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cDNA TRAP display for rapid and stable *in vitro* selection of antibody-like proteins

Taishi Kondo,[a] Minori Eguchi,[a] Seita Kito,[a] Tomoshige Fujino,[a] Gosuke Hayashi [a,b] and Hiroshi Murakami*[a,c]

[a] Graduate School of Engineering, Nagoya University, Nagoya, 464–8603, Japan.

[b] Japan Science and Technology Agency (JST), Kawaguchi, 332-0012, Japan.

[c] Institutes of Innovation for Future Society, Nagoya University, 464–8603, Japan

*E-mail: murah@chembio.nagoya-u.ac.jp

Materials and Methods

Materials: an3H18M, an5H16M, an3H18M, HS-$(SPC18)_n$-CC(Puromycin), and the original TRAP display PuL [(puromycin)CC-$(SPC18)_5$-an21M-HEX] were purchased from Nippon Bio Service (Japan). an3H18T was purchased from Hokkaido System Science Co., Ltd. (Japan), and the remaining oligonucleotides were purchased from FASMAC Co. Ltd. (Japan). The epidermal growth factor (EGF) receptor/Fc chimera was purchased from R&D systems (MN, USA). Ribonuclease H (RNase H) and SYBR® Green I Nucleic Acid Gel Stain were obtained from Takara Bio (Japan). *N*-(6-maleimidocaproyloxy)-succinimide was purchased from Dojindo Laboratories (Japan). Streptavidin–biotin-HRP was purchased from Abcam (UK). Deoxyribonuclease I (DNase I) was purchased from Roche Diagnostics (Germany). The preparation of the cell-free translation system, *Pfu-S* DNA polymerase[1], and Moloney murine leukemia virus reverse transcriptase (MMLV)[2] was described in a previous report[3]. The preparation of biotin–Phe-$tRNA^{ini}$ was described in a previous report[4].

3H18M sequence optimization: The 3H18M-X (X, 1–5; 0.6–1.2 μM) was annealed with the mRNA-X (X, 1–5; 1 μM) wild-type monobody in an annealing buffer (25 mM pH 7.8 HEPES-K, 200 mM potassium acetate) by heating the solution to 95°C for 2 min, followed by cooling to 25°C. The mixtures (1 μL) were mixed with 1 μL DNA loading buffer [20% (v/v) glycerol, 80 mM pH 8.3 Tris-AcOH, 2 mM EDTA] and loaded onto PAGE [6% acrylamide/bis mixed solution (37.5:1)]. The gel was analyzed using Pharos FX (Bio-Rad, USA).

Synthesis of the puromycin linker of the cDNA TRAP display: Five microliter of reaction mixture (100 mM HEPES-K pH 7.5, 50% DMSO, 270 μM an3H18M, and 90 mM EMCS) were mixed for 1 hour at 25℃. The reaction was stopped by adding 80 μL of cold isopropanol, and the resulting solution was kept at –20°C for 20 min. After centrifugation at 15,300 × *g* at 4°C, the pellet was recovered and dissolved in 1 μL of HEPES-K pH 7.5, followed by a second isopropanol precipitation. The resulting pellet (an3H18M-EM) was washed with 100 μL of cold acetone and then dissolved in 1 μL of 10 mM HEPES-K pH 7.5 buffer. The 11-μL reaction mixture containing 455 μM HS-$(SPC18)_n$-CC(puromycin), 1 mM TCEP, and 50 mM HEPES-K pH 7.5 was incubated at 37°C for 1 h. After completion of the reaction, the buffer was exchanged to HBST [50 mM HEPES-K pH 7.5, 300 mM NaCl, 0.05% (v/v) Tween 20] using P-2 gel (Bio-Rad, CA, USA). The reduced HS-$(SPC18)_n$-CC(puromycin) (1.5 eq) was mixed with an3H18M-EM. The solution was completely evacuated by a micro centrifugal vacuum concentrator (MV-100; Tomy Digital Biology, Japan). The dried pellet was dissolved in 1 μL of 10 mM HEPES-K pH 7.5 and incubated at 25°C for 1 h. After HepKCl gel [45 mM HEPES-K pH 7.8, 30 mM KCl, 18% acrylamide/bis mixed solution (19:1)]

electrophoresis purification, the PuL was eluted from the gel using ultrapure water. After concentration by freeze drying, the pellet was dissolved in ultrapure water and the concentration was determined by UV-Vis absorbance (using ε_{538} = 83,500 M^{-1} cm^{-1}). PuL with an3H18T was prepared by a similar method.

Preparation of monobody DNA and mRNA templates: The sequences of templates and primers used in this work are listed in Table S1. The preparation of the wild-type monobody (fibronectin type III domain) DNA and mRNA was described in a previous report[3]. The MC monobody (binding to EGFR1) DNA was amplificated by *Pfu-S* DNA polymerase using FN3-from-ExpVec.F40 and FN3-from-ExpVec.R40 as primers and the MC DNA as a template. The second polymerase chain reaction (PCR) was performed using T7SD8M2.F44 and G5S-4Gan21-3.R42 as primers. DNA was purified via phenol/chloroform extraction and isopropanol precipitation. The DNA was dissolved in 10 µL of 10 mM Tris-AcONa pH 7.8. The concentration was determined by 8% polyacrylamide gel electrophoresis (PAGE) using the ExcelBand 100 bp DNA Ladder (Smobio Technology) as a marker.

Analysis of the display efficiency of the monobody: The PuL (5 µM) was annealed with the wild-type monobody mRNA (5 µM) in an annealing buffer [25 mM HEPES-K pH 7.8, 200 mM potassium acetate] by heating the solution to 95°C for 2 min, followed by cooling down to 25°C. The PuL/mRNA (1 µM final concentration) was added to the cell-free translation system and the reaction mixture was incubated at 37°C for 30 min. The reaction mixtures (2.5 µL) were mixed with 27.5 µL of gel shift buffer [34.5% (v/v) formamide, 62.5 mM Tris-HCl pH 6.8, 5 mM dithiothreitol (DTT), 10 mM EDTA, and 0.05% (w/v) SDS] and loaded onto urea SDS–PAGE [0.05% (w/v) SDS, 6 M urea, 8% acrylamide/bis mixed solution (37.5:1)]. The gel was analyzed using Pharos FX (Bio-Rad, USA).

Model selection: The DNA (5 nM final concentration) of the MC monobody was added to the TRAP cell-free translation system containing either the original PuL or the new PuL (1 µM final concentration). The reaction mixture (8 µL) was incubated at 37°C for 30 min. After the reaction, 1.8 µL of 100 mM EDTA was added to the translation mixture. One microliter of translation mixture was dispensed and mixed with 20 µL of gel shift buffer (Fig. 3, lanes 1 and 5). The reverse transcription mixture [4.9 µL of 150 mM Tris-HCl pH 8.4, 225 mM KCl, 75 mM $MgCl_2$ and 16 mM DTT, 1.5 mM dNTPs, and 3.4 µM MMLV] was added to the remaining translation mixture (13.2 µL). For the original DNA TARP display, FN3S.R29 (0.83 µM) was added to the reaction mixture. The reaction mixture was incubated at 42°C for 15 min. The buffer was exchanged to HBST using Zeba™ Spin Desalting Columns 7K MWCO (Thermo Fisher Scientific, MA, USA). One microliter

of the mixture was mixed with 13 μL of gel shift buffer (Fig. 3, lanes 2 and 6). HBST solution (1.6 μL) with/without 20 U/μL RNase H were added to 4 μL of the buffer exchanged solution. The EGF receptor/Fc chimera (250 nM final concentration)-immobilized Protein G magnetic beads were added to the solution* and mixed at 25°C for 20 min. After the incubation, the beads were collected and washed with HBST thrice. The beads were mixed with the PCR premix [10 mM Tris-HCl pH 8.4, 100 mM KCl, 0.1% (v/v) Triton X-100, 2% (v/v) DMSO, 2 mM $MgSO_4$, and 0.2 mM each dNTPs] and heated at 95°C for 5 min. The recovered cDNA was quantified by SYBR® green-based quantitative PCR using T7SD8M2.F44 and FN3Lip.R20 as primers. Alternatively, 1 μL of the solution* was incubated at 25°C for 20 min and mixed with 9 μL of the gel shift buffer (Fig. 3, lanes 3, 4, 7, and 8). Two microliter of a sample mixed with the gel shift buffer was loaded onto urea SDS–PAGE. The resulting gel was analyzed using Pharos FX.

Western blot analysis of the displayed product: Biotin-$tRNA^{ini}$ (16 μM) was added to the translation mixture, and 10-formyl-5,6,7,8-tetrahydrofolic acid was removed from it. The product was analyzed by the same method used for model selection. Additionally, we performed Western blot analysis using streptavidin–biotin–HRP. The signal was analyzed using the ChemiDoc MP imaging system (Bio-Rad, USA). DNase I (1 U/μL, 3 μL) was added to the 3 μL RNase H-added solution for cDNA degradation. After incubation at 25°C for 20 min, the solution was mixed with 2 × gel shift buffer and loaded onto urea SDS–PAGE.

References:

1. Y. Wang, D. E. Prosen, L. Mei, J. C. Sullivan, M. Finney and P. B. Vander Horn, *Nucleic Acids Res.*, 2004, **32**, 1197-1207.
2. B. Arezi and H. Hogrefe, *Nucleic Acids Res.*, 2009, **37**, 473-481.
3. T. Kondo, Y. Iwatani, K. Matsuoka, T. Fujino, S. Umemoto, Y. Yokomaku, K. Ishizaki, S. Kito, T. Sezaki, G. Hayashi and H. Murakami, *Sci. Adv.*, 2020, **6**, eabd3916.
4. T. Ishizawa, T. Kawakami, P. C. Reid and H. Murakami, *J. Am. Chem. Soc.*, 2013, **135**, 5433-5440.

Supplementary Figures and Table

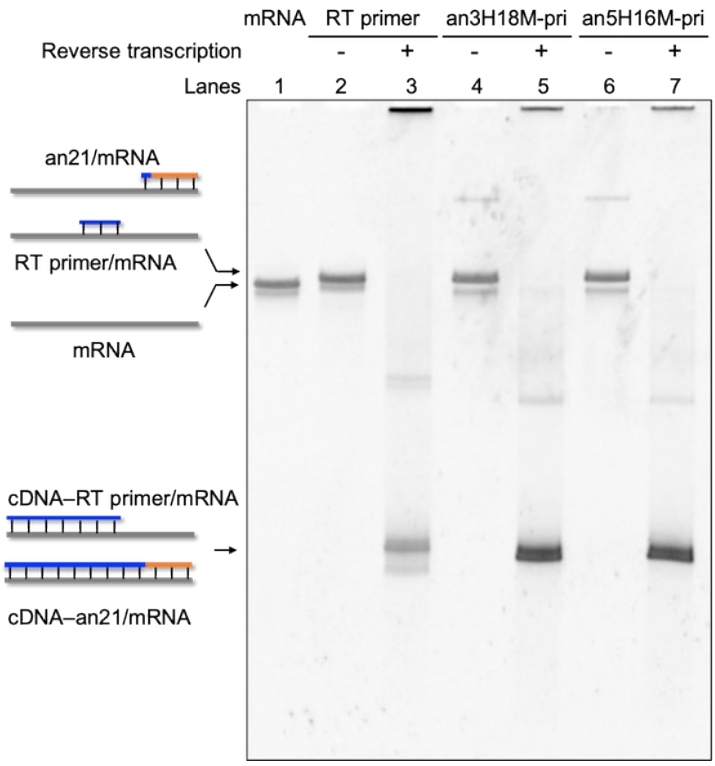


Fig. S1 Reverse transcription of mRNA using the reverse transcription (RT) primer, an3H18M-pri, or an5H16M-pri as a primer. an3H18M-pri, an5H16M-pri, or RT primer (FN3S.R29) was annealed with the wild-type monobody mRNA-1. After reverse transcription, the reaction mixture was analyzed by urea SDS–PAGE. The gel was stained with EtBr.

	Sequence (Blue; DNA, Orenge; 2′-OMe DNA)
3H18M-1	3′ CCTGCCCCCCGCCCTCCGCCC-HEX 5′
3H18M-2	3′ CCTGCCGCCCGCCGTCCGCCC-HEX 5′
3H18M-3	3′ CCTGCCGCCCGCGCTCCGCCC-HEX 5′
3H18M-4	3′ CCTGCCCGCCGCCGTCCGCCC-HEX 5′
3H18M-5	3′ CCTGCCCGCCGCGCTCCGCCC-HEX 5′

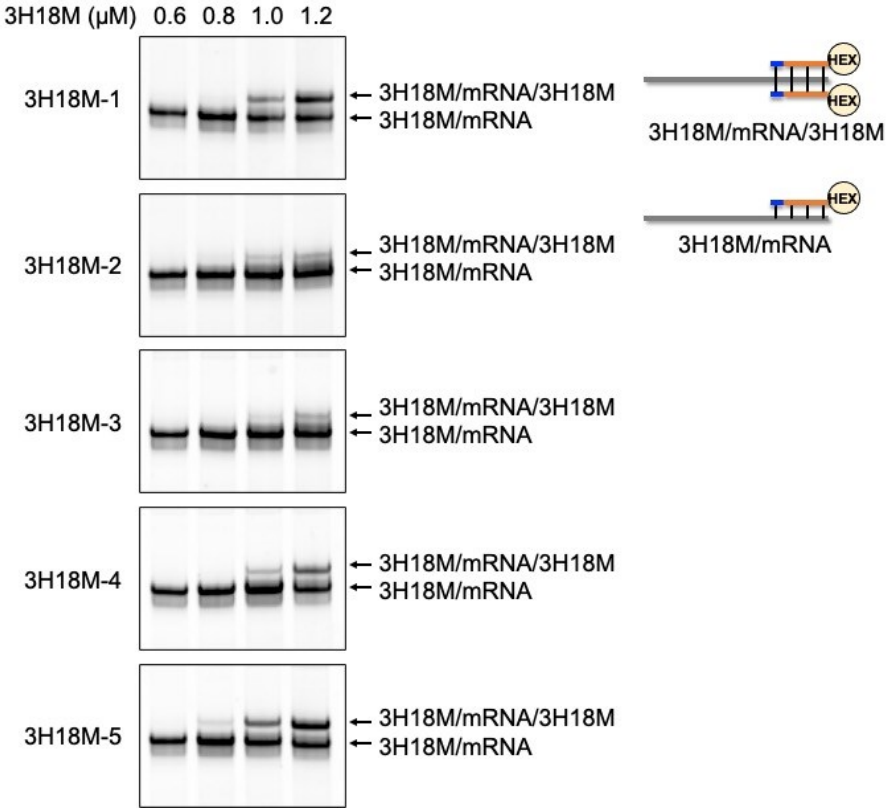
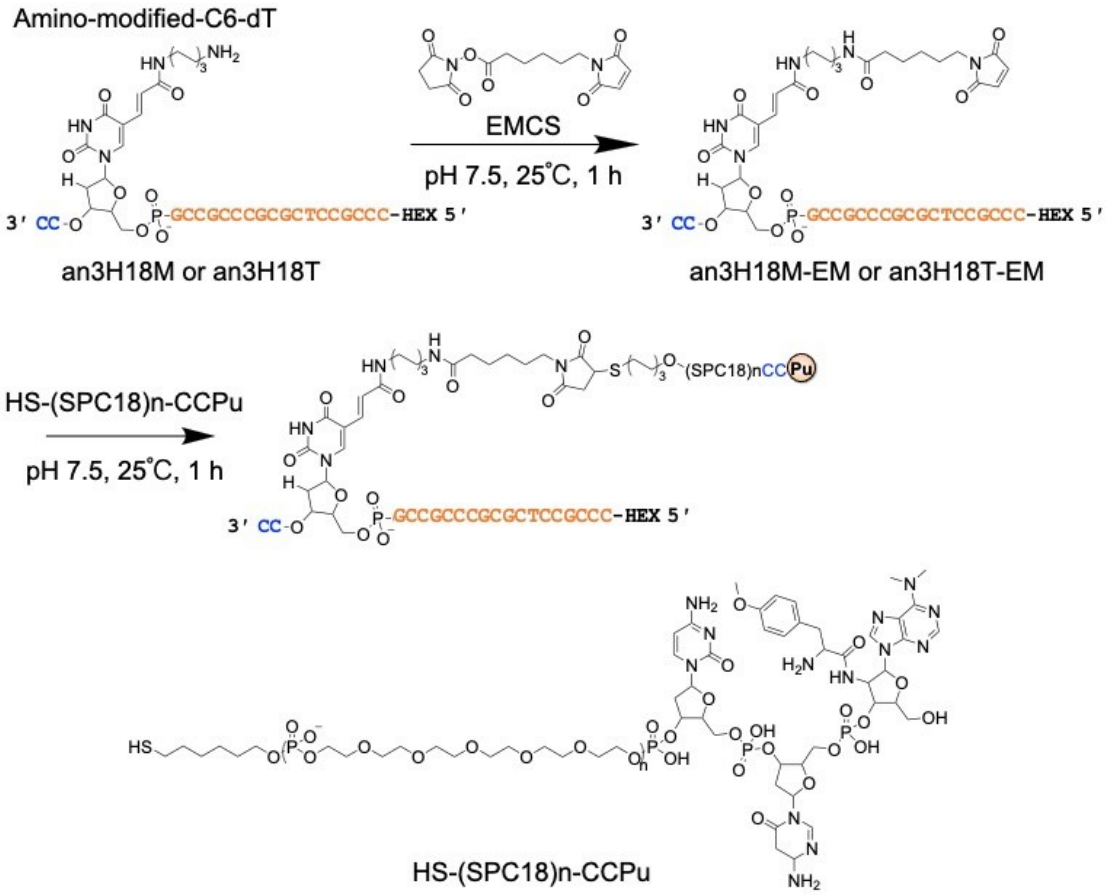


Fig. S2 3H18M sequence optimization. Hex-labelled 3H18M-X (X = 1–5, 0.6–1.2 μM) were annealed with wild-type mRNA-X (X = 1–5). The products were loaded onto 6% PAGE (1:37.5), followed by the measurement of the gel using a fluorescence gel imager.



an21	Spacer
an3H18M	HS-(SPC18)$_1$-CCPu
	HS-(SPC18)$_2$-CCPu
	HS-(SPC18)$_3$-CCPu
	HS-(SPC18)$_4$-CCPu
	HS-(SPC18)$_5$-CCPu
an3H18T	HS-(SPC18)$_2$-CCPu

Fig.S3 Schematic representation of the synthesis of the eight PuLs. The combinations of an21 and spacers in the PuLs are shown at the bottom. Unmodified 2′-H DNA is colored in blue. 2′-OMe DNA or L-αTNA are colored in orange. an3H18M-EM and an3H18T-EM are products of EMCS-reacted an3H18 and an3H18T oligonucleotides.

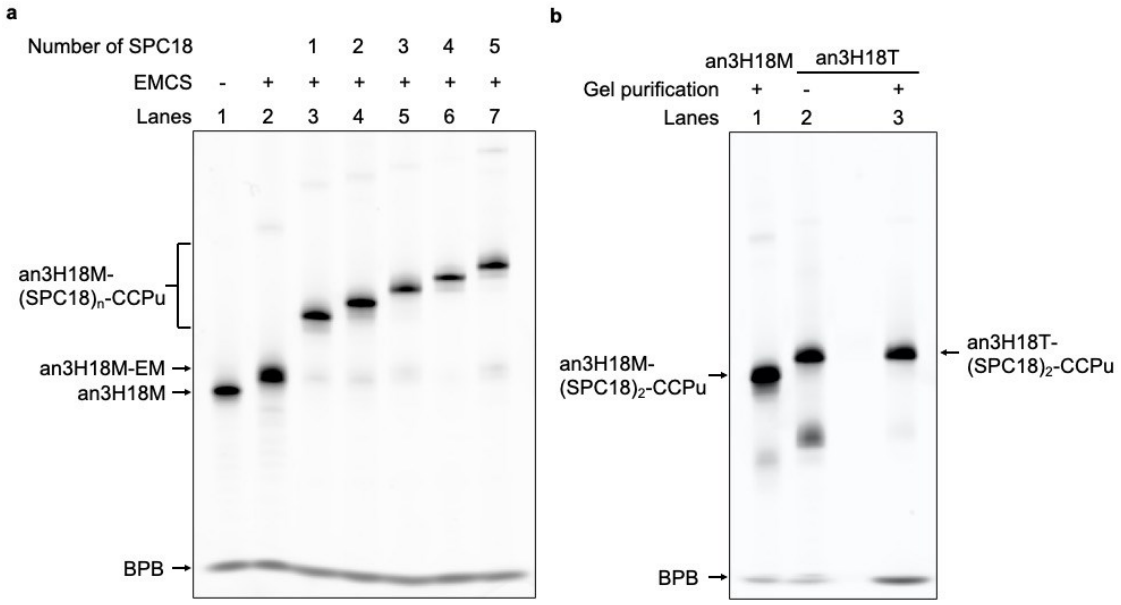


Fig. S4 PAGE analysis of the synthesized PuLs. (a) an3H18M PuLs. After reaction of an3H18M with EMCS, the product was reacted with HS-$(SPC18)_n$-CCPu. After gel purification, the purified products were loaded onto 6 M urea 18% PAGE (1:19), followed by the measurement of the gel using a fluorescence gel imager. (b) an3H18T PuL. an3H18M-$(SPC18)_2$-CCPu was used as a marker.

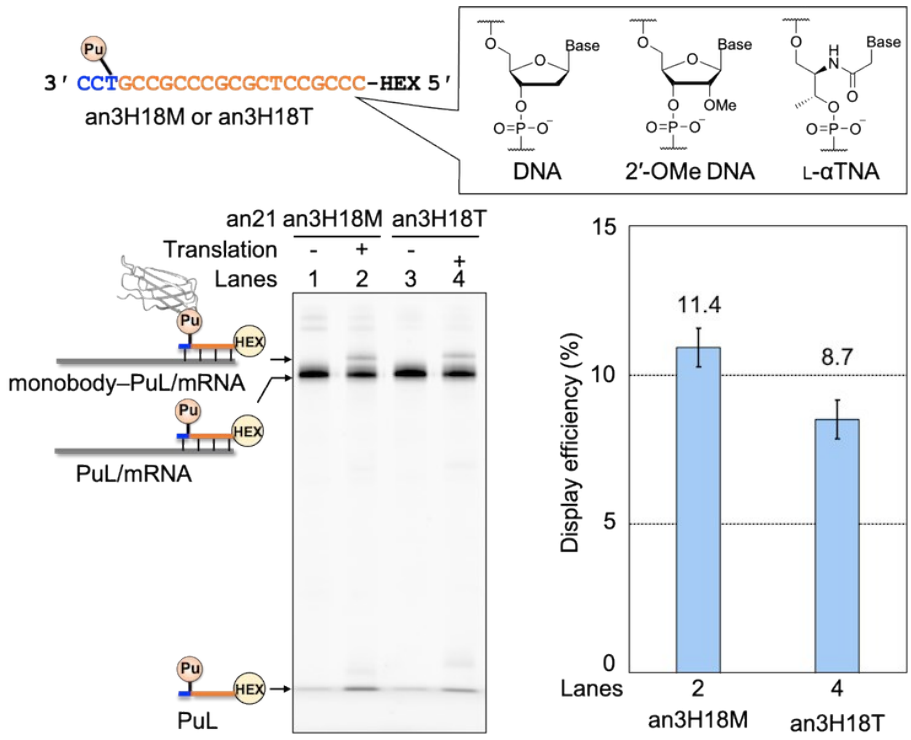


Fig. S5 Display efficiency of the monobody using a PuL with 2′-OMe-DNA or L-αTNA. an3H18M and an3H18T ("H," "M," and "T" represent 2′-H DNA, 2′-OMe DNA, and L-αTNA, respectively) were reacted with EMCS and attached to HS-$(SPC18)_2$-CC(puromycin). The PuLs were annealed with wild-type monobody mRNA and added to the cell-free translation system. After reaction at 37°C for 30 min, the reaction mixture was analyzed using urea SDS–PAGE, followed by measurement of the gel using a fluorescence gel imager. The panels on the right represent the efficiency of the ALPs/mRNA/PuL complex formation (lanes 2 and 4). Error bars, standard deviation of each experiment, in triplicate.

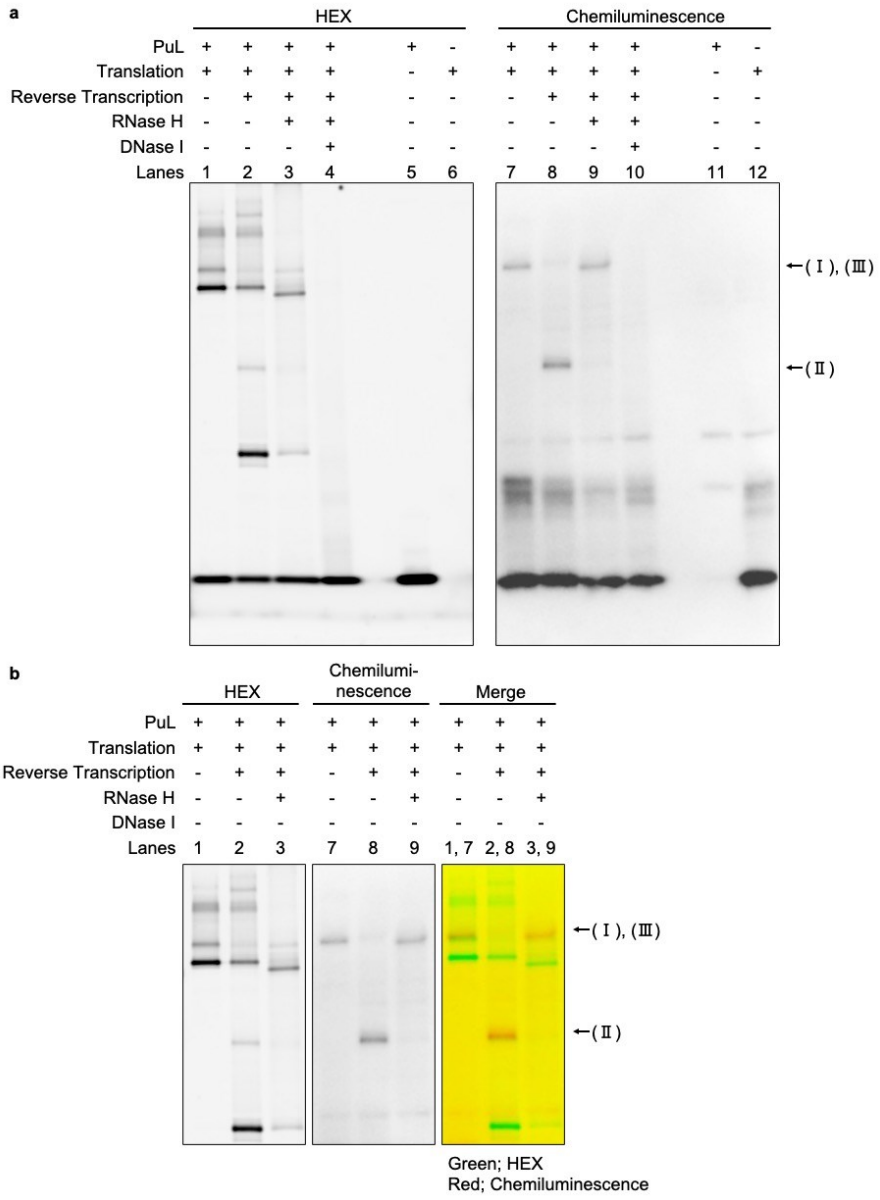


Fig. S6 Western blot analysis of monobody–PuL/mRNA (I), monobody–PuL–cDNA/mRNA (II), and monobody–PuL–cDNA complexes (III). (a) Biotin-Phe was incorporated at the *N*-terminal of the monobody. After urea SDS-PAGE, PuL was visualized by fluorescence from the HEX attached at the 5′ end (left). Alternatively, biotin at the *N*-terminal of the monobody was detected by Western blot using a streptavidin–HRP (right). (b) Expanded version of (a) and the superimposed image of the gel and Western blot.

Table S1 Nucleotide sequences used in this work. mT, mG, and mC represent 2′-OMe DNA. tT, tG, and tC represent L-αTNA. (T) represents amino-modified-C6-dT. Abbreviations: SPC18, spacer 18; Pu, puromycin; HEX, hexachloro-fluorescein

Name	Sequences (5′ to 3′)	Additional information
Wiled-type monobody (the 10th fibronectin type-III domain of human)	ATACTAATACGACTCACTATAGGATTAAGGAGGTGAT ATTTATGCAAGCCAATTCTGGTTCTCTGGAAGTTGTGG AAGCCAGCCCGACGAGCATTCAGATTTCTTGGGACGC TCCGGCGGTCACGGTTCGCTACTATCGCATTACCTATG GCGAAACCGGCGGTAACAGTCCGGTCCAGGAATTTAC GGTGCCGGGTTCAAAATCGACCGCGACGATTTCCGGC CTGAAACCGGGTGTTGATTATACCATCACGGTGTACG CAGTTACCGGTCGTGGTGACAGCCCGGCCAGCTCTAA ACCGATTTCTATCAACTACCGCACGGGTGGAGGAGGA GGTAGCTAGGGACGGCGGGCGCGAGGCGGG	
MC template	ATGCAAGCCAATTCTGGTTCTCTGGAAGTTGTGGAAG CCAGCCCGACGAGCATTCAGATTTCTTGGTGGATTAG GGGGTCGTTTTTGCTGTGGCGCTACTATCGCATTACCT ATGGCGAAACCGGCGGTAACAGTCCGGTCCAGGAATT TACGGTGCCGGGTTGGCGTAATACCGCGACGATTTCC GGCCTGAAACCGGGTGTTGATTATACCATCACGGTGT ACGCAGTTACCGCGGCGGCTATTTCGGTTTGGCGGCC GGTTGGGCCGATTTCTATCAACTACCGCACG	
FN3-from-ExpVec.F40	GGATTAAGGAGGTGATATTTATGCAAGCCAATTCTGG TTC	
FN3-from-ExpVec.R40	GCTACCTCCTCCTCCACCCGTGCGGTAGTTGATAGAA ATC	
T7SD8M2.F44	ATACTAATACGACTCACTATAGGATTAAGGAGGTGAT ATTTATG	
G5S-4Gan21-3.R42	CCCGCCTCGCGCCCGCCGTCCCTAGCTACCTCCTCCTC CACC	
FN3S.R29	TAGCTACCTCCTCCTCCACCCGTGCGGTA	
FN3L1p.R20	CCAAGAAATCTGAATGCTCG	
an3H18M	mCmCmCmGmCmCmTmCmGmCmGmCmCmCmGmCmCm G(T)CC	5'-HEX modification
an3H18T	tCtCtCtGtCtCtTtCtGtCtGtCtCtCtGtCtCtG(T)CC	5'-HEX modification

Hex-Pu-an21M (original TRAP display)	mCmCmCmGmCmCmTmCmGmCmGmCmCmCmGmCmCmGmTmCmC (SPC18) $_5$ CC (Pu)	5'-HEX modification, 3'-Pyuromycin modification
HS-(SPC18)$_n$-CCPu	HS-(SPC18)$_n$-CCPu	5'-Thiol modification, 3'-Pyuromycin modification
an3H18M-pri	mCmCmCmGmCmCmTmCmCmCmGmCmCmCmCmCmCmGTCC	Used in Fig. S1
an5H16M-pri	mCmCmCmGmCmCmTmCmCmCmGmCmCmCmCmCCGTCC	Used in Fig. S1
Wiled-type monobody mRNA-X	ATACTAATACGACTCACTATAGGATTAAGGAGGTGATATTTATGCAAGCCAATTCTGGTTCTCTGGAAGTTGTGGAAGCCAGCCCGACGAGCATTCAGATTTCTTGGGACGCTCCGGCGGTCACGGTTCGCTACTATCGCATTACCTATGGCGAAACCGGCGGTAACAGTCCGGTCCAGGAATTTACGGTGCCGGGTTCAAAATCGACCGCGACGATTTCCGGCCTGAAACCGGGTGTTGATTATACCATCACGGTGTACGCAGTTACCGGTCGTGGTGACAGCCCGGCCAGCTCTAAACCGATTTCTATCAACTACCGCACGGGTGGAGGAGGAGGTAGCTAG-XSeq X=1, XSeq = GACGGGGGGCGGGAGGCGGG X=2, XSeq = GACGGCGGGCGGCAGGCGGG X=3, XSeq = GACGGCGGGCGCGAGGCGGG X=4, XSeq = GACGGGCGGCGGCAGGCGGG X=5, XSeq = GACGGGCGGCGCGAGGCGGG	Used in Fig. S1 and S2
G5S-4an21-1.R41	CCCGCCTCCCGCCCCCCGTCCTAGCTACCTCCTCCTCCACC	
G5S-4an21-2.R41	CCCGCCTGCCGCCCGCCGTCCTAGCTACCTCCTCCTCCACC	
G5S-4an21-3.R41	CCCGCCTCGCGCCCGCCGTCCTAGCTACCTCCTCCTCCACC	
G5S-4an21-4.R41	CCCGCCTGCCGCCGCCCGTCCTAGCTACCTCCTCCTCCACC	
G5S-4an21-5.R41	CCCGCCTCGCGCCGCCCGTCCTAGCTACCTCCTCCTCCACC	

an3H18M-1	mCmCmCmGmCmCmTmCmCmCmGmCmCmCmCmCmCm GTCC	5'-HEX modification
an3H18M-2	mCmCmCmGmCmCmTmGmCmCmGmCmCmCmGmCmCm GTCC	5'-HEX modification
an3H18M-3	mCmCmCmGmCmCmTmCmGmCmGmCmCmCmGmCmCm GTCC	5'-HEX modification
an3H18M-4	mCmCmCmGmCmCmTmGmCmCmGmCmCmGmCmCmCm GTCC	5'-HEX modification
an3H18M-5	mCmCmCmGmCmCmTmCmGmCmGmCmCmGmCmCmCm GTCC	5'-HEX modification