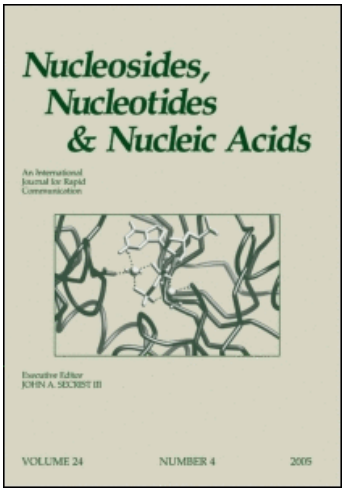

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Synthesis of DNA Dumbbells: Chemical vs. Enzymatic Ligation of Self-Complementary Oligonucleotides

Carol S. Lim[a]; C. Anthony Hunt[a]
[a] Departments of Biopharmaceutical Sciences and Pharmaceutical Chemistry, University of California, San Francisco, CA

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SYNTHESIS OF DNA DUMBBELLS: CHEMICAL VS. ENZYMATIC LIGATION OF SELF-COMPLEMENTARY OLIGONUCLEOTIDES

Carol S. Lim* and C. Anthony Hunt
Departments of Biopharmaceutical Sciences and Pharmaceutical Chemistry
University of California, San Francisco, CA 94143-0446

ABSTRACT: The chemical (cyanogen bromide) and enzymatic (T4 DNA ligase) ligation of five different self-complementary oligonucleotide sequences which form 14- or 16-base pair dumbbells are described and compared here. A review of both chemical and enzymatic methods is presented; an improved enzymatic method is described as well. While both methods of ligation are effective, chemical ligation may be preferred since it is faster and less costly.

INTRODUCTION

DNA dumbbells have historically been used as physical models for analyzing local thermal stability in DNA[1], for examining hairpins, cruciforms, and locally melted domains within naturally occurring DNA polymers[2], for studying DNA conformations as substrates for various enzymes[3,4] and for overcoming problems of double-strand oligomer dissociation when investigating nucleic acid drug targets [5]. In addition to their utility as physical models, DNA dumbbells have biological relevance as aptamers or decoys for trapping proteins such as transcription factors[6,7]. Synthesis of DNA dumbbells has been achieved by enzymatically ligating a self-complementary (intramolecularly annealing) phosphorylated oligonucleotide using T4 DNA ligase[1,2,7,8]. Another method of ligation stems from the chemical "template-directed cyclization" using cyanogen bromide described by Kool[9]. Template-directed polymerization of oligoadenylates on a poly (U) template was first described in 1986 by Kanaya and Yanagawa[10]. No template is required for our dumbbell-forming oligos since they self-anneal (i.e., form their own template). This paper describes and compares an optimized enzymatic method of ligation and a chemical (BrCN) method of ligation, and demonstrates proof of ligation.

RESULTS AND DISCUSSION

The sequences of the oligos used and their corresponding dumbbell structures are shown in TABLE 1. All oligos contain 4 T (thymidine) loops, with the exception of unligated 5 T-loop "e" and the 5 T-loop "e" dumbbell.

For either the chemical or enzymatic method of ligation, proof of synthesis (covalent closure or formation of the dumbbell duplex) can be verified by altered electrophoretic mobility of product [1,7,8,11] and resistance to various enzymes. These enzymes include exonucleases[2], single-strand specific nuclease (S1 nuclease)[7], alkaline phosphatase[2], or phosphodiesterase[7]. Also, recognition of duplex formation within the dumbbell can be verified by specific restriction enzyme cleavage[11]. Further proof of ligation is demonstrated by differences in melting temperature profiles between the ligated and unligated structures.

Chemically and enzymatically ligated dumbbells in FIGS. 1A and 1B migrate faster than their unligated counterparts for all sequences shown here (upper bands, unligated material; lower bands, ligated material). Upon ligation, these oligonucleotides form 16 (or 14, in one case) base-pair dumbbell structures.

Further proof of ligation is demonstrated by resistance to enzymatic degradation or by restriction enzyme cleavage (shown here for the 5 T-loop "e" sequence). The 5 T-loop "e" dumbbell was found to be more resistant to the exonuclease action of the Klenow fragment compared to its corresponding unligated sequence. After an 8-hour incubation with Klenow, the ligated sequence is unchanged while the unligated sequence is completely degraded (FIG. 2, lanes 2a and 2b, respectively). After 12 hours, the ligated sequence starts getting degraded as well (FIG. 2, lane 3a). The 5 T-loop "e" dumbbell was also degraded more slowly by S1 nuclease (a fairly single-strand specific nuclease) compared to the non-ligated sequence (FIG. 3). S1 nuclease however, will start degrading the loop ends of the dumbbell sequence (FIG. 3, lane 3a) after a 20 minute incubation.

The restriction enzyme *Mae* I cuts at "C↓TAG" (present in the 5 T-loop "e" dumbbell) and was used to show that the dumbbell DNA formed a cleavable, recognizable duplex. The "e" dumbbell DNA should be cleaved into 2 segments (a 15-mer and a 27-mer) by *Mae* I. Analysis of reaction products from *Mae* I treatment (FIG. 4, lane 1) does indeed show 2 smaller fragments. The smallest fragment (FIG. 4, lane 1, lower arrow) does not show up well since the intensity of staining by ethidium bromide is proportional to the size of the DNA[12].

In FIG. 5, shrimp alkaline phosphatase (SAP) cleaved the phosphate group from the phosphorylated unligated sequence, yet had no effect on the ligated dumbbell DNA. Two bands, phosphorylated and unphosphorylated (lane 2) are reduced to 1 band by SAP

TABLE 1. Oligonucleotides (underlined sequences are self-complementary).

unligated "a":

5'- TATACGGGTTTTCCCGTATACCACTCTGTTTTCAGAGTGG -3'

"a" dumbbell:

$_T{}^T$TCCCGTATACCACTCTGT$_T$
$^T{}_T$GGGCATATGGTGAGACT^T

unligated "b":

5'- TAACAACTTTTGTTGTTATAGTAACTTTTGTTACTA -3'

"b" dumbbell:

$_T{}^T$GTTGTTATAGTAACT$_T$
$^T{}_T$CAACAATATCATTGT^T

unligated "c":

5'- ATTTTTCCCTTTTGGGAAAAATTCCCCCCTTTTGGGGGGA -3'

"c" dumbbell:

$_T{}^T$GGGAAAAATTCCCCCCT$_T$
$^T{}_T$CCCTTTTTAAGGGGGGT^T

unligated "d":

5'- CTAGGGGTTTTCCCCTAGCAACAGATGTTTTCATCTGTTG -3'

"d" dumbbell:

$_T{}^T$CCCCTAGCAACAGATGT$_T$
$^T{}_T$GGGGATCGTTGTCTAC$_T{}^T$

unligated 5 T-loop "e":

5'- CTAGGGGTTTTTCCCCTAGCAACAGATGTTTTTCATCTGTTG -3'

5 T-loop "e" dumbbell:

$_T{}^T{}^T$CCCCTAGCAACAGATG$^T{}_T{}_T$
$^T{}_T{}_T$GGGGATCGTTGTCTAC$_T{}^T$

(lane 3). SAP has no effect on the dumbbell sequence (lanes 4 and 5). The results of these "proof of ligation" reactions are not affected by the impurities (shorter, failed sequences) which appear in the purchased, non-HPLC purified starting oligo as "smears" below main band (in FIGS. 2-5).

Lastly, for proof of ligation, a melting temperature experiment comparing purified unligated vs. ligated dumbbell was performed. As shown in FIG. 6, the unligated and the ligated dumbbell structures (shown for sequence "d") clearly have different melting temperature profiles, suggesting that they are structurally different. The melting profile of the ligated dumbbell structure exhibits a cleaner transition and little or no hysteresis, compared to the unligated structure, as expected.

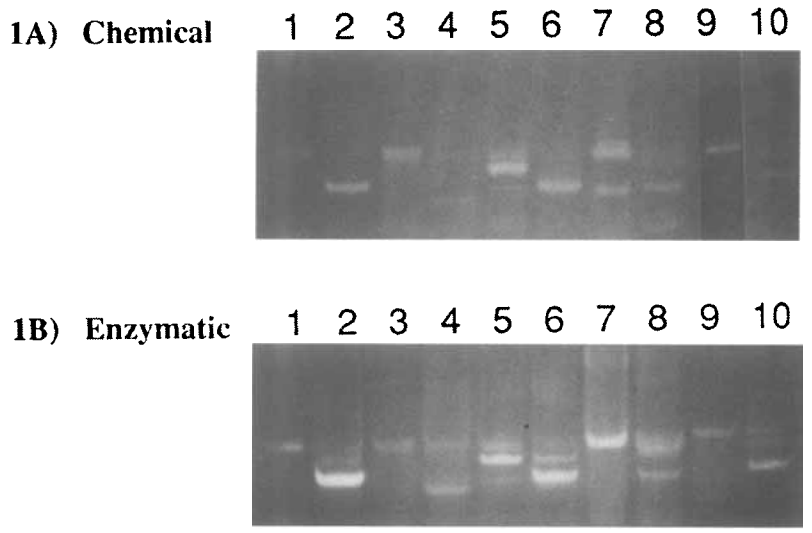

FIG. 1A (chemical ligations) and **FIG. 1B** (enzymatic ligations).
(1) unligated "a;" (2) ligated "a" dumbbell.
(3) unligated "b;" (4) ligated "b" dumbbell.
(5) unligated "c;" (6) ligated "c" dumbbell.
(7) unligated "d;" (8) ligated "d" dumbbell.
(9) unligated 5 T-loop "e;" (10) ligated 5 T-loop "e" dumbbell.
In all, upper band is unligated material; lower band is ligated material.
Note: Lanes 9 and 10 of the chemical ligation, FIG. 1A, were run on a separate gel. Occasionally, oligos of this length contain "shorter" bands (FIG. 1B, lane 5) which are either failed sequences or the full-length oligo folding back on itself. This is dependent on secondary structure formation of the newly synthesized oligo which sometimes is maintained despite highly denaturing (7 M Urea) conditions. Also, double bands may appear in the ligation product due to incomplete phosphorylation (FIG. 1B, lane 10).

Chemical vs. enzymatic ligation yields can be quantitated by scanning the gel photographs on an imaging densitometer as shown in FIG. 7 (shown for unligated "d" and "d" dumbbell). Due to the differential staining of double-stranded compared to single-stranded oligos (ethidium bromide stains double-stranded DNA more intensely[12]) only the relative yield of the ligated bands may be compared. Apparently for this oligo sequence, the chemical ligation is more efficient (approximately 20% higher yield). Percent yield of these reactions is dependent on the sequence of the oligos used.

The chemical method using cyanogen bromide described by Kool and others[9,13] ligates circular, *non*-self-complementary oligonucleotides by use of a template oligo to position the 3' and 5 ' ends of the oligo in close proximity. Since our self-complementary

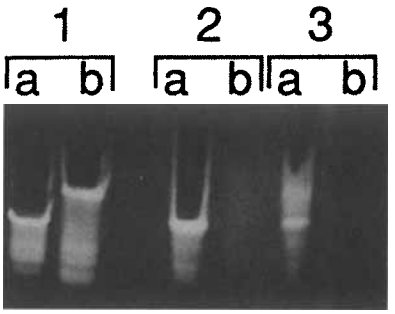

FIG. 2. Klenow fragment (exonuclease) reactions. (1) no enzyme treatment; (2) 8 hour enzyme treatment; (3) 12 hour enzyme treatment. In (a), 5 T-loop "e" dumbbell and in (b) unligated 5 T-loop "e."

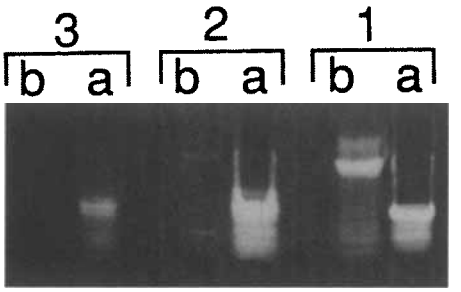

FIG. 3. S1 nuclease reactions. (1) no S1 enzyme treatment;(2) 5 minute S1 treatment; (3) 20 minute S1 treatment. In (a), 5 T-loop "e" dumbbell and in (b) unligated 5 T-loop "e."

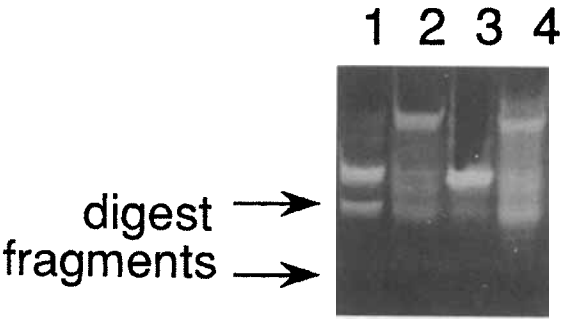

FIG. 4. *Mae* I cleavage. (1) *Mae* I digestion of 5 T-loop "e" dumbbell; (2) *Mae* I digest of unligated 5 T-loop "e;" (3) 5 T-loop "e" dumbbell; (4) unligated 5 T-loop "e."

oligos form their own "template," the 3'-OH and 5'-PO_4 ends are already near each other. This is therefore a simple, straightforward application of the cyanogen bromide method for DNA dumbbell synthesis. Recently, an improvement in the reaction rate (forms the product in minutes instead of 24 hours) of the cyanogen bromide method has been described[14], which uses N-morpholinoethanesulfonate instead of imidazole. An alternative chemical method for making DNA dumbbells uses 1-(3-dimethlyaminopropyl)-3-ethylcarbodiimide as the coupling agent[15], but requires longer

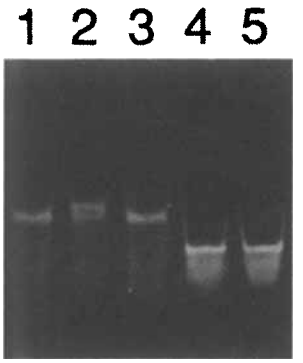

FIG. 5. Shrimp alkaline phosphatase (SAP) reactions. Odd-numbered lanes are SAP-treated. (1) unligated 5 T-loop "e" + SAP; (2) phosphorylated unligated 5 T-loop "e;" (3) phosphorylated unligated 5 T-loop "e" + SAP; (4) 5 T-loop "e" dumbbell; (5) 5 T-loop "e" dumbbell + SAP.

reaction times (2-3 days). Any of these methods requires a phosphate on one end of the oligo. While we enzymatically phosphorylated our oligos, pre-phosphorylated oligos may also be routinely purchased and can be used successfully for chemical or enzymatic ligations (data not shown).

While others have used an enzymatic method for ligating 5 T loop dumbbells[2,6,7] our enzymatic ligation method can successfully be used to ligate 4 T loop dumbbells from single, self-complementary oligos as well. In a previous study[2,16] failure to ligate a 4 T loop, 8 base-pair dumbbell has been attributed to improper alignment of the oligo ends due to the distortion by the 4 T loop ends[16]. Ashley and Kushlan attributed the failure to ligate this 4 T loop, 8 base-pair dumbbell on the substrate specificity of T4 DNA ligase[15], since they were able to ligate it chemically. However, *our* enzymatic method shows that 4 T loop dumbbells may be successfully ligated. This could reflect an improvement of the enzymatic reaction on our part, and/or it could reflect a possible substrate length requirement (i.e., a longer internal base-paired sequence) of T4 DNA ligase.

Additionally, the concentrations of T4 DNA ligase used in published enzymatic ligation reactions varied widely from 1 U enzyme (or less) per nmol oligo[2,7] to 50 U (or more) enzyme per nmol oligo[1,11] which could have an effect on the successful outcome of ligation. Our enzymatic ligation method used a low amount of enzyme, 3 U/nmol, thus minimizing the cost of the reaction. Amaratunga *et al.*[11] also ligated 4 T loop dumbbells but used a much higher concentration of ligase (over 15 times more).

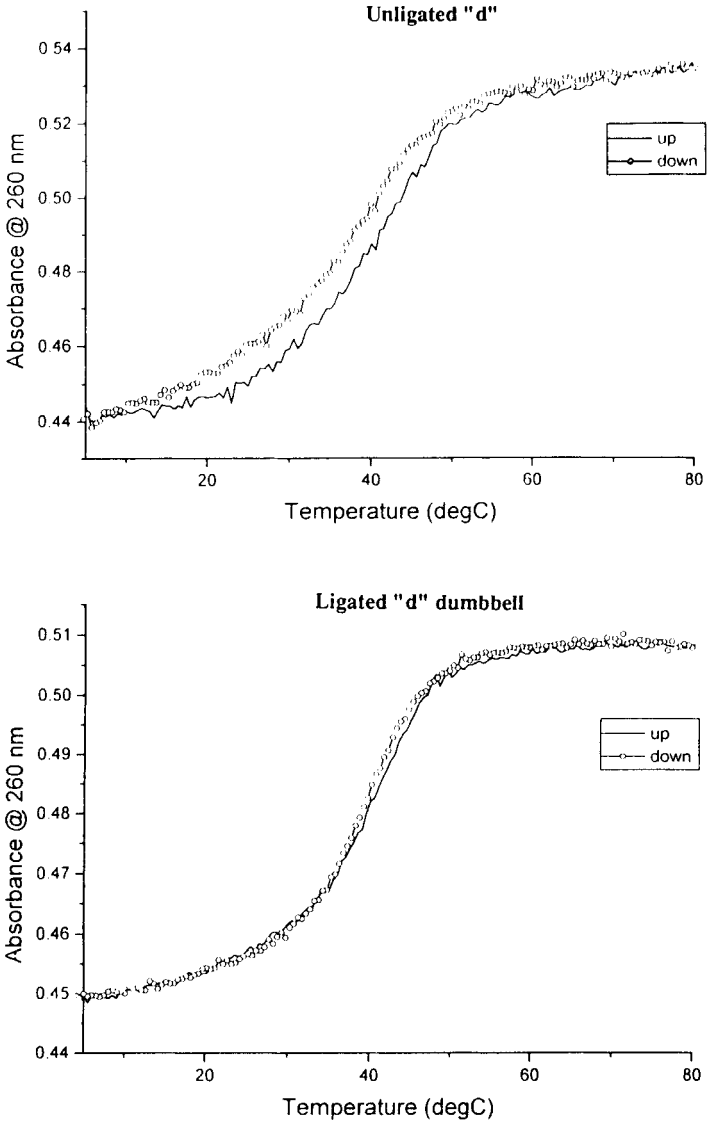

FIG. 6. Melting temperature experiment. The unligated "d" and "d" dumbbell sequences were subjected to heating/cooling (0.5°C/min.) from 20°C to 95°C to 20°C on a Cary 3E Spectrophotometer.

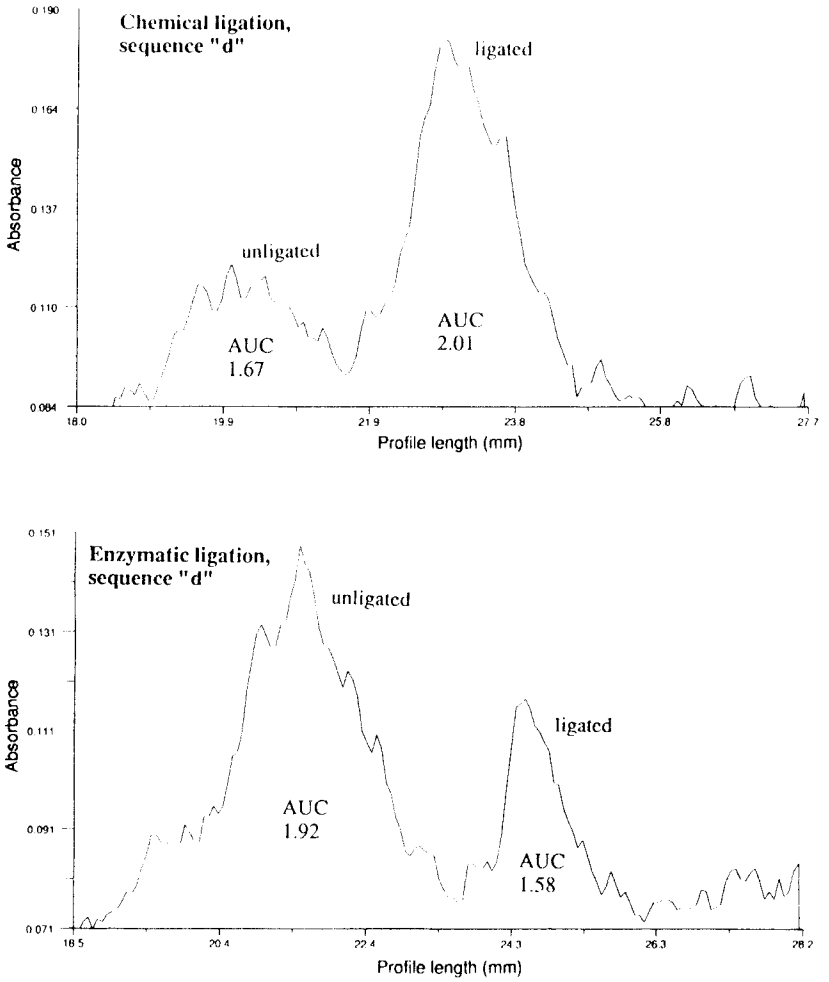

FIG. 7. Scanning densitometer results for the unligated "d" and "d" dumbbell sequences. In this case, the yield for the chemical method is slightly higher (compare ligated AUCs).

In general, yields of these ligation reactions may differ not only due to the method of ligation used but also due to the sequences or loop size/composition of the starting oligonucleotide. Whereas the enzymatic method ligates the 5 T-loop "e" better than the chemical method (FIGS. 1A and 1B, lanes 9 and 10), the chemical method ligates the "c" sequence better than the enzymatic method (FIGS. 1A and 1B, lanes 5 and 6). Another difference is that the enzymatic ligation reactions are performed on concentrated oligo solutions and may be directly gel purified if necessary; in contrast, the chemical ligation reactions are run in a larger volume (with respect to the amount of oligo used) and may

require lyophilization prior to purification. Nevertheless, the chemical ligation method may be advantageous overall since it is less time consuming and more cost effective. However, the potential toxicity of cyanogen bromide should be taken into account. Overall, this comparison of enzymatic and chemical methods may be useful for those interested in making dumbbell DNA.

MATERIALS AND METHODS

Self-complementary oligonucleotides (oligos) were obtained from Keystone Laboratories or from Oligos, Etc. The names, sequences, and corresponding ligated structures (e.g., dumbbells) are listed in TABLE 1. Concentrations of reactants in all of the following reactions are listed in terms of final concentrations, unless noted otherwise.

Oligos were phosphorylated as follows: 600 μM oligo, 3 units T4 polynucleotide kinase/nmol oligo, 2 mM ATP (Mg salt), 50 mM Tris HCl (pH 7.6), 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol were mixed together at 37°C. After 1 hour additional ATP and T4 polynucleotide kinase were added (same amounts as initially), and the mixture was incubated for 24 hours. After phosphorylation, oligos were heated to 90-100°C for 10 minutes then cooled to room temperature over several hours (to anneal).

Chemical ligation was performed with 50 μM phosphorylated oligo, 50 mM BrCN, 200 mM Imidazole (pH 7), 100 mM $NiCl_2 \cdot 6H_2O$ at 25°C for 24 hours[9]. A two-fold excess of BrCN could typically be used without alteration in yield. For the enzymatic ligation 400 μM phosphorylated oligo, 3 units T4 DNA ligase/nmol oligo, 5% polyethylene glycol (PEG), 66 mM Tris HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 66 μM ATP were incubated at 16°C for 48 hours.

Ligation reactions (8 μg starting material for the chemical ligations and 5 μg for the enzymatic ligations) were mixed with formamide, boiled for 2 minutes, and quickly loaded onto 12% denaturing polyacrylamide gels with ethidium bromide as described[8] (FIGS. 1A and 1B).

All "proof of synthesis" enzymatic reactions shown here were performed on the 5 T-loop "e" dumbbell product mixture at 37°C and were terminated by freezing to -20°C (unless otherwise indicated). For Klenow fragment reactions, 5 μg oligo, 6 units Klenow fragment, 66 mM Tris HCl (pH 7.6), 6.6 mM $MgCl_2$, and 1 mM DTT in a reaction volume of 6 μl were incubated for 8 or 12 hours (FIG. 2). For S1 nuclease reactions, in a 10 μl volume, 2.5 μg oligo, 2 units S1 nuclease, 250 mM NaCl, 30 mM sodium acetate (pH 4.5), 1 mM $ZnSO_4$, and 5% glycerol were incubated for 5 or 20 minutes (FIG. 3). *Mae* I reactions included 3 μg oligo, 10 units *Mae* I, 20 mM Tris HCl pH 8, 250 mM NaCl, 6 mM $MgCl_2$, and 7 mM mercaptoethanol in 25 μl total and were incubated for 24 hours (FIG. 4). Lastly, for shrimp alkaline phosphatase reactions, 1.6 μg oligo, 100 mM

Tris HCl pH 8, and 100 mM $MgCl_2$ in a total volume of 10 µl were incubated for 2 hours and terminated by heating to 65°C (FIG. 5). For analysis, all enzyme reactions (on ligated dumbbell DNA or unligated starting material) were loaded with formamide onto 12% (19% for *Mae* I) denaturing polyacrylamide gels.

0.7 OD of purified unligated "d" and "d" dumbbell were used for melting temperature experiments (FIG. 6). Each sample was mixed with 7 M urea and 1 M Tris HCl, pH 7.5. Samples were boiled for 5 minutes and cooled before running on a Cary 3E Spectrophotometer. Samples were heated/cooled at a rate of 0.5°C/min. from 20°C to 95°C to 20°C.

For a quantitative comparison of the two reactions, the unligated "d" and "d" dumbbell sequences (7.2 µg starting material for both) were run on gels as previously, photographed, and scanned using the Bio-Rad GS 670 Imaging Densitometer. For analysis using Bio-Rad Molecular Analyst version 1.1, the gel images were inverted to obtain absorbance peaks. Gel bands were analyzed, and absorbance vs. profile length (mm) was plotted to obtained a spectra with two major peaks (ligated and unligated, FIG. 7). Area under the curve, AUC (obtained using Molecular Analyst), for each peak is indicated in FIG.7.

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