Electronic Supplementary Material (ESI) for Chemical Communications

Detection of Bond Formations by DNA-Programmed Chemical Reactions and PCR Amplification

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SUPPLEMENTAL INFORMATION

Materials and general methods:

Unless otherwise noted, all reagents and solvents were purchased from commercial sources and used as received. DNA oligonucleotides were synthesized on an Applied Biosystems 394 DNA synthesizer using standard phosphoramidite protocols and purified by C18 reverse-phase HPLC with aqueous 0.1 M triethylammonium acetate (TEAA)/CH_3CN gradient on Agilent 1200 HPLC systems. For all phosphoramidites with unnatural bases, coupling time was modified to 999 seconds. Phosphoramidites with unnatural bases were either purchased or prepared in laboratory as described below. Oligonucleotides with 3'- amino group were synthesized using 3'-amino-modifier C7 CPG. 5'-amino was incorporated using 5'-amino-modifier 5. Oligonucleotides were quantitated by UV using a BioTek Epoch UV-Vis spectrometer based on extinction coefficient at 260 nm. Oligonucleotides were characterized by either a Bruker APEX IV (for ESI-MS) or a Bruker ultrafleXtreme [MALDI-MS, matrix: 8:1 (50 mg/mL 3-HPA in 1:1 water:acetonitrile):(50 mg/mL ammonium citrate in water)] mass spectrometer. All DNA sequences are written in the 5'- to 3'- orientation. DNAs in PAGE analysis were stained with ethidium bromide and visualized on a Tanon-1600 gel image system. Water was purified with a Thermo Scientific Barnstead Nanopure system. Photocleavage experiments were conducted by a UVP CL-1000L Ultraviolet crosslinker at 365 nm wavelength with an intensity of approximately 100 μJ /cm^2.

Abbreviations:

DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene
DCM: dichloromethane
DMF: *N*,*N*'-dimethylformamide
DMT: di-(4-methoxyphenyl)phenylmethyl
EDCI: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
3-HPA: 3-hydroxypicolinic acid
MES: 2-morpholinoethanesulfonic acid
MOPS: 3-(*N*-morpholino)propanesulfonic acid
NHS: *N*-hydroxysuccinimide
dNTP: deoxy-ribonucleoside triphosphate
PAGE: polyacrylamide gel electrophoresis
qPCR: quantitative polymerase chain reaction
PEG: polyethylene glycol
TEAA: triethylammonium acetate
T4 PNK: T4 polynucleotide kinase

General PCR and quantitative analysis methods:

All PCR reactions were carried out with RealMasterMix (0.1 μL/20 μL reaction volume, TIANGEN) in the buffer provided. PCR reactions include Mg^{2+} (3 mM), dNTPs (200 μM each), and primers (200 nM each). Templates were amplified from a standard initial concentration of 625 pM, unless otherwise noted. The thermal cycling sequence was as follows: 95 °C for 2 minutes, then iterated cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 68 °C for 15 seconds. In preparative PCR reactions, upon completion of the iterated cycles, a final incubation at 68 °C for 2 minutes was performed. Quantitative PCR experiments were performed in triplicates on a BioRad CFX96 Real-Time PCR Detection System with SYBR Green as the detection dye.

Quantitative qPCR analysis methods:

We used the quantitative feature of qPCR (amplification cycle threshold value, C_T) to determine the initial copy number of the amplifiable DNA templates, i.e. DNA templates from DNA ligase-mediated re-ligation; also we have used ΔC_T (C_T of bond-forming – C_T of non-bond-forming) to evaluate the selectivity of our system for the bond-forming events over non-bond-forming ones.

First, in order to verify our system performs well-behaved PCR amplification over a range of concentrations, we generated a standard curve by qPCR. Five different concentrations of the standard template (625 pM, 62.5 pM, 6.25 pM, 625 fM, 62.5 fM, and 6.25fM) were subjected to qPCR under standard conditions. The log of initial template concentration was plotted *vs.* threshold cycle, and a linear function was fit to the data.

The initial template concentration for individual experiments can be determined by plotting the measured C_T to the standard curve. For bond-forming events *vs.* non-bond-forming events, the difference of initial template concentrations is determined and the system's selectivity (Figure 1, S2c, S2d) and enrichment fold (mixed system, Figure 2c) are calculated.

Finally, considering the qPCR's variation from experiment to experiment, we conducted standard curve establishment and calculation for each experiment series respectively for better accuracy. As shown in the following sections, a standard curve is provided for every experiment series.

Sequences of the standard template:

GAG CTC GTT GAT ATC CGC AGT CAG CAA AGC TTTATAT CTT AGC CCA GAT GCC AGC TGC TAG CTT TAG GT

Sequences of primers:
P1: GAG CTC GTT GAT ATC CGC AG
P2: ACC TAA AGC TAG CAG CTG GC

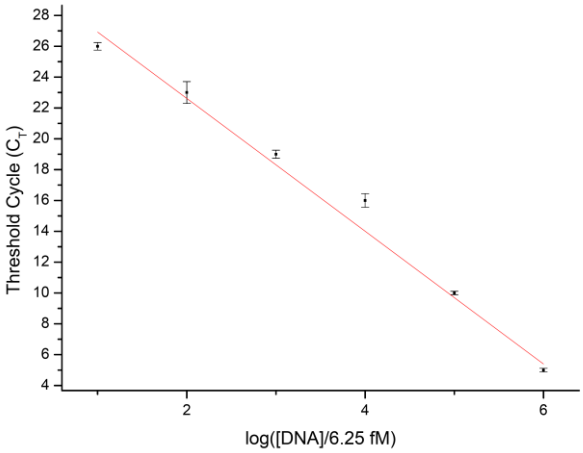


Figure S1: Standard curve plot for validation of qPCR performance over a range of initial template concentrations.

Equation: Y = 31.2 - 4.2*X (linear function), Y = C_T, X = log([DNA]/6.25fM) (in 20 µL PCR system)
Therefore: X = (31.2 - Y)/4.2
[template] = A = 6.25 fM*$10^{(X-1)}$
For example, if X = 1, then A = 6.25 fM

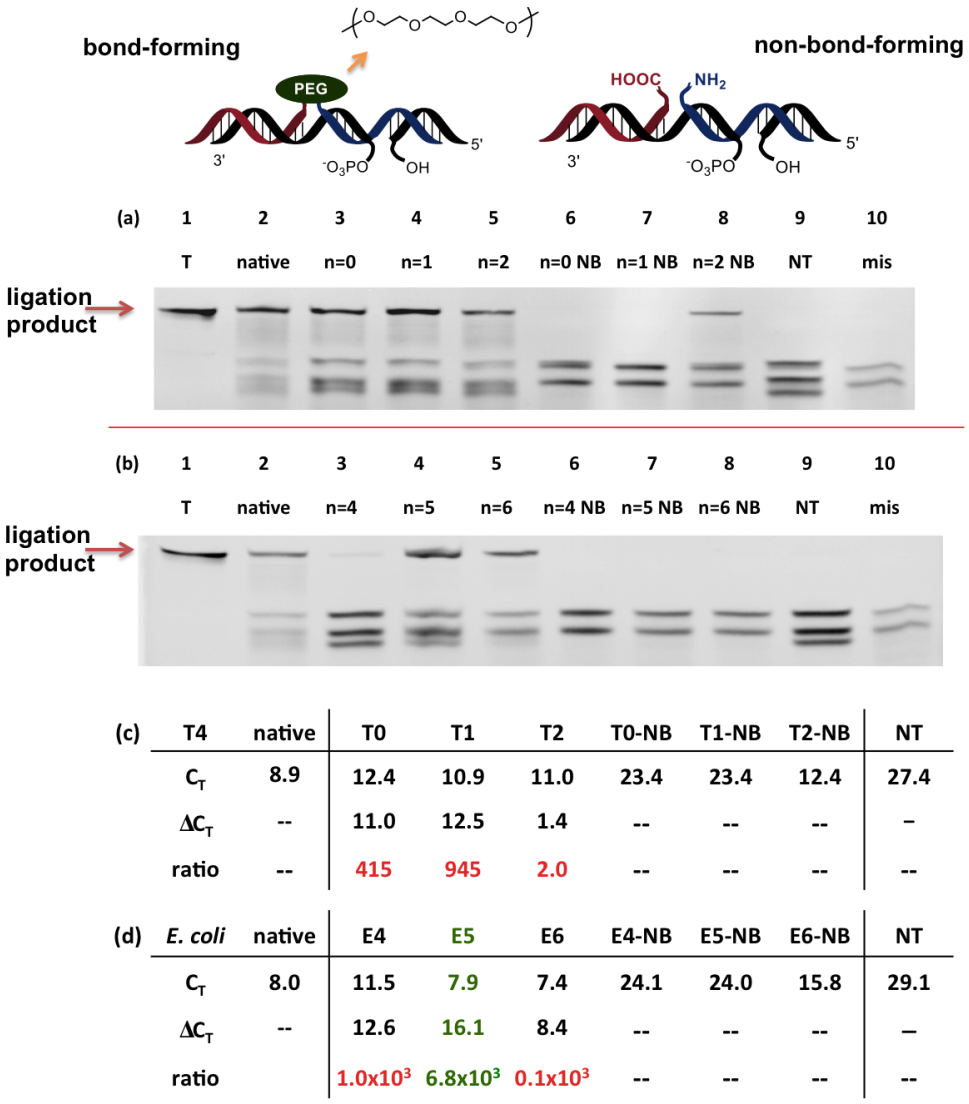
Note: this standard curve was generated along with and used for the n value studies described in the next section.

Evaluation of n value by DNA ligases:

We performed the n value studies (Scheme 1) with two DNA ligases: T4 DNA ligase and *E. coli* DNA ligase. A PEG linker is used as the synthetic structure in the opposite strand (Figure S2).

T4 DNA ligase is known to have distinct ligase activities, including ligations of very short DNAs,[S1] blunt-end DNA duplexes,[S2, S3] abasic site/gap joining,[S4] and even ligation of single strand DNA,[S5] therefore we expect a very small n value. Indeed, T4 ligase can re-ligate the template when the ligation site is very close (n = 1,2; Figure S2a, lane 4, 5) or even directly opposite to the reaction site (n = 0; Figure S2a: lane 3). However, ligation was also detected in the absence of bond formation when n = 2 (Figure S2a, lane 8), reflecting T4 DNA ligase's activity even with a very short 2-base sticky end. In contrast, *E. coli* DNA ligase usually requires a 6~8 DNA bases flanking the ligation site.[S1] We observed ligation product when n = 5 or 6 (Figure S2b, lane 4, 5). At n = 4, the ligation efficiency is much lower (Figure S2b, lane 3), consistent with the reported minimal base requirement.[S1]

These results are corroborated by the threshold values (C_T) from qPCR (using the standard curve generated in this experiment series, Figure S1). The data shown in Figure S2c and S2d are consistent with PAGE analysis; and it clearly shows *E. coli* DNA ligase with n = 5 provides the optimal selectivity with a large ΔC_T of 16.1, corresponding to a ratio of ~6.8×10^3 (E5/E5-NB in Figure S2d) for the "bond-forming" experiment. At n = 6, the selectivity ratio is low, possibly due to the 6-base sticky end duplex formation in the negative control (estimated T_m = 31.1°C, ligation reaction temperature: 37°C). Collectively, these data established an optimal n value of 5 with *E. coli* DNA ligase, which is used for all the following studies in this report.



(c)	T4	native	T0	T1	T2	T0-NB	T1-NB	T2-NB	NT
	C_T	8.9	12.4	10.9	11.0	23.4	23.4	12.4	27.4
	ΔC_T	--	11.0	12.5	1.4	--	--	--	–
	ratio	--	415	945	2.0	--	--	--	--

(d)	*E. coli*	native	E4	E5	E6	E4-NB	E5-NB	E6-NB	NT
	C_T	8.0	11.5	7.9	7.4	24.1	24.0	15.8	29.1
	ΔC_T	--	12.6	16.1	8.4	--	--	--	–
	ratio		$1.0x10^3$	$6.8x10^3$	$0.1x10^3$	--	--	--	--

Figure S2. Studies of optimal n value with T4 DNA ligase and *E. coli* DNA ligase-mediated re-ligation of DNA templates. (a) PAGE analysis results with T4 DNA ligase: Lane 1: template only; lane 2: re-ligation with native DNA (instead of the PEG-3 linker); lane 3-5: with PEG-3-linked template (upper left DNA duplex), n = 0, 1, 2; lane 6-8, non-bond-forming (NB) controls (upper right DNA duplex); lane 9: no template control (NT); lane 10: with mismatched 5'-OPO_3^- reagent DNA. (b) Results with *E. coli* DNA ligase: Lane sequence is the same as T4 DNA ligase. qPCR threshold value (C_T and ΔC_T) and selectivity ratio measurement and calculations for (c): T4 DNA ligase when n = 0, 1, 2 (T0, T1, T2); and for (d): *E. coli* DNA ligase when n = 4, 5, 6 (E4, E5, E6); native: with native DNA; NB: non-bond-forming negative controls (upper right DNA duplex); NT: no template, primer only. $\Delta C_T = C_T$ (bond-forming) - C_T (NB). Ratio: re-ligated DNA template for bond-forming *vs.* non-bond-forming. All C_T values are average of at least three replicates.

DNA sequences and characterizations (bond-forming):

n = 0: ATC TGG GCT AAG ATA TA (PEG-3) AAG CTT TGC TGA;

observed mass = 9167.9 (ESI), (expected: 9167.9).

n = 1: ATC TGG GCT AAG ATA T (PEG-3) A AAG CTT TGC TGA;

observed mass = 9167.2 (ESI), (expected: 9167.9).

n = 2: ATC TGG GCT AAG ATA (PEG-3) TA AAG CTT TGC TGA;

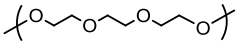
observed mass = 9166.5 (ESI), (expected: 9167.9).

n = 4: ATC TGG GCT AAG A (PEG-3) T ATA AAG CTT TGC TGA;

observed mass = 9166.5 (ESI), (expected: 9167.9).

n = 5: ATC TGG GCT AAG (PEG-3) ATA TA AAG CTT TGC TGA;

observed mass = 9166.2 (ESI), (expected: 9167.9).
n = 6: ATC TGG GCT AA (PEG-3) G ATA TA AAG CTT TGC TGA;
observed mass = 9167.7 (ESI), (expected: 9167.9).

PEG-3: 

DNA sequences and characterizations (non-bond-forming):

n = 0: ATC TGG GCT AAG ATA TA-3'-COOH;
observed mass = 5442.6 (ESI), (expected: 5442.5).
5'-NH_2-AAG CTT TGC TGA;
observed mass = 3826.9 (ESI), (expected: 3827.6).

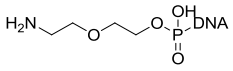
n = 1: ATC TGG GCT AAG ATA T-3'-COOH;
observed mass = 5128.7(ESI), (expected: 5129.3).
5'-NH_2-A AAG CTT TGC TGA;
observed mass = 4140.6 (ESI), (expected: 4140.7).

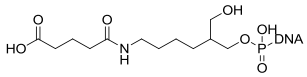
n = 2: ATC TGG GCT AAG ATA-3'-COOH;
observed mass = 4825.0 (ESI), (expected: 4825.1).
5'-NH_2-TA AAG CTT TGC TGA;
observed mass = 4444.0 (ESI), (expected: 4444.9).

n = 4: ATC TGG GCT AAG A-3'-COOH;
observed mass = 4207.1 (ESI), (expected: 4207.7).
5'-NH_2-T ATA AAG CTT TGC TGA;
observed mass = 5062.2 (ESI), (expected: 5062.3).

n = 5: ATC TGG GCT AAG-3'-COOH;
observed mass = 3893.9 (ESI), (expected: 3894.5).
5'-NH_2-ATA TA AAG CTT TGC TGA;
observed mass = 5374.6 (ESI), (expected: 5375.3).

n = 6: ATC TGG GCT AA-3'-COOH;
observed mass = 3565.2 (ESI), (expected: 3565.3).
5'-NH_2-G ATA TA AAG CTT TGC TGA;
observed mass = 5704.8 (ESI), (expected: 5704.7).

5'-NH_2-: 

3'-COOH: 

Other DNA sequences and characterizations:

Standard template: GAG CTC GTT GAT ATC CGC AGT CAG CAA AGC TTTATAT CTT AGC CCA GAT GCC AGC TGC TAG CTT TAG GT

Native DNA control: ATC TGG GCT AAG ATATA AAG CTT TGC TGA; observed mass = 8955.6 (ESI), (expected: 8955.9).

Ligase-mediated ligation DNA strands: 5'- $^{-}O_3$PO-TATAT CTT AGC CCA GAT GCC AGC TGC TAG CTT TAG GT; observed mass = 11410.3 (ESI), (expected: 11410.4); GAG CTC GTT GAT ATC CGC AGT CAG CAA AGC TT observed mass = 9823.9 (ESI), (expected: 9824.4).

PCR primer sequences:
P1: GAG CTC GTT GAT ATC CGC AG
P2: ACC TAA AGC TAG CAG CTG GC

T4 DNA ligase-mediated ligation condition (n =0, 1, 2):

A solution of 100 pmol splint DNA strands (bond-forming or none-bond-forming) and 100 pmol ligation DNA strands in 90 µL water was heated at 95 °C for 5 min and slowly cooled to 25 °C in 1 hour. To this solution, 400 units T4 DNA ligase and 10 µL ligation buffer provided by the manufacture (New England Biolabs) were added and the ligation reaction was incubated at 37 ºC. For PAGE analysis, the reaction time was 16 hours to ensure the formation of sufficient ligation products detectable by gel staining. The mixture was ethanol precipitated prior to subsequent analysis.

E. coli DNA ligase-mediated ligation condition (n =4, 5, 6):

A solution of 100 pmol splint DNA strands (bond-forming or none-bond-forming) and 100 pmol ligation DNA strands in 90 µL water was heated at 95 °C for 5 min and slowly cooled to 25 °C in 1 hour. To this solution, 10 units *E. coli DNA* ligase and 10 µL ligation buffer provided by the manufacture (New England Biolabs) were added and the ligation reaction was incubated at 37 ºC. For PAGE analysis, the reaction time was 16 hours to ensure the formation of sufficient ligation products detectable by gel staining. The mixture was ethanol precipitated prior to subsequent analysis.

qPCR condition for n value optimizations:

Samples used for qPCR analysis was carried out under the conditions described above, except the ligation time was 4 hours instead of 16 hours. The ligation reaction was stopped by heat at 95 ºC for 20 min. The mixture was diluted and subjected to qPCR without ethanol precipitation. The starting concentration of DNA was 625 pM. All C_T values are average of at least three replicates.

Quantitative qPCR analysis and calculation of the selectivity:

The ligation ratio between the bond-forming event and the non-bond-forming event represents the selectivity of the ligation system. Based on the linear function from the qPCR standard curve generated along with this experiment series (Figure S1), the selectivity ratio was calculated as following:

Equation: $Y = 31.2 - 4.2*X$ (linear function), $Y = C_T$, $X = \log([DNA]/6.25fM)$ (in 20 µL PCR system)
Therefore: $X = (31.2 - Y)/4.2$
[re-ligated DNA template] = $A = 6.25\ fM*10^{(X-1)}$
Ligation efficiency = $A/A_i*100\%$ (initial overall DNA concentration $A_i = 625$ pM)

For example, for n=5 with *E. coli DNA* ligase:

$C_{T(bond\text{-}forming)} = 7.9$, so X=5.55 and ligation efficiency $_{(bond\text{-}forming)} = 35.3\%$

$C_{T(non\text{-}bond\text{-}forming)} = 24.0$, so X=1.71 and ligation efficiency $_{(non\text{-}bond\text{-}forming)} = 5.18*10^{-3}\ \%$

Therefore, the selectivity ratio = ligation efficiency $_{(bond\text{-}forming)}$/ ligation efficiency $_{(non\text{-}bond\text{-}forming)}$ = $35.3\%/5.18*10^{-3}\ \% = 6.8 \times 10^3$

The same calculation method is used to evaluate the selectivity between bond-forming event and the non-bond-forming event through this report.

Optimization of ligase-mediated ligation conditions:

Optimization of the ligation time:

We performed template re-ligation at various reaction times and compared the corresponding ΔC_T with standard ligation/qPCR condition described above (Table S1). The reaction was stopped by heat at 95 ºC for 20 min. The reaction mixture was diluted and subjected to qPCR directly. The starting concentration of DNA was 625 pM. All C_T values are average of at least three replicates. As shown in Table S1, a 4-hour reaction time provides a large and optimal ΔC_T value of.16.1.

	bond forming				non-bond-forming			
Time	2 h	4 h	8 h	16 h	2 h	4 h	8 h	16 h
C_T	11.3	7.9	7.8	6.8	25.3	24.0	23.6	22.2
ΔC_T	14.0	16.1	15.8	15.4	--	--	--	--

Table S1: Reaction time optimization for ligase-mediated template re-ligation.

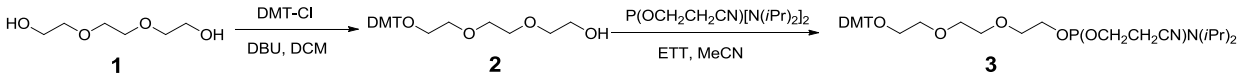
Optimization of the ligation temperature:

We performed template re-ligation at various temperatures and compared the corresponding ΔC_T with standard ligation/qPCR condition described above (Table S2). The reaction was stopped by heat at 95 ºC for 20 min. The reaction mixture was diluted and subjected to qPCR directly. The starting concentration of DNA was 625 pM. All C_T values are average of at least three replicates. As shown in Table S2, 37 ºC provides the optimal ΔC_T value of 16.1.

	bond forming					non-bond-forming				
Temperature	4 °C	16 °C	25 °C	37 °C	45 °C	4 °C	16 °C	25 °C	37 °C	45 °C
C_T	15.6	16.0	13.1	7.9	10.2	25.8	25.1	23.9	24.0	25.5
ΔC_T	10.2	9.1	10.8	16.1	15.3	--	--	--	--	--

Table S2: Reaction temperature optimization for ligase-mediated template re-ligation.

Substrate scope studies:



1 **2** **3**

Synthesis of the PEG-3 phosphoramidite:

To a stirred solution of diol **1** (10.0 mmol, 2.5 equiv.) in 10 mL DCM, DBU (8.0 mmol, 2.0 equiv.) was added, followed by the addition of DMT-Cl (4.0 mmol, 1.0 equiv.) at 25 ºC. The reaction was allowed to stir at 25 ºC for 24 hours and quenched by addition of 20 mL saturated Na_2CO_3 (aq.). The aqueous layer was further extracted by ethyl acetate (15 mL x 3) and the organic layer was combined and dried over Na_2SO_4, before the solvent was removed under reduced pressure. The residue was flash chromatographed on a silica gel column to give mono-DMT-protected diol intermediate **2** (yield: 33.1%). This product was used without further characterization.

To a stirred solution of mono-DMT-protected diol **2** (1.0 mmol, 1.0 equiv.) and 2-cyanoethyl bisdiisopropyl-phosphoramidite (2.0 mmol, 2.0 equiv.) in 10 mL dry CH_3CN was added 5-ethylthio-1H-tetrazole (ETT) (0.7 mmol, 0.7 equiv.) at 25 ºC. The reaction was complete after 90 min as shown by TLC. The solvent was removed under reduced pressure and the residue was dissolved by 20 mL DCM The organic layer was washed by 15 mL saturated $NaHCO_3$ (aq.) and dried over Na_2SO_4, before the solvent was removed under reduced pressure. The residue was flash chromatographed on a silica gel column to afford the product **3** (yield: 72.4 %).

Syntheses of DIP phosphoramidite and HEX phosphoramidite:

DIP and HEX phosphoramidites were synthesized with the same procedure as described above route from the corresponding symmetric diol.

Synthesis of DNA oligonucleotides with unnatural linkers:

DNA oligonucleotides were synthesized as described in the general method section. For the above prepared phosphoramidites with unnatural bases, coupling time was modified to 999 second to ensure complete coupling. PEG-9 linker was synthesized by 3 consecutive coupling of the PEG-3 phosphoramidite.

DNA sequences used in substrate scope studies:

ATC TGG GCT AAG (PEG-3) ATA TA AAG CTT TGC TGA;
observed mass = 9166.2 (ESI), (expected: 9167.9).
ATC TGG GCT AAG (PEG-9) ATA TA AAG CTT TGC TGA;
observed mass = 9592.6 (ESI), (expected: 9591.0).
ATC TGG GCT AAG (DIP) ATA TA AAG CTT TGC TGA;
observed mass = 9133.6 (ESI), (expected: 9133.9).
ATC TGG GCT AAG (HEX) ATA TA AAG CTT TGC TGA;
observed mass = 9255.7 (ESI), (expected: 9255.9).

Ligase-mediated ligation DNA strands:

5’- $^{-}O_3$PO-TATAT CTT AGC CCA GAT GCC AGC TGC TAG CTT TAG GT

GAG CTC GTT GAT ATC CGC AGT CAG CAA AGC TT

Primer sequences：

P1: GAG CTC GTT GAT ATC CGC AG
P2: ACC TAA AGC TAG CAG CTG GC

P2: ACC TAA AGC TAG CAG CTG GC

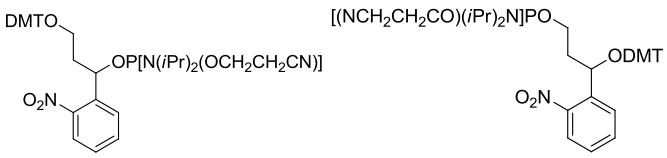
Quantitative qPCR analysis and calculation of the selectivity:

This series of experiments was performed along with the n value studies and the standard curve in Figure S1 was used. The qPCR analysis and selectivity calculation are as described above.

Preparation of DNA templates with photocleavable linker and photocleavage test

Synthesis of oligonucleotides with photocleavable linker:

The photocleavable oligonucleotides were synthesized by automated DNA synthesis with two special phosphoramidites as shown below. These phosphoramidites were prepared based on the synthesis procedures reported by Taylor *et al.*[S6,S7] The phosphoramidites were incorporated with extended coupling time (999 seconds). Note: one of the phosphoramidites (left structure) is already commercially available (Glen Research, PC linker phosphoramidite).



DNA sequences:

Photocleavable template: GAG CTC GTT GAT ATC CGC AG TCA GCA AAG CTT – linker – TAT ATC TTA GCC CAG ATG CCA GCT GCT AGC TTT AGG T; observed mass = 21734.1 (ESI), (expected: 21734.8). This batch was used for all experiments throughout this report.

Splint DNA: ATC TGG GCT AAG ATATA AAG CTT TGC TGA

Re-ligation DNA template: GAG CTC GTT GAT ATC CGC AG TCA GCA AAG CTT TAT ATC TTA GCC CAG ATG CCA GCT GCT AGC TTT AGG T

Photocleavage and T4 PNK/E. coli DNA ligase-mediated re-ligation test:

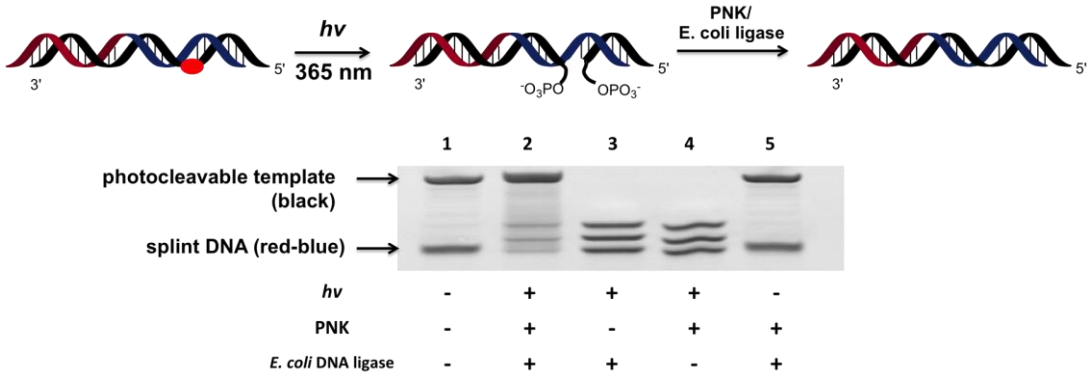


Figure S3. Photocleavage and T4 PNK/*E. coli* DNA ligase-mediated re-ligation test by denaturing PAGE. A native DNA is used as the splint (red-blue) along with the photocleavable DNA template (black) to perform the test. Lane 1: DNA only; lane 2-5: experiments with reaction conditions as indicated.

In order to validate the T4 PNK/*E. coli* DNA ligase-mediated re-ligation of the DNA template after photocleavage, we have performed a model test, analyzed by denaturing PAGE. As shown in Figure S3, clearly all

three factors, T4 PNK, *E. coli* DNA ligase, and irradiation, are required for the re-ligation of the DNA template. Experimentally, a 90 μL solution of 100 pmol splint DNA and 100 pmol reaction template was heated at 95 °C for 5 min and slowly cooled to 25 °C in 1 hour. The mixture was then irradiated at 365 nm for 30 min. After photocleavage, for positive experiment (lane 2), 10 μL *E. coli* DNA ligase buffer and 10 units T4 polynucleotide kinase (T4 PNK, New England Biolabs) were added and the reaction was incubated at 37 ºC for 3 hours, followed by the addition of 10 units *E. coli* DNA ligase (New England Biolabs). The ligation reaction was incubated at 37 ºC for 16 hours. For negative control experiments (lane 3-5), corresponding factors were omitted and replaced by water. The reaction mixture was precipitated with ethanol prior to PAGE analysis.

Full sequence experiments of DNA-templated reactions and bond formation detections:

For the labeled DNA reagents used for each DNA-templated reactions, functional groups were incorporated by direct acylation of 5'- or 3'-amine-bearing DNA strands with NHS-ester of corresponding carboxylic acids.

DNA sequences and characterizations:
Photocleavable template: GAG CTC GTT GAT ATC CGC AG TCA GCA AAG CTT – linker – TAT ATC TTA GCC CAG ATG CCA GCT GCT AGC TTT AGG T; observed mass = 21734.1 (ESI), (expected: 21734.8).

Reagent DNA 1: ATC TGG GCT AAG-3'-NH_2; observed mass = 3894.2 (ESI), (expected: 3894.6).

Reagent DNA 2: 5'-NH_2-ATA TA AAG CTT TGC TGA; observed mass = 5375.5 (ESI), (expected: 5375.4).

Primer sequences:
P1: GAG CTC GTT GAT ATC CGC AG
P2: ACC TAA AGC TAG CAG CTG GC

Modifications of reagent DNA with NHS-ester of corresponding acids:
For substrates that were commercially available as activated carboxylic acid derivatives, a 10 mg/mL stock solution in DMF was prepared and added directly to the labeling reaction, which typically consist 20 nmol of 5'- or 3'-amino-terminated oligonucleotide in 100 μL of 0.2 M phosphate buffer at pH 8.0, to which 50 μL NHS ester solution was added with sonication. The resulting solution was vigorously agitated for 2~6 hours; labeling reactions were passed through a NAP-5 Sephadex gel-filtration column and the labeled oligonucleotide was purified by reverse-phase HPLC, typically yielding 10-15 nmol of the desired product.

Glutaric acid NHS-ester was coupled to Reagent DNA 1 (R1) to give R3: ATC TGG GCT AAG-3'-COOH;
observed mass = 4025.5±4 (MALDI-TOF), (expected: 4026.6).
4-Azidobenzoic acid NHS ester was coupled to Reagent DNA 1 (R1) to give R4: ATC TGG GCT AAG-3'-N_3;
observed mass = 4056.8±4 (MALDI-TOF), (expected: 4057.6).
Hex-5-ynoic acid NHS-ester was coupled to Reagent DNA 2 (R2) to give R5: 5'-alkynyl-ATA TA AAG CTT TGC TGA;
observed mass = 5484.8±5 (MALDI-TOF), (expected: 5487.6).
4-Formylbenzoic acid was coupled to Reagent DNA 1 (R1) to give R6: ATC TGG GCT AAG-3'-CHO;
observed mass = 4042.1±4 (MALDI-TOF), (expected: 4044.6).

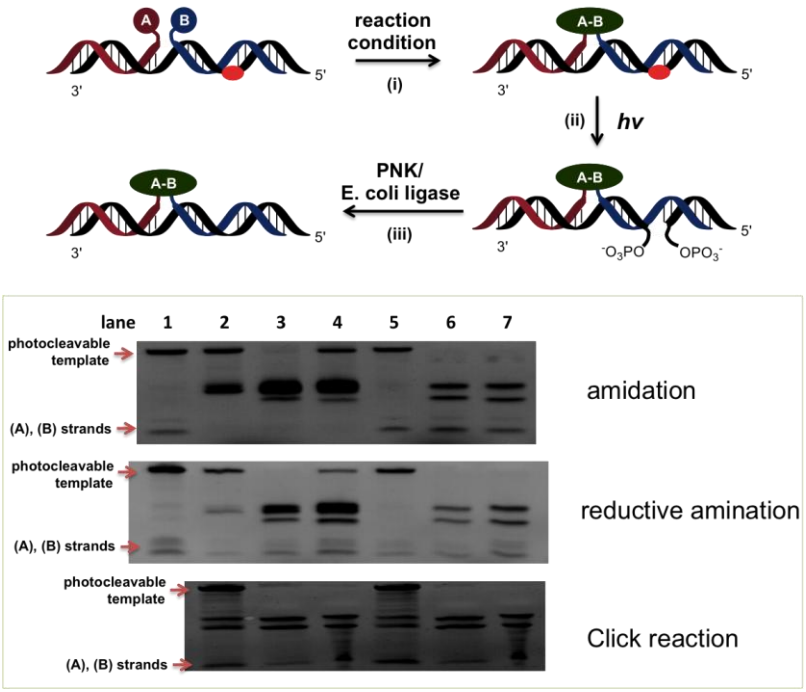


Figure S4: Photocleavage and re-ligation detected by denaturing PAGE (15% TBE-Urea). Lane 1: DNA only; lane 2: after addition of EDCI/NHS for amidation reaction, $NaBH_3CN$ for reductive amination and $CuSO_4$/sodium ascorbate for Click reaction; lane 3: after photocleavage; lane 4: after PNK/ligase-mediated template re-ligation; lane 5-7: same sequence as lane 2-4 but without respective reagent added (negative controls).

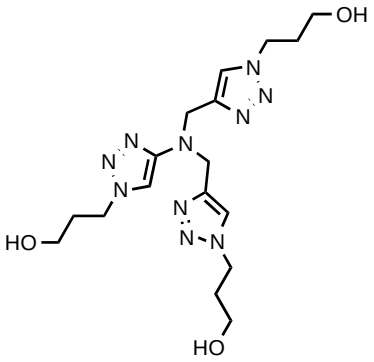
Reaction conditions of DNA-templated reactions:

Amidation: 400 pmol of the substrate DNAs (R2 and R3) and the photocleavable template in 300 µL buffer (100 mM MOPS, 1.0 M NaCl, pH 7.0) in a 1.5 mL Eppendorf tube was heated at 95 °C for 5 min and slowly cooled to 25 °C in 1 hour. To this solution, 100 µL of a solution of EDCI/NHS (0.8 M EDCI, 0.12 M NHS) was added and the resulting solution was briefly vortexed and then left at 25 ºC temperature for 14 hours; the DNA was then recovered by ethanol precipitation. For non-bond-forming negative control, the same experiment was performed under the same condition, except that 100 µL of H_2O was used instead of EDCI.

Reductive amination: 400 pmol of the substrate DNAs (R2 and R6) and photocleavable template in 400 µL buffer (100 mM MES, 1.0 M NaCl, pH 6.0) in a 1.5 mL Eppendorf tube was heated at 95 °C for 5 min and slowly cooled to 25 °C in 1 hour. To this solution, 2 µL of a 0.3 M solution of $NaBH_3CN$ in 1 M NaOH was added and the resulting solution was briefly vortexed and then left at 25 ºC for 14 hours; the DNA was then recovered by ethanol precipitation. For non-bond-forming negative control, the same experiment was performed under the same condition, except that 2 µL of H_2O was used instead of $NaBH_3CN$.

Cu(I)-catalyzed Click reaction: 400 pmol of the substrate DNAs (R4 and R5) and the photocleavable template in 300 µL buffer (100 mM MOPS, 1.0 M NaCl, pH 7.0) in a 1.5 mL Eppendorf tube was heated at 95 °C for 5 min and slowly cooled to 25 °C in 1 hour. To this solution, 50 µL of a 2.5 mg/100µL solution of ligand (see structure below), 40 µL of a 1 mg/1 mL solution of sodium ascorbate and 10 µL of a 20 mg/1 mL solution of $CuSO_4$, were added and the resulting solution was briefly vortexed and then left at 25 ºC temperature for 10 hours; the DNA was then recovered by ethanol precipitation. For non-bond-forming negative control, the same experiment was performed under the same condition, except that 10 µL of H_2O was used instead of $CuSO_4$.

Ligand structure:



Photocleavage and ligase-mediated re-ligation: 100 pmol of product from DNA-templated reactions was dissolved in 90 μL H_2O. This solution was irradiated at 365 nm for 30 min, then 10 μL *E. coli* DNA ligase buffer and 10 units T4 polynucleotide kinase (New England Biolabs) were added and the reaction was incubated at 37 ºC for 3 hours, followed by the addition of 10 units *E. coli* DNA ligase (New England Biolabs). The re-ligation reaction solution was incubated at 37 ºC for 4 hours.

qPCR experiments: The re-ligation reaction was stopped by heating at 95 ºC for 20 min. The reaction mixture was then subjected to qPCR as described above.

Experiment condition and enrichment fold determination:

DNA sequences:
Photocleavable template (bond-forming, RT1): GAG CTC GTT GAT ATC CGC AGT CAG CAA AGC TT – linker – TAT ATC TTA GCC CAG ATG CCA GCT GCT AGC TTT AGG T; observed mass = 21734.1 (ESI), (expected: 21734.8).

Bond-forming reagent DNA (R7): ATC TGG GCT AAG-(PEG-3)-ATA TA AAG CTT TGC TGA

Primer sequences for bond-forming event:
P1: GAG CTC GTT GAT ATC CGC AG
P2: ACC TAA AGC TAG CAG CTG GC

Photocleavable template (non-bond-forming, RT2): CAC GTC GTA AGA TTC GCC ACC ATG TCC ACG TA – linker – ATA TAG ATC ACA CCT GAG CGA CCT GGT CAC TAT ACG A; observed mass = 9946.7±10 and 11399.4±12 (MALDI-TOF), (expected: 9948.4, $[M+matrix+H]^+$ and 11406.4, $[M+H]^+$; analyzed as laser-cleaved fragments)

Non-bond-forming reagent DNA. R8: TCA GGT GTG ATC-3'-NH_2; observed mass = 3893.9 (ESI), (expected: 3894.5). R9: 5'-NH_2-TAT ATT ACG TGG ACA TG; observed mass = 5374.6 (ESI), (expected: 5375.3).

Primer sequences for non-bond-forming event:
P3: CAC GTC GTA AGA TTC GCC AC
P4: TCG TAT AGT GAC CAG GTC GC

A 90 μL solution containing 100 pmol of each non-bond-forming DNA strands (RT2, R8, R9) and varied amount of each bond-forming DNA strands (RT1, R7) (0 pmol, 10 pmol, 1 pmol, 100 fmol, 20 fmol) was heated at 95 °C for 5 min and slowly cooled to 25 °C in 1 hour. The photocleavage, PNK/ligase-mediated re-ligation, and qPCR were conducted as described above. The qPCR was performed with the bond-forming and the non-bond-forming primer pairs respectively. As a control for background, only 100 pmol of each non-bond-forming DNA strands (RT2, R8, R9) was subjected to qPCR. All C_T values are average of at least three replicates.

Calculation of enrichment fold:

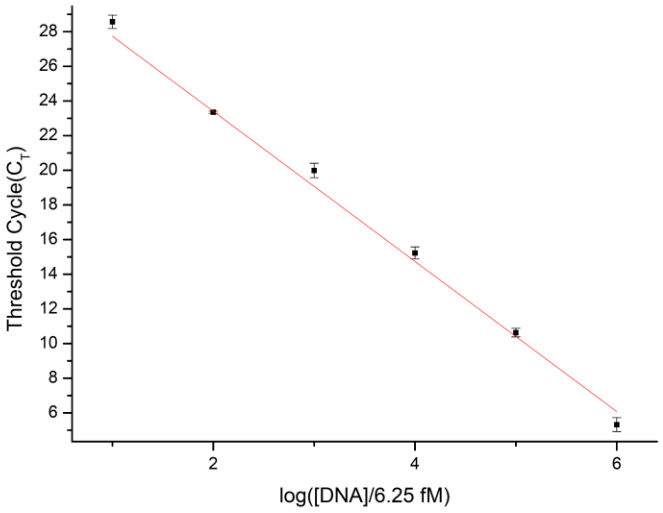


Figure S5: Standard curve (bond-forming) for enrichment fold calculation.

Standard curves were established for this series of qPCR experiments. Based on the linear function, the enrichment fold was calculated as follows:

Equation for bond-forming: $Y = 33.1 - 4.5*X$ (linear function), $Y = C_T$, $X = \log([DNA]/6.25fM)$
Therefore: $X = (33.1 - Y)/4.5$
[re-ligated DNA template] = $A = 6.25\ fM*10^{(X-1)}$ – [background]

ratio	0:1	1:10	1:100	1:1000	1:5000	primer only
C_T	26.4	12.6	16.5	22.4	27.4	27.0
[re-ligated template]/fM *	5.0	22.4×10^3	3.04×10^3	135	0 **	14.0

Table S3. qPCR results with the bond-forming primer pair. *: background deducted; **: negative value is entered as zero (equal to background).

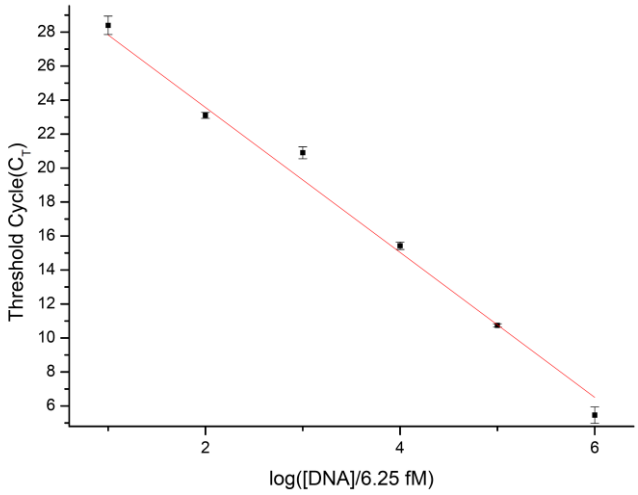


Figure S6: Standard curve (non-bond-forming) for enrichment fold calculation.

Equation for non-bond-forming: Y = 33.0 - 4.5*X (linear function), Y = C_T, X = log([DNA]/6.25fM)
Therefore: X = (33.0 - Y)/4.5
[re-ligated DNA template] = A = 6.25 fM*$10^{(X-1)}$ – [background]

ratio	0:1	1:10	1:100	1:1000	1:5000	primer only
C_T	19.0	19.0	19.1	19.1	19.3	19.7
[re-ligated template]/fM *	243	243	203	203	128	564

Table S4. qPCR results with the non-bond-forming primer. *: background deducted.

Enrichment fold = ratio $_{(\text{after selection})}$/ratio $_{(\text{before selection})}$
= {[re-ligated template] $_{(\text{bond-forming})}$ /[re-ligated template] $_{(\text{non-bond-forming})}$}/ratio $_{(\text{before selection})}$

When ratio $_{(\text{initial})}$ = 1:10, enrichment fold = [(22.4 x 10^3)/243]/(1:10) = 922
When ratio $_{(\text{initial})}$ = 1:100, enrichment fold = [(3.04 x 10^3)/203]/(1:100) = 1.50 x 10^3
When ratio $_{(\text{initial})}$ = 1:1000, enrichment fold = 135/203/(1:1000) = 665

Study of 3-component reactions mediated by photocleavable template:

DNA sequences:
Photocleavable template (RT1): GAG CTC GTT GAT ATC CGC AG TCA GCA AAG CTT – linker –TAT ATC TTA GCC CAG ATG CCA GCT GCT AGC TTT AGG T

Reagent DNA strands:

5’-aldehyde-ATA TA AAG CTT TGC TGA; observed mass = 5506.1±5 (MALDI-TOF), (expected: 5507.6).
R6 (m=0): ATC TGG GCT AAG-3’-CHO; observed mass = 4042.1±4 (MALDI-TOF), (expected: 4044.6).
R11 (m=2): GCA TCT GGG CTA-3’-CHO; observed mass = 4000.7±4 (MALDI-TOF), (expected: 4002.5).
R12 (m=5): CTG GCA TCT GGG-3’-CHO; observed mass = 4042.1±4 (MALDI-TOF), (expected: 4044.6).
R13 (m=10): AGC AGC TGG CAT-3’-CHO; observed mass = 4008.4±4 (MALDI-TOF), (expected: 4011.6).

Primer Sequences：

P1: GAG CTC GTT GAT ATC CGC AG

P2: ACC TAA AGC TAG CAG CTG GC

Reaction conditions:

400 pmol of the reagent DNAs and reaction template (RT1) in 400 μL buffer with ethylenediamine (100 mM MES, 1.0 M NaCl, 100 mM ethylenediamine, pH 7.5) in a 1.5 mL Eppendorf tube was heated at 95 °C for 5 min and slowly cooled to 25 °C in 1 hour. To this solution, 2 μL of a 0.3 M solution of $NaBH_3CN$ in 1M NaOH was added and the resulting solution was briefly vortexed and then left at 25 ºC temperature for 14 hours, and the DNA was then recovered by ethanol precipitation. For non-bond-forming negative control, the same experiments were performed, except that 2 μL of H_2O was used instead of $NaBH_3CN$. For diamine-free control, MES buffer without ethylenediamine was used. The photocleavage, re-ligation and qPCR were carried out as described above.

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