Supporting information

Turning on protein function inhibited by DNA aptamers employing a covalent DNA-binding protein

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FIGURES

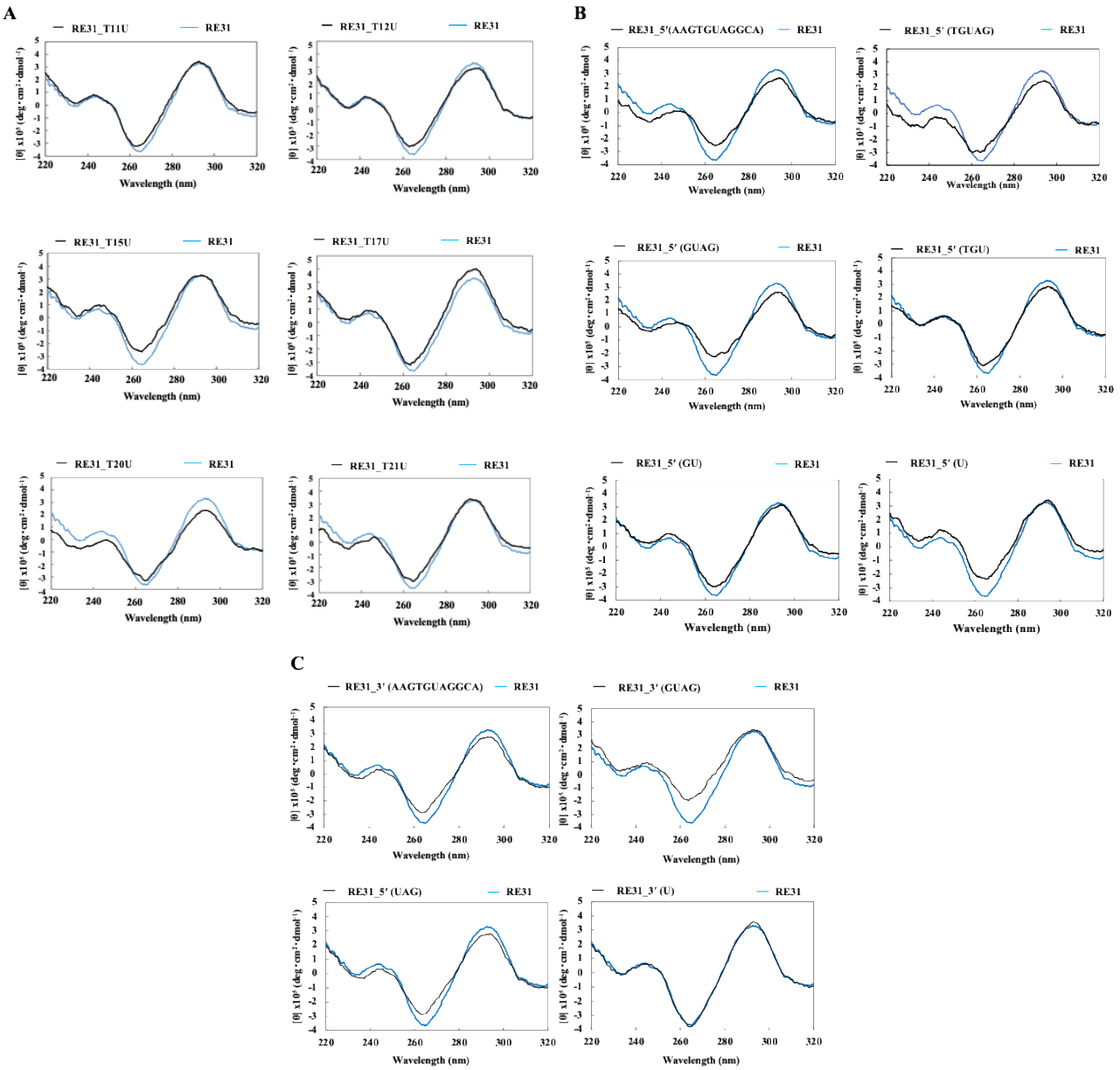
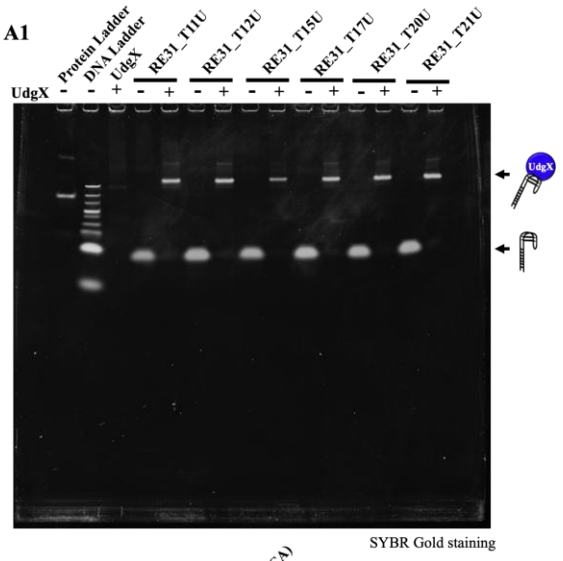


Figure S1: CD spectra of RE31 and RE31 mutants.

(A) CD spectra of RE31 (blue) and RE31 with uracil-containing sequences (black). (B) CD spectra of RE31 (blue) and RE31 with uracil-containing sequences introduced at the 5′ end (black). (C) CD spectra of RE31 (blue) and RE31 with uracil-containing sequences introduced at the 3′ end (black).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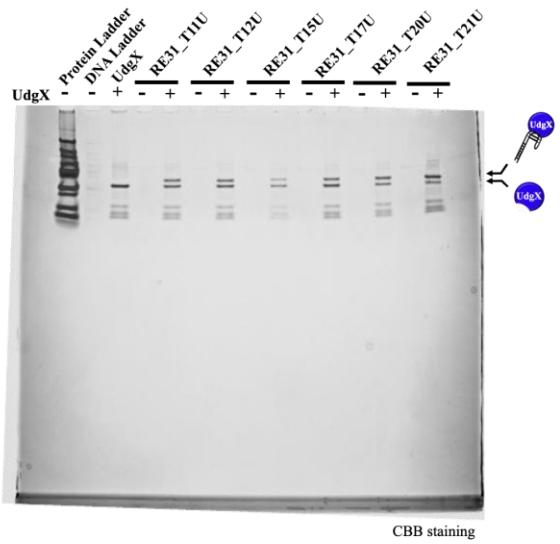
Protein Ladder
DNA Ladder
UdgX
RE31_T11U
RE31_T12U
RE31_T15U
RE31_T17U
RE31_T20U
RE31_T21U
UdgX
SYBR Gold staining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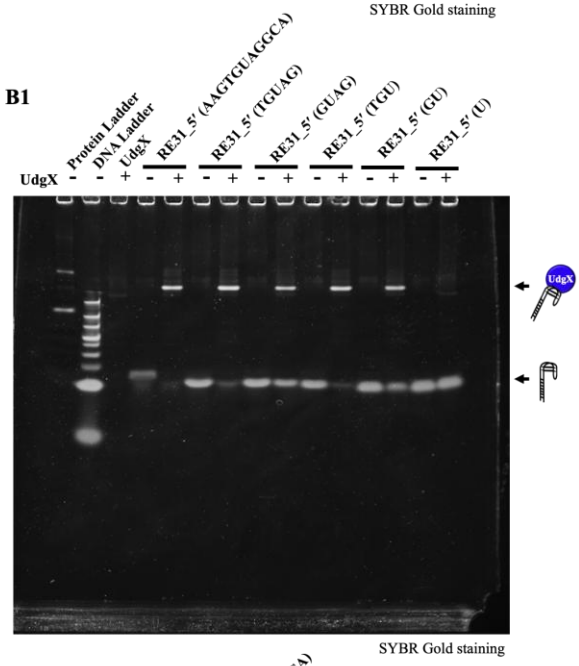
DNA Ladder
UdgX
RE31_T11U
RE31_T12U
RE31_T15U
RE31_T17U
RE31_T20U
RE31_T21U
UdgX
CBB staining


B1
Protein Ladder
DNA Ladder
UdgX
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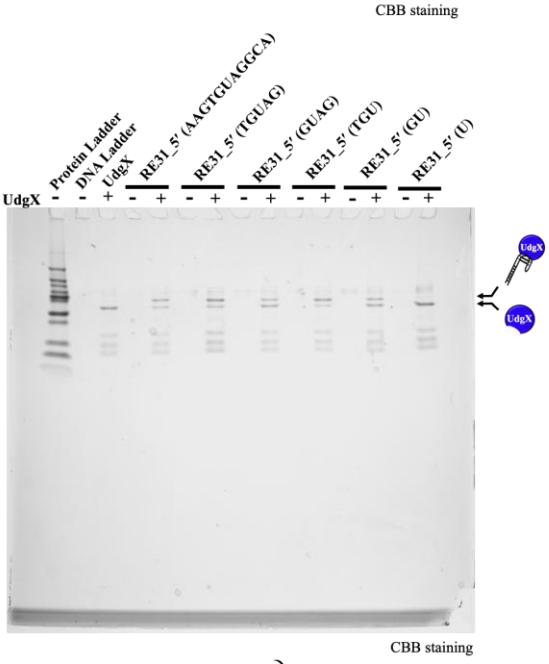
RE31_5′ (TGUAG)
RE31_5′ (GUAG)
RE31_5′ (TGU)
RE31_5′ (GU)
RE31_5′ (U)
UdgX
SYBR Gold staining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Protein Ladder
DNA Ladder
UdgX
RE31_5′ (AAGTGUAGGCA)
RE31_5′ (TGUAG)
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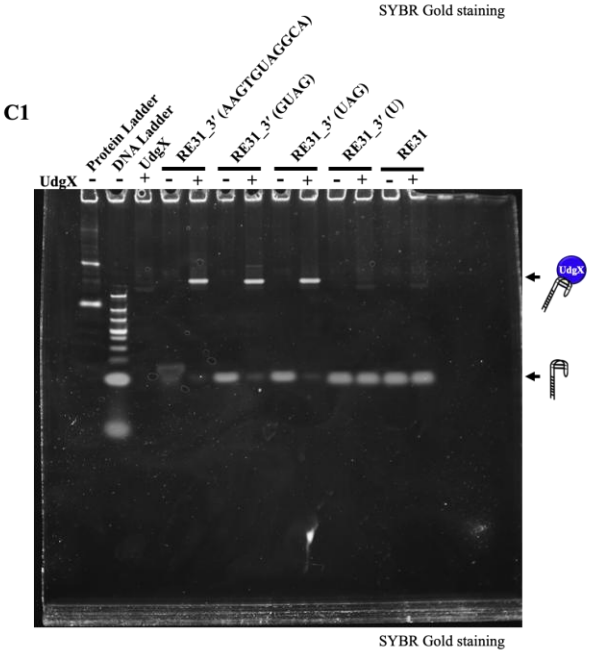
RE31_5′ (GU)
RE31_5′ (U)
UdgX
CBB staining


C1
Protein Ladder
DNA Ladder
UdgX
RE31_3′ (AAGTGUAGGCA)
RE31_3′ (GUAG)
RE31_3′ (UAG)
RE31_3′ (U)
RE31
UdgX
SYBR Gold staining

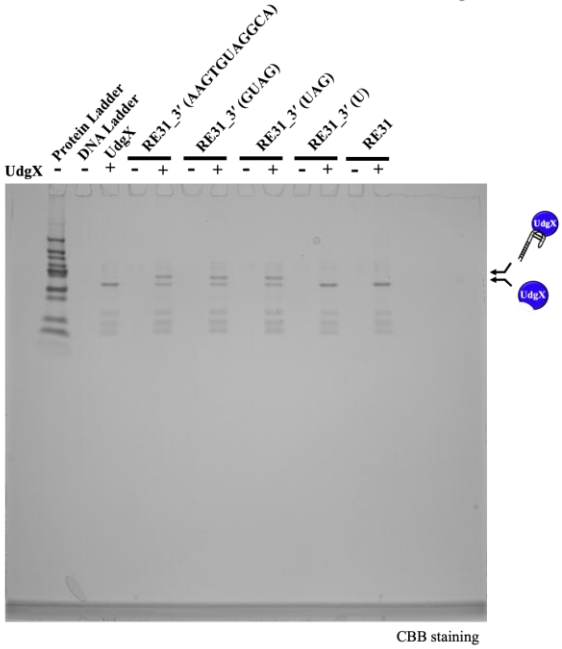

C2
Protein Ladder
DNA Ladder
UdgX
RE31_3′ (AAGTGUAGGCA)
RE31_3′ (GUAG)
RE31_3′ (UAG)
RE31_3′ (U)
RE31
UdgX
CBB staining

Figure S2: SDS-PAGE of RE31 mutant and UdgX.

(A) SDS-PAGE of RE31 with uracil-containing sequences. A1: SYBR Gold staining; A2: CBB staining. (B) SDS-PAGE of RE31 with uracil-containing sequences introduced at the 5′ end. B1: SYBR Gold staining; B2: CBB staining. (C) SDS-PAGE of RE31 with uracil-containing sequences introduced at the 3′ end and RE31. C1: SYBR Gold staining; C2: CBB staining.

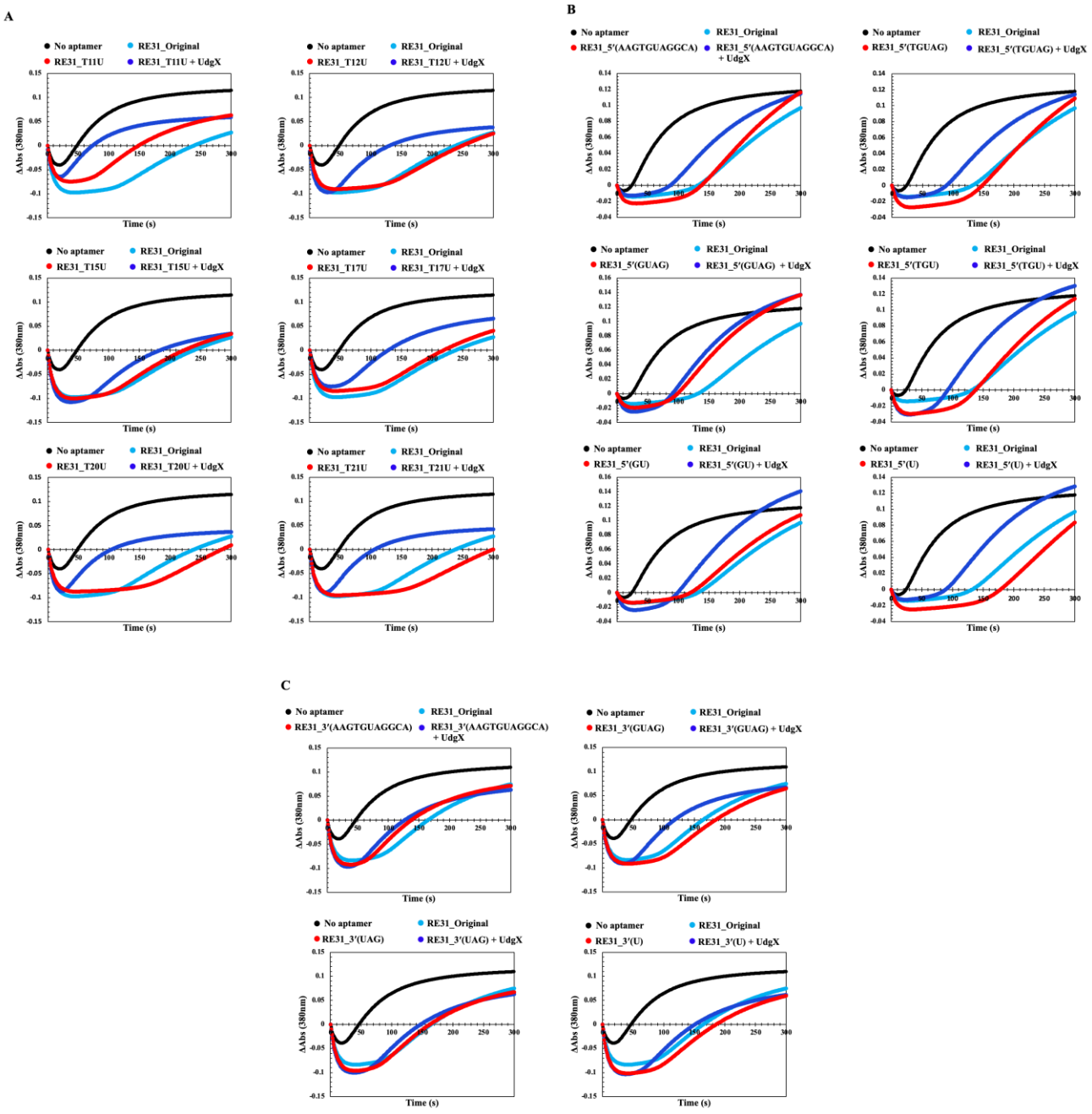


Figure S3: Time-course measurement of absorbance at 380 nm monitoring fibrinogen coagulation.

(A) No aptamer (black), RE31 (light blue), uracil-DNA-replaced RE31 (red), and uracil-DNA-replaced RE31 (with UdgX) (deep blue). (B) No aptamer (black), RE31 (light blue), uracil-DNA-containing sequences introduced at the 5′ end (red), and uracil-DNA-containing sequences introduced at the 5′ end (with UdgX) (deep blue). (C) No aptamer (black), RE31 (light blue), uracil-DNA-containing sequences introduced at the 3′ end (red), and uracil-DNA-containing sequences introduced at the 3′ end (with UdgX) (deep blue)

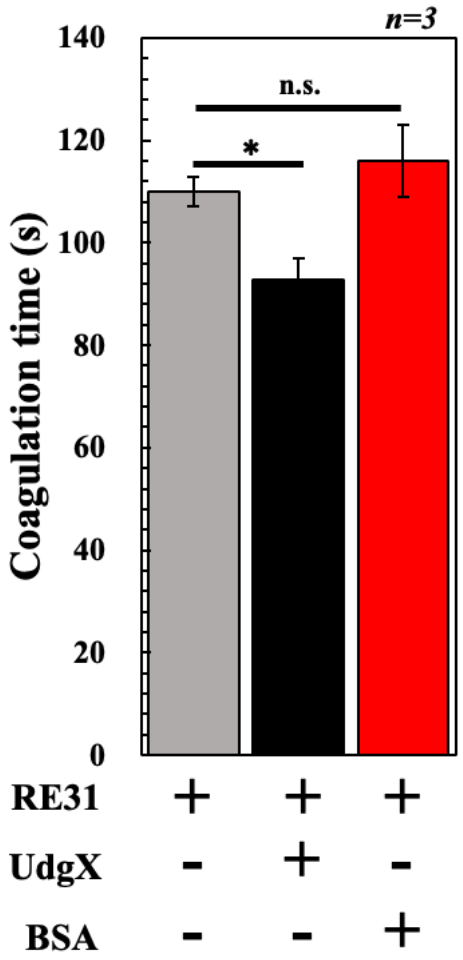


Figure S4: Coagulation time of only RE31 and RE31 with UdgX or BSA.
Coagulation time of RE31 (gray), in the presence of UdgX (black) or BSA (red). The results are presented as mean ± S.D. (*n* = 3). Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test**.** (*$p < 0.05$, n.s.: $p > 0.05$).

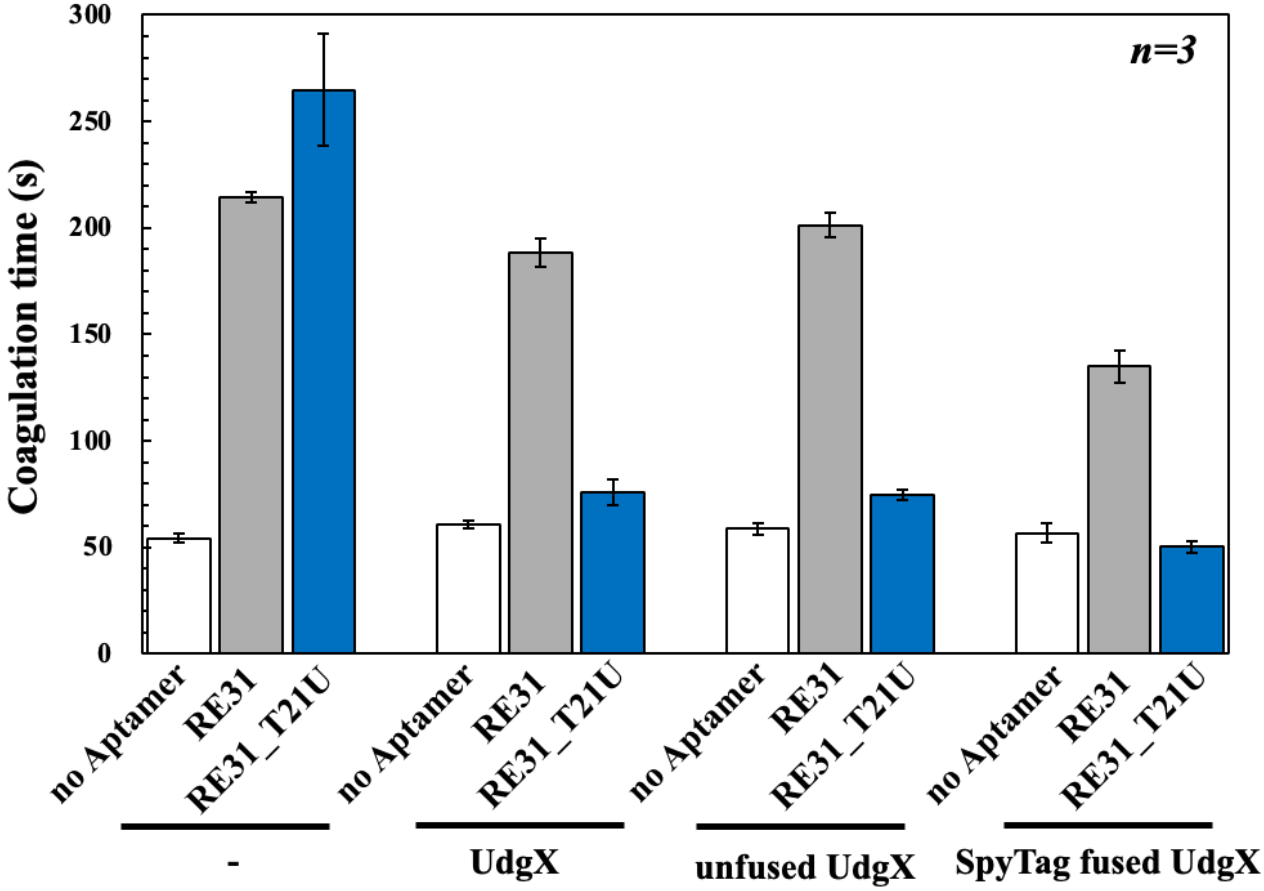


Figure S5: Coagulation times with unfused UdgX or SpyTag-fused UdgX.

Coagulation times without aptamer (white), or with RE31 (gray), and RE31_T21U. The inhibitory effect of RE31_T21U was not lost in any UdgX samples.

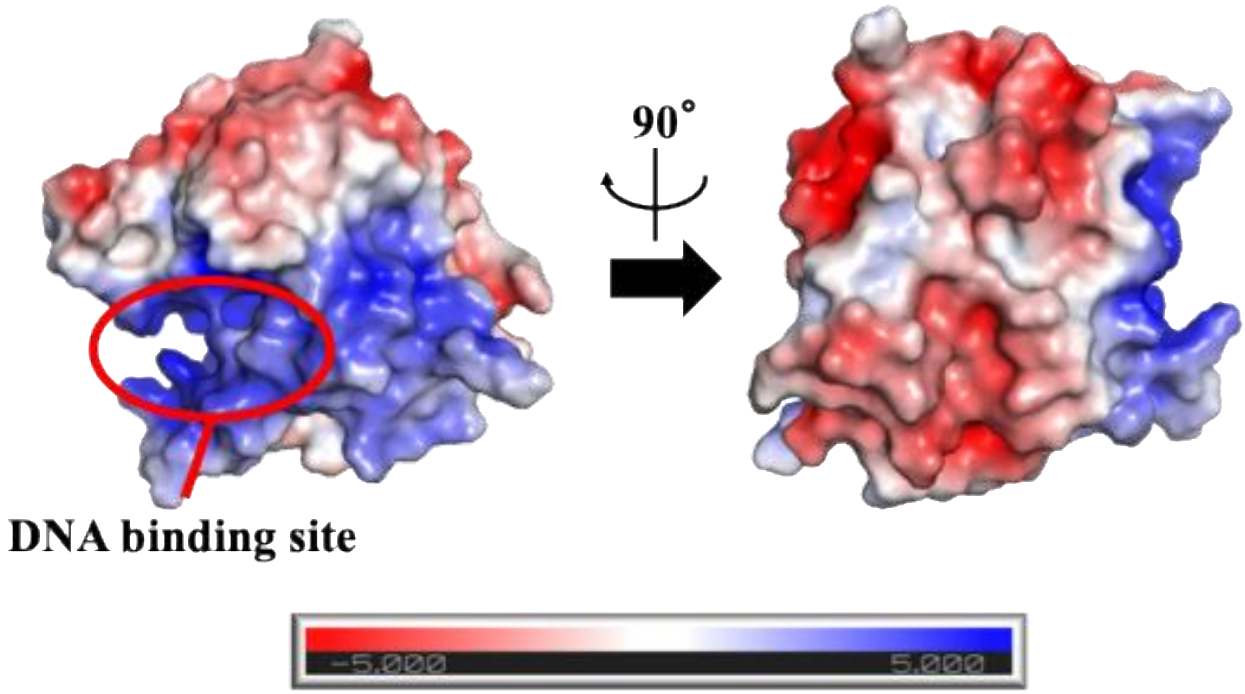


Figure S6: Surface electrification of UdgX.
Surface electrification of UdgX (PDB: 6AIL) at pH 7.0 as calculated using APBS in PyMOL. Blue indicates a basic surface with more positively charged residues, whereas red represents an acidic surface with more negatively charged residues.

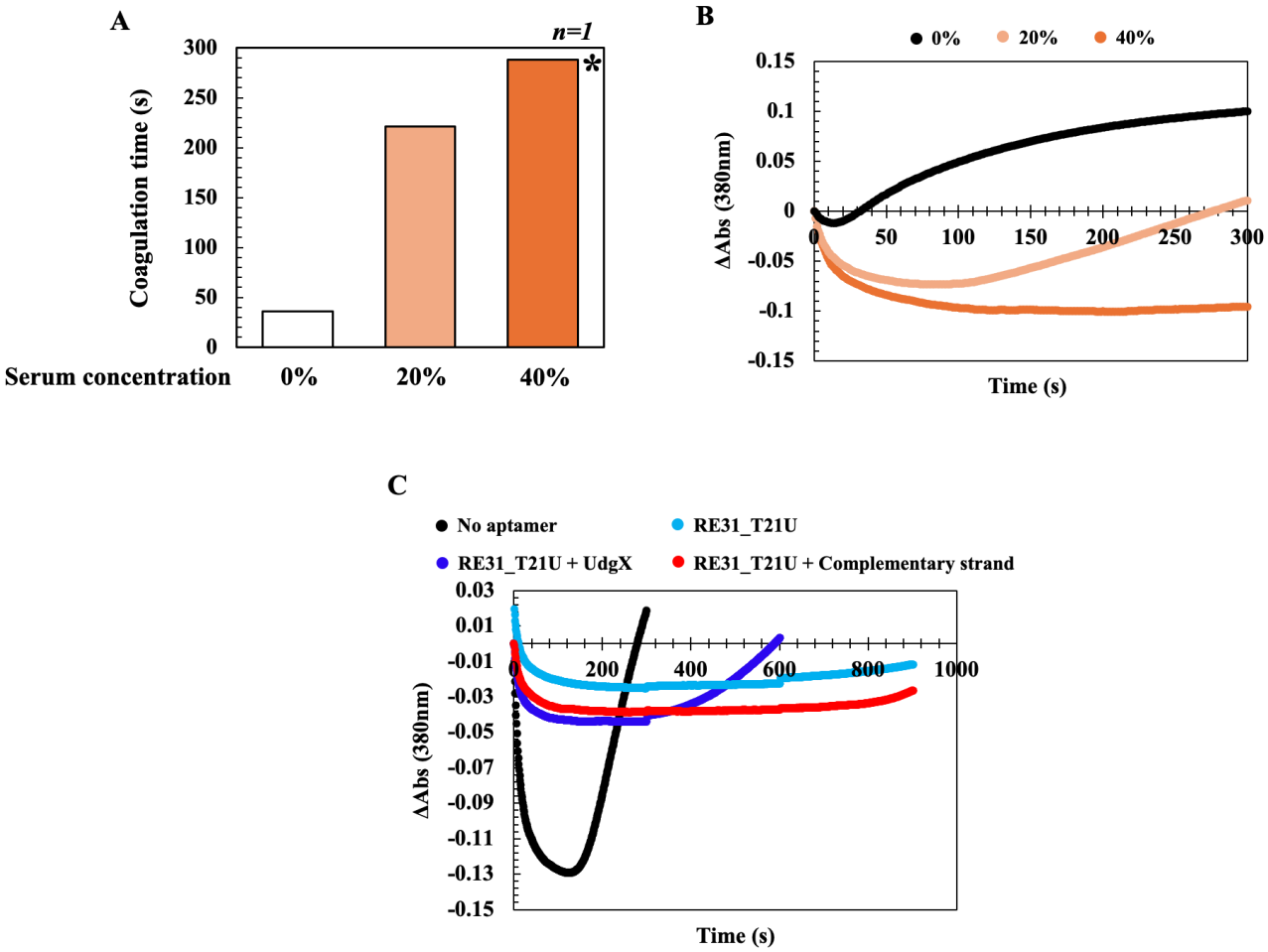


Figure S7: Effect of serum on fibrinogen coagulation reaction.

(A) Effect of serum on fibrinogen Coagulation time at serum concentrations of 0% (white), 20% (light orange), and 40% (deep orange); *= 300 s over. (B) Time-course measurement of absorbance at 380 nm monitoring fibrinogen coagulation at serum concentrations of 0% (black), 20% (light orange), and 40% (deep orange). (C) Time-course measurement of absorbance at 380 nm monitoring fibrinogen coagulation, No aptamer (black), RE31_T21U (light blue), RE31_T21U + UdgX (deep blue), RE31_T21U + Complementary strand (red).

Table S1: Oligonucleotide sequences for TBA, A14#1, and A14#1_T12U.

Sample	Sequence (5′ to 3′)
TBA	GGTTGGTGTGGTTGG
A14#1	GCGGTTGGTGGTAGTTACGTTCGC
A14#1_T12U	GCGGTTGGTGGUAGTTACGTTCGC

Table S2: TrSC-fused UdgX protein sequence.

Sample	Sequence (N term – C term)
TrSC-fused UdgX	MAGAQDFVPHTADLAELAAAAGECRGCGLYRDATQAVFGAGGRSARI MMIGEQPGDKEDLAGLPFVGPAGRLLDRALEAADIDRDALYVTNAVK HFKFTRAAGGKRRIHKTPSRTEVVACRPWLIAEMTSVEPDVVVLLGAT AAKALLGNDFRVTQHRGEVLHVDDVPGDPALVATVHPSSLLRGPKEER ESAFAGLVDDLRVAADVRPGSGDSATHIKFSKRDEDGKELAGATMELR DSSGKTISTWISDGQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNE QGQVTVNGLVPRGSKLAAALEHHHHHH

In this study, TrSC-fused UdgX was used as UdgX.

MATERIAL AND METHODS

Reagents

All DNA (Table1, S1) were purchased from Eurofins Genomics (Tokyo, Japan). Dulbecco's phosphate buffered saline (DPBS; Catalog No. 14249-95) and Rapid Stain CBB kit (Catalog No. 30035-14) were purchased from Nacalai Tesque (Kyoto, Japan). Sample buffer for electrophoresis, EzApply (Catalog No. AE-1430), was purchased from ATTO (Tokyo, Japan). Novex™ Shape Pre-Stained Protein Standard (Catalog No. LC5800), Ultra LowRange DNA Ladder (Catalog No. 10597012), SYBR™ Gold Nucleic Acid Gel Stain (Catalog No. S11494) and NeutrAvidin-HRP (Catalog No. 31001) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Thrombin from bovine plasma (Catalog No. 206-18411, CAS: 9002-04-4) and fibrinogen from bovine plasma (Catalog No. 066-06681, CAS: 9001-32-5) were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Unconjugated acceptor beads (Catalog No. 6772001) and streptavidin donor beads (Catalog No. 6760002S) were purchased from PerkinElmer (Shelton, CT, USA). TritonX-100 (CAS: 9036-19-5), Bovine serum albumin (CAS: 9048-46-8) and Immobilon® ECL UltraPlus Western HRP Substrate (Catalog No. WBULP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (Catalog No. 12603C) was purchased from JRH Biosciences (Lenexa, Kansas, USA). The human α-thrombin (Purity>95% by SDS-PAGE, Catalog no. HCT-0020) was purchased from Haematologic Technologies (Essex Junction, VT, USA))

Preparation of DNA aptamers

All aptamers were heat-treated at 95 ℃ for 5 min, then gradually cooled at a 1 ℃ min^{-1} to 30 ℃ in DPBS (Nacalai Tesque) to form G4 structures. The G4-folded oligodeoxynucleotide solutions were stored at 4 ℃ until further use.

Recombinant production of truncated SpyCatcher (TrSC)-fused UdgX

TrSC-fused UdgX was used as the UdgX. We developed a versatile and irreversible DNA–protein coupling module by constructing a SpyCatcher-fused UdgX at the C-terminal (1). As the structural stability of SpyCatcher was improved by truncating both terminal sequences (2), we designed TrSCs with further truncations and constructed a TrSC-fused UdgX protein (Table S2). The gene cassette was obtained via overlap extension polymerase chain reaction (PCR) and inserted into pET30c to construct the expression vector. TrSC-fused UdgX and unfused UdgX were recombinantly prepared and purified in a similar manner to the UdgX preparation method reported previously (1).

Circular dichroism (CD) spectroscopy analysis

CD spectroscopic analysis was performed using J-725 (JASCO Co., Ltd., Tokyo, Japan). CD spectra were recorded from 320 to 220 nm at a scanning rate of 50 nm/min, as previously described (3). The cumulative number of CD spectra was five at 25 ℃. The results were presented after subtracting the DPBS spectrum from the background.

Evaluation of UdgX binding to RE31 mutants

The RE31 mutant diluted to 8 μM by DPBS was mixed with an equal volume of 40 μM UdgX, and incubated at 25 ℃ for 1 h. Subsequently, EzApply was mixed with an equal volume of the nucleic acid-UdgX solution and treated at 70 ℃ for 10 min. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 25 mA and 180 V, with a pH of 8.3, using TBE-PAGE Mini 20% (TEFCO, Tokyo, Japan). Novex™ Shape Pre-Stained Protein Standard and Ultra LowRange DNA Ladder were used as the protein and nucleic acid markers,

respectively. Finally, the nucleic acids were stained using SYBR™ Gold Nucleic Acid Gel Stain, followed by protein staining using a Rapid Stain CBB kit.

Measurement of coagulation time

RE31 and its mutants were diluted to 0.5 μM with DPBS. Subsequently, 40 μL of 10 units mL^{-1} thrombin was added to 20 μL of each RE31 mutant, and 420 μL DPBS was pipetted, followed by incubation at 25 ℃ for 5 min. Thereafter, 20 μL UdgX (final concentration: 100 nM) or complementary strand (final concentration: 100 nM and 1000 nM) was added and incubated for 5 min. After incubation, 500 μL of 2 mg mL^{-1} fibrinogen solution was added and mixed for 5 s. Finally, absorbance at 380 nm was continuously monitored for 5 min using a U-2900 spectrometer (Hitachi, Ltd., Tokyo, Japan). The coagulation time was defined as the time to reach the maximum change in absorbance within 5 s during a 5-min measurement.

To investigate the effect of serum on anticoagulant activity, different concentrations of fetal bovine serum (FBS) were added instead of DPBS.

Briefly, 40 μL of 10 units mL^{-1} thrombin form bovine was added to 20 μL. Then, 220 μL DPBS and 200 μL FBS (JRH Biosciences) or 20 μL DPBS and 400 μL FBS (JRH Biosciences) was pipetted, followed by incubation at 25℃ for 5 min. Thereafter, 20 μL DPBS was added and incubated for 5 min. After incubation, 500 μL of 2 mg mL^{-1} fibrinogen solution was added and mixed for 5 s. Finally, absorbance at 380 nm was continuously monitored for 5 min using a U-2900 spectrometer. The coagulation time was defined as the time taken to reach the maximum change in absorbance in 5 s during the measurement time of 5 min. In the result, Extended thrombin coagulation time was confirmed in serum (Figure S7A, S7B).

To measure the inhibition of thrombin activity by aptamer, RE31_T21U, in 40% serum, thrombin concentration was increased 10 times, as the fibrinogen coagulation reaction catalyzed by thrombin is inhibited in serum. Specifically, 40 μL of 100 units mL^{-1} thrombin was added to 20

μL of RE31_T21U (diluted to 1 μM with DPBS), 20 μL DPBS, and 400 μL FBS–was pipetted, followed by incubation at 25℃ for 5 min. Thereafter, 20 μL UdgX (final concentration: 100 nM) or complementary strand (final concentration: 100 nM) was added and incubated for 5 min. After incubation, 500 μL of 2 mg mL^{-1} fibrinogen solution was added and mixed for 5 s. Finally, absorbance at 380 nm was continuously monitored for 15 min using a U-2900 spectrometer. The coagulation time was defined as the time taken to reach the maximum change in absorbance in 5 s during the measurement time of 15 min. (Figure 3B, S7C)

The thrombin activity recovery rate was calculated by setting the coagulation time without aptamers as 100% and the coagulation time with aptamers as 0%.

Measurement of aptamer affinity by AlphaAssay

The human α-thrombin immobilized acceptor beads were prepared via the Borch reaction, utilizing the aldehyde group on the unconjugated acceptor bead surface and the amino group of thrombin (4). The biotinylated RE31 and biotinylated RE31_T21U were diluted to 2 μM with the assay buffer, DPBS containing 0.5% (v/v) TritonX-100 and 0.1% (w/v) bovine serum albumin (BSA). An equal volume of UdgX (final concentration: 5 μM) was added to the diluted biotinylated RE31 (final concentration: 1 μM) or biotinylated RE31_T21U and incubated at 25 ℃ for 30 min. Thereafter, 10 μL of various concentrations of the aptamer–UdgX mixture (5, 2.5, 1.0, 0.50, 0.25, 0.20, 0.15, and 0.10 nM) as well as 10 μL of thrombin immobilized acceptor beads (final concentration: 60 μg/mL) were added to a well of 1/2 Area OptiPlate-96 (PerkinElmer) and gently shaken for 1 min, followed by incubation in the dark for 1 h. Subsequently, 10 μL of streptavidin donor beads (final concentration: 60 μg/mL) were added and mixed for 1 min, followed by incubation in the dark for 1 h. Finally, the chemiluminescence observed upon irradiation with 680 nm excitation light was measured using a plate reader (EnSight, PerkinElmer).

Binding ability analysis by aptamer blotting for evaluation of the versatility

Aptamer blotting was performed as described previously (5). Briefly, 1.5 μg of bevacizumab was spotted onto a nitrocellulose membrane. The membrane was subsequently dried for 5 min and blocked with 2% (w/v) BSA in PBS for 1 h. After washing with PBS containing 0.02% (v/v) Tween-20, the membrane was incubated with 1 μM biotinylated anti-bevacizumab aptamer, A14#1 (6) or A14#_T12U, for 1 h in PBS. Thereafter, the membrane was washed, and 5 μM UdgX (0, 2.5, 5, 10, 20 μM UdgX in S7A and S7B) or PBS was added. The membrane was washed once and incubated with 10,000-fold diluted NeutrAvidin-HRP for 30 min. After washing three times, the spots were visualized with Immobilon Western chemiluminescent HRP substrate and detected using ImageQuant™ 500 (Cytiva, MA, USA).

Statistical Analyses

Data were presented as the mean ± standard deviation (SD) unless otherwise stated. All statistical analyses were performed using MATLAB R2024a (MathWorks, Natick, MA, USA). The significance of the results was analysed using one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison test. A $p < 0.05$ was considered statistically significant.

References

(1) Komiya, E.; Takamatsu, S.; Miura, D.; Tsukakoshi, K.; Tsugawa, W.; Sode, K.; Ikebukuro, K.; Asano, R. Exploration and Application of DNA-Binding Proteins to Make a Versatile DNA–Protein Covalent-Linking Patch (D-Pclip): The Case of a Biosensing Element. *Journal of the American Chemical Society* **2024**.

(2) Li, L.; Fierer, J. O.; Rapoport, T. A.; Howarth, M. Structural analysis and optimization of the covalent association between SpyCatcher and a peptide Tag. *Journal of molecular biology* **2014**, *426* (2), 309-317.

(3) Tu, A. T. T.; Hoshi, K.; Ikebukuro, K.; Hanagata, N.; Yamazaki, T. Monomeric G-quadruplex-based CpG oligodeoxynucleotides as potent toll-like receptor 9 agonists. *Biomacromolecules* **2020**, *21* (9), 3644-3657.

(4) Takeuchi, S.; Yamazaki, T.; Yamaguchi, K.; Komura, F.; Tabata, T.; Nishi, H.; Azumai, S.; Miura, K.; Hirokawa, M.; Ikemoto, K. Toward the Establishment of a Harmonized Physicochemical Profiling Platform for Therapeutic Oligonucleotides: A Case Study for Aptamers Where the Higher-Order Structure Influences Physical Properties. *Molecular Pharmaceutics* **2024**, *21* (7), 3471-3484.

(5) Hasegawa, H.; Sode, K.; Ikebukuro, K. Selection of DNA aptamers against VEGF 165 using a protein competitor and the aptamer blotting method. *Biotechnology letters* **2008**, *30*, 829-834.

(6) Saito, T.; Shimizu, Y.; Tsukakoshi, K.; Abe, K.; Lee, J.; Ueno, K.; Asano, R.; Jones, B. V.; Yamada, T.; Nakano, T. Development of a DNA aptamer that binds to the complementarity-determining region of therapeutic monoclonal antibody and affinity improvement induced by pH-change for sensitive detection. *Biosensors and Bioelectronics* **2022**, *203*, 114027.