Improved Ligation Specificity with Chemically Modified Ligation Components

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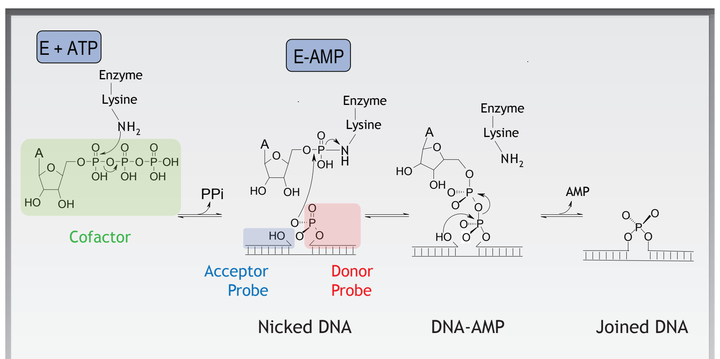
Abstract

Ligases are gaining utility in molecular biology applications, such as nucleotide sequence detection, single nucleotide polymorphism (SNP) detection, protein detection and “next generation” sequencing by ligation. With the increased demand for DNA ligases in the field of biotechnology, comes increased demand for ligation fidelity. Described approaches to improved ligation fidelity include ligases from different biological sources, point mutations of key amino acid residues within the ligase, modified reaction conditions and addition of crowding reagents, such as PEG. Although most approaches to improved ligation fidelity have focused on the ligase itself, further improvements are needed and may be attainable by a different approach. Herein a strategy to improve the discrimination between matched and mismatched targets is described which employs chemical modification to the nucleic acid components of the reaction, such as the donor probe, the acceptor probe and the ATP cofactor. The results demonstrate that chemically modified components increase the stringency of DNA ligase-mediated nucleic acid detection, providing a unique approach for SNP genotyping.

Figure 1

Proposed approach for improved DNA ligase specificity

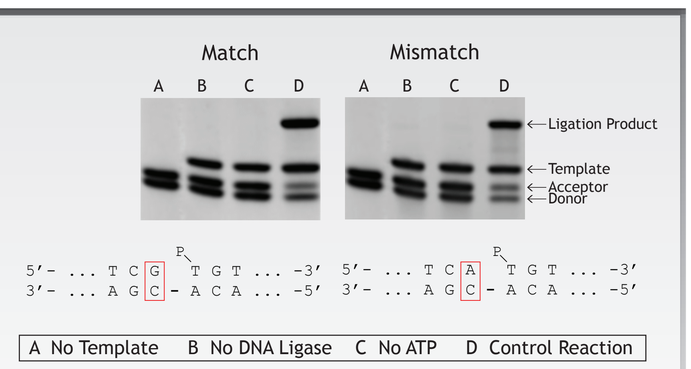


Three Potential Components to Modify:

1) ATP Cofactor 2) Acceptor Probe 3) Donor Probe

Figure 2

Limitations of T4 DNA ligase in mismatch discrimination

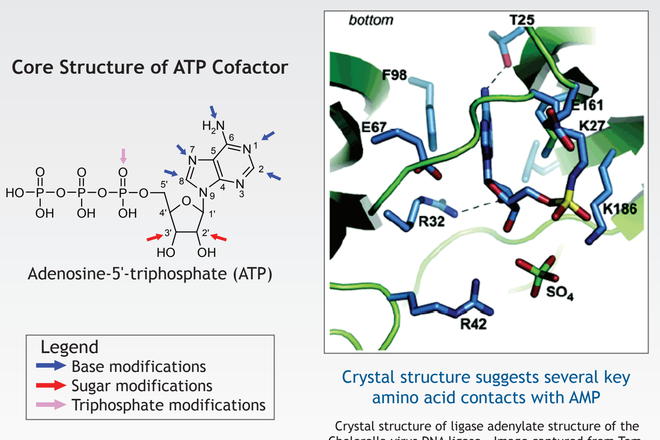


The strong propensity for T4 DNA ligase to join mismatched ligation probes necessitates a screening of modified ligation components for improved stringency of ligation

Ligation conditions: 1X Ligase Buffer (50 mM Tris (pH 7.5), 10 mM DTT, 10 mM $MgCl_2$), Ligation Probes (1 µM), ssDNA Template (1 µM), ATP analogs (1 mM), T4 DNA Ligase (0.5 Weiss units), 20 µL. Thermal cycling conditions: 95ºC (3 min), 4ºC (3 min), add Ligase, 22ºC (60 min), 65ºC (10 min).

Figure 3

Design of chemically modified ATP Cofactors



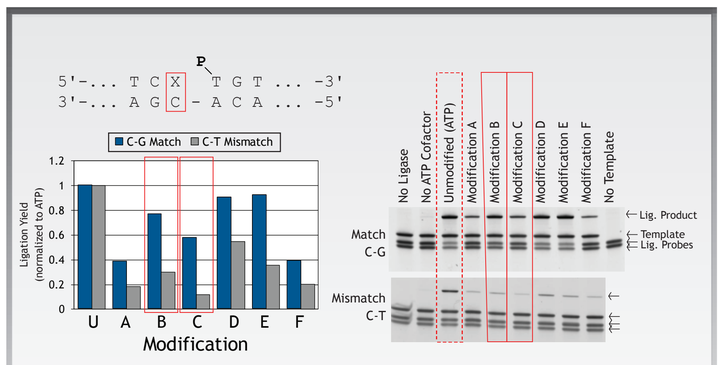
Crystal structure suggests several key amino acid contacts with AMP

Crystal structure of ligase adenylate structure of the Cholorella virus DNA ligase. Image captured from Tomkinson et. al. Chem Rev. (2006) 106, 687-99.

Chemical modifications to the cofactor may influence interactions at key ligase contacts to improve ligation fidelity

Figure 4

Evaluation of chemically modified cofactor analogs

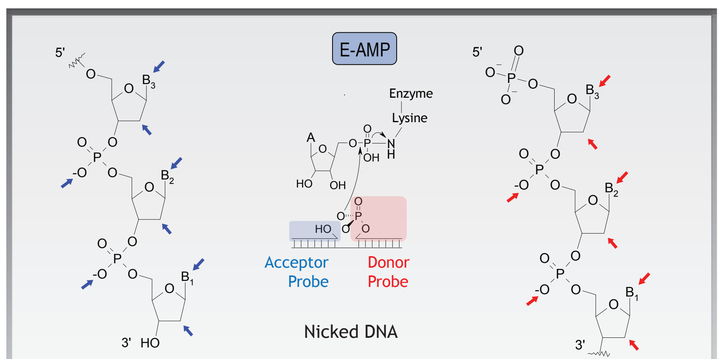


Modified Cofactors B and C provide robust ligation yield and improved match versus mismatch discrimination

Ligation conditions: 1X Ligase Buffer (50 mM Tris (pH 7.5), 10 mM DTT, 10 mM $MgCl_2$), Ligation Probes (1 µM), ssDNA Template (1 µM), ATP analogs (1 mM), T4 DNA Ligase (0.5 Weiss units), 20 µL. Thermal cycling conditions: 95ºC (3 min), 4ºC (3 min), add Ligase, 22ºC (20 min), 65ºC (10 min).

Figure 5

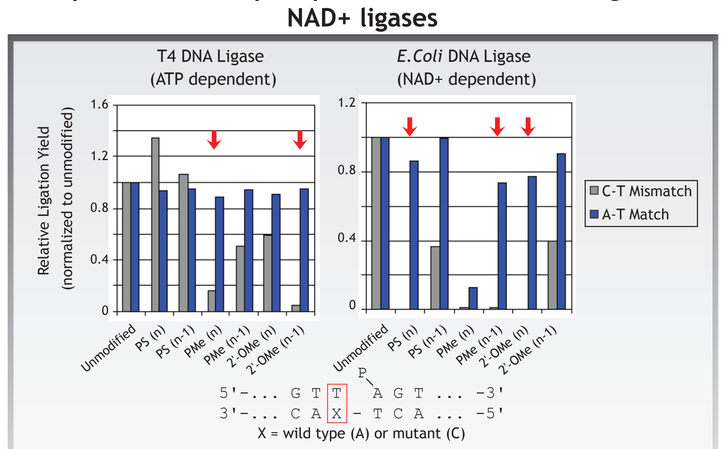
Acceptor and donor modifications evaluated



Chemical modifications to the base, sugar, or phosphate of the donor and acceptor probes were evaluated for their influence on improved mismatch discrimination

Figure 6

Comparison of acceptor probe modifications using ATP or NAD+ ligases



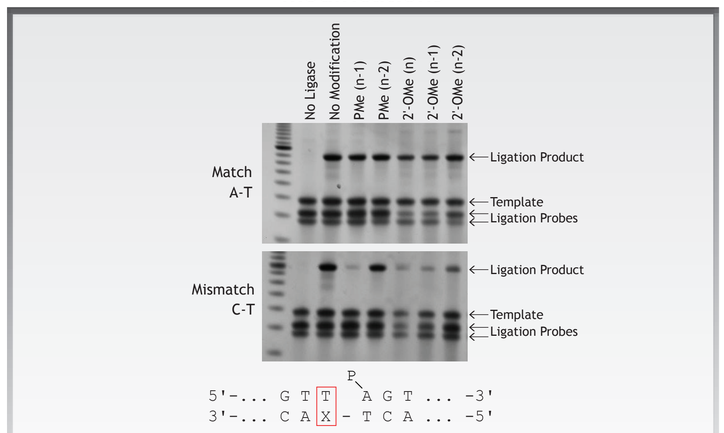
X = wild type (A) or mutant (C)

Different lead acceptor probe modifications identified depending on the DNA ligase family (ATP or NAD+)

Ligation conditions: 1X Ligase Buffer (50 mM Tris (pH 7.5), 10 mM DTT, 10 mM $MgCl_2$, 1 mM ATP), Ligation Probes (1 µM), ssDNA Template (1 µM), (0.5 Weiss units T4 DNA Ligase or 5 Units *E.Coli* DNA Ligase), 20 µL. T4 DNA Ligase Thermal cycling conditions: 95ºC (3 min), 4ºC (3 min), add Ligase, 22ºC (20 min), 65ºC (10 min). *E.Coli* DNA Ligase Thermal cycling conditions: 95ºC (3 min), 4ºC (3 min), add Ligase, 16ºC (20 min), 65ºC (20 min).

Figure 7

Investigation of chemically modified donor probe modifications



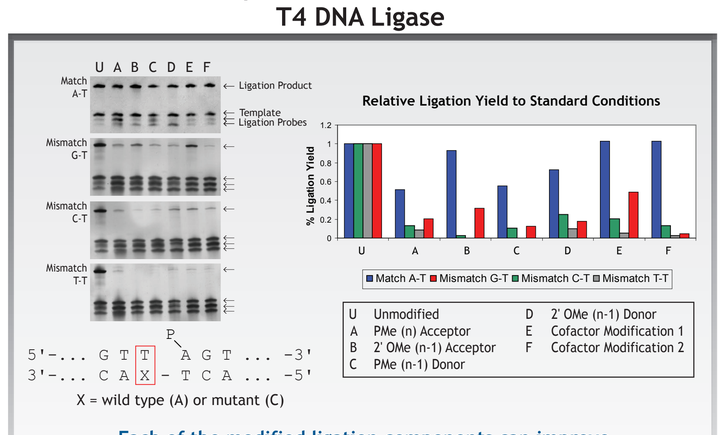
X = wild type (A) or mutant (C)

Several candidate modifications to the donor probe decrease mismatch ligation

Ligation conditions: 1X Ligase Buffer (50 mM Tris (pH 7.5), 10 mM DTT, 10 mM $MgCl_2$), Ligation probes 1 µM, ssDNA Template 1 µM, ATP 1 mM, T4 DNA Ligase 2 Units, 20 uL Thermal Cycling Conditions: 95°C (3min), 4°C (3 min), add Ligase, 16°C (20 min), 65°C (10 min)

Figure 8

Evaluation of lead probe and cofactor modifications with T4 DNA Ligase



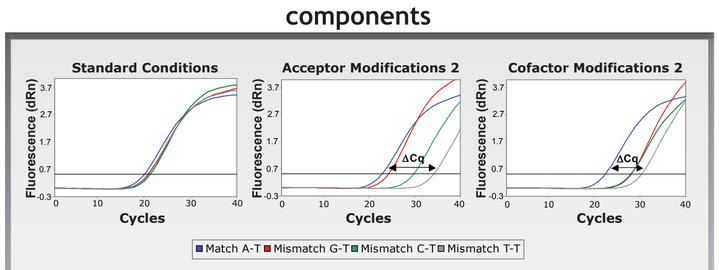
X = wild type (A) or mutant (C)

Each of the modified ligation components can improve discrimination between match and mismatched targets

Ligation conditions: 1X T4 Ligase buffer (50 mM Tris (pH 7.5), 10 mM DTT, 10 mM $MgCl2$), Ligation probes (1 µM), ATP analogs (1 mM), ssDNA template (1 µM), T4 DNA Ligase (0.5 Weiss units), 20 µL. Thermal cycling conditions: 22°C (1 hour), 65°C (10 min).

Figure 9

Real-time ligation-PCR using chemically modified ligation components



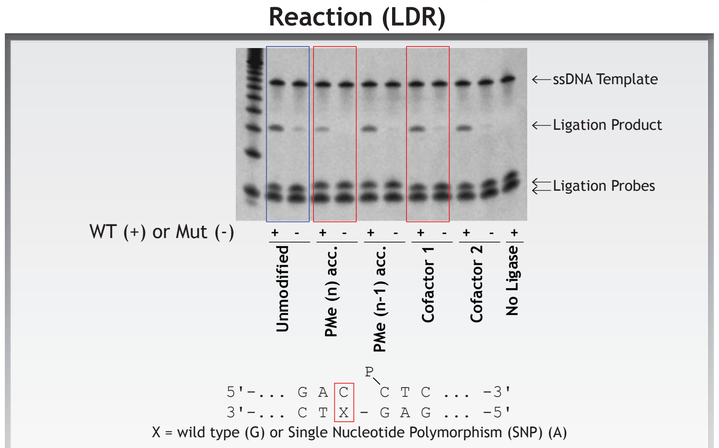
	Match A-T		Mismatch G-T		Mismatch C-T		Mismatch T-T	
	Cq (dRn)	ΔCq	Cq (dRn)	ΔCq	Cq (dRn)	ΔCq	Cq (dRn)	ΔCq
Standard Conditions	20.15	0	20.96	1	21.48	1	21.10	1
Acceptor Modifications 2	22.97	0	24.40	4	29.84	40	34.31	14
Cofactor Modifications 2	22.52	0	28.14	8	28.09	8	30.45	10

Chemically modified ligation components increase ΔCq's between matched and mismatched targets compared to standard conditions

PCR conditions: 1X PCR buffer (20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$), CleanAmp™ Precision Primers (0.1 µM), 0.2 mM dNTPs, 5 µL Ligation product (Diluted 10^5), 1.25 U IVGN *Taq* DNA polymerase, SYTO®9 Green (2 µM), ROX (0.03 µM), 25 µL. Thermal cycling conditions: 95ºC (10 min); [95ºC (30 sec), 56ºC (30 sec), 72ºC (1min)] 40X.

Figure 10

Cystic Fibrosis SNP detection by Ligase Detection Reaction (LDR)



X = wild type (G) or Single Nucleotide Polymorphism (SNP) (A)

Chemically modified components improve mismatch discrimination in LDR

LDR conditions: 1X Ligase Buffer (20 mM Tris (pH 7.5), 20mM KCl, 1 mM DTT, 10 mM MgCl2, 0.1% IGEPAL®), 0.01 mM Cofactors, 0.50 µM Donor probe, 0.25 µM Acceptor probe, ssDNA template (50 nM), 4 Units *Pfu* DNA Ligase, 20 µL. Cycling conditions: 94°C (2 min), Cycle 20x [94°C (30 sec), 65°C (4 min)]

Conclusion

1) Chemical modifications made to the ATP cofactor, donor probes and acceptor probes provide improved ligation specificity for T4 DNA ligase.

2) Optimal chemical modifications to acceptor probes vary depending on the DNA ligase family (ATP or NAD+).

3) Cofactor modifications provide the greatest improvement to ligation fidelity of T4 DNA ligase.

4) The lead ATP cofactor demonstrated the most consistent increase in ΔCq’s in real-time PCR.

5) LDR reactions employing chemically modified ligation components yield more specific match vs. mismatch discrimination.

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