A System for In Vitro Selection of Fully 2'-Modified RNA Aptamers

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Table of Contents

1. Materials

DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and purified using denaturing polyacrylamide gel electrophoresis (PAGE) according to the manufacturer's recommendations. All sequences purchased for the experiments herein can be found in Table S1. Enzymes including BST 2.0 WarmStart DNA polymerase, BST 2.0 DNA polymerase, Exonuclease I, Lambda Exonuclease, Therminator DNA Polymerase, and Phusion DNA Polymerase were purchased from New England Biolabs (Ipswich, MA). Additionally, dNTPs were purchased from New England Biolabs and all fNTPs except 2'-fluoro-ethynyluridine (fEdUTP) were purchased from TriLink BioTechnologies (San Diego, CA). fEdUTP was synthesized in-house using our published route.[1] Hydrophilic streptavidin M270-Dynabeads were purchased from Invitrogen (Waltham, MA). Sybr Gold nucleic acid gel stain was purchased from Life Technologies. CentriSep 10 and 20 spin columns were purchased from Princeton Separations (Princeton, NJ). All other reagents were purchased from National Diagnostics, Sigma- Aldrich, Acros Organics, New England Biolabs, or Fisher and used without further purification unless otherwise noted.

Table S1. Oligonucleotide Sequences

Name	Sequence
Library Template	CTTGTCGTCTCCTGTGTGCTTNNNNNNNN NNNNNNNNNNNNNNNNCCCGTACCCGT TAAAACTCCACCTCATAACCGCA
Regeneration Primer	/5Phos/CCCGTACCCGAATATAAAATAAAAA TATAAAATATAAAATTGCGGTTATGAGGTG GAGTT
Hairpin Poison Primer	TAAAATAAAAATATAAAATATAAAATTGCG GTTATGAGGTGGAGTTTTAACGGGTACGG G
Biotin Forward Primer	/5Biosg/TGCGGTTATGAGGTGGAGTT
Reverse Primer	CTTGTCGTCTCCTGTGTGCTT
NheI Template	CTTGTCGTCTCCTGTGTGCTTCGTTCTTAT GTTCTCACTCGCTAGCCCCGTACCCGTTA AAACTCCACCTCATAACCGCA
NheI Selection Primer	/5BiotinTEG/TTCTCACTCGCTAGC
Clone 1	CTTGTCGTCTCCTGTGTGCTTTATCCGTA GGTTGCACCGTGGGTCTCCCGTACCCGTT AAAACTCCACCTCATAACCGCA
Clone 2	CTTGTCGTCTCCTGTGTGCTTTGACCCAC GGTGCAACCTACGGATACCCGTACCCGTT AAAACTCCACCTCATAACCGCA
Clone 3	CTTGTCGTCTCCTGTGTGCTTCATAGGGA ACCCAGGTGATTGGGGCCCCGTACCCGT TAAAACTCCACCTCATAACCGCA
Click test template	CTTGTCGTCTCCTGTGTGCTTTATCCGTA GGTTGCACCGTGGGTCTCCCGTACCCG
Click test primer	CGGGTACGGGAGACC

* The fidelity template was not purchased from IDT. See Section 6 for information on how it was produced.

Table S2. Buffer Compositions

Buffer name	1X Component Concentrations
Hairpin Storage Buffer	2 mM $MgSO_4$ and 10 mM Tris-HCl pH 7.5
Click Buffer	2 mM $MgSO_4$ and 10 mM HEPES pH 8
Streptavidin Bead Buffer	1 M NaCl, 0.5 mM EDTA, 5 mM Tris-HCl pH 7.5 and 0.1% Tween 20
Binding Buffer with Tween (BBT)	150 mM NaCl, 2 mM $MgSO_4$, 20 mM Tris-HCl pH 7.5 and 0.1% Tween 20
SFM Wash Buffer	50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, pH 8.0
Equilibration Buffer	10 mM $(NH_4)_2SO_4$, 100 mM NaCl, 1mM DTT, 10% glycerol
KOD Exchange Buffer	1 mM DTT, 50mM Tris-HCl pH 8, 0.1% Nonidet P-40, 0.1% Tween 20, 10% glycerol
KOD Storage Buffer	1 mM DTT, 50mM Tris-HCl pH 8, 0.1% Nonidet P-40, 0.1% Tween 20, 90% glycerol
KOD Buffer #2	6 mM $(NH_4)_2SO_4$, 10 mM KCl, 120 mM Tris-HCl pH 8.8, 0.1% Triton X-100, 0.001% BSA

2. Enzyme Expression and Purification

2.1. SFM4-3 Expression and Purification

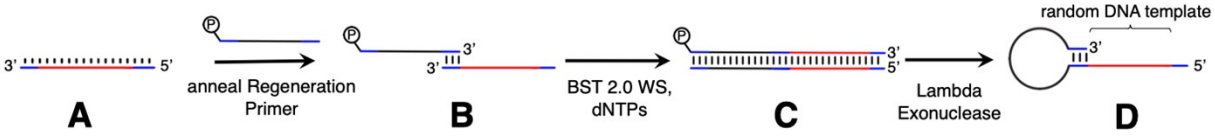
Plasmid for the SFM4-3 polymerase was obtained from the Romesburg lab (Scripps Research Institute) and competent BL21 (DE3) pLysS cells were purchased from Invitrogen. Pure plasmid was transformed into the competent cells through heat shock according to manufacturer's instructions for toxic gene expression. Transformed cells were then diluted into prewarmed SOC broth and incubated at 37 °C for 1 hour. These cells were then innoculated into 1 L of 2XYT growth media and left to grow at 37 °C. After reaching an OD of 0.6, transcription was induced by adding IPTG to a concentration of 0.4 mM and the incubation temperature lowered to 30 °C. After 18 hours, the cells were pelleted at 4000 x g for 20 minutes at 4 °C. The supernatant was poured off and pelleted cells were resuspended in 25 mL of 1X PBS + 1X Bug Buster Protein Extraction Reagent (Novagen). This suspension was incubated at 70 °C for 30 minutes. Cell debris was pelleted at 10,000 x g for 45 minutes at 4 °C and the supernatant was then tumbled with 1 mL of Ni-NTA resin for 1 hr at 4 °C. The slurry was then applied to an EconoPac column (BioRad) and the flow through collected. The resin was washed 3 times with 5 mL of the SFM Wash Buffer. SFM4-3 was eluted in 3 subsequent 5 mL washes of 50 mM, 100 mM, and 250 mM imidazole + 0.5 mM ß-ME respectively and collected in 1 mL fractions. SDS-PAGE was used to determine which fractions contained the polymerase and these were subsequently pooled. The collected fractions were then dialyzed in a 20K cassette with 50 mM Tris-HCl (pH 8.5) and 0.5 mM EDTA overnight. After dialysis, the enzyme was concentrated to roughly 150 µL using 30 kDa Vivaspin columns and 150 µL of glycerol was added for 50% glycerol stock storage. For subsequent use, the enzyme was diluted to the desired activity which was to extend 8 picomoles of single stranded hairpin in 30 minutes. This test and dilution was done for each aliquot of the enzyme between expressions.

2.2. KOD DGLNK Expression and Purification

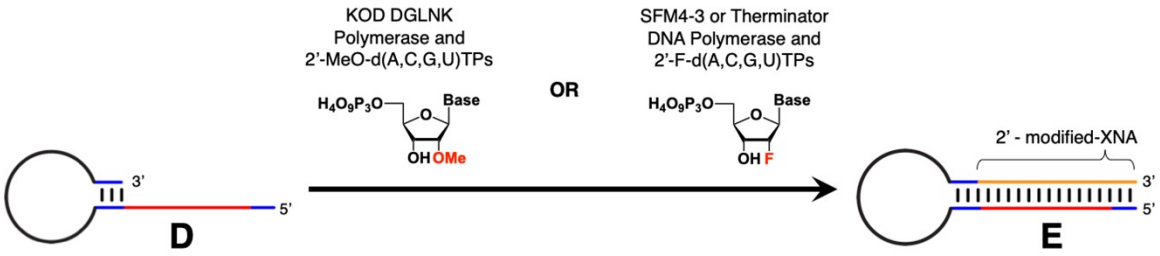
Plasmid for the KOD DGLNK polymerase was obtained from the Obika lab (Osaka University) and was transformed into BL21 (DE3) pLysS cells according to the manufacturer's instructions. A 250 mL culture was grown in 2XYT media containing 100 µg/mL kanamycin and 34 µg/mL chloramphenicol at 37 °C to an OD of 0.6 before inducing with 0.4 mM IPTG. Cells were pelleted at 4000 x g for 20 minutes at 4 °C. Pelleted cells were resuspended in 20 mL 1 X BugBuster Protein Extraction Reagent (Novagen) in PBS and incubated at 70 °C for 30 minutes. Cell debris was pelleted by centrifugation at 10000 x g for 40 minutes at 4 °C, and the supernatant was filtered with a 0.22 µm cellulose acetate filter before FPLC purification.

For FPLC purification of KOD DGLNK a 1 mL HiTrap Heparin HP column (Cytiva) was used. First the column was equilibrated with 10 mL of Equilibration Buffer. Next 8-10 mL of crude protein was applied onto the column using 3-4 injections of a 2 mL sample injection loop. A linear gradient of 0.1-1.5 M NaCl was applied for 20 minutes at a flow rate of 1 mL/minute, and 1 mL fractions collected. SDS-PAGE was used to identify the fractions containing KOD DGLNK. These were combined and buffer exchanged into KOD Exchange Buffer using a 30 kDa MWCO filter to 150 µL. The concentrated protein was combined with an equal volume of KOD Storage Buffer and stored at –20 °C. For subsequent use, the enzyme was diluted based to the desired activity which was to extend 8 picomoles of single stranded hairpin in 30 minutes. This test and dilution was done for each aliquot of the enzyme between expressions.

3. SELMA Library Construction



Installation of the hairpin involves three enzymatic reactions. Starting with 50 pmol of a single stranded 80 base pair template sequence (Form A) (either a single sequence or library), Thermopol Buffer (1X final concentration), 100 pmol of Regeneration Primer, and water were added to a final volume of 100 µL in a PCR tube. This mixture was heated to 95 °C for 1 minute, cooled to 45 °C for 1 minute, and then left at room temperature. dNTPs (200 µM each final concentration) and 2 U of BST 2.0 WarmStart DNA Polymerase were added to the mixture and then heated to 60 °C for 10 minutes to create a double stranded product with one strand phosphate labeled (Form C), then buffer exchanged into water using CentriSep-20 columns (Princeton Separations). Next, 1 U of Exonuclease 1 was added with Exonuclease 1 Buffer (1X final concentration) and the mixture heated to 37 °C for 30 minutes followed by 80 °C for 20 minutes. This digest was then buffer exchanged into Hairpin Storage Buffer using CentriSep-20 columns (Princeton Separations). Finally, 1 U of Lambda Exonuclease was added with Lambda Exonuclease Buffer (1X final concentration) and the mixture heated to 37 °C for 30 minutes followed by 75 °C for 10 minutes to generate Form D, and buffer exchanged into water using CentriSep-20 columns (Princeton Separations).



After creation of single-stranded hairpin by the steps above, we extended with 2' modified RNA using one of three enzymes: SFM4-3, Therminator, KOD DGLNK.

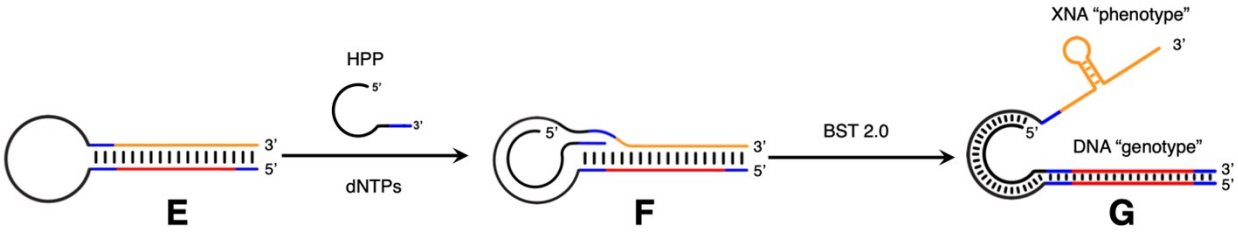
Standard SFM4-3 hairpin extension reactions included 1X Taq Buffer (NEB), 0.2 μM single stranded hairpin (Form D), 2 mM supplemental $MgCl_2$, 1 mM $MnCl_2$, and 0.4 mM F-NTPs. Optimal SFM4-3 concentration varied between enzyme purifications and was determined experimentally as the minimal amount of enzyme required to extend 8pmol of hairpin in 30 minutes. Hairpin template was heated to 95 ºC for 1 minute and cooled to 45 °C for 5 minutes to promote self-priming prior to the addition of salts, NTPs, and SFM4-3. The complete reaction mixture was then incubated at 70 ºC for 30 minutes to generate Form E.

Standard Therminator hairpin extension reactions included 1X Thermopol Buffer (NEB), 0.2 μM single stranded hairpin (Form D), 0.4 mM F-NTP mix, and 2 U of Therminator polymerase (NEB). Hairpin template was heated to 95 ºC for 1 minute and cooled to 45 °C for 5 minutes to promote self-priming prior to the addition of NTPs, and Therminator. The complete reaction mixture was then incubated at 60 ºC for 10 minutes to generate Form E.

Standard KOD DGLNK hairpin extension reactions included 1X KOD Buffer #2, 0.2 μM single stranded hairpin (Form D), 1 mM $MgSO_4$, and 0.2 mM OMe-NTP mix.[2, 3] Optimal KOD DGLNK concentration varied between enzyme purifications and was determined experimentally as the minimal amount of enzyme required to extend 8 pmol of hairpin in 30 minutes. Hairpin template was heated to 95 ºC for 1 minute and cooled to 45 °C for 5

minutes to promote self-priming prior to the addition of NTPs, and KOD DGLNK. The complete reaction mixture was then incubated at 75 ºC for 1 hour to generate Form E.

The hairpin extension, regardless of enzyme used, was followed by buffer exchanging into Hairpin Storage Buffer using CentriSep-10 columns (Princeton Separations).



Next, we strand displaced the modified RNA using the hairpin poison primer and BST 2.0 DNA polymerase. Typical reactions include 0.8 µM of product E, 1X Thermopol Buffer (NEB), 0.16 µM HPP, and 0.6 mM dNTPs in a final volume of 50 µL. The reaction is heated to 95 °C and cooled at 0.3 °C per minute to 25 °C to generate Form F. 8 U of BST 2.0 DNA polymerase are then added and the reaction is incubated at 55 °C for 40 minutes to produce Form G. This material was then buffer exchanged into Hairpin Storage Buffer using CentriSep-20 columns (Princeton Separations).

Interestingly, when strand displacement was performed using BST 2.0 WarmStart DNA polymerase (BST 2.0 is inhibited by the "WarmStart" aptamer, which binds the enzyme active site at cooler temperatures), we found that the NEB WarmStart aptamer forms an isolable complex with our strand-displaced library. To demonstrate this, the strand displacement product generated using BST 2.0 WarmStart Polymerase was run on an 8% native gel. Each band present was excised from the gel and crushed into small pieces. The DNA from each band was then extracted in 400 µL of 0.4 mM KOAc while rotating at room temperature overnight. The supernatant was concentrated with a speed vac to ~50 uL followed by buffer exchanging into water twice using CentriSep-20 columns (Princeton Separations) and then finally concentrated to ~20 µL. This concentrated DNA extract was then analyzed on a 10% denaturing urea PAGE gel and we were able to see that the

extracted species contain the WarmStart aptamer. Representative gel images are shown in Figure S1. In the Native gel, the full construct band is indicated with a red box, all lower bands are composed of the HPP. The warmstart aptamer is apparent in the denaturing gel and indicated with a bracket.

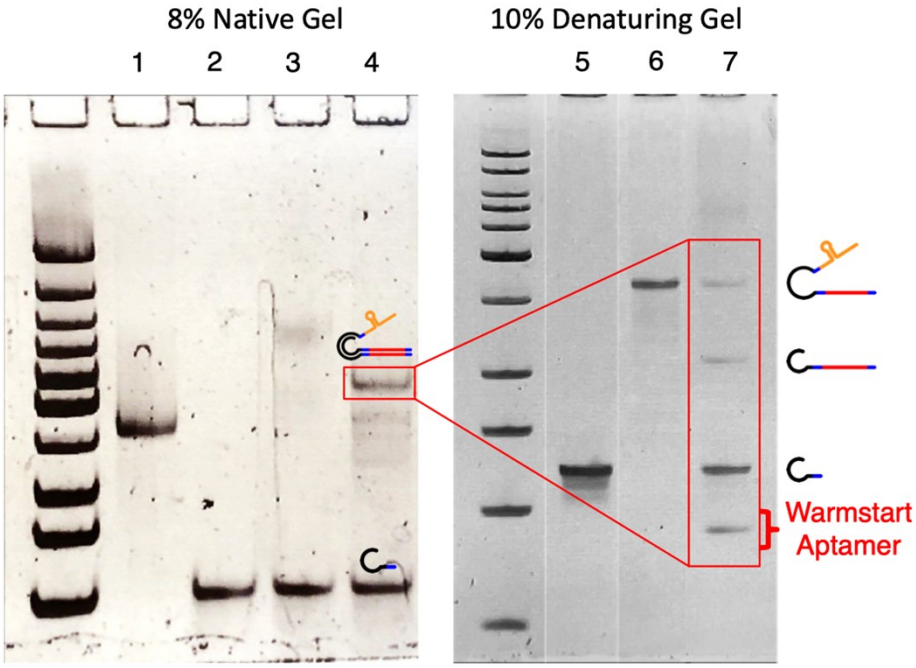


Figure S1. PAGE analysis of the displacement of F-RNA using WS BST 2.0 DNA polymerase. Lanes 1 and 6 contain Form E built from the library template (controls). Lanes 2 and 5 contain pure HPP in 4.5 times excess of Form E (controls). Lane 3 contains Form F, sampled immediately after the annealing protocol, with excess HPP visible at the bottom. Lane 4 contains the strand-displaced library (Form G) generated with WS BST 2.0 DNA polymerase (top band), with excess HPP visible at the bottom. In lane 7 of the denaturing gel the full HPP extension product (Form G) is shown.

We repeated the same procedure as described for Figure S1 with BST 2.0 DNA polymerase that contained no WarmStart aptamer to show that after gel excision no 40 bp aptamer remained.

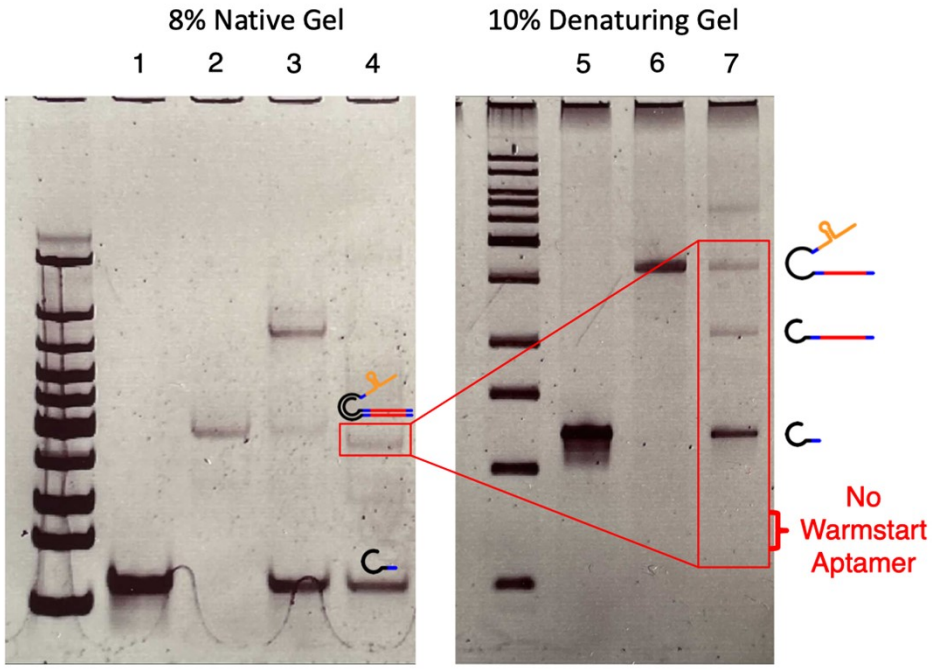


Figure S2. The topmost band from lane 4 containing Form G indicating the fully formed SELMA construct (Form G).

4. SFM4-3 and KOD DGLNK Temperature Optimization

To optimize extension temperature for SFM4-3 and KOD DGLNK reactions, standard hairpin reactions were performed as described previously (Section 3) with the exception of varying extension temperatures. For SFM4-3, three sequences identified in previous SELMA selections were used as control templates. Extension progress was visualized on a 10% Urea gel run at 300V for 15 minutes.

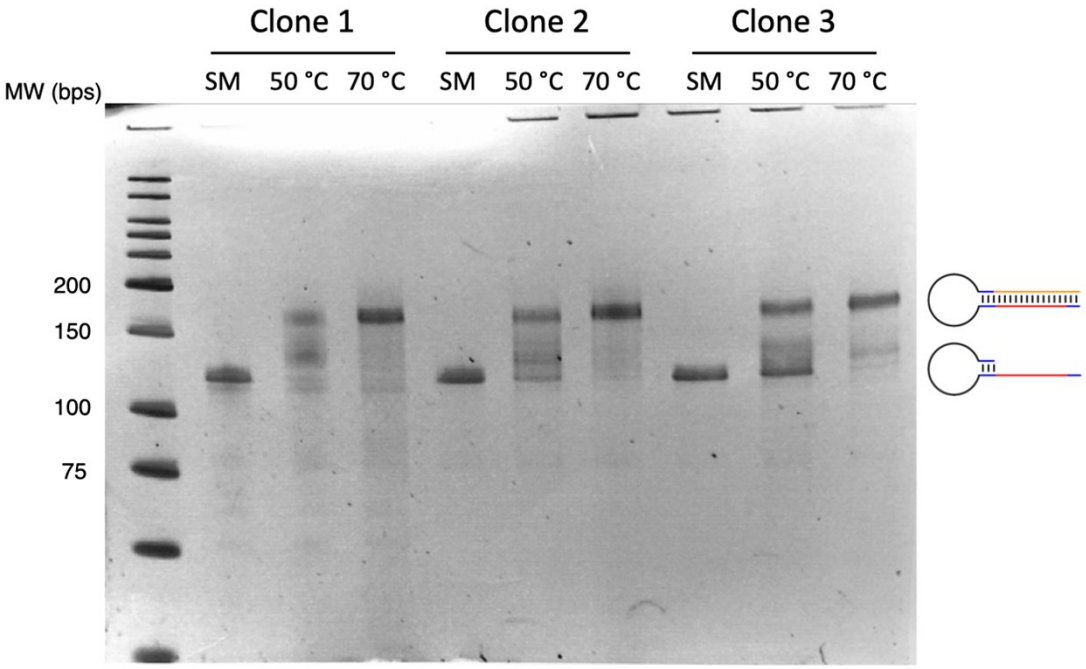


Figure S3. SFM4-3 hairpin extensions on various clones at 50 °C and 70 °C. Reactions were set up as described in Section 3. Extension progress after 30 minutes at 50 °C or 70 °C visualized on 10% Urea PAGE gel. SM = starting material. Clone sequences are presented in Table S1.

For KOD DGLNK, single stranded hairpin made from the library template was extended at various temperatures.

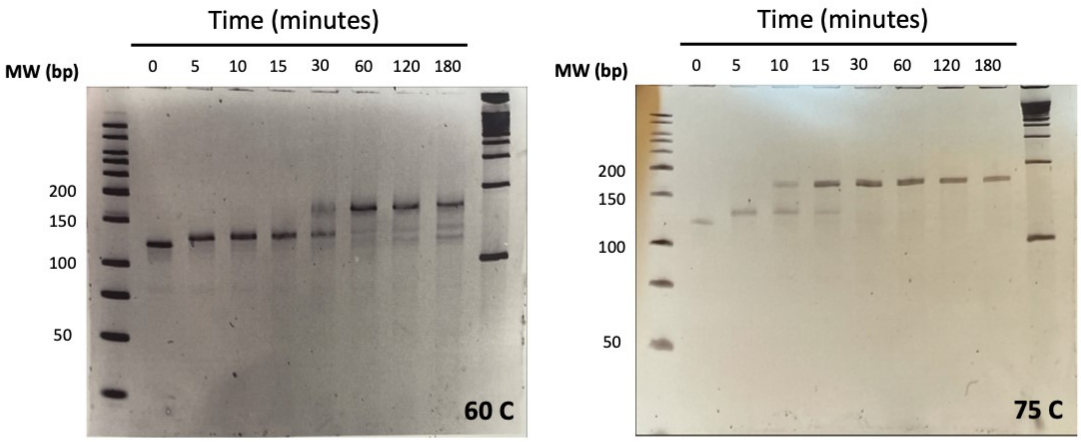
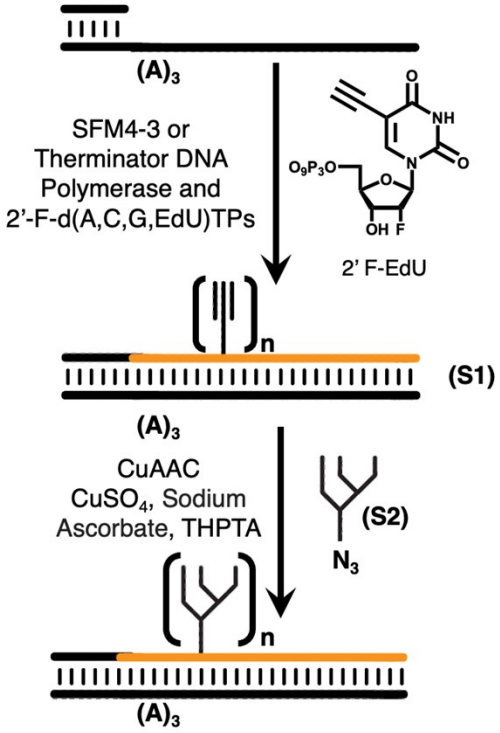
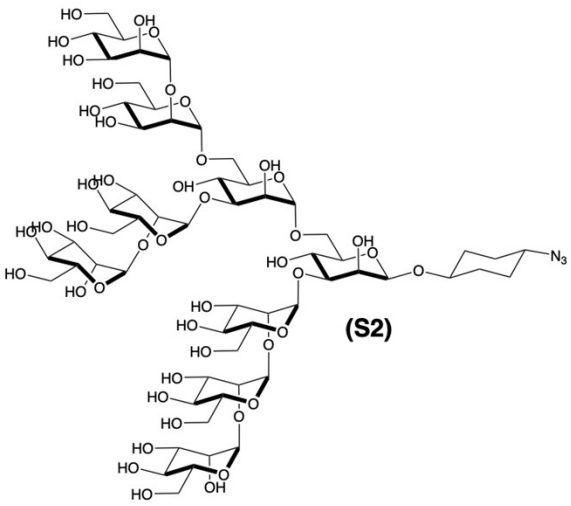


Figure S4. Extension temperature optimization of KOD DGLNK. The protocol described in Section 4 was performed and 2 µL samples removed and added to 8M Urea at different time points to evaluate KOD DGLNK's activity at 60 °C and 75 °C. Full conversion to the final product (Form E) was observed at 30 minutes during the 75 °C but not observed after 180 minutes at 60 °C.

5. SFM4-3 and Therminator Click Comparison





Our click comparison between SFM4-3 and Therminator extension products was done by a CuAAC with Man_9-cyclohexyl azide, as previously reported.[1, 4] To generate a clickable fluorinated RNA strand (**S1**), the Therminator and SFM4-3 extension protocols described in section 3 were completed using the Click test template and primer. After the extension the product was buffer exchanged into Click buffer using CentriSep-10 columns (Princeton Separations). Tubes containing dry or degassed reaction components were prepared as follows: 202 nmol sodium ascorbate solution was placed in a capless 0.5 mL Eppendorf tube. Next, 90 nmol $CuSO_4$ and 108 nmol THPTA solutions were added to a second capless tube. Lastly, 5 pmol of the Therminator or SFM4-3 extension product of the click test primer and template and 50 nmol of Man_9-cyclohexyl-N_3 (**S2**) were placed in a third tube. All tubes were concentrated via speed-vac until the $CuSO_4$/THPTA and sodium ascorbate were dry and the library-sugar mixture was approximately 20 µL in volume. All tubes were then transferred to a 2-neck flask with N_2 gas flowing through. After increasing the flow of nitrogen gas, 5 µL of degassed water from a separate 2-neck flask was used to redissolve the sodium ascorbate. Next the dissolved sodium ascorbate

was added to the tube with $CuSO_4$ and THPTA to reduce the Cu(II) to Cu(I). Subsequently, the mixture was added to the final tube of oligonucleotide-sugar mixture. The vessel was then flushed with nitrogen for 10 minutes before closing the septum while the reaction ran under slight N_2 pressure for 3 hours. The reaction was then diluted to 50 µL and buffer exchanged into MQ CentriSep-10 columns (Princeton Separations).

6. NheI Selection

Briefly, 2 pmol of SELMA library form G was made as described in Section 3, and 4 pmol of biotinylated NheI selection primer were diluted to a final volume of 50 µl in water and rotated for 10 minutes at room temperature. 0.1 mg streptavidin magnetic beads (Dynabeads M270) were then added, and the reaction was rotated for 20 minutes at room temperature. The supernatant was removed, and the beads were washed twice with Streptavidin Bead Buffer. Bound sequences were eluted by resuspending the beads in 50 µl 0.15 M NaOH for 4 minutes at room temperature. Supernatant was removed and neutralized with the addition of 5.5 µl 100mM Tris and 6.5 µl of 1.25 M HOAc. Eluted sequences were used as PCR template, and the resulting PCR products were digested with NheI according to manufacturer’s instructions.

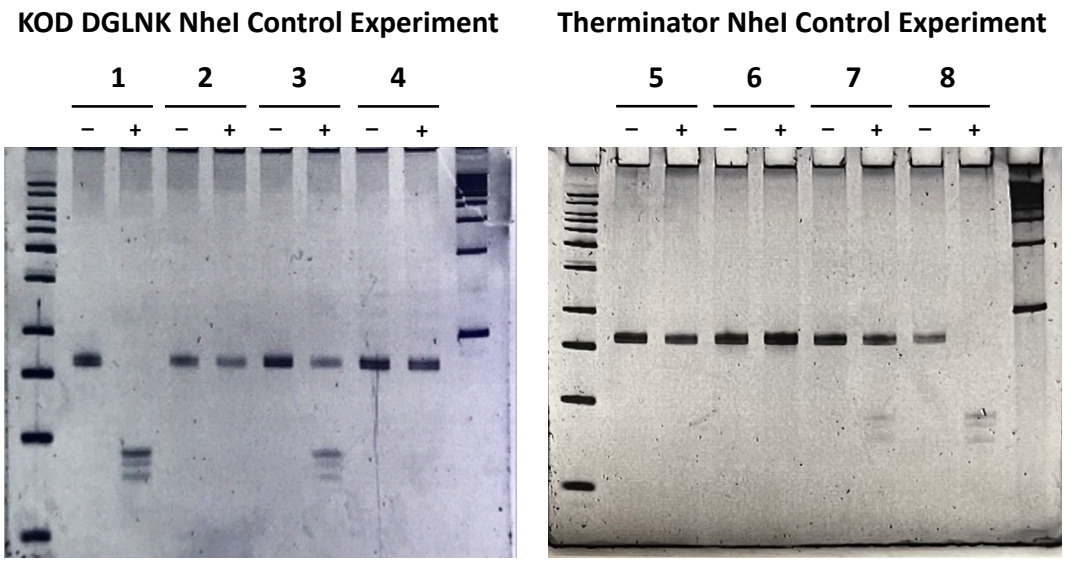


Figure S5. Complete PAGE of mock NheI selection gels from Main Text Figure 4. Random libraries produced by KOD DGLNK (with MeO-RNA) and Therminator DNA polymerase (with F-RNA) were spiked with NheI restriction sequence and subjected to 1 round of selection with a biotinylated oligo complementary to that sequence. (+) and (-) indicate samples with and without NheI treatment. Group 1 = pure NheI sequence; Group 2 = 1000:1 mixture of random and NheI sequence before selection; Group 3 = random:NheI mixture after selection; Group 4 = random library alone; Group 5 = random library alone; Group 6 = 1000:1 mixture of random and NheI sequence before selection; Group 7 = 1000:1 mixture of random and NheI sequence after selection; Group 8 = pure NheI sequence.

References:

(1) Redman, R. L.; Krauss, I. J. Directed Evolution of 2'-Fluoro-Modified, RNA-Supported Carbohydrate Clusters That Bind Tightly to HIV Antibody 2G12. *Journal of the American Chemical Society* **2021**, *143* (23), 8565-8571. DOI: 10.1021/jacs.1c03194.
(2) Hoshino, H.; Kasahara, Y.; Fujita, H.; Kuwahara, M.; Morihiro, K.; Tsunoda, S.-I.; Obika, S. Consecutive incorporation of functionalized nucleotides with amphiphilic side chains by novel KOD polymerase mutant. *Bioorganic & Medicinal Chemistry Letters* **2016**, *26* (2), 530-533. DOI: 10.1016/j.bmcl.2015.11.079.
(3) Hoshino, H.; Kasahara, Y.; Kuwahara, M.; Obika, S. DNA Polymerase Variants with High Processivity and Accuracy for Encoding and Decoding Locked Nucleic Acid Sequences. *Journal of the American Chemical Society* **2020**, *142* (51), 21530-21537. DOI: 10.1021/jacs.0c10902.
(4) Temme, J. S.; Macpherson, I. S.; Decourcey, J. F.; Krauss, I. J. High Temperature SELMA: Evolution of DNA-Supported Oligomannose Clusters Which Are Tightly Recognized by HIV bnAb 2G12. *Journal of the American Chemical Society* **2014**, *136* (5), 1726-1729. DOI: 10.1021/ja411212q.