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Comparative Analysis of Golden Gate and Classical Cloning Techniques in E. coli: A Study in Molecular Cloning Efficiency

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Abstract: Molecular cloning remains a cornerstone technique in genetic engineering and synthetic biology. In this study, we conducted a systematic comparative analysis between the classical cloning method and the Golden Gate assembly technique, utilizing Escherichia coli as the model organism. Through polymerase chain reaction (PCR) amplification, restriction enzyme digestion, ligation, transformation, and Sanger sequencing, we assessed the operational efficiency and cloning fidelity of both strategies. Our results demonstrated that Golden Gate assembly, leveraging type IIS restriction enzymes and simultaneous ligation, significantly enhanced cloning efficiency and precision, particularly for seamless multi-fragment assembly. In contrast, the classical cloning approach maintained certain advantages in simplicity and robustness for specific experimental conditions. Challenges encountered during transformation and sequencing highlighted the critical impact of technical accuracy on experimental outcomes. This study underscores the importance of selecting appropriate cloning methodologies tailored to experimental objectives and laboratory capabilities, providing a foundation for optimized molecular cloning workflows in future synthetic biology and biotechnology applications.

Keywords: Golden Gate Assembly; Classical Cloning; Escherichia Coli; Molecular Cloning; DNA Assembly; Recombinant DNA Technology; Transformation Efficiency; Synthetic Biology

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1.Introduction

DNA recombination and cloning techniques are among the most fundamental techniques in molecular biology and are achieved by artificial means. It is widely used in various fields related to genetic manipulation and is central to synthetic biology experiments[1]. This experimental study compares the methodologies of the Golden Gate technique and the classical technique for DNA assembly, revealing the characteristics of the two methods. Compared to Classic technology, Golden Gate technology is very effective in accurately assembling multiple DNA fragments, especially when precise and seamless DNA assembly is required, such as in protein engineering; however, the method's dependence on specific types of restriction enzymes may limit its flexibility[2]. This will help to provide a basis for the selection of the most appropriate method based on the needs of the specific project and the expertise of the laboratory.

In addition, E. coli was used as the growth environment in this experiment. As an important tool in scientific research, E. coli has simple cultivation and genetic manipulation, its fast growth rate, its low cost, and its importance for the study of many basic biological processes[3]. E. coli strains commonly used in laboratories are usually harmless and fall into a low biosafety

risk category, facilitating safe laboratory practices. The goal of this experiment was to utilize recombinant DNA methods to insert two gene segments into a plasmid vector—employing classic cloning and Golden Gate technology respectively. The modified vectors were then introduced into Escherichia coli, with the ultimate step involving the examination of the DNA sequences obtained.

2.Methods and Material

2.1 Amplification of DNA fragments

PCR reactions were set, reactants were made by adding the following reagents: 1× Q5 reaction buffer (New England Biolabs), 0.2 mM dNTP mix, 0.5 μM Forward primer, 0.5 μM Reverse primer; 0.01 ng/μL Template DNA (provided by University of Edinburgh); 20 units/mL Q5 DNA polymerase (New England Biolabs). Other unlabeled chemicals used were of the high grade available commercially.

2.2 Digestion of pGEX-6p-1 vector with restriction-enzyme

For restriction enzyme digest reaction: 5 tubes were set up following the given protocol, 3 remained incubating at 37°C overnight, then heat-inactivated at 65°C for 15 minutes; 2 tubes were heat- inactivated after 37°C water bath for 30 minutes. Tube 1 was set up with 3.7 μL sterile H2O, 3 μL 10× CutSmart Buffer (New England Biolabs) and 3 μL 10× HcoRI-HF. Tube 2 was set up with 3.7 μL sterile H2O, 3 μL 10× CutSmart Buffer (New England Biolabs) and 3 μL 10× Xhol. Tube 3 was set up with 10 μL each from both Tube 1 and Tube 2, and centrifuged briefly. These three tubes were incubated in a waterbath at 37 °C for 30 minutes. After 30 minutes, Tube 4 was set up with 10 μL Tube 1 and Tube 5 was set up with 10 μL Tube 2, then these two were heat inactivated at 65 °C for 15 minutes. The three remaining tubes remained incubating at 37 °C overnight and was heat- inactivated at 65 °C for 15 minutes the other day.

2.3 Preparation of agarose gel

0.8% w/v gels were assigned and made, the ratio was 0.8 mg agarose and 10 mL distilled water. When making gels, the given protocol was followed to ensure all agarose was fully dissolved, cooled for at least 10 minutes (cooled but still in a form of liquid) before adding gel stain. The given gel stain was diluted from 20000× to 1×, therefore, 5 μL of gel stain was added into the system.

2.4 Visualization of PCR product and vector after digestion

When visualizing and analyzing PCR product and vector digested by restriction enzymes by agarose gel electrophoresis, 0.8% w/v gels were assigned and made, the ratio was 0.8 mg agarose and 10 mL distilled water. When making gels, the given protocol was followed to ensure all agarose was fully dissolved, cooled for 10 minutes (cooled but still in a form of liquid). During the visualization of PCR product, 30 μL of PCR product, 10 μL of ladder and 10 μL of positive control group were added respectively. During the visualization of digested vectors, 5 μL of dye was added in each tube; therefore, the overall amount of solution injected s was more than expected. The detailed amount that was added in the cube injected was 25 μL and 15 μL respectively.

2.5 Gel extraction of PCR products and digested vector; restriction-enzyme digest of PCR product for classical cloning; ligation of insert into pGEX-6P-1 vector

With the help of QIAquick Gel Extraction Kit from QIAGEN[4, 5], DNA was extracted and purified from the gel slices from your PCR products and from the vector that was digested overnight with both restriction enzymes. Noted that ethanol was already been added to Buffer PE; collection tube was used instead of vacuum manifold; lid of a sterile Eppendorf tube should be carefully snipped off by scissors and kept safe and clean; volume of Buffer EB was changed to 35 μL and left for at least 2 minutes; do not discard the sample from the last step; keep the purified PCR products on ice.

6 μL H2O (sterile, molecular biology grade), 15 μL purified PCR product, 3 μL 10× CutSmart Buffer (New England Biolabs), 3 μL 10× EcoRI-HF (New England Biolabs) and 3 μL 10× Xhol (New England Biolabs) were used during the restriction-enzyme digest of PCR product. After setting up the reaction, it was incubated overnight at 37 °C, then heated inactivated at 65 °C for 15 minutes.

After that, the PCR product was purified for classical cloning using a QIAquick PCR Purification Kit from QIAGEN. Noted that ethanol had already been added to Buffer PE; pH indicator had already been added to Buffer PB; collection tube was

used instead of vacuum manifold; use scissors to carefully snip off the lid of a sterile 1.5 Ml Eppendorf tube; lid of a sterile Eppendorf tube should be carefully snipped off by scissors and kept safe and clean for ligation and placed on ice to pre-chill; the volume of Buffer EB was changed 35 μL during the last step and left for at least 2 minutes; flow-through was kept.

2.6 Golden Gate Assembly

Four reactions (one reaction and three controls) were set, each with a final volume of 20 μL, using the table below: one reaction and three controls. One reaction was set up with everything needed, including 1 μL vector (provided by the University of Edinburgh), 1 μL PCR product (provided by the University of Edinburgh), 14 μL sterile H2O, 2 μL 10× DNA ligase buffer (New England Biolabs), 1 μL Bsal and 1 μL T4 DNA Ligase. For the other three control groups, one was set up with everything needed except for PCR product, one was set up with everything needed except for Basl enzyme, one was set up with everything needed except for T4 DNA Ligase. Each reaction was mixed gently by pipetting up and down slowly (the pipette was set at no more than 10 μL), then centrifuged briefly. PCR thermal cycler program was carried out for 90 minutes.

2.7 Transformation of both ligated vectors into E.coli

For each ligation reaction: 10 μL of ligation reaction was transferred to new pre-chilled Eppendorf and returned to ice; 100 μL of competent cells were transferred carefully and returned to ice.

For classical cloning only, two control groups were prepared similarly: For negative control, only 50 μL cells was added; for positive control: 50 μL competent cells was transferred to tube containing 1 μL uncut vector .

Then, each ligation reaction was incubated on ice for 15 minutes. The cells were heat stocked at 42 °C for 1 minute and returned to ice for 1 minute.

After that, 250 μL SOC was added and the sample was mixed intermittently by gently inverting the tube.

During the procedure of pouring LB agar, 0.25 mL volume of each antibiotic (kanamycin for pETGG and carbenicillin for pGEX-6P-1) was added to LB agar and mixed by swirling or rolling the bottle gently, avoiding introduction of air-bubbles, after that, each plate was poured and contained about 10 mL LB (provided by University of Edinburgh). Then, waited for about 15 minutes for the agar to chill, and 50 μL of competent cells was pipetted carefully into the center of a plate and spread the solution carefully with the sterile blue spreaders. The remaining cells were concentrated and spined for 1 minute at 6000 rpm to pellet; cells were resuspended and spread onto a fresh plate. Finally, plates were transferred to a 37 °C incubator and colonies were picked to set up overnight 5 mL LB/antibiotic in a 50 ml Falcon tube for further sequencing step.

2.8 Extraction of DNA and Preparation for Sanger Sequencing

From the 5 mL cultures using a QIAprep Spin Miniprep Kit from QIAGEN, DNA was extracted and purified. Noted that there were some differences made in the handbook: LyseBlue reagent was used; RNase A has already been added to Buffer P1; Ethanol has already been added to Buffer PE; in step 1, the centrifugation conditions were changed to 4000 rpm for 10 minutes; discard the supernatant when it was clear without disturbing the cell pellet; then the pellet was centrifuged again at 4000 rpm for 10 minutes; the remaining supernatant was removed carefully with a pipette; in step 2, the cell pellet was resuspended by gently pipetting the liquid up and down; after step 9, the column was left to dry in air for approximately 15 minutes; the lid off a sterile Eppendorf tube was snipped and the QIAquick column was place in the tube; the lid was stored somewhere clean; in step 10 Buffer EB was changed to 35 μL and left for at least 2 minutes, before eluting DNA by centrifuging; flow-through was kept this time. Before sanger sequencing, samples were prepared by setting two tubes per pair. The concentration of purified DNA using the Nanodrop was 83.381 μg/mL for Classic technology and 159.26 μg/mL for Golden Gate method. Therefore, 15 μL and 7.8 μL of sterile water were added respectively.

For the sequencing reaction, each plasmid requires two primers, one forward (For) and one reverse (Rev): pGEX 6P-1 (For) and pGEX 6P-1(Rev) for the pGEX 6P-1 vector; T7 promoter (For) and T7 terminator (Rev) for the pET28GG vector. Labelled samples and these primers were sent for sequencing at DNA sequencing and Services at the Medical Sciences Institute in Dundee.

3.Results

3.1 Overall molecular cloning using Classic technology

3.1.1 Restriction-enzyme digest of pGEX-6P-1 vector by both EcoRI-HF and Xhol enzymes as well as its

visualization by agarose gel electrophoresis

Compare the visualization results of PCR products with predicted length: The control group of Classic method (C150) ran faster than that from Golden Gate method (GG150). Moreover, in Lane 1, the band was almost invisible, only a very faint band was visible at slightly above 100 bp. There were no bright band in Lane 3, Lane 4. For Lane 5 and Lane 6, Lane 5 was close to the control group of GG150 at 100 bp, while the band in Lane 6 was much wider than that in Lane 5. As for the ladder, the bands were diffuse and smeared, which was not ideal. (Figure 3).

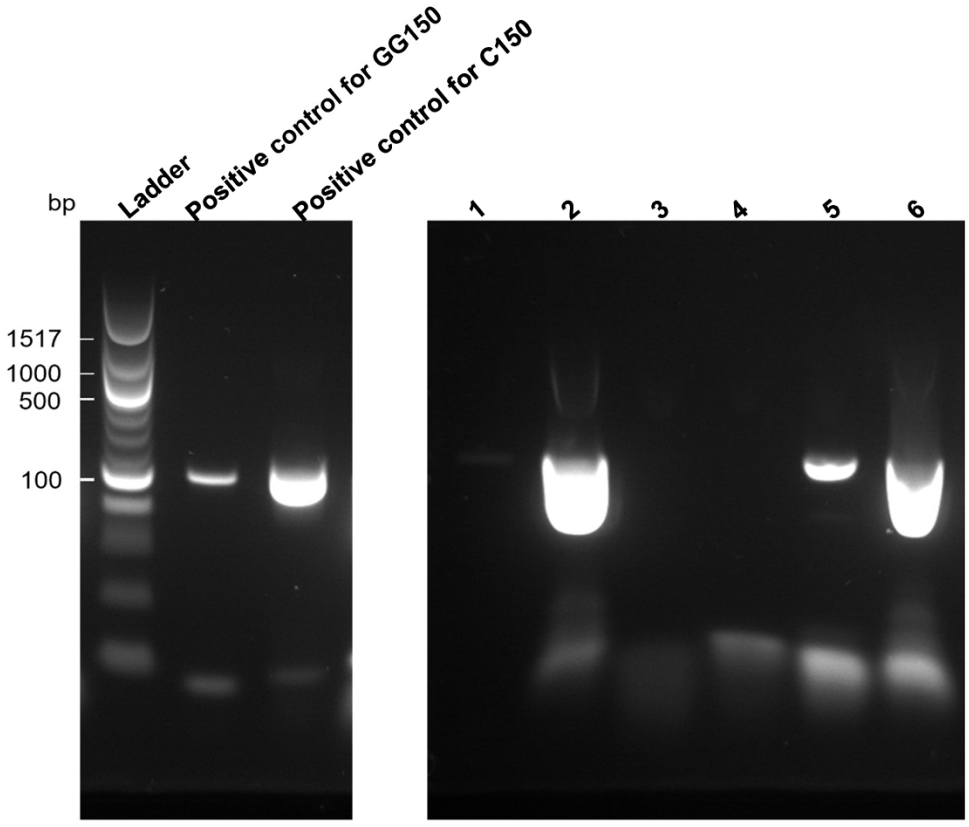


Figure 3. 1.2 % agarose for PCR products Lane 1, Lane 2 stood for PCR products gained from Classic technology; Lane 3, Lane 4 stood for PCR products gained from Golden Gate technology; while Lane 1 and Lane were added with excess dNTPs.

With the given length of pGEX-6P-1 vector of 4984 bp, the digested pGEX-6P-1 vector by both EcoRI-HF and XhoI enzymes was expected to be 4969 bp (4984 bp minus 15 bp in Figure 4).

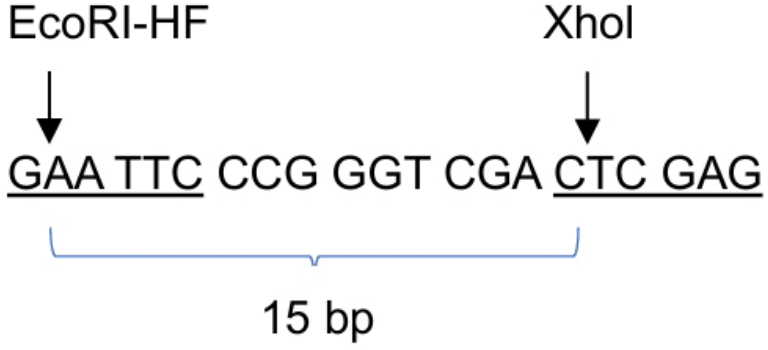
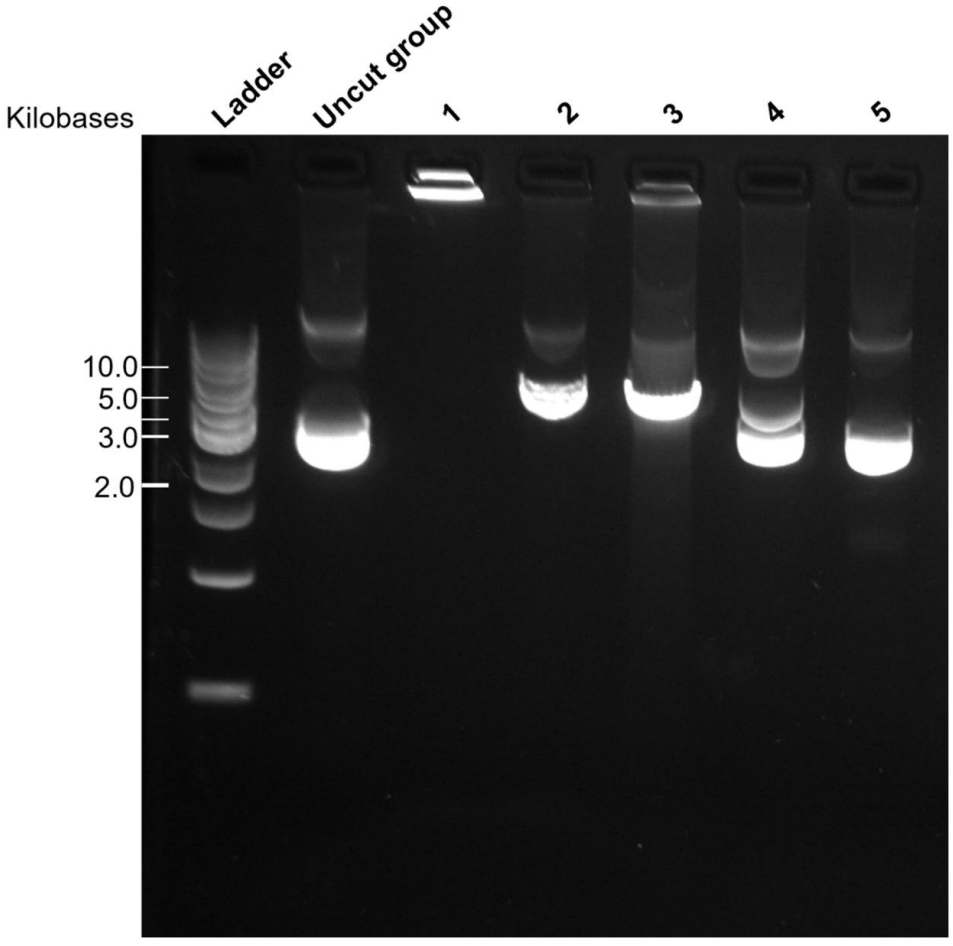


Figure 4.Agarose gel electrophoresis for vector analysis typically included five sample lanes alongside a ladder and an uncut pGEX-6p-1 (4984 bp) vector control. Lanes 1 to 5 represented: EcoRI digested overnight, XhoI digested overnight, EcoRI and XhoI co- digested overnight, EcoRI digested for 30 minutes, and XhoI digested for 30 minutes, respectively. Expected outcomes were: the fastest migration for the uncut control, followed by the 30-minute single enzyme digests (Lanes 4 and 5), then the overnight co-digest (Lane 3), and lastly, the overnight single enzyme digests.

The uncut group in Lane 2 showed a band below 3000 bp; Lanes 4 and 5 displayed bands parallel to the uncut control; in

Lane 1 and 2, with overnight incubation, Lane 1's sample was immobile, and Lane 2 migrated slower due to linearization. Lane 3 contained a mix of linear and smaller base-paired structures, with an approximate size of 4.5 kilobases, migrating similarly to the 30-minute incubation groups (Figure 5). The trailing phenomenon could be seen in every lane.

Figure 5. 0.8 % agarose for vectors, Lane 1 represented EcoRI digested overnight, Lane 2 represented XhoI digested overnight, Lane 3 represented EcoRI and XhoI co- digested overnight, Lane 4 represented EcoRI digested for 30 minutes, and Lane 5 represented XhoI digested for 30 minutes.



3.1.2 Extraction and ligation of digested PCR product into digested pGEX-6P-1 vector

Follow the instruction of QIAquick Gel Extraction Kit, PCR product and vector were extracted from the gel; two control groups were set to ensure the success and accuracy of transformation.

3.1.3 Transformation of pGEX-6P-1 ligated vectors into TOP strain of E.coli

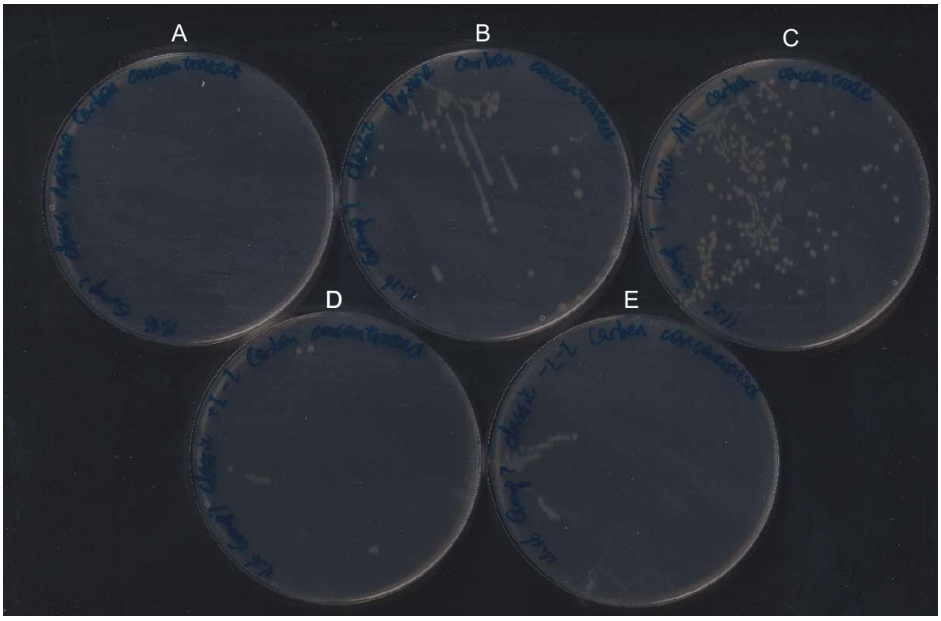
pGEX-6P-1 ligated vectors into TOP strain of E.coli. Five groups were set, and during the procedure, the negative group showed no bacterial growth; the positive group exhibited a small amount of colony growth and was unevenly distributed, with a linear dense presence in the middle portion, and only a few individual colonies were visible on the right side, the shape those separated bacteria was in regular rounded circles, white in color.

The plate grown with everything needed (both PCR product and T4 DNA ligase) showed higher densities of colonies and was segregated better, but it was still less homogeneous, mainly concentrated in the upper left corner, and the separated ones were in in regular rounded circles, white in color.

For the plate grown with T4 DNA ligand but without PCR product, only a few scattered bacteria were seen on the plate, and were not very well grown; the shape was in regular rounded circles, white in color.

For the plate grown without both T4 DNA ligand and PCR product, few scattered bacteria were seen on the plate, unevenly shaped and poorly separated (Figure 6).

Figure 6.Plate demonstrating the transformation of pGEX-6P-1 ligated vectors into TOP strain of E.coli. A. Negative group with only half the amount of the cell B. Positive group half the amount of cells and 1 μL uncut vector C. ALL grown with everything needed (both PCR product and T4 DNA ligase) D. +L-I with T4 DNA ligand but without PCR product E. -L-I grown without both T4 DNA ligand and PCR product.



3.2 Overall molecular cloning using Golden Gate technology

3.2.1 Restriction-enzyme digest of pETGG vector by BsaI enzyme as well as visualization by agarose gel electrophoresis

In the Golden Gate cloning method, cleavage by the BsaI enzyme and ligation of DNA fragments into pETGG vector was performed simultaneously in a single reaction. As this method only one enzyme to cleave, the length of vector remained unchanged; the length of PCR product was calculated as 469 bp (the length of PCR product for Golden Gate method was 493 bp shown in Appendix 1, and the number of base pairs cleaved was 24 bp, therefore it could be calculated that PCR products after digestion was 493 minus 24 bp).

3.2.2 Extraction and ligation of digested PCR product into pETGG vector.

Follow the instruction of QIAquick Gel Extraction Kit, PCR product was extracted from the gel; two control groups were set to ensure the success and accuracy of transformation.

3.2.3 Transformation of pETGG ligated vectors into TOP strain of E.coli

With three control group, a total of four groups were set during this procedure.

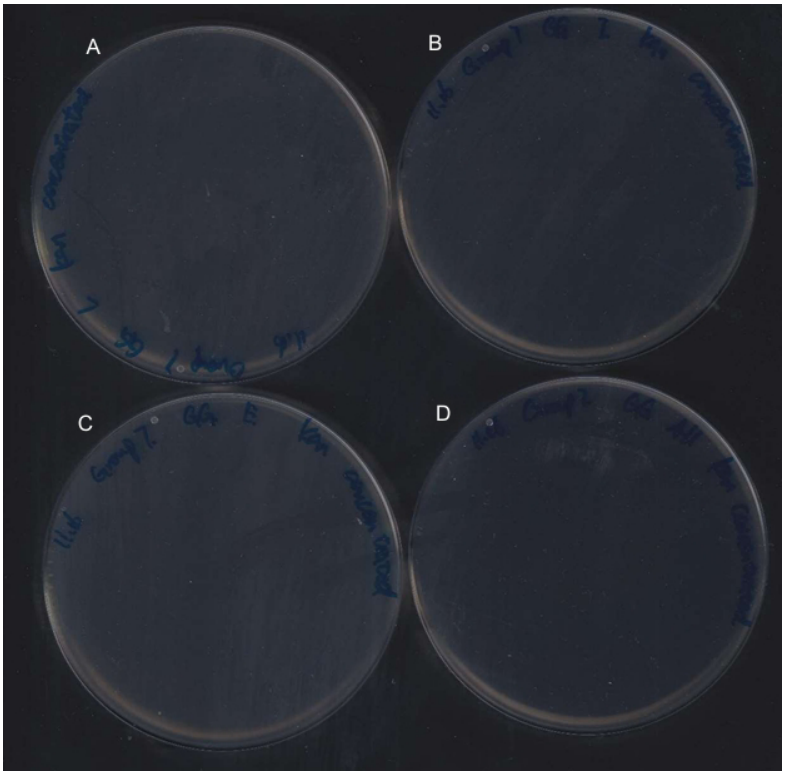
For the plate grown with everything needed, no bacteria growth can be seen; for the plate grown with everything needed except for PCR product for Golden Gate, no bacteria growth can be seen; for the plate grown with everything needed except for Bsal enzyme, no bacteria growth can be seen; for the plate grown with everything needed except for T4 DNA ligase, no bacteria growth can be seen.

A. Grown with everything needed but T4 DNA Ligase B. Grown with everything needed but PCR product C. Grown with everything needed but Basl enzyme. D. Grown with everything needed/

3.3 Extraction of DNA and Sanger Sequencing

The concentration of purified DNA was determined (83.381 μg/mL for Classic technology and 159.26 μg/mL for Golden Gate method and samples were formulated accordingly. A total of four sequences were measured by sanger sequencing, including PCR product from Golden Gate technology using forward primer and reverse primer; PCR product from Classic technology using forward primer and reverse primer (Appendix 2). By comparing the clarity and definiteness of chromatography, PCR product from Classic technology using reverse primer and PCR product from Golden Gate technology using forward primer, compared to the expected sequences, no similarity was found for both methods.

Figure 7. Plate demonstrating the transformation of pETGG ligated vectors into TOP strain of E.coli.



4.Discussion

4.1 Analysis through Agarose Gel Electrophoresis

Gel electrophoresis sorts DNA fragments by size: smaller fragments migrate faster and further. A control comprising 517 bp and 493 bp fragments was used, alongside a ladder for size benchmarking. In this experiment, samples in various lanes contained PCR products from previous steps. Lane 1 had a Classic method PCR product with excess dNTPs; Lane 2 and Lane 3 had Classic method PCR products; Lane 4 had a Golden Gate method PCR product with excess dNTPs; Lanes 5 and 6 had Golden Gate method PCR products.

The separation of PCR protein using Agarose Gel Electrophoresis depends on size. The agarose gel matrix acts like a sieve through which smaller molecules can move more easily and therefore travel faster, while larger molecules move more slowly. Therefore, for the separation of PCR products (from Classic and Golden Gate method, 517 bp and 493 bp respectively), the PCR product from Golden Gate was expected to run faster than that from Classic method. And the base pairs expected in PCR products after they have been digested with restriction enzymes as 501 bp (the length of PCR product for Classic method was 517 bp shown in Figure 1, and the number of base pairs cleaved was 16 bp, therefore it could be calculated that PCR products after digestion was 517 minus 16 bp)

However, the actual result turned out to be the opposite of what was expected. In Lane

1, the band was almost invisible, this might be caused by operation error. During the injection of Lane 1 the injecting speed was too fast, causing the formation of bubbles, which took away many samples and resulting in the inadequate amount of sample in Lane1. For Lane 5 and Lane 6, despite that they were duplicates of the same method amplifying products, great difference could be detected. Lane 5 was close to the control group for Golden Gate method at 100 bp, while Lane 6 might indicate error. As for the ladder, the ladder in agarose gel electrophoresis should appear as distinct bands of different sizes, each corresponding to DNA fragments of known lengths. While in this image, the ladder bands were diffuse and smeared, this could be caused by too much sample loaded; uneven gel; degraded or contaminated DNA ladder. It was also noted that there were no bright band in Lane 3, Lane 4. After recalling the experimental procedure and comparing it with the group that also used the instrument to rule out sample degradation, poor well formulation, electrophoresis issues, it was considered that the samples might not have been loaded into the wells properly due to an error in the sample loading process.

4.2 Analyzing Plasmid DNA Morphologies and Electrophoresis Outcomes in Enzyme Digest Experiments

Plasmid DNA is normally present in vivo in 3 forms: supercoiled, liner and relaxed circular or nicked. The superhelix morphology is the most compact and has the fastest migration rate in electrophoresis. Linear morphology followed. Therefore, the expectation was that overnight groups with linear structure ran slower than uncut group, which had superhelix structure; and groups that were incubated for 30 minutes (partially linear).

Expected outcomes were: the fastest migration for the uncut control, followed by the 30-minute single enzyme digests (Lanes 4 and 5), then the overnight co-digest (Lane 3), and lastly, the overnight single enzyme digests.

The uncut group in Lane 2 showed a band below 3000 bp, possibly due to structural conformation. Lanes 4 and 5 displayed bands parallel to the uncut control, suggesting that within 30 minutes, the DNA remained largely undigested with only minor successful cuts.

In Lane 1 and 2, with overnight incubation, Lane 1's sample was immobile, suggesting errors (this will be further analyzed in Discussion 5.3), and Lane 2 migrated slower due to linearization. Lane 3 contained a mix of linear and smaller base-paired structures, with an approximate size of 4.5 kilobases, migrating similarly to the 30-minute incubation groups (Figure 5). The sample of vector in Lane 2 (single enzyme EcoRI incubated for 30 minutes) was stuck in the well during electrophoresis analysis after PCR, possible reasons are as follows: the sample was too sticky; the amount of loading buffer added was insufficient; the sample might be loaded incorrectly; the agarose might be uneven, resulting in small bubbles in the pore or uneven structure, which affects the movement of the sample.

Moreover, the trailing phenomenon can be seen in every lane and may be caused by the introduction of impurities in the process.

4.3 Assessing pGEX-6P-1 Vector Integration and Control Group Efficacy

During the transformation of pGEX-6P-1 ligated vectors into TOP strain of E.coli, the result observed was not exactly as expected. For the positive group which exhibited a small amount of colony growth and was unevenly distributed, it was probably due to the fact that during the fact that when spreading the solution on the plate, it didn't spread very evenly; the fact that few individual bacteria could be seen indicating poor separation.

The plate grown with everything needed (both PCR product and T4 DNA ligase) showed higher densities of colonies and was segregated better, suggesting that a homogeneous inoculation might have been carried out, possibly through the coating method of liquid suspension media. The spacing between colonies showed that they were well separated, which helped in the subsequent selection of individual colonies for further analysis.

For the plate grown with T4 DNA ligand but without PCR product, few scattered bacteria could be seen and the shape was in regular rounded circles, white in color. In the absence of only PCR product but in the presence of T4 DNA ligase, the colonies should not have grown, but here a small number of colonies appeared, probably because of the presence of a small number of free base pairs spelled on the vector and the vector closed on itself with the help of the T4 DNA ligase, but this situation was more idealized.

For the plate grown without both T4 DNA ligand and PCR product, bacteria were seen on the plate, unevenly shaped and poorly separated. However, there should be no growth of colonies at all, but colonies appeared on this plate. Observe the shape of the bacteria, which is irregular, and consider that there may have been contamination during the operation that led to the growth of stray bacteria

Control groups were set for different reasons, for positive group, half of the cells and 1 μL uncut vector was added, which was used for the conformation of plate efficient transformation. Negative group with only half the amount of the cell should not grow anything, it was set to verify the validity of the experiment to ensure that changes in the results are due to the experimental treatment and not to other variables in the experimental process. For the plate grown with T4 DNA ligand but without PCR product, the role and impact of PCR products in this experiment could be observed. For the plate grown without both T4 DNA ligand and PCR product, the chance or non- experimental factors on experimental results might be excluded to ensure the reliability of experimental results.

4.4 Assessing Anomalies in E. coli Transformation with pETGG Vectors

During the transformation of pETGG ligated vectors into TOP strain of E.coli, no bacteria growth could be seen, which was unreasonable. Comparing with other groups' results, it seemed that most of groups gained reasonable results with detectable bacteria growth. During the procedure, many steps could go wrong, improper storage of enzymes at too high a temperature; improper operation and so on. Here is the prediction of the reasonable results: The mCherry gene (mCherry is a red fluorescent protein commonly used as a marker protein) with flanking BsaIsites was cloned between the NcoI and XhoI sites to generate pETGG vector from the commercial vector pET28b, therefore, if the PCR product was ligated successfully, the bacteria should have grown in white color; on the other hand, if the PCR product was not ligated, then depending on the situation, the bacteria might have grown in pink color or did no grow at all. When it was grown with everything needed except for Basl enzyme, it should have grown bacteria in pink color; when it was grown with everything needed except for T4 DNA ligase, it shouldn't have grown anything; when it was grown with everything needed except for PCR product, it shouldn't have grown anything, but there was a slight chance that the vector closed on itself with the help of T4 DNA ligase and free base pair.

4.5 Troubleshooting Primer Mix-Up in Sanger Sequencing Experiment

Sanger Sequencing was not a success with no similarity found in both methods. Then, there were indeed clear and well-defined sequences displayed in the mapping that just didn't match our target gene fragments. This happened in three groups of students, so the instructor assumed that it might have happened that the Golden Gate group was labeled backwards from the Classic method group during the final preparation for sanger sequencing, and the wrong primer was used, which led to the failure of the experiment, which was very likely to happen.

Specifically, the sequence was obtained from the Golden Gate ligated vector using the classic method primer, presumably because there happened to be a recognition site for the classic method primer in the vector.

In order to verify this, the Golden Gate sequence was compared with the complete pETGG ligated vectors sequence, but still no similarity found.

There are many possible causes of errors in sanger sequencing, which may be due to operational errors such as sample cross-contamination, volume errors, etc., and it is not possible to clearly determine the specific steps that went wrong

4.6 Efficiency and Specificity in Golden Gate Cloning

Compared with traditional methods, in the Golden Gate cloning method, cleavage by the BsaI enzyme and ligation of DNA fragments can be performed simultaneously in a single reaction. This is an advantage of the Golden Gate cloning technique, which allows multiple fragments to be constructed simultaneously with high efficiency and fidelity. Golden Gate cloning takes advantage of the properties of Type IIS restriction enzymes (such as BsaI), which cut on the outside of their recognition sequences, leaving excessively long fragments with sticky ends. These sticky ends can be designed to be complementary to each other, allowing multiple fragments to be ligated into the vector in the correct orientation in the same reaction. With the addition of a DNA ligase, cutting and ligation can be performed in the same tube and under the same conditions, simplifying the process and allowing for the orderly assembly of multiple fragments in one step. Comparing longer sticky ends (overhangs) formed by Bsal with shorter sticky ends; it offers several advantages: Longer overhangs can provide more specificity during the ligation step, reducing the chance of self-ligation or non-specific assembly; efficiency oof this process is improved, as longer overhangs can increase the probability of correct fragment binding and assembly; ensure accuracy as an unique overhangs created by the restriction enzymes ensure that DNA fragments can only join in one orientation and so on.

5.Conclusion

In conclusion, this report successfully highlights the importance of using Golden Gate and classical cloning techniques in scientific research. Using Escherichia coli as a model organism, the results of this study, obtained from accurate experiments, provide important insights into the efficacy and practical application of these DNA assembly methods. By comparing these techniques, this report not only fulfils its aims and objectives, but also enhances our understanding of the role of molecular cloning in the development of scientific knowledge. It highlights the importance of selecting appropriate cloning strategies according to specific experimental requirements and the potential of E. coli as a versatile tool for biotechnological research.

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no

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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