A Sequential Strand-Displacement Strategy Enables Efficient Six-Step DNA-Templated Synthesis

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Supporting Information

General Methods. All oligonucleotides were synthesized on a PerSeptive Biosystems Expedite 8090 DNA synthesizer using standard phosphoramidite chemistry and purified by reverse-phase HPLC using Eclipse XDB-C18 (Agilent) columns and an acetonitrile/0.1 M triethylammonium acetate (TEAA) gradient. Modified phosphoramidites and CPG for DNA synthesis were purchased from Glen Research. The 3’-amino and quencher modified oligonucleotides were synthesized using 3’-PT Amino-Modifier C6 CPG and Epoch Eclipse™ Quencher CPG, respectively (Glen research). The 5’-thiol and fluorescein modified oligonucleotides were synthesized using 1’-[2-cyanoethyl]- (N,N-diisopropyl)]-phosphoramidite (Thiol-Modifier C6 S-S) and 2-Dimethoxytrityloxymethyl-6-(3’, 6’-dipivaloylfluorescein-6-yl)-hexyl-1-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (6-Fluorescein Phosphoramidite), respectively. Oligonucleotides were quantified by 260 nm UV absorbance. All modified DNA molecules were purified by HPLC and characterized by LC/MS on Waters Acquity UPLC equipped with a Waters Acquity UPLC BEH C18 column, using an aqueous 6 mM tetraethyl ammonium bicarbonate (TEAB)/MeOH mobile phase. Electrospray mass spectrometry was carried out on a Waters Q-TOF Premier instrument. All chemicals were purchased from Sigma-Aldrich, unless otherwise noted.

Oligonucleotide Sequences

T: 5’- ACA TAC TGT TGT AGT GAT AAA TAG GTA CGC TTG CTA AGA GAT TGA TGG TGT TCG GT /C6 amine/

T_fluo: 5’- ACA TAC TGT TGT AGT GAT AAA TAG GTA CGC TTG CTA AGA GAT T(Fluorescein)GA
TGG TGT TCG GT /C6 amine/

\( T_{mis}: 5'-\text{GTA ATG TCT TAA TGA GAG TGT GGA CGT GGA GCA TCT GTA TAT CTA GCT GTA ATG TA /C6 amine/} \)

\( S1: 5'/-\text{SThioMC6-D/ ACC GAA CAC CAT CAA T} \)

\( S2: 5'/-\text{SThioMC6-D/ ACC GAA CAC CAT CAA TCT CTT AGC} \)

\( S3: 5'/-\text{SThioMC6-D/ ACC GAA CAC CAT CAA TCT CTT AGC AAG CGT AC} \)

\( S4: 5'/-\text{SThioMC6-D/ ACC GAA CAC CAT CAA TCT CTT AGC AAG CGT ACC TAT TTA T} \)

\( S5: 5'/-\text{SThioMC6-D/ ACC GAA CAC CAT CAA TCT CTT AGC AAG CGT ACC TAT TTA TCA CTA CAA} \)

\( S6: 5'/-\text{SThioMC6-D/ ACC GAA CAC CAT CAA TCT CTT AGC AAG CGT ACC TAT TTA TCA CTA CAA CAG TAT GT} \)

\( \text{F104: /5'Fluo/ TGC TCC ATT TCG GTC AGT GGG TCA CAG CTA GTT GTG GAT TGG ATT GCC ACA TAC} \)

\( \text{TGT TGT AGT GAT AAA TAG GTA CGC TTA AGA GAT TGA TGG TGT TCG GT} \)

\( \text{Q64: 5' - CAC TAC AAC AGT ATG TGG CAA TCC AAT CCA CAA CTA GCT GTG ACC CAC TGA CCG AAA TGG AGC A /3'Quen/} \)

\( \text{D72: 5' - CTA TTT ATC ACT ACA ACA GTA TGT GGC AAC CAA CAC TAC CAC AAT TAG CTG TGA CCC ACT GAC CGA AAT GGA GCA} \)

\( \text{Q80: 5' - AAG CGT ACC TAT TTA TCA CTA CAA CAG TAT GTG GCA ATC CAA TCC ACA ACT AGC TGT GAC CCA CTG ACC GAA ATG GAG CA /3'Quen/} \)

\( \text{D88: 5' - CTC TTA GCA AGC GTA CCT ATT TAT CAC TAC AAC AGT ATG TGG CAA TCC AAT CCA CAA CTA GCT GTG ACC CAC TGA CCG AAA TGG AGC A} \)

\( \text{Q96: 5' - CCA TCA ATC TCT TAG CAA GCG TAC CTA TTT ATC ACT ACA ACA GTA TGT GGC AAT CCA ATC CAC AAC TAG CTG TGA CCC ACT GAC CGA AAT GGA GCA /3'Quen/} \)

\( \text{D104: 5' - ACC GAA CAC CAT CAA TCT CTT AGC AAG CGT ACC TAT TTA TCA CTA CAA CAG TAT GTG GCA ATC CAA TCC ACA ACT AGC TGT GAC CCA CTG ACC GAA ATG GAG CA} \)

**Synthesis of NHS Ester-Linked Substrates.** All substrate oligonucleotides were synthesized on CPG resin and purified by reverse-phase HPLC. To append the NHS group to the 5’ thiol modification on each substrate oligonucleotide, the synthesized oligonucleotide was first treated with 100 mM DTT (pH 8.5) at room temperature for 30 min to cleave the S-S bond and generate free
terminal thiol group. After cleavage, the reaction was desalted by gel filtration with a NAP-5 columns (GE Healthcare Life Sciences) and added directly to 250 μL of a 40 mg/mL solution of N-hydroxymaleimide in 0.5 M MOPS (pH 7.5). After 30 min, the reaction was desalted by gel filtration and purified by reverse-phase HPLC to generate a 5’ NHS-linked oligonucleotide.

NVOC-protected amino acids were prepared using a previously reported protocol.\textsuperscript{1-3} Briefly, an amino acid (0.3 mmol) was mixed with Na\textsubscript{2}CO\textsubscript{3} (0.3 mmol) in 8 mL H\textsubscript{2}O. An equimolar amount of 4,5-dimethoxy-2-nitrobenzyl chloroformate (0.3 mmol) dissolved in 8 mL dioxane was added slowly with stirring to the aqueous solution. After stirring at room temperature for 1 h, the reaction was diluted with 7.5 mL dichloromethane followed by acidification with 5 mL of 1 M NaHSO\textsubscript{4}. The organic phase was collected and the aqueous phase was washed with dichloromethane. The combined organic extracts were dried over anhydrous Na\textsubscript{2}SO\textsubscript{4} and then concentrated in vacuo to give a solid crude product. The crude protected amino acids were used directly for DNA conjugation.

To conjugate NVOC-protected amino acids or biotin with DNA, the desired amino acid or biotin (~2 mg) was mixed with ~0.5 mg EDC·HCl in 200 μL DMF. After 5 min, 20 μL of the mixture was added directly to an aliquot of NHS-linked oligonucleotide in 100 μL 0.1 M MES (pH 6.0). After 5 min, the reaction was desalted with a NAP-5 column and purified by reverse-phase HPLC. Amino acid (E)-4-Amino-2-butoenoic acid was conjugated to the appropriate DNA oligonucleotide to generate S1. 4-(aminomethyl) benzoic acid was conjugated to the appropriate DNA oligonucleotide to generate S2 and S4. Trans-4-(aminomethyl) cyclohexane carboxylic acid was conjugated to the appropriate DNA oligonucleotide to generate S3. 4-(2-aminoethoxy)-3-methoxybenzoic acid (Alinda Chemical Inc.) was conjugated to the appropriate DNA oligonucleotide to generate S5. Biotin was conjugated to the appropriate DNA oligonucleotide to generate S6. After HPLC purification, 1% TFA was added into each fraction before lyophilization to prevent hydrolysis of the NHS ester. The lyophilized oligonucleotide samples were stored at -80 °C before using. Exposure of the redissolved oligonucleotides (in 0.1 M NaOAc, pH 5.0) to a Spectroline® E-Series handheld UV lamp (365 nm) for 1.5 h at 4 °C resulted in the quantitative deprotection of the NVOC protection group as evaluated by HPLC. The photodeprotected DNA-linked substrates, after desalting (Princeton Separations) and UV quantification, were directly combined with the other components for multistep DNA-templated reactions.
Multistep DNA-Templated Reactions. Each photodeprotected substrate (S1-S6) was quantified by UV absorbance at 260 nm and mixed with T with a 1.2:1 stoichiometric ratio of S:T in aqueous buffer containing 50 mM MOPS and 10 mM Mg(OAc)$_2$, pH 7.5. The initial concentration of T was 1 µM. After 30 min incubation at 23 °C, the second photodeprotected substrate S2 was added into the system, followed by another 30 min incubation at 23 °C. Thereafter, the same amount of each remaining substrate was sequentially added into the solution every 30 min. During the entire process, the reaction buffer remained 50 mM MOPS and 10 mM Mg(OAc)$_2$, pH 7.5. After the last substrate addition and incubation was complete, the crude reaction mixture was desalted by gel filtration (Princeton Separations), and directly subjected to high-resolution LC/MS analysis.

LC/MS Analysis. Oligonucleotide reaction mixtures were characterized by LC/MS on a Waters Acquity UPLC using a Waters BEH C18 (1.7 µm, 1.0x50 mm) column and an aqueous 6 mM tetraethyl ammonium bicarbonate (TEAB)/MeOH mobile phase. The DNA-templated reaction mixture was desalted and injected in the LC/MS. Under experimental conditions, the -5 charged ion species exhibited best signal:noise ratio for the DNA template and all products.

Calculation of Multistep Reaction Yields. DNA template T-fluo (containing a single fluorescein group) was subjected to no reaction, or to 1-, 2-, 3-, 4-, 5-, and 6-step reaction sequences in different vials. After reaction, NHS-linked S6 was added to each reaction mixture to hybridize with T-fluo for 30 min at room temperature. The resulting solutions were each desalted by gel filtration (Princeton Separations), then digested with DdeI (NEB) in NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl$_2$, 1 mM DTT, pH 7.9) at 37 °C for 1 h. The digested mixtures were subjected to 25% denaturing polyacrylamide gel electrophoresis (PAGE, 18x16 cm in size) analysis (Figure 4 and Figure S1). Electrophoresis was performed at 23 °C (300 V) for 16 hours and then imaged with Typhoon scanner (GE Healthcare; excitation: 488 nm; emission: 520 nm). Densitometry was performed with ImageQuant. The densitometry analysis revealed that the three-step (Lane 4) and six-step (Lane 7) DNA-templated reactions have an overall yield of 55% and 35%, respectively.
**Figure S1.** 25% denaturing PAGE analysis of *DdeI*-digested reaction products. A fluorescein group was incorporated into the template DNA to facilitate visualization. Lanes 1 and 8: unreacted template; lanes 2-7: DNA template undergoing 1-, 2-, 3-, 4-, 5-, and 6-step reaction sequences, respectively. Fluorescein-linked species were visualized using a Typhoon fluorescence image scanner.
An independent experiment was also performed to determine reaction yields in which non-fluorescein-labeled T was used for all the reactions. After reaction, NHS-linked S6 was added into each reaction mixture to hybridize with T. The resulting solution was desalted by gel filtration (Princeton Separations), and then treated with Exonuclease I (in ExoI buffer, NEB) at 37 °C for 1 h to remove all single-stranded DNA (leaving only the S6:T duplex). After ExoI treatment, the sample was heated to 95 °C for 15 min to denature the ExoI, and then treated with endonuclease Ddel in NEBuffer 2 at 37 °C for 1 h. The Ddel-treated samples were analyzed by 25% denaturing polyacrylamide gel electrophoresis. Finally, the gel was stained with Sybr Green II (VWR) and imaged with the Typhoon scanner. According to the densitometry analysis of digested T-linked species, the three-step (Lane 4) and six-step (Lane 7) DNA-templated reactions have an overall yield of 59% and 38%, respectively, similar to the yields determined using fluorescein-labeled T.

Figure S2. 25% denaturing PAGE analysis of the digested reaction products. The gel was stained with Sybr green II and imaged with Typhoon scanner. Lane 1: unreacted template; Lane 2-7: DNA template underwent 1, 2, 3, 4, 5, and 6-step reaction, respectively; 10 bp DNA ladder was added at the both sides to track the DNA position.
**Toehold Displacement of Longer DNA Strands.** In order to test the toehold displacement of longer DNA strands in duplexes, we designed a 104-mer single strand DNA template (F104), which carries a fluorescein group at its 5' terminus. F104 was hybridized with a complementary 64-nt (Q64), 80-nt (Q80), or 96-nt (Q96) DNA oligonucleotide, each of which has a quencher group at 3’ terminus. The three duplexes were then each treated with a longer displacement DNA oligonucleotide of length 72-nt (D72), 88-nt (D88), or 104-nt (D104), respectively. The fluorescence signals over time were recorded starting from the addition of the displacement DNA (Figure S3, t=0). The red, blue, and green lines in Figure S3 report the Q64+D72, Q80+D88, and Q96+D104 signals, respectively. The effective recovery of fluorescence suggests effective toehold strand displacement in each case. We note slower displacement kinetics as the strand length increases, as expected. As a negative control, the F104/Q80 complex was also treated with the shorter displacement DNA oligonucleotide D72, with little change in fluorescence signal (black line in Figure S3). The fluorescence signal was recorded using Tecan Safire2 plate reader (Ex: 495 nm, Em: 520 nm). The experiments were performed in 50 mM aqueous MOPS, 10 mM Mg(OAc)$_2$, pH 7.5 at a final concentration of 500 nM.

![Figure S3. Kinetics of toehold strand displacement with different lengths. Red line: F104/Q64 + D72; blue line: F104/Q80 + D88; green line: F104/Q96 + D104; black line: F104/Q80 + D72. The data were recorded every five seconds at Ex: 495 nm and Em: 520 nm.](image)

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(29) Clark, M. A.; Acharya, R. A.; Arico-Muendel, C. C.; Belyanskaya, S. L.; Benjamin, D. R.; Carlson, N. R.; Centrella, P. A.; Chiu, C. H.; Creaser, S. P.; Cuzzo, J. W.; Davie, C. P.; Ding, Y.; Franklin, G. J.; Franzen, K. D.; Gefter, M. L.; Hale, S. P.; Hansen, N. J. V.; Israel, D. I.; Jiang, J. W.; Kavara, M. J.; Kelley, M. S.; Kollmann, C. S.; Li, F.; Lind, K.; Mataruse, S.; Medeiros, P. F.; Messer, J. A.; Myers, P.; O'Keefe, H.; Oliff, M. C.; Rise, C. E.; Satz, A. L.; Skinner, S. R.; Svendsen, J. L.; Tang, L. J.; van Vloten, K.; Wagner, R. W.; Yao, G.; Zhao, B. G.; Morgan, B. A. *Nat Chem Biol* **2009**, *5*, 647.
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