Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Efficiency and fidelity of T3 DNA ligase in ligase-catalysed oligonucleotide polymerisations

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Supporting Information

Supporting Methods

General information

Unless otherwise noted, water was purified with Milli-Q purification system. DNA oligonucleotides without amine modification were purchased from Integrated DNA Technologies. DNA oligonucleotides with amine modification were synthesized on a Bioautomation Mermade12 synthesizer. All materials and reagents used for oligonucleotide synthesis were purchased from Glen Research. All oligonucleotides were synthesized and deprotected according to the manufacturer's protocols. Oligonucleotides were purified by reverse-phase high-pressure liquid chromatography (HPLC, Agilent 1260) using a C18 stationary phase (Eclipse-XDB C18, 5 µm, 9.4 x 200 mm) and an acetonitrile/100 mM triethylammonium acetate gradient. Oligonucleotide concentrations were quantitated by UV spectroscopy using a Nanodrop ND2000 spectrophotometer.

DNA sequences

The sequences below are written from 5'→3'. <Aam> = Amino-modifier C6 dA, <N>=A/T/C/G, <Y>=C/T, <R>=G/A, <Yam> = Amino-modifier C6 dC/dT

Templates

TP(NNN)13P /5Phos/GA TTC GCC TGC CGT CGC ANN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NCA CGT GGA GCT CGG ATC CT

TP(NNR)13P /5Phos/GA TTC GCC TGC CGT CGC ANN RNN RNN RNN RNN RNN RNN RNN RNN RNN RNN RNN RCA CGT GGA GCT CGG ATC CT

TP(NNNN)10P /5Phos/GA TTC GCC TGC CGT CGC ANN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNC ACG TGG AGC TCG GAT CCT

TP(NNNR)10P /5Phos/GA TTC GCC TGC CGT CGC ANN NRN NNR NNN RNN NRN NNR NNN RNN NRN NNR NNN RNN NRC ACG TGG AGC TCG GAT CCT

TP(NNNNN)8P /5Phos/GA TTC GCC TGC CGT CGC ANN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNC ACG TGG AGC TCG GAT CCT

TP(NNNNT)8P /5Phos/GA TTC GCC TGC CGT CGC ANN NNT NNN NTN NNN TNN NNT NNN NTN NNN TNN NNT NNN NTC ACG TGG AGC TCG GAT CCT

TP(NNNNR)8P /5Phos/GA TTC GCC TGC CGT CGC ANN NNR NNN NRN NNN RNN NNR NNN NRN NNN RNN NNR NNN NRC ACG TGG AGC TCG GAT CCT

Primers

PR5 /5Phos/GG ATC CGA GCT CCA CGT G

PR6 /5Phos/TG CGA CGG CAG GCG AAT CT

iTruS_i7_D701 CAA GCA GAA GAC GGC ATA CGA GAT ATT ACT CGG TGA CTG GAG TTC AG

iTruS_i7_D702 CAA GCA GAA GAC GGC ATA CGA GAT TCC GGA GAG TGA CTG GAG TTC AG

iTruS_i7_D703 CAA GCA GAA GAC GGC ATA CGA GAT CGC TCA TTG TGA CTG GAG TTC AG

iTruS_i7_D704 CAA GCA GAA GAC GGC ATA CGA GAT GAG ATT CCG TGA CTG GAG TTC AG

iTruS_i7_D705 CAA GCA GAA GAC GGC ATA CGA GAT ATT CAG AAG TGA CTG GAG TTC AG

iTruS_i7_D706 CAA GCA GAA GAC GGC ATA CGA GAT GAA TTC GTG TGA CTG GAG TTC AG

iTruS_i7_D707 CAA GCA GAA GAC GGC ATA CGA GAT CTG AAG CTG TGA CTG GAG TTC AG

iTruS_i7_D708 CAA GCA GAA GAC GGC ATA CGA GAT TAA TGC GCG TGA CTG GAG TTC AG

iTruS_i7_D709 CAA GCA GAA GAC GGC ATA CGA GAT CGG CTA TGG TGA CTG GAG TTC AG

iTruS_i7_D710 CAA GCA GAA GAC GGC ATA CGA GAT TCC GCG AAG TGA CTG GAG TTC AG

iTruS_i7_D711 CAA GCA GAA GAC GGC ATA CGA GAT TCT CGC GCG TGA CTG GAG TTC AG

Adapters

AdapterA AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T

AdapterB /5phos/AC TGN NNN NNN NNN NNN NAG ATC GGA AGA GCA CAC GTC TGA ACT CCA GTC AC

Building blocks

NNN /5Phos/NNN

YNN /5Phos/YNN

NH_2-YNN /5Phos/<Yam>NN

NNNN /5Phos/NNNN

YNNN /5Phos/YNNN

NH_2-YNNN /5Phos/<Yam>NNN

NNNNN /5Phos/NNNNN

ANNNN /5Phos/ANNNN

NH_2-ANNNN /5Phos/<Ama>NNNN

YNNNN /5Phos/YNNNN

NH_2-YNNNN /5Phos/<Yam>NNNN

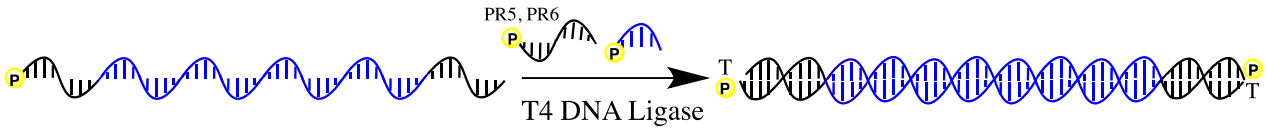
Synthesis of building blocks

Building blocks were synthesized on a Mermaid12 DNA synthesizer using a DMT-ON protocol on a 1µmol scale (1000 Å CPG column). Amino-modifier C6 dA (Glen Research 10-1089), amino-modifier C6 dC (Glen Research 10-1019), amino-modifier C6 dT (Glen Research 10-1039), dA+dC+dG+dT-CE Phosphoramindite (Glen Research 10-1000, 10-1010, 10-1020, 10-1030), Chemical Phosphorylation Reagent II (Glen Research 10-1901) were incorporated as specified by the manufacturer. Following synthesis, the oligonucleotide was cleaved from the resin by incubation at 25°C in 400µl of a 1:1 mixture of ammonium hydroxide and methylamine for 25 min. Then the resin was removed by filtration and cleaved DNA was incubated in the same solution at 65°C for 30min. The cleaved resin was filtered off, and the oligonucleotide was concentrated under reduced pressure using a speedvac. The residue was then taken up into 100 µl of H_2O and purified using reverse-phase HPLC purification using a [10 % acetonitrile in 0.1 M TEAA, pH 7] to [80 % acetonitrile in 0.1 M TEAA, pH 7] solvent gradient with a column temperature of 45 °C. The purified oligonucleotide was then incubated at room temperature in 800 µl of 40 % aqueous acetic acid for 1 hour to cleave the DMT group, and then frozen and lyophilized. The oligonucleotide was incubated in 400 µl 30 % ammonium hydroxide at room temperature for 15 min to cleave the CPRII linker. Following deprotection, the oligonucleotide was concentrated under reduced pressure using a speedvac. The dried product was dissolved into 100 µl H_2O and subjected to reverse-phase HPLC purification using a [10 % acetonitrile in 0.1M TEAA, pH 7] to

[80 % acetonitrile in 0.1M TEAA, pH 7] solvent gradient with a column temperature of 45 °C. The purified oligonucleotide was resuspended in H_2O for subsequent use in LOOPER.

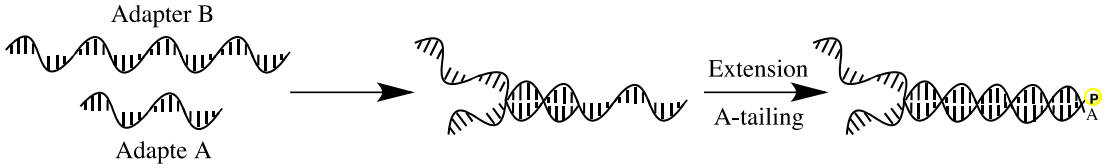
Ligase-mediate adapter ligation protocols

T3-DNA Ligase-mediate polymerization protocols



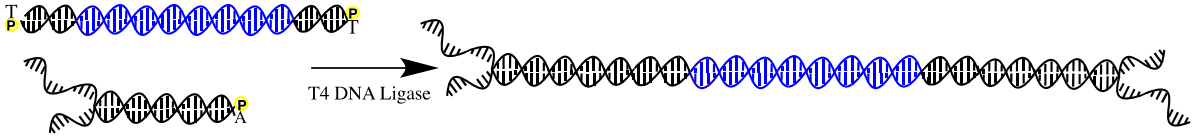
1µl DNA template (10µM in H_2O), 1.5µl PR5 (10µM in H_2O), 1.5µl PR6 (10µM in H_2O, FAM label at 3'-end for polymerization efficiency test), 1µl T4 RNA ligase reaction buffer (10X, New England Biolabs, B0216L), and 10 equiv. of building blocks to the template for each occurrence of the corresponding codon were mixed in a total volume of 8µl in a PCR tube. The mixture was incubated at 95°C for 10s, 65 °C for 4min and a ramp from 65°C to 4°C at 0.1°C every ten seconds. Then 1µl ATP (10mM) and 1µl of T3 DNA ligase (3,000U/µl, New England Biolabs, M0317S) were added to the mixture. The reaction was incubated at 4 °C for 12 h and then at 16°C for 2h. The FAM labeled primers and polymerization products are imaged without Ethidium Bromide stain using Gel Doc™ EZ Gel imager.

Adapter duplex synthesis

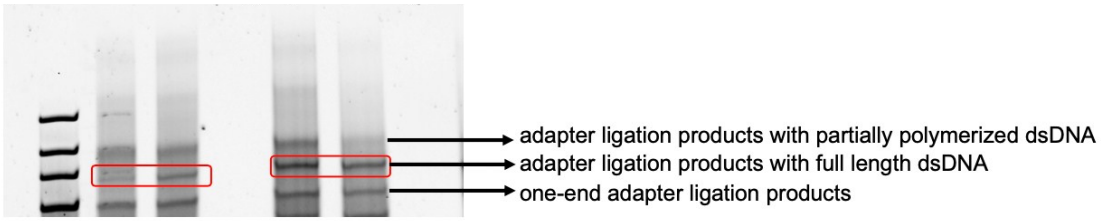


15µl adapterA (100µM in H_2O) and 15µl adapter (100µM in H_2O) were mixed in a PCR tube. The mixture was heated to 95 °C for 5 min and cooled to room temperature over 1 h. Then 4 µl NEBuffer2 (10X, New England Biolabs, M0212L), 25U Klenow Fragment (3'→5' exo$^-$, New England Biolabs, M0212L), 1µl dNTP mix (10mM each, Thermo Scientific, R0191) were added to the mixture. The extension was performed at 37°C for 1h. The adapter duplex was purified with QIAquick® Nucleotide Removal Kit eluted with 30µl H_2O.

Adapter ligation



In a PCR tube was added 10 pmol polymerization products, 200 pmol A-tailing adapter duplex, 10µl NEBNext® Quick Ligation Reaction Buffer (5X, New England Biolabs, B6058S), 2.5µl BSA (2mg/mL in H_2O), 1000 U T4 DNA ligase (New England Biolabs, M0202L). The total volume of reaction was adjusted to 50µl with H_2O. The ligation was performed at 16°C for 16h. The ligated products were then gel purified by PAGE. The polymerization yields were calculated based on the adapter ligation products on the native gel. The gel intensity was determined by the Gel Doc™ EZ Gel imager and the yield was calculated by the ratio of partially polymerized products and fully polymerized products with adapter duplex attached.



PCR protocols

Each purified adapter ligation product was amplified with a different primer from iTrus_D701 to iTrus_D711.

In a PCR tube was added 50 attmole purified adapter ligation product in 20µl H_2O, 2.5µl 10µM PrimerB, 2.5µl 10µM corresponding iTrus_D7XX primer, and 25µl Q5® High-Fidelity Master Mix (2X, New England Biolabs, M0492S). The tube was then transferred to a preheated themocycler (98°C). The PCR cycle was 10 s of 98 °C denature step, 30 s of primer annealing step (annealing temperature was 55 °C for the first two cycles, and 71 °C for the rest of the cycles), and 30 s of 72 °C extension step. The dsDNA PCR products were then loaded on 10% denaturing polyacrylamide gel and run at 55°C for 60min. The gel was then stained by SYBR™ Safe gel stain at room temperature for 15mins. The bands were imaged by Pearl Biotech Blue Transilluminator and the desired length (237bp) was marked with GeneRuler Low Range DNA Ladder (Thermo Scientific™, SM1193). The desired bands were cut out and soaked in 100µl 0.3M NaCl at 37°C overnight. The extracted dsDNA products were purified with CENTRI-SEP columns (PRINCETON separations, CS-901).

Sequencing

The concentrations of gel purified samples were determined with Kapa library quantification kit for Illumina® libraries (KK4845) on Roche LightCycler 480. Paired-end Illumina® sequencing was performed on an Illumina® MiSeq system using the kit v2 with 300 cycles (150bp PE sequencing) at the Georgia Genomics and Bioinformatics Core, University of Georgia, Athens, GA, USA.LOOPER fidelity was analyzed using the program *analooper*: https://github.com/HiliLab/analooper.

Supporting Tables

buffer	control	A	B	C	D	E	F	G
Tris-HCl (66mM)	pH 7.6	pH 6.9	pH 8.5	pH 7.6	pH 7.6	pH 7.6	pH 7.6	pH 7.6
PEG6000	6%	6%	6%	0	6%	6%	6%	6%
Mg^{2+}	10mM	10mM	10mM	10mM	5mM	15mM	10mM	10mM
ATP	25μM	25μM	25μM	25μM	25μM	25μM	250μM	1mM

Table S1 Various buffer conditions for T4 DNA ligase

buffer	control	T3_A	T3_B	T3_D	T3_E	T3_F	T3_G
Tris-HCl (50mM)	pH 7.5	pH 6.9	pH 8.5	pH 7.5	pH 7.5	pH 7.5	pH 7.5
PEG6000	0	0	0	0	0	0	0
Mg^{2+}	10mM	10mM	10mM	5mM	15mM	10mM	10mM
ATP	1mM	1mM	1mM	1mM	1mM	250μM	25μM
yield	65%	64%	60%	66%	62%	60%	65%
fidelity	61.4%	66.9%	57.5%	58.3%	60.9%	56.0%	58.3%

Table S2 Yield and fidelity of various buffer conditions for T3 DNA ligase

	building block (5'->3')	codon (5'->3')	total counts	mismatched counts
1	CAA	TTG	3704	89
2	CAC	GTG	5327	117
3	CAG	CTG	2417	44
4	CAT	ATG	2967	225
5	CCA	TGG	6372	160
6	CCC	GGG	10446	211
7	CCG	CGG	5197	37
8	CCT	AGG	5053	200
9	CGA	TCG	3026	107
10	CGC	GCG	4762	90
11	CGG	CCG	2193	39
12	CGT	ACG	2368	311
13	CTA	TAG	3709	46
14	CTC	GAG	4656	80
15	CTG	CAG	2887	29
16	CTT	AAG	2956	61
17	TAA	TTA	2954	106
18	TAC	GTA	3987	143
19	TAG	CTA	1965	42
20	TAT	ATA	2569	121
21	TCA	TGA	4340	116
22	TCC	GGA	5882	129
23	TCG	CGA	3107	33
24	TCT	AGA	2851	64
25	TGA	TCA	2684	68
26	TGC	GCA	3479	68
27	TGG	CCA	1789	21
28	TGT	ACA	2185	136
29	TTA	TAA	2792	39
30	TTC	GAA	3107	62
31	TTG	CAA	2041	26
32	TTT	AAA	2202	50
Total counts			115974	3070
Fidelity				97.4%

Table S3 Example of fidelity analysis results (NH_2-YNN codon sets)

Supporting Figures

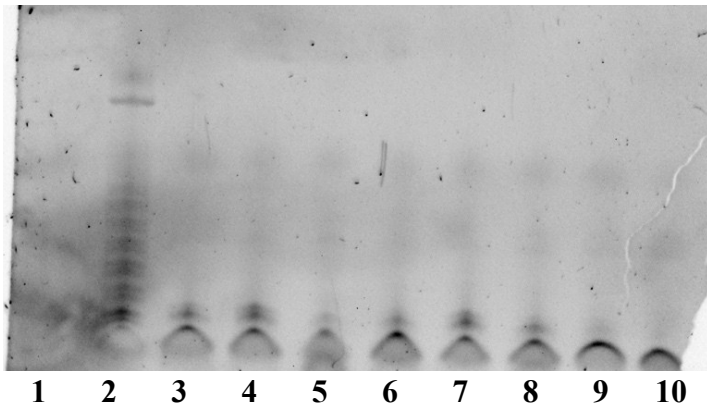


Figure S1 T4 DNA ligase-catalyzed polymerization with A*NNN using FAM labeled primers: Lane1: template without FAM primer; Lane2: ANNN in control buffer; Lane3: NH_2-ANNN in control buffer; Lane4: NH_2-ANNN pH6.9; Lane5: NH_2-ANNN pH8.5; ; Lane6: NH_2-ANNN without PEG; ; Lane7: NH_2-ANNN 5mM Mg^{2+}; Lane8: NH_2-ANNN 15mM Mg^{2+}; Lane9: NH_2-ANNN 250μM ATP; Lane10: NH_2-ANNN 1mM ATP

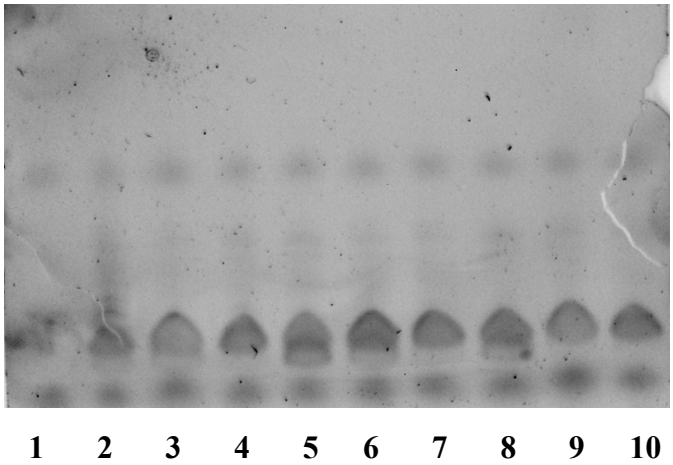


Figure S2 T4 DNA ligase-catalyzed polymerization with A*NN using FAM labeled primers: Lane1: template without FAM primer; Lane2: ANN in control buffer; Lane3: NH_2-ANN in control buffer; Lane4: NH_2-ANN pH6.9; Lane5: NH_2-ANN pH8.5; ; Lane6: NH_2-ANN without PEG; ; Lane7: NH_2-ANN 5mM Mg^{2+}; Lane8: NH_2-ANN 15mM Mg^{2+}; Lane9: NH_2-ANN 250μM ATP; Lane10: NH_2-ANN 1mM ATP

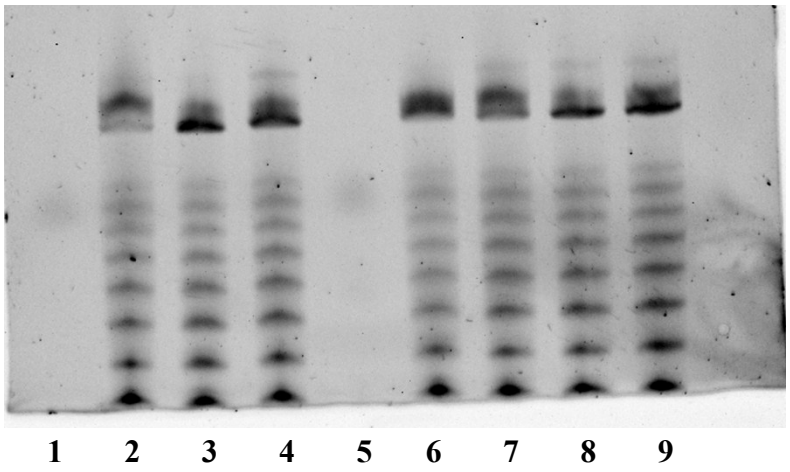


Figure S3 T3 DNA ligase-catalyzed polymerization with NNNNN codon sets using FAM labeled primers: Lane1: template without FAM primer; Lane2: control buffer; Lane3: pH6.9; Lane4: pH8.5; Lane5: 5% PEG; Lane6: 5mM Mg^{2+}; Lane7: 15mM Mg^{2+}; Lane8: 250μM ATP; Lane9: NNNNN 1mM ATP

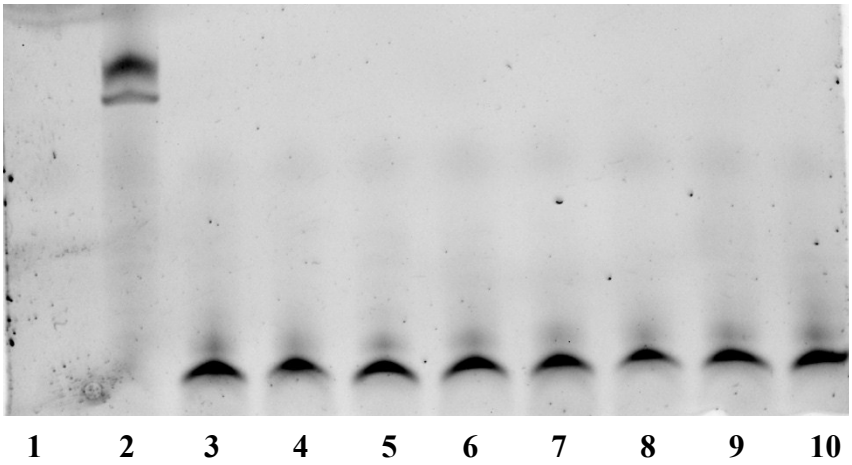


Figure S4 T3 DNA ligase-catalyzed polymerization with A*NN codon sets using FAM labeled primers: Lane1: template without FAM primer; Lane2: ANN in control buffer; Lane3: NH_2-ANN in control buffer; Lane4: NH_2-ANN pH6.9; Lane5: NH_2-ANN pH8.5; ; Lane6: NH_2-ANN with PEG; ; Lane7: NH_2-ANN 5mM Mg^{2+}; Lane8: NH_2-ANN 15mM Mg^{2+}; Lane9: NH_2-ANN 250μM ATP; Lane10: NH_2-ANN 1mM ATP

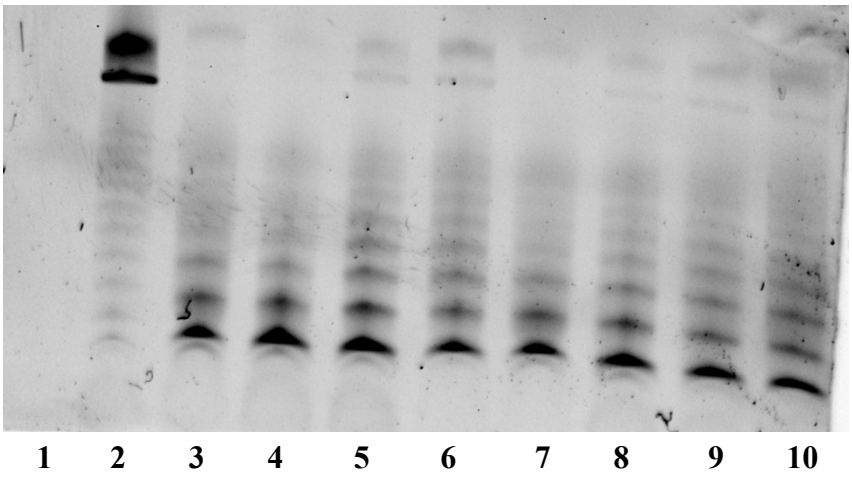


Figure S5 T3 DNA ligase-catalyzed polymerization with A*NNN codon sets using FAM labeled primers: Lane1: template without FAM primer; Lane2: ANNN in control buffer; Lane3: NH_2-ANNN in control buffer; Lane4: NH_2-ANNN pH6.9; Lane5: NH_2-ANNN pH8.5; ; Lane6: NH_2-ANNN with PEG; ; Lane7: NH_2-ANNN 5mM Mg^{2+}; Lane8: NH_2-ANNN 15mM Mg^{2+}; Lane9: NH_2-ANNN 250μM ATP; Lane10: NH_2-ANNN 1mM ATP