Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Selectivity and Efficiency of the Ligation of The Pyrene:Abasic Base Pair by T4 and PBCV-1 DNA Ligases

Hansol Park,[a] and Julianne M. Gibbs[a*]

[a]Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada.

[*]julianne.gibbs@ualberta.ca

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Material and Methods

General Materials

T4 DNA ligase (400,000 CEU/mL, M0202S), PBCV-1 DNA ligase (25,000 units/mL, M0375S), 10X T4 DNA or PBCV-1 ligase buffer, and 10 mM ATP solution were obtained from New England Biolabs (NEB, Ipswich, MA). 100 mM ATP solution was purchased from ThermoFisher Scientific (Waltham, MA). For the ligation experiments performed at ATP concentrations greater than 1 mM, a buffer was prepared instead of using that provided from New England Biolabs. This corresponding ligation buffer was prepared (50 mM Tris-HCl, pH 7.5 at 25 °C and 10 mM $MgCl_2$) and stored as a 10X stock. Tris-HCl (BP152-1) was purchased from Fisher Scientific. $MgCl_2$ (M4880) and 28-30% ammonium hydroxide solution (221228) was purchased from Sigma Aldrich.

DNA Synthesis and Purification

Oligonucleotides were synthesized on an Applied Biosystems Model 392 DNA synthesizer using reagents from Glen research (Sterling, VA) following their recommendation procedure. The DNA (dA, dT, dG, dC, dSpacer, and dT-Fluorescein) phosphoramidite and controlled porous glass (CPG) were used. To cleave the synthesized DNA strand from the solid support, the CPGs were incubated in a ~30% ammonia solution at room temperature overnight, or at 55 °C for two hours. The DMT-on purification protocol with Glen-pak cartridges was used to purify the oligonucleotide.

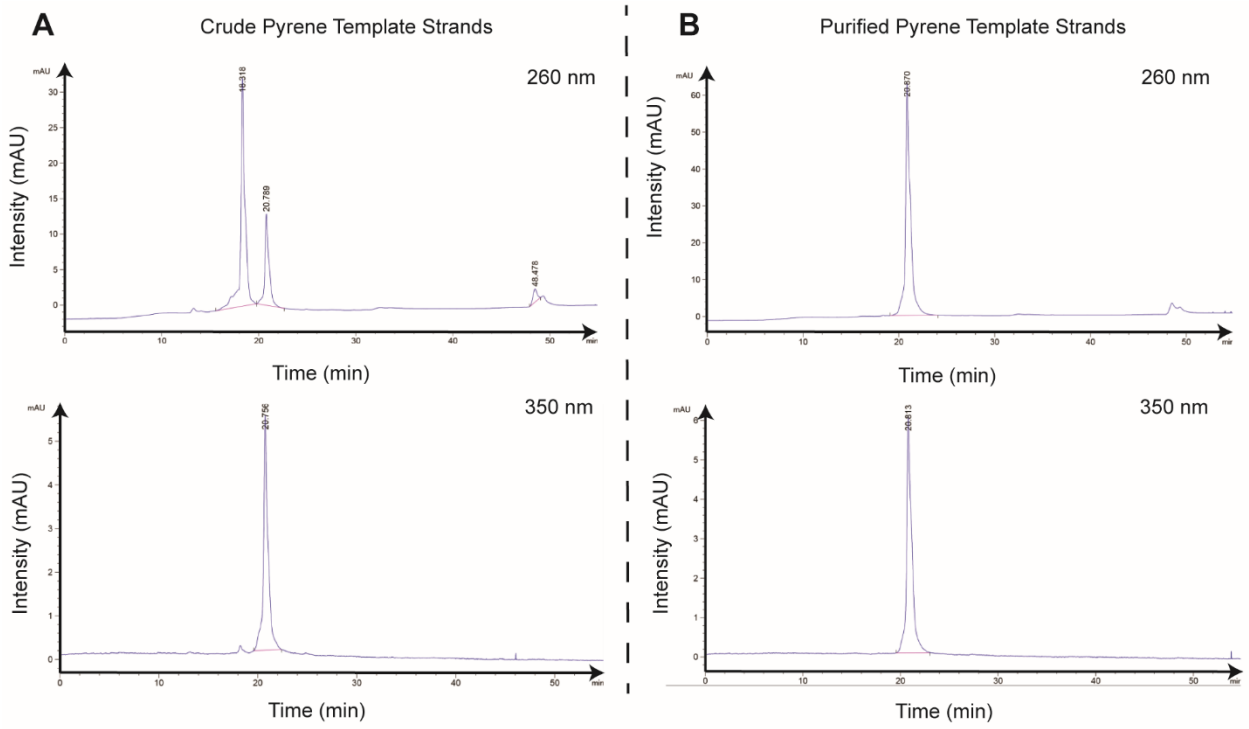
Synthesis of Pyrene Nucleotide on DNA Strands

5'-DMT 3'-cyanoethyl 1-pyrene phosphoramidite was synthesized following the literatures,[1] with slight modifications in the 5' DMT protection[2] and phosphorylation procedures.[3] The characterization by ^{1}H, ^{13}C and ^{31}P NMR of the nucleoside precursors and final product matched with the reported values in the literature.[1,2] The DNA strands (5'-phosphate pyrene and pyrene-containing template) were synthesized from 1.0 μmol CPG using the standard DNA synthesis cycle up to the position where the pyrene was to be included and then paused. Freshly synthesized pyrene phosphoramidite in $CDCl_3$ was installed in the 5th position of the solid-phase synthesizer immediately after NMR analysis. The addition of the pyrene phosphoramidite was performed with a standard DNA synthesis cycle with an extended coupling time (20 min). The synthesis was continued based on the sequence. For the 5'-phosphate pyrene strand, 5'-phosphorylation was performed using solid chemical phosphorylation reagent II (10-1902-90). For the pyrene-containing template, the addition of bases was continued as designed. The standard DNA purification using a Gel-Pak cartridge was performed to purify the pyrene-modified strands. The pyrene-modified oligonucleotides were further purified by RP-HPLC. The pyrene modified strands were analyzed by MALDI-TOF and polyacrylamide gel electrophoresis with Stains-All.

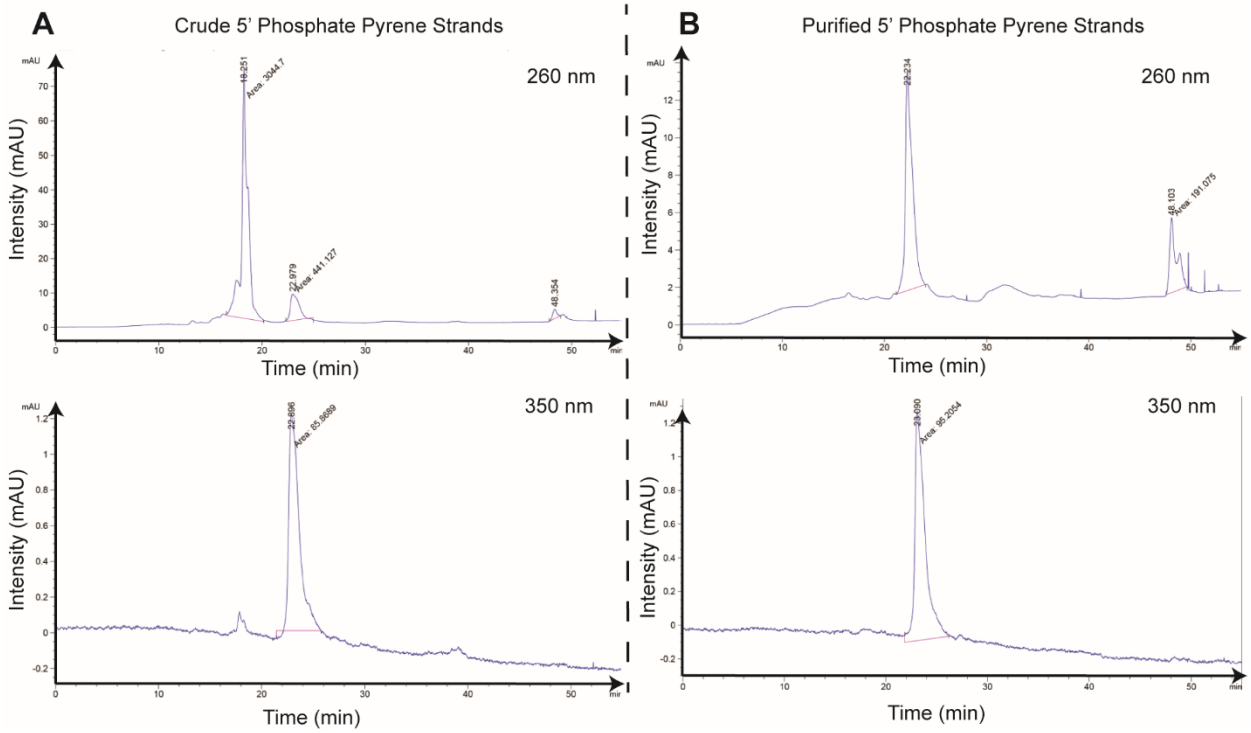
HPLC Purification of Pyrene Modified Oligonucleotides

The crude pyrene-modified strands were analyzed by RT-HPLC after DMT-on purification by Glen-Pak cartridge. The solvent system used was described in the following. A solvent: 0.03 M triethylammonium acetate (TEAA) buffer and B solvent: acetonitrile with 5% of 0.03 M TEAA. The flow rate was consistent at 3 mL per minute. For the analysis, the sample (1 nmol in 20 μL

H_2O, injection of 7 µL) was run through the C18 column (Dynamax Microsorb 300-10 C18) with a gradient of B solvent, 1% to 50% over 55 minutes followed by 5 minutes of post-run. The analysis was performed both at 260 nm wavelength where the oligonucleotide was observed and 350 nm wavelength where the pyrene moiety was observed. For the purification, the crude product (40 nmol in 180 µL H_2O) was run through the column with the same procedure. The injection of the sample was 60 µL per run. The UV detector was set at 260 nm wavelength to monitor the oligonucleotide. The average purification yield was 20-23% for pyrene modified strands based on the initial amount of crude material.



Analytical HPLC chromatogram of pyrene containing template. The detection wavelength is at 260 nm (top) and 350 nm (bottom). (**A**) Crude pyrene containing template (**B**) Purified pyrene containing template. Based on analytical HPLC, a semi-preparatory run was performed, and the product was collected from the peak (RT ~20 min from 260 nm wavelength).



Analytical HPLC chromatogram of 5'-phosphate pyrene strand. The detection wavelength is at 260 nm (top) and 350 nm (bottom). (**A**) Crude 5' phosphate pyrene strand (**B**) Purified 5' phosphate pyrene strand. Based on analytical HPLC, a semi-preparatory run was performed, and the product was collected from the peak (RT ~22 min from 260 nm wavelength).

Ligation Assay

The pyrene modified strand (5'-phosphate pyrene strand and pyrene-containing template) was aliquoted as 0.041 nmol per tube and lyophilized. All other DNA strands were diluted with Millipore water to reach a concentration of 16.3 μM. For the reaction using 5'-phosphate pyrene strand, 2.5 μL of Millipore water was added to the lyophilized tube containing the 5'-phosphate pyrene strand and combined with 1.25 μL of each of the diluted fluorescein-labeled 3'-OH strand and the template. For the reactions using the pyrene-containing template after adding 2.5 μL of Millipore water, 1.25 μL was removed and used in the reaction with 2.5 μL of the phosphate strand

and 1.25 μL of the fluorescein-labeled 3'-OH strand. A master mix was prepared by combining 10X Tris-HCl buffer supplied by New England Biolabs or prepared in house buffer for the ATP concentration variation experiments, 10X ATP (for the ATP concentration variation experiments), and T4 DNA ligase (400,000 CEU/mL, 1 μL per total 15 μL of reaction volume) or PBCV-1 DNA ligase (25,000 units/mL, 1.5 μL per total 15 μL of reaction volume) and then incubated at the reaction temperature for 10 minutes. The master mix was then added into each reaction tube; 2.5 μL of master mix for ligation reactions totalling 7.5 μL volume or 5 μL master mix for a ligation reactions totalling 15 μL volume. In each reaction, the final concentration of the phosphate modified strands was 2.8 μM as excess and the final concentration of the other fluorescein-labeled strands and the templates were 1.4 μM. in 50 mM Tris-HCl buffer containing 10 mM $MgCl_2$, and various ATP concentrations. The reaction was collected at each data point by removing a 1.2 μL aliquot from the reaction and quenching it with 1.2 μL solution containing 0.5 M EDTA and 10% (w/v) 1-bromophenol/sucrose.

Competitive Ligation Assay

In the competitive ligation assay, either abasic template or dA template or both templates were added. When either template was used, the final concentration of template was 1.4 μM. When both templates were added, each template was 0.7 μM, to make the sum of template concentration consistent as 1.4 μM. Final concentration of both 5'-phosphate strands (pyrene or thymine) were 2.8 μM, and fluorescein-labeled strands were 1.4 μM. The DNA fragments and templates were mixed and incubated at the reaction temperature while preparing the master mix. The master mix was prepared by adding a 10X Tris-HCl buffer containing $MgCl_2$, 10X ATP, and DNA ligase. For

T4 DNA ligase (400,000 CEU/mL) 1 μL per 15 μL total volume was used, while PBCV-1 DNA ligase (25,000 units/mL) 1.5 μL per 15 μL total volume was used. Then the master mix was incubated at the reaction temperature for 10 minutes and then added to the reaction mixture. To stop ligation after 10 minutes of reaction, the reaction mixture (3 μL) was mixed with 0.5 M EDTA (3 μL) containing 1-bromophenol dye and sucrose. The reaction was analyzed by 15% denaturing PAGE.

Analysis by Polyacrylamide Gel Electrophoresis

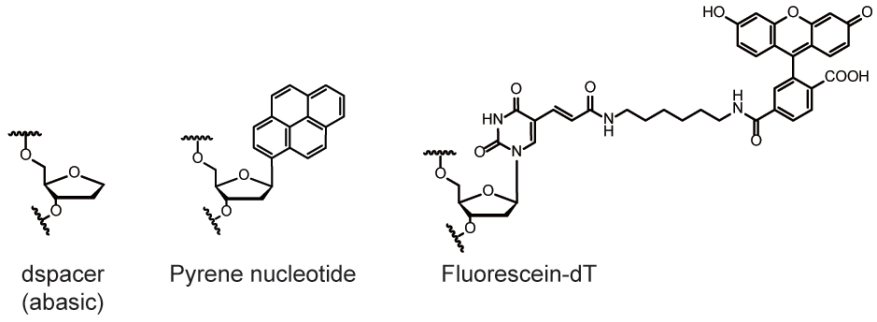
A 15% denaturing polyacrylamide gel (0.75 nm, 10 wells) was prepared using 8 M urea mixed with 5X TBE buffer and 40% acrylamide/bis solution 19:1 (Bio-Rad, 161-0144). To analyze the purity of the synthesized DNA strand, we used Stains-All to visualize each oligonucleotide band (Aldrich, E9379). After electrophoretic separation, the gel was soaked in Stains-All solution (1:1 Formamide/water) (Formamide, Fisher F841) for 10 mins and visualized by white illumination via ImageQuant RT ECL instrument from GE Healthcare life science. To analyze the ligation reaction, we visualized the fluorescein-labeled strand using UV transillumination via ImageQuant RT ECL instrument from GE Healthcare life science.

Matrix-Assisted Laser Desorption Ionization (MALDI)

The mass of synthesized oligonucleotides was analyzed by MALDI using a Voyager Elite (Applied Biosystems, Foster City, CA) time-of-flight in linear negative mode. The dried oligonucleotides (1~3 nmol) were dissolved in a 1:1 ratio of water and matrix (about 0.6 mM to 1 mM solution). Matrix solutions were prepared with a 9:1 ratio of 25 mg/mL of 2,4,6-tridoxyacetophenole in 1:1 acetonitrile: water solution to 25 mg/mL ammonium citrate.

Table S1. Oligonucleotide sequence and corresponding MALDI

Oligonucleotides	Sequence (5' → 3')	Expected	Found
Template(Ab)	TTG TTA AAT **Ab**TT GAT AAG	5419	5425
Template(A)	TTG TTA AAT **A**TT GAT AAG	5552	5555
Template(T)	TTG TTA AAT **T**TT GAT AAG	5543	5549
Template(G)	TTG TTA AAT **G**TT GAT AAG	5568	5574
Template(C)	TTG TTA AAT **C**TT GAT AAG	5528	5532
3'-OH fluorescein	$T_{fluorescein}$ CTT ATC AA	3184	3188
5'-phosphate pyrene	phosphate (**Pyrene**) ATT TAA CAA	3166	3170
5'-phosphate thymine	phosphate **T** ATT TAA CAA	3090	3094
5'-phosphate thymine (+3nt)	phosphate T ATT TAA CAA TAA	4020	4021
Complementary template(Pyrene)	CTT ATC AA(**Pyrene**) ATT TAA CAA	5517	5520
Complementary template(T)	CTT ATC AA**T** ATT TAA CAA	5441	5445
Complementary 3'-OH fluorescein	$T_{fluorescein}$TG TTA ATT	3239	3243
Complementary 5'-phosphate abasic	phosphate (**Abasic**)TT GAT AAG	2708	2712
Complementary 5'-phosphate adenine	phosphate **A**TT GAT AAG	2842	2845



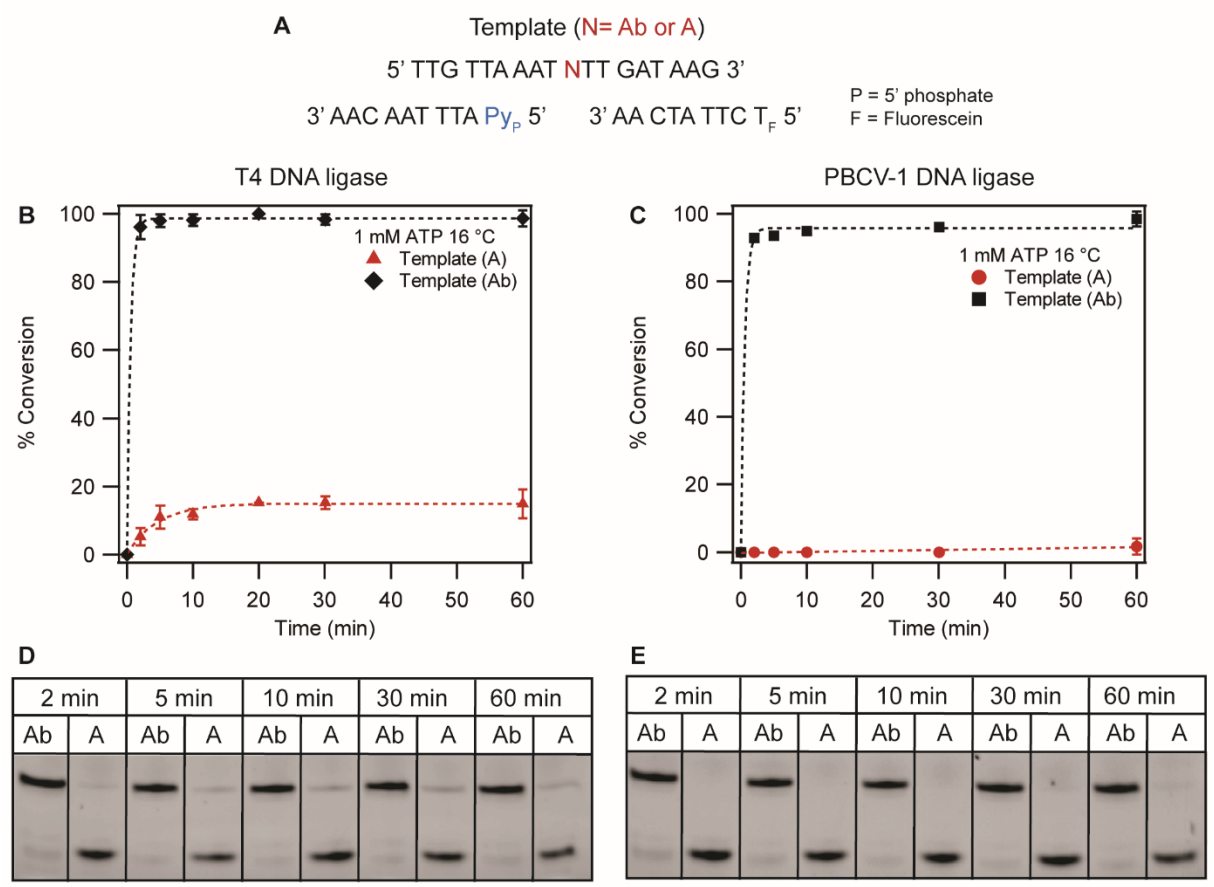


Figure S1. The time-course of the ligation reaction of the 5'-phosphate pyrene strand. (**A**) Template and fragments sequences that were used in the experiments. Time course of enzymatic ligation of 5'-phosphate pyrene strand (**B**) with T4 DNA ligase. (**C**) with PBCV-1 DNA ligase. Representative PAGE gel image of stoichiometric ligation (**D**) with T4 DNA ligase (**E**) with PBCV-1 DNA ligase. *Experimental conditions*: 2.8 µM of the 5'-phosphate pyrene strand, 1.4 µM of the fluorescein-labeled strand and the template strands. T4 DNA ligase (400,000 CEU/mL, 1 µL per 15 µL total volume) or PBCV-1 DNA ligase (25,000 units/mL, 1.5 µL per 15 µL total volume) in 50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM ATP concentrations.

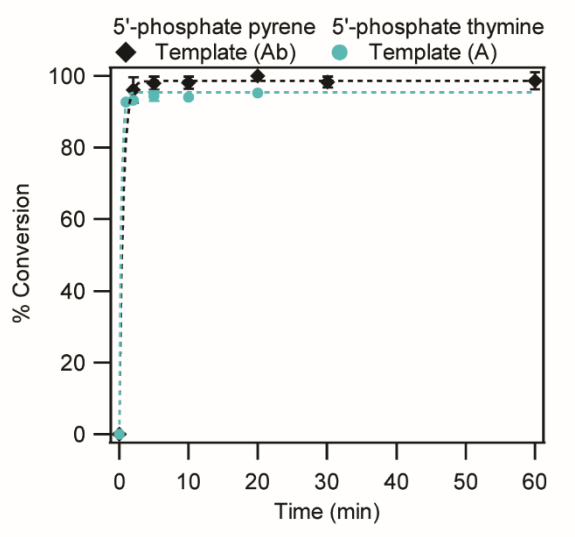


Figure S2. Ligation comparison with Py:Ab base pair with A:T base pair. *Experimental conditions:* 2.8 µM of the 5'-phosphate strand, 1.4 µM of the fluorescein-labeled strand and the template strands. T4 DNA ligase (400,000 CEU/mL, 1 µL per 15 µL total volume) in 50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM ATP concentrations at 16 °C.

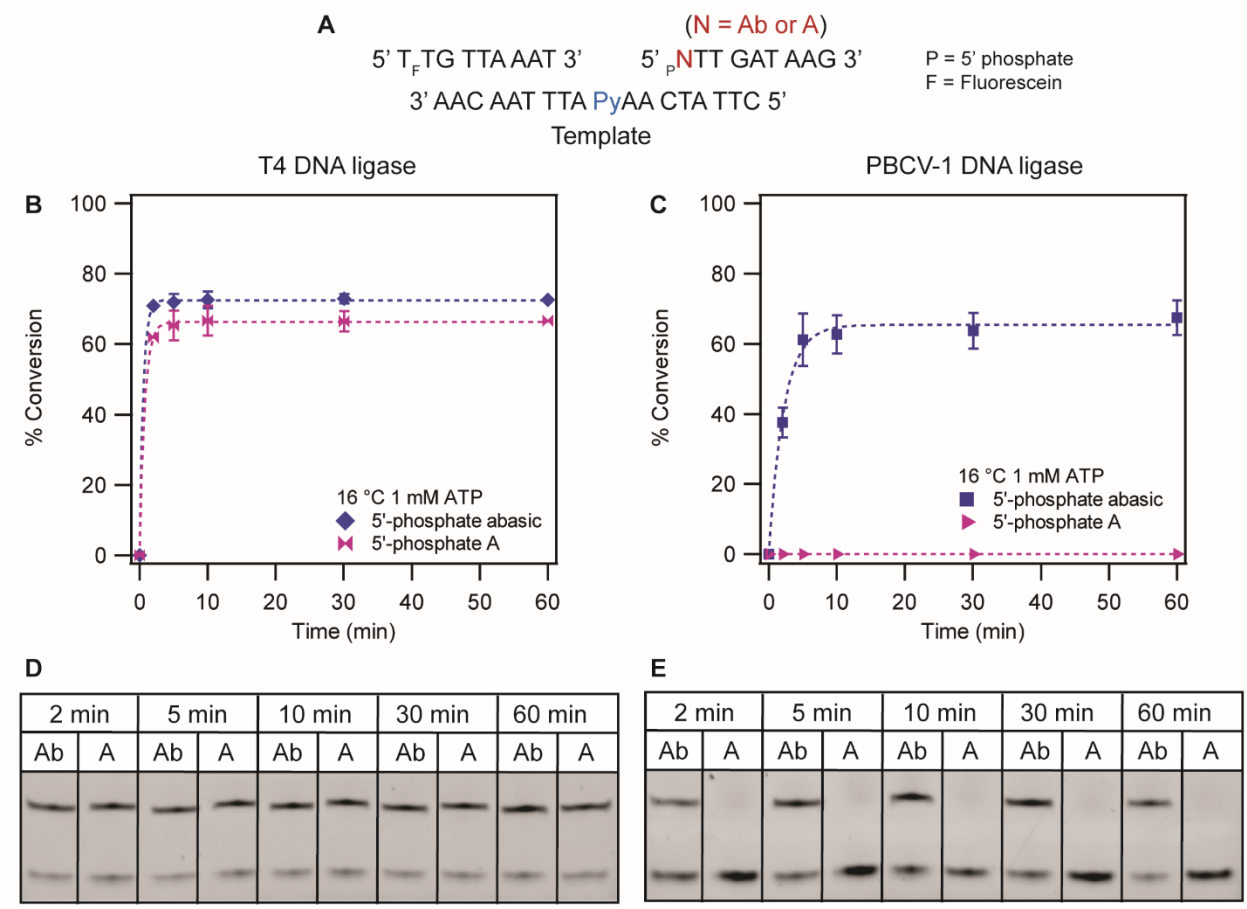


Figure S3. Ligation of 5'-phosphate abasic strand or 5'-phosphate adenine strand on the pyrene-containing template. (**A**) The DNA strands that were used in this experiment. Time course of the ligation reaction (**B**) with T4 DNA ligase (**C**) with PBCV-1 DNA ligase. Representative PAGE gel image of stoichiometric ligation (**D**) with T4 DNA ligase. (**E**) with PBCV-1 DNA ligase. *Experimental conditions*: 2.8 µM of the 5'-phosphate strand, 1.4 µM of the fluorescein-labeled strand and the template strands. T4 DNA ligase (400,000 CEU/mL, 1 µL per 15 µL total volume) or PBCV-1 DNA ligase (25,000 units/mL, 1.5 µL per 15 µL total volume) in 50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM ATP concentrations.

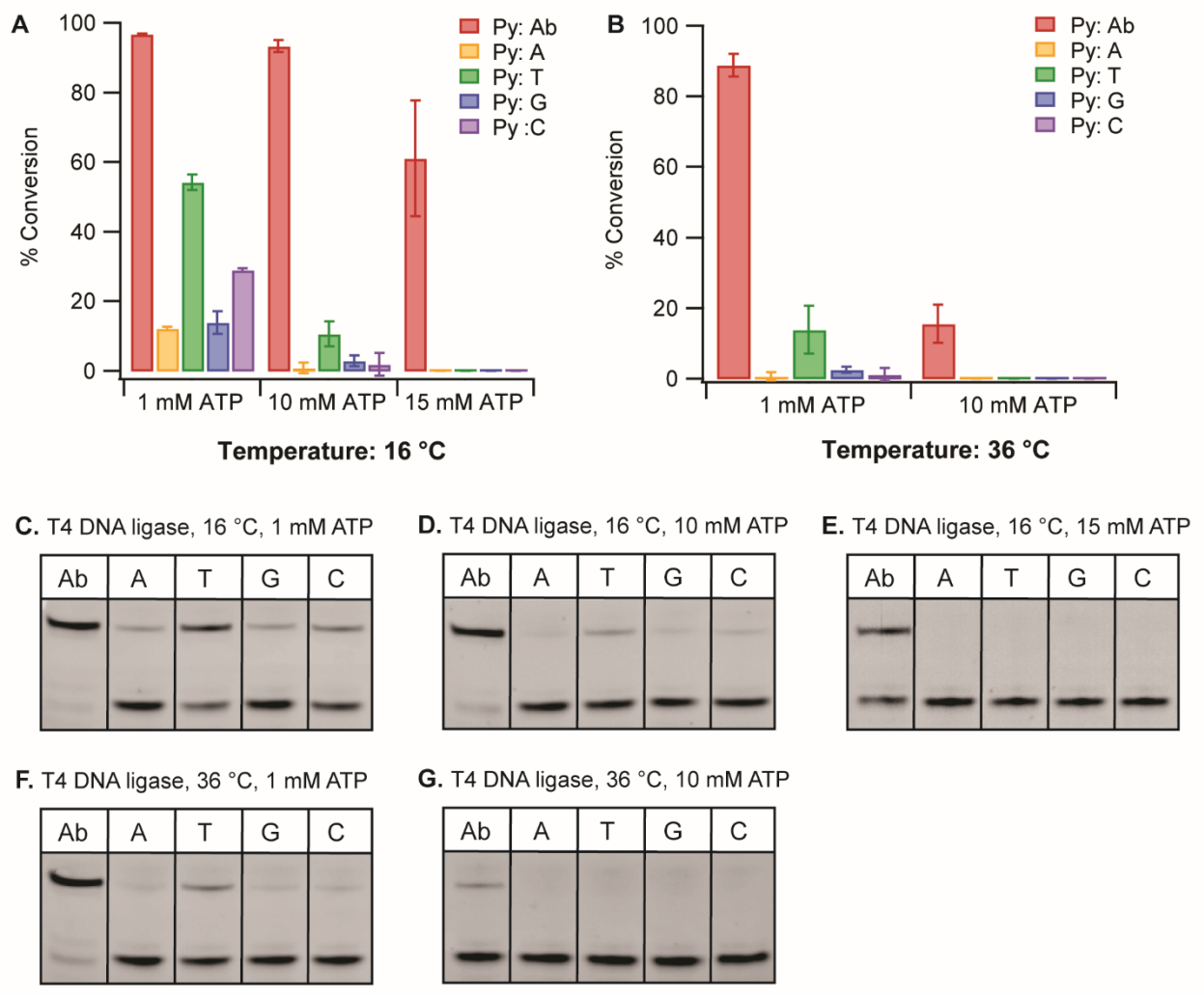


Figure S4. Ligation efficiencies of 5'-phosphate pyrene strand with abasic or natural bases containing template with T4 DNA ligase. (**A**) Comparison of ATP concentration, 1mM, 10 mM, versus 15 mM at 16 °C. (**B**) Comparison of ATP concentrations, 1 mM versus 10 mM at 36 °C. Representative PAGE image of (**C**) 16 °C, 1 mM ATP (**D**) 16 °C, 10 mM ATP (**E**) 16 °C, 15 mM ATP (**F**) 36 °C, 1 mM ATP and (**G**) 36 °C, 10 mM ATP. *Experimental conditions*: 2.8 µM of the 5'-phosphate pyrene strand, 1.4 µM of the fluorescein-labeled strand and the template strands. T4 DNA ligase (400,000 CEU/mL, 1 µL per 15 µL total volume) in 50 mM Tris-HCl, 10 mM $MgCl_2$ and various ATP concentrations. The data evaluation was at 10 minute after the reaction was mixed.

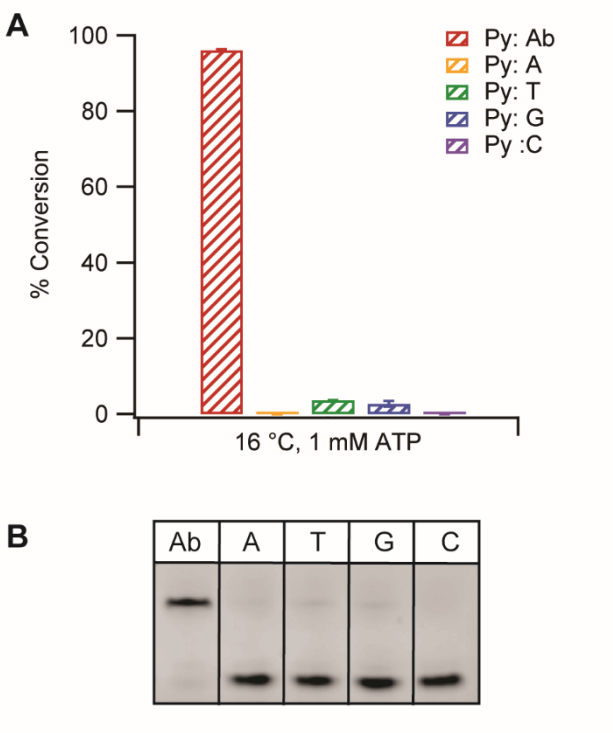


Figure S5. Ligation efficiencies of the unnatural pyrene base at the ligation site with abasic and natural bases with PBCV-1 DNA ligase at 10 minutes. (**A**) Standard ATP concentration (1 mM) at 16 °C. (**B**) Representative PAGE images. *Experimental conditions*: 2.8 µM of the 5'-phosphate pyrene strand, 1.4 µM of the fluorescein-labeled strand and the template strands. PBCV-1 DNA ligase (25,000 units/mL, 1.5 µL per 15 µL total volume) in 50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM ATP concentrations. The data evaluation was at 10 minute after the reaction was mixed.

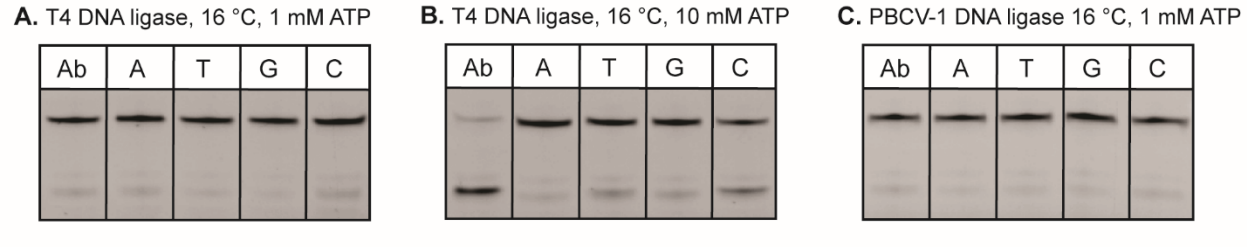


Figure S6. Representative PAGE image of template selectivity using 5'-phosphate thymine strand by T4 DNA ligase or PBCV-1 DNA ligase. (**A**) T4 DNA ligase at 16 ℃, 1 mM ATP (**B**) T4 DNA ligase at 16 ℃, 10 mM ATP, and (**C**) PBCV-1 DNA ligase at 16 ℃, 1 mM ATP from Figure 3.

Reference

(1) Wojciechowski, F.; Lietard, J.; Leumann, C. J. 2-Pyrenyl-DNA: Synthesis, Pairing, and Fluorescence Properties. *Org. Lett.* **2012**, *14* (20), 5176–5179. https://doi.org/10.1021/ol302150a.

(2) Ren, R. X.-F.; Chaudhuri, N. C.; Paris, P. L.; Rumney; Kool, E. T. Naphthalene, Phenanthrene, and Pyrene as DNA Base Analogues: Synthesis, Structure, and Fluorescence in DNA. *J. Am. Chem. Soc.* **1996**, *118* (33), 7671–7678. https://doi.org/10.1021/ja9612763.

(3) Kausar, A.; McKay, R. D.; Lam, J.; Bhogal, R. S.; Tang, A. Y.; Gibbs-Davis, J. M. Tuning DNA Stability To Achieve Turnover in Template for an Enzymatic Ligation Reaction. *Angew. Chem. Int. Ed.* **2011**, *50* (38), 8922–8926. https://doi.org/10.1002/anie.201102579.