Supplementary Information

Evolution of Sequence-Defined Highly Functionalized Nucleic Acid Polymers

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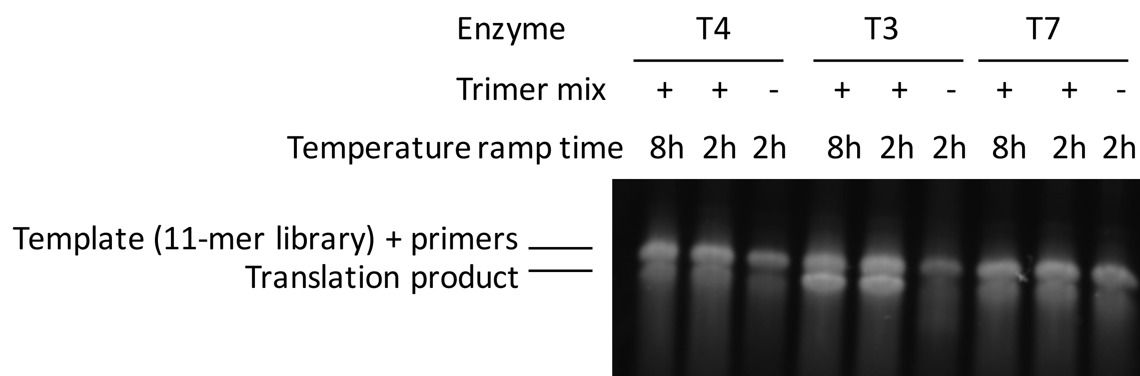
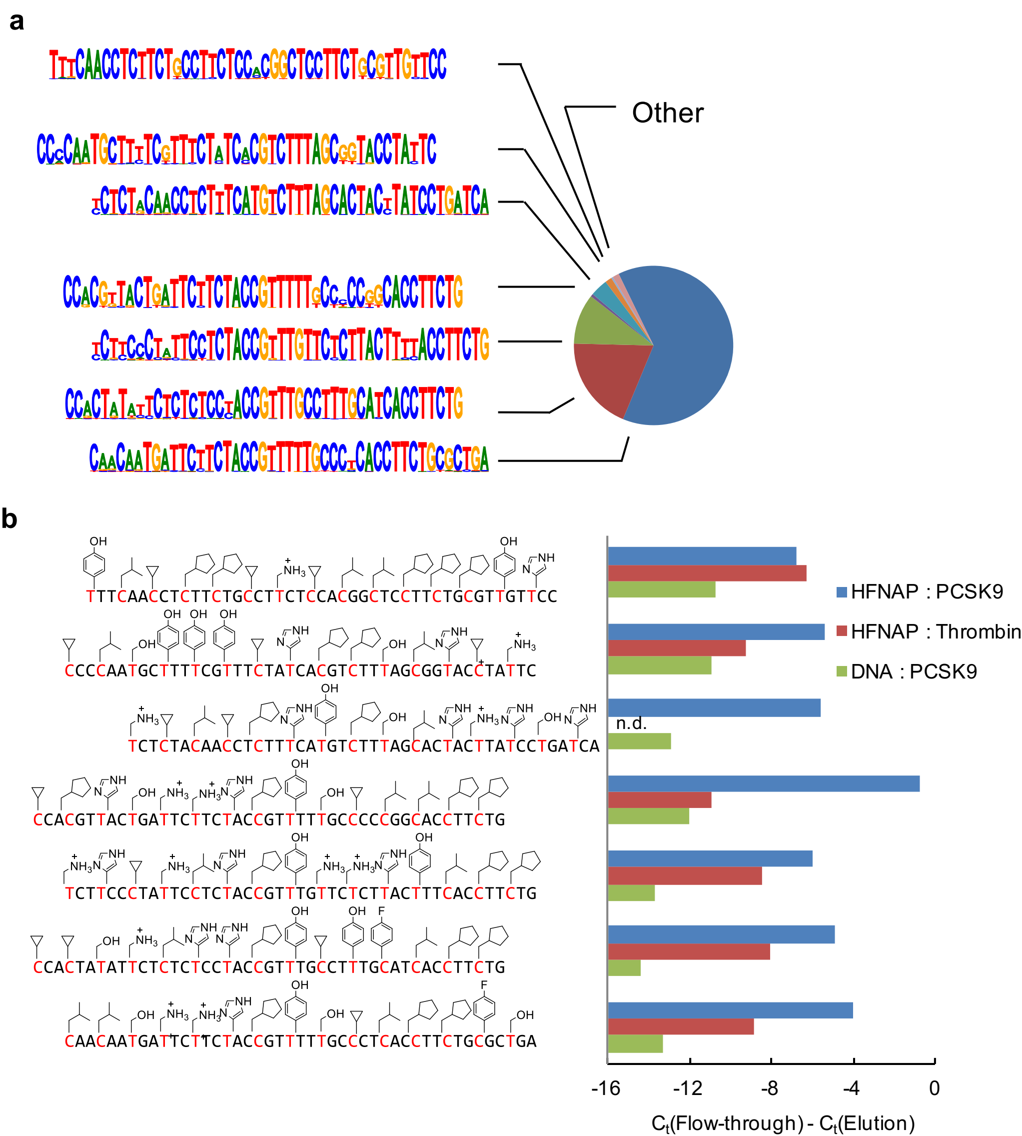
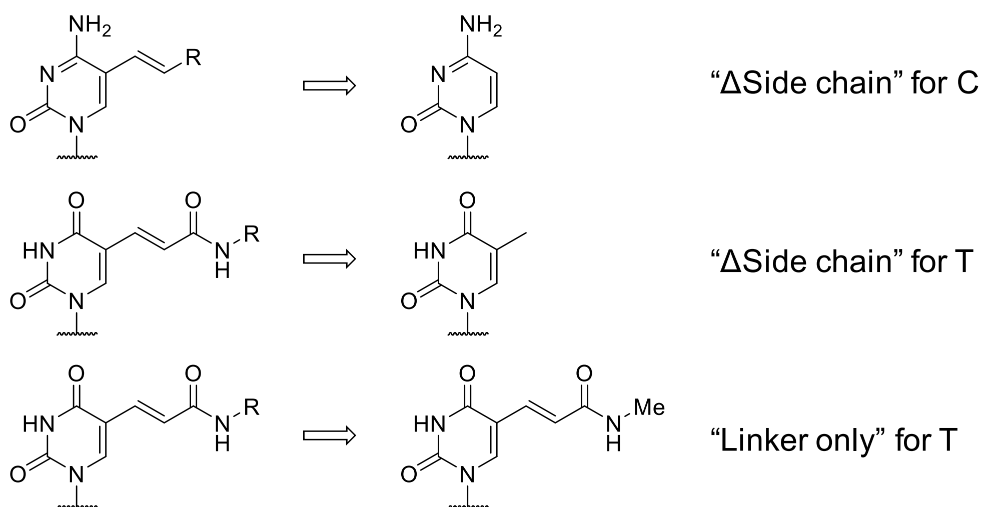


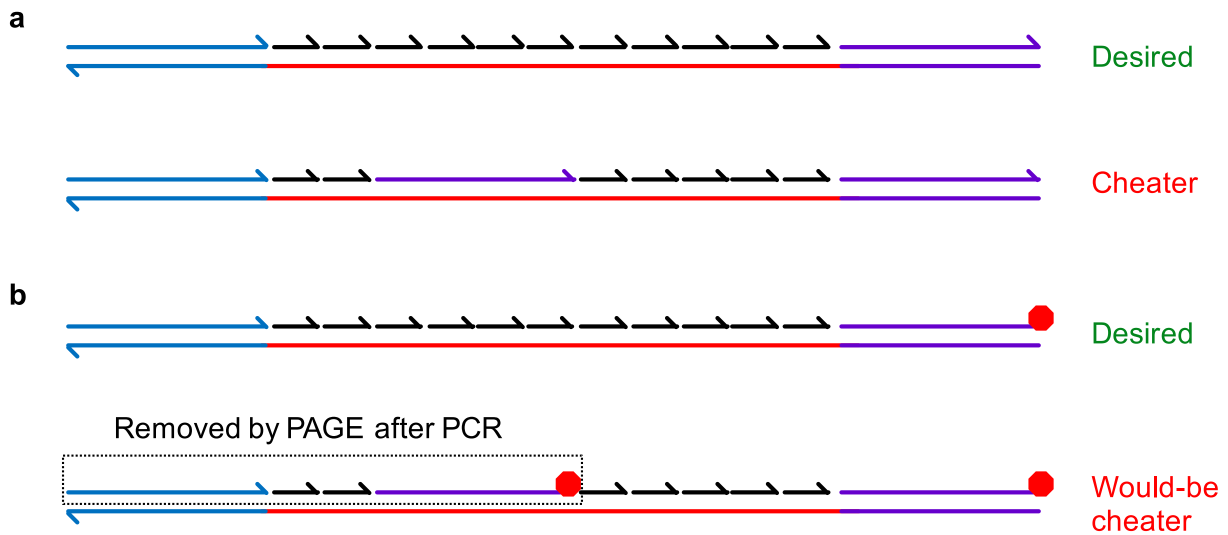
Supplementary Figure 1. Screening of ligases for polymerization. The translation reactions, as well as control reactions from which the trinucleotide building blocks were omitted, were analyzed by polyacrylamide gel electrophoresis on a non-denaturing 10% TBE gel and imaged by SYBR Gold staining. T3 DNA ligase mediated higher translation efficiency than T4 and T7 DNA ligases. Increasing the temperature ramp-down time from 2 hours to 8 hours had little effect on translation yield.



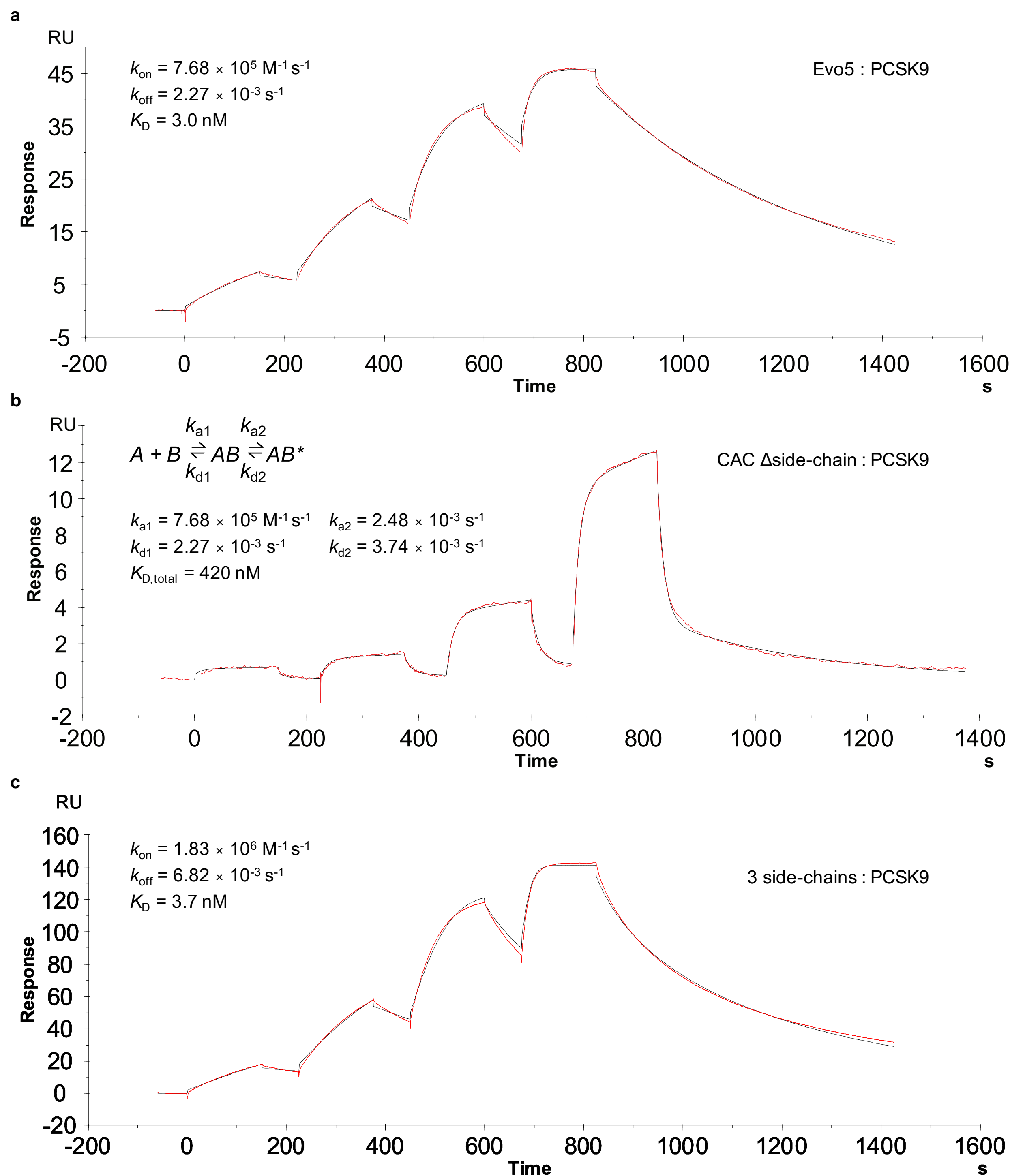
Supplementary Figure 2. Sequence homology and characterization of selection-enriched PCSK9-binding HFNAPs. (a) Relative abundances and sequence logos of the dominant seven sequence clusters found in 3.1 million sequences from high-throughput DNA sequencing after nine rounds of PCSK9-binding selection from a naïve library. (b) Retention of selection-enriched HFNAPs (only the variable, functionalized parts of the sequences are shown) on immobilized PCSK9 (target; blue bars) and immobilized thrombin (non-target; red bars) and of sequence-matched unfunctionalized DNA on immobilized PCSK9 (green bars), as quantified by qPCR. n.d. = not determined.



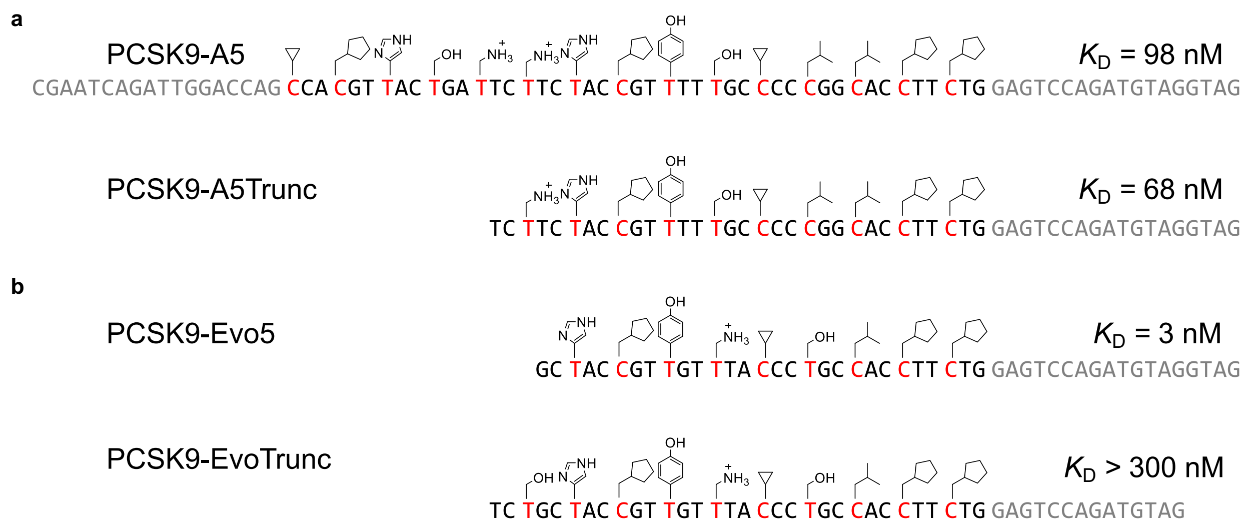
Supplementary Figure 3. Structures of bases in side-chain mutants characterized during structure-activity relationship studies of PCSK9-A5 and PCSK9-Evo5.



Supplementary Figure 4. Cheater suppression strategy. (a) In addition to the desired polymerization reaction (top), incorporation of a polymerization primer in the coding region can generate undesired cheaters (bottom) that rapidly amplify during PCR due to their small size and eventually dominate the sequence pool over rounds of selection. While our first PCSK9-binding selection campaign was not substantially affected by cheaters, our initial attempt at evolving PCSK9-A5 for higher affinity was unsuccessful because the cheaters eventually took over the pool. (b) Using a non-extendable 2',3'-dideoxyribose-terminated 3' polymerization primer addresses this problem, as truncated cheaters can be removed during PAGE purification.

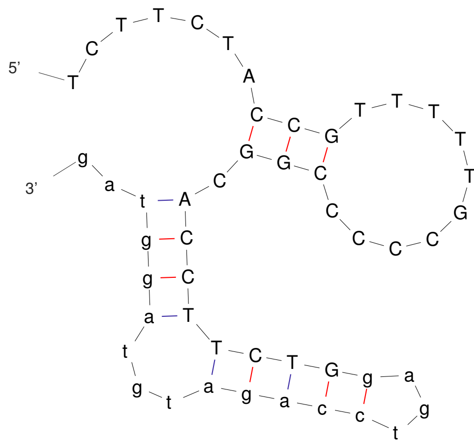


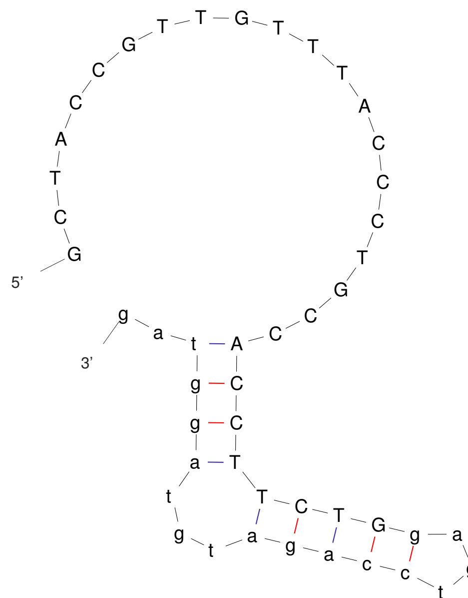
Supplementary Figure 5. Binding kinetics characterization between PCSK9 protein and PCSK9-Evo5 or a side-chain mutant. (a) SPR sensogram characterizing binding kinetics between PCSK9 protein and surface-immobilized biotinylated PCSK9-Evo5. (b) SPR sensogram characterizing binding kinetics between PCSK9 protein and surface-immobilized biotinylated PCSK9-Evo5-"CACΔSide chain". The concentrations of injected PCSK9 were 2, 6, 20, and 60 nM. The raw sensograms are shown in red and the fitted curves with the kinetic parameters listed are shown in black.



Supplementary Figure 6. Effects of truncating HFNAPs on their binding affinity to PCSK9 protein. (a) Effect of truncating PCSK9-A5 from the 5' end. (b) Effects of truncating PCSK9-Evo5 from the 3' end. No binding of up to 300 nM solution-phase PCSK9 to surface-immobilized PCSK9-EvoTrunc was observed by SPR.

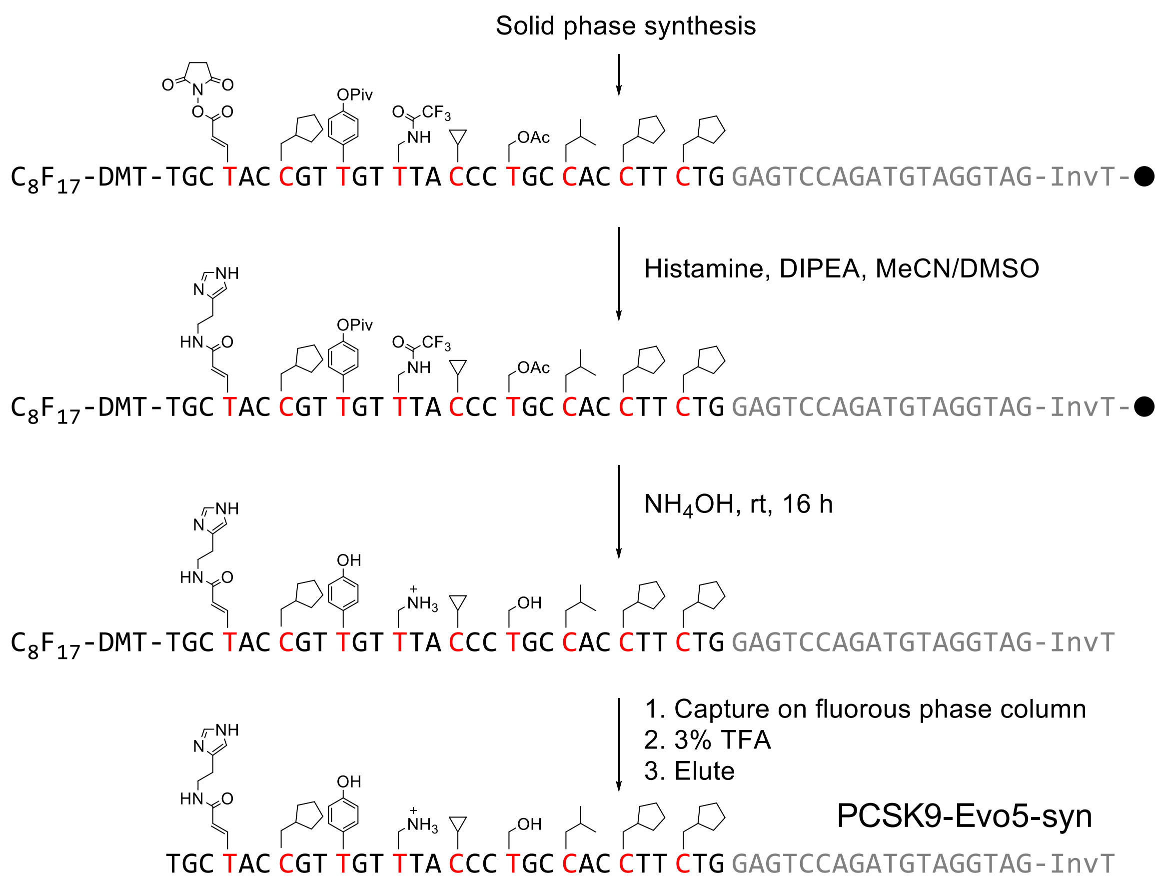
a **b**



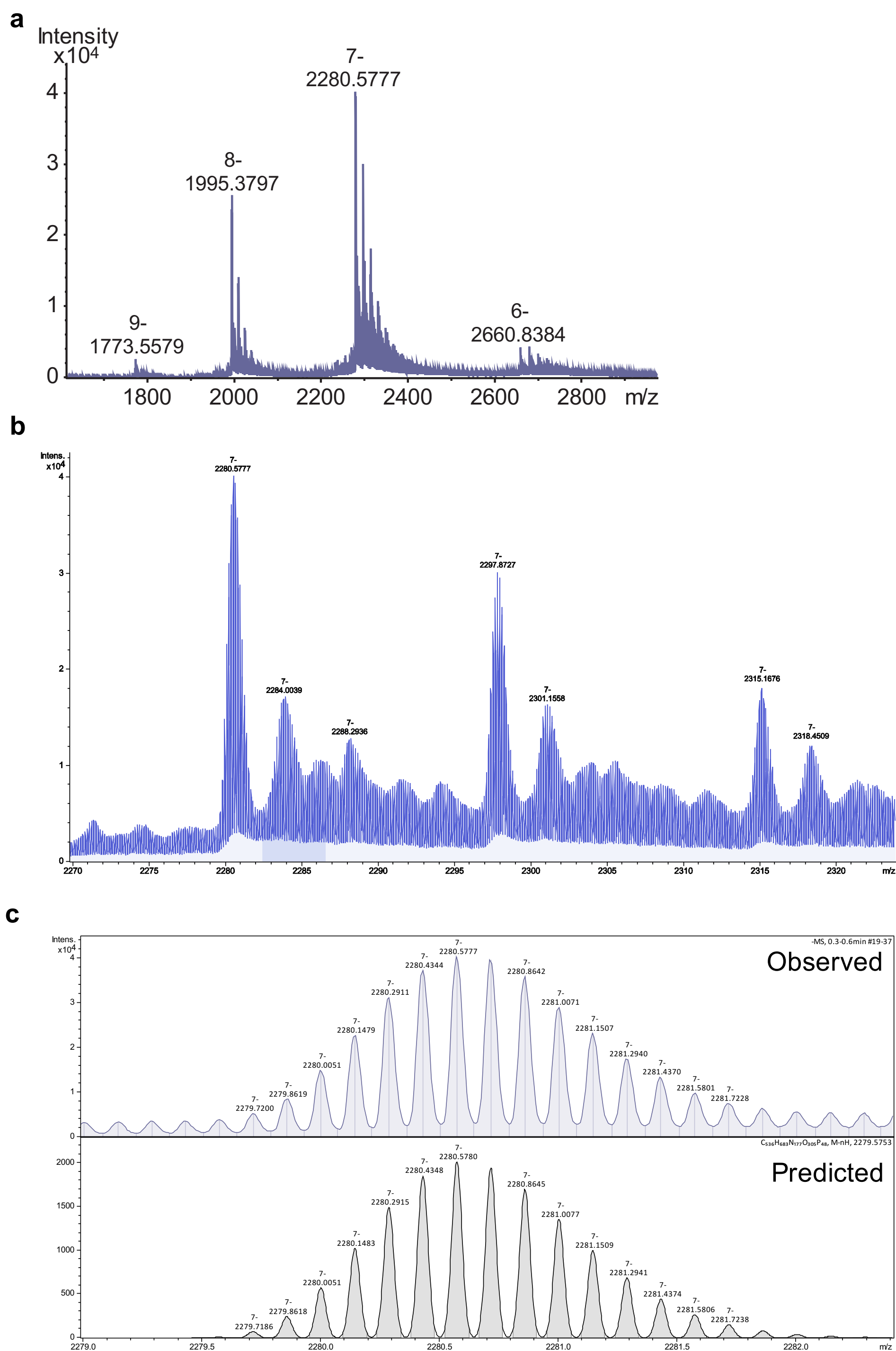


PCSK9-A5Trunc (DNA) PCSK9-Evo5 (DNA)

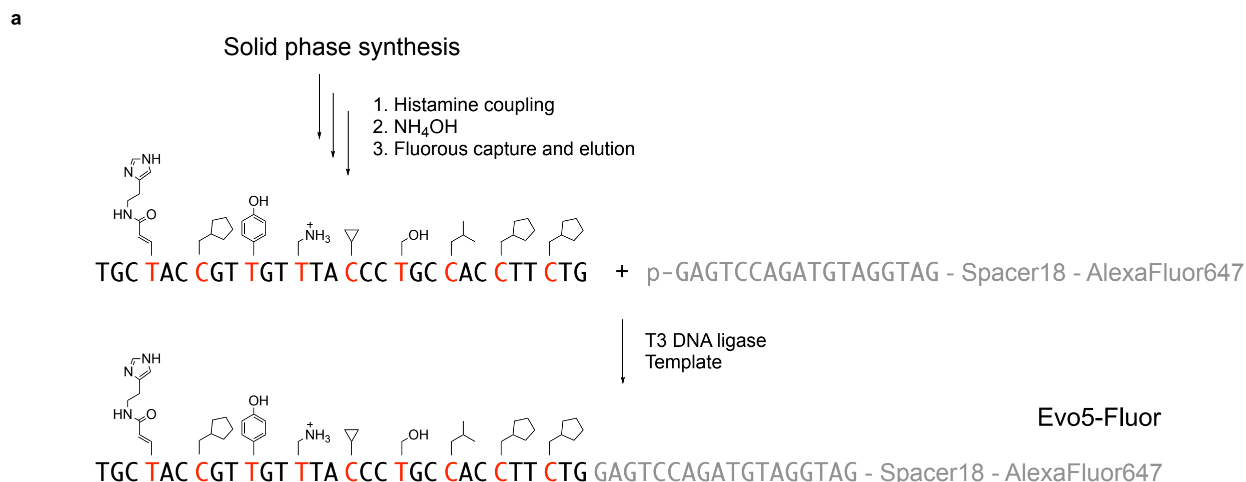
Supplementary Figure 7. Secondary structure predictions of HFNAPs based on their DNA sequences. (a) Predicted minimum free energy secondary structure based on the DNA sequence of PCSK9-A5Trunc. (b) Predicted minimum free energy secondary structure based on the DNA sequence of PCSK9-Evo5. Nucleotides in the constant, unfunctionalized primer-binding region are shown in lowercase letters, and nucleotides in the variable, functionalized region are shown in uppercase letters. Note that predictions do not consider side chains, which are likely to be crucial in HFNAP folding.

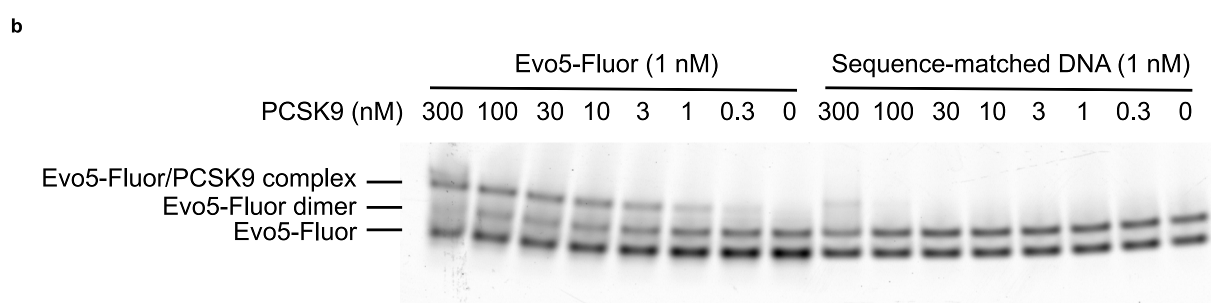


Supplementary Figure 8. Multi-milligram-scale synthesis scheme for PCSK9-Evo5-syn.

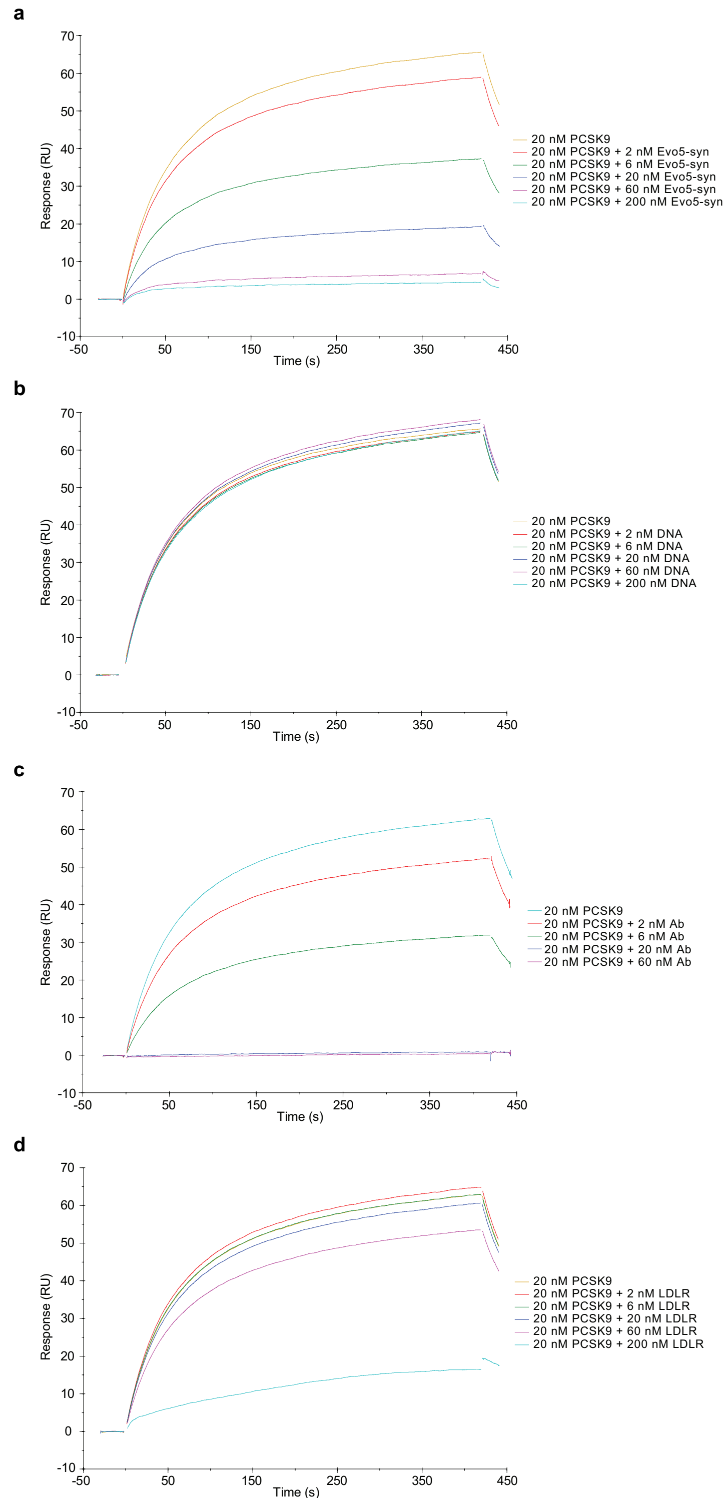


Supplementary Figure 9. ESI-MS spectrum of PCSK9-Evo5-syn. Molecular formula of PCSK9-Evo5-syn is $C_{536}H_{683}N_{177}O_{305}P_{48}$. (a) Full spectrum. (b) Spectrum detailing the "7-" group of peaks, showing the peak clusters for $[M-7H]^{7-}$ (around m/z 2280.5777), as well as the sodium adduct (around m/z 2284.0039), the Tris adduct (around m/z 2297.8727), and other multiple adducts. (c) Top: observed spectrum of the $[M-7H]^{7-}$ peak cluster. Bottom: predicted spectrum based on the molecular formula and isotopic abundances.

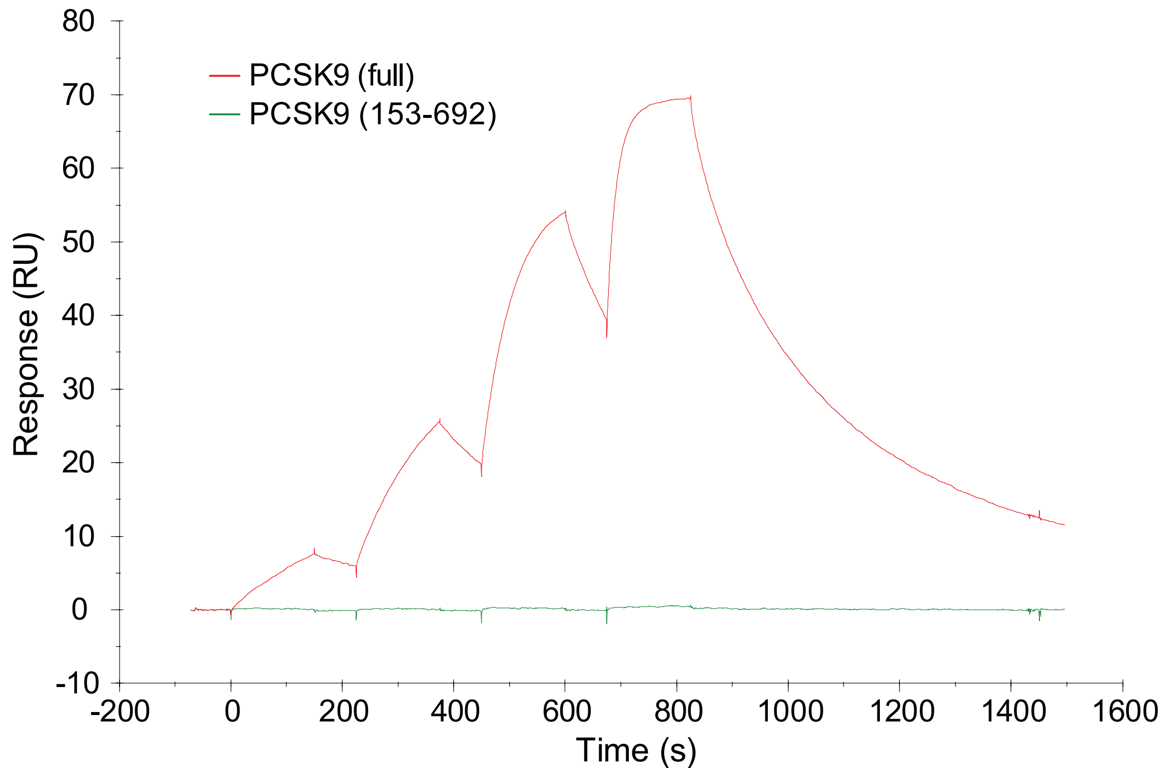




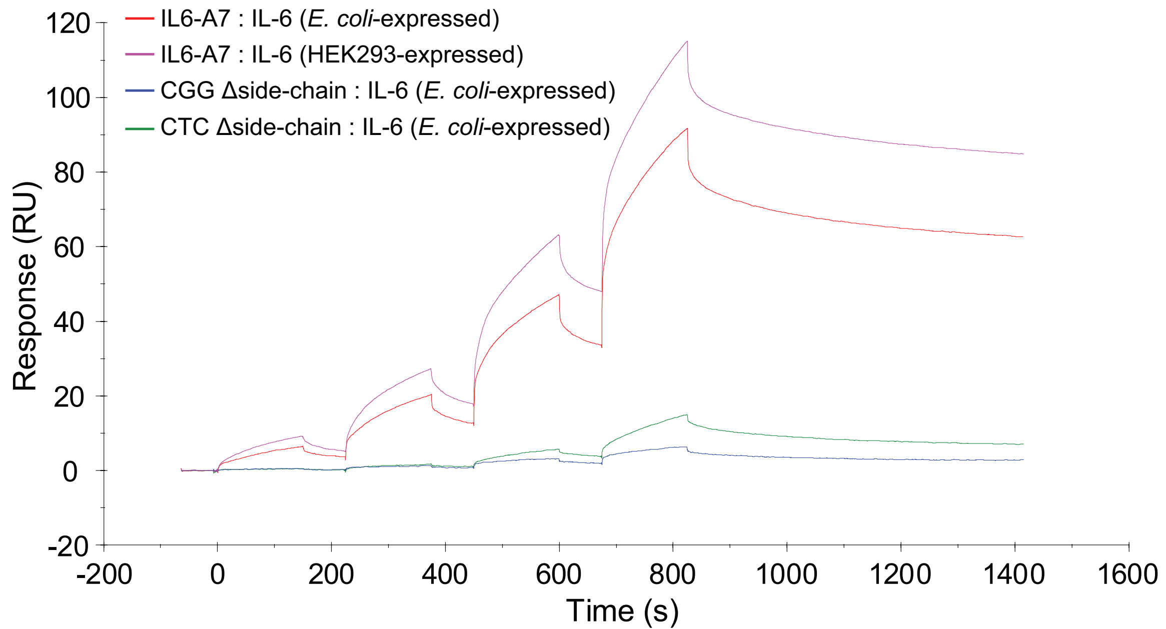
Supplementary Figure 10. Electrophoretic mobility shift assay (EMSA) confirms binding of Evo5-Fluor to PCSK9 protein. (a) Synthesis of the Alexa Fluor 647-labeled Evo5-Fluor. (b) EMSA results testing Evo5-Fluor binding to PCSK9. Evo5-Fluor (1 nM) could form a complex with PCSK9 protein at concentrations down to 0.3 nM, while a sequence-matched DNA (1 nM) formed a small amount of complex only at 300 nM PCSK9. Both Evo5-Fluor and the sequence-matched DNA dimerize under the assay conditions (4 °C), resulting in the extra band. We note that the EMSA gel electrophoresis experiment takes place on a slower time scale (~15 min) than dissociation of the polymer:PCSK9 complex ($k_{off} = 2.42 \times 10^{-3}$ s^{-1} by SPR, which corresponds to $t_{½}$ ~5 min), and therefore underestimates binding affinity.



Supplementary Figure 11. SPR characterization of PCSK9-LDLR binding in the presence of PCSK9-Evo5-syn or various controls. The sensograms show the interaction between surface-immobilized LDLR and solution-phase PCSK9 pre-incubated with varying concentrations of (a) PCSK9-Evo5-syn, (b) sequence-matched unfunctionalized DNA, (c) a known PCSK9-neutralizing antibody, or (d) unlabeled LDLR.



Supplementary Figure 12. Effect of truncating PCSK9 on its binding affinity to PCSK9-Evo5-syn. SPR sensograms show binding kinetics between surface-immobilized biotinylated PCSK9-Evo5 and either full-length PCSK9 protein (red) or a truncated PCSK9 protein missing the prodomain (green). The concentrations of injected protein were 2, 6, 20, and 60 nM.



Supplementary Figure 13. SPR characterization of binding between IL-6 and HFNAPs. The SPR sensograms show binding kinetics between biotinylated IL6-A7 (red) or two side-chain variants (blue and green) and *E. coli*-expressed IL-6 protein, and between biotinylated IL6-A7 and HEK293 cell-expressed IL-6 protein (purple). For all experiments, comparable amounts of the biotinylated HFNAPs (all between 90 and 120 RU) were immobilized on the active flow cell. The concentrations of injected IL-6 were 10, 30, 100, and 300 nM.

Materials and Methods

1. Oligonucleotide sequences

All occurrences of U below are 2'-deoxy-U. Commercially available oligonucleotide modifiers are denoted by shorthand notations used by IDT.

Evaluation of translation yield on template libraries

Name	Sequence
Template-11codon	CGTACGGTCGACGCTAGCNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRCACGTGGAGCTCGGATCC
Template-13codon	CGTACGGTCGACGCTAGCNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRCACGTGGAGCTCGGATCC
Template-15codon	CGTACGGTCGACGCTAGCNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRNNRCACGTGGAGCTCGGATCC
pp1-library	/5Phos/GCTAGCGTCGACCGTACG
pp2-library	GGATCCGAGCTCCACGTG

Validation of sequence specificity of translation and amplification

Name	Sequence
Template-Bt-CATA	/52-Bio//iSp18/CGTACGGTCGACGCTAGCTTGAAAGTGCAAGAGACACCGCGACACGTGGAGCTCGGATCC
Template-Bt-CBTB	/52-Bio//iSp18/CGTACGGTCGACGCTAGCATGTTACTGGTATCGTGAGCGGGACACGTGGAGCTCGGATCC
Template-Bt-CCTC	/52-Bio//iSp18/CGTACGGTCGACGCTAGCTAGATATGGCTAGGGTCAAGGGCACACGTGGAGCTCGGATCC
Template-Bt-CDTD	/52-Bio//iSp18/CGTACGGTCGACGCTAGCAAGTAACAGGAAACGAGACGGCCACACGTGGAGCTCGGAUCC
pp1-library-T7	/5Phos/GCTAGCGTCGACCGTACGAGCGTCGCTACGCGTGAC
pp2-library	GGATCCGAGCTCCACGTG
T7-out-PCR2	TAATACGACTCACTATAGGGCTCGATTTAATTTCGCCGACGTGATGACATTCCAGGCAGTGTCACGCGTAGCGACGCT

PCSK9 binder selection and evolution

Name	Sequence
Naïve library AZ15	CGA ATC AGA TTG GAC CAG YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN GAG TCC AGA TGT AGG TAG
BtBt-ExtA	/52-Bio//iSp18/CTA CCT ACA TCT GGA CTC
ExtA	CTA CCT ACA TCT GGA CTC
pp1A	/5Phos/GAG TCC AGA TGT AGG TAG

pp2Z	CGA ATC AGA TTG GAC CAG
MiSeqA	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNNN CTA CCT ACA TCT GGA CTC
MiSeqZ	TGG AGT TCA GAC GTG TGC TCT TCC GAT CT NNNN CGA ATC AGA TTG GAC CAG
IlluminaAdapterFwd	AATGATACGGCGACCACCGAGATCTACAC[8-base barcode]ACACTCTTTCCCTACACGAC
IlluminaAdapterRev	CAAGCAGAAGACGGCATACGAGAT[8-base barcode] GTGACTGGAGTTCAGACGTGTGCT
Rediv library AZ15	CGA ATC AGA TTG GAC CAG XZP XFO JPZ JFP JOZ JOZ JPZ XFO JOO JFZ XZZ XFF XPZ XOO XOF GAG TCC AGA TGT AGG TAG X = 79% dC, 21% T Z = 7% dA, 79% dC, 7% dG, 7% T P = 79% dA, 7% dC, 7% dG, 7% T F = 7% dA, 7% dC, 79% dG, 7% T O = 7% dA, 7% dC, 7% dG, 79% T J = 21% dC, 79% T
pp1A-3ddC	/5Phos/GAG TCC AGA TGT AGG TAG/3ddC/

Synthesis of putative PCSK9 binders for bead retention assay

Name	Sequence
pp1A	/5Phos/GAG TCC AGA TGT AGG TAG
pp2Z	CGA ATC AGA TTG GAC CAG
PCSK9A1-BtTempl	/52-Bio//iSp18/CTA CCT ACA TCT GGA CTC CAG AAG GTG ATG CAA AGG CAA ACG GTA GGA GAG AGA ATA TAG TGG CTG GTC CAA TCT GAT TCG
PCSK9A1-DNA	CGA ATC AGA TTG GAC CAG CCA CTA TAT TCT CTC TCC TAC CGT TTG CCT TTG CAT CAC CTT CTG GAG TCC AGA TGT AGG TAG
PCSK9A2-BtTempl	/52-Bio//iSp18/CTA CCT ACA TCT GGA CTC TCA GCG CAG AAG GTG AGG GCA AAA ACG GTA GAA GAA TCA TTG TTG CTG GTC CAA TCT GAT TCG
PCSK9A2-DNA	CGA ATC AGA TTG GAC CAG CAA CAA TGA TTC TTC TAC CGT TTT TGC CCT CAC CTT CTG CGC TGA GAG TCC AGA TGT AGG TAG
PCSK9A3-BtTempl	/52-Bio//iSp18/CTA CCT ACA TCT GGA CTC TGA TCA GGA TAA GTA GTG CTA AAG ACA TGA AAG AGG TTG TAG AGA CTG GTC CAA TCT GAT TCG
PCSK9A3-DNA	CGA ATC AGA TTG GAC CAG TCT CTA CAA CCT CTT TCA TGT CTT TAG CAC TAC TTA TCC TGA TCA GAG TCC AGA TGT AGG TAG
PCSK9A4-BtTempl	/52-Bio//iSp18/CTA CCT ACA TCT GGA CTC GAA TAG GTA CCG CTA AAG ACG TGA TAG AAA CGA AAA GCA TTG GGG CTG GTC CAA TCT GAT TCG
PCSK9A4-DNA	CGA ATC AGA TTG GAC CAG CCC CAA TGC TTT TCG TTT

	CTA TCA CGT CTT TAG CGG TAC CTA TTC GAG TCC AGA TGT AGG TAG
PCSK9A5-BtTempl	/52-Bio//iSp18/CTA CCT ACA TCT GGA CTC CAG AAG GTG CCG GGG GCA AAA ACG GTA GAA GAA TCA GTA ACG TGG CTG GTC CAA TCT GAT TCG
PCSK9A5-DNA	CGA ATC AGA TTG GAC CAG CCA CGT TAC TGA TTC TTC TAC CGT TTT TGC CCC CGG CAC CTT CTG GAG TCC AGA TGT AGG TAG
PCSK9A6-BtTempl	/52-Bio//iSp18/CTA CCT ACA TCT GGA CTC GGA ACA ACG CAG AAG GAG CCG TGG AGA AGG CAG AAG AGG TTG AAA CTG GTC CAA TCT GAT TCG
PCSK9A6-DNA	CGA ATC AGA TTG GAC CAG TTT CAA CCT CTT CTG CCT TCT CCA CGG CTC CTT CTG CGT TGT TCC GAG TCC AGA TGT AGG TAG
PCSK9A7-BtTempl	/52-Bio//iSp18/CTA CCT ACA TCT GGA CTC CAG AAG GTG AAA GTA AGA GAA CAA ACG GTA GAG GAA TAG GGA AGA CTG GTC CAA TCT GAT TCG
PCSK9A7-DNA	CGA ATC AGA TTG GAC CAG TCT TCC CTA TTC CTC TAC CGT TTG TTC TCT TAC TTT CAC CTT CTG GAG TCC AGA TGT AGG TAG

Synthesis of biotinylated PCSK9-A5 and variants for surface plasmon resonance assay

Name	Sequence
pp1A	/5Phos/GAG TCC AGA TGT AGG TAG
BtBt-pp2Z	/52-Bio//iSp18/CGA ATC AGA TTG GAC CAG
PCSK9A5-Templ	CTA CCT ACA TCT GGA CTC CAG AAG GTG CCG GGG GCA AAA ACG GTA GAA GAA TCA GTA ACG TGG CTG GTC CAA TCT GAT TCG

Synthesis of biotinylated PCSK9-Evo5 and variants for surface plasmon resonance assay

Name	Sequence
pp1A-3primeBtBt	/5Phos/GAG TCC AGA TGT AGG TAG/iSp18/iBiodT/3Bio/
pp1A-3primeBtBt-14nt	/5Phos/GAG TCC AGA TGT AG/iSp18/iBiodT/3Bio/
Evo5-pp2-dU	CCA CGT TAC TGA TTC UGC
PCSK9Evo5-Templ	CTA CCT ACA TCT GGA CTC CAG AAG GTG GCA GGG TAA ACA ACG GTA GCA GAA TCA GTA ACG TGG

Synthesis of PCSK9-Evo5-Fluor and negative control for EMSA assay

Name	Sequence
pp1A-Alexa647	/5phos/GAG TCC AGA TGT AGG TAG /iSp18//3AlexF647N/
Evo5_DNA-LeftHalf	TGCTACCGTTGTTTACCCTGCCACCTTCTG
BtBt_Evo5-Template	/52-Bio//iSp18/CTA CCT ACA TCT GGA CTC CAG AAG GTG GCA GGG TAA ACA ACG GTA GCA GAA TCA GTA ACG TGG

	CTG

Negative control for PCSK9-Evo5-syn in SPR assay

Name	Sequence
Evo5DNA-InvdT	TGC TAC CGT TGT TTA CCC TGC CAC CTT CTG GAG TCC AGA TGT AGG TAG /3InvdT/

IL-6 binder selection

Name	Sequence
Naïve library CW15	CTC GGA TGA ACC TGG ACT YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN YNN GGA CTG AGT CCA GAG TAA
BtBt-ExtC	/52-Bio//iSp18/TTA CTC TGG ACT CAG TCC
ExtC	TTA CTC TGG ACT CAG TCC
pp1C	/5Phos/GGA CTG AGT CCA GAG TAA
pp2W	CTC GGA TGA ACC TGG ACT
MiSeqC	ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNNNNN TTA CTC TGG ACT CAG TCC
MiSeqW	TGG AGT TCA GAC GTG TGC TCT TCC GAT CT NNNN CTC GGA TGA ACC TGG ACT
IlluminaAdapterFwd	AATGATACGGCGACCACCGAGATCTACAC[8-base barcode] ACACTCTTTCCCTACACGAC
IlluminaAdapterRev	CAAGCAGAAGACGGCATACGAGAT[8-base barcode] GTGACTGGAGTTCAGACGTGTGCT

Synthesis of putative IL-6-binding HFNAPs for bead retention assay

Name	Sequence
pp1C	/5phos/GGA CTG AGT CCA GAG TAA
pp2W	CTC GGA TGA ACC TGG ACT
IL6-A1-BtTempl	/52-Bio//iSp18/TTA CTC TGG ACT CAG TCC TGG TGG CCA CTG GCA GCA CCG TCA CGG AGG CTG AGA CTG CCG CAA AGT CCA GGT TCA TCC GAG
IL6-A2-BtTempl	/52-Bio//iSp18/TTA CTC TGG ACT CAG TCC ACA GGA GGG ATA CCG GAA GGG CAA AGG CCG TAG CCA GAA CAA AAA AGT CCA GGT TCA TCC GAG
IL6-A3-BtTempl	/52-Bio//iSp18/TTA CTC TGG ACT CAG TCC ACA ACG CAG CCG GAA GCA CCA CAG GAG AGG CGG TAA CCA GCA ACA AGT CCA GGT TCA TCC GAG
IL6-A4-BtTempl	/52-Bio//iSp18/TTA CTC TGG ACT CAG TCC TCG GAA GCG TCA CGA CGG TAA CCG GCA CTG AGA GCA ACA CCA CAA AGT CCA GGT TCA TCC GAG
IL6-A5-BtTempl	/52-Bio//iSp18/TTA CTC TGG ACT CAG TCC TGG CTA AGG CGA CCA CGG GCA CTG CAA CCA CAG CCA AAG GGG TGA

	AGT CCA GGT TCA TCC GAG
IL6-A6-BtTempl	/52-Bio//iSp18/TTA CTC TGG ACT CAG TCC CAA TGA GGG AGA GAG GGG GAA GGG CGA CAA AGG CCG TAA CCA GGA AGT CCA GGT TCA TCC GAG
IL6-A7-BtTempl	/52-Bio//iSp18/TTA CTC TGG ACT CAG TCC CCG GAG GAA TGA GTA CGA GGA AGG GCA ACG AAA TAA ACA GCA GCA AGT CCA GGT TCA TCC GAG

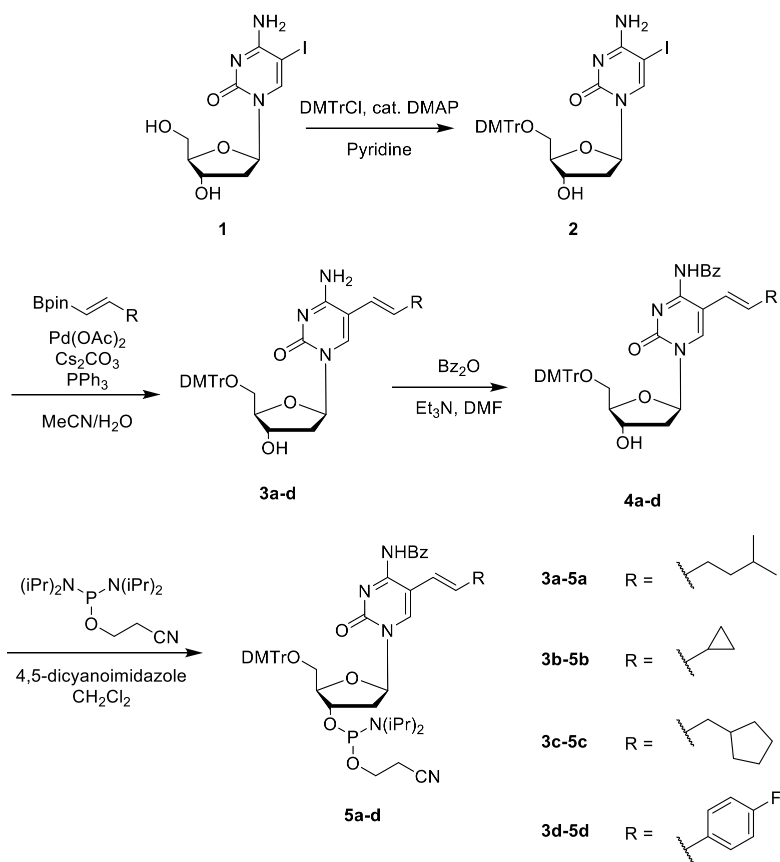
Synthesis of biotinylated IL6-A7 and variants for surface plasmon resonance assay

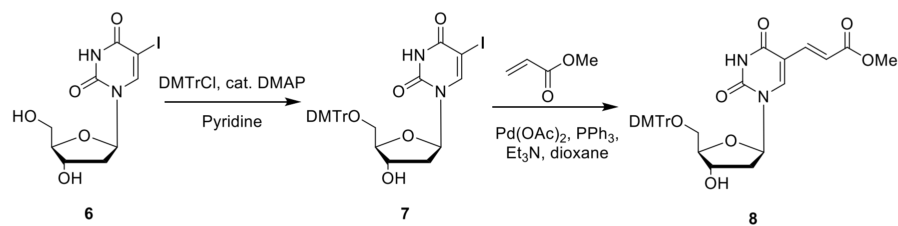
Name	Sequence
pp1C-3ddC	/5Phos/GGACTGAGTCCAGAGTAA/3ddC/
BtBt-pp2W	/52-Bio//iSp18/CTCGGATGAACCTGGACT
IL6-A7-Templ	TTA CTC TGG ACT CAG TCC CCG GAG GAA TGA GTA CGA GGA AGG GCA ACG AAA TAA ACA GCA GCA AGT CCA GGT TCA TCC GAG

2. Synthesis and characterization of Phosphoramidite Intermediates.

Compounds were prepared and characterized by Wuxi AppTec Co. under the direction of Xun Hong. The protocols and characterization furnished along with these compounds are printed here. NMR spectra were recorded on a Bruker Avance 400 MHz for ^{1}H NMR. Chemical shifts are reported in ppm (δ). Chromatographic purifications were by flash chromatography using 100~200 mesh silica gel. Anhydrous solvents were pre-treated with 3 Å MS column before use. All commercially available reagents were used as received unless otherwise stated.

General synthetic routes:

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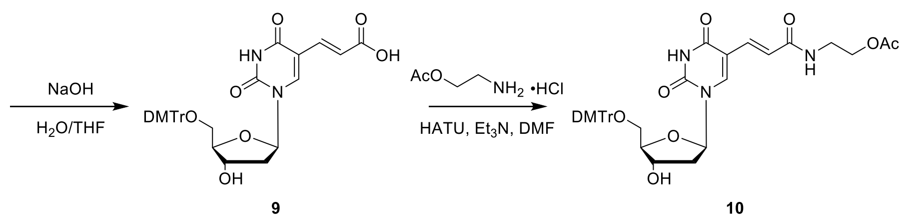
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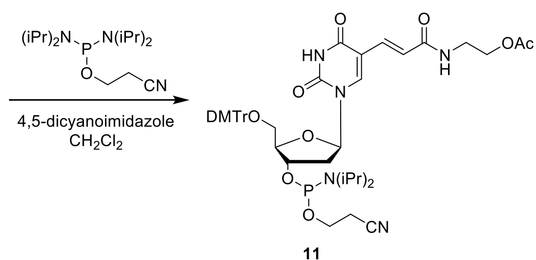
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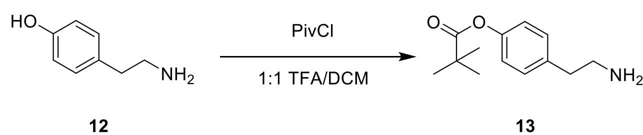
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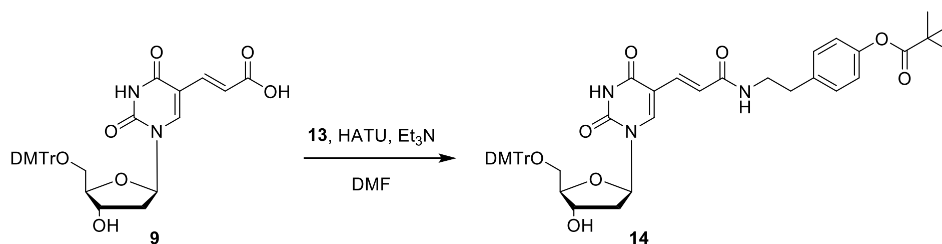
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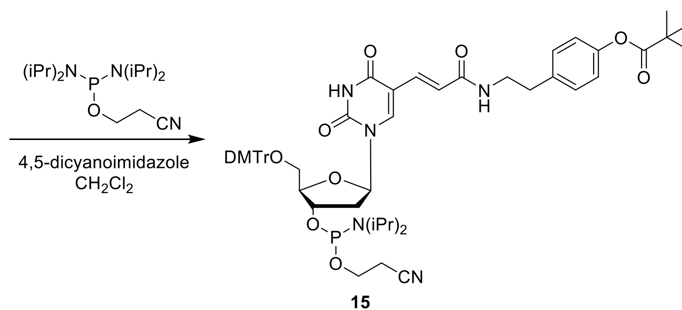
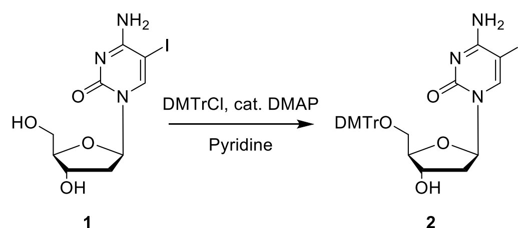
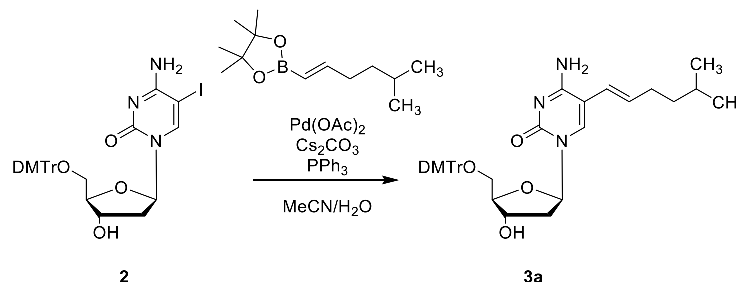
9
14


(iPr)2N
P
N(iPr)2
CN
4,5-dicyanoimidazole
CH2Cl2
15

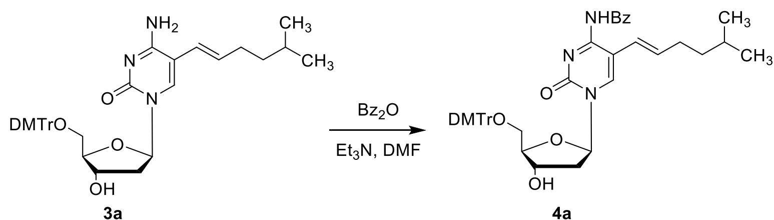
*Synthesis of phosphoramidites **5a-d***



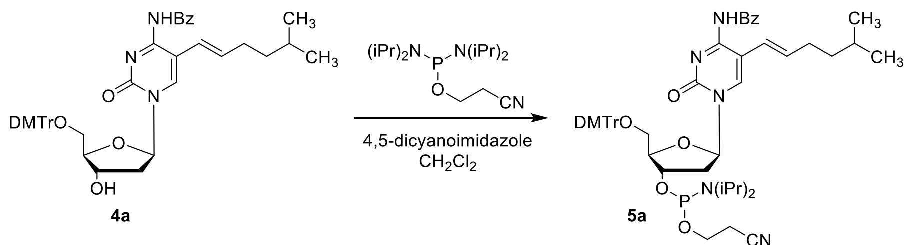
To a solution of **1** (40.0 g, 113.2 mmol, 1 equiv) and DMAP (0.113 g, 1.13 mmol, 0.01 equiv) in pyridine (400 mL) was added dropwise DMTrCl (40.2 g, 119 mmol, 1.05 equiv) and at 0 °C. The mixture was stirred at 25 °C for 16 h. TLC (DCM/MeOH = 20/1) indicated that **1** was consumed completely. The reaction mixture was concentrated with MeOH (50 mL) under reduced pressure to remove pyridine. The residue was purified by column chromatography (SiO_2, DCM/MeOH=50/1 to 20/1) to give the **2** (62 g, yield 84%) as a white foam. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.65 (d, *J*=4.02 Hz, 1 H), 7.99 (s, 1 H), 7.53 (dd, *J*=7.28, 5.77 Hz, 1 H), 7.36 - 7.42 (m, 2 H), 7.20 - 7.35 (m, 6 H), 6.90 (d, *J*=9.03 Hz, 4 H), 6.09 (t, *J*=6.78 Hz, 1 H), 4.15 - 4.24 (m, 1 H), 3.91 (d, *J*=3.51 Hz, 1 H), 3.74 (s, 6 H), 3.18 (d, *J*=3.01 Hz, 2 H), 2.22 (ddd, *J*=13.30, 5.77, 3.01 Hz, 1 H), 2.06 - 2.16 (m, 1 H).



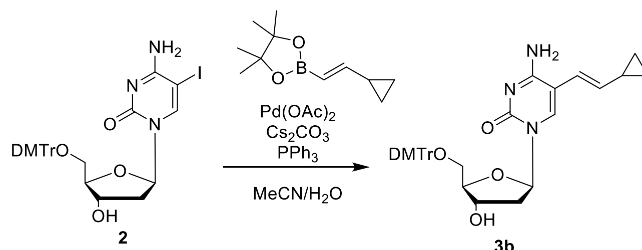
To a solution of **2** (6 g, 9.15 mmol, 1 equiv), Cs_2CO_3 (8.95 g, 27.5 mmol, 3 equiv), (*E*)-4,4,5,5-tetramethyl-2-(5-methylhex-1-en-1-yl)-1,3,2-dioxaborolane (2.46 g, 11 mmol, 1.2 equiv) and PPh_3 (1.2 g, 4.58 mmol, 0.5 equiv) in dioxane (700 mL) and water (30 mL) was added $Pd(OAc)_2$ (2.35 g, 10.5 mmol, 0.1 equiv) at 25 °C under N_2 current. The mixture was heated to 90 °C and stirred for 16 h. TLC (ethyl acetate/MeOH=20/1) showed **2** was consumed completely. The reaction mixture was diluted with water 50 mL and extracted with ethyl acetate (100 mL x 2). The combined organic layers were washed with sat. aqueous NaCl (50 mL), dried over $MgSO_4$**,** filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, DCM/MeOH=50/1 to 20:1) to give compound **3a** (5.1 g, 8.15 mmol, 89% yield) was obtained as a light-yellow solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.86 (s, 1 H), 7.42 (d, *J*=7.53 Hz, 2 H), 7.18 - 7.35 (m, 7 H), 6.81 (d, *J*=7.53 Hz, 4 H), 6.46 (t, *J*=6.53 Hz, 1 H), 5.53 - 5.70 (m, 2 H), 4.47 - 4.57 (m, 1 H), 4.13 (d, *J*=3.01 Hz, 1 H), 3.79 (s, 5 H), 3.47 (dd, *J*=10.54, 3.01 Hz, 1 H), 3.28 (dd, *J*=10.54, 3.01 Hz, 1 H), 2.70 (ddd, *J*=13.55, 5.52, 3.01 Hz, 1 H), 2.24 (dt, *J*=13.55, 6.78 Hz, 1 H), 1.56 - 1.83 (m, 4 H), 1.13 - 1.40 (m, 3 H), 0.91 (dtd, *J*=9.47, 6.43, 6.43, 3.26 Hz, 2 H), 0.75 (dd, *J*=6.78, 2.76 Hz, 6 H).



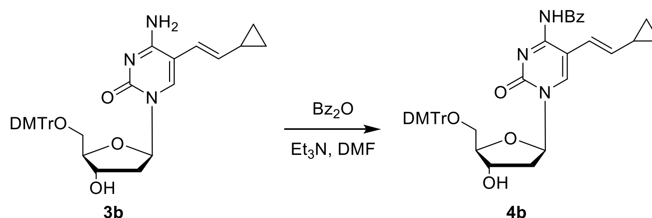
To a solution of **3a** (4.23 g, 6.76 mmol, 1.00 equiv) in DMF (40.00 mL) was added Et_3N (1.03 g, 10.14 mmol, 1.41 mL, 1.50 equiv) and benzoic anhydride (1.84 g, 8.11 mmol, 1.53 mL, 1.20 equiv). The mixture was stirred at 0 to 25 °C for 16 h. TLC (petroleum ether/ethyl acetate =1/1) indicated **3a** was consumed completely and the reaction was clean. The reaction mixture was quenched by addition water 20 mL at 0-5 °C, and then extracted with ethyl acetate (50 mL). The combined organic layers were washed with sat. aqueous NaCl (20 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (Basic SiO_2, petroleum ether/ethyl acetate=5/1 to 2/1) to a yellow solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 13.52 (br. s., 1 H), 8.30 (d, *J*=7.03 Hz, 2 H), 7.96 (s, 1 H), 7.50 - 7.57 (m, 1 H), 7.40 - 7.50 (m, 4 H), 7.21 - 7.37 (m, 7 H), 6.84 (dd, *J*=8.53, 1.51 Hz, 4 H), 6.40 (t, *J*=6.78 Hz, 1 H), 6.21 (d, *J*=16.06 Hz, 1 H), 5.92 - 6.03 (m, 1 H), 4.55 (d, *J*=3.01 Hz, 1 H), 4.10 (d, *J*=3.01 Hz, 1 H), 3.79 (s, 6 H), 3.56 (dd, *J*=10.54, 3.01 Hz, 1 H), 3.31 (dd, *J*=10.54, 3.01 Hz, 1 H), 2.52 (ddd, *J*=13.55, 5.77, 2.76 Hz, 1 H), 2.35 (dt, *J*=13.68, 6.96 Hz, 1 H), 2.09 (d, *J*=3.51 Hz, 1 H), 1.72 - 1.91 (m, 2 H), 1.41 (dt, *J*=13.43, 6.59 Hz, 1 H), 0.83 - 1.00 (m, 2 H), 0.77 (dd, *J*=6.53, 1.51 Hz, 6 H).



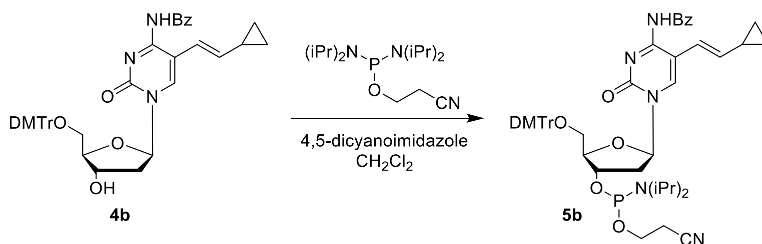
To a solution of **4a** (2.87 g, 3.93 mmol, 1 equiv) and 4,5-dicyanoimidazole (0.696 g, 5.90 mmol, 1.5 equiv) in DCM (30 mL) was added drop wise of 3-bis(diisopropylamino) phosphanyloxypropanenitrile (1.42 g, 4.72 mmol, 1.2 equiv) at 0 °C under N_2 current. Then the mixture was stirred at 0-25 °C for 2 h under N_2 current. A clear yellow solution was obtained. TLC (petroleum ether/ethyl acetate =2/1) showed **4a** was consumed completely. The reaction mixture was concentrated under reduced pressure to give a residue. The resulting residue was purified by column chromatography (basic SiO_2, petroleum ether/ethyl acetate =10/1 to 5/1) to give phosphoramidite **5a** (1.75 g, 1.88 mmol, 48% yield) as a light-yellow foam. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 13.51 (br. s., 1 H) 8.30 (d, *J*=7.53 Hz, 2 H) 7.99 (d, *J*=18.57 Hz, 1 H) 7.50 - 7.58 (m, 1 H) 7.41 - 7.50 (m, 4 H) 7.21 - 7.37 (m, 8 H) 6.84 (ddd, *J*=6.65, 4.64, 2.26 Hz, 4 H) 6.36 - 6.48 (m, 1 H) 6.18 (d, *J*=16.06 Hz, 1 H) 5.88 - 6.02 (m, 1 H) 4.62 (td, *J*=6.65, 3.26 Hz, 1 H) 4.15 - 4.26 (m, 1 H) 3.79 (d, *J*=2.51 Hz, 7 H) 3.50 - 3.66 (m, 4 H) 3.25 (dt, *J*=10.79, 3.39 Hz, 1 H) 2.54 - 2.70 (m, 2 H) 2.27 - 2.44 (m, 2 H) 1.66 - 1.86 (m, 2 H) 1.38 (dd, *J*=13.30, 6.78 Hz, 1 H) 1.12 - 1.22 (m, 9 H) 1.04 (d, *J*=7.03 Hz, 3 H) 0.79 - 0.93 (m, 3 H) 0.75 (t, *J*=5.77 Hz, 6 H). **^{31}P NMR** (162 MHz, $CDCl_3$) δ 148.40 - 149.28 (m, 1 P). **TLC** petroleum ether/ethyl acetate =2/1 (R_f = 0.43).



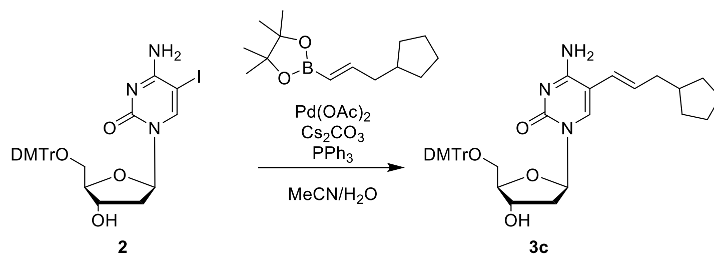
To a solution of **2** (8.00 g, 12.2 mmol, 1 equiv), Cs_2CO_3 (11.9 g, 36.6 mmol, 3 equiv), (*E*)-2-(2-cyclopropylvinyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (2.84 g, 14.7 mmol, 1.2 equiv) and PPh_3 (1.60 g, 6.10 mmol, 0.5 equiv) in dioxane (60 mL) and water (30 mL) was added $Pd(OAc)_2$ (0.274 g, 1.22 mmol, 0.1 equiv) at 25 °C under N_2 current. The mixture was heated to 90 °C and stirred for 16 h. TLC (ethyl acetate/MeOH = 20/1) showed compound **2** was consumed completely. The reaction mixture was diluted with water 50 mL and extracted with ethyl acetate (100 mL x 2). The combined organic layers were washed with sat. aqueous NaCl (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, DCM/MeOH=50/1 to 20**:**1) to give **3b** (7.00 g, 11.8 mmol, 96% yield), obtained as a light-yellow solid.



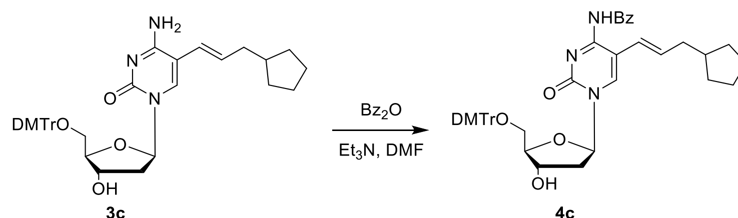
To a solution of **3b** (3.4 g, 5.71 mmol, 1.00 equiv) in DMF (30.00 mL) was added Et_3N (0.693 g, 6.85 mmol, 1.50 equiv) and benzoic anhydride (1.42 g, 6.28 mmol, 1.20 equiv). The mixture was stirred at 0-25 °C for 16 h. TLC (DCM/MeOH=20/1) indicated **3b** was consumed completely and the reaction was clean. The reaction mixture was quenched by addition sat. aqueous NaCl 20 mL at **25** °C, and then extracted with ethyl acetate (50 mL). The combined organic layers were washed with sat. aqueous NaCl (20 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (Basic SiO_2, petroleum ether/ethyl acetate = 3/1 to 2**:**1) to give **4b** (2.6 g, yield 66%) as a yellow solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 13.53 (br. s., 1 H), 8.29 (d, *J*=7.53 Hz, 2 H), 7.92 (s, 1 H) 7.50 - 7.57 (m, 1 H), 7.41 - 7.49 (m, 4 H), 7.21 - 7.37 (m, 8 H), 6.85 (d, *J*=7.53 Hz, 4 H), 6.39 (t, *J*=6.53 Hz, 1 H), 6.30 (d, *J*=16.06 Hz, 1 H), 5.60 (dd, *J*=15.56, 9.03 Hz, 1 H), 4.48 - 4.56 (m, 1 H), 4.08 (d, *J*=3.01 Hz, 1 H), 3.80 (s, 6 H), 3.56 (dd, *J*=10.54, 3.01 Hz, 1 H), 3.28 (dd, *J*=10.79, 3.26 Hz, 1 H), 2.51 (ddd, *J*=13.55, 6.02, 3.01 Hz, 1 H), 2.32 (dt, *J*=13.93, 6.84 Hz, 1 H), 2.19 (d, *J*=4.02 Hz, 1 H), 1.28 (td, *J*=8.41, 4.77 Hz, 2 H), 0.43 - 0.61 (m, 2 H), -0.22 - 0.01 (m, 2 H).



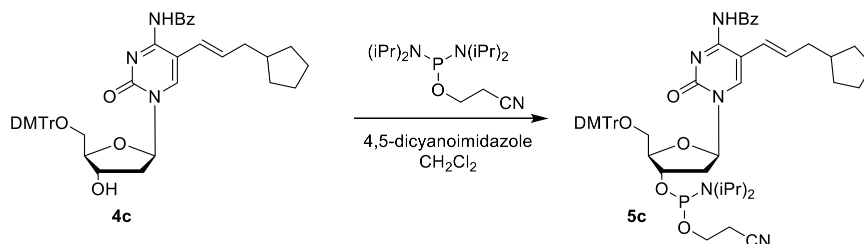
To a solution of **4b** (2.25 g, 3.22 mmol, 1 equiv) and 4,5-dicyanoimidazole (0.570 g, 4.83 mmol, 1.5 equiv) in DCM (20 mL) was added dropwise of 3-bis(diisopropylamino) phosphanyloxypropanenitrile (1.16 g, 3.86 mmol, 1.2 equiv) at 0 °C under N_2 current. Then the mixture was stirred at 0-25 °C for 2 h under N_2 current. A clear yellow solution was obtained. TLC (DCM/MeOH=20/1) showed **4b** was consumed completely. The reaction mixture was concentrated under reduced pressure to give a residue. The resulting residue was purified by column chromatography (basic SiO_2, petroleum ether/ethyl acetate =6/1 to 5/1) to give phosphoramidite **5b** (2.20 g, 2.44 mmol, 75.9% yield) as a light-yellow foam. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 13.53 (br. s., 1 H) 8.29 (d, *J*=7.53 Hz, 2 H) 7.95 (d, *J*=18.57 Hz, 1 H) 7.50 - 7.57 (m, 1 H) 7.41 - 7.49 (m, 4 H) 7.21 - 7.38 (m, 7 H) 6.85 (dd, *J*=7.53, 4.52 Hz, 4 H) 6.36 - 6.46 (m, 1 H) 6.28 (dd, *J*=15.81, 3.76 Hz, 1 H) 5.57 (dd, *J*=15.81, 9.29 Hz, 1 H) 4.59 (td, *J*=6.53, 3.01 Hz, 1 H) 4.19 (dd, *J*=15.56, 2.01 Hz, 1 H) 3.80 (d, *J*=2.51 Hz, 6 H) 3.48 - 3.66 (m, 4 H) 3.22 (dt, *J*=10.54, 3.01 Hz, 1 H) 2.52 - 2.70 (m, 2 H) 2.27 - 2.43 (m, 2 H) 1.21 - 1.31 (m, 2 H) 1.17 (dd, *J*=6.78, 2.76 Hz, 10 H) 1.04 (d, *J*=6.53 Hz, 3 H) 0.78 - 0.92 (m, 1 H) 0.39 - 0.56 (m, 2 H) –0.29 - –0.08 (m, 2 H). **^{31}P NMR** (162 MHz, $CDCl_3$) δ 148.36 - 149.25 (m, 1 P). **TLC** 20:1 DCM:methanol (R_f = 0.85).



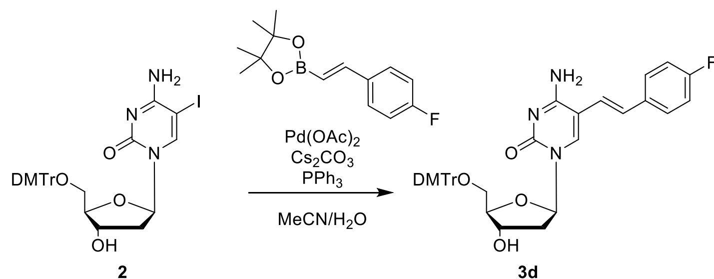
To a solution of **2** (6.00 g, 9.15 mmol, 1 equiv), Cs_2CO_3 (8.95 g, 27.5 mmol, 3 equiv), (*E*)-2-(2-cyclopropylvinyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (2.59 g, 11.0 mmol, 1.2 equiv) and PPh_3 (1.20 g, 4.58 mmol, 0.5 equiv) in dioxane (40 mL) and water (20 mL) was added $Pd(OAc)_2$ (0.274 g, 1.22 mmol, 0.1 equiv) at 25 °C under N_2 current. The mixture was heated to 90 °C and stirred for 16 h. TLC (DCM/ MeOH=20/1) showed **2** was consumed completely. The reaction mixture was diluted with water (50 mL) and extracted with ethyl acetate (100 mL x 2). The combined organic layers were washed with sat. aqueous NaCl (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, DCM/MeOH=50/1 to 20:1) to give the **3c** (4.90 g, 7.68 mmol, 83% yield) was obtained as a light-yellow solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.82 (s, 1 H), 7.43 (d, *J*=7.53 Hz, 2 H), 7.19 - 7.36 (m, 8 H), 6.82 (d, *J*=8.53 Hz, 4 H), 6.52 (t, *J*=6.53 Hz, 1 H), 5.55 - 5.69 (m, 2 H), 4.46 - 4.55 (m, 1 H), 4.16 (d, *J*=2.51 Hz, 2 H), 3.72 - 3.84 (m, 6 H), 3.45 (dd, *J*=10.04, 3.01 Hz, 1 H), 3.29 (dd, *J*=10.29, 3.26 Hz, 1 H), 3.09 (q, *J*=7.19 Hz, 1 H), 2.74 (dt, *J*=10.79, 2.89 Hz, 1 H), 2.21 (dt, *J*=13.80, 6.65 Hz, 1 H), 1.72 - 1.86 (m, 2 H), 1.35 - 1.59 (m, 9 H), 0.92 (br. s., 2 H).



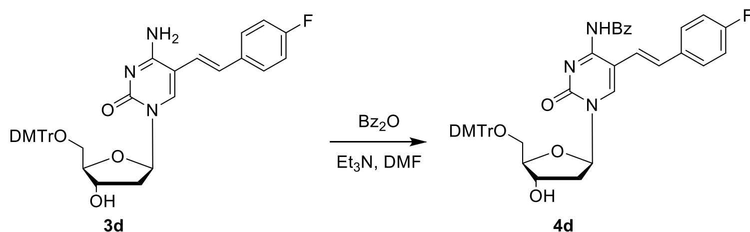
To a solution of compound **3c** (4.8 g, 7.53 mmol, 1.00 equiv) in DMF (50.0 mL) was added Et_3N (1.14 g, 11.3 mmol, 1.50 equiv) and benzoic anhydride (2.04 g, 9.04 mmol, 1.20 equiv). The mixture was stirred at 0-25 °C for 16 h. TLC (DCM/MeOH = 20/1) indicated **3c** was consumed completely and the reaction was clean. The reaction mixture was quenched by addition water 20 mL at 25 °C, and then extracted with ethyl acetate (50 mL). The combined organic layers were washed with sat. aqueous NaCl (20 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (basic SiO_2, petroleum ether/ethyl acetate = 5/1 to 2:1) to give compound **4c** (3.60 g, yield 64%) as a yellow solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 13.52 (br. s., 1 H), 8.30 (d, *J*=7.53 Hz, 2 H), 7.90 (s, 1 H), 7.50 - 7.57 (m, 1 H), 7.40 - 7.48 (m, 4 H), 7.20 - 7.37 (m, 8 H), 6.84 (d, *J*=8.03 Hz, 4 H), 6.39 (t, *J*=6.53 Hz, 1 H), 6.13 (s, 2 H), 4.51 - 4.58 (m, 1 H), 4.10 (d, *J*=3.01 Hz, 1 H), 3.79 (s, 6 H), 3.53 (dd, *J*=10.54, 3.51 Hz, 1 H), 3.33 (dd, *J*=10.54, 3.51 Hz, 1 H), 2.52 (ddd, *J*=13.68, 5.90, 3.01 Hz, 1 H), 2.33 (dt, *J*=13.55, 6.78 Hz, 1 H), 2.18 (d, *J*=3.51 Hz, 1 H), 1.79 - 1.94 (m, 2 H), 1.37 - 1.61 (m, 7 H), 1.00 (br. s., 2 H). **TLC** DCM/MeOH = 20/1 (R_f = 0.43).



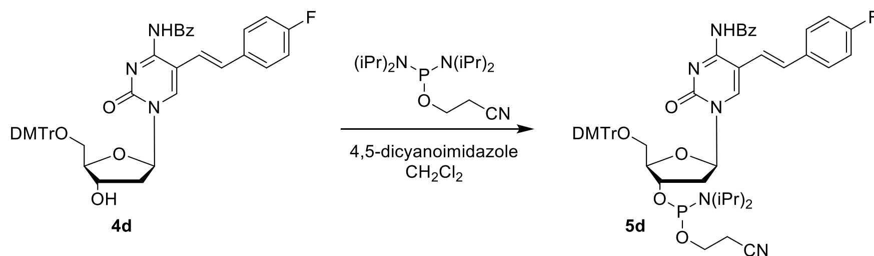
To a solution of **4c** (2.40 g, 3.24 mmol, 1 equiv) and 4,5-dicyanoimidazole (0.574 g, 4.86 mmol, 1.5 equiv) in DCM (20 mL) was added dropwise of 3-bis(diisopropylamino) phosphanyloxypropanenitrile (1.17 g, 3.89 mmol, 1.2 equiv) at 0 °C under N_2 current. Then the mixture was stirred at 0-25 °C for 2 h under N_2 current. A clear yellow solution was obtained. TLC (petroleum ether/ethyl acetate = 2/1) showed **4c** was consumed completely. The reaction mixture was concentrated under reduced pressure to give a residue, which was purified by column chromatography (basic SiO_2, petroleum ether/ethyl acetate = 10/1 to 6/1) to give phosphoramidite **5c** (1.70 g, 2.44 mmol, 56% yield) as a white foam. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 13.51 (br. s., 1 H), 8.30 (d, *J*=7.03 Hz, 2 H), 7.93 (d, *J*=18.57 Hz, 1 H), 7.50 - 7.57 (m, 1 H), 7.41 - 7.49 (m, 4 H), 7.22 - 7.37 (m, 8 H), 6.84 (dd, *J*=7.53, 5.02 Hz, 4 H), 6.36 - 6.46 (m, 1 H), 6.03 - 6.18 (m, 2 H), 4.62 (td, *J*=6.78, 3.01 Hz, 1 H), 4.16 - 4.26 (m, 1 H), 3.79 (d, *J*=2.51 Hz, 6 H), 3.49 - 3.66 (m, 4 H), 3.27 (dt, *J*=10.54, 3.76 Hz, 1 H), 2.53 - 2.70 (m, 2 H), 2.27 - 2.44 (m, 2 H), 1.72 - 1.91 (m, 2 H), 1.35 - 1.56 (m, 6 H), 1.14 - 1.23 (m, 9 H), 1.05 (d, *J*=6.53 Hz, 2 H), 0.97 (d, *J*=3.01 Hz, 3 H). **^{31}P NMR** (162 MHz, $CDCl_3$) δ 148.49 - 149.26 (m, 1 P).



To a solution of **2** (6.00 g, 12.211.0 mmol, 1 equiv), Cs_2CO_3 (8.95 g, 27.5 mmol, 3 equiv), (*E*)-2-(4-fluorostyryl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (2.72 g, 11.0 mmol, 1.2 equiv) and PPh_3 (1.20 g, 4.58 mmol, 0.5 equiv) in dioxane (60 mL) and water (30 mL) was added $Pd(OAc)_2$ (0.206 g, 0.915 mmol, 0.1 equiv) at 25 °C under N_2 current. The mixture was heated to 90 °C and stirred for 16 h. TLC (DCM/ MeOH=20/1) showed **2** was consumed completely. The reaction mixture was diluted with water (50 mL) and extracted with ethyl acetate (100 mL x 2). The combined organic layers were washed with sat. aqueous NaCl (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, DCM/MeOH = 50/1 to 20:1) to give **3d** (3.10 g, 4.77 mmol, 52% yield) as a light-yellow solid. **TLC** DCM/MeOH = 20/1 (R_f = 0.20).

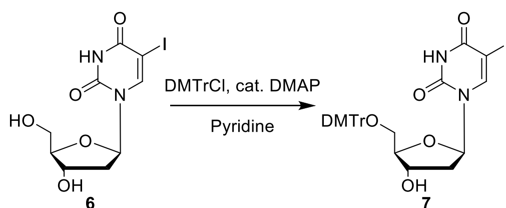


To a solution of **3d** (2.7 g, 4.16 mmol, 1.00 equiv) in DMF (30.00 mL) was added Et_3N (0.631 g, 6.24 mmol, 1.50 equiv) and benzoic anhydride (1.13 g, 4.99 mmol, 1.20 equiv). The mixture was stirred at 0-25 °C for 16 h. TLC (petroleum ether/ethyl acetate = 1/1) indicated **3d** was consumed completely and the reaction was clean according to TLC. The reaction mixture was quenched by addition water 100 mL at 0-5 °C, and then extracted with ethyl acetate (50 mL). The combined organic layers were washed with sat. aqueous NaCl (20 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (basic SiO_2, petroleum ether/ethyl acetate = 2/1 to 1**:**1) to give **4d** (2.00 g, yield 64%) as a yellow solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 13.59 (br. s., 1 H), 8.32 (d, *J*=7.53 Hz, 2 H), 8.22 (s, 1 H), 7.52 - 7.59 (m, 1 H), 7.42 - 7.51 (m, 4 H), 7.33 (dd, *J*=8.78, 1.76 Hz, 4 H), 7.22 - 7.29 (m, 3 H), 7.14 - 7.21 (m, 1 H), 7.05 (d, *J*=16.56 Hz, 1 H), 6.71 - 6.89 (m, 9 H), 6.43 (t, *J*=6.53 Hz, 1 H), 4.58 (br. s., 1 H), 4.15 (d, *J*=2.51 Hz, 1 H), 3.59 - 3.75 (m, 7 H), 3.29 (dd, *J*=11.04, 3.01 Hz, 1 H), 2.59 (ddd, *J*=13.55, 6.02, 3.01 Hz, 1 H), 2.41 (dt, *J*=13.55, 6.78 Hz, 1 H), 2.14 (br. s., 1 H); **^{19}F NMR** (376 MHz, $CDCl_3$) δ -114.32 (s, 1 F)

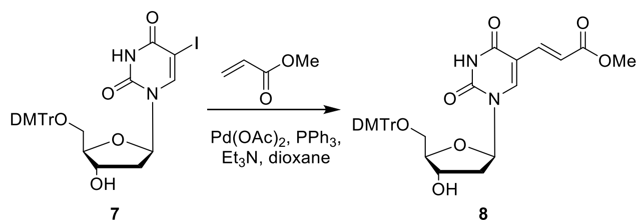


To a solution of **4d** (2.30 g, 3.05 mmol, 1 equiv) and 4,5-dicyanoimidazole (0.570 g, 4.27 mmol, 1.5 equiv) in DCM (20 mL) was added drop wise of 3-bis(diisopropylamino) phosphanyloxypropanenitrile (1.10 g, 3.66 mmol, 1.2 equiv) at 0 °C under N_2 current. Then the mixture was stirred at 0-25 °C for 3 h under N_2 current. A clear yellow solution was obtained. TLC (petroleum ether/ethyl acetate = 2/1) showed **4d** was consumed completely. The reaction mixture was concentrated under reduced pressure to give a residue, which was purified by column chromatography (basic SiO_2, petroleum ether/ethyl acetate =6/1 to 5/1) to give phosphoramidite **5d** (2.10 g, 2.20 mmol, 72% yield) as a light-yellow foam. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 13.59 (s, 1 H), 8.33 (d, *J*=7.53 Hz, 2 H), 8.25 (d, *J*=19.07 Hz, 1 H), 7.52 - 7.59 (m, 1 H), 7.41 - 7.51 (m, 4 H), 7.22 - 7.38 (m, 7 H), 7.18 (dd, *J*=7.03, 4.02 Hz, 1 H), 7.03 (d, *J*=16.06 Hz, 1 H), 6.66 - 6.85 (m, 9 H), 6.45 (q, *J*=6.53 Hz, 1 H), 4.65 (td, *J*=6.53, 3.01 Hz, 1 H), 4.19 - 4.30 (m, 1 H), 3.69 (s, 6 H), 3.49 - 3.65 (m, 3 H), 3.19 - 3.28 (m, 1 H), 2.58 - 2.76 (m, 2 H), 2.36 - 2.46 (m, 2 H), 1.51 (d, *J*=6.53 Hz, 1 H), 1.17 (d, *J*=7.03 Hz, 8 H), 1.04 (d, *J*=6.53 Hz, 3 H). **^{31}P NMR** (162 MHz, $CDCl_3$) δ 148.45 - 149.26 (m, 1 P). **^{19}F NMR** (376 MHz, $CDCl_3$) δ -114.45 (s, 1 F). **TLC** 2:1 pentane ether: ethyl acetate (R_f = 0.43).

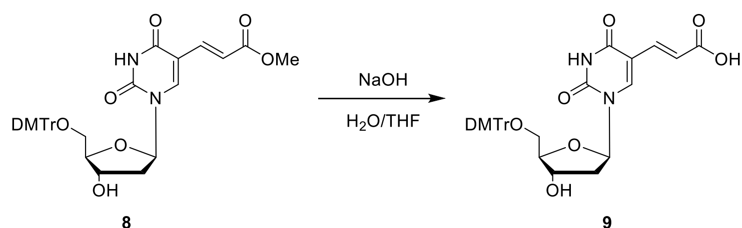
*Synthesis of phosphoramidite **11***



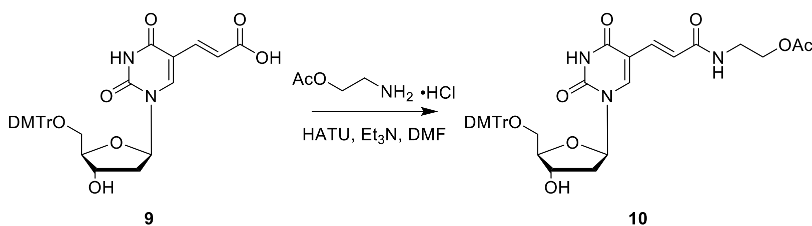
To a solution of **6** (50.0 g, 141.2 mmol, 1 equiv) and DMAP (0.172 g, 1.41 mmol, 0.01 equiv) in pyridine (500 mL) was added dropwise DMTrCl (50.2 g, 148.2 mmol, 1.05 equiv) and at 0°C. The mixture was stirred at 25°C for 16 h. TLC (Petroleum ether /Ethyl acetate = 1/1) indicated compound **6** was consumed completely. The reaction mixture was concentrated with MeOH (5 mL) under reduced pressure to remove pyridine. The residue was purified by column chromatography (SiO_2, petroleum ether/ethyl acetate = 2/1 to 1/2) to give **7** (85.0 g, yield 92%) as a white foam. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.16 (s, 1 H), 7.44 (d, *J*=7.53 Hz, 2 H), 7.22 - 7.38 (m, 7 H), 6.87 (d, *J*=8.53 Hz, 4 H), 6.35 (dd, *J*=7.78, 5.77 Hz, 1 H), 4.53 - 4.62 (m, 1 H), 4.11 - 4.18 (m, 2 H), 3.81 (s, 6 H), 3.35 - 3.48 (m, 2 H), 2.53 (ddd, *J*=13.55, 5.52, 2.51 Hz, 1 H), 2.26 - 2.37 (m, 1 H). **TLC** petroleum ether/ethyl acetate = 1/1 (R_f = 0.15).



To a solution of **7** (68.7 g, 105 mmol, 1 equiv), methyl acrylate (54 g, 627 mmol, 2 equiv), PPh_3 (5.5 g, 20.9 mmol, 0.2 equiv), and trimethylamine (21.2 g, 209 mmol, 2 equiv) in dioxane (700 mL) was added $Pd(OAc)_2$ (2.35 g, 10.5 mmol, 0.1 equiv) at 25°C under N_2 current. The mixture was heated to 90 °C and stirred for 16 h. TLC (petroleum ether/ethyl acetate =1/1) showed **7** was consumed completely. The reaction mixture was filtered under reduced pressure to give a residue. The residue was purified by column chromatography (SiO_2, petroleum ether/ethyl acetate =1/1 to 1.5:1) to give the compound **8** (42 g, 68.3 mmol, 65.3% yield) as a light-yellow solid.

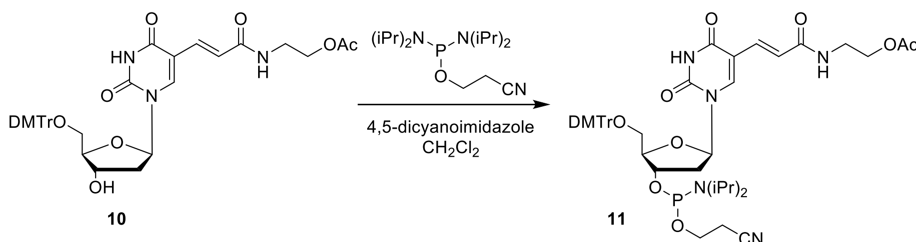


To solution of **8** (42 g, 68.3 mmol, 1 equiv) in THF (500 mL) was added NaOH aqueous (1N, 102.5 mL, 1.5 equiv) at 25 °C, and then the resulting mixture was stirred at 25 °C for 16 h. TLC (DCM/MeOH=10/1) indicated **8** was consumed completely. The reaction mixture was partitioned between ethyl acetate (200 mL) and water (100 mL), the water phase was separated, acidified with sat. aqueous citric acid to pH7, the white suspension was filtered and dried under reduced pressure to give the compound **9** (30.5 g, 50.8 mmol, 74% yield) as a white solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.67 (s, 1 H), 7.40 (d, *J*=7.53 Hz, 2 H), 7.24 - 7.33 (m, 8 H), 7.15 - 7.22 (m, 1 H), 6.91 (d, *J*=15.56 Hz, 1 H), 6.82 (d, *J*=9.04 Hz, 4 H), 6.29 (t, *J*=6.27 Hz, 1 H), 4.47 (d, *J*=6.02 Hz, 1 H), 3.99 (d, *J*=5.02 Hz, 1 H), 3.75 (s, 5 H), 3.45 (dd, *J*=10.29, 5.27 Hz, 1 H), 3.34 (dd, *J*=10.04, 4.52 Hz, 1 H), 2.41 - 2.51 (m, 1 H), 2.26 (dt, *J*=13.80, 6.65 Hz, 1 H). **TLC** DCM /MeOH = 10/1 (R_f = 0.15).



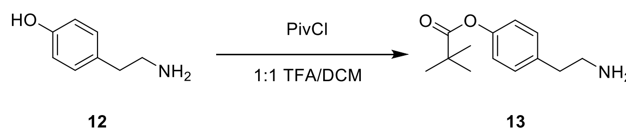
A solution of **9** (5 g, 8.32 mmol, 1 equiv), Et_3N (4.21 g, 41.6 mmol, 5 equiv) and HATU (4.75 g, 12.5 mmol, 1.5 equiv) in DMF (60 mL) was stirred for 30 min at 25 °C. Then to this mixture was added 2-aminoethyl acetate hydrochloride (1.39 g, 9.99 mmol, 1.2 equiv) at 25°C. The mixture was stirred at 25 °C for 16 h. TLC (DCM/MeOH=20/1) showed the acid was consumed completely. The reaction mixture was quenched by addition of sat. aqueous $NaHCO_3$ (50 mL) at 25 °C, and then extracted with ethyl acetate (100 mL x 2). The combined organic

layers were concentrated under reduced pressure to give a residue. The residue was purified by column chromatography (basic SiO_2, DCM/MeOH = 50/1 to 30/1) to give compound **10** (2.7 g, yield 47%) as a white foam. **TLC** DCM/MeOH = 20/1 (R_f = 0.30).

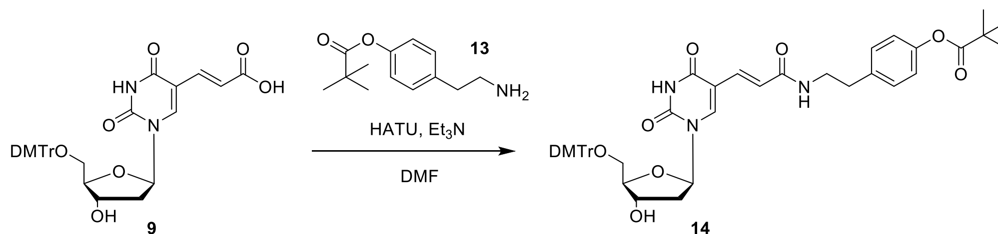


To a solution of **10** (2.10 g, 3.06 mmol, 1 equiv) and 4,5-dicyanoimidazole (0.543 g, 4.59 mmol, 1.5 equiv) in DCM (30 mL) was added drop wise of 3-bis(diisopropylamino)phosphanyloxypropanenitrile (1.11 g, 3.67 mmol, 1.2 equiv) at 0 °C under N_2 current. Then the mixture was stirred at 0-25 °C for 2 h under N_2 current. A clear yellow solution was obtained. TLC (DCM/MeOH=20/1) showed **10** was consumed completely. The reaction mixture was concentrated under reduced pressure to give a residue, which was purified by column chromatography (basic SiO_2, DCM/Acetone= 15/1 to 8/1) to give phosphoramidite **11** (1.7 g, 1.44 mmol, 75% yield) as a white foam. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.79 - 7.90 (m, 1 H) 7.43 (d, *J*=7.53 Hz, 2 H), 7.20 - 7.36 (m, 7 H), 7.04 (d, *J*=15.56 Hz, 1 H), 6.82 - 6.92 (m, 4 H), 6.71 - 6.80 (m, 1 H), 6.28 (t, *J*=6.53 Hz, 1 H), 5.41 - 5.55 (m, 1 H), 4.57 (dt, *J*=6.53, 3.26 Hz, 1 H), 4.18 - 4.29 (m, 1 H), 4.07 (q, *J*=5.02 Hz, 2 H), 3.79 (s, 6 H), 3.54 - 3.70 (m, 3 H), 3.39 - 3.51 (m, 3 H), 3.28 - 3.37 (m, 1 H), 2.55 - 2.80 (m, 2 H), 2.45 (t, *J*=6.27 Hz, 1 H), 2.28 (dt, *J*=13.68, 6.96 Hz, 1 H), 2.06 (s, 3 H), 1.25 - 1.31 (m, 1 H), 1.18 (t, *J*=6.27 Hz, 9 H), 1.09 (d, *J*=7.03 Hz, 2 H).

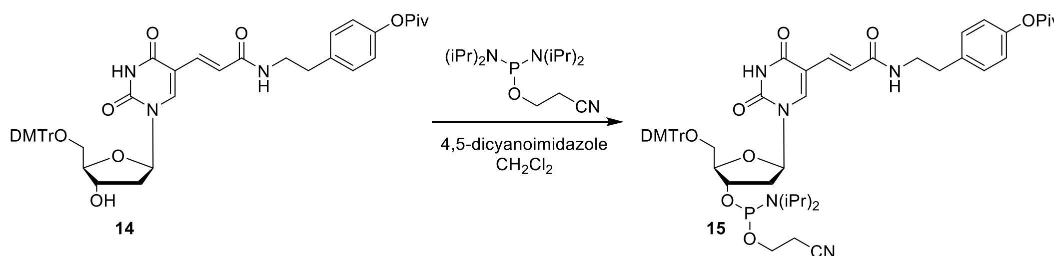
*Synthesis of phosphoramidite **15***



Pivaloyl chloride (7.25 g, 61.1 mmol, 1 equiv) was added drop wise to a solution of 4-(2-aminoethyl) phenol (7.5 g, 54.5 mmol, 1 equiv) in DCM (50 mL) and TFA (50 mL) at 25 °C, then the resulting brown mixture was stirred for 12 h. LCMS showed reactant was consumed completely and one main peak with desired MS was detected. The reaction mixture was concentrated under reduced pressure to give a residue, which was purified by column chromatography (SiO_2, DCM: MeOH = 20/1 to 1:1) to give compound **13** (9.5 g, 42.9 mmol, 79% yield) as a brown solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.21 (d, *J*=8.53 Hz, 2 H), 6.95 (d, *J*=8.53 Hz, 2 H), 3.18 (t, *J*=7.28 Hz, 2 H), 2.90 - 2.99 (m, 2 H), 1.34 (s, 9 H). **^{19}F NMR** (376 MHz, CDCl3) δ -75.82 (s, 1 F).



A solution of **9** (2.8 g, 4.66 mmol, 1 equiv), Et_3N (0.94 g, 9.32 mmol, 2 equiv) and HATU (3.54 g, 9.32 mmol) in DMF (50 mL) was stirred for 30 min at 25°C. Then to this mixture was added **13** (1.13 g, 5.13 mmol, 1.1 equiv) at 25°C. The mixture was stirred at 25 °C for 16 h. TLC (DCM/MeOH = 20/1) showed the acid was consumed completely. The reaction mixture was concentrated under reduced pressure to remove solvents. The residue was purified by column chromatography (basic SiO_2, DCM/MeOH = 20/1 to 10/1) to give compound **14** (2 g, yield 86%) as a white foam. **TLC** DCM/MeOH=20/1 (R_f = 0.20).



To a solution of **14** (2.90 g, 3.61 mmol, 1 equiv) and 4,5-dicyanoimidazole (0.639 g, 5.41 mmol, 1.5 equiv) in DCM (30 mL) was added drop wise of 3-bis(diisopropylamino)phosphanyloxypropanenitrile (1.30 g, 4.33 mmol, 1.2 equiv) at 0 °C under N_2 current. Then the mixture was stirred at 0-25 °C for 2 h under N_2 current. A clear yellow solution was obtained. TLC (DCM/MeOH=20/1) showed **14** was consumed completely. The reaction mixture was concentrated under reduced pressure to give a residue, which was purified by column chromatography (basic SiO_2, DCM/acetone = 15/1 to 10/1) to give phosphoramidite **15** (1.95 g, 1.94 mmol, 54% yield) as a light-brown gum. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.76 - 7.91 (m, 2 H), 7.45 (d, *J*=6.02 Hz, 2 H), 7.27 - 7.38 (m, 7 H), 7.13 - 7.26 (m, 3 H), 6.97 - 7.09 (m, 3 H), 6.87 (dd, *J*=8.78, 3.76 Hz, 3 H), 6.61 (dd, *J*=15.56, 11.04 Hz, 1 H), 6.28 - 6.35 (m, 1 H), 6.18 (s, 1 H), 5.01 - 5.12 (m, 1 H), 4.59 (br. s., 1 H), 4.10 - 4.28 (m, 4 H), 3.80 (s, 5 H), 3.28 - 3.62 (m, 9 H), 2.78 (td, *J*=6.15, 1.76 Hz, 3 H), 2.60 - 2.72 (m, 10 H), 2.45 (t, *J*=6.27 Hz, 1 H), 2.28 (dt, *J*=12.92, 6.34 Hz, 1 H), 1.37 (s, 7 H), 1.30 (t, *J*=6.27 Hz, 15 H), 1.16 - 1.22 (m, 7 H), 1.09 (d, *J*=6.53 Hz, 3 H). **^{31}P NMR** (162 MHz, $CDCl_3$) δ 148.76 - 149.28 (m, 1 P) 14.16 (s, 2 P).

3. Synthesis and characterization of functionalized oligonucleotides.

Standard phosphoramidite reagents and 1000-Å controlled-pore glass (CPG) supports for dA, Ac-dC, dmf-dG, and dT were purchased from Glen Research, as were chemical phosphorylation reagent II (10-1901) and the phosphoramidite for NHS-carboxy-dT (10-1535). The phosphoramidite reagent for the incorporation of the aminoallyl side-chain-functionalized nucleotide (BA 0311) was purchased from Berry and Associates, as was the perfluoroalkyl-DMT dT phosphoramidite (FL 1300). The phosphoramidite reagents for the incorporation of isopentyl (**5a**), cyclopropyl (**5b**), cyclopentyl (**5c**), fluorophenyl (**5d**), ethanolamine (**11**), and tyramine (**15**) side-chain-functionalized nucleotides were custom synthesized by WuXi AppTec as detailed in the previous section.

Solid-phase synthesis of side-chain-functionalized DNA was performed on a PerSeptive Biosystems Expedite 8909 DNA synthesizer. All side-chain-functionalized phosphoramidites were incubated with molecular sieves overnight before use. Syntheses were performed on 1-μmol columns using standard coupling cycles, except for 5'-phosphorylation, which required 7 minutes of coupling with chemical phosphorylation reagent II. When the histamine side-chain-functionalized nucleotide was called for, NHS-carboxy-dT was incorporated in its place, and after the full-length synthesis was completed, a solution of histamine (free base; 5 mg) and diisopropylethylamine (1 μl) in 200 μl of 10% DMSO in acetonitrile was manually injected into the column and allowed to react overnight, and then the column was washed with acetonitrile and dried. Similarly, when a methylamine-functionalized nucleotide was required (for probing side chain SAR; see Supplementary Fig. 3), NHS-carboxy-dT was incorporated in its place, and methylamine (30 equiv. with respect to solid-phase synthesis scale, from a 2M stock in THF) and diisopropylethylamine (1 μl) in 200 μl of acetonitrile was added to the column and allowed to react overnight before washing and drying. The functionalized DNA was then deprotected and cleaved from solid support by incubation in 30% ammonium hydroxide overnight at room temperature. The phosphorylated trinucleotide building blocks were synthesized DMT-off and, after deprotection, cleavage, and evaporation of ammonium hydroxide, were purified by reverse-phase HPLC using a gradient of 0-20 % acetonitrile in 0.1 M TEAA, pH 7, over 24 minutes, followed by 20-40 % acetonitrile in 0.1 M TEAA, pH 7, over 10 minutes. The full length HFNAP Evo5-syn was synthesized DMT-on and the 5'-most nucleotide was incorporated with a perfluoroalkyl-DMT phosphoramidite (Berry and Associates, FL 1300). After deprotection/cleavage, the polymer was purified and deprotected on column with a fluorous phase purification cartridge (Fluoro-Pak II from Berry and Associates) according to manufacturer's instructions. (See Supplementary Fig. 8 for synthetic scheme.) The Evo5-syn used for mass spectrometry characterization and for SPR experiments was further purified by denaturing PAGE on a 10% TBE-urea gel.

Oligonucleotide samples were analyzed in negative ion mode using a Bruker Impact II q-TOF mass spectrometer equipped with an Agilent 1290 uHPLC using flow injection analysis. The purified samples were introduced at a constant flow rate of 0.200 mL/minute using 50% acetonitrile and 0.1% formic acid. Each individual data file was calibrated for the m/z scale using a plug of sodium formate clusters introduced through a secondary isocratic pump and introduced using a 6-port valve. Using this internal calibration method, less than 2 ppm relative error was obtained on all samples. Bruker Data Analysis software was used to simulate the isotope pattern for each target ion.

Name	Expected m/z ($[M-2H]^{2-}$)	Observed m/z	Expected m/z ($[M-H]^{-}$)	Observed m/z
phos-CAA-Isopentyl	513.6257	513.6253	1028.2588	1028.2580
phos-CAC-Isopentyl	501.6201	501.6198	1004.2475	1004.2467
phos-CTC-Isopentyl	497.1143	497.1142	995.2360	995.2355
phos-CGG-Isopentyl	529.6207	529.6204	1060.2486	1060.2478
phos-CAT-Fluorophenyl	521.0918	251.0914	1043.1908	1043.1896
phos-CAG-Fluorophenyl	533.5950	533.5946	1068.1973	1086.1961
phos-CGA-Fluorophenyl	533.5950	533.5946	1068.1973	1068.1960
phos-CGC-Fluorophenyl	521.5894	521.5890	1044.1861	1044.1849
phos-CTA-Cyclopropyl	494.0965	494.0961	989.2003	989.1990
phos-CCA-Cyclopropyl	486.5967	486.5961	974.2006	974.1993
phos-CCT-Cyclopropyl	482.0909	482.0907	965.1890	965.1885
phos-CCC-Cyclopropyl	474.5910	474.5907	950.1894	950.1885
phos-CTT-Cyclopentyl	510.6142	510.6137	1022.2356	1022.2343
phos-CTG-Cyclopentyl	523.1174	523.1170	1074.2421	1047.2406
phos-CGT-Cyclopentyl	523.1174	523.1169	1047.2421	1047.2406
phos-CCG-Cyclopentyl	515.6176	515.6170	1032.2425	1032.2408
phos-TTT-Phenol	551.5987	551.5983	-	-
phos-TTG-Phenol	564.1020	564.1019	1129.2112	1129.2099
phos-TGT-Phenol	564.1020	564.1015	1129.2112	1129.2094
phos-TCG-Phenol	556.6021	556.6017	1114.2115	1114.2103
phos-TAA-Imidazole	547.6081	547.6079	1096.2234	1096.2227
phos-TAC-Imidazole	535.6025	535.6023	1072.2122	1072.2117
phos-TCA-Imidazole	535.6025	535.6021	1072.2122	1072.2113
phos-TCC-Imidazole	523.5969	523.5964	1048.2010	1048.1999
phos-TAT-Primary alcohol	518.0889	518.0883	1037.1850	1037.1837
phos-TAG-Primary alcohol	530.5921	530.5917	1062.1915	1062.1904
phos-TGA-Primary alcohol	530.5921	530.5917	1062.1915	1062.1903
phos-TGC-Primary alcohol	518.5865	518.5862	1038.1802	1039.1793
phos-TTA-Allylamine	489.0861	489.0857	979.1795	979.1786
phos-TTC-Allylamine	-	-	955.1683	955.1672
phos-TCT-Allylamine	-	-	955.1683	955.1673
phos-TGG-Allylamine	509.5868	509.5864	1020.1809	1020.1799

Mass spectrometry data for PCSK9-Evo5-syn are given in Supplementary Figure 9.

4. Additional experimental procedures

Isolation of single-stranded HFNAP following ligase-mediated polymerization

To synthesize and isolate an unbiotinylated HFNAP, unbiotinylated primers and a doubly biotinylated ssDNA template was used in a solution phase polymerization reaction. After the reaction incubation period, the reaction mixture was combined with MyOne Streptavidin C1 magnetic beads (ThermoFisher Scientific, 65002; 1 µL of the stock 1% suspension per 4 pmol of biotinylated template), and then an equal volume of 2x bind-and-wash buffer (2M NaCl, 2mM EDTA, 20 mM Tris-HCl, pH 7.5) was added. After 30 minutes of incubation on a rotor, the supernatant was removed by magnetic separation, and the beads were suspended 18 µL of 20 mM NaOH. The supernatant was combined with 12 µL of formamide denaturing mix (95% formamide, 1 mM EDTA) and run on a 10% TBE-urea PAGE gel. Desired product was visualized by UV shadowing at 265 nm against a TLC plate (with F254 indicator), excised from the gel, eluted in 200 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) overnight, filtered, mixed with 2 mL of ssDNA column loading mix (40:60:0.5 v/v/v of saturated aqueous guanidinium chloride/isopropanol/3M sodium acetate, pH 5.2) and cleaned up with a Qiagen QiaQuick column. Typical isolated yield of HFNAP from 200 pmol of template was between 5 and 15 pmol as determined by Nanodrop or quantitative PCR. For the validation of sequence specificity of translation and amplification (Fig. 1D), a small sample (~1 fmol) was amplified by PCR using Q5 DNA polymerase and the primers T7-out-PCR2 and pp2-library. The amplicon was purified by PAGE on a nondenaturing 10% TBE gel and subjected to Sanger sequencing.

Synthesis and isolation of a biotinylated HFNAP followed the same procedure as above, except that an unbiotinylated ssDNA template was used, and one of polymerization primers was doubly biotinylated. After polymerization reaction, streptavidin bead capture, and alkaline denaturation, the bead-bound biotinylated molecules were eluted by heating in 95% formamide, 1 mM EDTA at 95 °C for 30 min. After magnetic separation, desired product was isolated from the supernatant by PAGE on a denaturing TBE-urea gel. Typical isolated yield of HFNAP from 200 pmol of template was between 5 and 15 pmol. Synthesis and isolation of a biotinylated, truncated HFNAP (such as PCSK9-Evo5) followed the same procedure, except that the primer contained a 2'-deoxy-U nucleotide, and the ligation reaction mixture was treated with USER enzyme (New England Biolabs) at 37 °C for 2 h before proceeding to streptavidin bead capture.

During the selections, polymerization reactions were performed with templates immobilized on streptavidin beads and processed as detailed in the main text Methods section.

High-throughput DNA sequencing and data analysis

Small samples from the elution pool of selection rounds were amplified by PCR using Q5 Hot Start High-Fidelity 2X Master Mix to sub-saturation number of cycles (determined during the selection by qPCR) with primers that install flanking sequences. The amplicons were PAGE-purified and amplified by PCR with Illumina adapter primers. The amplicons were again PAGE-purified and subjected to high-throughput sequencing on an Illumina MiSeq.

The FASTQ files from high-throughput sequencing were first processed with CutAdapt[1] for the following operations: a quality-based trim (with a threshold Phred score of 30), removal of constant regions (with a one-base error tolerance in each region; sequences were discarded if either constant region was not found), and filtering for the correct length (45) in the remaining

sequence. Sequences that could not be completely parsed into trimer codons (whose first nucleobase should always be C or T) were discarded.

For the initial PCSK9 selection and the IL-6 selection, the copy numbers of all unique sequences were tallied and the unique sequences above 5 reads per million were clustered using FASTAptamer[2]. Sequence logos for PCSK9 selection-enriched sequences were then generated for individual clusters with WebLogo 3[3]. For the PCSK9-A5 evolution, sequence logos were generated directly from sequencing data with WebLogo 3.

Affinity characterization by bead retention assay

Candidate PCSK9-binding HFNAPs (from ligase-catalyzed polymerization reaction) or sequence-matched unfunctionalized DNA (0.5 pmol) in 50 µL of DPBS (with calcium and magnesium) containing 0.1 mg/ml BSA and 0.01 % Tween-20 was incubated with 1 µL of either PCSK9 beads or thrombin beads (1 mg protein per mL resin) in a micro-spin filtration column at room temperature for 1 h on a rotor. Following the same procedure described for the selection, flow-through was collected, the beads were washed three times and the retained HFNAP or DNA was eluted by heating, and the amount of amplifiable HFNAP or DNA in the flow-through, wash, and elution samples were quantified by qPCR.

Candidate IL-6-binding HFNAPs and sequence-matched DNA were similarly assayed on PCSK9 beads (prepared as above, but serving as negative control) or on IL-6 beads (0.25 mg protein per mL resin).

Detailed procedures for surface plasmon resonance (SPR) assays

All SPR assays were performed at 25 °C on a Biacore X100 or Biacore T200 (GE Healthcare Life Sciences).

Binding kinetics between enzymatically synthesized biotinylated HFNAPs and unlabeled PCSK9 (ACROBiosystems, PC9-H5223) were measured using single-cycle kinetics with the Biotin CAPture kit (GE Life Sciences, 28920233 or 28920234). HBS-EP buffer (GE Life Sciences, BR100188), diluted by MilliQ water to 0.9x, was used as the bulk running buffer. Each experiment consisted of three start-up cycles followed by multiple data collection and blank cycles. In each data collection cycle, the CAP reagent was injected onto both active and control flow cells of the CAP chip to generate streptavidin-coated surfaces, and then a doubly biotinylated HFNAP was injected onto the active flow cell as the immobilized ligand. Afterwards, four ascending concentrations of PCSK9 protein [10, 30, 100, 300 nM protein supplemented with 1 mg/ml salmon sperm DNA (Invitrogen, 15632-011) as nonspecific binding reducer for PCSK9-A5 and its variants; 2, 6, 20, 60 nM protein without salmon sperm DNA for PCSK9-Evo5 and its variants] in 0.9x HBS-EP were injected onto both flow cells in series at 30 µL/min for 150 seconds each, followed by 240 seconds of dissociation. Both flow cells were then regenerated with a 3:1 mixture of 8 M guanidinium chloride and 1 M NaOH following manufacturer's instructions. Blank cycles were run similarly except that 0.9x HBS-EP (containing 1 mg/ml salmon sperm DNA when PCSK9-A5 and its variants were assayed) without PCSK9 protein was injected. As signals from blank cycles were similar regardless of the immobilized HFNAP, one blank cycle was run for every two data collection cycles. Kinetic parameters were fitted to double-blank-subtracted sensograms using BIAEvaluation software under a 1:1 binding model, unless stated otherwise. Binding between biotinylated Evo5 and truncated PCSK9 protein missing the prodomain ("human mature PCSK9", ACROBiosystems, PC9-H5226) was also assayed using this protocol.

Binding kinetics between enzymatically synthesized biotinylated HFNAPs and unlabeled IL-6 protein, expressed in either *E. coli* (PeproTech, 200-06) or human HEK293 cells (ACROBiosystems, IL6-H4218), were assayed similarly with the following differences. Four ascending concentrations (10, 30, 100, 300 nM) of IL-6 without additional nonspecific binding reducer were injected in the single-cycle kinetic runs. As the binding kinetics did not fit a classical 1:1 binding model, a heterogeneous ligand model was used to fit the double-blank-subtracted sensograms.

Binding kinetics between chemically synthesized Evo5-syn and biotinylated Avi-tagged PCSK9 (ACROBiosystems, PC9-H82E7) were measured using single-cycle kinetics with on a Series S SA chip (GE Life Sciences, BR100531) using 0.9x HBS as the bulk running buffer. Both active and control flow cells were conditioned with three consecutive one-minute injections of 1 M NaCl in 50 mM NaOH. Biotinylated PCSK9 in 0.9x HBS buffer was immobilized onto the active flow cell to ~1000 RU. Either five portions of buffer or five ascending concentrations of Evo5-syn (1.8, 6, 18, 60, 180 nM) were injected onto both flow cells in series at 30 μL/min for 150 seconds each, followed by 600 seconds of dissociation. Kinetic parameters were fitted to double-blank-subtracted sensograms under a 1:1 binding model using BIAEvaluation software.

Binding of PCSK9 on surface-immobilized LDLR in the presence of various competing agents was measured on a Series S SA chip. The bulk running buffer was 10 mM HEPES, 150 mM NaCl, 0.1 mM $CaCl_2$, 0.005% Tween-20, pH 7.5. Both active and control flow cells were conditioned with three consecutive one-minute injections of 1 M NaCl in 50 mM NaOH, and then biotinylated Avi-tagged LDLR (BPS Bioscience, 71206) was immobilized onto the active flow cell to ~2000 RU. In each data collection cycle, a solution consisting of PCSK9 (20 nM final), a carboxymethyl dextran-based non-specific binding reducer (GE Healthcare, BR-1006-91, 1 mg/ml final), and varying concentrations (0, 2, 6, 20, 60, or 200 nM final) of PCSK9-Evo5-syn, Evo5DNA-InvdT, unlabeled LDLR (AcroBiosystems, LDR-H5224), or a known PCSK9-neutralizing antibody (BPS Bioscience, 71207) in bulk running buffer was injected at 10 μL/min for 420 s, followed by 15 s of dissociation. The surface was regenerated using two consecutive one-minute injections of 50 mM HCl. Blank cycles were run similarly except that running buffer containing 1 mg/ml non-specific binding reducer without protein was injected. Response levels at the end of the injection periods from double-blank-subtracted sensograms were recorded.

Electrophoretic mobility shift assay (EMSA)

A 7.5% Tris-Glycine polyacrylamide gel (Bio-rad, 5671024) was pre-run at 150 V for 1 hour at 4 °C in a cold room. Mixtures (12 μl each) of PCSK9-Evo5-Fluor or a sequence-matched DNA (1 nM final), PCSK9 protein (ACROBiosystems, PC9-H5223, between 0.3 and 300 nM final), and salmon sperm DNA (Invitrogen, 15632-011, 30 μg/ml final) in 0.5x HBS-EP buffer (diluted from HBS-EP buffer, GE Life Sciences, BR100188) containing 3% v/v glycerol was incubated at 25 °C for 30 minutes, and then at 4 °C for 5 minutes. The mixtures were loaded onto the pre-run gel and run at 150 V for 15 minutes at 4 °C. The gel was imaged with a Typhoon imager using the Cy5 channel.

DNA secondary structure prediction

DNA secondary structure prediction was performed on the mfold Web server[4] using parameters that reflect conditions used in SPR assays: the folding temperature was set to 25 °C, the sodium concentration to 0.15 M, and the magnesium concentration to zero.

Supplementary References

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