Application of Circular Ligase to Provide Template for Rolling Circle Amplification of Low Amounts of Fragmented DNA

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Abstract:

Compromised biological evidence, particularly those samples that contain a very small amount of DNA or may have a significant amount of degradation, may not yield a complete or useful DNA profile. One approach that shows promise to overcome the limited quantity of DNA issue is the use of Whole Genome Amplification (WGA). The WGA method amplifies all the DNA in a sample, yielding higher levels of DNA template that can be subsequently analyzed using standard forensic technologies. One WGA technique termed Rolling Circle Amplification (RCA) involves the amplification of circular DNA fragments such as those of plasmids and viral genomes. With a highly processive polymerase, RCA can yield microgram quantities of DNA from circular templates. However, with the exception of intact mitochondrial DNA (mtDNA), circular DNA fragments do not occur naturally in humans. Consequently, RCA would have limited application as would most WGA methods for fragmented or degraded DNA evidence. However, a commercially-available circular ligase enzyme (CircLigase™, EPICENTRE® Biotechnologies) which possesses a catalytic activity that circularizes single-stranded DNA (ss-DNA) offers the potential for RCA-WGA to be used on compromised DNA materials.

This paper describes a study focused on optimizing the circularization reaction of several DNA templates, including ss-DNA and denatured double-stranded DNA (ds-DNA). In addition, a detection method was developed to identify the circular products of various templates in order to monitor optimization as circular DNA ladders are not commercially available. Optimization studies involved varying experimental conditions such as incubation time and concentrations of manganese chloride, ATP, and enzyme. Results show that the optimal manganese chloride concentration varies depending on the size of the template. However high concentrations (5 mM) of manganese chloride appears to induce non-specific degradation of the template. Neither higher than manufacturer's suggested ATP concentrations nor increased incubation periods yield any discernible benefit or detriment to circularization. However, higher circular ligase concentrations appear to increase the yield of circular products. Templates with different terminal nucleotides were used to examine the efficiency of circularization, and the results demonstrate that the efficiency of circularization is dependent on the specific base present at both the 5' and 3' terminal ends. The activity of the enzyme using denatured, ds-DNA template was also examined.

The methods described should form the foundation for developing protocols to circularize human DNA fragments that would be suitable for RCA. Thus, it may be possible to obtain partial or perhaps complete mtDNA and/or nuclear DNA profiles from compromised forensic evidence.

Introduction:

DNA has many characteristics that make it a robust material for individual identification. However, DNA evidence can be degraded to the point that analysis of the samples is challenging or even impossible. Whole Genome Amplification (WGA), a novel method that increases the concentration of high quality templates may overcome the aforementioned problem. However, WGA is most successful when using high quality template and can fail to yield sufficient representative amplified DNA when the initial template is highly degraded (1). However, Rolling Circle Amplification (RCA) and Multiple Displacement Amplification (MDA), variations of WGA, offer the potential to yield results even from highly degraded samples (2). Both techniques use Φ29 polymerase, an enzyme with a low error rate (1 in 10^6 bp) when amplifying DNA (3, 4 and 5).

The MDA and RCA methodologies are based on amplification of any template using degenerate primers. But the RCA technique exploits the efficiency of the polymerase with circular templates. Circular templates in essence are infinite length templates and thus are not as refractory to WGA as linear degraded template DNA. Since circular template, other than intact mtDNA, are not typically found in the human

genome, other techniques have been applied to achieve template circularization so that RCA can be used effectively. One of these techniques uses T4 DNA ligase. This enzyme forms phosphodiester bonds between the 5' phosphate and the 3' hydroxyl groups of ds-DNA. However, the enzyme will ligate ds-template forming concatamers; the ligation of ds-DNA is for the most part inefficient and the ligation of ss-template is very poor (6). Because of these features subsequent analyses are problematic with T4 DNA ligase treated templates.

Epicentre Biotechnologies offers a new enzyme called CircLigase™ that circularizes single stranded DNA. This enzyme ligates the template by the 5'phosphate group and the 3' hydroxyl group. It circularizes fragments larger than 30 nucleotides and up to 1.9 kb. Use of CircLigase can provide circular products from ss-DNA template for RCA; however its activity on ds-DNA template is unknown (7). Because this is a novel technique, optimization studies need to be performed.

Materials and Methods:

Circularization Assay

Templates used were synthetic, phosphorylated oligonucleotides with known sequences and sizes (Integraded DNA Technology (Coralville, IA), PAGE purified). The DNA final concentration for the circular ligase reaction was 0.5μM, the amount recommended by the manufacturer. Ligation was performed according to the manufacturer's recommendations. Master mix for samples treated with CircLigase™ was composed of 1X reaction buffer, 50μM ATP, 2.5 mM $MnCl_2$, 5 Units/μL of CircLigase™ (Madison, WI) in a final volume of 20μl. For untreated samples, the enzyme was substituted with deionized water. The positive control provided in the kit was run with all samples.

After ligation, samples were treated with exonuclease I or III (Exo I, Exo III), enzymes that digest ss- and ds-DNA respectively (8 and 9). For the exonuclease digestion, 10μl from the 20 μl circular ligase reaction were taken and treated with 1μl of exonuclease and the remaining 10μl of the reaction were mixed with 1μL of molecular grade water.

T4 DNA ligase

The same templates used for the circularization assay with CircLigase™ were treated in parallel with T4 DNA ligase (10) to compare ligase efficiencies. Samples were treated with T4 ligase following Invitrogen's (Carlsbad, CA) protocol: 5X ligase reaction buffer, 2μM of template, 1U T4 DNA ligase and water up to 20 μl. Samples were incubated at 14°C for ~17 hours. After ligation, samples were treated with Exo I or III depending on ss- or ds- template and separated by 2% agarose and 6% polyacrylamide gel electrophoresis systems.

Optimization of CircLigase Enzyme Reaction

Reaction parameters recommended by the manufacturer were varied to ascertain what are optimal for the enzymatic reaction. During this study, concentrations were varied for $MnCl_2$, ATP and the ligase on 55, 100 and 200 nucleotide-long templates as mimics of degraded DNA. Reaction time was also studied. Even though the concentrations of the reagents were changed, the final volume of reaction was not altered.

- CircLigase (CL): concentrations of CircLigase™ ranged from 2.5 U/μl to 15 U/μl with intermediates of 5 and 10 U/μl.
- ATP and $MnCl_2$: concentrations ranged from 25, 50 and 100 μM for ATP and 1.25, 2.5 and 5 mM for $MnCl_2$.
- Temperature and time: the manufacturer's recommended reaction time and temperatures are 60 minutes at 60ºC, respectively. For this study, the incubation time ranged from 60 to 240 minutes, with an intermediate time of 120 minutes. The enzyme was heat-inactivated for 10 minutes at 80ºC to stop the reaction.

The optimization study was done changing only one component or parameter (variable) at a time. In other words, after determination of optimal concentration for any given reagent, the concentration of the next reagent studied, was varied using the determined optimum from the previous evaluation until all variables were studied. The parameters were evaluated in the following order: ligase enzyme, ATP, $MnCl_2$, and time.

Phosphorylation Assay

Taking in consideration that not all DNA evidence would have the 5' phosphate required for ligation, the same templates used for the other studies were used for this study without the 5'phosphate group. Phosphorylation was performed following Epicentre's recommendations for use of T4 polynucleotide kinase (PNK) (11). The mixture of reagents and sample was incubated for 30 minutes at 37 ºC and stopped by heating for 5 minutes at 70 ºC. After phosphorylation, samples were treated with CircLigase™ followed by exonuclease digestion where indicated.

Optimal Terminal Nucleotides

Complementary DNA sequences were used for this study. The forward strand had terminal 5'G and 3'T and the reverse strand had a 5'A and 3'C as terminal nucleotides. These sequences were used to examine whether the ligase enzyme efficiency is sequence dependent. Samples were treated under the previously determined circular ligase optimal conditions.

After each study, samples were analyzed by agarose or denaturing polyacrylamide gel electrophoresis. Percentages of acrylamide ranged from 6% up to 15% and agarose gel concentrations ranged from 0.6 – 2% depending on template size. For visualization of DNA, gels were stained with SYBR Gold (Molecular Probes) for 40 minutes with shaking.

Results and Discussion:

Circularization Assay

Complete circularization of ss-DNA template from various length templates was achieved with CircLigase™ (CL), as depicted by the products in *Figure 1* that are enclosed by the ovals. The substrate treated with the enzyme results in complete ligation with no evidence of concatamerization.

Within the rectangle of *Figure 1* are displayed the results of four reactions for the 55 nucleotide long template (55mer). In the first lane within the rectangle from left to right, the substrate was treated with CL and exonuclease. A single band is observed, because the product is Exo I resistant, as expected for a circular product. In the second lane to the right, the sample was treated with CL but not with Exo I. A single band is observed that migrated the same distance as the band in the previous lane. This observation supports that the reaction results in complete ligation of all template. In the third lane to the right, the sample was not treated with CL, but treated with Exo I. Due to the digestive action of the exonuclease on linear ss-DNA, the substrate is no longer present. In the fourth and last lane within rectangle, the substrate in its native, linear state is observed, since the sample was not treated with any enzyme. On the rest of the gel, similar results can be observed within the four lanes depicted for each substrate (100mer and 200mer, respectively). The migration of the circular products differs for the different templates on this 6% polyacrylamide gel matrix. For the 55mer, the product migrates faster, whereas for the 100mer and 200mer, the product migrates slower than the linear substrate.

T4 DNA Ligase

The same four reactions performed for the CL studies and displayed in *Figure 1* were carried out with T4 DNA ligase. Analysis of reaction products demonstrates that T4 DNA ligase has very poor or inefficient ligase efficiency *(Figure 2)*. Samples that were treated with T4 DNA ligase did not result in circular DNA products. This enzyme was not able to ligate ss-DNA, and the reaction for ds-template was inefficient, as well. The results indicate that T4 DNA ligase is not as robust and efficient as CircLigase™. From consideration of the products formed by the ligase enzymes, it can be concluded that CircLigase™ is the better enzyme to use for producing circular DNA.

Optimization Study

Variation of reagents was tested to asses the efficiency of circularization of 35, 55 and 100mer templates. It was observed that not all size templates have the same optimal conditions by comparison of circular product and unused substrate, but a standard set of conditions could be established leading to complete circularization (*Table 1*). The following conditions were determined:

- ATP: ligation was unaffected by variation of ATP concentration. Circular product was observed even for samples that were not treated with ATP. Circularization in the absence of added ATP could be due to the presence of *adenylated* circular ligase in the enzyme mix obtained from the

manufacturer. The ATP concentration used in subsequent reactions is the one recommended by Epicenter, 50 μM.

- $MnCl_2$: the optimal concentration range is 1.25 - 2.5mM. Concentrations higher than 2.5 mM seemed to promote degradation of the circular product. The $MnCl_2$ concentration used in subsequent reactions was that of the manufacturer's recommendation, 2.5 mM.
- CL: 10U/μl appears optimal for reactions. Using more than 10U/μl does not enhance ligation of the template. Decreasing enzyme concentration results in incomplete circularization. CL optimal concentration is 10 U/μl, an increase from the manufacturer's recommendation (5U/μl), and was used in subsequent reactions.
- Incubation time: circularization appears to be unaffected by length of incubation. Therefore the incubation time for the reaction is the same as the manufacturer's recommendation of one hour.

Phosphorylation Assay

To study the effect of the 5' phosphate on the enzyme activity, unphosphorylated nucleotides were tested in the ligation reaction both before and after treatment with polynucleotide kinase *(Figure 3)*. Oligonucleotides lacking a 5' phosphate were successfully circularized by circular ligase after treatment with polynucleotide kinase. Templates without the 5'phosphate were not circularized. The results demonstrated that samples previously treated with PNK are able to ligate when treated with CircLigase™. The phosphorylation reaction conditions were studied by increasing PNK concentration in the reaction. No difference was observed between increased PNK concentrations (12.5 U) and the recommended concentration of 10 U.

Optimal Terminal Nucleotides

Templates having 5'G and 3'T as terminal nucleotides were compared with complementary templates with 5'A and 3'C terminal nucleotides *(Figures 4 and 5)*. The arrow on the gel image indicates the position of the circular product. The band below the circular product is the un-circularized substrate. When ligation is compared with the two different terminal end nucleotides, based on concentration of linear product (observed within the oval on the gel image)., it can be determined that G and T ligate better than the complementary sequence obtaining complete circularization of substrate.

Conclusions:

This study describes the initial efforts to develop a method that will provide suitable template for RCA. CircLigase is an enzyme that can circularize a single linear DNA molecule. With the optimal conditions for CircLigase™ that have been established for synthetic templates, experiments with forensic samples can commence. In addition, it was shown that CircLigase™ is much more efficient in producing circular DNA than T4 DNA. Therefore, foundations have been laid such that protocols may be developed for typing degraded DNA samples that currently are not typeable. Further studies include optimization of amplification of circularized product by RCA of control and mock and adjudicated forensic samples. Additional work will include studies with adapter sequences to provide optimal terminal nucleotides for circularization and development of post WGA analytical conditions.

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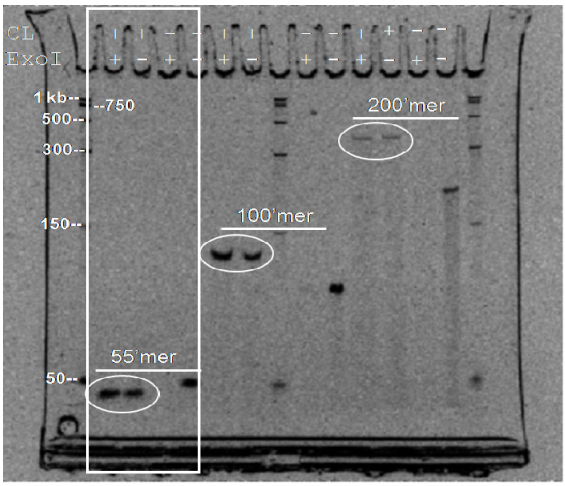


Figure 1: Circular ligase reaction
Ligation of linear DNA of different lengths using CircLigase™.

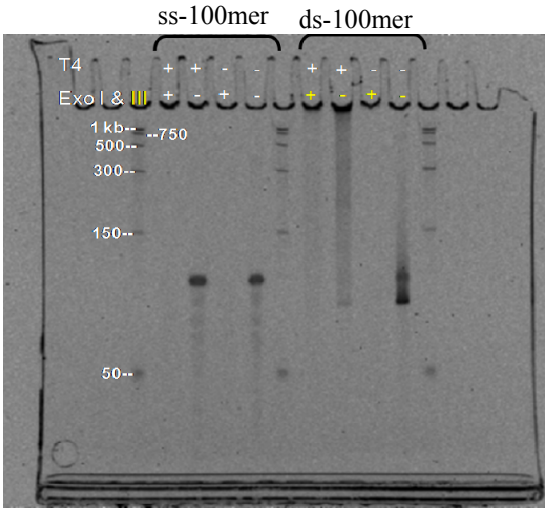


Figure 2: T4 DNA Ligase
Ligation of linear ss- and ds- DNA using T4 DNA ligase.

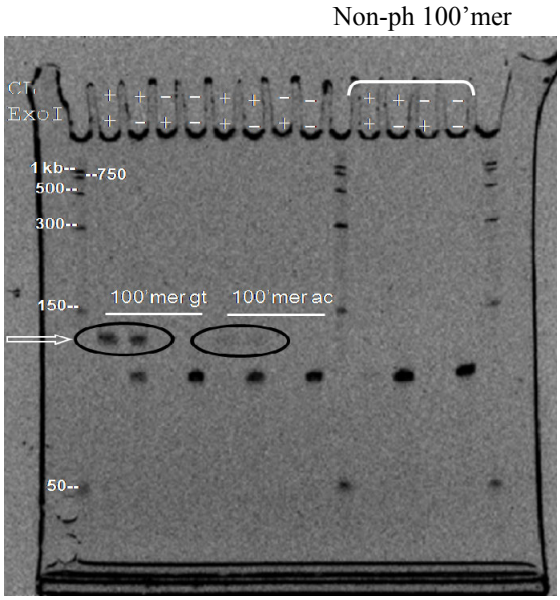
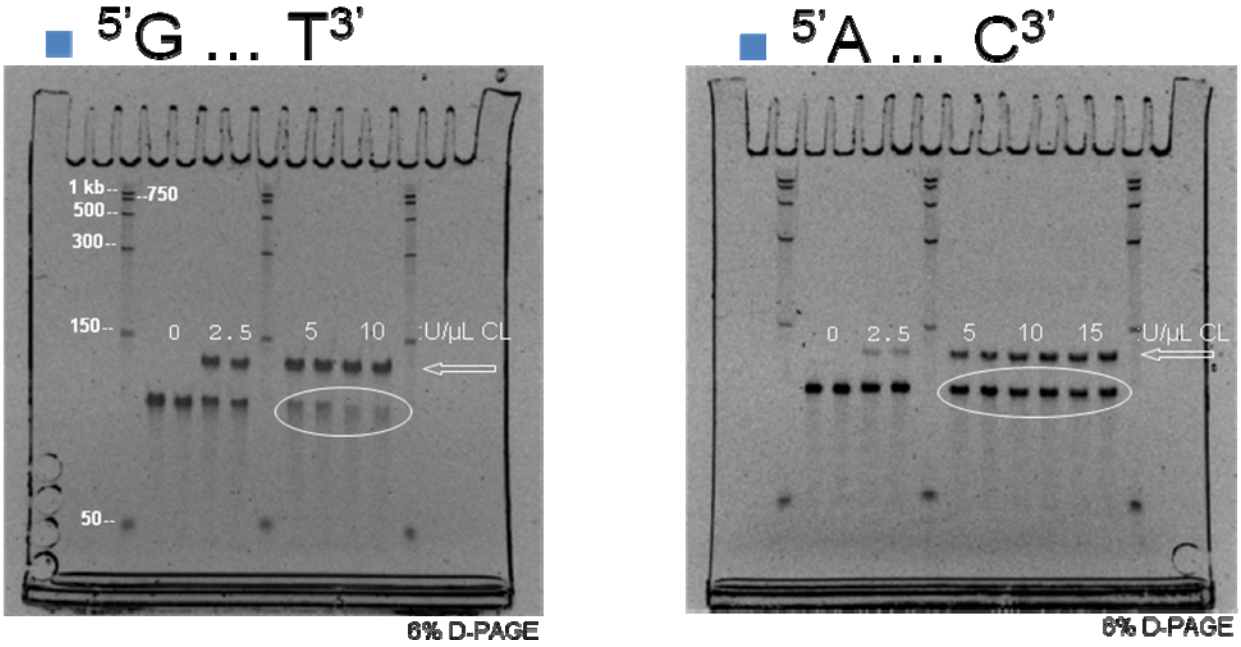


Figure 3: Phosphorylation reaction
100mer ss-template was treated with polynucleotide kinase for phosphorylation of 5' ends.

Table 1: **Optimized conditions for CircLigase™ reaction**

Template	*$MnCl_2$ (mM)*	*ATP (µM)*	*CL (U/µl)*	*Time (hr)*
55-mer	*0 – 2.5*	*25*	*5 – 10*	*1*
100-mer	*1.25 - 2.5*	*25 - 50*	*10*	*1*
200-mer	*5*	*50*	*5 - 10*	*1*
Optimal Protocol	*2.5*	*50*	*10*	*1*



Figures 4 and 5: Terminal nucleotide study
CircLigase™ enzyme efficiency was studied by comparing reactions with complementary strands of ss-DNA