UCC_Ireland_SeaDNA_Subproject_Protocols

1. Making Agarose Gels

We decided to make 1% agarose gels for the duration of our project as they separate DNA whose sizes are between 250 base pairs to 12 Kb. This is optimal for our working range. This required us to make 5X TBE and gel loading buffer.

For the 5X TBE stock per litre:

- 54g Tris Base
- 27.5g Boric Acid
- 3.7 grams EDTA
  This was then dissolved in 800mL of DH2O and then mad up to one litre by adding water. It was then diluted 5 fold to 1X TBE working solution when required.

For the 6X gel Loading Buffer:

- 0.25% bromophenol blue
- 0.25% xylene cyanol FF
- 30% (w/v) glycerol and H2O

A safeview stain was used to visualize the DNA under U.V light. 5µL was used for a 50mL gel.

2. Preparing the pCDF vector for cloning

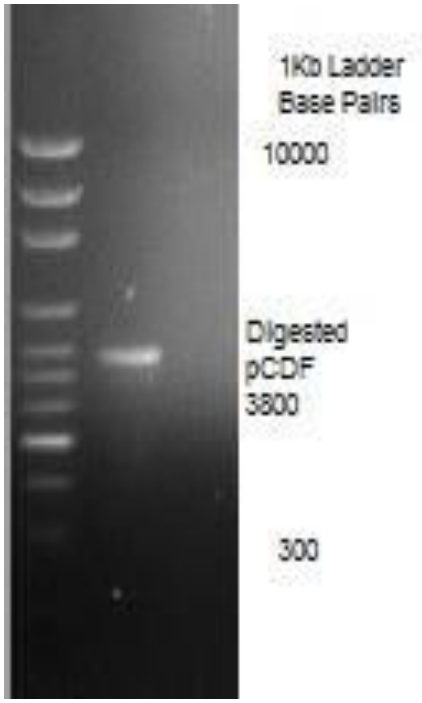
We started off with cutting the pCDF-duet and pRSF-duet plasmids with two restriction enzymes EcoRI and HindIII. The hagfish DNA insert has the sites MFeI and HindIII which would then be compatible with both the cut vectors.

We made a restriction digest using the following protocol:

	pCDF restriction digest	pRSF restriction digest
Vector	13 µL	8 µL
EcoRI	1.5 µL	1.5 µL
HindIII	1.5 µL	1.5 µL
Green Buffer	4 µL	4 µL
Water	20 µL	25 µL
Total volume	40 µL	40 µL

The digest was then incubated at 37°C for 2 hours. A 1% agarose gel was then made up. All the 40 µL of the digest was then loaded up into one well alongside a 1Kb Marker at 100 Volts for 1 hour. The gel was then viewed under ultraviolet light in an LED illuminator to see if there was a bind the size of the cut vector separated from its cut our piece of DNA. This bright band was then cut out of the gel and the DNA was extracted using a Gel extraction kit. A nanodrop test was then done to measure the concentration of DNA which was found to be 40.6 ng/µL for the pCDF plasmid, and 37.8 ng/µL for the pRSF plasmid.

Figure 1: Digested pCDF-duet vector



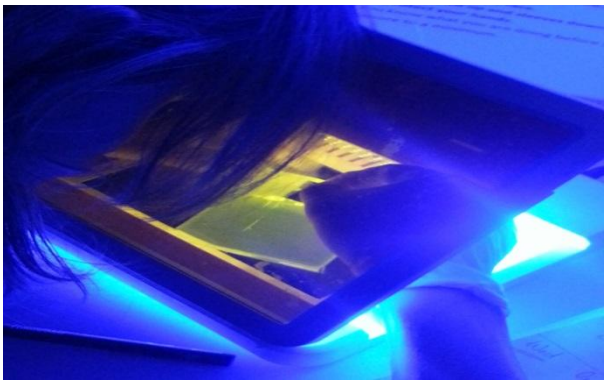


Figure 2: Cutting out the gel to extract DNA

A ligation was also carried out to verify that we had the cut vector using the following protocol:

	Ligation
Water	14 µL
10x Buffer	2 µL
Cut pCDF / pRSF	3 µL
T4 Ligase	1 µL
Total Volume	20 µL

The Ligation digest was then incubated at 22°C for 30 minutes. It was then transformed in 100 µL of the DH5α strain of E.coli cells and plated up on an agar plate with Streptomycin and incubated overnight at 37°C and checked the next morning.

The reason for this ligation was to verify that the vectors had been cut at the EcoRI and HindIII sites. If these cuts had been made there would be no colonies on the plates the next morning as the vector would be linear instead of its original circular shape. We can then continue with the cloning of the hagfish target genes into the pCDF vector.

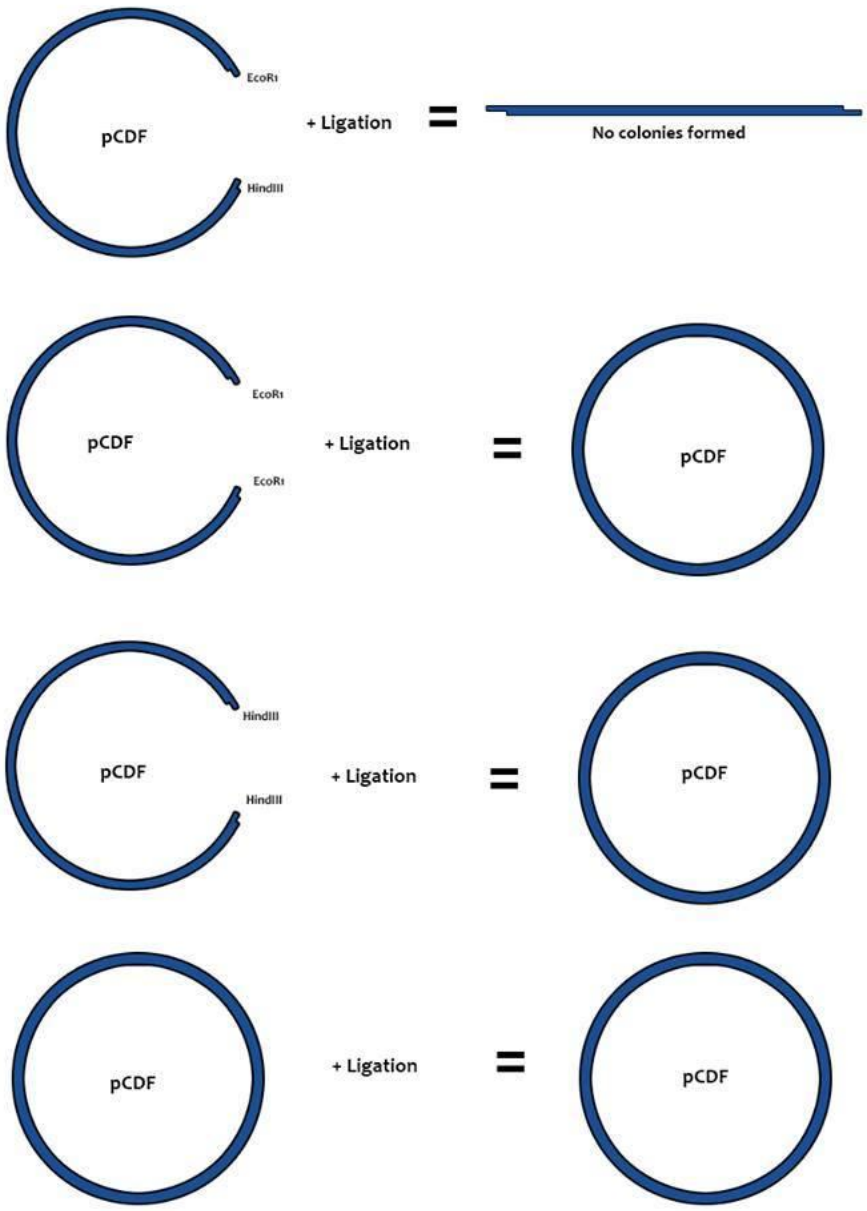


Figure 3: Verification of restriction digest.

3. Digestion of the Hagfish DNA

The hagfish slime consist of two subunits, Alpha and Gamma. We ordered the genes for these two subunits and digested them using the following protocol:

	Alpha subunit	Gamma subunit
Hagfish DNA	10 µL	10 µL
Cut Smart buffer	2 µL	2 µL
Water	6.5 µL	6.5 µL
MFeI	0.5 µL	0.5 µL

HindIII	1 µL	1 µL
Total Volume	20 µL	20 µL

The DNA samples were digested for 2 hours at 37°C and the heated for 20 minutes at 65°C to inactivate the enzymes. 5 µL of this digest was then used in the ligation with pCDF.

4. Ligation of the Alpha and Gamma Subunits with the pCDF vector

At this stage we have the cut pCDF vector and the digested Hagfish DNA. We ligated the Alpha and Gamma subunits separately in the pCDF vector using the following protocol:

	Alpha subunit	Gamma subunit
pCDF vector	3 µL	3 µL
Hagfish DNA digest	5 µL	5 µL
T4 Ligase	1 µL	1 µL
10x Buffer	2 µL	2 µL
Water	9 µL	9 µL
Total Volume	20 µL	20 µL

The ligation digest was incubated for 30 minutes at 22°C.

5. Transformation pCDF Alpha and pCDF Gamma into DH5α cells

For the transformation our team chose the DH5α E. coli cells. They are a non-pathogenic and were developed for laboratory cloning use. This strain enables high-efficiency transformations. The mutations that the DH5-Alpha strain has are: dlacZ Delta M15 Delta (lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1. These mutations correspond to the distinct characteristics that make the DH5-Alpha strain excel in laboratory cloning procedures.

The protocol used for the transformation:

- Incubate agar plates with streptomycin at 37°C, use one plate for control.
- Use 100µL of DH5α cells for each transformation, make sure everything is on ice.
- Add 1-5µL of vector to cells and mix gently
- Incubate on ice for 25 minutes.
- Heat shock at 42°C for 1 minute maximum.
- Put cells back on ice for 5 minutes.
- Add 1ml of LB broth as a medium.
- Incubate shaking at 37°C for 1 hour.
- Plate up cells- Spin cells at 3k for 3 minutes in an eppie spinner, quickly pour off supernatant, leaving 100µL of broth and suspend cells.
- After plating up cells, incubate plates at 37°C overnight and check for colonies the next morning.

The morning after the transformation, there were colonies present on both the DH5α pCDF Alpha subunit and DH5α pCDF Gamma subunit plates. There were no colonies on the control.

6. Colony PCR for both Alpha and Gamma subunits in DH5α cells

The plated up cells with the DH5α pCDF Alpha subunit and the DH5α pCDF Gamma subunit contained colonies which is a sign of a successful transformation and cloning. We then carried out colony PCR on 17 colonies on the Gamma plate and 10 colonies on the Alpha plate along with 3 control reactions. PCR will verify if the insert was cloned and if the PC is successful, the structure we hoped for is present.

Figure 4: Gel showing successful colony PCR on the alpha subunit.

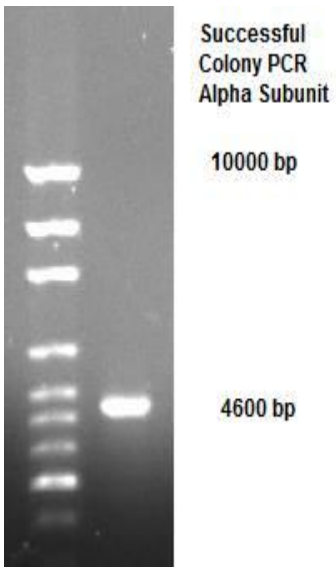
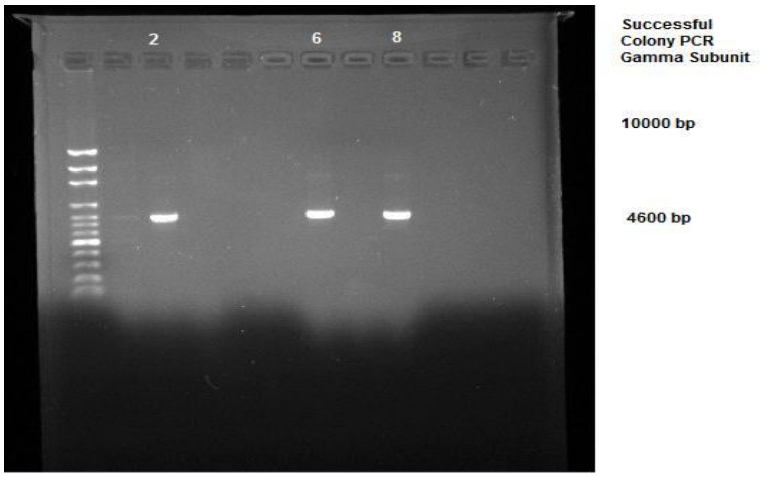


Figure 5: Gel showing successful colony PCR on the gamma subunit.



The protocol used for each individual colony sample were as follows, there were 30 tubes in total (17 Gamma, 10 Alpha, 3 control).

Forward Primer	0.5 µL
Reverse Primer	0.5 µL
PCR Buffer	2 µL
Water	16.25 µL
Enzyme	0.25 µL
dNTP	0.5 µL
Total volume/tube	20 µL

A master mix was made for each subunit separately and 20 µL aliquots were then made. Finally, the colonies were labelled on the plate, a toothpick was used to touch the colony lightly and inserted into the appropriate tube.

After the PCR reaction, agarose gels were ran with each individual samples, 3 of the brightest bands from the Gamma samples and 1 of the brightest bands from the Alpha sample were noted. We went back to these colonies on the respective plates and grew up these colonies in a 10ml L.B broth with Streptomycin (10µL) overnight.

7. Plasmid mini-preps were carried out on the DH5α pCDF Alpha and Gamma overnight cultures.

The GenElute Plasmid mini prep kit was used, the protocol used is as follows:

- Use 5ml of the overnight culture.
- Pellet cells at maximum speed for 1 minute, discard supernatant.
- Resuspend pellets in 200 µL of resuspension solution, pipette up and down well to resuspend.
- Lyse cells, add 200 µL of lysis reagent, invert gently 5-6 times.
- Immediately, add 350 µL of neutralisation solution, invert gently 5-6 times.
- Centrifuge at top speed for 10 minutes. If there are floaty white bits, spin again.
- Apply clear lysate to spin column.
- Centrifuge at top speed for 1 minute, discard flow through.
- Add 750 µL of wash solution, centrifuge at top speed for 1 minute, and discard flow through.
- Centrifuge empty column for 1 minute more at maximum speed.
- Transfer column to a fresh collection tube.
- Add 100 µL of elution buffer/sterile water, incubate for 1 minute.
- Centrifuge at top speed for 1 minute.
- Measure DNA concentration.

The DNA concentration for the pCDF Alpha was 53.1ng/µl

The DNA concentration for the pCDF Gamma was 55 ng/µl

8. **Performing a restriction digest to re check if we have the correct insert**

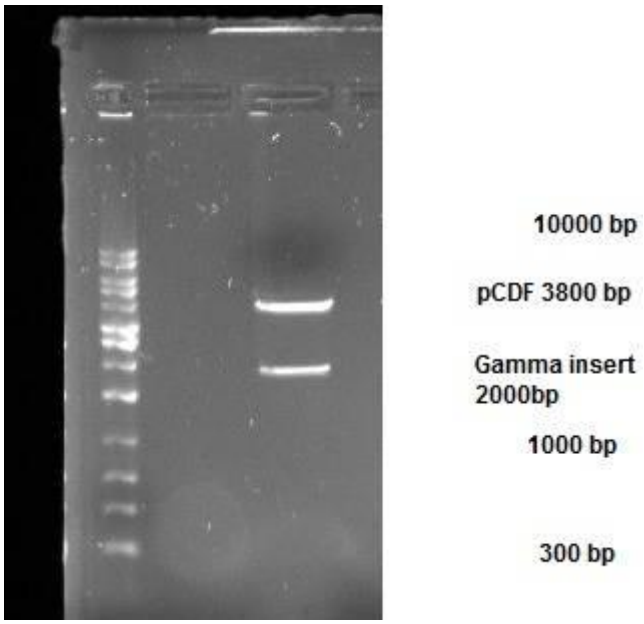


Figure 6: Products of a restriction digest showing the pCDF backbone and the cut out gamma insert.

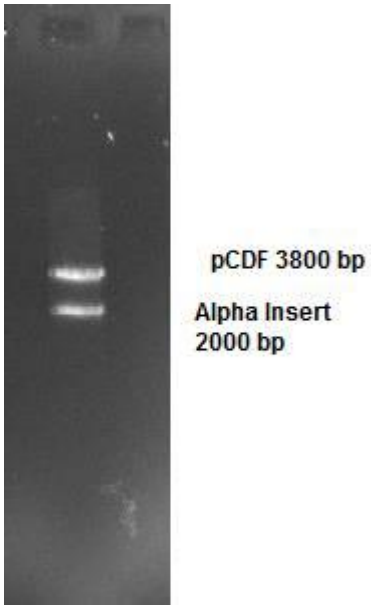


Figure 7: Products of a restriction digest showing the pCDF backbone and the cut out alpha insert.

9. Transforming the cloned plasmids into BL21 cells

After we completed the plasmid mini preps, we transformed the cloned plasmids separately in BL21 cells. BL21 is a strain of E.coli cells that are chemically competent and suitable for protein expression.

The protocol used for the transformation:

- Incubate agar plates with streptomycin at 37°C, use one plate for control.
- Use 100µL of DH5α cells for each transformation, make sure everything is on ice.
- Add 1-5µL of vector to cells and mix gently
- Incubate on ice for 25 minutes.
- Heat shock at 42°C for 1 minute maximum.
- Put cells back on ice for 5 minutes.
- Add 1ml of LB broth as a medium.
- Incubate shaking at 37°C for 1 hour.
- Plate up cells- Spin cells at 3k for 3 minutes in an eppie spinner, quickly pour off supernatant, leaving 100µL of broth and suspend cells.
- After plating up cells, incubate plates at 37°C overnight and check for colonies the next morning.

There were colonies present the next morning on both the Alpha and Gamma plates and there were no colonies on the control. Now, we are ready to start the process of protein purification.

10. Purification of Histidine-tagged protein

The purification process we used was split into 3 days.

DAY 1

- Prepared 2 10 ml LB broth in a glass bottle, I was autoclaved at 121°C for 15 minutes.
- Using aseptic techniques 10µL of Streptomycin was pipetted into each glass bottle.
- Next, one glass bottle was inoculated with E.coli BL21 pCDF Gamma and the other with E.coli BL21 pCDF Alpha colonies.
- They were incubated in the shaking incubator overnight.
- 2 500 ml broths were prepared in 2.5 litre conical flasks and autoclaved at 121°C for 15 minutes and left at room temperature until the next day.

DAY 2

- Using aseptic techniques 0.5 ml of Streptomycin was pipetted into both the 500ml LB broth that was prepared the previous day.
- Aseptically 5ml of both the alpha and gamma cultures were added separately into the flasks.
- They were incubated for 2-3 hours in the shaking incubator.
- A 1ml sample was taken from each culture after 2.5 hours and the absorbance was measured using a spectrophotometer using the OD600 measurement (optical density at 600nm), it needs to be 0.4.
- Once the correct OD600 was reached, the exact reading was made note of.
- 1ml of each culture was taken and pipetted into a 1.5ml Eppendorf. Using a microfuge, the eppendorfs were spun down for 10 minutes at 10,000 rpm.

- The supernatant was removed and the pellet was frozen. They was labelled T_0 Alpha and T_0 Gamma respectively. These samples were used later on in PAGE analysis.
- We then induced expression of the fusion protein by the addition of 0.1M IPTG o a final concentration of 1mM (1/100 dilution) to the cultures.
- The flasks were returned to the shaking incubator for 3 hours at 37°C.
- After 3 hours, the OD 600 was noted for both samples. 1ml cultures were then taken and spun down for 10 minutes at 10,000 rpm. The supernatant was removed the pellets was frozen. They were labelled T_3 Gamma and T_3Alpaha respectively and were then used for PAGE analysis.
- The remainder of the 500 ml culture was also spun down at 4000 rpm for 30 minutes in the Sorvall RC-5B centrifuge.

The procedure above was repeated again at 30°C to check if the protein was more soluble at a lower temperature.

DAY 3

- We set up two affinity chromatography columns.
- Attach two empty columns to clamps on two retort stands.

We carried out the following procedure for each of the columns simultaneously:

- Pipette 1ml “Probond” affinity resin (shake bottle before use to resuspend beads in solution) into the column. Allow the alcohol o run of, close the column.
- Wash the column with Millipore water to remove ethanol from the resin; 20ml is sufficient. Collet flowthrough into a beaker. When finished replace the stopper at the column outlet to prevent resin from drying out.
- Wash he column with 10ml loading buffer, it is now ready for use.
- Following the centrifugation of bacterial culture, pour supernatant back into the original culture flask (for autoclaving later).
- Keeping everything on ice. Resuspend the bacterial pellets in 5ml loading buffer (use a 1ml, 5ml or 10ml pipette to ensure even homogenisation of the mixture).
- Transfer the resuspend cells to a 10ml glass beaker. To ensure constant low temperature for the culture, place the beaker in a 50 ml beaker of slushy ice.
- Disrupt bacterial cells to release cell contents (lysate) by use of an ultrasonicator (Soniprep)
- If the total volume of resuspended culture is 10ml or less use a narrow probe. If it is more than 10 mls use a wide probe.
- Before beginning the sonicationg step have a good look at the suspension, note the milkyness.
- Begin sonication, set power to mid-range and place the probe into the ice insulated beaker. Keep the probe well under the surface (to prevent bubbles). Teal cells for 10 seconds, Stop, and allow the cells to cool for at least 1 minute before proceeding.
- Repeat the sonicating step 4 more times. Don’t forget the cooling step each time. If the solution froths, stop.
- Once the bacterial cells have lysed the solution should change colour it will still be opaque but will have lost the milkyness.
- Transfer disrupted cells into a centrifuge tube. Using an appropriate counterweight, spin at 12,000g for 30 minutes using a Sorvall centrifuge with Sa600 rotor.

- Remove the lysate (supernatant) and transfer into a universal tube, label and place on ice, Discard cell debris pellet. Pipette 50µL supernatant into a 0.5 Eppendorf, label “clear lysate” and place on ice.
- Pour the lysate supernatant onto the column, collect the flow through in a Universal tube. Label “flow through”, place on ice.
- Run 10ml of loading buffer through the column, collect the fraction in a falcon tube and label “wash 1”.Place on ice.
- Pour 25ml of wash buffer through column, collect the fraction in a labelled falcon tube; wash 2. Place on ice.
- Run 1ml elution buffer through column and collect 1ml fractions, repeat 10 times.
- Add 5ml elution buffer, run through the column and collect 5ml in one tube.
- Label fractions as E1, E2, E3, E4 and E5.
- Place all fractions on ice. Store at 4°C.
- Run polyacrylamide gels to check for purity of the target protein.

11. SDS PAGE

We used the BioRad system. We used a discontinuous buffer system which includes a non-restrictive large pore gel called a stacking gel which is layers on top of the separating gel known as the resolving gel. We used an acrylamide concentration of 10% for our resolving gel as it separates proteins between 20-80 kilodaltons

4% Stacking Gel	20 mL
Water	12.2 mL
1.0M Tris pH 6.8	5 mL
30% Acrylamide	2.6 mL
10% SDS	200 µL
APS	100 µL
TEMED	20 µL

10% Resolving Gel	40 mL
Water	16.26 mL
1.5M Tris pH 8.8	10 mL
30% Acrylamide	13.34mL
10% SDS	400 µL
APS	200 µL
TEMED	20 µL

12. Making Glycerol Stocks

- 750 µL of 40% glycerol
- 700 µL of the culture

Cultures were then stored in the -80 °C