LSCI 3000 Synthetic Biology Workshop
Case Study 1

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05-04-2023 ~ 21-04-2023

Preface

This section compiles the primary objective, initial timetable, and procedures to preface the case study report. However, the main purpose of this preface is to demonstrate how astray we have ended up from our initial plans in practice. More to be discussed in the reflection and discussion.

Primary Objective

To create a ready-to-use DNA gene ladder consisting of bands at 250bp, 500bp, 750bp, 1000bp, 1500bp, 3000bp, and 6000bp from pLadder6K samples.

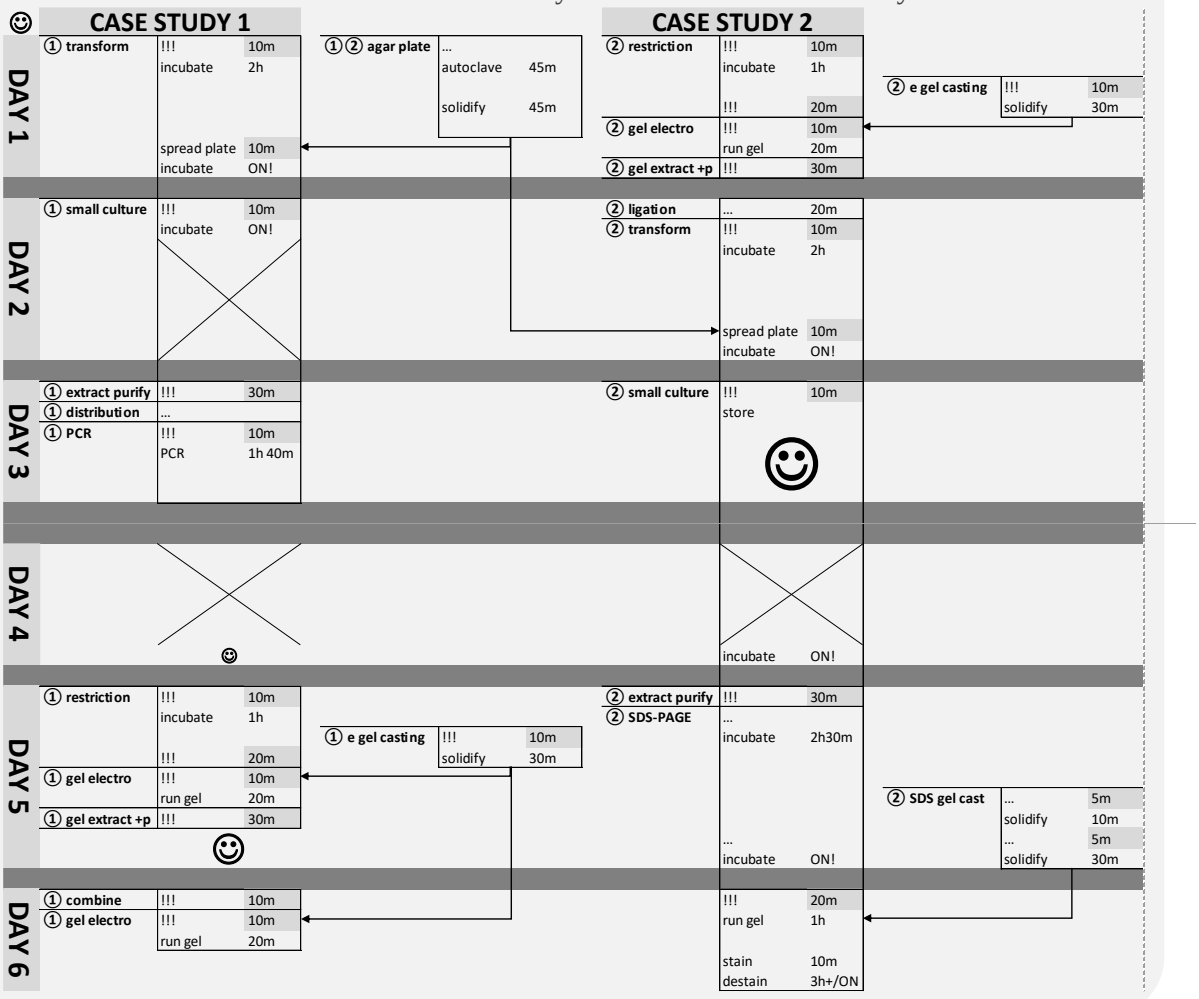
Initial Procedures Flow

Note that secondary steps (e.g., making agar plates and agarose gels) are omitted here.

1. Transform pLadder6K into competent DH5-α E. coli and culture it in an agar plate.
2. Extract colonies of transformed E. Coli to make a small culture.
3. Extract and purify the pLadder6K form the E. coli.
4. Perform PCR on the extracted pLadder6K to create the smaller band sizes.
5. Restrict the extracted pLadder6K to create the larger band sizes.
6. Perform gel electrophoresis on the created bands.
7. Extract and purify the DNA bands from the electrophoresed gel.
8. Combine the DNA of different sizes in accordance with their resultant concentration. The final product was designed with the 1000bp band being brighter than the rest.
9. Perform gel electrophoresis on the final product for results.

Initial Timetable

Procedures in the same row denotes concurrency. The timetable for case study 2 is also included.



Day 1 (05-04-2023)

Note

There was only limited pre-made pLadder6K at the time, and the nanodrop results (6.4 & 6.6 ng/μL) indicated a very poor concentration from the sample. Hence, we have decided to extract some from the previously transformed colonies. However, we still opted to perform PCR on the samples since there was little to do on day 1 due to an unforeseen rearrangement for case study 2.

1. **Experiment 1** *(Prerequisite: Lab 1 Experiment 2)*

1.1. Objective

- To create small cultures of pLadder6K-transformed E. coli.

1.2. Precautions

- Wear gloves.
- Dispose of the pipette tips after each transfer.
- Complete procedures with a flame nearby to ensure aseptic conditions.
- Lightly flame the openings of the flacon tubes with each opening and closing.

1.3. Materials

- 20mL of LB cell culture broth
- 20μL of kanamycin (1000x)
- 1 petri dish of DH5α E. coli colonies transformed with pLadder6K

1.4. Equipment

1.4.1. Machineries

- 37℃ incubator
- 4℃ refrigerator
- Bunsen burner

1.4.2. Apparatus

- Pipette (P200) and appropriate pipette tips

1.4.3. Containers

- 4 falcon tubes

1.4.4. Miscellaneous

- Marker pen
- Lighter
- 70% ethanol
- Paper towel

1.5. Preparations

- Label the falcon tubes appropriately.
- Light a flame to generate an aseptic convection current.

1.6. Procedures

1. Pipette 20μL of kanamycin to the LB cell culture broth.
2. Gently mix the LB broth.
3. Pour 4.5mL of the LB broth mixture into each falcon tube.
4. Circle and label four non-satellite colonies the petri dish that will be extracted. Choose colonies that are far apart from each other.
5. Clean the pipette with 70% ethanol and paper towel.
6. Mount a suitable pipette tip to a P20 pipette and lightly flame the tip.

7. Using the tipped P20 pipette, gently scrape a labelled colony from a petri dish while the dish is still upside-down.
8. Eject the entire pipette tip into the respective flacon tube.
9. Repeat steps 5-8 until four colonies has been isolated and put into their separate flacon tubes.
10. Loosen the caps slightly.
11. Use tape to secure the caps such that they would not unscrew during incubation while allowing air flow.
12. Put the tubes in an incubator at 37℃ with 220rpm overnight (12-16 hours).

1.7. Products

- 4 small cultures of pLadder6K-transformed E. coli in falcon tubes

2. Experiment 2 *(Prerequisite: Lab 3 Experiment 2)*

2.1. Objective

- To partially amplify the pLadder6K to obtain the desired short DNA strands (250bp, 500bp, 750bp, 1000bp).

2.2. Precautions

- Wear gloves.
- Dispose of the pipette tips after each transfer.

2.3. Materials

2.3.1. Master mix solution

Note: The shown master mix material volumes are 150% of the necessary amount to allow for pipette errors.

- 187.5μL of *Taq* polymerase (TaKaRa) premix
- 7.5μL of primer E
- 157.5μL of ddH_2O

2.3.2. Other PCR reagents

- 1μL of primer A
- 1μL of primer B
- 1μL of primer C
- 2μL of primer D
- 10μL of pLadder6K from Lab 3 Experiment 2

2.3.3. Miscellaneous

- Ice

2.4. Equipment

2.4.1. Machineries

- PCR thermocycler

2.4.2. Apparatus

- Pipettes (P10/P20/P200) and appropriate pipette tips

2.4.3. Containers

- 1 centrifuge tube
- 10 PCR tubes
- Ice box

2.4.4. Miscellaneous

- Marker pen

2.5. Preparations

- Transfer all materials into separate centrifuge tubes and pre-chill them with an ice box.
- Label each experimental centrifuge tube appropriately. (2 for primers A+E, 2 for primers B+E, 2 for primers C+E, 4 for primers D+E)

2.6. Procedures

1. Pipette 187.5μL of Taq polymerase premix to the master mix centrifuge tube.
2. Pipette 7.5μL of primer E to the master mix.
3. Pipette 157.5μL of ddH_2O to the master mix.
4. Pipette 24μL of the master mix to each PCR tube.
5. Pipette 1μL of DNA to each PCR tube.
6. Pipette 1μL of primer A to two of the PCR tubes.
7. Pipette 1μL of primer B to two of the PCR tubes.
8. Pipette 1μL of primer C to two of the PCR tubes.
9. Pipette 1μL of primer D to four of the PCR tubes.
10. Mix all the tubes gently.
11. Put the tubes in the PCR thermocycler and set the parameters as follows.

2.7. PCR thermal cycle

Phase	Temperature	Duration	Cycles
Initial denaturation	98℃	5m	1
Denaturation	98℃	30s	34
Annealing	56℃	30s	
Extension	72℃	1m 30s	
Final extension	72℃	7m	1
Sample keeping	4℃	∞	1

Day 2 (06-04-2023)

Note

Here omitted, we have performed gel electrophoresis on the PCR products of case study 1 experiment 2 and no bands can be observed at all. We then proceeded to redo the PCR for the second time this day using the newly purified pLadder6K.

3. Experiment 3 *(Prerequisite: Case Study 1 Experiment 1)*

3.1. Objective

- To extract and purify pLadder6K from the E. coli small culture.

3.2. Precautions

- Wear gloves.
- Dispose of the pipette tips after each transfer.
- When placing tubes into the centrifuge, position the tubes such that their hinges face outwards to allow consistent pellet settling behaviour.

3.3. Materials

3.3.1. Small cultures

- 2 pRSFDuet-1 transformed DH5α E. coli small cultures in LB broth in falcon tubes
- 2 pLadder6K transformed DH5α E. coli small cultures in LB broth in falcon tubes

3.3.2. iNtRON easy-spin total RNA extraction kit

- 1000μL of resuspension buffer
- 1000μL of lysis buffer
- 1400μL of neutralisation buffer
- 2800μL of washing buffer B

3.3.3. Miscellaneous

- 160μL of ddH_2O
- Ice

3.4. Equipment

3.4.1. Machineries

- Centrifuge
- -20℃ refrigerator

3.4.2. Apparatus

- Pipettes (P200/P1000) and appropriate pipette tips

3.4.3. Containers

- 8 centrifuge tubes
- 4 spin columns (from the iNtRON kit)
- 4 collection tubes (from the iNtRON kit)
- Ice box

3.4.4. Miscellaneous

- Marker pen

3.5. Preparations

- Add RNase A solution to the resuspension buffer as per the instructions on the bottle, then chill the buffer with ice until use.
- Add ethanol to washing buffer B as per the instructions on the bottle.
- Centrifuge the E. coli small culture before use.
- Label all centrifuge tubes and columns appropriately.

- Mount the columns into the collection tubes as necessary before use.

3.6. Procedures

1. Pipette 250μL of resuspension buffer to each small culture falcon tube.
2. Gently mix the samples by vortexing until all bacteria clumps are resuspended.
3. Transfer all samples into separate centrifuge tubes with a pipette.
4. Pipette 250μL of lysis buffer to each tube.
5. Gently mix the samples by inverting the tubes 10 times.
6. Let the samples sit at room temperature for 3 minutes.
7. Pipette 350μL of neutralisation buffer to each tube.
8. Gently mix the samples by inverting the tubes 10 times.
9. Let the samples sit in ice for 5 minutes.
10. Centrifuge the samples at 13000rpm for 10 minutes.
11. Pipette the supernatant fluids into separate columns. Avoid transferring the pellets.
12. Centrifuge the samples at 13000rpm for 1 minute.
13. Discard the filtrate fluids in the collection tubes and remount the columns into the same collection tubes.
14. Pipette 700μL of washing buffer B to each column.
15. Centrifuge the samples at 13000rpm for 1 minute.
16. Discard the filtrate fluids in the collection tubes and remount the columns into the same collection tubes.
17. Centrifuge the samples at 13000rpm for 1 minute or until the filter membranes are dry.
18. Put the columns into separate centrifuge tubes. Remove and discard the used collection tubes.
19. Pipette 40μL of ddH_2O to each column.
20. Let the samples stand for 1 minute.
21. Centrifuge the samples at 13000rpm for 1 minute.
22. Discard the columns and store the plasmids at -20℃ for future use.

3.7. Products

- 4 centrifuge tubes each with 40μL of purified pLadder6K

Interlude

We did nanodrop test on the 4 purified pLadder6K samples to determine their concentrations.

Results

Sample number	1	2	3	4
Density (ng/μL)	254.6	306.5	199.4	132.2

The necessary volumes were calculated and adjusted as necessary. For the restrictions (experiment 4), the volumes are adjusted such that we have 60ng/μL of DNA in the mixtures.

4. Experiment 4 *(Prerequisite: Case Study 1 Experiment 3)*

4.1. Objective

- To restrict the purified pLadder6K with *Eco*RI, *Pst*I, and *Xba*I to obtain the desired long DNA strands (1500bp, 3000bp, 6000bp).

4.2. Precautions

- Wear gloves.
- Dispose of the pipette tips after each transfer.

4.3. Materials

4.3.1. *Restriction digestion*

- 16μL of purified pLadder6K sample 1
- 7μL of purified pLadder6K sample 2
- 12μL of Anza red buffer (10×)
- 4μL of *Eco*RI enzyme
- 4μL of *Pst*I enzyme
- 4μL of *Xba*I enzyme
- 79μL of ddH_2O

4.3.2. *iNtRON MEGAquick-spin plus total fragment DNA purification kit*

- 600μL of BNL buffer
- 2250μL of washing buffer

4.3.3. *Miscellaneous*

- 90μL of ddH_2O
- Ice

4.4. Equipment

4.4.1. *Machineries*

- Centrifuge
- 37℃ incubator
- Vortex mixer
- -20℃ refrigerator

4.4.2. *Apparatus*

- Pipettes (P10/P20/P200/P1000) and appropriate pipette tips

4.4.3. *Containers*

- 9 centrifuge tubes
- 3 spin columns (from the iNtRON kit)
- 3 collection tubes (from the iNtRON kit)
- Ice box

4.4.4. *Miscellaneous*

- Marker pen

4.5. Preparations

- Add ethanol to the washing buffer as per the instructions on the bottle.
- Pre-chill the DNA samples in ice.
- Pre-chill the enzymes in ice.
- Pre-chill the Anza red buffer in ice.
- Label all centrifuge tubes and columns appropriately.
- Mount the columns into the collection tubes as necessary before use.

4.6. Procedures

1. Pipette 4μL of pLadder6K sample 1 and 13μL of ddH_2O to each of four centrifuge tubes.
2. Pipette 3.5μL of pLadder6K sample 2 and 13.5μL of ddH_2O to each of two centrifuge tubes.
3. Pipette 2μL of Anza red buffer to each sample.
4. Pipette 1μL of *Eco*RI enzyme to each of two samples.
5. Pipette 1μL of *Pst*I enzyme to each of two sample.
6. Pipette 1μL of *Xba*I enzyme to each of two sample.
7. Centrifuge the samples at 1000rpm for 15 seconds to mix.
8. Incubate the samples at 37℃ for 1 hour.
9. Transfer one *Eco*RI-restricted sample to the other with a pipette to combine both samples.

10. Transfer one *Pst*I-restricted sample to the other with a pipette to combine both samples.
11. Transfer one *Xba*I-restricted sample to the other with a pipette to combine both samples.
12. Vortex the samples for 5 seconds or until mixed.
13. Pipette 200μL of BNL buffer to each sample.
14. Gently mix the samples by inverting the tubes 10 times.
15. Transfer the sample mixtures to separate columns with a pipette.
16. Centrifuge the samples at 11000×g for 30 seconds.
17. Discard the filtrate fluids in the collection tubes and remount the columns into the same collection tubes.
18. Pipette 750μL of washing buffer to each column.
19. Centrifuge the samples at 11000×g for 30 seconds.
20. Discard the filtrate fluids in the collection tubes and remount the columns into the same collection tubes.
21. Centrifuge the samples at 16000×g for 3 minute or until the filter membranes are dry.
22. Put the columns into separate sterile centrifuge tubes. Remove and discard the used collection tubes.
23. Pipette 30μL of ddH_2O directly onto the filter membrane of each column.
24. Let the samples stand for 2 minutes.
25. Centrifuge the samples at 16000×g for 1 minute.
26. Discard the columns and store the purified DNA at -20℃ for future use.

4.7. Products

- 1 centrifuge tube with 30μL of *Eco*RI-restricted pLadder6K
- 1 centrifuge tube with 30μL of *Pst*I-restricted pLadder6K
- 1 centrifuge tube with 30μL of *Xba*I-restricted pLadder6K

5. Experiment 5 *(Prerequisite: Case Study 1 Experiment 3)*

Note

Considering the PCR failure in case study 1 experiment 2, we have concluded that the failure was most likely due to the low concentration of the DNA template. However, we opted to err on the side of caution and decided to no longer use a master mix, but instead pipette all the reagents directly into the PCR tubes individually. We have also made sure to only add the *Taq* premix at the very last moment before PCR.

5.1. Objective

- To partially amplify the pLadder6K to obtain the desired short DNA strands (250bp, 500bp, 750bp, 1000bp).

5.2. Precautions

- Wear gloves.
- Dispose of the pipette tips after each transfer.

5.3. Materials

- 125μL of Taq polymerase (TaKaRa) premix
- 1μL of primer A
- 1μL of primer B
- 1μL of primer C
- 2μL of primer D
- 5μL of primer E
- 105μL of ddH_2O
- 10μL of purified pLadder6K sample 1

- Ice

5.4. Equipment

5.4.1.Machineries

- PCR thermocycler

5.4.2.Apparatus

- Pipettes (P10/P20/P200) and appropriate pipette tips

5.4.3.Containers

- 10 PCR tubes
- Ice box

5.4.4.Miscellaneous

- Marker pen

5.5. Preparations

- Transfer all materials into separate centrifuge tubes and pre-chill them with an ice box.
- Label each experimental centrifuge tube appropriately. (2 for primers A+E, 2 for primers B+E, 2 for primers C+E, 4 for primers D+E)

5.6. Procedures

1. Pipette 10.5μL of ddH_2O to each tube.
2. Pipette 0.5μL of primer E to each tube.
3. Pipette 0.5μL of primer A to two of the tubes.
4. Pipette 0.5μL of primer B to two of the tubes.
5. Pipette 0.5μL of primer C to two of the tubes.
6. Pipette 0.5μL of primer D to four of the tubes.
7. Pipette 1μL of DNA to each tube.
8. Pipette 12.5μL of Taq polymerase premix to each tube. Wait until the PCR machine is set up before doing this step to minimise unwanted prior reaction.
9. Mix all the tubes gently.
10. Put the tubes in the PCR thermocycler and set the parameters as follows.

5.7. PCR thermal cycle

Phase	Temperature	Duration	Cycles
Initial denaturation	98℃	5m	1
Denaturation	98℃	30s	34
Annealing	56℃	30s	
Extension	72℃	1m 30s	
Final extension	72℃	7m	1
Sample keeping	4℃	∞	1

Day 3 (07-04-2023)

Interlude

Here omitted, we have performed gel electrophoresis on the restriction and PCR products of case study 1 experiment 4 and 5 respectively.

Results

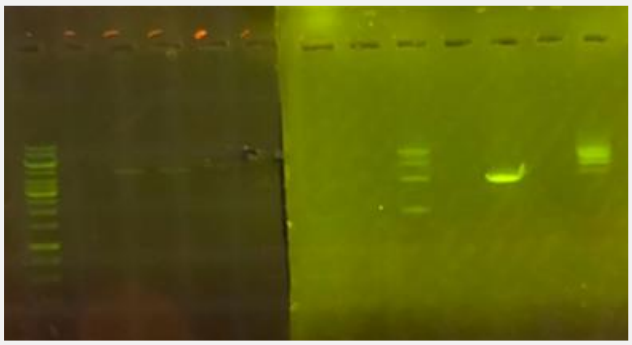


Figure 1 – Electrophoresed gel with restriction products under UV light
From left to right: DNA ladder, nulls, EcoRI-restricted sample, PstI-restricted sample, XbaI-restricted sample

Bands of desired sizes (1500bp, 3000bp, 6000bp) can be observed. For the *Eco*RI-restricted sample, some by-products (3000bp, 4500bp, 6000bp) can be observed. We have decided to also isolate the 3000bp and 6000bp bands for use.

Here omitted, the PCR samples showed only some bands of ~6000bp while the others were not visible.

6. Experiment 6 *(Prerequisite: Case Study 1 Experiment 4)*

6.1. Objective

- To extract and purify DNA of desired sizes (1500bp, 3000bp, 6000bp) with gel extraction.

6.2. Precautions

- Wear gloves.
- Dispose of the pipette tips after each transfer.
- Do not look directly into the UV light without the protective shield.

6.3. Materials

6.3.1. Electrophoresed gel

- 1 electrophoresed gel

6.3.2. iNtRON MEGAquick-spin plus total fragment DNA purification kit

- 2250μL of BNL buffer
- 2250μL of washing buffer

6.3.3. Miscellaneous

- 120μL of ddH_2O

6.4. Equipment

6.4.1. Machineries

- UV transilluminator
- 55℃ heat block
- Centrifuge
- -20℃ refrigerator

6.4.2.Apparatus

- Pipettes (P200/P1000) and appropriate pipette tips
- Scalpel

6.4.3.Containers

- 8 centrifuge tubes
- 3 spin columns (from the iNtRON kit)
- 3 collection tubes (from the iNtRON kit)

6.4.4.Miscellaneous

- Marker pen

6.5. Preparations

- Add ethanol to the washing buffer as per the instructions on the bottle.
- Put plastic wrap on the UV transilluminator sample surface before use.
- Label the centrifuge tubes and columns appropriately.
- Mount the column into the collection tube as necessary before use.

6.6. Procedures

1. Place the gel on a UV transilluminator.
2. Isolate all the desired bands with a sterile scalpel.
3. Transfer each gel slice into a separate centrifuge tube.
4. Pipette 450μL of BNL buffer to each tube.
5. Gently mix the sample by vortexing.
6. Place the tubes in a heat block at 55℃ for 7 minutes or until the gel slices are completely dissolved.
7. Allow the sample to cool to room temperature.
8. Transfer one sample mixture of each bp size to separate columns with a pipette.
9. Centrifuge the samples at 11000×g for 30 seconds.
10. Discard the filtrate fluids in the collection tubes and remount the columns into the same collection tubes.
11. Transfer the remaining sample mixtures to the respective columns of that bp size with a pipette.
12. Repeat steps 9-10.
13. Pipette 750μL of washing buffer to each column.
14. Centrifuge the samples at 11000×g for 30 seconds.
15. Discard the filtrate fluids in the collection tubes and remount the columns into the same collection tubes.
16. Centrifuge the samples at 16000×g for 3 minute or until the filter membranes are dry.
17. Put the columns into sterile centrifuge tubes. Remove and discard the used collection tubes.
18. Pipette 40μL of ddH_2O directly onto the filter membranes of each column.
19. Let the samples stand for 2 minute.
20. Centrifuge the samples at 16000×g for 1 minute.
21. Discard the columns and store the purified DNA at -20℃ for future use.

6.7. Products

- 1 centrifuge tube with 40μL of 1500bp DNA strands
- 1 centrifuge tube with 40μL of 3000bp DNA strands
- 1 centrifuge tube with 40μL of 6000bp DNA strands

Day 4 (12-04-2023)

7. Experiment 7 *(Prerequisite: Case Study 1 Experiment 3)*

Note

Considering the PCR failure of case study 1 experiment 5, we have decided to lower the PCR annealing temperature to 52℃.

7.1. Objective

- To partially amplify the pLadder6K to obtain the desired short DNA strands (250bp, 500bp, 750bp, 1000bp).

7.2. Precautions

- Wear gloves.
- Dispose of the pipette tips after each transfer.

7.3. Materials

- 125µL of Taq polymerase (TaKaRa) premix
- 1µL of primer A
- 1µL of primer B
- 1µL of primer C
- 2µL of primer D
- 5µL of primer E
- 105µL of ddH_2O
- 10µL of purified pLadder6K sample 2
- Ice

7.4. Equipment

7.4.1. Machineries

- PCR thermocycler

7.4.2. Apparatus

- Pipettes (P10/P20/P200) and appropriate pipette tips

7.4.3. Containers

- 10 PCR tubes
- Ice box

7.4.4. Miscellaneous

- Marker pen

7.5. Preparations

- Transfer all materials into separate centrifuge tubes and pre-chill them with an ice box.
- Label each experimental centrifuge tube appropriately. (2 for primers A+E, 2 for primers B+E, 2 for primers C+E, 4 for primers D+E)

7.6. Procedures

1. Pipette 10.5µL of ddH_2O to each tube.
2. Pipette 0.5µL of primer E to each tube.
3. Pipette 0.5µL of primer A to two of the tubes.
4. Pipette 0.5µL of primer B to two of the tubes.
5. Pipette 0.5µL of primer C to two of the tubes.
6. Pipette 0.5µL of primer D to four of the tubes.
7. Pipette 1µL of DNA to each tube.

8. Pipette 12.5µL of Taq polymerase premix to each tube. Wait until the PCR machine is set up before doing this step to minimise unwanted prior reaction.
9. Mix all the tubes gently.
10. Put the tubes in the PCR thermocycler and set the parameters as follows.

7.7. PCR thermal cycle

Phase	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98℃	5m	1
Denaturation	98℃	30s	34
Annealing	52℃	30s	
Extension	72℃	1m 30s	
Final extension	72℃	7m	1
Sample keeping	4℃	∞	1

Day 5 (13-04-2023)

Interlude

Here omitted, we have performed gel electrophoresis on the PCR products of case study 1 experiment 7. The samples showed only some bands of ~6000bp while the others were not visible. We then proceeded to redo the PCR for the third time this day.

8. Experiment 8 *(Prerequisite: Case Study 1 Experiment 3)*

Note

Considering the PCR failure of case study 1 experiment 7, we have decided to run a gradient PCR at 4 different temperatures.

8.1. Objective

- To partially amplify the pLadder6K to obtain the desired short DNA strands (250bp, 500bp, 750bp, 1000bp).

8.2. Precautions

- Wear gloves.
- Dispose of the pipette tips after each transfer.

8.3. Materials

- 200µL of Taq polymerase (TaKaRa) premix
- 2µL of primer A
- 2µL of primer B
- 2µL of primer C
- 2µL of primer D
- 8µL of primer E
- 168µL of ddH_2O
- 16µL of purified pLadder6K sample 3
- Ice

8.4. Equipment

8.4.1. Machineries

- PCR thermocycler

8.4.2. Apparatus

- Pipettes (P10/P20/P200) and appropriate pipette tips

8.4.3. Containers

- 16 PCR tubes
- Ice box

8.4.4. Miscellaneous

- Marker pen

8.5. Preparations

- Transfer all materials into separate centrifuge tubes and pre-chill them with an ice box.
- Label each experimental centrifuge tube appropriately. (1 of each primer combination at each temperature)

8.6. Procedures

1. Pipette 10.5µL of ddH_2O to each tube.
2. Pipette 0.5µL of primer E to each tube.
3. Pipette 0.5µL of primer A to four of the tubes.

4. Pipette 0.5µL of primer B to four of the tubes.
5. Pipette 0.5µL of primer C to four of the tubes.
6. Pipette 0.5µL of primer D to four of the tubes.
7. Pipette 1µL of DNA to each tube.
8. Pipette 12.5µL of Taq polymerase premix to each tube. Wait until the PCR machine is set up before doing this step to minimise unwanted prior reaction.
9. Mix all the tubes gently.
10. Put the tubes in the PCR thermocycler and set the parameters as follows.

8.7. PCR thermal cycle

Phase	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98℃	5m	1
Denaturation	98℃	30s	34
Annealing	52.6℃ 51.4℃ 49.7℃ 48.3℃	30s	
Extension	72℃	1m 30s	
Final extension	72℃	7m	1
Sample keeping	4℃	∞	1

Day 6 (14-04-2023)

Interlude

Here omitted, we have performed gel electrophoresis on the PCR products of case study 1 experiment 8.

Figure 2 - Electrophoresed gel under UV light
From left to right: DNA ladder, null, A+E primer-samples, nulls, B+E primer-samples

Figure 3 - Electrophoresed gel under UV light
From left to right: DNA ladder, null, C+E primer-samples, nulls, D+E primer-samples

All the samples showed only a band of ~6000bp. We decided to redo the PCR for the fourth time at a later date.

Day 7 (19-04-2023)

Note

We focused on working on case study 2 and did not do any work on this case study on this day.

Day 8 (20-04-2023)

9. Experiment 9 *(Prerequisite: Case Study 1 Experiment 3)*

Note

Considering the PCR failure of case study 1 experiment 8, we have decided to increase the concentrations of the primers and DNA template extend the annealing time to 45s.

9.1. Objective

- To partially amplify the pLadder6K to obtain the desired short DNA strands (250bp, 500bp, 750bp, 1000bp).

9.2. Precautions

- Wear gloves.
- Dispose of the pipette tips after each transfer.

9.3. Materials

- 200µL of Taq polymerase (TaKaRa) premix
- 4µL of primer A
- 4µL of primer B
- 4µL of primer C
- 4µL of primer D
- 16µL of primer E
- 152µL of ddH_2O
- 24µL of purified pLadder6K
- Ice

9.4. Equipment

9.4.1. Machineries

- PCR thermocycler

9.4.2. Apparatus

- Pipettes (P10/P20/P200) and appropriate pipette tips

9.4.3. Containers

- 16 PCR tubes
- Ice box

9.4.4. Miscellaneous

- Marker pen

9.5. Preparations

- Transfer all materials into separate centrifuge tubes and pre-chill them with an ice box.
- Label each experimental centrifuge tube appropriately. (1 of each primer combination at each temperature)

9.6. Procedures

1. Pipette 9µL of ddH_2O to each tube.
2. Pipette 1µL of primer E to each tube.
3. Pipette 1µL of primer A to four of the tubes.
4. Pipette 1µL of primer B to four of the tubes.
5. Pipette 1µL of primer C to four of the tubes.
6. Pipette 1µL of primer D to four of the tubes.
7. Pipette 1.5µL of DNA to each tube.

8. Pipette 12.5µL of Taq polymerase premix to each tube. Wait until the PCR machine is set up before doing this step to minimise unwanted prior reaction.
9. Mix all the tubes gently.
10. Put the tubes in the PCR thermocycler and set the parameters as follows.

9.7. PCR thermal cycle

Phase	**Temperature**	**Duration**	**Cycles**
Initial denaturation	98℃	5m	1
Denaturation	98℃	30s	34
Annealing	52.6℃ 51.4℃ 49.7℃ 48.3℃	45s	
Extension	72℃	1m 30s	
Final extension	72℃	7m	1
Sample keeping	4℃	∞	1

Day 9 (21-04-2023)

Interlude

Here omitted, we have performed gel electrophoresis on the PCR products of case study 1 experiment 8. All the samples showed only a band of ~6000bp. Due to time constraints, we are unable to redo the PCR.

We decided to combine only the purified DNA strands of large sizes (1500bp, 3000bp, 6000bp) with dye and perform gel electrophoresis to produce half of the desired ladder as a final measure.

Finale

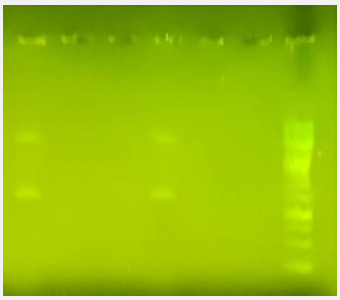


Figure 4 - Electrophoresed gel with partial ladder
From left to right: partial DNA ladder product samples, nulls, commercial DNA ladder

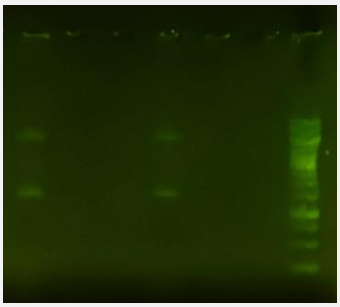


Figure 5 - Same gel as Figure 4 but captured with lower exposure

As can be observed, the 6000bp and 1500bp bands can clearly be seen. The 3000bp band while there, was quite faint and was difficult to observe. We believe this is due to the final mixture concentration not being regulated due to product volume constraints.

Discussion

This case study was not fully successful, although a partial DNA ladder was achieved in the end. The largest contributing factor in this failure is due to our poor PCR results despite numerous trials and modifications to the parameters. The other groups faced the same problems to various extents. While initially we have planned this case study to be finished on day 6 (later re-evaluated to be finished on day 3 when we realised no transformation is necessary) with room to spare for buffer, it had turned out that there was not enough time for the multitude of trials we require. Looking back, I believe one possible improvement of the procedures is to immediately extend the annealing time and try a very low annealing temperature to generate data points. Such data points can be used to give an upper and lower bound for the working temperature more efficiently than blindly decreasing temperature by an arbitrary amount each successive trial.

Despite the failure, I believe that this does not reflect poorly about our time management and planning regarding this case since we were already doing daily PCRs without much wasted time.