Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

Insertion of Target DNA in Vector and Performing DNA Sequencing Using Transformed Bacteria

Introduction

In this experiment, DsRed (the gene of interest), a red fluorescent protein, was used as the insert to be inserted into the plasmids. Plasmids are circular pieces of double-stranded DNA. They can be replicated independently in the host prokaryotic cells. pET28a vector was used in this experiment.

Since the source of the template that contained the desired DNA sequence was often contaminated or limited in amount, the desired DNA sequence was marked and amplified using polymerase chain reaction (PCR) to produce an abundant amount or later use.

PCR works in a series of reactions that can be summarized in 3 main steps: denaturation, annealing, and extension. PCR requires a polymerase from a microbe Thermus aquaticus (Taq), this bacterium can work in high temperatures (up to 90°C) required for denaturing the protein. The PCR includes cycles of temperature alteration. It is first heated to around 95°C to separate the double-stranded DNA template. The two single strands of DNA template will then be annealed with their unique complementary DNA sequences by the polymerase when the temperature is lowered in step 2. In step 3, the temperature is raised again to make it optimal for the Taq polymerase and the DNA strands to synthesize. After this whole cycle, there are a total of 2 double-stranded DNA templates. The reaction is ready to go onto the second round. The temperature is raised to 95°C again to separate the two strands again and the reaction goes on. At the end of n cycles, there will be a total of 2^n products accumulated and ready for use.

The temperature of PCR was determined based on the length of primers and the amount of GC content in the DNA sequence due to the extra bond between G and C base pairs compared to A and T base pairs. Higher the GC content in the DNA sequence, the higher the temperature required for the step of annealing.[1]

The overall purpose of this experiment was to insert the DNA of interest, DsRed, into the pET28a vector. To identify if the insertion was successful or not, bacteria in different controlled groups that had taken in the modified pET28a plasmids were transformed onto agar plates. After screening the transformed colonies, colonies were selected to perform plasmid miniprep to isolate the plasmids from the bacteria to be sent for DNA sequencing.

Methods

The whole experiment was split into 5 days. During the 1st day, PCR for amplifying the desired DsRed insert was performed. A gel electrophoresis was run to visualize the size of DNA fragments of the two amplified DsRed insert PCR products. A PCR clean-up step was performed to purify the PCR product of the DsRed insert using 3 types of buffers with the final one eluting the DNA into the microcentrifuge tube. Due to the high ethanol residual in the two samples made

[1] Rychlik W, Spencer WJ, Rhoads RE. Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Res. 1990 Nov 11;18(21):6409-12. doi: 10.1093/nar/18.21.6409. Erratum in: Nucleic Acids Res 1991 Feb 11;19(3):698. PMID: 2243783; PMCID: PMC332522.

Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

on day 1, one sample with normal purity was given to perform the procedures for the next few days.

Day 2 aimed to purify the DsRed insert sample and pET28a samples from the electrophoresis gel. Prior to purifying the samples, compatible ends of the DsRed insert and the pET28a vector were created by utilizing the 2 restriction enzymes EcoRI and Xhol to digest the 2 samples. The bands of digested samples on the gel were cut and purified using different Buffers. The concentration and wavelength of samples were measured at the end of Day 2 to ensure the purity of both samples.

After the first two days, the two samples were ready to be ligated together. Since self-ligation of the linearized pET28a vector was more favored than the ligation between the insert and the vector, the 5' phosphates on the vector were removed through one dephosphorylation reaction. 2 samples with ligated samples were prepared with different pET28a:DsRed ratios. Both ratios were greater than 1:3 to ensure the DsRed insert was properly inserted into the plasmids. Another control group was prepared with only the pET28a vector. Competent bacteria cells prepared in Ca^{2+} were used to take in the ligated plasmids. 5 transformations of bacteria including the controls were performed on agar plates.

After 2 days of growing, colonies needed to be selected for expansion. However, only transformations #4 and #5 showed colonies whereas all the transformations should have colonies growing except for transformation #1. Given that the originally prepared plates did not have any colonies, the colonies from transformations #2 and #3 prepared by the instructor were used for expansion.

On Day 5, a plasmid miniprep was performed to ensure the purity of DNA samples was high enough to perform the following applications. The plasmid miniprep involved lysing the bacteria cells to release the intracellular components. After balancing and washing the sample solution using buffers, the plasmid DNA was eluted from the column. Restriction digestion was performed to cut the inserted DsRed from the pET28a vectors. A gel electrophoresis with all the digested and undigested plasmid DNA samples was run to visualize the insertion of DsRed. The rest of the undigested samples of plasmid DNA were used to prepare sequencing samples. The final sequencing results were used for alignment with the known DsRed sequence.

Results

1. PCR results

Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

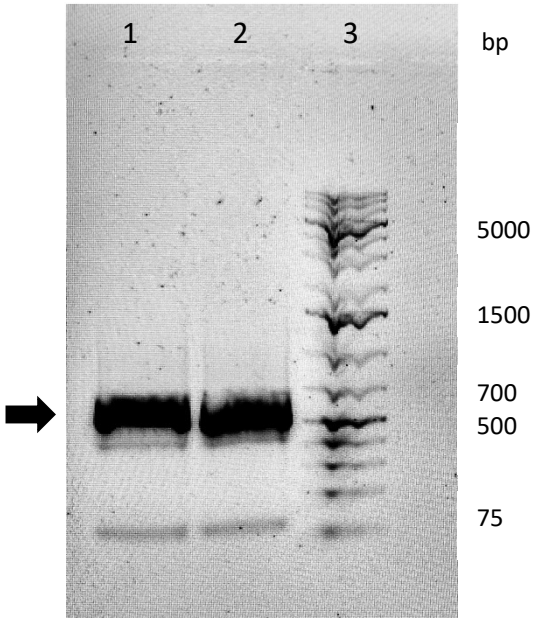

Figure 1 Amplification of DsRed PCR Product. Lane 1, the PCR product of DsRed insert #1; Lane 2, the PCR product of DsRed insert #2; Lane 3, the 1kb DNA ladder used for size reference. The labels above represent the lane number. The numbers on the right represent the size of the bands in terms of the number of base pairs. The arrowhead indicates the position of the band representing the DsRed PCR product fragment. Product fragment size is roughly 500-700 base pairs.

The Intensity of the bands for both samples indicated that the PCR products were successfully obtained. The 2 bands at the position ~ 700 bp appeared to be darker and much wider than the 1 kb DNA ladder. The position of the bands on the gel indicated that the observed results matched the expectation. There were two unexpected bands at the bottom of the gel that were around 75 base pairs. The best guess was that these bands were formed the primers added to the DsRed DNA solution prior to running the PCR reaction.

The final concentration of PCR product was 125.1 ng/μL for sample #1, and 150.5 ng/μL for sample #2.

Due to the high ethanol residual in the samples produced on Day 1, a different DsRed sample was used from another group. The concentration of the newly given DsRed sample was 103.9 ng/μL. Since only one new sample of DsRed was given, all the subsequence applications that required both samples of DsRed were performed with only one sample of DsRed.

2. Restriction Digestion Results

Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

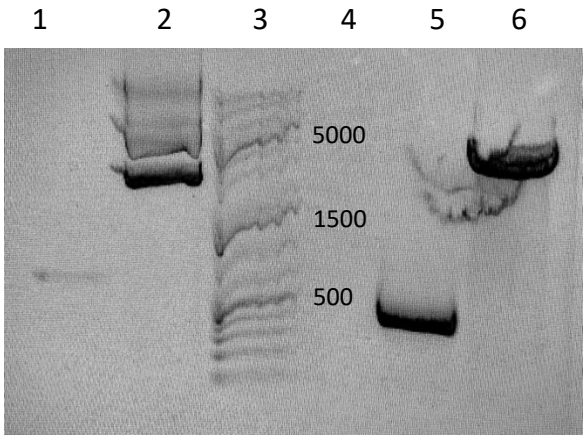

Figure 2 Result From Digestion Restriction of DsRed and pET28a. Lane 1, the undigested DsRed DNA fragment; Lane 2, undigested pET28a vector; Lane 3, 1kb DNA ladder for reference; Lane 5, digested DsRed insert product; Lane 6, Digested pET28a product. Labels in Lane 4 indicates the size of the bands in terms of number of base pairs.

Table 1. Comparison between the expected and observed sizes of insert and vector samples

Sample name	**Expected number of bands**	**Observed number of bands**	**Expected band size (bp)**	**Observed band size (bp)**
Undigested DsRed	1	1	500 – 700	500 – 700
Digested DsRed	1	1	650	650
Undigested pET28a	3	3	Around 5000	1: >20000, 2: ~5000, 3: ~4000
Digested pET28a	1	1	One aligned with the middle band of undigested pET281	~5000

3 bands were expected for the undigested pET28a vector. The digested pET28a was expected to run a band in the position aligned with the middle band from the undigested pET28a sample. The undigested and digested DsRed bands should be roughly in the same position.

The band run by using the digested pET28a was a bit slanted. This might be due to some of the samples flowing in the TAE Buffer when it was added into the well. Although being a bit off position, the band still matched with the expected base pairs numbers, which was around 500 base pairs and aligned with the middle band from the undigested sample.

After gel purification, the concentration for digested DsRed sample was 48.6 ng/μL. pET28a had a concentration of 83.7 ng/L. Both samples did not show ethanol contamination and were used in the subsequent applications.

3. Transformation Results

Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

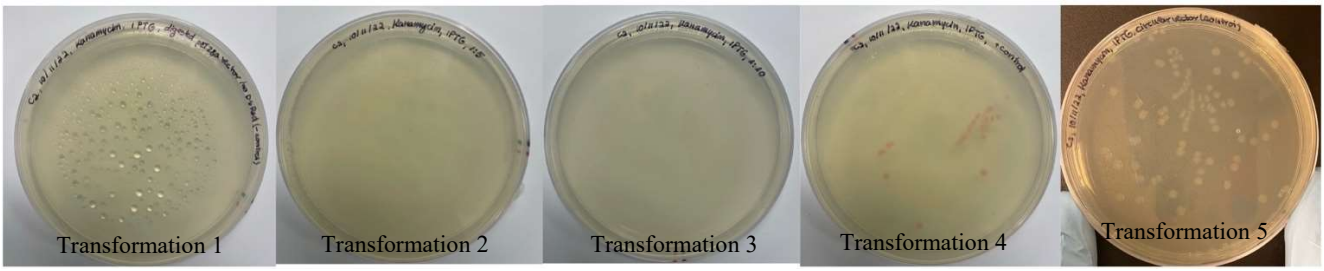

Figure 3 Transformation Results on Agar Plates. Transformation 1 was the negative control that only included the digested pET28a sample with the addition of DsRed. No colonies were observed because the pET28a vectors were linearized and not functional. Transformation 2 was the 1:5 ratio of pET28a:DsRed. No colonies were observed. Transformation 3 was the 1:10 ratio of pET28a:DsRed. No colonies were observed. Transformation 4 was the positive control using the pET28a – DsRed plasmid given in the lab. A few red colonies were observed. Transformation 5 was the control plate with circular pET28a vectors. Many white colonies were observed.

Table 2 Comparison Between Transformation Plates

Plate	Conditions	Expect colonies (yes/no)	Relative number of colonies
1	Digested pET28a vector with no DsRed (- control)	No	None
2	1:5 (pET28a:DsRed)	Yes	None
3	1:10 (pET28a:DsRed)	Yes	None
4	pET28a – DsRed plasmid (+ control)	Yes	¼ the amount of #5
5	Circular pET28a vector (control)	Yes	~80 colonies

Only transformations #4 and #5 showed results as expected. Given that no DsRed sequence was inserted into the pET28a vector used for transformation #5, white colonies were grown on the plate instead of red colonies. Transformation #4 used the plasmids that were already inserted with the DsRed sequence. So, the colonies appeared to be red in color.

Unfortunately, transformations #2 and #3, which were the two plates that were expected to have red colonies growing on them did not grow any colonies at all. The instructor later went through the transformation procedures again and found out that the phosphatase that was used in the dephosphorylation step was competing with the ligase, so the two samples were not successfully ligated together. Thus, no functional plasmids were produced for the bacteria to take in.

4. Sequencing Results

Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

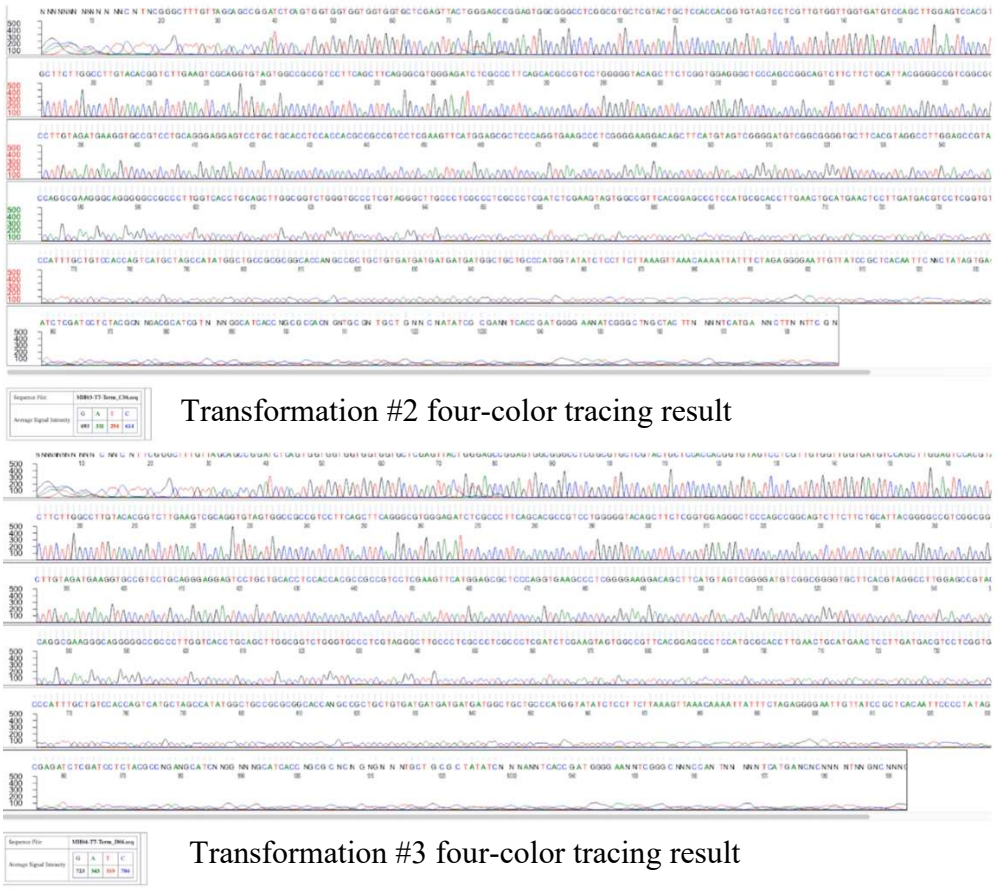

Figure 4 Four-color Tracing Results. The upper graph represents the color tracing result for

Transformation #2
Green highlight – XhoI sites
Blue highlight – EcoRI sites
Yellow highlight – DsRed nucleotides
ATG – ATG start sites

NNNNNNNNNNNNNNCNTNCGGGCTTTGTTAGCAGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGA
GTTACTGGGAGCCGGAGTGGCGGGCCTCGGCGTGCTCGTACTGCTCCACCACGGTGTAGTCCTCGTTG
TGGTTGGTGA**ATG**TCCAGCTTGGAGTCCACGTAGTGGTTGCCGGGCAGCTGCACGGGCTTCTTGGCCTT
GTACACGGTCTTGAAGTCGCAGGTGTAGTGGCCGCCGTCCTTCAGCTTCAGGGCGTGGGAGATCTCGC
CCTTCAGCACGCCGTCCTGGGGGTACAGCTTCTCGGTGGAGGGCTCCCAGCCGGCAGTCTTCTTCTGCA
TTACGGGGCCGTCGGCGGGGAAGTTCACGCCCTTGAACTTCACCTTGTAGATGAAGGTGCCGTCCTGC
AGGGAGGAGTCCTGCTGCACCTCCACCACGCCGCCGTCCTCGAAGTTCATGGAGCGCTCCCAGGTGAA
GCCCTCGGGGAAGGACAGCTTCATGTAGTCGGGGATGTCGGCGGGGTGCTTCACGTAGGCCTTGGAGC
CGTACTGGAACTGGGGGGACAGGATGTCCCAGGCGAAGGGCAGGGGGCCGCCCTTGGTCACCTGCAG
CTTGGCGGTCTGGGTGCCCTCGTAGGGCTTGCCCTCGCCCTCGCCCTCGATCTCGAAGTAGTGGCCGTT
CACGGAGCCCTCCATGCGCACCTTGAACTGCATGAACTCCTTGATGACGTCCTCGGTGTTGTCCATGAA
TTCGGATCCGCGACCCATTTGCTGTCCACCAGTCATGCTAGCCATATGGCTGCCGCGCGGCACCANGC
CGCTGCTGTGATGATGATGATGATGGCTGCTGCCCATGGTATATCTCCTTCTTAAAGTTAAACAAAATT
ATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCNNCTATAGTGAGTCGTANTAATTTCGCGGGATCG
AGATCTCGATCCTCTACGCNNGACGCATCGTNNNGGCATCACCNGCGCCACNGNTGCGNTGCTGNNCN
ATATCGCGANNTCACCGATGGGGAANATCGGGCTNGCTACTTNNNNTCATGANNCTTNNTTCGNNNNN

Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

Transformation #3

NNNNNNNNNNNCNNCNTTCGGGCTTTGTTAGCAGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGA
GTTACTGGAGCCGGAGTGGCGGGCCTCGGCGTGCTCGTACTGCTCCACCACGGTGTAGTCCTCGTTGT
GGTTGGTG**ATG**TCCAGCTTGGAGTCCACGTAGTGGTTGCCGGGCAGCTGCACGGGCTTCTTGGCCTTG
TACACGGTCTTGAAGTCGCAGGTGTAGTGGCCGCCGTCCTTCAGCTTCAGGGCGTGGGAGATCTCGCC
CTTCAGCACGCCGTCCTGGGGGTACAGCTTCTCGGTGGAGGGCTCCCAGCCGGCAGTCTTCTTCTGCAT
TACGGGGCCGTCGGCGGGGAAGTTCACGCCCTTGAACTTCACCTTGTAGATGAAGGTGCCGTCCTGCA
GGGAGGAGTCCTGCTGCACCTCCACCACGCCGCCGTCCTCGAAGTTCATGGAGCGCTCCCAGGTGAAG
CCCTCGGGGAAGGACAGCTTCATGTAGTCGGGGATGTCGGCGGGGTGCTTCACGTAGGCCTTGGAGCC
GTACTGGAACTGGGGGGACAGGATGTCCCAGGCGAAGGGCAGGGGGCCGCCCTTGGTCACCTGCAGC
TTGGCGGTCTGGGTGCCCTCGTAGGGCTTGCCCTCGCCCTCGCCCTCGATCTCGAAGTAGTGGCCGTTC
ACGGAGCCCTCCATGCGCACCTTGAACTGCATGAACTCCTTGATGACGTCCTCGGTGTTGTCCATGAAT
TCGGATCCGCGACCCATTTGCTGTCCACCAGTCATGCTAGCCATATGGCTGCCGCGCGGCACCANGCC
GCTGCTGTGATGATGATGATGATGGCTGCTGCCCATGGTATATCTCCTTCTTAAAGTTAAACAAAATTA
TTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCCCTATAGTGAGTCGTATTAATTTCGCGGGATCGA
GATCTCGATCCTCTACGCCNGANGCATCNNGGNNNGCATCACCNGCGCNCNGNGNNNTGCTGCGCTAT
ATCNNNANNTCACCGATGGGGAANNTCGGGCNNNCCANTNNNNNTCATGANCNCNNNNTNNGNCNN
NGGTNNNG

Alignment Results:

Score	Expect	Identities	Gaps	Strand
1253 bits(678)	0.0	678/678(100%)	0/678(0%)	Plus/Minus

```
Query  69   TTACTGGGAGCCGGAGTGGCGGGCCTCGGCGTGCTCGTACTGCTCCACCACGGTGTAGTC  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  678  TTACTGGGAGCCGGAGTGGCGGGCCTCGGCGTGCTCGTACTGCTCCACCACGGTGTAGTC  619

Query  129  CTCGTTGTGGTTGGTGATGTCCAGCTTGGAGTCCACGTAGTGGTTGCCGGGCAGCTGCAC  188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  618  CTCGTTGTGGTTGGTGATGTCCAGCTTGGAGTCCACGTAGTGGTTGCCGGGCAGCTGCAC  559

Query  189  GGGCTTCTTGGCCTTGTACACGGTCTTGAAGTCGCAGGTGTAGTGGCCGCCGTCCTTCAG  248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  558  GGGCTTCTTGGCCTTGTACACGGTCTTGAAGTCGCAGGTGTAGTGGCCGCCGTCCTTCAG  499

Query  249  CTTCAGGGCGTGGGAGATCTCGCCCTTCAGCACGCCGTCCTGGGGGTACAGCTTCTCGGT  308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  498  CTTCAGGGCGTGGGAGATCTCGCCCTTCAGCACGCCGTCCTGGGGGTACAGCTTCTCGGT  439

Query  309  GGAGGGCTCCCAGCCGGCAGTCTTCTTCTGCATTACGGGGCCGTCGGCGGGGAAGTTCAC  368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  438  GGAGGGCTCCCAGCCGGCAGTCTTCTTCTGCATTACGGGGCCGTCGGCGGGGAAGTTCAC  379

Query  369  GCCCTTGAACTTCACCTTGTAGATGAAGGTGCCGTCCTGCAGGGAGGAGTCCTGCTGCAC  428
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  378  GCCCTTGAACTTCACCTTGTAGATGAAGGTGCCGTCCTGCAGGGAGGAGTCCTGCTGCAC  319

Query  429  CTCCACCACGCCGCCGTCCTCGAAGTTCATGGAGCGCTCCCAGGTGAAGCCCTCGGGGAA  488
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  318  CTCCACCACGCCGCCGTCCTCGAAGTTCATGGAGCGCTCCCAGGTGAAGCCCTCGGGGAA  259

Query  489  GGACAGCTTCATGTAGTCGGGGATGTCGGCGGGGTGCTTCACGTAGGCCTTGGAGCCGTA  548
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258  GGACAGCTTCATGTAGTCGGGGATGTCGGCGGGGTGCTTCACGTAGGCCTTGGAGCCGTA  199

Query  549  CTGGAACTGGGGGGACAGGATGTCCCAGGCGAAGGGCAGGGGGCCGCCCTTGGTCACCTG  608
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  198  CTGGAACTGGGGGGACAGGATGTCCCAGGCGAAGGGCAGGGGGCCGCCCTTGGTCACCTG  139

Query  609  CAGCTTGGCGGTCTGGGTGCCCTCGTAGGGCTTGCCCTCGCCCTCGCCCTCGATCTCGAA  668
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  138  CAGCTTGGCGGTCTGGGTGCCCTCGTAGGGCTTGCCCTCGCCCTCGCCCTCGATCTCGAA  79

Query  669  GTAGTGGCCGTTCACGGAGCCCTCCATGCGCACCTTGAACTGCATGAACTCCTTGATGAC  728
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78   GTAGTGGCCGTTCACGGAGCCCTCCATGCGCACCTTGAACTGCATGAACTCCTTGATGAC  19

Query  729  GTCCTCGGTGTTGTCCAT  746
            ||||||||||||||||||
Sbjct  18   GTCCTCGGTGTTGTCCAT  1
```

Figure 5 Alignment Result for Transformation #2. The alignment result is 100% matched with 678 base pairs of matching

Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

Score	Expect	Identities	Gaps	Strand
1245 bits(674)	0.0	677/678(99%)	1/678(0%)	Plus/Minus

```
Query  70   TTACT-GGAGCCGGAGTGGCGGGCCTCGGCGTGCTCGTACTGCTCCACCACGGTGTAGTC  128
            |||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  678  TTACTGGGAGCCGGAGTGGCGGGCCTCGGCGTGCTCGTACTGCTCCACCACGGTGTAGTC  619

Query  129  CTCGTTGTGGTTGGTGATGTCCAGCTTGGAGTCCACGTAGTGGTTGCCGGGCAGCTGCAC  188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  618  CTCGTTGTGGTTGGTGATGTCCAGCTTGGAGTCCACGTAGTGGTTGCCGGGCAGCTGCAC  559

Query  189  GGGCTTCTTGGCCTTGTACACGGTCTTGAAGTCGCAGGTGTAGTGGCCGCCGTCCTTCAG  248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  558  GGGCTTCTTGGCCTTGTACACGGTCTTGAAGTCGCAGGTGTAGTGGCCGCCGTCCTTCAG  499

Query  249  CTTCAGGGCGTGGGAGATCTCGCCCTTCAGCACGCCGTCCTGGGGGTACAGCTTCTCGGT  308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  498  CTTCAGGGCGTGGGAGATCTCGCCCTTCAGCACGCCGTCCTGGGGGTACAGCTTCTCGGT  439

Query  309  GGAGGGCTCCCAGCCGGCAGTCTTCTTCTGCATTACGGGGCCGTCGGCGGGGAAGTTCAC  368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  438  GGAGGGCTCCCAGCCGGCAGTCTTCTTCTGCATTACGGGGCCGTCGGCGGGGAAGTTCAC  379

Query  369  GCCCTTGAACTTCACCTTGTAGATGAAGGTGCCGTCCTGCAGGGAGGAGTCCTGCTGCAC  428
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  378  GCCCTTGAACTTCACCTTGTAGATGAAGGTGCCGTCCTGCAGGGAGGAGTCCTGCTGCAC  319

Query  429  CTCCACCACGCCGCCGTCCTCGAAGTTCATGGAGCGCTCCCAGGTGAAGCCCTCGGGGAA  488
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  318  CTCCACCACGCCGCCGTCCTCGAAGTTCATGGAGCGCTCCCAGGTGAAGCCCTCGGGGAA  259

Query  489  GGACAGCTTCATGTAGTCGGGGATGTCGGCGGGGTGCTTCACGTAGGCCTTGGAGCCGTA  548
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258  GGACAGCTTCATGTAGTCGGGGATGTCGGCGGGGTGCTTCACGTAGGCCTTGGAGCCGTA  199

Query  549  CTGGAACTGGGGGGACAGGATGTCCCAGGCGAAGGGCAGGGGGCCGCCCTTGGTCACCTG  608
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  198  CTGGAACTGGGGGGACAGGATGTCCCAGGCGAAGGGCAGGGGGCCGCCCTTGGTCACCTG  139

Query  609  CAGCTTGGCGGTCTGGGTGCCCTCGTAGGGCTTGCCCTCGCCCTCGCCCTCGATCTCGAA  668
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  138  CAGCTTGGCGGTCTGGGTGCCCTCGTAGGGCTTGCCCTCGCCCTCGCCCTCGATCTCGAA  79

Query  669  GTAGTGGCCGTTCACGGAGCCCTCCATGCGCACCTTGAACTGCATGAACTCCTTGATGAC  728
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78   GTAGTGGCCGTTCACGGAGCCCTCCATGCGCACCTTGAACTGCATGAACTCCTTGATGAC  19

Query  729  GTCCTCGGTGTTGTCCAT  746
            ||||||||||||||||||
Sbjct  18   GTCCTCGGTGTTGTCCAT  1
```

Figure 6 Alignment Result for Transformation #3. There is a 99% alignment between the unknown sequence and the provided DsRed sequence.

Both unknown sequences from Transformation #2 and #3 showed a high matching score. The unknown sequence from Transformation #3 had one mismatch with the provided DsRed sequence which was caused by a missing nucleotide.

5. Diagnostic Restriction Enzyme Digestion

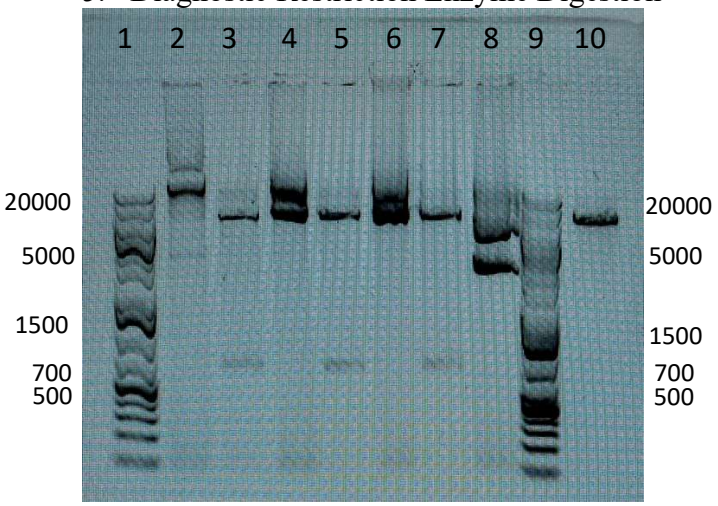

Figure 7 restriction digestion result. Lane 1, 1kb DNA ladder, Lane 2, Transformation #2: pET28a-DsRed (1:5) plasmid undigested; Lane 3, Transformation #2: pET28a-DsRed (1:5) plasmid digested; Lane 4, Transformation #3: pET28a-DsRed (1:10) plasmid undigested; Lane 5, Transformation #3: pET28a-DsRed

Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

(1:10) plasmid digested; Lane 6, Transformation #4: pET28a-DsRed + control undigested; Lane 7, Transformation #4: pET28a-DsRed + control digested; Lane 8, Transformation #5: pET28a vector undigested; Lane 9, 1kb DNA Ladder; Lane 10, Transformation #5, pET28a vector digested.

Table 3 Comparison Between Expected and Observed Band Sizes from Transformation Samples

Transformation #	Condition	Digested/undigested	Expected band size (bp)	Observed band size (bp)
#2	1:5 (pET28a:DsRed)	Undigested	3 bands >5000	20000
#2	1:5 (pET28a:DsRed)	Digested	Upper: >5000 Lower: ~700	Upper: 10000 Lower: 750
#3	1:10 (pET28a:DsRed)	Undigested	3 bands > 5000	1: >20000, 2: 20000
#3	1:10 (pET28a:DsRed)	Digested	Upper: >5000 Lower: ~700	Upper: 10000 Lower: 750
#4	pET28a – DsRed plasmid (+ control)	Undigested	3 bands >5000	1: >20000, 2: 20000
#4	pET28a – DsRed plasmid (+ control)	Digested	Upper: >5000 Lower: ~700	Upper: 10000 Lower: 750
#5	Circular pET28a vector (control)	Undigested	Upper: ~ 20000 Middle: >5000 Lower: 4000	Upper: 20000 Middle: 5000 Lower: 4000
#5	Circular pET28a vector (control)	Digested	>5000	~10000

Since DsRed was inserted into the pET28a vectors for transformations #2 and #3 prior to running the gel, 2 bands with different sizes were expected to be visualized on the gel for the digested samples. Transformation #4, which was the positive control, was also expected to run 2 bands on the gel because of the presence of DsRed in the pET28a vector.

Discussion

The purpose of the experiment was to insert the DsRed gene into the pET28a vector and utilized various applications to verify the success of inserting the gene.

1. PCR Discussion

2 bands were visualized for the DsRed PCR product. The band with higher intensity indicated the DNA template produced by PCR. The band that represented the PCR product was expected to show up at a position around 700 bp since the DsRed DNA sequence has a size of 678 base pairs. The other band (the lower one) might represent the primer that was added for PCR given that the band was relatively lighter and had a small size (75 bp) compared to the upper band.

The intensity of the band was quite high which tells that the DNA template was amplified, which indicated that the PCR reactions were quite reproducible.

The whole PCR reaction performed on Day 1 had 30 cycles. 2^{30} copies of DNA were produced from the original input. 2^{30} = 1073741824 copies of DNA.

2. Restriction Digest Discussion

Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

The plasmid digestion linearized the pET28a vector. This could be visualized in the gel electrophoresis result. There were 3 bands for the undigested plasmid sample and only 1 band for the digested plasmid sample. There were 3 shapes of the undigested plasmid sample which could be described as "nicked", supercoiled, and linearized. However, after restriction digestion was performed, only one shape of the plasmid was left, which was linearized. This shape made the fragment difficult to go through the pores of the agarose gel as compared to undigested. Although running more slowly compared to the supercoiled plasmids on the gel, it still ran faster than the "nicked" shape plasmids.

The two restriction enzymes recognize some designated places on the DNA samples and cut at these 2 sites to create "sticky ends" at both ends of the DNA sample. Both the DsRed insert and pET28a vector needed to be cut to create sites for both "nicked" strands to be ligated together. There were two sites that could be recognized by the digestion enzymes EcoRI and Xhol. The two sites were cut by the enzymes indicated in the labeled sites for both transformations in the result section. There was no sign of incomplete digestion given that enough digestion enzymes were used. The results from the gel electrophoresis ran using the digested samples could prove it.

The 2 bands that had the size of 75 base pairs from the gel on Day 1 might indicate the primers used for PCR. There were no other bands that cannot be accounted for.

3. Transformation Discussion

Transformations #2 and #3 were not able to produce any colonies due to the phosphatase in the dephosphorylation solution was competing with the ligase. As result, the inserts and the vectors were not ligated together to form complete plasmids.

Both ratios (1:5 and 1:10) worked similarly because transformations #2 and #3 prepared from another lab produced roughly the same number of colonies.

The negative control, transformation #1, developed no colonies, which indicated that the plasmid did not self-ligate. The positive control transformation, #4, developed red colonies that indicated the intake of the DsRed plasmid into the bacteria. The control, transformation #5, developed white colonies that indicated the intake of pET28a plasmids.

The size of the plasmid probably did have a bit of an impact on the expression. When comparing transformations #4 and #5, a very different number of colonies were observed. The colonies from transformation #4 were much separated and the number of colonies was lower compared to transformation #4. So, the bigger plasmid, which is the pET28a-DsRed plasmid, took more effort for the bacteria to take in compared to the regular pET28a.[2]

4. Diagnostic Restriction Digest & Sequencing Discussion

The cloning project did work according to the diagnostic digestion results. DsRed was properly inserted into the pET28a vector indicated by the transformation result obtained from the

[2] Ohse M, Takahashi K, Kadowaki Y, Kusaoke H. Effects of plasmid DNA sizes and several other factors on transformation of Bacillus subtilis ISW1214 with plasmid DNA by electroporation. Biosci Biotechnol Biochem. 1995 Aug;59(8):1433-7. doi: 10.1271/bbb.59.1433. PMID: 7549093.

instructor's transformation plates. Both sequencing data obtained from the 4-colour tracing graph showed that the sequences for transformations #2 and #3 were in alignment with the known DsRed sequence.

There was one difference in the sequencing alignment between the two transformation samples when an alignment was performed between the transformation sample and the known DsRed sequence. The 75th nucleotide was missing for Transformation #3. The most possible reason would be that the ratio of 1:10 made the ligation happen too rapidly compared to the 1:5 ratio. Eventually, tiny mistakes were resulted when the DsRed was ligated with the pET28a vector.

If the experiment is repeated one more time, the dephosphorylation step could be modified. The phosphatase used on Day 3 competed with the activity of the ligase[3] which made the two strands unable to be ligated together. It was crucial to have the insert and the vector ligated together to produce functional plasmids.

Questions such as how the different sizes of plasmid affect the expression of the gene and why phosphatase used in the lab competed with the activity of the ligase could be asked based on the results. For the first question, an additional experiment could be performed with another type of fluorescent protein that functions similarly to the DsRed protein. However, the two proteins should have a significant difference in sizes so that the ligated plasmids would be different after the insertion of the two proteins respectively. Then the same types of transformation could be performed using the same bacteria. The number of colonies from different transformations could be compared to see the expression of the gene using different sizes of plasmids.

[3] Baker ST, Opperman KJ, Tulgren ED, Turgeon SM, Bienvenut W, Grill B. RPM-1 uses both ubiquitin ligase and phosphatase-based mechanisms to regulate DLK-1 during neuronal development. PLoS Genet. 2014 May 8;10(5):e1004297. doi: 10.1371/journal.pgen.1004297. PMID: 24810406; PMCID: PMC4014440.

Name: Zhiyu Wei
Date: 10/25/2022
Lab Partner: Crina Maria Curca

References:

Baker ST, Opperman KJ, Tulgren ED, Turgeon SM, Bienvenut W, Grill B. RPM-1 uses both ubiquitin ligase and phosphatase-based mechanisms to regulate DLK-1 during neuronal development. PLoS Genet. 2014 May 8;10(5):e1004297. doi: 10.1371/journal.pgen.1004297. PMID: 24810406; PMCID: PMC4014440.

Ohse M, Takahashi K, Kadowaki Y, Kusaoke H. Effects of plasmid DNA sizes and several other factors on transformation of Bacillus subtilis ISW1214 with plasmid DNA by electroporation. Biosci Biotechnol Biochem. 1995 Aug;59(8):1433-7. doi: 10.1271/bbb.59.1433. PMID: 7549093.

Rychlik W, Spencer WJ, Rhoads RE. Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Res. 1990 Nov 11;18(21):6409-12. doi: 10.1093/nar/18.21.6409. Erratum in: Nucleic Acids Res 1991 Feb 11;19(3):698. PMID: 2243783; PMCID: PMC332522.