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Development of a Real-Time Polymerase Chain Reaction Method to Measure Ligation Efficiency

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An efficient method to measure ligation efficiency would be a useful tool in experiments that use the ligase enzyme. In this study, real-time polymerase chain reaction (qPCR) was used to determine whether the ligation of synthetic oligonucleotides by T4 DNA ligase had occurred. qPCR is a technique used to simultaneously amplify and quantify DNA, and is widely used in many research settings for gene expression analysis. As an alternative to gel electrophoresis, qPCR was used to confirm ligation since it not only requires less time to produce results, but also is a more sensitive assay capable of detecting a few femtogram quantities of DNA. A second objective was to determine the effect of cohesive ends' GC (guanine and cytosine) content on the ligation efficiency of T4 DNA ligase. The results of the initial experimental setup resulted in no amplification, which may be due to complementary 5' and 3' ends of ligated oligonucleotides forming concatemers. Modified oligonucleotide sequences resulted in DNA amplification during qPCR, indicating that ligation had occurred. qPCR can be used as a method to analyze ligation by T4 DNA ligase, and can be utilized in further experiments to determine ligation efficiencies of cohesive ends with different GC content. However, caution must be taken when designing oligonucleotides to avoid unfavourable formation of DNA structures that may hinder the amplification of DNA during qPCR.

Restriction endonucleases are enzymes that cut double-stranded DNA at specific recognition sequences known as restriction sites. Restriction enzymes such as EcoRI and XmaI produce 4-nucleotide overhangs of 5'-AATT-3' and 5'-CCGG-3', respectively, which differ not only in their sequence, but also in their GC content. DNA fragments with complementary cohesive ends generated by the same enzyme can be joined together by T4 DNA ligase, an enzyme originating from *Escherichia coli* phage T4. This allows for molecular cloning and the formation of recombinant DNA, which is widely used in basic research for applications such as identifying, mapping and determining the functions of genes. It is important to measure ligation efficiency so that the amount of successful recombination can be determined and efforts can be made to improve ligation efficiency to increase the yield of recombinant DNA if needed. In previous studies, Chang *et al.* and Bola have observed that DNA fragments cut by different restriction enzymes, which have overhangs with different nucleotide sequences and GC (guanine and cytosine) content, have different ligation efficiencies when using T4 DNA ligase (1, 2). Generally, a higher GC content in recognition sequences with varying overhang lengths appears to improve ligation efficiency (2). However, the effect of GC content on ligation efficiency independent of the overhang length has not yet been determined.

Real-time polymerase chain reaction (qPCR) is a technique used to simultaneously amplify and quantify DNA. Fluorescent dyes that intercalate with double-stranded DNA can be used to label PCR products and the measurement of the fluorescent signal during thermal cycling allows for precise quantification of real-time PCR products (3). Quantification cycle (Cq) is the fractional cycle number at which the fluorescence of the accumulated DNA passes the fixed threshold NTC (no template control) and is used to determine relative quantification for qPCR (3). Fluorescence is measured in relative fluorescence units (RFU) and the greater the initial amount of the DNA template, the sooner an increase in fluorescence is detected and the lower the quantification cycle (Cq).

In this study, we examined the feasibility of using qPCR as an alternative to gel electrophoresis to determine whether ligation of complementary double-stranded DNA had occurred. Furthermore, we attempted to use qPCR to determine the ligation efficiency of T4 DNA ligase when used to ligate overhangs with different GC content. Since qPCR can be used for a relative quantitation assay, it should be possible to quantify the differences in the amount of ligated DNA between different samples relative to a reference sample. We tested this through a synthetic approach by using designed oligonucleotides that when annealed together, would form short DNA duplexes that could be ligated together at their complementary 4-nucleotide overhangs (4). The results of this study showed that qPCR can indeed be used to determine whether ligation occurred. Care should be taken when designing oligonucleotide and primer sequences in order to ensure their feasibility for qPCR analysis.

MATERIALS AND METHODS

Synthesizing oligonucleotides and primers. 25 nmole of DNA primers and 100 nmole of DNA oligonucleotides with varying GC content for the 4-nucleotide overhang at the 3' end were designed and ordered from Integrated DNA Technologies. Refer to Table 1 for the exact sequences used.

Annealing of synthetic oligonucleotides. 200 uM of each oligonucleotide (Eta2-7) were annealed to Eta1 using 50 ul of annealing buffer (50 mM Tris pH 7.5, 5 mM EDTA, and 250 mM NaCl). The annealing protocol was followed from Sigma-Aldrich (5). The Biometra T-gradient Thermocycler was programmed at 95 °C for 2 minutes and gradually cooled to 25 °C over a period of 45 minutes. PCR tubes with annealed DNA were stored at 4 °C throughout the experiment.

Ligation of cohesive ends. Annealed synthetic oligonucleotides were phosphorylated at the 5' end prior to ligation using T4

polynucleotide kinase (New England Biolabs, Cat no. M0201S) following the protocol derived from Cawood (6). The kinase reaction was set at 37 °C for 30 minutes then 63 °C for 20 minutes. The complementary double-stranded fragments with cohesive ends were ligated together using T4 DNA ligase (Invitrogen, Cat no. 15224-025). The ligation reaction was set at 16 °C for 1 hour and 65 °C for 10 minutes to inactivate the T4 DNA ligase (7). PCR tubes with ligated DNA were stored at 4 °C throughout the experiment.

TABLE 1 Synthesized DNA oligonucleotide sequences with varying four-nucleotide GC content in bold and DNA primers.

Primer Name	# Base Pairs	Primer Sequence
Eta1	48	5'ATATTCAGTTACGAGG ACAGATCAGTGAGAGAC AGTAGTGCATGCGAT-3'
Eta2-CCGC	52	5'ATCGCATGCACTACTG TCTCTCACTGATCTGTCC TCGTAACTGAATAT**CCG C**-3'
Eta3-GCGG	52	5'ATCGCATGCACTACTG TCTCTCACTGATCTGTCC TCGTAACTGAATAT**GCG G**-3'
Eta4-AATA	52	5'ATCGCATGCACTACTG TCTCTCACTGATCTGTCC TCGTAACTGAATAT**AAT A**-3'
Eta5-TATT	52	5'ATCGCATGCACTACTG TCTCTCACTGATCTGTCC TCGTAACTGAATAT**TAT T**-3'
Eta6-AGTC	52	5'ATCGCATGCACTACTG TCTCTCACTGATCTGTCC TCGTAACTGAATAT**AGT C**-3'
Eta7-GACT	52	5'ATCGCATGCACTACTG TCTCTCACTGATCTGTCC TCGTAACTGAATAT**GAC T**-3'
Main Primer	20	5'ATCGCATGCACTACTG TCTC-3'
Short Primer	20	5'ATATTCAGTTACGAGG ACAG-3'

Initial qPCR. The controls used in this experiment were the Main primer without template (Control A1), non-phosphorylated non-ligated annealed DNA (Control B1), phosphorylated non-ligated annealed DNA (Control C1), and Main and Short primers without template (Control D). Controls A1 through C1 were negative controls and Control D was the positive control. Main and Short primers were diluted to 4 uM. DNA template for samples and Controls B1 and C1 were diluted to 12.5 pg/ul. 10 ul of SsoAdvanced Universal SYBR Green Supermix (BioRad, Cat no. 1725265), 4 ul of sterile distilled water, 4 ul of 4 uM Main primer, and 2 ul of DNA template (25 pg) were added to each PCR tube for the 1 hour ligated samples with varying GC content and Controls B1 and C1. For Control A1, 2ul sterile distilled water replaced the 2 ul of DNA temple. For Control D, 2 ul of Main primer and 2 ul of Short primer were added instead of 4 ul of Main primer. Three replicates from the same dilution were used for each control and 1 hour ligated samples with varying GC content. qPCR conditions were 3 minutes at 95°C, then 35 cycles of 95°C for 1 minute and 45°C for 7 seconds on the CFX Connect™ Real-Time PCR Detection System.

Analysis of qPCR data. qPCR analysis was done using Bio-Rad's CFX Manager™ software on automatic baseline settings. Cq determination mode was set to single threshold. A melt curve was run between 65°C and 95°C, in 0.5°C increments.

PCR conditions. Each PCR reaction consisted of the following: 1 ul of template DNA (107 ng), 5 ul of 10X Pfx Amplification Buffer (Invitrogen, Cat no. 11708-013), 1.5 ul of 10 mM dNTP mixture (Invitrogen, Cat no. 11708-013), 1 ul of 50 mM $MgSO_4$ (Invitrogen, Cat no. 11708-013), 1.5 ul of each of the 10 uM primers, 0.4 ul of Platinum Pfx DNA Polymerase (Invitrogen, Cat no. 11708-013), and sterile water to make up to a total volume of 50 ul. A positive control that consisted of a 700 bp GFP fragment and its forward and reverse primers was also included. PCR was carried out using a Biometra T-gradient Thermocycler. Cycling parameters were initial denaturation at 95°C for 3 minutes, followed by 35 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 45 seconds.

Heat denaturation and agarose gel electrophoresis. Aliquots of ligated DNA were denatured at 100 °C for 1 minute and ran on a 3% agarose gel along with non-denatured ligated DNA. The gel was made using UltraPure™ agarose (Invitrogen, Cat no. 16500-500) and 1X TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). 10 bp DNA ladder (Invitrogen, Cat no. 10821-015) and 100 bp DNA ladder (Invitrogen, Cat no. 15628-050) were loaded along with samples. 6X DNA loading dye (Thermo Scientific, Cat no. R0611) was added to the DNA prior to running the gel at 50 V for 1 hour. The gel was post-stained in 0.5 ug/ml ethidium bromide for 15 minutes before using the AlphaImager to image the gel.

Synthesizing revised oligonucleotide. Two new oligonucleotides and a new reverse primer were designed and synthesized. Absence of likely hairpins and primer-dimers was confirmed using the online OligoAnalyzer 3.1 tool from Integrated DNA Technologies. Refer to Table 2 for the exact sequences used.

TABLE 2 Synthesized DNA oligonucleotide sequences and a reverse primer used in qPCR to form double-stranded DNA with an overhang in bold that is complementary to the DNA duplex formed by Eta1 and Eta4.

Primer Name	# Base Pairs	Primer Sequence
O48	48	5'ATGGCCTCGACGGATGA GAAGCTGTTGCGTGGATA TACTCGTAACTGG-3'
O52	52	5'CCAGTTACGAGTATATC CACGCAACAGCTTCTCAT CCGTCGAGGCCAT**TATT**-3'
P2	22	5'CCAGTTACGAGTATATC CACGC-3'

qPCR on modified sequences. qPCR conditions were the same as the initial qPCR, with more controls included. Control A2 had 2 ul of the Main and 2 ul of the P2 primers without template. 2 ul of Eta1/Eta4 doubled-stranded DNA template (25 pg) was added to each of Controls B2 and C2. 2 ul of O48/O52 double-stranded DNA template (25 pg) was added to each of Controls B3 and C3. Eta1/Eta4 is used to denote the double-stranded DNA molecule that resulted from single-stranded Eta1 annealing to single-stranded Eta4. O48/O52 is used to denote the double-stranded DNA that resulted from single-stranded O48 annealing to single-stranded O48. qPCR data analysis methods were kept constant.

RESULTS

Heterodimerization of Main and Short primers. qPCR was performed on controls to determine if the primers could dimerize in the absence of DNA template. The 3' ends of

the Main and Short primers were complementary, and thus can heterodimerize. When used in qPCR, amplification was observed for the Main and Short primers in the absence of template (supplementary Table 1). The Main and Short primers were used as a positive control for subsequent qPCRs.

No amplification of ligated DNA samples in initial qPCR. qPCR was performed in order to determine the ligation efficiency of DNA fragments with overhangs containing 0%, 50%, and 100% GC content. In Figure 1, Controls A1, B1 and C1 were the negative controls, which consisted of either the primer or the template, while Control D was the positive control. There was no amplification for Controls A1 through C1, which was as expected. The Main and Short primers in Control D had complementary 3' ends, which allowed them to bind together and amplify, giving Cq of 18.54, 18.21, and 21.37 for the three replicates. The mean of Cq for Control D was 19.4 with a standard error (SE) of 1.0, which suggested that the three replicates were precise. All of the 1 hour ligated DNA samples, with 0%, 50%, or 100% GC content for the 4-nucleotide cohesive ends, did not amplify. There were no melt peaks for the negative controls and the 1 hour ligated DNA samples (Fig. 2). For Control D, the melt peak was at approximately 76°C (Fig. 2).

Gel electrophoresis did not confirm ligation of initial oligonucleotides. The DNA samples used in the initial qPCR were run in a 3% agarose gel to confirm ligation occurred between the complementary cohesive ends. Aliquots of the samples were heated to 100°C for 1 minute to denature any concatemers that may have formed so that single-stranded DNA of 100 bp could be visualized in a gel if ligation occurred. Gel electrophoresis after PCR (Fig. 3) showed that the negative PCR control lane had no bands and the positive PCR control gave a band with a size of approximately 700 bp, which was as expected. However, thick bands were also seen at where the wells were located for all lanes except for the negative control in Lane 3. Bands of approximately 50 bp and streaks of higher molecular weight bands appeared in all the lanes with synthetic oligonucleotides (Fig. 3).

Amplification of ligated DNA samples with modified oligonucleotide sequences in qPCR. Modified oligonucleotides were synthesized after discovering that the 5' and 3' ends of the initial ligated oligonucleotides were complementary, and qPCR was performed using these modified sequences to confirm ligation of DNA samples. In Figure 4, Controls A2 through C3 were the negative ontrols, while Control D was the positive control. Control A2, which consisted of the Main and P2 primers, gave no amplification as expected. However, the remaining negative controls gave inconsistent results. Only one replicate from Control B2, which contained non-phosphorylated non-ligated Eta1/Eta4, had a Cq value of 17.6, while the other two replicates had no amplification. Two replicates from Control C2, which contained phosphorylated non-ligated Eta1/Eta4, had Cq values of 23.0 and 28.3, while the third replicate had no amplification. Two replicates from Control B3, which contained non-phosphorylated non-ligated

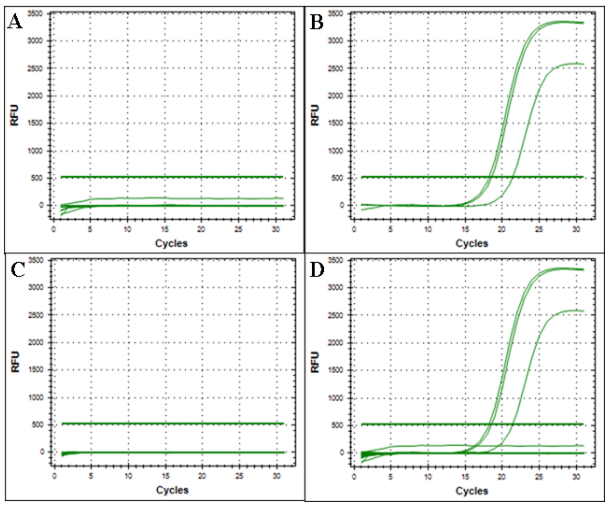

FIG 1 RFU values from qPCR on controls and 1 hour ligated DNA with 0%, 50%, and 100% GC content for the 4-nucleotide cohesive ends after 35 cycles. The auto-calculated single threshold value of 518 RFU was determined using Bio-Rad's CFX Manager™ software. (A) Negative Controls A1 to C1. (B) Positive Control D. (C) 1 hour ligated DNA samples with 0%, 50%, and 100% GC content. (D) Summation of graphs in (A) to (C).

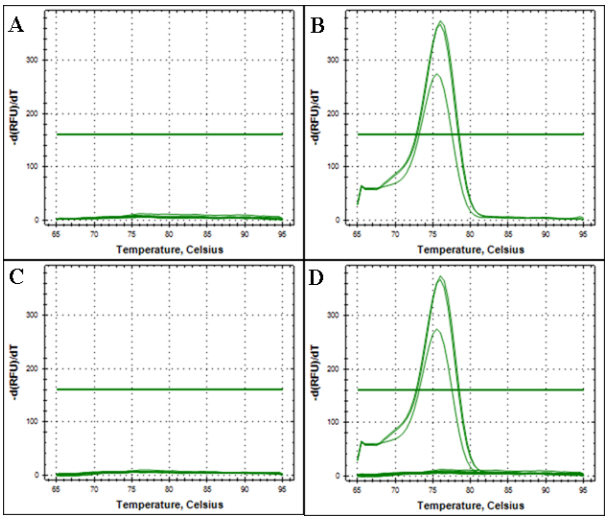

FIG 2 Melt peaks from qPCR on controls and 1 hour ligated DNA with 0%, 50%, and 100% GC content for the 4-nucleotide cohesive ends after 35 cycles. (A) Negative Controls A1 to C1. (B) Positive Control D. (C) 1 hour ligated DNA samples with 0%, 50%, and 100% GC content. (D) Summation of graphs in (A) to (C).

O48/O52 had Cq values of 23.8 and 29.1, while the third replicate had no amplification. Only one replicate from Control C2, which contained phosphorylated non-ligated O48/O52, had a Cq value of 26.5, while the other two replicates had no amplification. Control D was the positive control since it contained the Main and Short primers, and it gave consistent Cq values of 21.8, 23.8, and 20.4. The 1 hour ligation sample with ligated Eta1/Eta4 and O48/O52 had the smallest Cq values of 14.2, 14.4, and 14.4, when compared to all other positive results in Figure 4. Refer to supplementary Table 1 and supplementary Table 2 for further information.

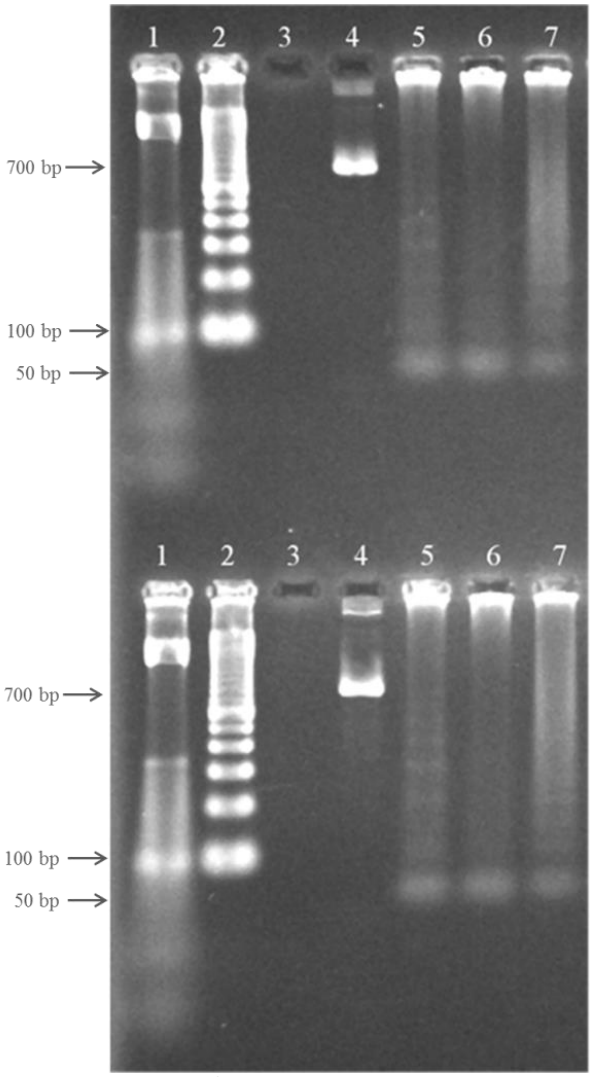

FIG 3 Electrophoresis of 1 hour ligated synthetic oligonucleotides in a 3% agarose gel. Top lanes are non-denatured DNA products. Bottom lanes are heat denatured (100°C for 1 minute) DNA products. Lane 1: 3.5 ul of 10 bp DNA ladder (Invitrogen). Lane 2: 3.5 ul of 100 bp DNA ladder (Invitrogen). Lane 3: 5 ul of negative control PCR product. Lane 4: 5 ul of positive control PCR product. Lane 5: 5 ul of 1 hour ligated 100% GC content overhang PCR product. Lane 6: 5 ul of 1 hour ligated 0% GC content overhang PCR product. Lane 7: 5 ul of 1 hour ligated 50% GC content overhang PCR product.

There was no melt peak for Control A2, which was as expected since it was a negative control (Fig. 5A). However, Controls B2 through C3 each had at least one replicate with a melt peak at approximately 80°C even though they were negative controls (Fig. 5B-E). Control D was expected to have a melt temperature of approximately 76°C as it was the same positive control used in the initial qPCR (Fig. 2B); this was observed in Figure 5. The 1 hour ligated DNA sample also had a melt peak at approximately 80°C (Fig. 5G). Thus, the two distinct peaks seen at approximately 76°C and 80°C (Fig. 5H) correspond to Control D, and Controls B2 through C3 as well as the 1 hour ligated DNA sample, respectively.

DISCUSSION

Ligation is an essential process in the joining and recombination of nucleic acids, which is an important molecular biology tool used for applications such as cloning of DNA fragments generated by restriction enzyme digestion as well as the construction of genomic and cDNA libraries (8). As ligation is often a critical intermediate step in many experimental procedures, being able to evaluate the efficiency of ligation is important. The main advantage of using synthetic oligonucleotides is that it allows for the construction of desired base sequences. We used synthetic oligonucleotides in our study to control the length and GC content of the overhangs for the DNA oligonucleotides that would be ligated together. This would allow for easy comparison of the effect that overhang GC content has on ligation efficiency. However, the main focus of the study was on whether qPCR could be used as a method to determine ligation efficiency, which would be faster than transformation of recombinants and would not require as much of the ligation product be sacrificed as gel electrophoresis (8). The results from the initial qPCR showed no amplification of our samples so we were not able to confirm ligation of our synthesized DNA (Fig. 1C). The melt curve showed a peak for only the positive control (Control D), which indicates that it was the only amplicon produced (Fig. 2A-D). Therefore, it could not be confirmed that there was ligation of the cohesive ends of the double-stranded DNA.

In order to confirm whether ligation occurred, gel electrophoresis was performed following a PCR run. However, ligation could not be confirmed from the results observed in Figure 3. Thick bands located at the wells of the gel may indicate significant amounts of DNA stuck in the wells containing the synthetic oligonucleotide samples (Fig. 3). The streaks of higher molecular weight bands seen may indicate ligated DNA products (Fig. 3). Several possible structures (Fig. 6A,B) can be formed from synthetic oligonucleotides that have complementary 5’ and 3’ ends after ligation. The pattern of discrete bands observed (Fig. 3) indicates structures with different molecular weights, which suggests that the structures formed contained different numbers of strands of oligonucleotides (9).

Heat denaturation was conducted in an attempt to separate the DNA strands, so that 100 bp single-stranded DNA fragments could be visualized in an agarose gel to confirm ligation occurred. The DNA structures may have been too stable to denature at 100°C for 1 minute, hence the electrophoresis bands for heat denatured DNA looked similar to non-denatured DNA (Fig. 3). As the 5’ end of the annealed oligonucleotides is complementary to the 3’ end, stable double-stranded DNA concatemers may have formed after ligation due to the recognition of complementary bases (10). The DNA template may have

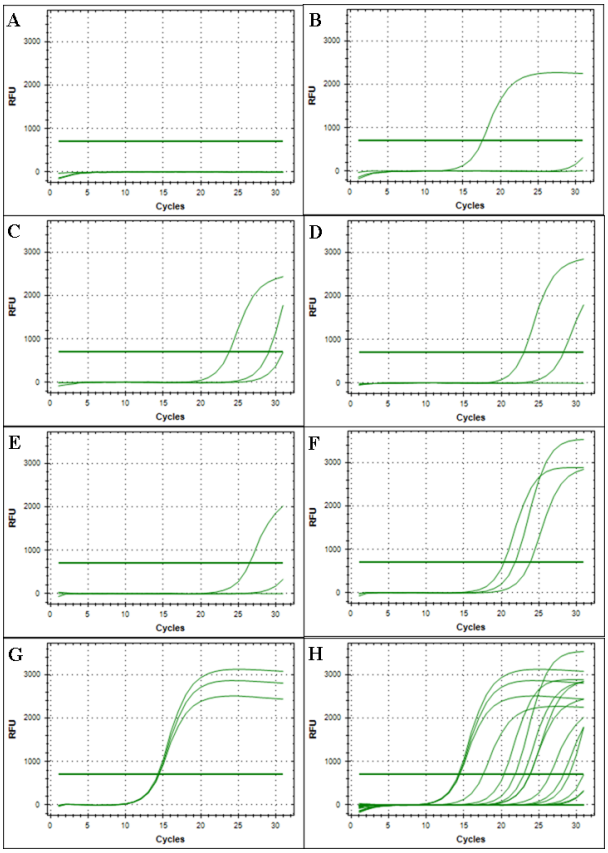

FIG 4 RFU values from qPCR on the 1 hour ligation with One Eta 1/Eta 4 and O48/O52 after 35 cycles. The auto-calculated single threshold value of 703 RFU was determined using Bio-Rad's CFX Manager™ software. (A) Control A2. (B) Control B2. (C) Control B3. (D) Control C2. (E) Control C3. (F) Control D. (G) 1 hour ligated DNA samples with One Eta 1/Eta 4 and O48/O52. (H) Summation of graphs in (A) to (G).

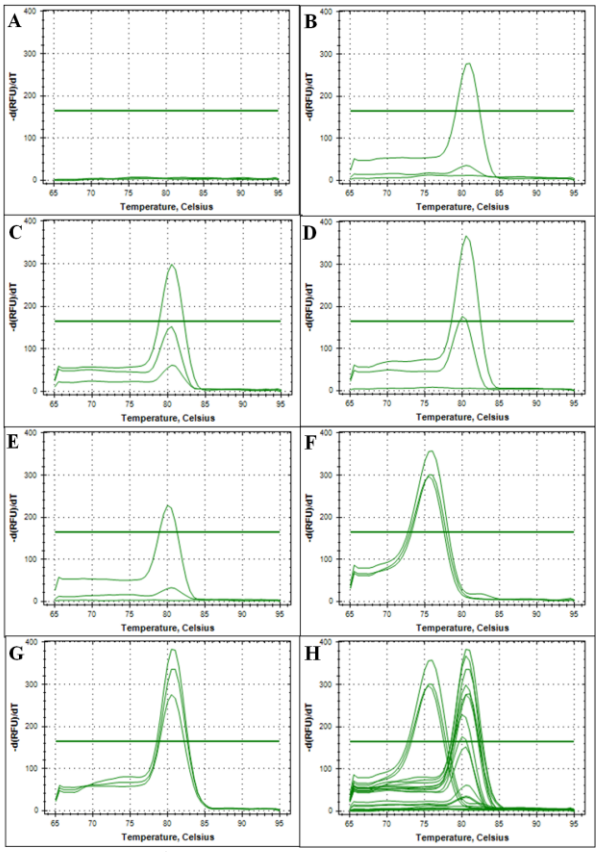

FIG 5 Melt peaks from qPCR on the 1 hour ligation with One Eta 1/Eta 4 and O48/O52 after 35 cycles. (A) Control A2. (B) Control B2. (C) Control B3. (D) Control C2. (E) Control C3. (F) Control D. (G) 1 hour ligated DNA samples with One Eta 1/Eta 4 and O48/O52. (H) Summation of graphs in (A) to (G).

folded up onto itself or formed large concatemers that prevented the polymerase from binding and amplifying the template, thereby preventing the amplification of DNA in the initial qPCR. The bands seen in gel electrophoresis may not be amplification products from the PCR, but distinct species of DNA structures that formed during the PCR run. However, this needs to be confirmed by running the PCR protocol without the polymerase to determine if the same bands would appear in the gel. Because the synthesized oligonucleotides had identical 5' ends, ligation could not be confirmed and sequences had to be redesigned to avoid self-dimerization.

The results from the qPCR with the modified sequences showed amplification of our samples (Fig. 4G). Control A2 showed no amplification (Fig. 4A) along with the absence of a melt peak (Fig. 5A), which means that there was no formation of primer dimers. Therefore, the Main primer and P2 primer only bind to Eta1 of Eta1/Eta4 and O48 of O48/O52, respectively, and not to each other. Considering that Controls B2 and C2 only contained Eta1/Eta4 and Controls B3 and C3 only contained O48/O52, theoretically, amplification would be unlikely or be very low and result in a non-detectable or a very large Cq value. For this reason, Controls B2, B3, C2, and C3 were denoted as the negative controls in qPCR. However, some replicates for Controls B2 through C3 have amplification, where the Cq values ranged from 17.8 to 29.1 within the 35 cycles (Fig. 4B-E). This would still occur 2^3 to 2^{15} cycles after the Cq for the 1 hour ligation sample with Eta 1/Eta 4 and O48/O52, where threshold was reached at an average of 14.3. In addition, replicates with a Cq value for Controls B2 through C3 all had a melt peak at 80°C, which indicated that the amplicon was the same in all of these cases (Fig. 5B-E). Amplification for the negative controls would not be due to contamination with the primers (Main and Short primers bind to Eta1/Eta4) from the positive control, but may have occurred due to contamination with the DNA template from the 1 hr ligated sample. Another possibility was that the low annealing temperature of 45°C used during the qPCR could have resulted in nonspecific amplification.

Since Control D and the qPCR conditions were the same for both qPCR, the Cq values and the melt

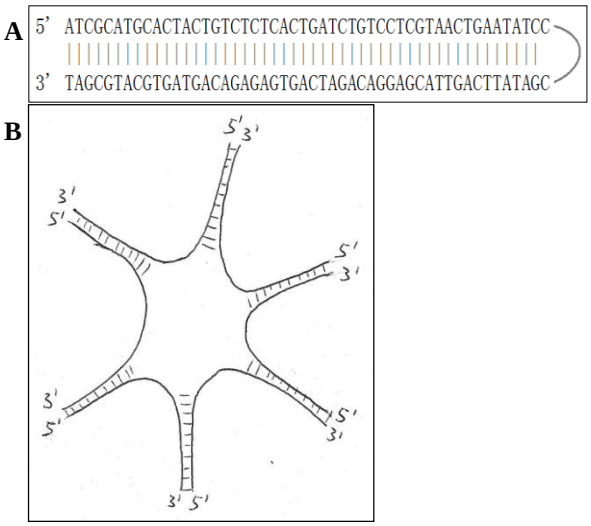

FIG 6 Hypothetical DNA structures due to complementary 5' and 3' ends of synthesized oligonucleotides. (A) Hairpin structure formed from ligated Eta2 and Eta1. (B) Concatemer structure formed spontaneously after mixing of double-stranded synthetic oligonucleotides.

temperature for Control D were expected to be similar. The melt temperature for Control D in Figure 2B and Figure 5F were the same. However, the mean Cq value for Control D in Figure 1B was 19.4 with a SE of 1.0 but it was 22.0 with a SE of 1.0 in Figure 4F. There was also a difference of 1 to 3 cycles between the Control D replicates in both Figure 1B and Figure 4F. This variation among the technical replicates may have been due to the lack of a master mix, meaning that the volumes of the reagents dispersed directly into the reaction tubes could have been imprecise (11). The pipet tips used may not efficiently repel negatively-charged molecules, such as DNA, and there could also be some liquid retention, both of which would contribute to the variation among the replicates made from the same dilution (11). The negative controls, Control A1 with the Main primer in the initial qPCR (Fig. 1A) and Control A2 with the Main and P2 primers (Fig. 4A), confirmed that no amplification occurred. As some of the negative controls and the positive control gave expected results, it suggested that the qPCR was working and the data for the 1 hour ligation with Eta1/Eta4 and O48/O52 was reliable. Furthermore, the Cq values of 14.2, 14.4, and 14.4, with a mean of 14.3 and a SE of 0.0, indicates precision for the 1 hour ligation replicates. Since amplification occurred and a mean Cq value of 14.3 was obtained, which indicates that the threshold cycle was reached early during the qPCR compared to the positive control, it demonstrates that ligation of the cohesive ends of Eta1/Eta4 and O48/O52 had occurred.

In conclusion, we found that ligation of synthetic oligonucleotides by T4 DNA ligase and the use of qPCR to determine ligation efficiency is feasible. qPCR gives amplification relative to the initial amount of ligated DNA and the resulting Cq values can be used to determine ligation efficiency. Given that the sequences are well-designed and contamination is not present, qPCR is a quicker and more quantitative method for determining ligation efficiency that requires less DNA than gel electrophoresis. The results from this experiment support the hypothesis that ligation of synthetic oligonucleotides using T4 DNA ligase can be measured by qPCR as long as the DNA strands are incapable of folding or forming concatemers. This study provides a foundation for future experiments to investigate and compare ligation efficiencies under specific conditions such as varying GC content.

FUTURE DIRECTIONS

Guanine and cytosine base pairs are known to bind more strongly together than adenine and thymine base pairs because GC base pairs form three hydrogen bonds instead of just two hydrogen bonds. Therefore, it can be speculated that increasing the GC content of cohesive ends may increase ligation efficiency of T4 DNA ligase. Future experiments using qPCR to investigate the ligation efficiency of synthetic oligonucleotides should ensure that each synthetic strand has different sequences to avoid self-annealing and concatemer formation. In addition, sequences can be designed for future experiments with reference to the bolded sequences in Table 1 for varying GC content. These sequences can then be annealed and ligated before comparison of the ligation efficiency of oligonucleotides with different GC content, which is done using qPCR. This would test the hypothesis that the GC content of cohesive ends has an effect on the ligation efficiency of T4 DNA ligase.

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