RNA Lab Book

02.08.17

Aim: To assemble longer sequences of CAG repeats of varying lengths so which will be used to construct RNA scaffolds of different sizes.

Materials & Methods:

- 10µM 10xCAG Oligo (Tm = 86.8°C)
- 10µM 10xCTG Oligo (Tm = 86.8°C)
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

PCR Protocol p020801 (20µl Reactions):

1. 95°C – Initial Denaturation (2 Minutes)
   ----------*60 x Cycles*----------
2. 95°C – Denaturation (30 Seconds)
3. 80°C – Annealing (30 Seconds)
4. 68°C – Extension (45 Seconds)
   ----
5. 68°C – Final Extension (5 Minutes)
6. 12°C – HOLD

Reactions for p020801

COMPONENT	20µl REACTION	FINAL CONCENTRATION
5x Phusion HF	4µl	1x
10mM dNTPs	0.4µl	200µM
Phusion DNA Polymerase	0.2µl	1.0 units/50µl PCR
10µM 10xCAG Oligo	1µl | 2µl | 0µl	<250ng
10µM 10xCTG Oligo	1µl | 2µl | 0µl	<250ng
Nuclease-Free Water		to 20µl

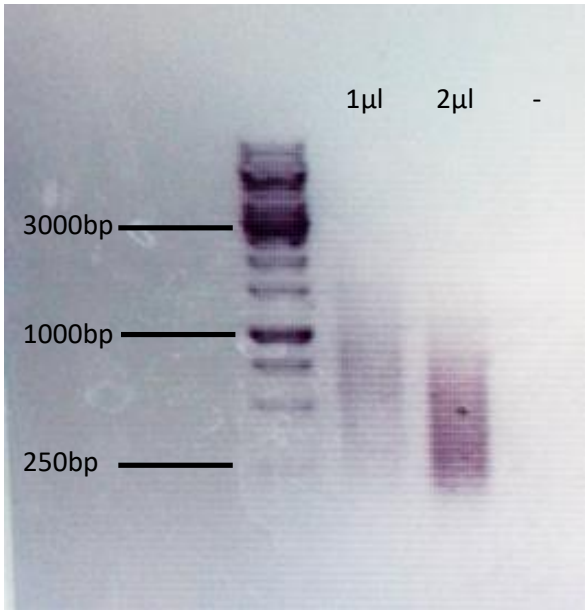
	13.4µl 11.4µl 15.4µl	

1. Three reactions were set up for the PCR cycles:
    1. 1µl volume of each oligo (10xCAG and 10xCTG)
    2. 2µl volume of each oligo (10xCAG and 10xCTG)
    3. A Negative control that did not contain any oligo
2. While the PCR reaction was undergoing, a 1% agarose gel to determine the size of the product.

    1% Agarose Gel Methods

    1. 0.5g of Agarose was mixed with 50ml of 1x TAE Buffer.
    2. The solution was heated until agarose is completely dissolved.
    3. 5µl of SyberSafe was added to the solution once it has been cooled for 5-10 minutes.
    4. Solution is to be poured into the mould and left to cool.
3. The PCR products were added to the wells with 6x loading dye (1µl of dye to 5µl of PCR Product).

Results & Evaluations:



The PCR products show the expected "smears" of the oligo nucleotides, which indicates that repeats of varying lengths have been assembled. As can be seen in *fig. 1*, the 1µl of each oligo nucleotide produced fragments varying in sizes of <250bp to over 1000bp. The 2µl of each oligo seems to have produced less repeats of similar lengths but of higher concentration at about 300bp.

Although the expected smear was achieved, the PCR protocol used was not completely suited for the Phusion DNA Polymerase, as described by the NEB

protocol. Both the annealing and denaturing temperatures were too high and too low respectively. Thus, the PCR experiment was repeated with the parameters more optimally set.

Materials & Methods:

- 10µM 10xCAG Oligo (Tm = 86.8°C)
- 10µM 10xCTG Oligo (Tm = 86.8°C)
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

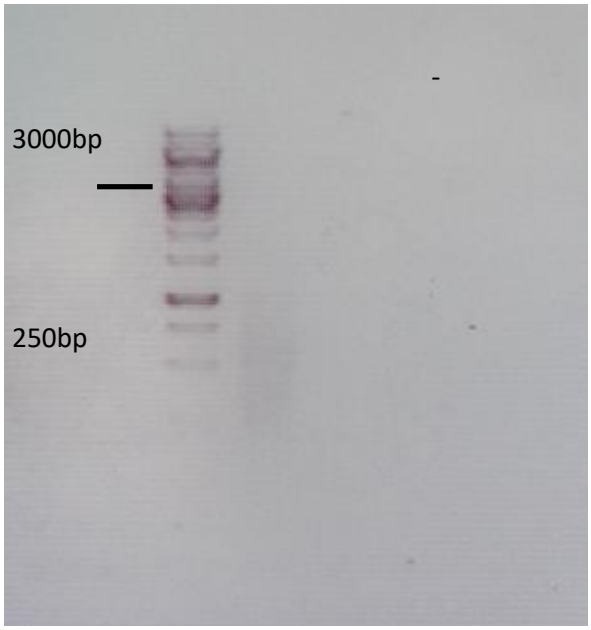
PCR Protocol p020802 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*35 x Cycles*----------
2. 98°C – Denaturation (10 Seconds)
3. 72°C – Annealing & Extension (1 Minute)
   ----
4. 72°C – Final Extension (5 Minutes)
5. 12°C – HOLD

Reactions for p020802

COMPONENT	25µl REACTION	FINAL CONCENTRATION
5x Phusion HF	5µl	1x
10mM dNTPs	0.5µl	200µM
Phusion DNA Polymerase	0.25µl	1.0 units/50µl PCR
10µM 10xCAG Oligo	1µl | 2µl | 0µl	<250ng
10µM 10xCTG Oligo	1µl | 2µl | 0µl	<250ng
Nuclease-Free Water	17.25µl | 15.25µl | 19.25µl	to 25µl

Results & Evaluations:



A repeat of the experiment with more optimal settings of the Phusion High Fidelity PCR Protocol surprisingly produced less effective results from the prior protocol. As can be seen in *fig. 2*, the PCR product which had 1µl of each oligo shows the appearance of a very faint "smear" indicating that there was assembly of the repeats. Although, there is no visible product in lane 2, containing the result of 2µl of each oligo.

The results seem to indicate that the annealing and extension temperatures will need to be further optimised. Also, based on the sizes of the smears in *fig. 1* the product reaches sizes that are higher than 1000bps, so we will also look to optimise the length of time for both the annealing and the extension periods in order to assemble smaller fragments.

03.08.17

Aim: To continue testing different PCR parameters in order to assemble smaller CAG/CTG fragments.

In order to assemble smaller CAG/CTG fragments two different PCR protocols were tried, where the length of time at 80°C (Annealing and Extension steps) were done for 10 and 20 seconds, compared to the 30 seconds done in *p020801*.

Materials & Methods:

- 10µM 10xCAG Oligo (Tm = 86.8°C)
- 10µM 10xCTG Oligo (Tm = 86.8°C)
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

PCR Protocol p030801 (20µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*60 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 80°C – Annealing & Extension (10 Seconds)
   ----
4. 72°C – Final Extension (5 Minutes)
5. 12°C – HOLD

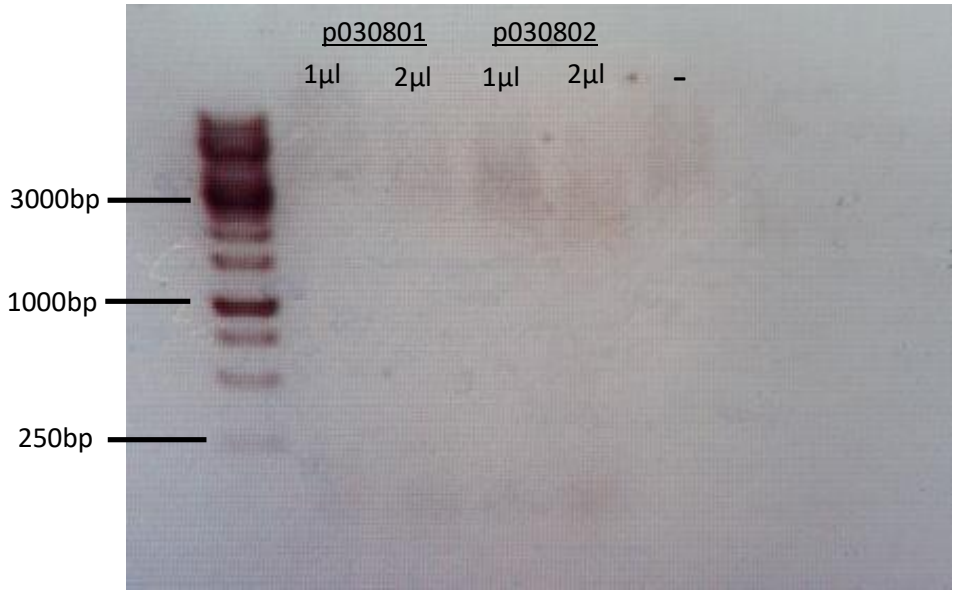
PCR Protocol p030802 (20µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*60 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 80°C – Annealing & Extension (20 Seconds)
   ----
4. 72°C – Final Extension (5 Minutes)
5. 12°C – HOLD

Reactions for p030801 & p030802

COMPONENT	20µl REACTION	FINAL CONCENTRATION
5x Phusion HF	4µl	1x
10mM dNTPs	0.4µl	200µM
Phusion DNA Polymerase	0.2µl	1.0 units/50µl PCR
10µM 10xCAG Oligo	1µl | 2µl | 0µl	<250ng
10µM 10xCTG Oligo	1µl | 2µl | 0µl	<250ng
Nuclease-Free Water	13.4µl | 11.4µl | 15.4µl	to 20µl

Results & Evaluations:



As can be seen in *fig. 3*, there were no observable products from both *p030801* and *p030802*. It is difficult to assess if the assembled repeats were so small that it was undetectable or whether the reaction didn't not occur in the first

place. If the latter is the case, then perhaps we the annealing and extension observed in *p020801* occurred during the 68°C step.

In continuation of the optimisation of the repeats assembly 72°C and 68°C will be adopted for both the annealing and extension temperatures respectively.

04.08.17

Aim: To continue the assembly of oligo repeats by changing the annealing and extension temperatures in an attempt to construct smaller fragments. Higher concentrations of the oligos were also tested to improve the signal.

The extension step will be varied at 10 and 25 seconds as a means of reducing the size of the smear. Additionally, the number of cycles will also be reduced in an attempt to reduce the size of the repeat sequence formation.

Materials & Methods:

- 10µM 10xCAG Oligo (Tm = 86.8°C)
- 10µM 10xCTG Oligo (Tm = 86.8°C)
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

PCR Protocol p040801 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*35 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 72°C – Annealing (30 Seconds)
4. 68°C – Extension (10 Seconds)
   ----
5. 72°C – Final Extension (5 Minutes)
6. 12°C – HOLD

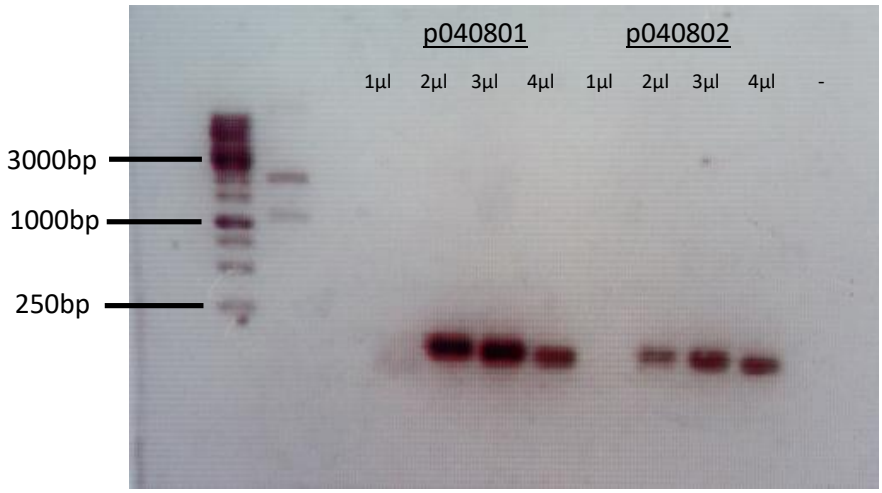
PCR Protocol p040802 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*35 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 72°C – Annealing (30 Seconds)
4. 68°C – Extension (25 Seconds)
   ----
5. 72°C – Final Extension (5 Minutes)
6. 12°C – HOLD

Reactions for p040801 & p040802

COMPONENT	25µl REACTION	FINAL CONCENTRATION
5x Phusion HF	5µl	1x
10mM dNTPs	0.5µl	200µM
Phusion DNA Polymerase	0.25µl	1.0 units/50µl PCR
10µM 10xCAG Oligo	1µl | 2µl | 3µl | 4µl | 0µl	<250ng
10µM 10xCTG Oligo	1µl | 2µl | 3µl | 4µl | 0µl	<250ng
Nuclease-Free Water	17.25 µl | 15.25 µl | 13.25 µl | 11.25 µl | 19.25 µl	to 25µl

Results & Evaluations:



The products of *p040801* and *p040802* indicate that there is an assembly of the oligos occurring, although it is very small and difficult to determine with the gel image. The samples were run again on a gel with a 100bp ladder (not shown) but the products were still undefinable. We can assume that the repeats sequences that were assembled were smaller than 100bp, which would be ideal for the experiment.

07.08.17

Aim: Increase the extension period to try and determine if it will increase the size of the smear.

By increasing the period of extension we can compare with the results of *p040801/02* to understand the step involved in assembling the repeats to different lengths.

Materials & Methods:

- 10µM 10xCAG Oligo (Tm = 86.8°C)
- 10µM 10xCTG Oligo (Tm = 86.8°C)
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

PCR Protocol p070801 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*40 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 72°C – Annealing (30 Seconds)
4. 68°C – Extension (30 Seconds)
   ----
5. 72°C – Final Extension (5 Minutes)
6. 12°C – HOLD

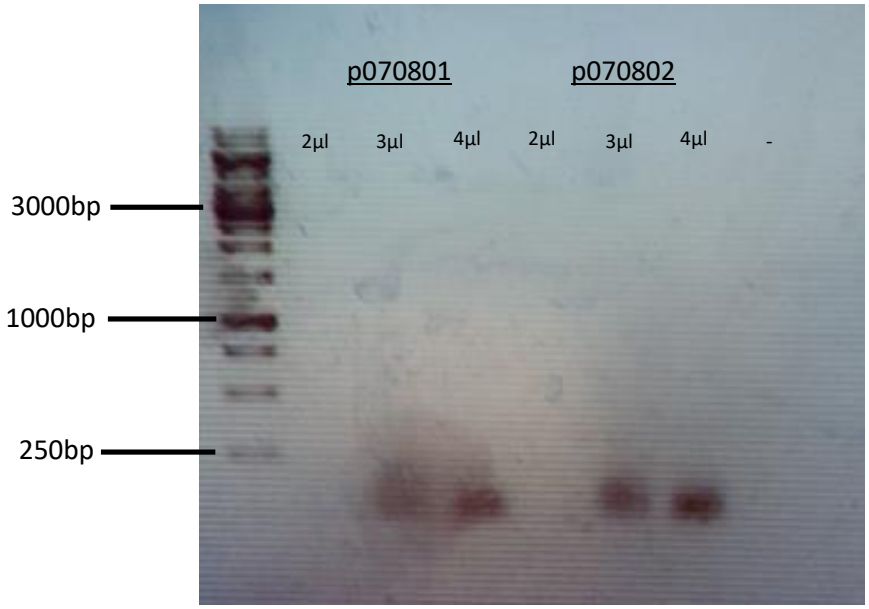
PCR Protocol p070802 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*40 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 72°C – Annealing (30 Seconds)
4. 68°C – Extension (45 Seconds)
   ----
5. 72°C – Final Extension (5 Minutes)
6. 12°C – HOLD

Reactions for p070801 & p070802

COMPONENT	25µl REACTION	FINAL CONCENTRATION
5x Phusion HF	5µl	1x
10mM dNTPs	0.5µl	200µM
Phusion DNA Polymerase	0.25µl	1.0 units/50µl PCR
10µM 10xCAG Oligo	2µl | 3µl | 4µl | 0µl	<250ng
10µM 10xCTG Oligo	2µl | 3µl | 4µl | 0µl	<250ng
Nuclease-Free Water	15.25 µl | 13.25 µl | 11.25 µl | 19.25 µl	to 25µl

Results & Evaluations:



The results of the increase of extension time to 30 seconds and 45 seconds for *p070801* and *p070802* respectively formed fragments that were much smaller than 250bp. It is once again difficult to determine the size of the fragments.

For the next step, primers with Bsa1 sites will be used with the products of *p020801* and *p070802* to test the effectiveness in both reducing the size of the fragments and preparing them for cloning.

Aim: Using primers containing Bsa1 sites and 5xCAG/5xCTG, the products *p020801a* (yellow), *p020801b* (blue), *p070801a* (blue) and *p070801b* (grey), we expect to see a reduction of the sample sizes. The addition of the Bsa1 sites will be used to clone the repeats for sequencing, which will determine the sizes.

For the primer extension of the samples, both 5x HF Buffer and GC Buffer were used in order to see try and improve the quality of the product, these will be named p070803 and p070804 respectively.

Materials & Methods:

- 10µM o.17.011 primer (Tm = 73.3°C)
- 10µM o.17.012 primer (Tm = 71.1°C)
- *p020801a*, *p020801b*, *p070801a*, *p070801b* samples
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

PCR Protocol p070803 & p070804 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*35 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 66°C – Annealing (30 Seconds)
4. 72°C – Extension (15 Seconds)
   ----
5. 72°C – Final Extension (5 Minutes)
6. 12°C – HOLD

Reactions for p070801 & p070802

COMPONENT	25µl REACTION	FINAL CONCENTRATION
5x Phusion HF/GC Buffer	5µl	1x
10mM dNTPs	0.5µl	200µM

Phusion DNA Polymerase	0.25µl	1.0 units/50µl PCR
p020801a / *p020801b* / *p070801a* / *p070801b*	1µl	<250ng
10µM *o.17.011*	1.25µl	0.5µl
10µM *o.17.012*	1.25µl	0.5µl
Nuclease-Free Water	15.75µl | 16.75µl	to 25µl

Results & Evaluations:

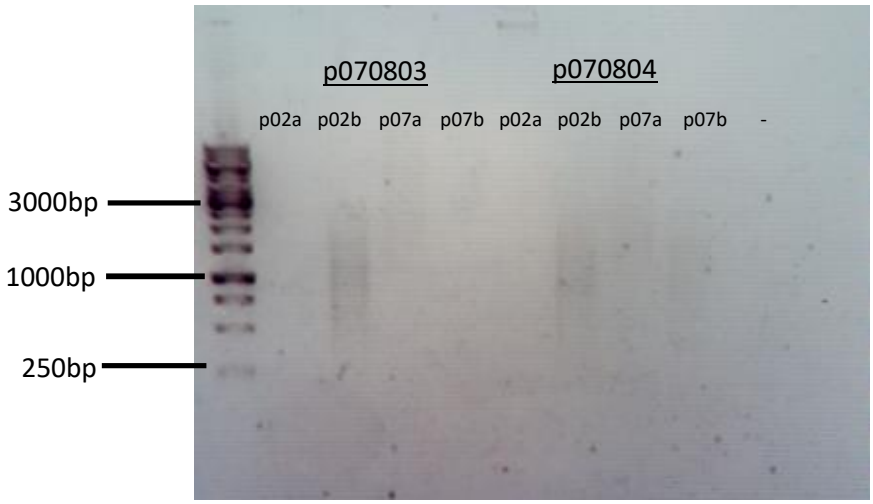


Figure 6 Products of p070803 and p070804 which used 5x HF buffer and GC buffer respectively

As can be seen on *fig. 6* there were no discernible products produced from the PCR of what was expected. There are some smears that are present in some of the lanes, which are very large. This is most likely just the extension of the previous PCR product as upon evaluation the annealing temperature for the PCR was too low for the primers. Thus, the experiment will need to be repeated.

11.08.17

Aim: Increase the percentage of agarose of the gel and rerun *p040801b*, *p040801c*, *p040801d*, *p040802b*, *p040802c* and *p040802d* with 100bp ladder to determine the size of the fragments.

2% Agarose Gel Methods

1. 1g of Agarose was mixed with 50ml of 1x TAE Buffer.
2. The solution was heated until agarose is completely dissolved.
3. 5µl of SyberSafe was added to the solution once it has been cooled for 5-10 minutes.
4. Solution is to be poured into the mould and left to cool.

Results & Evaluations:

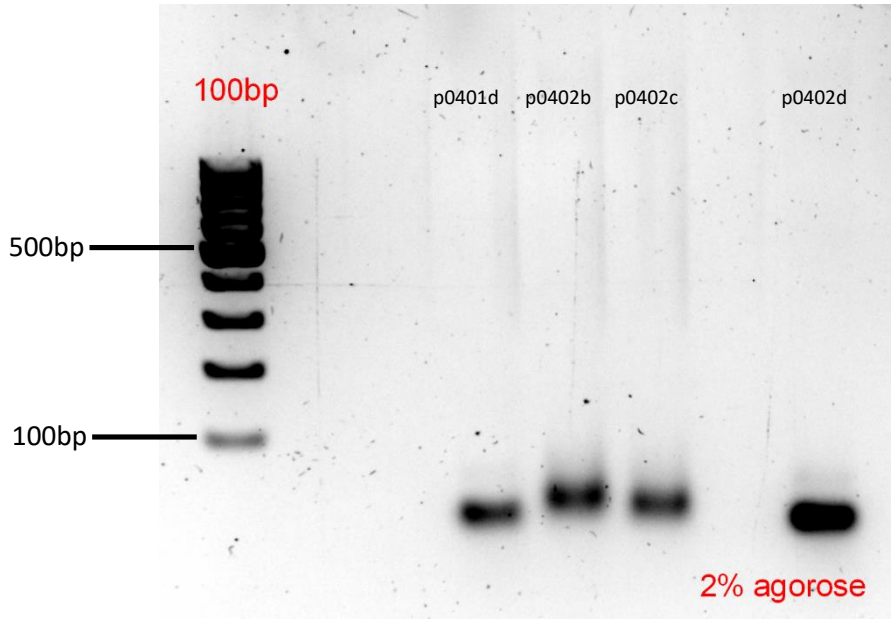


Figure 7 Resizing of samples p040801d, p040802b, p040802c and p040802d with 100bp ladder.

The size of the fragments from *p040801d, p040802b, p040802c* and *p040802d* show variable differences in sizes and there is an indication of a smear that is below 100bp. This implies that the assembly of the oligos have been constructed of repeats consisting of ideal lengths of repeats. The samples will be recreated and transformed for cloning purposes. *p040801b, p040801c, p040802b* were not measured as there was no more product available.

Aim: Assemble new oligo repeats to be used to clone into a vector with Bsa1 sites. The Bsa1 sites will be added to the repeats using primers *o.17.011* and *o.17.012*.

Materials & Methods:

- 10µM 10xCAG Oligo (Tm = 86.8°C)
- 10µM 10xCTG Oligo (Tm = 86.8°C)
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

PCR Protocol p110801 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*35 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 72°C – Annealing (30 Seconds)
4. 68°C – Extension (10 Seconds)
   ----
5. 72°C – Final Extension (5 Minutes)
6. 12°C – HOLD

PCR Protocol p110802 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*35 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 72°C – Annealing (30 Seconds)
4. 68°C – Extension (25 Seconds)
   ----
5. 72°C – Final Extension (5 Minutes)
6. 12°C – HOLD

Reactions for p110801 & p110802

COMPONENT	25µl REACTION	FINAL CONCENTRATION
5x Phusion HF	5µl	1x
10mM dNTPs	0.5µl	200µM
Phusion DNA Polymerase	0.25µl	1.0 units/50µl PCR
10µM 10xCAG Oligo	2µl | 3µl | 4µl | 0µl	<250ng
10µM 10xCTG Oligo	2µl | 3µl | 4µl | 0µl	<250ng
Nuclease-Free Water	15.25 µl | 13.25 µl | 11.25 µl | 19.25 µl	to 25µl

Results & Evaluations:

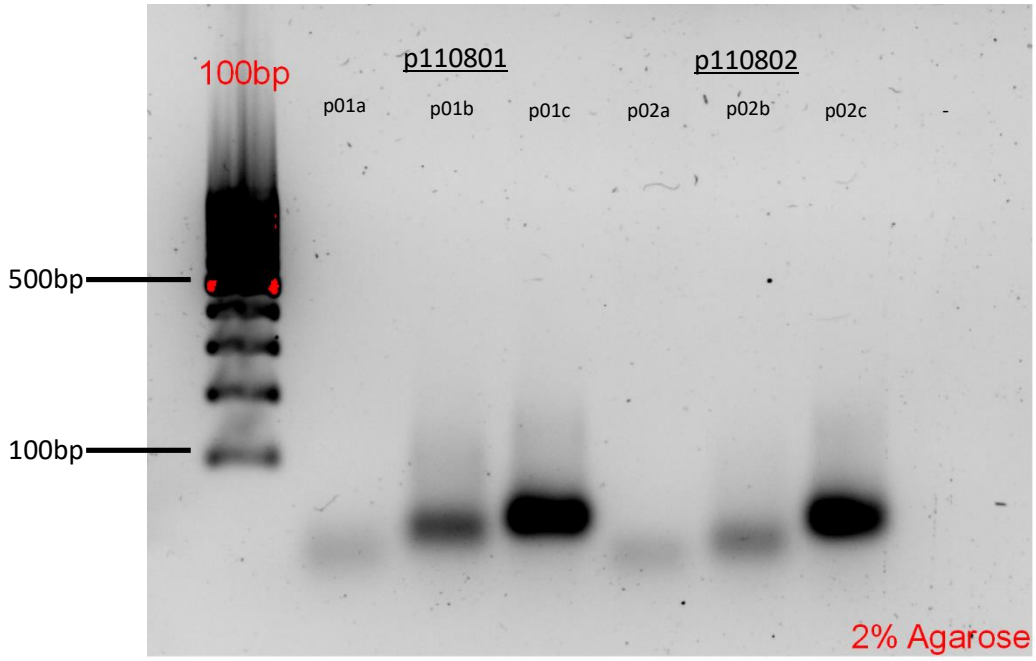


Figure 8 Product of p110801 and p110802. Replication of p070801/2 PCR protocol.

13.08.17

Aim: Bsa1 site need to be added to the assembled repeats for cloning into a vector. Primers have been designed that consist of Bsa1 sites followed by either 5xCAG or 5xCTG, which would amplify the repeats with the addition of the restriction sites. The assembled repeats from *p110801* and *p110802* will be used as the DNA template.

Due to the random annealing of the primers, it is expected that the assembled repeats would be on average smaller upon addition of the Bsa1 sites via the primers.

Materials & Methods:

- 10µM o.17.011 primer (Tm = 73.3°C)
- 10µM o.17.012 primer (Tm = 71.1°C)
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

PCR Protocol p130803 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*35 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 72°C – Annealing (30 Seconds)
4. 72°C – Extension (15 Seconds)
   ----
5. 72°C – Final Extension (5 Minutes)
6. 12°C – HOLD

Reactions for p070801 & p070802

COMPONENT	25µl REACTION	FINAL CONCENTRATION
5x Phusion HF	5µl	1x
10mM dNTPs	0.5µl	200µM
Phusion DNA Polymerase	0.25µl	1.0 units/50µl PCR
p110801a / *p110801b* /		

p110801c / *p110802a* / *p110802b* / *p110802c*	1µl	<250ng
10µM *o.17.011*	1.25µl	0.5µl
10µM *o.17.012*	1.25µl	0.5µl
Nuclease-Free Water	15.75µl / 16.75µl	to 25µl

Results & Evaluations:

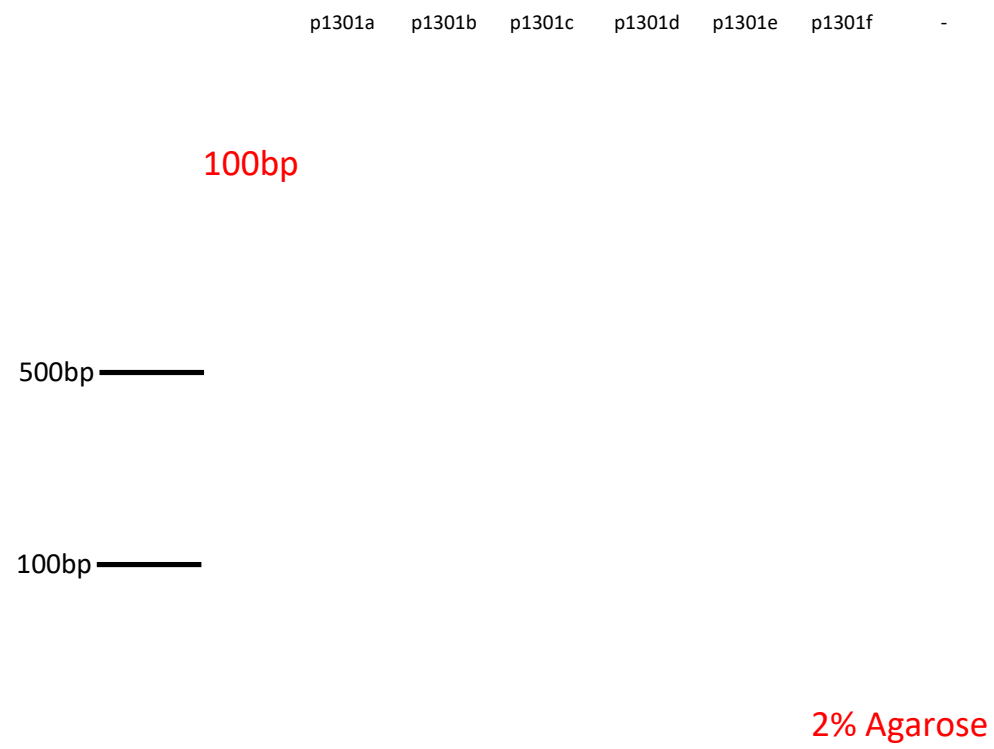


Figure 9 Products of p130801. p1301a, p1301b, p1301c, p1301d, p1301e, p1301f are amplifications of 110801a, 110801b, 110801c, 110802a, 110802b and 110802c respectively.

The product of *p130801* shows an increase in the size of the smears, which was unexpected. The size of the repeats increased considerably and seem to show random annealing. One explanation of the result could be that the Tm calculated for the primers was incorrect since the Bsa1 site should not be considered in the binding region. Secondly, the time of the extension was very long and could be reduced to make smaller fragments.

14.08.17

Aim: Using Bsa1 containing primers, the restriction site will once again be added to the repeats. For the repeat experiment the Tm will be recalculated for the primers and the extension timing will be reduced.

Materials & Methods:

- 10µM o.17.011 primer (Tm = 59.8°C)
- 10µM o.17.012 primer (Tm = 59.8°C)
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

PCR Protocol p130803 (25µl Reactions):

7. 98°C – Initial Denaturation (30 Seconds)
   ----------*30 x Cycles*----------
8. 98°C – Denaturation (30 Seconds)
9. 59.8°C – Annealing (20 Seconds)
10. 72°C – Extension (10 Seconds)
    ----
11. 72°C – Final Extension (5 Minutes)
12. 12°C – HOLD

Reactions for p070801 & p070802

COMPONENT	25µl REACTION	FINAL CONCENTRATION
5x Phusion HF	5µl	1x
10mM dNTPs	0.5µl	200µM
Phusion DNA Polymerase	0.25µl	1.0 units/50µl PCR
p110801a / *p110801b* / *p110801c* / *p110802a* / *p110802b* / *p110802c*	1µl	<250ng
10µM *o.17.011*	1.25µl	0.5µl
10µM *o.17.012*	1.25µl	0.5µl
Nuclease-Free Water	15.75µl / 16.75µl	to 25µl

Results & Evaluations:

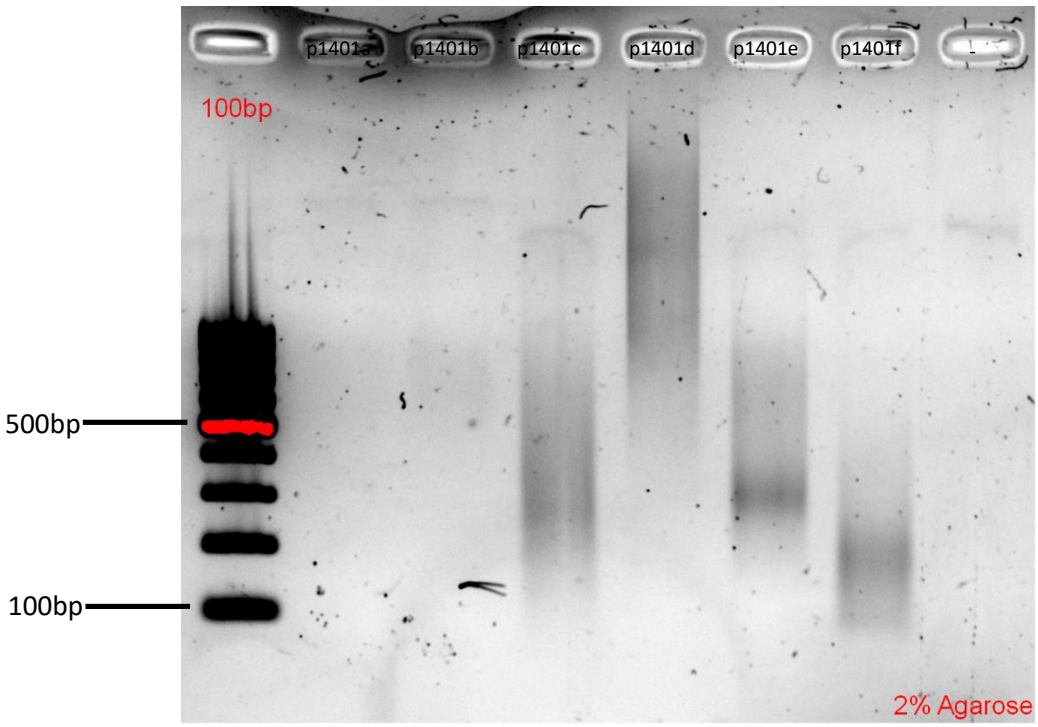


Figure 10 p140801 was a repeat of the protocol used in p130801. The results show a slight decrease in the size of repeats formed but not a considerably large difference.

Following the results of *p140801*, the next step would be to execute a restriction enzyme digest for the *Bsa1* restriction sites. As the products of *p140801* are large treatment with restriction enzymes should break up the longer repeats containing the *Bsa1* site into smaller fragments.

Aim: To treat products of *p140801* with restriction enzymes for the *Bsa1* sites by a fast digest, with the goal of segmenting the large repeats into smaller fragments.

Materials & Methods*:

- Nuclease-free water
- 10X FastDigest Green Buffer
- DNA
- Eco31I

1. Combine the following reaction components at room temperature in the order indicated:

Component	PCR Product
Water	17µl
10x FastDigest Green Buffer	2µl
DNA	10µl (~0.2ng)
FastDigest Enzyme	1µl
Total	30µl

2. Mix gently and spin down.
3. Incubate at 37°C in a heat block or water thermostat for 5 min. Optional: Inactivate the enzyme by heating for 5min at 65°C.
4. Load an aliquot of the reaction mixture directly on a gel.

Results & Evaluations:

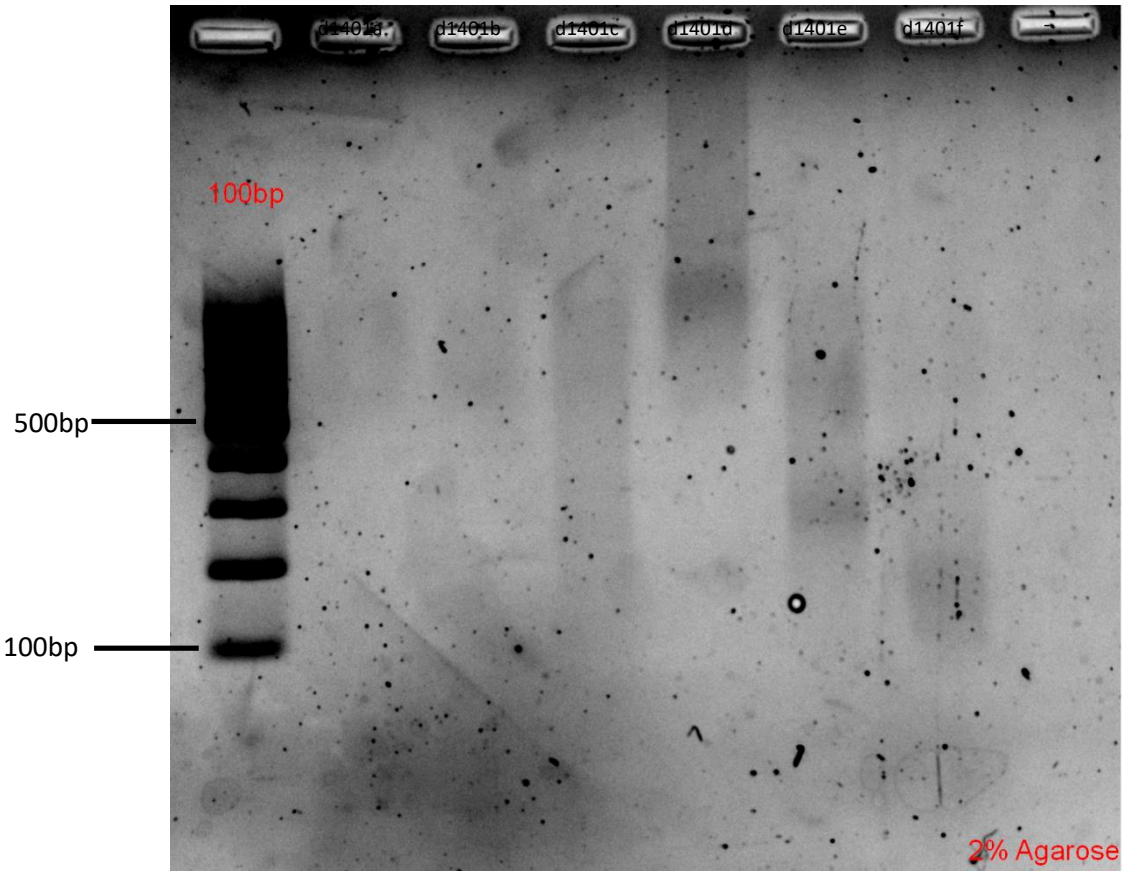


Figure 11 The product of d150801 shows that the digest was unsuccessful

The digest of the products of *p130801* proved to be unsuccessful. The bands in *d140801* are very faint and they show no change in size. The steps will be repeat from *p110801* & *p110802*, although this time a PCR purification step will be done in order to remove any unbound oligos from product, which will hopefully improve the product on the second round of PCR.

15.08.17

Aim: Purify the product of *p110801* & *p110802* to reduce the size of the repeat assembly by removing any unbound oligos. The purified product will undergo a PCR reaction with the *Bsa1* primers.

Materials & Methods:

- Buffer PB
- Buffer PE
- Nuclease-free water
- QIAquick column

PCR Purification Kit

1. Add 5:1 ratio of buffer PB to PCR reaction and mix. If the colour of the mixture is orange or violet, add 10µl 3M sodium acetate, pH 5.0, and mix. The colour of the mixture will turn yellow.
2. Place a QIAquick column in centrifuge a 2ml tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750µl buffer PE to the QIAquick column and centrifuge for 30-60s. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2ml collection tube for 1min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5ml microcentrifuge tube.
7. To elute DNA, add 50µl water (pH7.0-8.5) to the center of the QIAquick membrane and centrifuge the column for 1min.

Results & Evaluations:

Purified Sample	**DNA Conc. (ng/µl)**	**Notes**
pp1101a	3	Noisy signal
pp1101b	5.4	Low peak
pp1101c	7.9	Noisy signal
pp1102a	2.5	Noisy signal
pp1102b	32.5	High contamination
pp1102c	10.1	Slightly noisy signal

The results of the purification were generally very poor. Aside from the high contamination observed in *pp1102b*, all other samples had very low concentrations of DNA. Upon evaluation, the purification kit was most likely the issue for the observed results, as the kit is effective from a minimum of 100bp samples thus a lot of the sample would have been lost in the purification process, explaining the low DNA concentration.

For the next round of PCR with the primers, sample *pp110802c* will be amplified as it had the best concentration of DNA with the lowest contamination.

Aim: PCR amplify *pp110802c* with *Bsa1* primers along at different concentration of 10, 1 and 0.1 ng/µl. Additionally, PCR amplify *PQ18 #1* and *PQ18 #2* (Plasmid DNA containing 18xCAG repeats).

Materials & Methods:

- 10µM o.17.011 primer (Tm = 59.8°C)
- 10µM o.17.012 primer (Tm = 59.8°C)
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

PCR Protocol p150801 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*30 x Cycles*----------
2. 98°C – Denaturation (10 Seconds)
3. 59.8°C – Annealing (10 Seconds)
4. 72°C – Extension (5 Seconds)
   ----
5. 72°C – Final Extension (5 Minutes)
6. 12°C – HOLD

Reactions for p070801 & p070802

COMPONENT	25µl REACTION	FINAL CONCENTRATION
5x Phusion HF	5µl	1x
10mM dNTPs	0.5µl	200µM

Phusion DNA Polymerase	0.25µl	1.0 units/50µl PCR
p150801a / *p150801b* / *p150801c* / *p150802d* / *p150802e*	1µl	<250ng
10µM *o.17.011*	1.25µl	0.5µl
10µM *o.17.012*	1.25µl	0.5µl
Nuclease-Free Water	15.75µl / 16.75µl	to 25µl

Results & Evaluations:

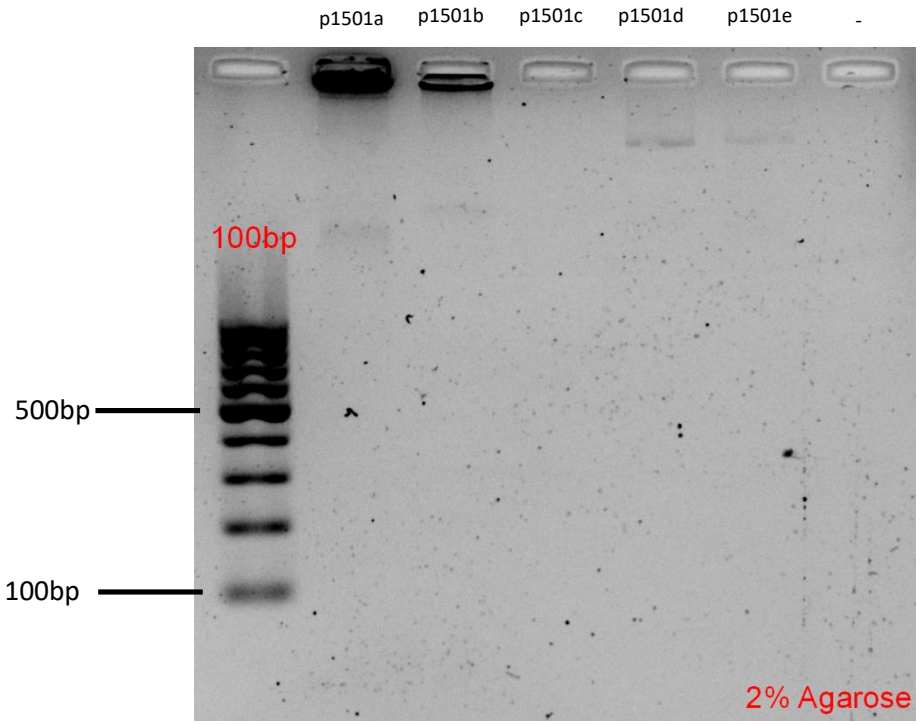


Figure 12 The products of p150801a. The PCR steps were altered to produce smaller fragments, the results show that was not the case.

Products *p1501a* and *p1501b* seem to indicate fragment sizes that are so big they were unable to leave their respective wells. This was unexpected as both the extension time and annealing times were reduced in an attempt to reduce the size of the fragments. A restriction digest of the *Bsa1* sites will be done to try and reduce the sizes of the fragments. Product *p1501c* shows no formation of product. Finally, *p1501d* and *p1501e,* which contain the plasmids *PQ18 #1*

and *PQ18 #2*, show two very large faint bands, which once again was unexpected under these PCR conditions.

Although, the most likely explanation for the large fragment sizes would be that during the purification process only very large fragments remained thus those were repeats that were amplified.

As the results of this experiment are not progressive to assembling repeat under 100bp, the conditions of *p110801* will be executed once again only with the inclusion of both *o.17.011* and *o.17.012* primers.

16.08.17 & 17.08.17

Aim: *Bsa1* digest the products *p150801a* and *p150801b* to see if there is any change to the size of the fragments. Secondly, redo a PCR of *p110801* with the primers included.

Materials & Methods:

- 10µM 10xCAG Oligo (Tm = 86.8°C)
- 10µM 10xCTG Oligo (Tm = 86.8°C)
- 10µM o.17.011 primer (Tm = 59.8°C)
- 10µM o.17.012 primer (Tm = 59.8°C)
- 10mM dNTPs
- Phusion DNA Polymerase
- 5x Phusion HF Buffer
- Nuclease-Free Water

PCR Protocol p160801 (25µl Reactions):

1. 98°C – Initial Denaturation (30 Seconds)
   ----------*35 x Cycles*----------
2. 98°C – Denaturation (30 Seconds)
3. 72°C – Annealing (30 Seconds)
4. 68°C – Extension (10 Seconds)
   ----
5. 72°C – Final Extension (5 Minutes)
6. 12°C – HOLD

Reactions for p160801

COMPONENT	25µl REACTION	FINAL CONCENTRATION
5x Phusion HF	5µl	1x
10mM dNTPs	0.5µl	200µM
Phusion DNA Polymerase	0.25µl	1.0 units/50µl PCR
10µM 10xCAG Oligo	2µl | 3µl | 4µl | 0µl	<250ng
10µM 10xCTG Oligo	2µl | 3µl | 4µl | 0µl	<250ng
10µM *o.17.011*	1.25µl	

10µM *o.17.012*	1.25µl	
Nuclease-Free Water	12.75 µl | 10.75 µl | 8.75 µl | 16.75 µl	to 25µl

Results & Evaluations:

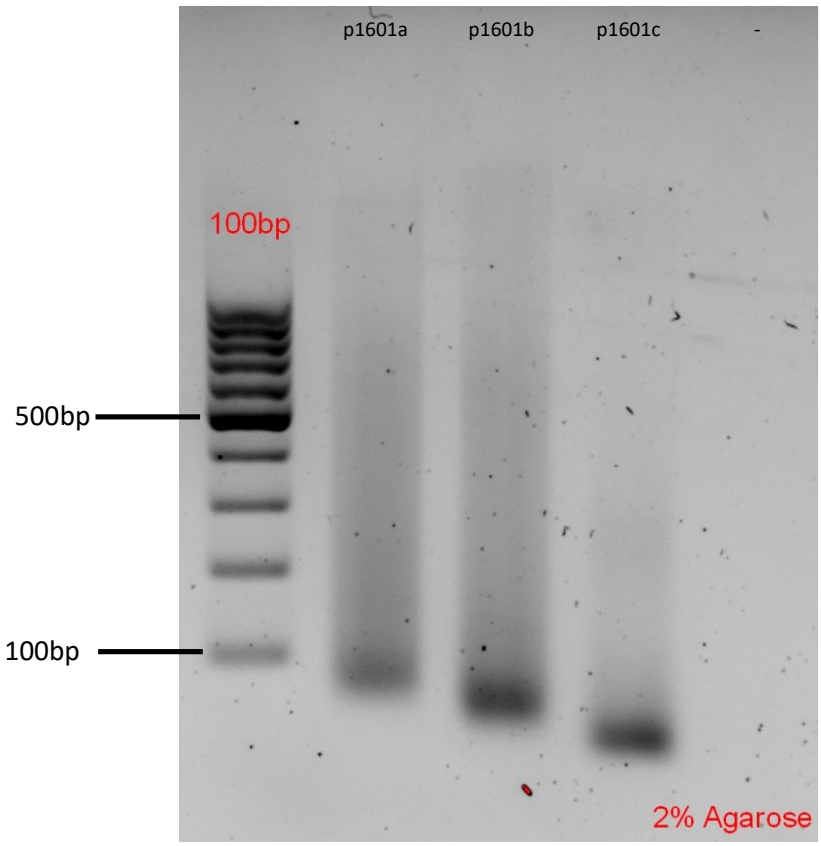


Figure 13 The products of the p110801 PCR conditions with both the different oligos and primers.

The *Bsa1* digest of *p150801a* and *p150801b* showed no fragments (results not shown). Significantly though, the products of *p160801* as seen in *fig. 13* show the amplification of repeats under 100bp. It shows the inclusion of the primers with the oligos provide the ideal conditions for repeat extensions. These samples will be cut using restrictions enzymes and ligated into Ap6 vector.

Aim: To ligate *Bsa1* digested *p1601a, p1601b* and *p1601c* into a Ap6 vector.

Materials & Methods:

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA (4kb)
- Insert DNA (1kb)
- Nuclease-free water

Digestion of *Bsa1* Containing Repeats

1. Combine the following reaction components at room temperature in the order indicated:

Nuclease-free Water	17µl
10x FastDigest Buffer	2µl
DNA (PCR Product)	10µl (~0.2µg)
FastDigest Enzyme (*Eco31I*)	1µl
Total Volume	30µl

2. Mix gently and spin down.
3. Incubate at 37°C for 1 hour
4. Stop reactions by heating at 65°C for 30min.

Digestion of Ap6 Vector

1. Prepare the following reaction mixture:

Plasmid DNA	1µg
10x FastDigest Buffer	2µl
Eco31I Restriction Enzyme	1µl
FastAP Alkaline Phosphatase	1µl
Nuclease-free Water	To 20µl
Total Volume	20µl

2. Mix thoroughly, spin briefly and incubate at 37°C for 10min.
3. Stop reactions by heating at 65°C for 15min.

T4 DNA Ligase Reaction

1. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.

2. Set up the following reaction in a microcentrifuge tube on ice:

Component	**Volume (µl)**
10x T4 DNA Ligase Buffer	2
Vector DNA: 50 ng (0.020 pmol)	1
Insert DNA: 37.5 ng (0.060 pmol)	1
Nuclease-free water	15
T4 DNA Ligase	1
Total	20

3. Gently mix the reaction by pipetting up and down and microfuge briefly.
4. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours.
5. Heat inactivate at 65 degrees C for 10 minutes.
6. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.
   Use 25 uL DH5α cells, and add 2 uL of reaction mixture.

Figure 14 pSB1C3 plasmid map. Assembled repeats containing Bsa1 overhangs were ligated into the plasmid.

Results & Evaluations:

Numerous colonies had appeared on the plates