Supporting Information

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SI Methods

1. Denaturing, Native, and Biotin Purification of Chemically Synthesized DNA Hairpins. Chemically synthesized hairpins were purified in-house with 8% (wt/vol) PAGE with 7 M Urea. To perform native (N) purification, 1 nmol $\mathbf{M}_i$ and 1 nmol of $\mathbf{A}_i$, each in ~30 μL 1× TNaK buffer [20 mM Tris (pH 7.5) at 25 °C, 140 mM NaCl, 5 mM KCl], were separately refolded. The two hairpins were then mixed and incubated at room temperature overnight. The mixture was then resolved with 8% native PAGE. The gel was stained with SYBR Gold and imaged using a STORM scanner (GE Healthcare). The bands corresponding to monomeric $\mathbf{M}_i$ and $\mathbf{A}_i$ were excised, crushed, and eluted with ~1 mL 1× TNaK at 37 °C overnight with shaking. The eluted DNA was then filtered through a 0.45-μm NanoSep filter (Pall) to remove gel debris and concentrated using an AmiconUltra-0.5 (3K MWKO) concentrator (Millipore).

To perform biotin (B) purification of $\mathbf{M}_1$, the "capture construct" (Fig. S3*B*) was prepared from 10 μM of **S_2mimic-L.Bio** and 20 μM of **S_2mimic-S**, using a procedure similar to that used to prepare the fluorescent reporters (*SI Methods*, section 3). Roughly 100 μL of High-Capacity NeutrAvidin Agarose Resin (Pierce) was loaded on a 10-mL column (Bio-Rad) and washed with ~30 mL of 1× TNaK. The resin was then removed from the column and incubated with 1 nmol of the capture construct (with **M_2mimic-S** in excess) in a 400-μL slurry at 37 °C for 45 min. The charged resin was transferred to the same Bio-Rad column and washed with ~30 mL 1× TNaK, recollected into a 1.5-mL tube, and incubated with 250 pmol of DN-pure $\mathbf{M}_2$ at 37 °C overnight with gentle shaking. DNB-pure $\mathbf{M}_1$ was recovered by filtering through a 0.45-μM NanoSep filter. The concentration of $\mathbf{M}_1$ was estimated by running an aliquot of DNB-pure $\mathbf{M}_1$ with a gradient of known quantity of D-pure $\mathbf{M}_1$ on an 8% denaturing polyacrylamide gel and comparing the intensity of SYBR gold staining.

2. Enzymatic Synthesis of DNA Hairpins. The plasmid containing the hairpin sequence and flanking sequences were constructed using a modified version of inside-out gene assembly (1) (with particular caution that oligonucleotides and intermediate assembly products should not self-prime) and sequence- and ligation-independent cloning (SLIC) (2). Oligonucleotides used to construct these sequences are listed in Table S2. The upstream flanking sequence contains an Nt.BstNBI recognition site 4 bp away from the hairpin sequence so that the nicking site is immediately upstream of the hairpin sequence. The last 3 bp of the hairpin sequence and the first 3 bp of the downstream flanking sequence constitute a PvuII site.

Roughly 750 ng of sequence-verified plasmid was used as the template of a 34.56-mL PCR carried out in three 96-well PCR plates, with each well containing a single 120-μL reaction. The DNA polymerases used in these reactions were commercial Vent(exo$^-$) (New England Biolabs) for $\mathbf{eM}_1$ and $\mathbf{eM}_2$ or laboratory-made DNA polymerase His6-Pfu-Sso7d (3) for $\mathbf{eM}_3$ and $\mathbf{eA}_3$, with the PCR buffers being commercial 1× ThermoPol (New England Biolabs) or laboratory-made 1× Buffer S1.5 [200 mM Tris·H_2SO_4 (pH 8.8) at 25 °C, 50 mM K_2SO_4, 100 mM $(NH_4)_2SO_4$, 15 mM $MgSO_4$, 1% Triton X-100, 1 mg/mL nuclease-free BSA], respectively. dNTP concentrations in both PCR reactions were 0.2 mM each.

The PCR products were mixed with 3 vol of Buffer QG (Qiagen) and column-purified using 24 EconoSpin columns (Epoch Life Science). The DNA in each column was eluted with 50 μL 1× TE (10 mM Tris·HCl, pH 7.5 at 25 °C, 1 mM EDTA). Typically ~500 μg of DNA was recovered.

The recovered DNA was digested with 3,000 units of PvuII-HF (New England Biolabs) in a 1.5-mL reaction in 1× NEBuffer 4 (New England Biolabs) at 37 °C for ~3 h. The reaction was then quenched with the addition of 300 μL of 100 mM EDTA. The quenched reaction was concentrated with an AmiconUltra-0.5 (3K MWKO) concentrator to ~60 μL, all of which was loaded on a 4% NuSieve GTG agarose gel (Lonza) containing 10 μg/mL Ethidium Bromide (EtBr). The upper band was excised under blue light (to avoid UV damage). The gel slice was melted with 6 vol of Buffer QG at 50 °C for ~15 min and purified with 20 EconoSpin columns. The DNA on each column was eluted with 50 μL 1× TE. Typically 250–300 μg of DNA was recovered.

The recovered DNA was digested with 1,000 units of Nt. BstNBI (New England Biolabs) in a 2-mL reaction in 1× NEBuffer 3 (New England Biolabs) at 55 °C for ~3 h, after which 6 mL of Buffer QG was added to the reaction and the DNA was column-purified using 12 EconoSpin columns. The DNA on each column was eluted with 50 μL 1× TE. Typically 180–200 μg of DNA was recovered.

The recovered DNA was then treated with 300 units of Vent (exo$^-$) (New England Biolabs) in a 1.5-mL reaction in the presence of 1× ThermoPol and 0.2 mM of each dNTP at 72 °C for 30 min. The reaction was then quenched, concentrated, and resolved in a 4% NuSieve GTG gel in the same procedure described above. The lower band, which contains the DNA hairpin, was excised. The gel slice was crushed via passing through an 18-gauge needle and eluted with ~10 mL 1× TNaK supplemented with 0.5 mM EDTA in a 50-mL conical tube at 37 °C on a rotator for 12–16 h. The slurry was then filtered through a piece of folded Kimwipe (Kimberly-Clark), and the filtrate was concentrated using an AmiconUltra-15 (3K MWCO) concentrator to ~400 μL. The EtBr in the DNA was extracted three times with equal volumes of water-saturated isoamyl alcohol. The aqueous phase was then subjected to ethanol precipitation. The pellet was resuspended with ~60 μL 1× TNaK and heated at 60 °C for ~10 min to melt residual agarose gel [which did not appear to affect catalyzed hairpin assembly (CHA) reactions]. DNA hairpin was quantified using a NanoDrop spectrophotometer. Typically 500–900 pmol of DNA hairpin was recovered.

3. Kinetic Characterization of CHA Reactions and Cascades. All reactions containing a fluorescent reporter were set up at 37 °C, in 40-μL volume with the following buffer composition: 1× TNaK supplemented with 0.5× TE [5 mM Tris (pH 7.5) at 25 °C, 0.5 mM EDTA] and 1 μM oligonucleotide $(dT)_{21}$. Two 18-μL aliquots were drawn from this mixture and each was transferred to 1 well of a 384-well plate (shallow well, black, polypropylene; Nalge Nunc International) prewarmed at 37 °C. The plate was then immediately transferred to a TECAN Safire plate reader. All fluorescent reporters were prepared by annealing 10 μM of the fluorophore strand with 20 μM of the quencher strand in 1× TNaK. For each $\mathbf{S}_i$ at 150 nM concentration, the fluorescent signals in the absence and the presence of 100 nM $\mathbf{C}_i$ (which should completely displace the quencher-bearing strand of $\mathbf{S}_i$) were recorded and served as a calibration standard to calculate the concentration of $\mathbf{M}_i$:$\mathbf{A}_i$ by linear interpolation. D-pure and enzymatically synthesized hairpins were stored at −20 °C and separately refolded immediately before use by heating to 90 °C for 1 min followed by slowly cooling down

at the rate of 0.1 °C/s. DN- and DNB-pure hairpins were stored at 4 °C and did not undergo refolding before use.

The catalytic efficiency (k_{app}) was calculated by fitting the kinetics of the single-layer reaction [i.e., relative fluorescence unit (RFU) vs. time] to a single-exponential function: RFU = $A(1 - e^{-kt})$, where k and A are the rate constant and the amplitude of the reaction. k is then divided by the concentration of catalyst to obtain k_{app}.

1. Cox JC, Lape J, Sayed MA, Hellinga HW (2007) Protein fabrication automation. *Protein Sci* 16(3):379–390.
2. Li MZ, Elledge SJ (2007) Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nat Methods* 4(3):251–256.
3. Wang Y, et al. (2004) A novel strategy to engineer DNA polymerases for enhanced processivity and improved performance in vitro. *Nucleic Acids Res* 32(3):1197–1207.

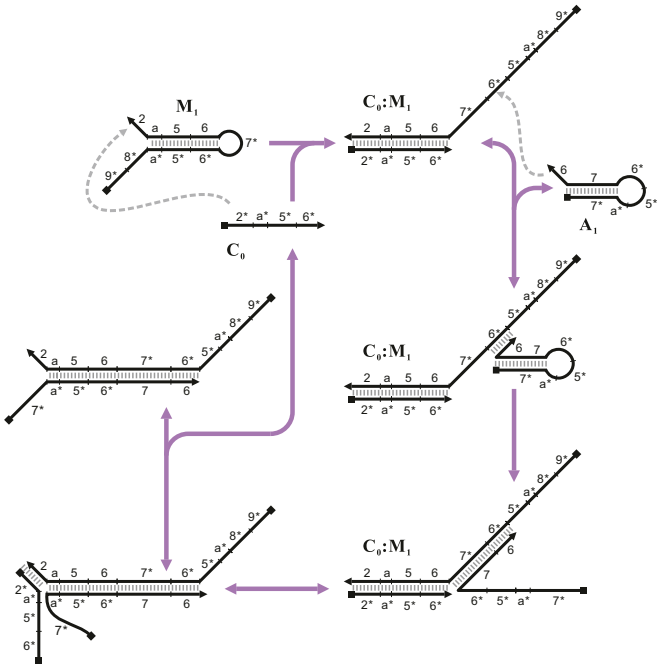

Fig. S1. Detailed pathway of CHA reaction $\mathbf{M_1} + \mathbf{A_1} \rightarrow \mathbf{M_1{:}A_1}$.

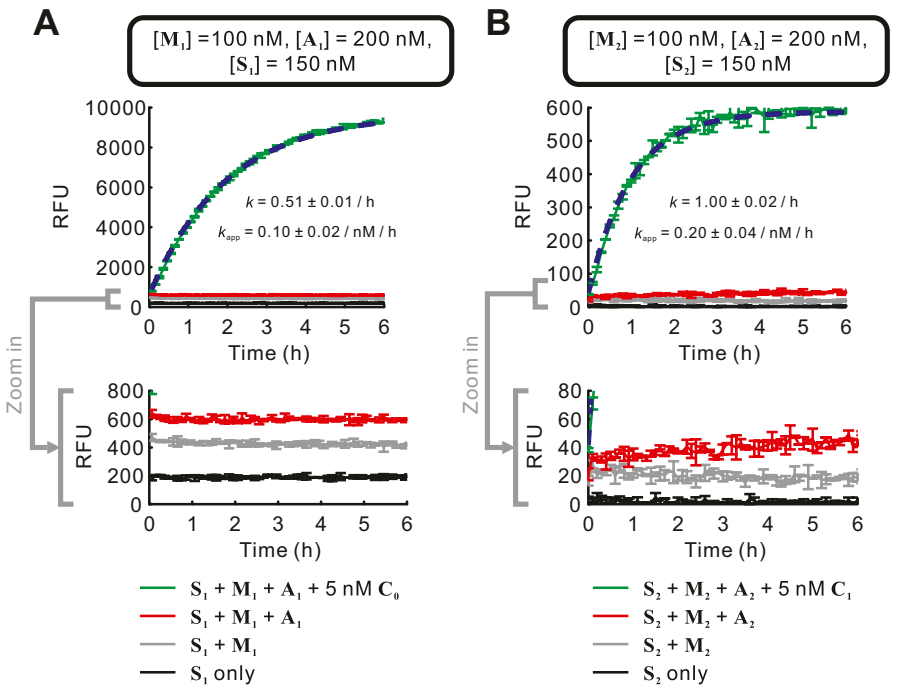

Fig. S2. Kinetic characterization of single-layer CHA reactions. (*A*) $\mathbf{M_1} + \mathbf{A_1} \rightarrow \mathbf{M_1{:}A_1}$, catalyzed by $\mathbf{C_0}$. (*B*) $\mathbf{M_2} + \mathbf{A_2} \rightarrow \mathbf{M_2{:}A_2}$, catalyzed by $\mathbf{C_1}$. Both sets of experiments were carried out with chemically synthesized, D-pure hairpins.

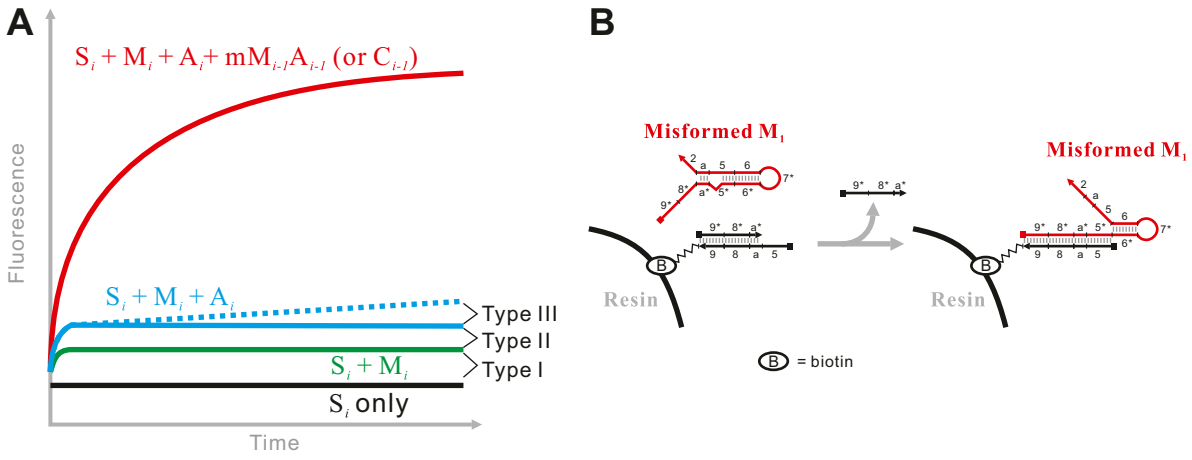

Fig. S3. Classification and reduction of circuit leakage. (*A*) Kinetic characteristics of type I, type II, and type III leakage revealed by different combinations of reagents. (*B*) Scheme of column-based purification (i.e., B purification) to reduce type I leakage.

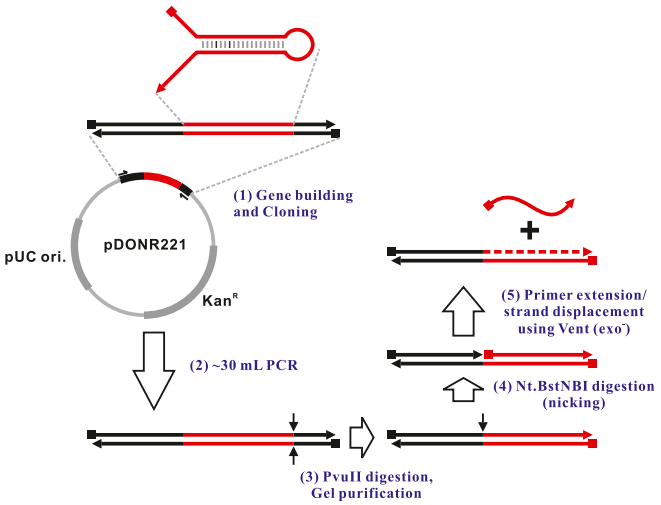

Fig. S4. Route of large-scale enzymatic hairpin synthesis. (1) The double-stranded DNA containing the hairpin sequence flanked by spacer sequences is built from oligonucleotides and cloned into a pDONR221-based vector. (2) The plasmid with confirmed sequence serves as the template of a large-scale (~30 mL) PCR. (3) The PCR product is then digested with PvuII to remove the downstream flanking sequence. (4) A nick in the sense strand between the upstream flanking sequence and the hairpin sequence is introduced by Nt.BstNBI. (5) The desired ssDNA is displaced by Vent(exo$^-$) during primer extension.

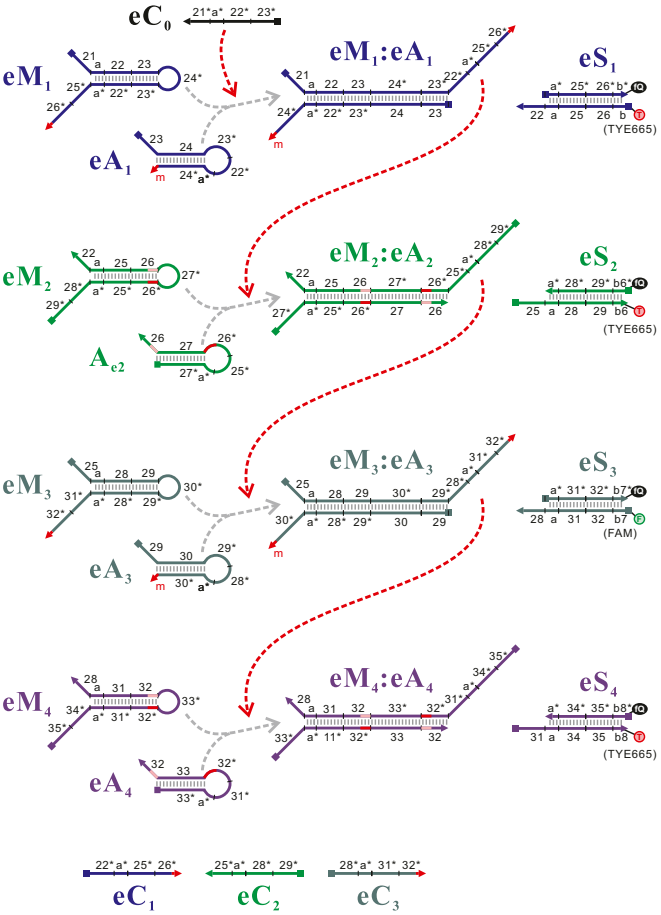

Fig. S5. Secondary structure and domain organization of the components of the four-layer CHA cascade compatible with enzymatic synthesis. Intermediate catalysts (**eC$_1$**, **eC$_2$**, and **eC$_3$**) and fluorescent reporters (**eS$_1$**, **eS$_2$**, and **eS$_3$**) used to test individual CHA reactions are also shown. Red and pink segments on DNA molecules show the position of CAG and CTG trinucleotides due to the introduction of the PvuII sites, respectively. Gray dashed arrows connect reactants and products, whereas red dashed arrows connect the catalysts and the catalyzed reactions. Hairpins of the same layer are shown in the same color.

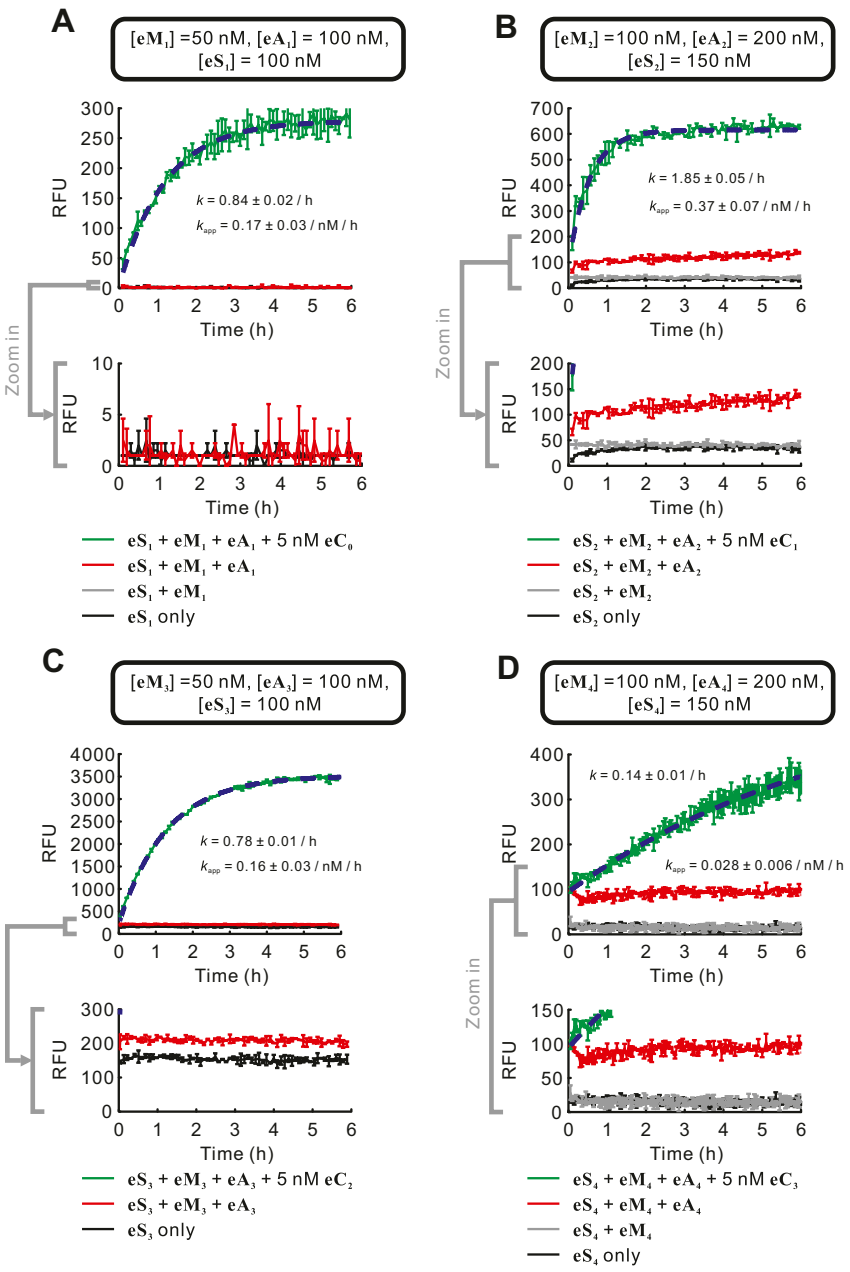

Fig. S6. Kinetic characterization of single-layer CHA reactions (*A*) $\mathbf{eM_1} + \mathbf{eA_1} \rightarrow \mathbf{eM_1{:}eA_1}$, catalyzed by $\mathbf{eC_0}$. (*B*) $\mathbf{eM_2} + \mathbf{eA_2} \rightarrow \mathbf{eM_2{:}eA_2}$, catalyzed by $\mathbf{eC_1}$. (*C*) $\mathbf{eM_3} + \mathbf{eA_3} \rightarrow \mathbf{eM_3{:}eA_3}$, catalyzed by $\mathbf{eC_2}$. (*D*) $\mathbf{eM_4} + \mathbf{eA_4} \rightarrow \mathbf{eM_4{:}eA_4}$, catalyzed by $\mathbf{eC_3}$. Experiments shown in *A* and *C* were carried out with enzymatically synthesized hairpins; those shown in *B* and *D* were carried out with chemically synthesized, D-pure hairpins.

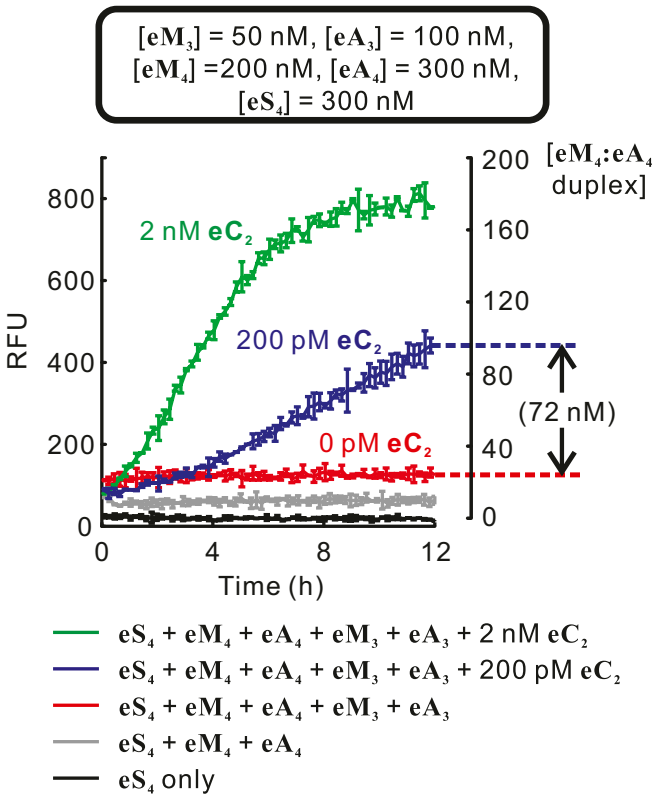

Fig. S7. Performance of the two-layer linear cascade consisting of enzymatically synthesized **eM$_3$** and **eA$_3$** and chemically synthesized DN-pure **eM$_4$** and **eA$_4$**.

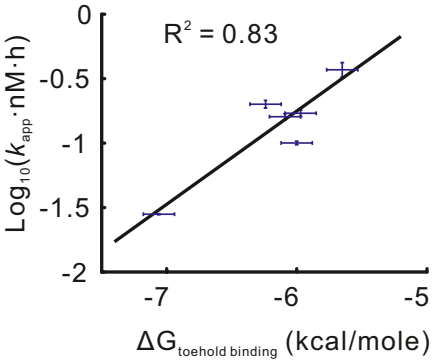

Fig. S8. Negative correlation between CHA speed and toehold strength. A more negative ΔG indicates a stronger toehold binding. The k_{app} values are taken from the results shown in Figs. S2 and S6. The error of the k_{app} measurement is dominated by error of catalyst concentration. We estimated that this should be within 15% and therefore applied a 15% relative error in the plotting. The error of ΔG estimation has been shown to be within 0.14 kcal/mol (1), and we therefore applied this estimation in the plot.

1. SantaLucia J, Jr. (1998) A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc Natl Acad Sci USA* 95(4):1460–1465.

Table S1. Summary of domain sequences

Domain ID	Sequence
a	GAG
b	TCAG
m	CAG
b6	GTG
b7	GTTG
b8	GAAG
2	GTTTCACC
3	CACATACG
4	GATCACCCCAAGTAG
5	GCACTTTC
6	GCTACTAC
7	GTGTTTTAGCGAGCG
8	CATCTTCG
9	GCCATTTC
10	CGTGGAGGATATAGC
11	CACATCAG
12	CTTTAGCC
21	GTCACTCT
22	TATCCCTG
23	GACGTAAG
24	CCATTAGAGAGAGAG
25	CGGTAGAA
26	CTGAAGTG
27	CAAGGGGTGATAAAC
28	AAAGTGCG
29	AGTAGAGC
30	CGATAAGGAAAGGTC
31	GGACGAAT
32	CTGGAAAG
33	GTTGAAATTGAGCCG
34	GGAAGGTA
35	GAATGAGC

Table S2. Sequences of oligonucleotides used in this work

Strand	Sequence
Layer 1	
C_0	GGTGAAAC CTC GAAAGTGC GTAGTAGC
M_1	GAAATGGC CGAAGATG CTC GAAAGTGC GTAGTAGC CGCTCGCTAAAACAC GCTACTAC GCACTTTC GAG GTTTCACC
A_1	CGCTCGCTAAAACAC CTC GAAAGTGC GTAGTAGC GTGTTTTAGCGAGCG GCTACTAC
S_1-F	GCACTTTC GAG CATCTTCG GCCATTTC CCAT/36-FAM/
S_2-Q	/5IAbFQ/ATGG GAAATGGC CGAAGATG CTC
B purification	
S_2mimic-L.Bio	GCACTTTC GAG CATCTTCG GCCATTTC TTTTTT/3Bio/
S_2mimic-S	GAAATGGC CGAAGATG CTC
Layer 2	
C_1	GAAATGGC CGAAGATG CTC GAAAGTGC
M_2	GCACTTTC GAG CATCTTCG GCCATTTC GCTATATCCTCCACG GAAATGGC CGAAGATG CTC CTGATGTG GGCTAAAG
A_2	GCCATTTC CGTGGAGGATATAGC GAAATGGC CGAAGATG CTC GCTATATCCTCCACG
S_3-F	/5TYE665/TCAG CTTTAGCC CACATCAG GAG CATCTTCG
S_3-Q	CTC CTGATGTG GGCTAAAG CTGA/3IAbRQSp/
Layer 1 (compatible with enzymatic synthesis)	
eC_0	CTTACGTC CAGGGATA CTC AGAGTGAC
eM_1	GTCACTCT GAG TATCCCTG GACGTAAG CTCTCTCTCTAATGG CTTACGTC CAGGGATA CTC TTCTACCG CACTTCAG
eA_1	GACGTAAG CCATTAGAGAGAGAG CTTACGTC CAGGGATA CTC CTCTCTCTCTAATGG CAG
eS_1-F	/5TYE665/TCAG CTGAAGTG CGGTAGAA GAG TATCCCTG
eS_1-Q	CTC TTCTACCG CACTTCAG CTGA /3IAbRQSp/
Layer 2 (compatible with enzymatic synthesis)	
eC_1	CAGGGATA CTC TTCTACCG CACTTCAG
eM_2	GCTCTACT CGCACTTT CTC TTCTACCG CACTTCAG GTTTATCACCCCTTG CTGAAGTG CGGTAGAA GAG TATCCCTG
eA_2	GTTTATCACCCCTTG CTC TTCTACCG CACTTCAG CAAGGGGTGATAAAC CTGAAGTG
eS_2-F	CGGTAGAA GAG AAAGTGCG AGTAGAGC TCAG /3TYE665/
eS_2-Q	/5IAbRQ/CTGA GCTCTACT CGCACTTT CTC
Layer 3 (compatible with enzymatic synthesis)	
eC_2	GCTCTACT CGCACTTT CTC TTCTACCG
eM_3	CGGTAGAA GAG AAAGTGCG AGTAGAGC GACCTTTCCTTATCG GCTCTACT CGCACTTT CTC ATTCGTCC CTTTCCAG
eA_3	AGTAGAGC CGATAAGGAAAGGTC GCTCTACT CGCACTTT CTC GACCTTTCCTTATCG CAG
eS_3-F	/56FAM/GTTG CTGGAAAG GGACGAAT GAG AAAGTGCG
eS_3-Q	CTC ATTCGTCC CTTTCCAG CAAC/3IABkFQ/
Layer 4 (compatible with enzymatic synthesis)	
eC_3	CGCACTTT CTC ATTCGTCC CTTTCCAG
eM_4	GCTCATTC TACCTTCC CTC ATTCGTCC CTTTCCAG CGGCTCAATTTCAAC CTGGAAAG GGACGAAT GAG AAAGTGCG
eA_4	CGGCTCAATTTCAAC CTC ATTCGTCC CTTTCCAG GTTGAAATTGAGCCG CTGGAAAG
eS_4-F	GGACGAAT GAG GGAAGGTA GAATGAGC GAAG /3TYE665/
eS_4-Q	/5IAbRQ/ CTTC GCTCATTC TACCTTCC CTC
Common primers for SLIC	
Insert-F	CAGTCTTAAGCTCGGGC
Insert-R	CGACTCACTATAGGGGATAT
Vector-F	ATATCCCCTATAGTGAGTCGTATTACATGGT
Vector-R	GGGGCCCGAGCTTAAGACT
To build **eM_1**	
eM1.s1	CAGTCTTAAGCTCGGGCCCC GAGTC AAGC GTCACTCT
eM1.s2	GAGTC AAGC GTCACTCT GAG TATCCCTG GACGTAAG CTCTCTCTCTAATGG
eM1.as2	TCAGCTGAAGTGCGGTAGAAGAGTATCCCTGGACGTAAGCCATTAGAGAGAGAG
eM1.as1	CGACTCACTATAGGGGATATCAGCTGAAGTGCGGTAGAA
To build **eA_1**	
eA1.s1	CAGTCTTAAGCTCGGGCCCC GAGTC AAGC GACGTAAG
eA1.s2	GAGTC AAGC GACGTAAG CCATTAGAGAGAGAG CTTACGTC CAGGGATA CTC
eA1.as1	CGACTCACTATAGGGGATATCAGCTGCCATTAGAGAGAGAGGAGTATCCCTGGACGTAAG

Table S2. Cont.

Strand	Sequence
To build **eM$_3$**	
eM3.s1	CAGTCTTAAGCTCGGGCCCC GAGTC AAGC CGGTAGAA
eM3.s2	GAGTC AAGC CGGTAGAA GAG AAAGTGCG AGTAGAGC GACCTTTCCTTATCG
eM3.as2	TCAGCTGGAAAGGGACGAATGAGAAAGTGCGAGTAGAGCCGATAAGGAAAGGTC
eM3.as1	CGACTCACTATAGGGGATATCAGCTGGAAAGGGACGAAT
To build **eA$_3$**	
eA3.s1	CAGTCTTAAGCTCGGGCCCC GAGTC AAGC AGTAGAGC
eA3.s2	GAGTC AAGC AGTAGAGC CGATAAGGAAAGGTC GCTCTACT CGCACTTT CTC
eA3.as1	CGACTCACTATAGGGGATATCAGCTGCGATAAGGAAAGGTCGAGAAAGTGCGAGTAGAGC
Common primers for large-scale PCR	
LS-PCR-F	TTTATTTGATGCCTGGCAGTTC
LS-PCR-R	CCATGTAATACGACTCACTATAGGGGATAT