Yang (KIST). For gene-silencing experiment, EGFP-positive cells were used. To generate the EGFP-positive cells, the bovine fetal fibroblasts, which are previously isolated[1], were used. The cDNA for EGFP and puromycin resistance gene were linked by 2A peptide sequences and amplified by PCR. The cDNAs were inserted into piggybac (PB) transposon expression vector including CAG promoter by Infusion cloning (Invitrogen, USA).[1-2] The EGFP plasmids with transposase were transfected into the fibroblasts by electroporation (Neon®, Invitrogen, USA). The transfected cells were cultured with the culture medium (DMEM supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acid, 1% penicillin-streptomycin, and 100 mM β-mercaptoethanol) containing 2 μg/mL puromycin (Thermo Fisher Scientific, USA) for 4 days. Survived GFP positive cells were sub-cultured into the new culture dishes. Both NIH3T3 and EGFP-positive fibroblast cell lines were propagated in DMEM supplemented with 100 U/ml penicillin, 100 ug/ml streptomycin and 10% FBS. Cells were grown to confluence of 80-90% in a humidified incubator supplied with 5% CO_2 and used for experiments between passages second and sixth.

Cellular uptake

NIH3T3 cells (8×10^4) were seeded on a 12-well plate and incubated for 24 h at 37 ˚C with 5% CO_2. Then, the cells were treated with FAM-labeled DNAzymes (300 nM) in DMEM supplemented with 0.5% FBS for 48 h. The cells were washed with DPBS and treated with 0.01% trypsin (1 mL). The resulting cell suspensions were centrifuged for 3 min at 1500 g. The supernatant was removed, and the cell pellets were resuspended in DPBS (250 μL, 2.67 mM KCl, 1.47 mM KH_2PO_4, 138 mM NaCl, 8.06 mM $Na_2HPO_4.7H_2O$). The cellular uptake efficiency was estimated by measuring fluorescence intensity of the cells by a flow cytometry (Guava, Millipore, USA). Samples of 10,000 cells were analyzed in three independent experiments to obtain the average and the standard deviation.

Gene silencing

EGFP-positive fibroblast cells (4×10^4) were seeded on 12 well-plate to 60-70% confluency. After 24 h, cells were treated with DNAzymes (2 μM) in DMEM supplemented with 0.5% FBS at 37 ˚C for 48 h. The cells were washed twice with DPBS and then detached by 0.01% trypsin (1 ml). After two rounds of centrifugation (3 min, 1,500 g), cells were resuspended in DPBS (250 μL). EGFP-positive cells remaining were quantified by a flow cytometry (Guava, Millipore, USA). Samples of 10,000 cells were analyzed in three independent experiments to obtain the average and the standard deviation.

Western blot

EGFP-positive fibroblast cells (4×10^4) cultured on 12 well-plate to 60-70% confluency were treated with DNAzymes (2 μM) in DMEM supplemented with 0.5% FBS at 37 ˚C for 48 h,

trypsinized, and washed with DPBS twice. The cells were treated with RIPA cell lysis buffer for 1 h on ice, followed by centrifugation (12,000 rpm, 20 min) at 4 ˚C. The supernatant containing soluble proteins were quantified by the Bradford assay. The protein extract (20 μg) was separated on 5 - 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membrane (100 min, 350 mA, 100V). The membrane was treated with 5% (w/v) bovine serum albumin (BSA) in TBST buffer (100 mM Tris-base, pH 7.6, 150 mM NaCl and 0.1% Tween 20) for 20 min at room temperature. The anti-GFP antibody (1:1,000) and anti-β-actin antibody (1:1,000) was added to the membrane and incubated overnight at 4 ˚C. Finally, the membrane was treated with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) for 2 h at room temperature. The bands were stained by SuperSignal™ West Pico Chemiluminescent (Thermo Fischer Scientific, USA) and imaged by WSE-6100 Luminograph system (Atto, Japan). The amounts of the expressed EGFP were quantified using ImageJ.

Reverse transcriptase PCR

After treatment of EGPF-positive cells with DNAzymes as described above, cellular RNA was subsequently isolated from the cells using an Easy Blue total mRNA extraction kit (iNtrON Biotechnology, Korea) according to the manufacturer's protocol. All RNA samples were quantified by Nanodrop Spectrophotometer (Thermo Fischer Scientific, USA) and reverse transcribed to cDNA using Accupower Rocket kit (Bioneer, Korea). The primers used for PCR are as follows: 5′-AAGCTGACCCTGAAGTTCATCTGC (EGFP mRNA, forward), 5′-CTTGTAGTTGCCGTCGTCCTTGAA (EGFP mRNA, reverse) and, for normalization, 5′-AGAGCTACGAGCTGCCTGAC (β-actin mRNA, forward), and 5′-AGCACTGTGTTGGCGTACAG (β-actin mRNA, reverse). The PCR reaction mixture (20 μL)

was prepared in 1 × Taq reaction buffer (2 μL, Bioneer, Korea) containing forward and reverse primers (250 nM), cDNA (45 ng), dNTP (500 μM), Taq polymerase (1 unit, New England Biolabs, USA). The cDNA of EGFP mRNA was amplified in Applied Biosystem 2720 thermocycler (Applied Biosystems, USA) through the thermocycling process composed of initial denaturation (95 ˚C, 5 min), followed by 30 cycles of amplification (denaturation at 95 ˚C for 30 sec, annealing at 66 ˚C for 30 sec, and extension at 72 ˚C for 45 sec) and a final elongation step at 72 ˚C for 10 min. For PCR amplification of cDNA of β-actin mRNA, the mixture was subject to initial denaturation (95 ˚C, 5 min), followed by 25 cycles of amplification (denaturation at 95 ˚C for 30 sec, annealing at 56 ˚C for 30 sec, and extension at 72 ˚C for 90 sec) and a final elongation step at 72 ˚C for 10 min. The PCR products were analyzed on a 1% agarose gel and stained using SYBR® Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, USA). The mRNA levels were quantified using ImageJ.

Table S1. Oligonucleotide sequences used in this study. The sequences of the Dz catalytic domain are underlined. iSp18 denotes hexa(ethylene glycol) linker. Each target sequence in the RNA substrate is indicated with the same color as that of the complementary Dz sequence. The T to G mutation at the loop region in the mutant strands (m-S3, m-S4, and m-S5) is indicated with the red colored bold style.

Strand	**Sequence (5′→ 3′)**
S1	GCTACACGAGAAGAGCCGCCATAGTAACACATTCCTAAGTCTGAAA CATTACAGC
S2	GCTCTTCACTCAACTGCCTGGTGATAAAACGACACTACGTGGGAATC TACTATGG

S3	GTGTCGTTTGTTGTACTTCCGAGCCGGTCGAAAGCTTGTGACTTCAG ACTTAGGAATGTGCTTCCCACG
m-S3	GTGTCGTTTGTTGTACTTCCGAGCCGGTCTAAAGCTTGTGACTTCAG ACTTAGGAATGTGCTTCCCACG
S4	GTCTGAAACGGATGTTGTCCGAGCCGGTCGAAGTCCTCCTGAGAAG AGCCGCCATAGTAACACATTCCT
m-S4	GTCTGAAACGGATGTTGTCCGAGCCGGTCTAAGTCCTCCTGAGAAGA GCCGCCATAGTAACACATTCCT
S5	GCTCTTCACGTCGATGCTCCGAGCCGGTCGAATTCAGCTCAAACGAC ACTACGTGGGAATCTACTATGG
m-S5	GCTCTTCACGTCGATGCTCCGAGCCGGTCTAATTCAGCTCAAACGAC ACTACGTGGGAATCTACTATGG
S6	GTGTCGTTTGTTGTACTTAGCTTGTGACTTCAGACTTAGGAATGTGCT TCCCACG
ssDz-A	GTTGTACTTCCGAGCCGGTCGAAAGCTTGTG
ssDz-B	GGATGTTGTCCGAGCCGGTCGAAGTCCTCCT
ssDz-C	GTCGATGCTCCGAGCCGGTCGAATTCAGCTC
RNA substrate	GAGCUGAAGGGCAUCGAC/iSp18/AGGAGGACGGCAACAUCC/iSp18/C ACAAGCUGGAGUACAAC-fluorescein

Table S2. Combination of DNA strands to assemble TDzs

TDz	**Strand**

TDz_1-A	S1/S2/S3
TDz_1-B	S2/S4/S6
TDz_1-C	S1/S5/S6
TDz_2-AB	S2/S3/S4
TDz_2-BC	S4/S5/S6
TDz_2-AC	S1/S3/S5
TDz_3-ABC	S3/S4/S5

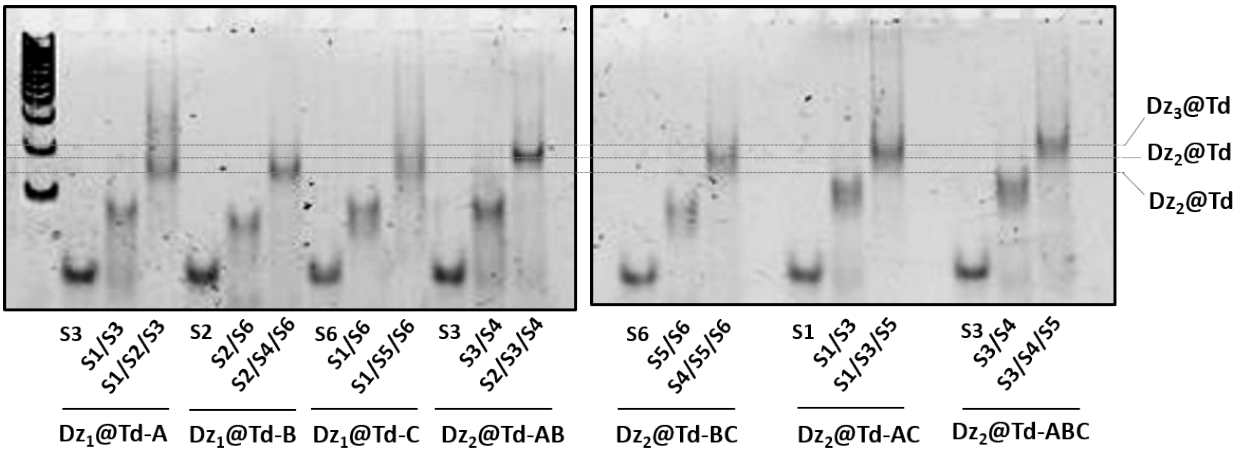


Figure S1. Native PAGE (8%) to analyze self-assembled TDzs. The 100-bp size ladder is shown in the left lane.

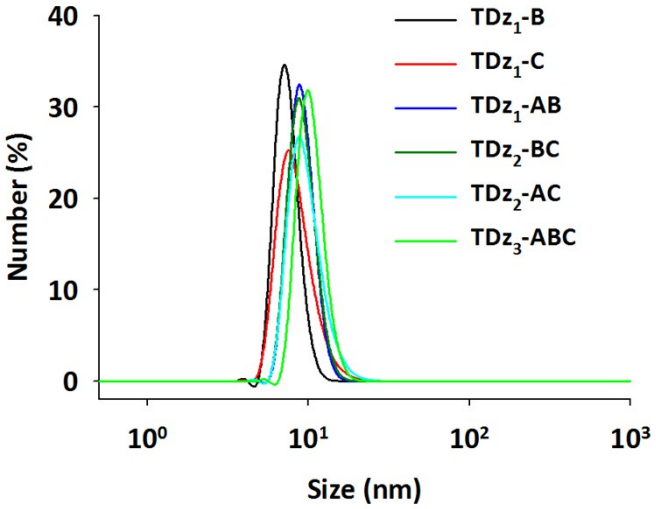


Figure S2. Dynamic light scattering analysis to measure hydrodynamic sizes of TDz_1-A (8.1 ± 0.3 nm), TDz_1-B (8.1 ± 0.8 nm), TDz_1-C (8.4 ± 0.6 nm), TDz_2-AB (8.7 ± 0.6 nm), TDz_2-BC (8.7 ± 0.7 nm), TDz_2-AC (8.9 ± 0.6 nm), and TDz_3-ABC (9.1 ± 0.6 nm).

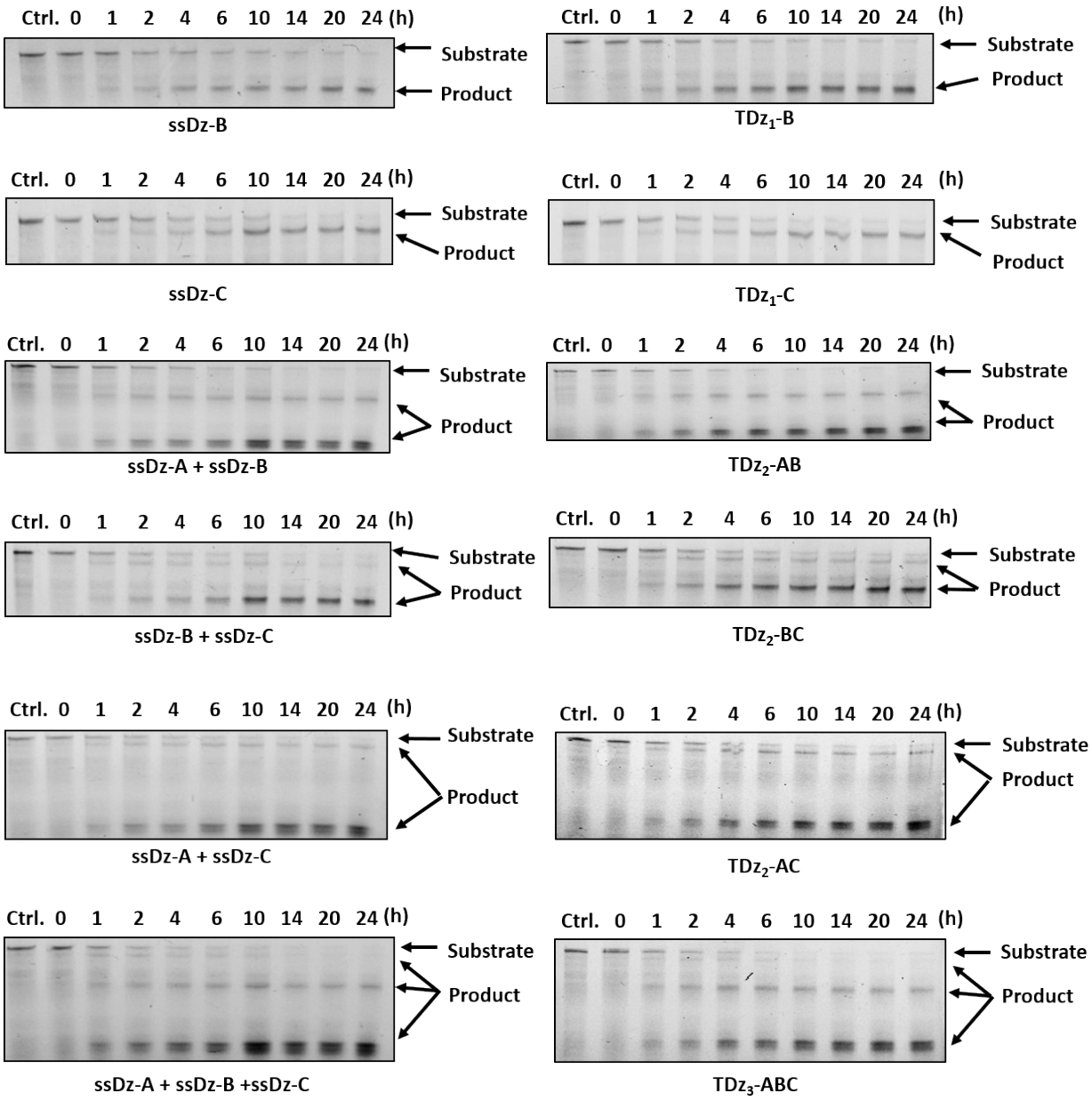
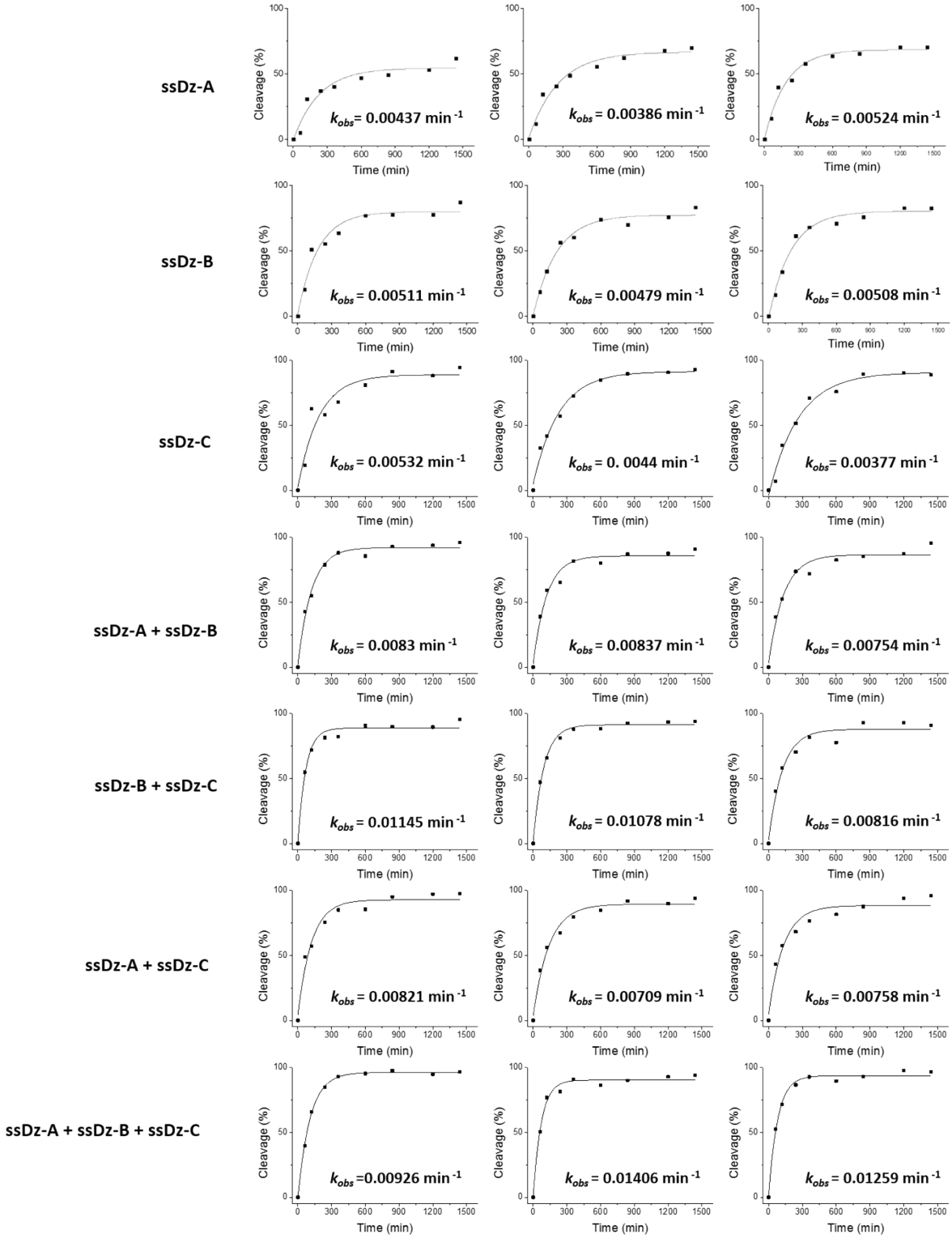
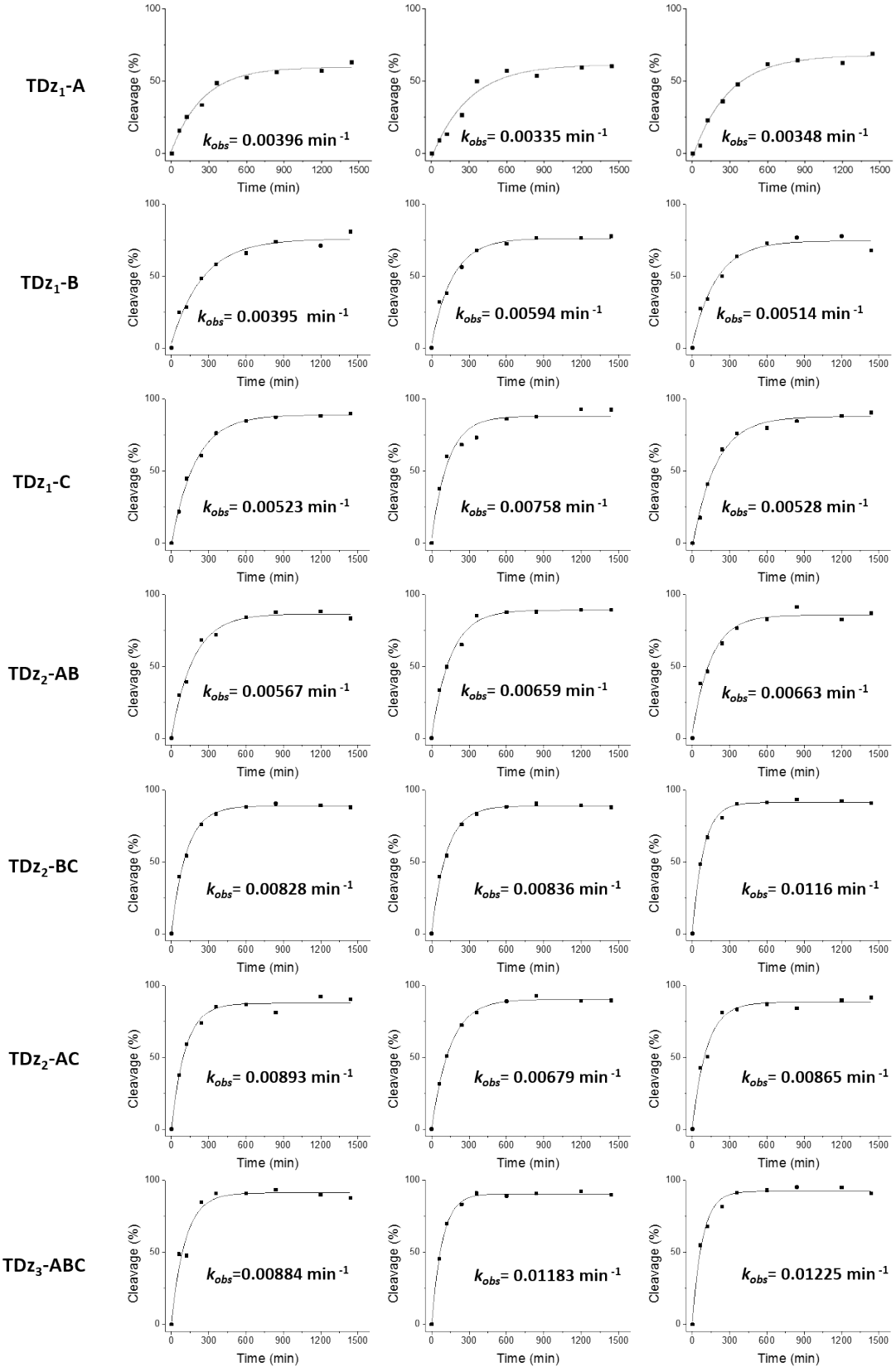


Figure S3. Denaturing PAGE (20%) analysis of RNA cleavage reactions by Dzs.

a


ssDz-A
ssDz-B
ssDz-C
ssDz-A + ssDz-B
ssDz-B + ssDz-C
ssDz-A + ssDz-C
ssDz-A + ssDz-B + ssDz-C
Cleavage (%)
Time (min)
k_{obs}= 0.00437 min^{-1}
k_{obs} = 0.00386 min^{-1}
k_{obs} = 0.00524 min^{-1}
k_{obs} = 0.00511 min^{-1}
k_{obs} = 0.00479 min^{-1}
k_{obs} = 0.00508 min^{-1}
k_{obs} = 0.00532 min^{-1}
k_{obs} = 0. 0044 min^{-1}
k_{obs}= 0.00377 min^{-1}
k_{obs} = 0.0083 min^{-1}
k_{obs} = 0.00837 min^{-1}
k_{obs} = 0.00754 min^{-1}
k_{obs} = 0.01145 min^{-1}
k_{obs} = 0.01078 min^{-1}
k_{obs} = 0.00816 min^{-1}
k_{obs} = 0.00821 min^{-1}
k_{obs} = 0.00709 min^{-1}
k_{obs} = 0.00758 min^{-1}
k_{obs} =0.00926 min^{-1}
k_{obs} = 0.01406 min^{-1}
k_{obs} = 0.01259 min^{-1}

b


TDz1-A
kobs= 0.00396 min-1
kobs= 0.00335 min-1
kobs= 0.00348 min-1
TDz1-B
kobs= 0.00395 min-1
kobs= 0.00594 min-1
kobs= 0.00514 min-1
TDz1-C
kobs= 0.00523 min-1
kobs= 0.00758 min-1
kobs= 0.00528 min-1
TDz2-AB
kobs= 0.00567 min-1
kobs= 0.00659 min-1
kobs= 0.00663 min-1
TDz2-BC
kobs= 0.00828 min-1
kobs= 0.00836 min-1
kobs= 0.0116 min-1
TDz2-AC
kobs= 0.00893 min-1
kobs= 0.00679 min-1
kobs= 0.00865 min-1
TDz3-ABC
kobs=0.00884 min-1
kobs= 0.01183 min-1
kobs= 0.01225 min-1
Cleavage (%)
Time (min)
0
50
100
0
300
600
900
1200
1500

c

DNAzyme	$k_{obs} \times 10^3$ (min^{-1})
ssDz-A	4.5 (0.7)
ssDz-B	5.0 (0.2)
ssDz-C	5.5 (2.4)
ssDz-A + ssDz-B	8.1 (0.5)
ssDz-B + ssDz-C	10.1 (1.7)
ssDz-A + ssDz-C	7.9 (0.4)
ssDz-A + ssDz-B + ssDz-C	12.0 (2.5)
TDz_1-A	3.5 (0.3)
TDz_1-B	5.0 (1.0)
TDz_1-C	6.3 (1.7)
TDz_2-AB	6.3 (0.5)
TDz_2-BC	9.4 (1.9)
TDz_2-AC	8.1 (1.2)
TDz_3-ABC	11.0 (1.8)

Figure S4. The k_{obs} values of (a) ssDzs and (b) TDzs estimated from three independent experiments to provide the averaged values and standard deviations as shown in (c) and Figure 3b.

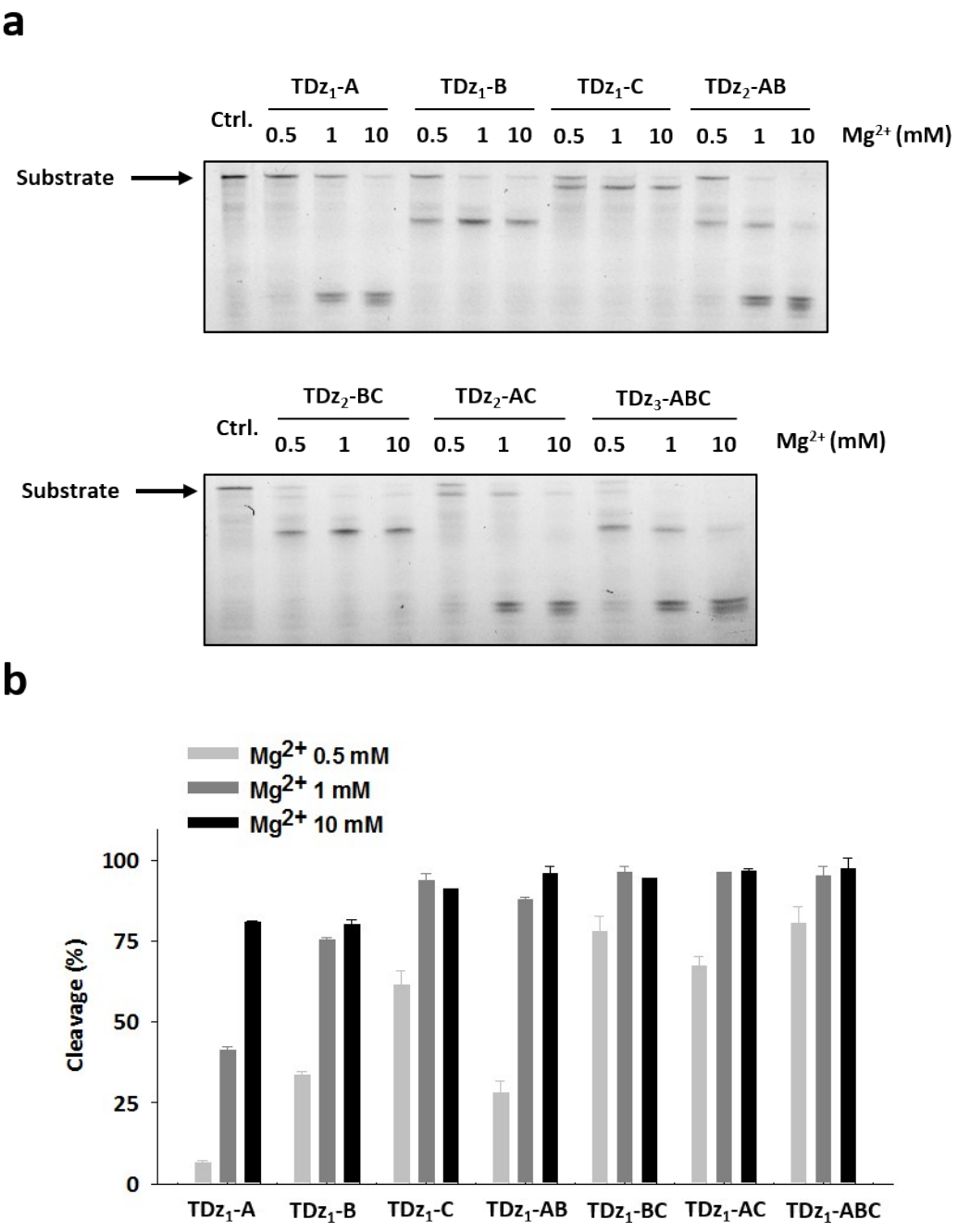


Figure S5. (a) PAGE analysis of the activity of TDzs at various magnesium concentrations. The control (Ctrl.) indicates the RNA substrate without TDzs. (b) The RNA cleavage activity of TDzs is displayed in a bar graph after quantifying the amount of the remaining substrate using ImageJ as described in the method section. All values are the results of two independent experiments.

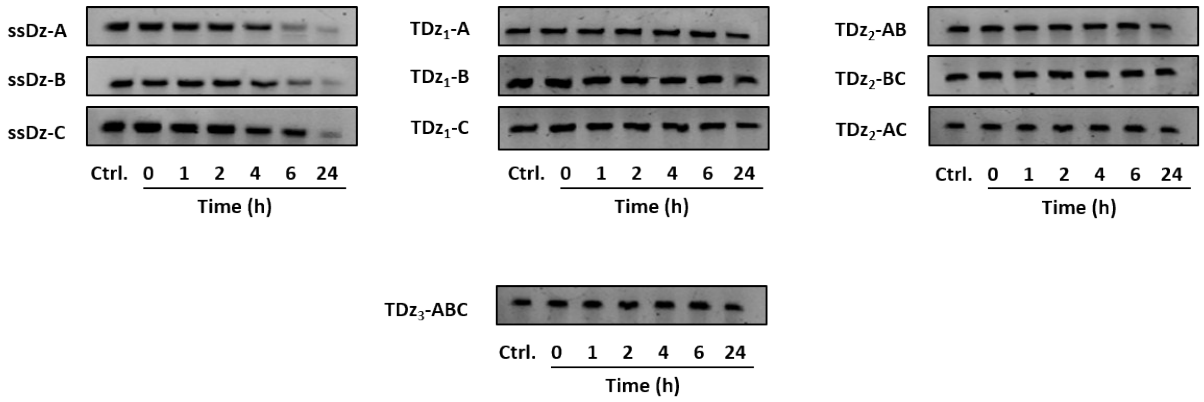


Figure S6. Stability of Dzs in 0.5% FBS.

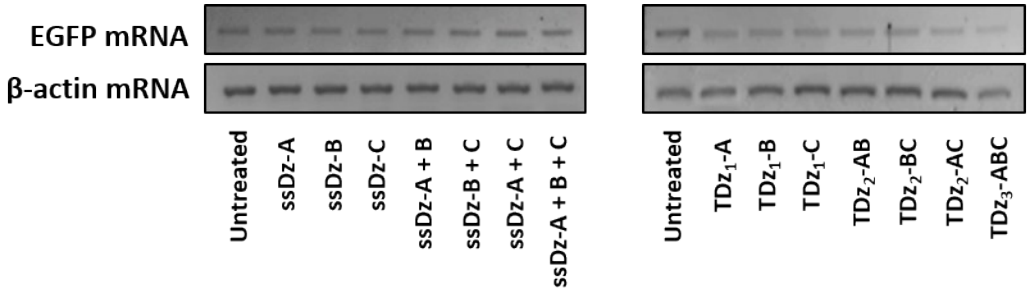


Figure S7. Reverse transcriptase PCR of EGFP mRNA in the cells treated with Dzs.

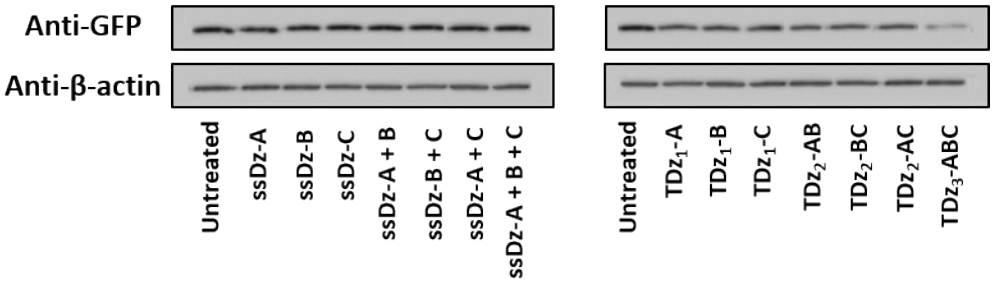


Figure S8. Western blotting of EGFP protein in the cells treated with Dzs.

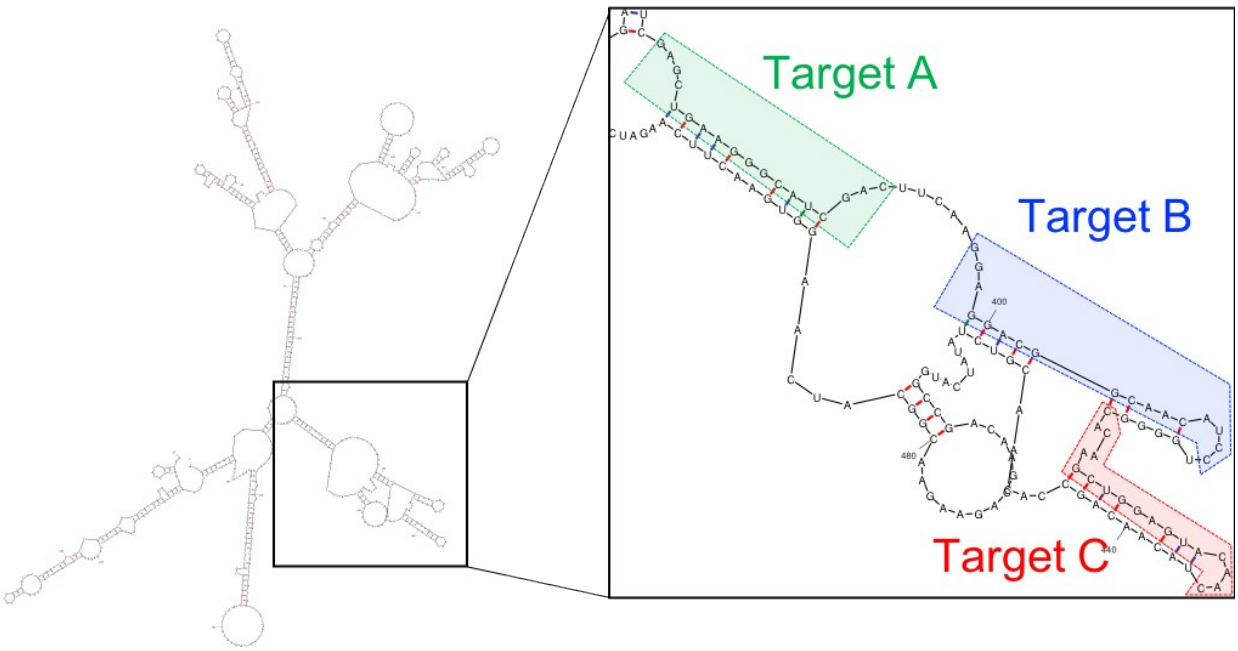


Figure S9. Energy minimized conformation of EGFP mRNA coding sequence by mfold (http://unafold.rna.albany.edu/?q=mfold/rna-folding-form). The zoomed in window shows the predicted secondary structures of the target sequences.

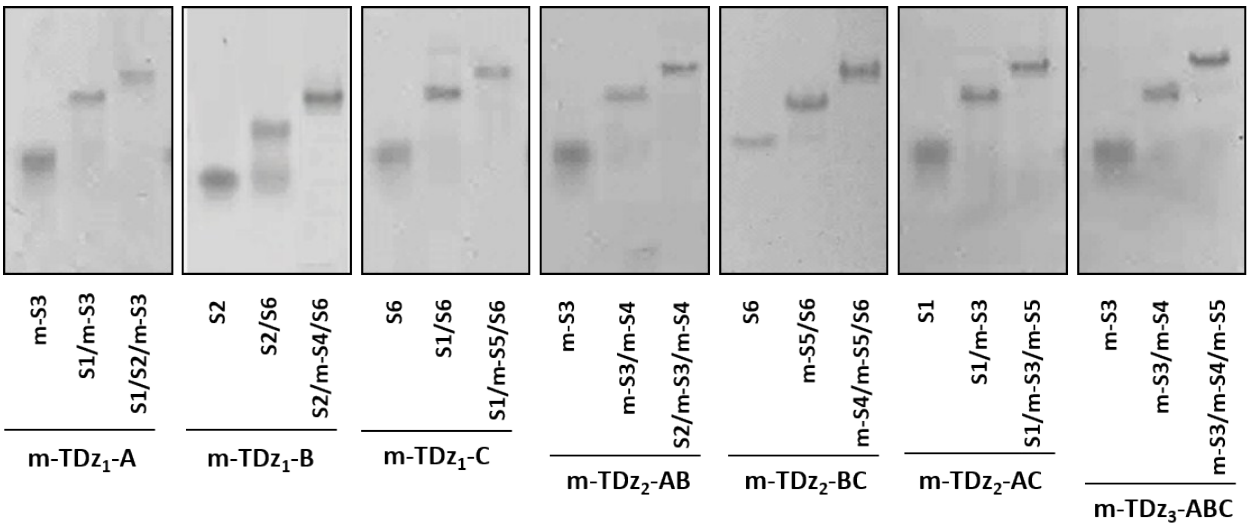


Figure S10. Native PAGE (8%) to analyze self-assembled m-TDzs.

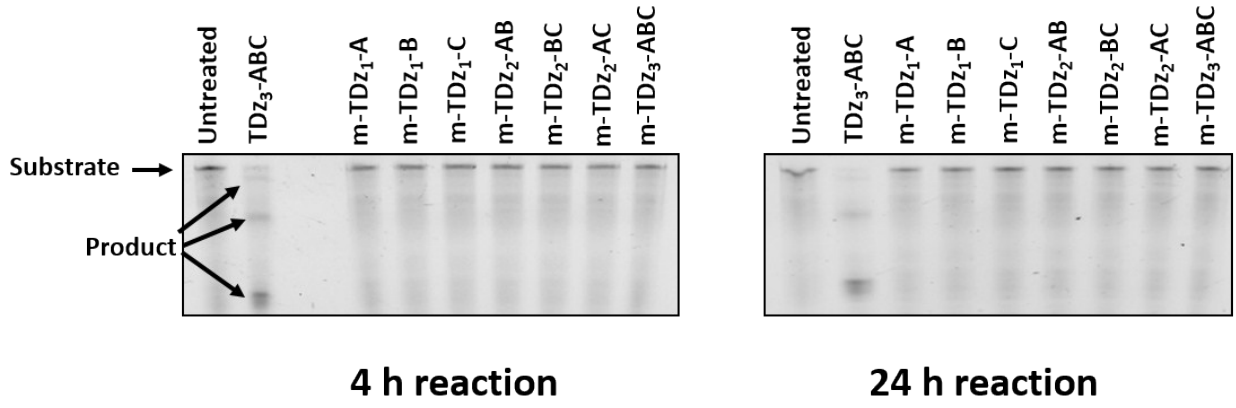


Figure S11. Denaturing PAGE (20%) analysis of RNA cleavage activity of m-Dzs.

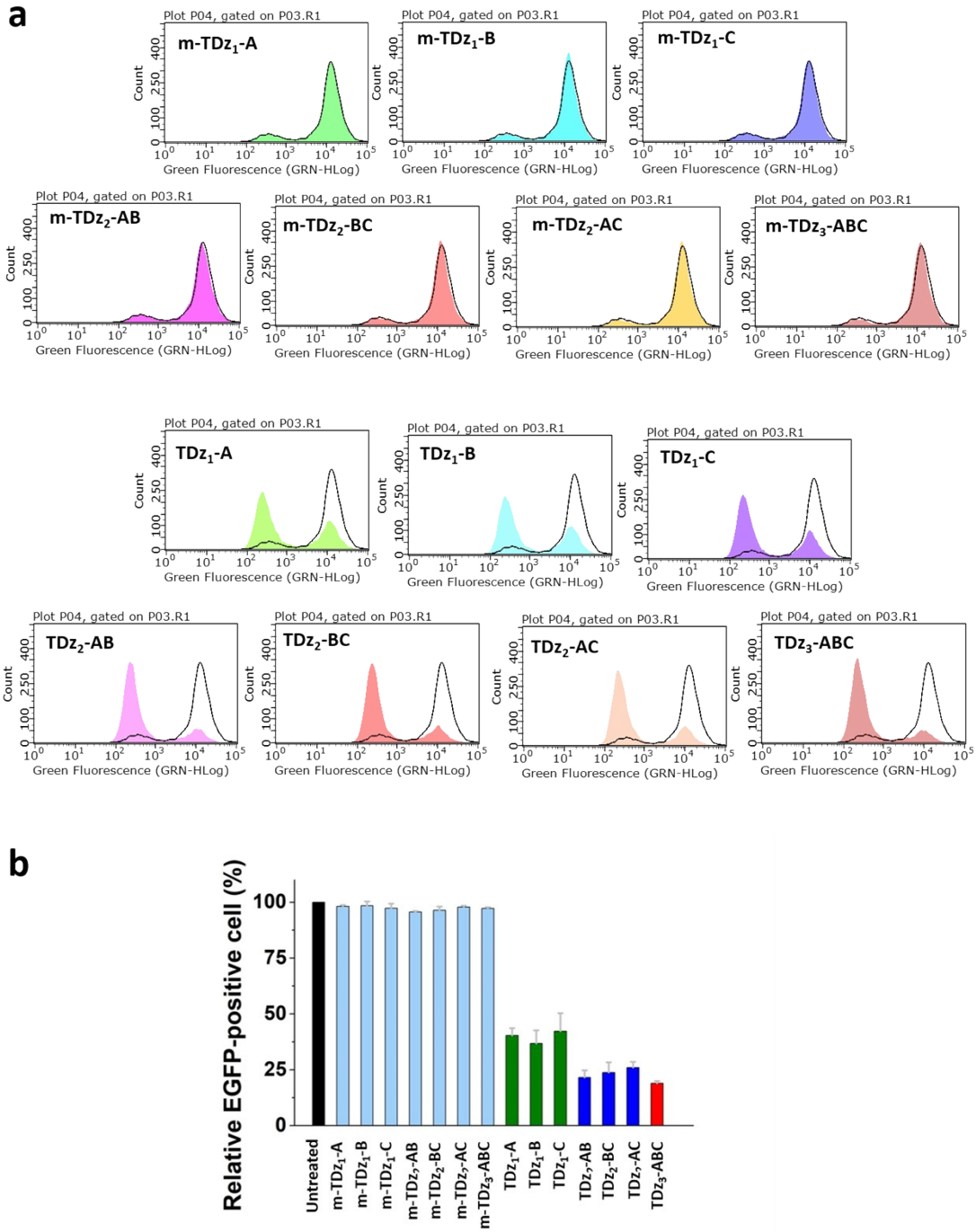


Figure S12. (a) Flow cytometry profiles of EGFP-positive cells before and after treatment with TDzs and m-TDzs for 48 h. The solid black line indicates untreated cells. Colored area indicates the cells treated with TDzs or m-TDzs. (b) Relative EGFP positive cells (%) before and after treatment with TDz and m-TDzs estimated based on the profiles in (a).

References

1. Kim S, Saadeldin IM, Choi WJ, Lee SJ, Lee WW, Kim BH, Han HJ, Bang du H, Lee BC, Jang G. Production of transgenic bovine cloned embryos using piggybac transposition. J Vet Med Sci. 2011 Nov;73(11):1453-7.
2. Yum SY, Lee SJ, Kim HM, Choi WJ, Park JH, Lee WW, Kim HS, Kim HJ, Bae SH, Lee JH, Moon JY, Lee JH, Lee CI, Son BJ, Song SH, Ji SM, Kim SJ, Jang G. Efficient generation of transgenic cattle using the DNA transposon and their analysis by next-generation sequencing. Sci Rep. 2016 Jun 21;6:27185. doi: 10.1038/srep27185.