

Directed assembly of DNA molecules via simultaneous ligation and digestion

Gregory J. Cost and Nicholas R. Cozzarelli
University of California-Berkeley, Berkeley, CA, USA

BioTechniques 42:84-89 (January 2007)
doi 10.2144/000112283

DNA ligation is a routine laboratory practice, yet the yield of the desired product is often very low due to competing off-pathway reactions. The sensitivity of subsequent manipulations (e.g., selection via bacterial transformation) often obviates the need for a high yield of correctly ligated products. However, the ability to perform high-yield, preparative-scale DNA ligations would benefit a number of downstream applications ranging from standard molecular cloning to biophysics and DNA computing. We describe here a ligation technique that specifically converts off-pathway ligation products back into substrate. We term this second-chance strategy enzymatic ligation assisted by nucleases (ELAN) and demonstrate the ordered assembly of four DNA fragments via simultaneous ligation and digestion in the presence of eight restriction enzymes. Use of ELAN increased the yield of the desired product by more than 30-fold.

INTRODUCTION

Enzymes lower the activation energies of chemical reactions, inherently catalyzing both the forward and reverse reactions. Many enzymes consume energy during catalysis. This is true of T4 DNA ligase, which converts ATP into pyrophosphate and a covalent AMP-enzyme intermediate, making the reverse reaction energetically unfavorable (1). Energetic considerations are sufficient to prevent reversal of ligation (nicking), except with high enzyme and AMP concentrations (1). In addition, ligation reversal at the same phosphodiester bond is confounded by dissociation of the ligase-DNA complex. Even in the absence of an energetic barrier, rebinding of the ligase to the exact site of prior ligation is simply unlikely.

In the forward reaction, two ligatable DNA ends can be made by the same restriction enzyme (identical ends) or by different enzymes that produce different ends but have compatible overhangs (different-but-compatible ends). Ligation of identical ends recreates the site for the enzyme used to create the end; ligation of different-but-compatible ends does

not recreate the site of either enzyme. If the desired ligation product results from the joining of different-but-compatible ends, then ligation in the presence of both restriction enzymes used to make the reactant fragments will convert unwanted identical-end

ligation products back into substrate molecules. Thus, only the desired product will accumulate. We term this technique enzymatic ligation assisted by nucleases (ELAN). ELAN permits the exact reversal of ligation and is specific for unwanted off-pathway molecules.

MATERIALS AND METHODS

The DNA fragment used in Figure 1 is a 3242-bp piece of pRS303 cut with *Bgl*II and *Bam*HI. The ELAN reaction in Figure 1 contained 20 U *Bam*HI, 10 U *Bgl*II, 400 U T4 ligase, 1 mM ATP, 100 µg/mL bovine serum albumin (BSA), 100 mM NaCl, 10 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, and 1 mM dithiothreitol (DTT). Fragment A is a 1066-bp piece of pGC255 cut with *Spe*I and *Pst*I; fragment B is a 1755-bp piece of pGC238 cut with *Nsi*I and *Bam*HI; fragment C is a 1567-bp piece of pGC228 cut with *Bgl*II and *Xho*I; and fragment D is a 3520-bp piece of pACYC184 cut with *Sal*I and *Xba*I. Equimolar amounts of each fragment comprising a total of 1.5 µg were used in the ligation. The sequences of the fragments used are available upon request. We normalized the amount

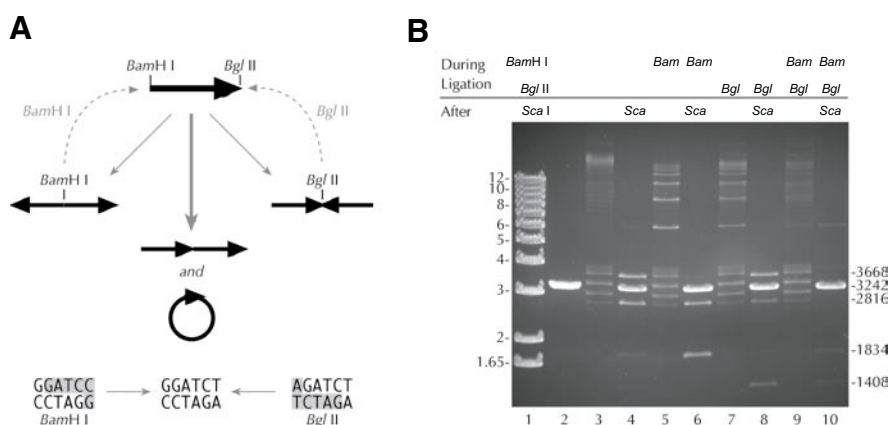


Figure 1. (A) Enzymatic ligation assisted by nucleases (ELAN). The end of a DNA fragment cut with *Bam*HI and *Bgl*II (black arrows) has a 50% chance of making a *Bam*HI-*Bgl*II junction and a 25% chance of making either identical-end junction. Self-ligation will produce a circular molecule. Restriction digestion converts identical-end ligation products back into substrate (dashed gray arrows). (B) ELAN reaction. Lane 1 contains molecular weight standards annotated in kilobase pairs on the left of the gel. Lane 2 contains only the substrate molecule. The ligation diagrammed in panel A was performed in the presence of neither (lane 3), either (lanes 5 and 7), or both restriction enzymes (lane 9). The reaction was heat-inactivated, and half of the reaction was digested with *Sca*I (lanes 4, 6, 8, and 10). *Sca*I digestion of *Bam*HI-*Bam*HI junctions produces a 3668-bp fragment; *Bam*HI-*Bgl*II junctions a 3242-bp fragment; *Bgl*II-*Bgl*II junctions, a 2816-bp fragment. A minor amount of *Bgl*II-*Bgl*II ligation occurred in the presence of *Bgl*II (lane 8, 2816-bp band). Digestion of the unligated ends of the high molecular weight arrays produces 1834- and 1408-bp bands.

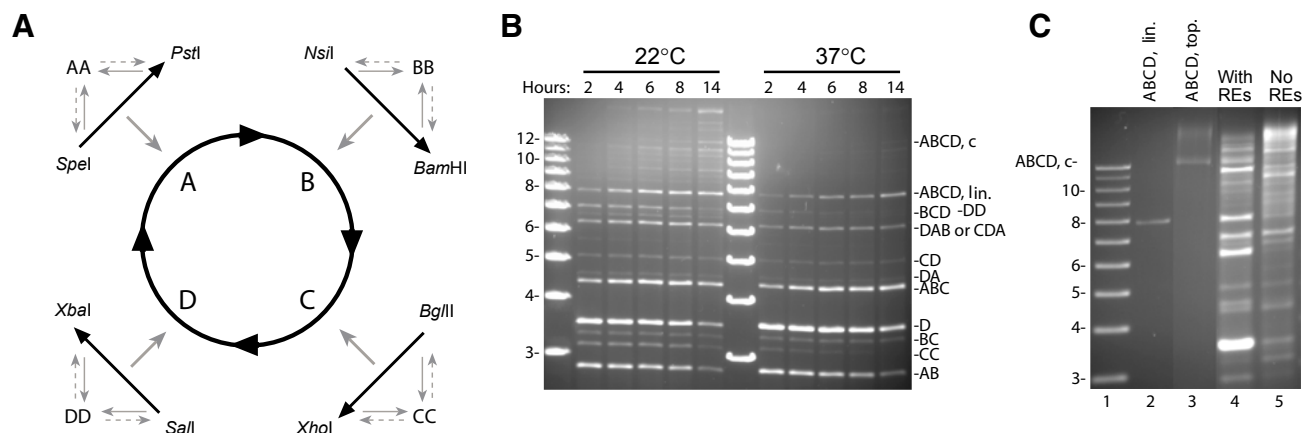


Figure 2. Multi-piece enzymatic ligation assisted by nucleases (ELAN) reaction. (A) The eight restriction enzyme ELAN reaction forms the DNA circle ABCD. Each fragment can ligate two ways with an identical end (thin gray arrows), and two ways with different-but-compatible ends (thick gray arrow). Ligation to identical ends is reversed by restriction digestion (dashed gray arrows). The restriction enzymes that created the fragment ends are shown. (B) The reaction diagrammed in panel A was performed at 22° and 37°C for 2, 4, 6, 8, or 14 h. The size of the molecular weight standards is shown in kilobase pairs on the left of the gel, with the fragment assignments on the right. Assignments were made by comparison of the predicted and computer-determined molecular weights for each band and digestion of the 7924-bp ABCD linear band. The predicted size of the CDA intermediate is 6169 bp; the DAB intermediate is 6353 bp; the computer-determined molecular weight of this fragment is 6243 bp. This band may be a mixture of co-electrophoresing fragments, or perhaps the steady-state level of one intermediate is not detectable. ABCD, 1 indicates the linear form of ABCD; ABCD, c indicates the circular topoisomers. (C) The linearized ABCD plasmid has the expected size 7924 bp (lane 2). Circular topoisomers of this plasmid (lane 3) co-migrate with a prominent band produced in the ELAN reaction in lane 4. Lane 5 is identical to lane 4, but without the addition of restriction enzymes during ligation. ABCD, c indicates the circular topoisomers of ABCD in lane 3. Very high molecular weight concatamers of the ABCD plasmid are also present in this lane. RES, restriction enzymes.

of restriction enzyme added by its activity in 100 mM NaCl at 37°C and its half-life. The ELAN reactions in Figure 2 contained 40 U *Bam*HI, 10 U *Bgl*III, 20 U *Sal*I, 20 U *Xho*I, 20 U *Nsi*I, 20 U *Pst*I, 80 U *Xba*I, 40 U *Spe*I, 1200 U T4 ligase, 1 mM ATP, 100 µg/mL BSA, 100 mM NaCl, 10 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, and 1 mM DTT in a volume of 200 µL. All enzymes were from New England Biolabs (Ipswich, MA, USA).

The topoisomer ladder was formed by digestion of the circular ABCD plasmid with *Eag*I, heat-inactivation of the restriction enzyme, and addition of 400 U T4 ligase and ATP to 1 mM. Both the ELAN reaction in Figure 2C and formation of the topoisomer ladder were done at 22°C. Electrophoresis in Figure 1 was performed in a 1% agarose gel; in Figure 2, 0.8% agarose gels were used. Electrophoresis was at 15–20 V/cm in 1× Tris-Taurine-EDTA with buffer recirculation.

RESULTS AND DISCUSSION

Figure 1A diagrams a simple ELAN reaction with a DNA fragment cut with *Bam*HI and *Bgl*III. *Bam*HI and *Bgl*III

generate different-but-compatible ends. Ligation is possible between both identical ends and between the different-but-compatible ends, but identical-end products will be converted back into substrate by *Bam*HI and *Bgl*III digestion. This ligation is performed in Figure 1B. Standard ligation of the *Bam*HI- and *Bgl*III-cut DNA fragment in lane 2 produced a high molecular weight product array consisting of a mixture of identical-end and different-but-compatible-end junctions (Figure 1B, lane 3, >12 kb). In addition, circularization produced a distribution of topoisomers migrating near the starting fragment (Figure 1B, lane 3, 2.8–3.8 kb range). When either *Bam*HI or *Bgl*III was added to the reaction to eliminate ligation of their respective identical ends, the size of the reaction products was reduced, and multiples of the starting fragment became apparent (Figure 1B, lanes 5 and 7). The same was true when both enzymes were included during ligation (Figure 1B, lane 9). The larger ligation products in this reaction are less distinct, presumably because of circularization.

The internal structure of the product arrays should be random with respect to the orientation of

its constituent fragments in the absence of ELAN and ordered when ligated with ELAN. The fragment used in Figure 1B contains a unique, asymmetrically positioned *Sca*I site. Postligation digestion of the products with *Sca*I will produce a 3668-bp band from each *Bam*HI-*Bam*HI junction, a 3242-bp band from each *Bam*HI-*Bgl*III junction, and a 2816-bp band from each *Bgl*III-*Bgl*III junction. When ligation was performed in the absence of restriction enzymes, *Sca*I digestion revealed that the array was randomly ordered, containing equal amounts of both identical-end junctions, as indicated by the equal amounts of 3668- and 2816-bp bands (Figure 1B, lane 4). (The amount of *Bam*HI-*Bgl*III product in the high molecular weight array is obscured by the abundance of monomer *Bam*HI-*Bgl*III circles.) Inclusion of *Bam*HI or *Bgl*III in the ligation specifically removed the appropriate identical-end junction from the products (Figure 1B, lanes 6 and 8). When both restriction enzymes were present during ligation, only an ordered array of *Bam*HI-*Bgl*III products formed (Figure 1B, lane 10).

Further evidence of order in the product arrays came from analysis

of the ends of the arrays. *ScaI* cutting produces 1408- and 1834-bp products from unligated *BglII* and *BamHI* ends. In both ordered and random arrays, there were equal amounts of both 1408- and 1834-bp bands (Figure 1B, lanes 4 and 10). Biasing the array order by inclusion of only one restriction enzyme biased the composition of the end fragments (Figure 1B, lanes 6 and 8).

The real advantage of using ELAN comes from extension of this two restriction enzyme reaction to multiple enzyme reactions, allowing for the one-tube assembly of DNA molecules that would otherwise require a series of laborious stepwise reactions and intermediate low-yield purifications. Figure 2A diagrams the ELAN strategy used in Figure 2B to assemble four separate fragments (A, B, C, and D) in the presence of eight restriction enzymes. The four piece ligation was performed at both 22° and 37°C and sampled at intervals of 2 h. This kinetic analysis revealed the accumulation of intermediate ligation products (Figure 2B). All possible correct intermediates were visible but one (the CDA fragment may overlap the DAB band); only two incorrect intermediates were visible. The abundance of correct intermediates increased with time, whereas the levels of the unwanted CC and DD products decreased with time. Due to the different temperature optima of T4 ligase (16°C) and the restriction enzymes (37°C), ligation at 22°C favored yield over specificity, and ligation at 37°C favored specificity over yield (Figure 2B).

To ensure that the band assignments in Figure 2B were correct, we analyzed the putative ABCD linear intermediate by restriction enzyme digestion. *ApaI* cuts once in fragment B, and *ScaI* cuts once in fragments A, C, and D. Digestion with both enzymes will produce a unique fragment from each of the four correct junctions. Digestion of the excised 7924-bp band by *ApaI* and by *ApaI* + *ScaI* gave the products expected of all four linear permutants of ABCD (data not shown).

The final product of the ligation in Figure 2A is a circular molecule. Similar to the circular molecules formed from self-ligation in Figure

1, circular ABCD molecules will be present in multiple topological forms. ABCD topoisomers (Figure 2C, lane 3) co-migrated with a prominent band from an ELAN reaction (Figure 2C, lane 4), unambiguously identifying the final product of ligation. Only a high molecular weight smear of products is produced when ligation was performed without the restriction enzymes (Figure 2C, lane 5).

Fragment D contains an *Escherichia coli* origin of replication and a chloramphenicol resistance gene. The presence of these in the ABCD plasmid allowed quantitation of the increase in ligation efficiency attributable to ELAN. Transformation of bacteria with DNA ligated without (Figure 2C, lane 5) or with ELAN (Figure 2C, lane 4) resulted in 320 and 4370 colony-forming units (cfu)/μg on chloramphenicol-containing plates, respectively. Transformation with fragment D alone gave 580 cfu/μg. Examination of plasmids from individual colonies showed that all (20/20) resulting from the ELAN ligation were correct ABCD molecules (data not shown). In contrast, only 9/20 plasmids from the standard ligation were correct ABCD molecules (data not shown). When combined, the increased number of colonies and the higher fraction that contain correct ABCD plasmids indicate that ELAN produced about a 30-fold enhancement of correct ligation.

Ligation of a fifth fragment to a linear four-piece molecule instead of circularization produces irreversibly off-pathway molecules. Such products can be seen in the 8–12 kb range in Figure 2, B and C, and account for 22% of ligation products. Seventy-eight percent of the ligated molecules are either the correct ligation product or other on-pathway precursors. These reactions are unavoidable, but can be minimized by ligation at low DNA concentrations.

Table 1 lists restriction enzyme pairs suitable for ELAN. The table is not meant to be an exhaustive list of compatible ends, but rather a guide to enzyme pairs likely to work well together.

Our technique, ELAN, achieves one-step, preparative-scale assembly of DNA molecules. Several technical

points of ELAN are worthy of mention. First, the quality of the fragment ends must be very high. Ligation with ELAN destroys unwanted products by recutting those formed from identical ends. While the large majority of non-cutable junctions are produced by ligation of different-but-compatible ends, ligation of degraded or chewed-back molecules will not be reversed. This nonreversal, plus an inability to ligate degraded molecules, will poison the assembly of molecules with multiple precursors. For example, if 5% of ends are unligatable, the theoretical yield of the ABCD ligation drops to only 66% (0.95⁸). As damaged-end linear ABCD molecules will not circularize, the nondamaged end will further depress yield by consuming substrate to form greater than four piece molecules. The end quality produced by a restriction enzyme can be measured by the ability to ligate and recut the end. Table 1 lists the fold overdigestion possible while retaining the ability to ligate and recut >95% of ends.

The success of ELAN is critically dependent on the competition between digestion and ligation. In principle, the activity of the restriction enzymes should be as high as possible. An excess of ligation activity will produce molecules that are incorrectly assembled. The balance of ligation and digestion is determined by the amount of each enzyme used, the activity and half-life of each enzyme, and the reaction temperature. Table 1 lists the activity of each enzyme under each buffer condition, the number of units of each enzyme required to give complete digestion of 1 μg DNA in 16 h, and the activity of each enzyme at 37°C. There are likely several combinations of these variables that will give satisfactory results. In reactions with many different enzymes, the maximum amount of enzymes usable may be constrained by the need to keep the final glycerol concentration below 5% to avoid restriction enzyme star activity.

Judicious choice of DNA fragments used in an ELAN reaction is imperative. Clearly, all restriction enzymes included in the reaction must cut only at the site of ligation. While we used suitable fragments of plasmids for

Short Technical Reports

ELAN, in practice it may be advantageous to PCR-amplify one common template devoid of relevant restriction sites with different primers containing the appropriate sites. Of the enzymes listed in Table 1, only *PstI* and *BspEI* are known to have any difficulty cutting near the very end of DNA.

We anticipate the ELAN technique will be most useful for the preparative-scale production of large DNA molecules in which selection by bacterial transformation

is not possible. In particular, many biophysical experiments require the generation of multi-piece derivatized molecules, a process that could be facilitated by use of ELAN (2). ELAN might also be used to speed the assembly of synthetic DNA fragments during gene construction (3). More speculatively, ELAN may find use in DNA computing. The products of the ELAN reactions in Figure 1B correspond to the logical operators AND (lane 4), XOR (lanes 6 and 8), and

NOR (lane 10). Coupling of ELAN with a fluorescence-resonance energy transfer (FRET) detection system appears well-suited for adaptation into current microfluidics-based DNA computation devices (4,5).

ACKNOWLEDGMENTS

This paper is dedicated in memoriam to N.R.C. Members of the Cozzarelli laboratory and Kristin

Table 1. Restriction Enzyme Pairs Suitable for Enzymatic Ligation Assisted by Nucleases (ELAN)

Four-Base Overhangs								
	<i>XhoI</i>	<i>SalI</i> ^a	<i>BamHI</i> ^a	<i>BglII</i>	<i>BclI</i> ^{b, 50}	<i>PstI</i>	<i>NsiI</i>	
Site	CTCGAG	GTCGAC	GGATCC	AGATCT	TGATCA	CTGCAG	ATGCAT	
Buffer 2	100	0	100	75	100	75	75	
Buffer 3	100	100	50	100	100	100	100	
End quality	10	50	40	50	200	50	–	
Half-life	0.13	0.13	0.5	0.25	0.5	0.5	0.25	
	<i>SpeI</i>	<i>XbaI</i> ^b	<i>AvrII</i>	<i>NheI</i>	<i>EcoRI</i> ^a	<i>MfeI</i> ^a	<i>MluI</i>	<i>BssHI</i> ⁷⁵
Site	ACTAGT	TCTAGA	CCTAGG	GCTAGC	GAATTC	CAATTG	ACGCGT	GCGCGC
Buffer 2	100	100	100	100	100	50	75	100
Buffer 3	25	75	50	10	100	10	100	100
End quality	20	20	100	100	100	20	10	100
Half-life	0.5	0.13	0.13	0.25	0.13	0.25	0.13	0.5
	<i>XmaI</i>	<i>AgeI</i>	<i>NgoMI</i>	<i>BspEI</i>	<i>BsrGI</i>	<i>Acc65I</i>	<i>BsiWI</i> ^{a, 50}	
Site	CCCGGG	ACCGGT	GCCGGC	TCCGGA	TGTACA	GGTACC	CGTACG	
Buffer 2	50	50	50	0	100	75	100	
Buffer 3	0	10	10	100	10	100	100	
End quality	10	20	20	10	20	2	–	
Half-life	0.5	1	0.13	0.13	0.13	0.5	0.13	
Two-Base Overhangs								
	<i>AclI</i>	<i>ClaI</i> ^b	<i>NarI</i>	<i>BstBI</i> ¹⁰	<i>PvuI</i>	<i>PacI</i>	<i>NdeI</i>	<i>AseI</i>
Site	AACGTT	ATCGAT	GGCGCC	TTCGAA	CGATCG	TTAATTAA	CATATG	ATTAAT
Buffer 2	10	50	75	50	75	75	100	75
Buffer 3	0	50	75	25	100	10	75	100
End quality	10	10	10	10	5	10	20	20
Half-life	0.5	0.5	1	0.13	–	1	0.13	0.13

Buffer 2 is the percent activity in 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), pH 7.9. Buffer 3 is the percent activity in 100 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9. End quality is the fold overdigestion possible while still giving >95% ligation and recutting. Half-life are units of enzyme required to digest 1 μg DNA in 16 h. Vertical lines indicate enzymes grouped by overhang. All data are from the 2005–2006 New England BioLabs catalog.

^aKnown to have star activity.
^bInhibited by methylation.
 Superscripted numbers are the percent activity at 37°C.

Robrock provided helpful comments on the manuscript. This work was funded by grant no. GM31657 to N.R.C. from the National Institutes of Health. G.J.C. was the Amgen Fellow of the Damon Runyon Cancer Research Foundation (grant no. 1702-02) and is currently funded via a Kirschstein National Research Service Award from the National Institutes of Health (NIH; F32 GM075645-01).

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. **Lehman, I.R.** 1974. DNA ligase: structure, mechanism, and function. *Science* 186:790-797.
2. **Bustamante, C., Z. Bryant, and S.B. Smith.** 2003. Ten years of tension: single-molecule DNA mechanics. *Nature* 421:423-427.
3. **Kodumal, S.J., K.G. Patel, R. Reid, H.G. Menzella, M. Welch, and D.V. Santi.** 2004. Total synthesis of long DNA sequences: synthesis of a contiguous 32-kb polyketide synthase gene cluster. *Proc. Natl. Acad. Sci. USA* 101:15573-15578.
4. **McCaskill, J.S.** 2001. Optically programming DNA computing in microflow reactors. *Biosystems* 59:125-138.
5. **Grover, W.H. and R.A. Mathies.** 2005. An integrated microfluidic processor for single nucleotide polymorphism-based DNA computing. *Lab Chip* 5:1033-1040.

Received 15 June 2006; accepted 22 August 2006.

Address correspondence to Gregory J. Cost, University of California-Berkeley, Department of Molecular and Cell Biology, 16 Barker Hall, Berkeley, CA 94720, USA. e-mail: gregcost@berkeley.edu

To purchase reprints of this article, contact: Reprints@BioTechniques.com