Chapter 6 - Final Discussion

6.1 Conclusions

DNA Ligases are essential repair enzymes. Consequently, they are required to quickly locate and seal a nicked site in the DNA backbone. The aim of the work presented in this thesis was to determine if EcoLigA could adopt a facilitated diffusion mechanism to locate a nicked site in various DNA substrates. Facilitated diffusion is important during cellular processes such as restriction and repair. Enzymes required in these processes, including the restriction enzyme EcoRV (Stanford et al. 2000, Bonnet et al. 2008) have been observed adopting a hopping motion along DNA to locate a target site. It is possible that Ligase, as repair rather than a restriction enzyme, could also adopt a similar method to locate a target site. In this thesis three key sets of experiments were carried out. The first (Chapter 3) concluded the EcoLigA reaction fitted the double displacement (ping-pong) mechanism to locate a nick. This was an important mechanism to establish at the outset, since the enzyme was proven to be adenylated first, and the nick ligation step second. The second set of experiments (Chapter 4) showed that it was possible for EcoLigA to use DNA flanking a target site as a guide to locating a nick. The third main result (Chapter 5) showed that EcoLigA is a weakly processive enzyme, but in the presence of Beta Clamp and clamp-loader molecules, the processivity was greatly increased. These are discussed in turn below.

In Chapter 3, initial experiments revealed that during the Ligase preparation, 12.4% of the protein was pre-adenylated. This was important to know as it would affect the results when trying to calculate initial rates. When pre-adenylated Ligase was added to a nicked DNA timecourse, 12.4% of the reaction would be completed almost instantly. This is known as burst kinetics. So to minimise the effect this had on reaction rate
experiments it was decided to use a limiting concentration of Ligase, set at 0.2 nM throughout the entire thesis. This meant that at time zero, 12.4% of 0.2 nM (0.025 nM) of *Eco* LigA was pre-adenylated. Therefore only 0.025 nM DNA was sealed immediately, a concentration so small that it can not affect the results when calculating the much larger initial rates of reaction.

It is widely accepted that *Eco* LigA requires NAD\(^+\) to perform its nick-sealing function (Gellert, *et al.* 1967). The next part of Chapter 3 investigated whether Ligase could also use other cofactors just as efficiently. The results concluded that NAD\(^+\) was the most efficient cofactor used. However, when the concentration was increased to from 25 to 100 µM, NADH was just as efficient, giving an initial reaction similar to that of NAD\(^+\) at 1.4 nMmin\(^{-1}\). It is possible that oxidation of NADH to NAD\(^+\) was responsible from this observation, or whether NADH in its own right participated in the reaction. The order of preference for cofactors tested was NAD\(^+\)>NADH>NADP\(^+\)>>NADPH=ATP.

The main aim of Chapter 3 was to determine if Ligase had an obligatory off-step from DNA once a nick has been sealed. That is, does Ligase have to first react with free NAD\(^+\) and release nicotinamide adenine mononucleotide (NMN) before then locating and sealing a nick in DNA? The results confirm those from previous work by the Lehman group (1974). Ligase first binds NAD\(^+\), reacts and then must release NMN before it can locate a DNA molecule. On a 40 bp duplex, the $V_{\text{max}}$ of Ligase for NAD\(^+\) increased from 0.8 to 4.4 nMmin\(^{-1}\). A similar value was achieved by Lehman (1974) at saturating NAD\(^+\) concentrations of 4.0 nMmin\(^{-1}\).

More recent studies on DNA Ligases have revealed Ligase undergoes conformational changes during the enzymatic reaction. Before adenylation, Ligase is in an open conformation (Popov *et al.* 2012, Mills *et al.* 2012). After reacting with its corresponding cofactor, the adenylated-Ligase adopts a more closed conformation (Suh
It is now thought that the binding of the AMP group to Ligase drives the conformational change, allowing a more specific tertiary fold for interaction with a DNA substrate (Samai and Shuman 2011). This is supported by the kinetic results in Chapter 3, in which Ligase appears to first bind and react with cofactor before releasing product. The adenylated-Ligase then locates a nicked site and reacts, releasing free enzyme and nick-sealed DNA. One aspect that remained unknown despite this double displacement mechanism is whether the Ligase has to depart from a sealed DNA nick into free solution for re-adenylation, before searching for another nick.

The main aim in Chapter 4 was to answer the question above and determine if Ligase could locate a nicked site in DNA using the flanking DNA lengths as a guide. This was achieved by conducting length-dependency competition experiments. Previous studies on EcoRI (Jack et al. 1982) showed that when two different DNA lengths are within the same reaction mix, the enzyme, given the 'choice', would locate the restriction site on the longer DNA length faster than that on the shorter DNA length. In this chapter, the results showed that when two different DNA lengths were in the same reaction mix, the initial reaction rate of EcoLigA were nearly always faster for longer DNA lengths. For example, for the 100 vs 301 bp competition reaction (Figure 4.7A), the rate at which Ligase locate the nicked site on the 301 bp substrate was 0.55 nMmin⁻¹ (average result), which was 1.77 times faster than the initial rate for the 100 bp substrate.

Figure 4.7C shows a graph of all the ratios of initial rates against the ratios of DNA lengths. A total of 15 different length ratios were investigated, with 8 being repeated. A simple line of best fit showed a clear positive correlation between the ratio or lengths and the ratio of rates. Essentially, it can be seen that Ligase locates a nicked site far faster for longer DNA lengths in a given length competition. The results suggest that Ligase is indeed able to use flanking DNA as a guide to locate a nicked site. This is a
definite example of a protein (that is not a restriction enzyme) undergoing facilitated diffusion along a DNA chain. If Ligase wasn’t associating with flanking DNA, we would expect to see initial rates for both DNA substrates in a competition to be nearly the same, according to random diffusion. Further competition experiments would be interesting using DNA lengths of 5000 bp. This would also help determine which method of facilitated diffusion Ligase adopts, whether 1D sliding or 3D hopping. On very long lengths of DNA it may be an efficient search strategy for a DNA repair enzyme to adopt a 3D hopping mechanism (Halford and Marko 2004).

The main aim of Chapter 5 was to determine if Ligase was a processive enzyme. That is, was it able to move between two well defined nicks without leaving the DNA molecule. This was achieved using a series of 471 bp DNA substrates, each containing two nicked sites separated by 21 to 75 bases. The first experiments conducted used DNA substrates containing two nicks on the same DNA strand (directly-repeated nicks). Results showed that Ligase was a very weakly processive enzyme, showing about 32% processivity. However, the fraction of processive reactions ($f_p$) increased slightly as the intersite distance between nicks increased (ranging overall from 16-39% processivity).

Using inverted nick sites, that is one nick on each strand of the same DNA molecule, the initial association rate of Ligase was slower than on the directly-repeated nicked DNA substrates. This suggested that Ligase adopts a 1D sliding motion to locate a nick, with the protein remaining on the same DNA strand, unable to hop to the other strand. However no conclusive answers can be obtained from the inverted repeat experiments, since no intermediate singly-sealed species was separable on polyacrylamide gels. That is, the singly-sealed species (471 b) were the same length as the doubly-sealed species (471 bp). For example, for the 24inv DNA substrate with one nick in the top strand and one nick in the bottom strand, the top strand fragments a (268 b) and b (203 b) produced
a 471 bp product when the nick was sealed. Bottom strand fragments C (244b) and D (227 b) also produced a 471 b DNA intermediate. Figure 5.15 showed an alternative strategy to the above, where either the top or the bottom DNA strand was 5'-radiolabelled instead of labelling throughout the DNA molecule. This allowed each intermediate in the reaction to be visualised individually. However, this method does not show the formation of the final DNA product (471 bp) and therefore also can not be fitted to a processivity calculation. An experimental design to overcome the problem of distinguishing intermediate and final product species has been devised subsequently (discussions with Prof. Steve Halford, Bristol University). In this new strategy, the use of a hairpin at one end of the double stranded substrate is introduced, and this links fragment b with fragment D. This converts what is a 2-body problem into a 3-body problem and processivity can be fully measured. This would allow all DNA fragments and products to be seen on a denaturing polyacrylamide gel and therefore a processivity calculation could be made to determine the $fp$ for both the directly and inverted nick repeats. This work will be carried out using hairpin molecules in the near future.

The final section of work in Chapter 5 concerned the interaction of *E. coli* beta sliding-clamp (beta-clamp) and gamma clamp-loading complex (gamma-loader) with Ligase and doubly-nicked DNA. Beta-clamp and gamma-loader were kindly provided by Professor Mike O’Donnell (Rockefeller Institute, New York). In combination, these proteins increase the processivity rates of enzymes at the replication fork in bacteria, playing a leading role in lagging and leading strand synthesis (Lopez and O’Donnell 2001).

In Chapter 5, it was determined that beta-clamp, once loaded onto a 471 bp doubly-nicked DNA clearly increased the processivity of Ligase. The reaction was conducted at 0.2, 5, 20 and 200 nM of the gamma-loader, with 5 nM beta-clamp present in all reactions. The initial reaction rate appeared to be lower than that previously observed
for Ligase alone (Figure 5.6), and also when in solution with beta-clamp but with no gamma-loader present; the reaction rate being about one quarter of the reaction of Ligase alone in solution. The reason for this drop in Ligase activity was unknown. It could be that Ligase associates with the beta-clamp in free solution and was unable to ‘get onto’ DNA without the presence of gamma-loader. Further experiments are required to determine the actual effect. However, at high gamma-loader concentrations (200 nM) the reaction was notably faster; processivity increased from 0.06% at 0.2 nM gamma-loader to 34% at 200 nM gamma-loader.

6.2 Future work

The results in the first part of Chapter 5 indicated that when alone in solution, Ligase is weakly processive. However, preliminary results showed that in the presence of beta-clamp and gamma-loader, processivity was increased by a factor of nearly 500. Further work needs to be conducted before any firm conclusions can be made. Firstly, the initial experiments conducted here using Ligase with beta-clamp and gamma-loader must be repeated. Secondly, the intersite distance between nicks should be varied more widely. Results in Chapter 5 showed an increase in processivity as the distance between nicks increased. If Ligase – beta-clamp proves to be a highly processive enzyme complex, the intersite distance could be increased to 100’s and also 1000’s of base pairs. This would allow us to answer the question: does processivity increase when intersite distance increases? Chapter 5 did however support there being a distinct interaction between Ligase and sliding clamp.

Samples of Ligase and ligase:clamp were kindly taken by Dr. John McGeehan (Portsmouth University) to the ESRF, Grenoble to be analysed by small-angle X-ray scattering (SAXS). Figure 6.1 below shows the preliminary data obtained, and this indicates that both proteins do interact with each other. Further work to establish the
Figure 6.1. Preliminary shape (SAXS) work on ligase, clamp and ligase-clamp complex.
nature of the interacting components (Ligase enzyme, NAD\(^+\), Mg\(^{2+}\), nicked-DNA, beta-clamp and clamp-loader) is under way.

Results in this thesis suggest that Ligase does bind non-specifically to DNA and uses the flanking sequences as an antenna to locate a nick site. As described in the section above, we have devised a new strategy using hairpin DNA molecules that will allow unambiguous determination of 1D vs 3D routes using the same processivity approach as Stanford et al. 2000. Another experimental approach that would help deduce the mechanism is that of DNA catenane studies as have previously been used to show that a restriction enzyme adopts a 3D mechanism to locate a specific sequence (Gowers and Halford, 2003). Briefly, catenanes comprise two interlinked DNA rings, one much larger than the other (The smaller circle is termed a minicircle). This is shown in Figure 6.2 below. If ligase is only able to adopt a 1D sliding motion, then once associated with the larger DNA circle, a nick site in the minicircle would rarely be found by sliding alone. However, 3D hopping motion would be revealed by rapid sealing of the minicircle. One further method is by direct visualisation of the Ligase on stretched DNA molecules as has been observed for some restriction enzymes (Bonnet et al, 2008).

### 6.2.1 Future publications

Other than one protein:protein gel shift by the O’Donnell group (Lopez 2001) there have been no other papers about NAD\(^+\) ligase associating with beta-clamp. It is well known that in eukaryotic cells, ATP ligase associates with the PCNA sliding clamp (Wei et al, 2009). Therefore the work in this thesis will be published. Data to be included will be from Chapter 4 (length dependency studies). Figure 4.7A and C will be included to show the competition experiments. These results showed that Ligase is able to use flanking DNA to locate a nick; rates were faster than if Ligase locates a nick by
Figure 6.2 Making catenanes

The plasmid pL1 contains the recognition site for BbvCI and two sites for the Tn21 resolvase. Recombination by Tn21 resolvase produces a catenane with two interlinked rings. The BbvCI recognition site is located on the minicircle.
random diffusion alone. Figures 5.7 and 5.16 will also be included. These figures show that Ligase is weakly processive without the presence of beta-clamp. After further analysis and repeated experiments, Figure 6.1 will also be included as this gives a visual of ligase:beta clamp interaction. Additionally, the hairpin experiments discussed above will be conducted and prepared for publication after a conclusion can be drawn about whether 1D or 3D pathways operate.
References


Cong, P. and Shuman, S. (1993). Covalent catalysis in nucleotidyl transfer. A KTDG motif essential for enzyme-GMP complex formation by mRNA capping enzyme is conserved at the active sites of RNA and DNA ligases. *JBC** 268, 7256-7260


Appendices

Appendix I

DNA sequence of plasmid pRB20 (pET-16b + EcLigA) for LigaseA expression in E.coli. From Dr Richard Bowater, UEA. Cloned and sequenced in 2000.

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TTCTTGAGACGAAAGGCGCTCTGCTGATACGGCGCTTCTCTCTTTATAGGTAATGTGAGTATAATGGTTTCT
TAGACGTCAGGCGCATTCTTGGGGGAAATGTCGAGCGGAGACCAATCTTCCTCTCTCTCTTTGTCGGGTTTTTTC
CCAGAACACCTGTTGAGAATGAAGAAGCAGTGGGACGCTGATCAGGCCTGCAGAAGTTGAGTTTTTTCTA
AGTTCTGCTAGTGGCGCGTATTTTCCCTTGCTGAGTGAAGAGTTGATCTTCTTAGCGCAGCTCAGCACAGGT
GACAAATATTAACTACGGTTGACCCTAATACAGTGATGATAACCTGCGAACCTTACTTCTGACAACGATCGGA
AGGACCGGAGACGAACCATCTCCCATTTCTGGCACAAACATGTTAATGGGTAATGACGCGGCTTCTTCTGA
CCGAGCTGTAATAGCAACCACTACCAAAACACAGAAGGCTGACACGCTGACGAATCTGGACGAAAGGACTG
TGCCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
CCGAAACTATTAACTACGGTGCTTCTTCTCTTCTGAGCGGGGTATTTTCTTGGGTTAGTTGAGGGTTACGA
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**Appendix II**

**Plasmid pL1**

Only the top strand is shown, starting at the 5’-end. The single BbvCI site is shown underlined in blue. Length of supercoiled circular plasmid = 2707 bp.

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**Plasmid pL2**

Only the top strand is shown, starting at the 5’-end.
The two BbvCI sites are shown underlined in blue.
Length of supercoiled circular plasmid = 2728 bp.

TCGCCGGTTTGCGGTAGCAGGTGAAAAACCTCTGACATGCAACTCCCCCCAGACGTCACAGCTTGTCGTGTC
GTAACGGAGGATCCGGAGAACAGCACAGGATCCACGCGAATTTGGGTTTGGGGAAAGGATGTAACAGGTGCCGA
CTTAATCGTGGCGATTACGAGATTTGTTATTACGAGATTACGACCATTGTCGCTGTTGGAATACCCGAGACG
GGTAAAGGAGAAAAATATCGCATTACGCCGATCCCACCTACTGCGTGAGCTGCGGGTTTGGGGAGCTGGCT
GTCGCTGCTGCCTGCCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGACGGGCCAACGCG
GGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCGCTCACTGACTCGCTGCGCTCGGTCGTTCGG
GGCTGCGCGAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCCAGGAAAGAACAT
GTGAGCAAAAGGCCAGAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGGTTTCCATAGGCTCCGC
CCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAAGGTTGCGAAGCTGTAATCATGGTCATAGCTGGT
TTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAAATACGAGCCGGAAGCATAAAGTGTAAAGCCT
GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGC
CGCTCACTGCCGCTTTCCAGCGGGAAATCTGGCGAGTGCTCAATGGAAGGCGCGGATCCCGGGGCCTCTTCG
CTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGAT
GCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATC
GGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAA
ACGCCAGGGTTTTCCCATCGCAGATGTTAAACAGCGGCCAGTGAATTTGCCACATCGTGTTGCTC
Plasmid pL4

Only the top strand is shown, starting at the 5’-end.
The two BbvCI sites are shown underlined in blue.
Length of supercoiled circular plasmid = 2731 bp.

TCGCCGGTTTGCGGTAGCAGGTGAAAAACCTCTGACATGCAACTCCCCCCAGACGTCACAGCTTGTCGTGTC
GTAACGGAGGATCCGGAGAACAGCACAGGATCCACGCGAATTTGGGTTTGGGGAAAGGATGTAACAGGTGCCGA
CTTAATCGTGGCGATTACGAGATTTGTTATTACGAGATTACGACCATTGTCGCTGTTGGAATACCCGAGACG
GGTAAAGGAGAAAAATATCGCATTACGCCGATCCCACCTACTGCGTGAGCTGCGGGTTTGGGGAGCTGGCT
GTCGCTGCTGCCTGCCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGACGGGCCAACGCG
GGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCGCTCACTGACTCGCTGCGCTCGGTCGTTCGG
GGCTGCGCGAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCCAGGAAAGAACAT
GTGAGCAAAAGGCCAGAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGGTTTCCATAGGCTCCGC
CCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAAGGTTGCGAAGCTGTAATCATGGTCATAGCTGGT
TTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAAATACGAGCCGGAAGCATAAAGTGTAAAGCCT
GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGC
CGCTCACTGCCGCTTTCCAGCGGGAAATCTGGCGAGTGCTCAATGGAAGGCGCGGATCCCGGGGCCTCTTCG
CTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGAT
GCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATC
GGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAA
ACGCCAGGGTTTTCCCATCGCAGATGTTAAACAGCGGCCAGTGAATTTGCCACATCGTGTTGCTC

178
Only the top strand is shown, starting at the 5’-end. The two BbvCI sites are shown underlined in blue.
Length of supercoiled circular plasmid = 2731 bp.
Plasmid pL6

Only the top strand is shown, starting at the 5'-end.
The two BbvCI sites are shown underlined in blue.
Length of supercoiled circular plasmid = 2731 bp.

Plasmid pL7

180
Only the top strand is shown, starting at the 5’-end. The two BbvCI sites are shown underlined in blue. Length of supercoiled circular plasmid = 2731 bp.

TCGCGCGTTTCGGGATGACGGTGAACAACCTCTGACACATGCACGCTCCCGAGAGGTGTCACAGCTTGTCTGTAAGCGAGAGGAGGATCGACCTGGCGCGTCCCGCTGCGGTCTGCGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTCCATAGGCTCCGCCCCCTGGACGAAAACTACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCCATAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTGGCTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCGAAGTTAATGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCTGCCTCCGGTTCCCAACGATCAAGGCGAGTACATGATCCCCCATGGTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTACTCATGCTATTTACGCTTAAGCTGTTATAGTTAGATGCCAGATTTAATCACTCCTGTTGAGGATGATGGTCCGAGAAAACCATGCGGCTGCATCGGCGAGGCGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTCCAGCATCTTTCTCTGGTGAGACAAAACAGGAAGGCAAAATGCCGCAGAAGGGCGACACGGAAATGTTGAATACTCATACTCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAACAAATAGGGGTTCCGCGCACATTCTCCCCGAAAAGTGGCCACTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTGCGT

Plasmid pL8

Only the top strand is shown, starting at the 5’-end. The two BbvCI sites are shown underlined in blue. Length of supercoiled circular plasmid = 2731 bp.

TCGCGCGTTTCGGGATGACGGTGAACAACCTCTGACACATGCACGCTCCCGAGAGGTGTCACAGCTTGTCTGTAAGCGAGAGGAGGATCGACCTGGCGCGTCCCGCTGCGGTCTGCGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTCCATAGGCTCCGCCCCCTGGACGAAAACTACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCCATAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTGGCTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCGAAGTTAATGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCTGCCTCCGGTTCCCAACGATCAAGGCGAGTACATGATCCCCCATGGTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTACTCATGCTATTTACGCTTAAGCTGTTATAGTTAGATGCCAGATTTAATCACTCCTGTTGAGGATGATGGTCCGAGAAAACCATGCGGCTGCATCGGCGAGGCGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTCCAGCATCTTTCTCTGGTGAGACAAAACAGGAAGGCAAAATGCCGCAGAAGGGCGACACGGAAATGTTGAATACTCATACTCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAACAAATAGGGGTTCCGCGCACATTCTCCCCGAAAAGTGGCCACTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTGCGT
Plasmid pL9

Only the top strand is shown, starting at the 5′-end. The two BbvCI sites are shown underlined in blue. Length of supercoiled circular plasmid = 2731 bp.
Only the top strand is shown, starting at the 5'-end.

The two BbvCI sites are shown underlined in blue.

Length of supercoiled circular plasmid = 2731 bp.
Plasmid pL11

Only the top strand is shown, starting at the 5’-end.
The two BbvCI sites are shown underlined in blue.
Length of supercoiled circular plasmid = 2731 bp.

TCGCGCGTTTCCGTTGATGACGGTGAAAACCTCTCTGACATGACGCTCCCGGAGACGGTCACAGCTTGTCTGTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGATCGGGCCTC
TTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTTGCATCCTCAGCTGACTAAT
TCGAGCTCGGTACCCGGGGATCCTGCTAGGCTGACCTGCAGGCATGCAAGCTACGTGCATCCTCAGCCTGA

Plasmid pL12

Only the top strand is shown, starting at the 5’-end.
The two BbvCI sites are shown underlined in blue.
Length of supercoiled circular plasmid = 2731 bp.

TCGCGCGTTTCCGTTGATGACGGTGAAAACCTCTCTGACATGACGCTCCCGGAGACGGTCACAGCTTGTCTGTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGATCGGGCCTC
TTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTTGCATCCTCAGCTGACTAAT
TCGAGCTCGGTACCCGGGGATCCTGCTAGGCTGACCTGCAGGCATGCAAGCTACGTGCATCCTCAGCCTGA

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