University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. Student

   Surname: **Tuck** Forename: **Benjamin**

   E-mail address: **2080442t@student.gla.ac.uk**

2. Supervisor:

   Surname: **Adam** Forename: **West**

   E-mail address: **Adam.West@glasgow.ac.uk**

2.1 Research Project Report

2.2 Project Title (maximum 20 words):

Genetic modification of the human AAVS1 locus using CRISPR dual nickase strategies

2.3 Project Lay Summary (copied from application):

Epigenetic mechanisms govern the cell type-specific transcription of genes during development and cellular differentiation. These mechanisms can be used in synthetic biology strategies for regenerative medicine or to restore gene activity in disease.

Recently, a mutant version of an RNA-guided nuclease protein has been developed. This system, known as the CRISPR/Cas9 system, induces a double strand break at the target location. This project aims to use mutant Cas9 proteins which nick only a single strand of target DNA with high potency. We will use dual CRISPR/Cas9 nickases and various guide RNA's to induce double strand breaks at the AAVS1 locus. Successful strategies could be incorporated into future gene therapy strategies

2.4 Start Date: **08/07/15** Finish Date: **31/08/15**

2.5 Original project aims and objectives (100 words max):

The aim and objective of this project was to assess the efficiency of RNA-guided Cas9D10A double nicking at the adeno-associated viral integration site 1 (AAVS1) locus.

Double nicking of DNA on opposite strands will lead to the formation of a double strand break which in turn causes error-prone cellular repair machinery to induce mutations (Fig 1). RFLP analysis will then be used to assess CRISPR-mediated mutagenesis

The results of this study will act as a preliminary investigation into a more specific gene targeting system with little to no off target effects. It provides Adam West's group with important data regarding the efficiency of nickase pairs, which can then be used to make decisions about future projects.

2.6 Methodology: summarise and include reference to training received in research

methods etc.

Five distinct pairs of CRISPR/D10A nickases targeting the AAVS1 locus were constructed from a panel of eight single guide RNA's (See appendix for oligo sequences). Individual top and bottom oligonucleotides were annealed together to give each gRNA, and ligated into their respective linearised expression vectors. Each ligation was transformed into Life Technologies DH5α E.coli cells. Colony PCR using the top oligo as a forward primer and a vector backbone primer as the reverse was performed on transformant colonies. A mini prep (Qiagen) was performed to extract DNA, which was quantified by Nanodrop. Subsequent sequencing confirmed the presence of each protospacer.

The next step was assembly of promoter-gRNA cassettes into tandem inverted repeat units of the pMulti-Cas9D10A-GFP plasmid to form an all-in-one expression vector. The sgRNA constructs were assembled into the desired destination vector using golden gate assembly (Fig 2), and each ligation was transformed into Agilent SURE 2 (Stop Unwanted Rearrangement Events) cells. Due to cell viability issues, DH5α were used instead. Colony PCR with blue-white IPTG/X-Gal screening and suitable primers identified transformant colonies, and the DNA was isolated by mini prep. Triple digests of GGA plasmids was performed to be certain, along with sequencing. Large stocks of plasmid DNA was extracted by midi prep (Qiagen).

Calcium phosphate-mediated DNA precipitation was used to transfect HEK293 cells with either a single guide + WT Cas9, or with Golden Gate Assembled D10A nickase pairs. A Cas9 only, no DNA and WT control were used. Cells were incubated for 48-72 hours, with frequent media changes. The DNA was extracted with the DNEasy DNA extraction Kit (Qiagen) and the transfection efficiency and cell viability was checked with the Attune follow cytometer.

Following DNA extraction, genomic PCR was performed using the HsAAVS1 primer set to give a 458 base pair product. A new primer set to give a 536 base pair product was instead used. Subsequent PCR clean up (Qiagen) and RFLP analysis was performed to assess dual Cas9D10A nickase mutagenesis.

2.7 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

1. Top and bottom oligonucleotides were successfully ligated to form guide RNAs.
2. Annealed oligos were successfully cloned under specific promoters in relevant guide vectors and confirmed by colony PCR and DNA sequencing.
3. Golden gate assembly of ligated plasmids into an all-in-one Cas9 nickase pair vector was successful, which was verified by colony PCR and triple digest (see fig 3). There is MIDI prepped plasmids of nickase pairs available.
4. Transfection of HEK293 was very efficient. The transfection efficiencies were always >70% and levels of GFP were high (See appendix). The transfected cells were also healthy and present in a good amount.
5. Genomic DNA was successfully extracted from HEK293 cells for both single guides, nickase pairs, Cas9 only, WT and no DNA controls and are also available in the lab.
6. Genomic PCR on extracted DNA was eventually successful. Various issues with primer sets lead to testing of many new primer sets and the use of a new primer set (see fig 4).
7. An RFLP analysis was designed using the genomic workshop CLC program.
8. RFLP analysis of single guides and nickase pairs was successful. The StuI digested single guide 2 unfortunately leaked from the agarose gel well, and there was no time for a repeat. All other guides however showed mutagenesis, as did all nickase pairs.

2.8 Discussion (500 words max):

Over the course of two months a great deal was completed in a short space of time. The annealing of the oligonucleotides was straight forward, and guide RNAs were prepared and readily available in stocks within the first few days. Vectors pGuide 6003, pGuide 6004, pGuide 6005, pGuide 4007, and pGuide 4008 were the first to be constructed. Upon first attempt no ligated plasmids were present. Upon second attempt only pGuide 6004 and 6005 showed ligations. We decided to mini prep two of each from the successful ligations, and repeat the experiment from the start for the rest. This time we also planned to anneal and ligate pGuide 1005, pGuide 1006 and pGuide 5005. After colony PCR all plasmids possessed the ligation, and two mini preps of each were prepared.

After the analysis of the sequences, it was determined that pGuide 5005 actually hadn't worked. This meant that we could only assemble pMulti 2005, 2006 and 2008. The assembled plasmid was to be transformed into SURE 2 cells. After transformation, there were no colonies present on the plate. Blue white IPTG/X-Gal screening was used, and still nothing was visible. The transformation was repeated with the same assembly, and the same result ensued. After discussing this issue with the lab, it appears there was a possibility that the SURE 2 cells were not as viable as they should be, and to confirm this, we plated 5µl directly onto an agar plate. Very few colonies grew overnight. It was because of this we decided to transform the golden gate assembly into DH5α cells. Since this protocol only involved multiplex expression of two guides and not four, DH5α cells were possible. After transformation and colony PCR, there were various bands, most at the correct size but some

slippage due to recombination. In order to be sure, we conducted a triple digest with NotI-Hf, AscI and XbaI which showed three bands of the correct size, showing that the golden gate was successful. Upon successful ligation and mini prep of plasmid DNA for pGuide 5005, golden gate assembly to construct pMulti 2007 and 2009 was being carried out, and unfortunately it was unsuccessful. We decided to construct pGuide5005-hH1-1/2-AAVSA64 from the beginning, since it was unsuccessful many times.

The next week, HEK293 cells were transfected with pMulti2005, 2006, 2008, 7 single guides, Cas9 only and WT no DNA. Only 7 guides were transfected instead of 8, as pGuide 5005 was not yet successfully constructed. Beginning week 5 we analysed the transfections, and all were healthy with high transfection efficiencies. Genomic DNA extraction was carried out via the DNEasy kit, and genomic PCR was performed. The genomic PCR was unsuccessful a multitude of times. This genomic PCR used the Herculase fusion set instead of *Taq* polymerase. After each genomic PCR including repeats, there were very large smears present on each agarose gel, with no band in the correct position (see fig 5), which we determined was due to interactions between our Herculase and gel loading dye. By switching to a lab-made 40% glycerol loading dye, the issue was solved and the genomic PCR worked. The first primer set used was showing unanticipated primer dimer bands. We decided to design new primer sets for the genomic PCR, and so after consulting recent literature we decided to use a primer pair designated Hockemeyer_For and Hockemeyer_Rev. Multiple primer sets were tested with the WT genomic DNA, and it was decided that the Hockemeyer set was the most suitable. These primers gave a 536pb product.

By the final week, the genomic PCR was optimised for pMulti2005, 2006 and 2008. pGuide 5005 had successfully been ligated, and pMulti 2007 and 2009 constructed, transfected, extracted and genomic PCR had been performed. The RFLP was then carried out on all pairs and 7 guides, and was mostly successful. The RFLP with the Hockemeyer primer sets was designed with specific restriction enzymes, so that mutant bands could be identified. If a mutant band was present on the gel, this meant that both nickases had cut, and a mutagenesis event had occurred. High intensity of mutant bands would mean that the dual nickases cut with high efficiency.

3. Reflection by the student on the experience and value of the studentship (300 words max):

Working over summer using the CRISPR/Cas9 system has been an invaluable experience. I thoroughly enjoyed my time in Adam's lab and I am extremely grateful for the opportunity, as well as appreciative for the supervision provided by Laura Richmond. By working with CRISPR/Cas9, I feel like I have taken part in the most current and up to date area of genetic and epigenetic research.

Over the course of 2 months I learned a huge variety of new techniques and experimental procedures such as colony and genomic PCR, RFLP analysis and more. I also learned many helpful tips from Laura. As well as this, I received valuable practice in methods which I was also familiar with, which has increased my understanding and will further help me in my future studies.

Another aspect which I thoroughly enjoyed was how stimulating the content of my project was. Epigenetics is a fascinating field, the background reading of scientific literature and relevant books was very interesting, as was the bi-weekly journal club meetings I was invited to attend within the lab. When any undesired results occurred, I had to think theoretically and creatively to identify and prevent the result recurring upon repeat. Unexpected results due to unforeseen factors was perhaps the most unanticipated aspect of my project.

Overall, I think choosing to apply for a placement was an excellent choice. I greatly enjoyed my time in Adam's lab, and I know this choice will benefit me hugely in the future.

5. Dissemination: (note any presentations/publications submitted/planned from the work):

6. Signatures:

Supervisor Date Student  Date **09/09/15**

Adam West
2015.09.14
09:08:17 +01'00'

Appendix

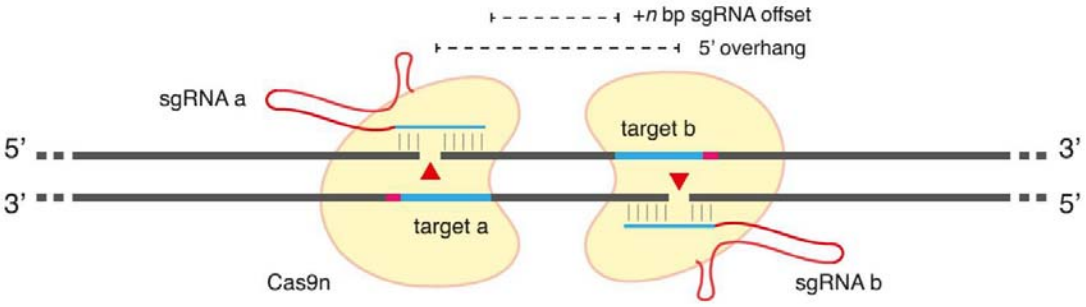


Figure 1: General overview of dual nickase strategies. A Cas9 D10A nickase (Cas9n) is guided to its target sequence via single guide RNAs. The mutant protein can only nick sequences complimentary to its sgRNA. A double strand break is generated. From (Ran et al., 2013)

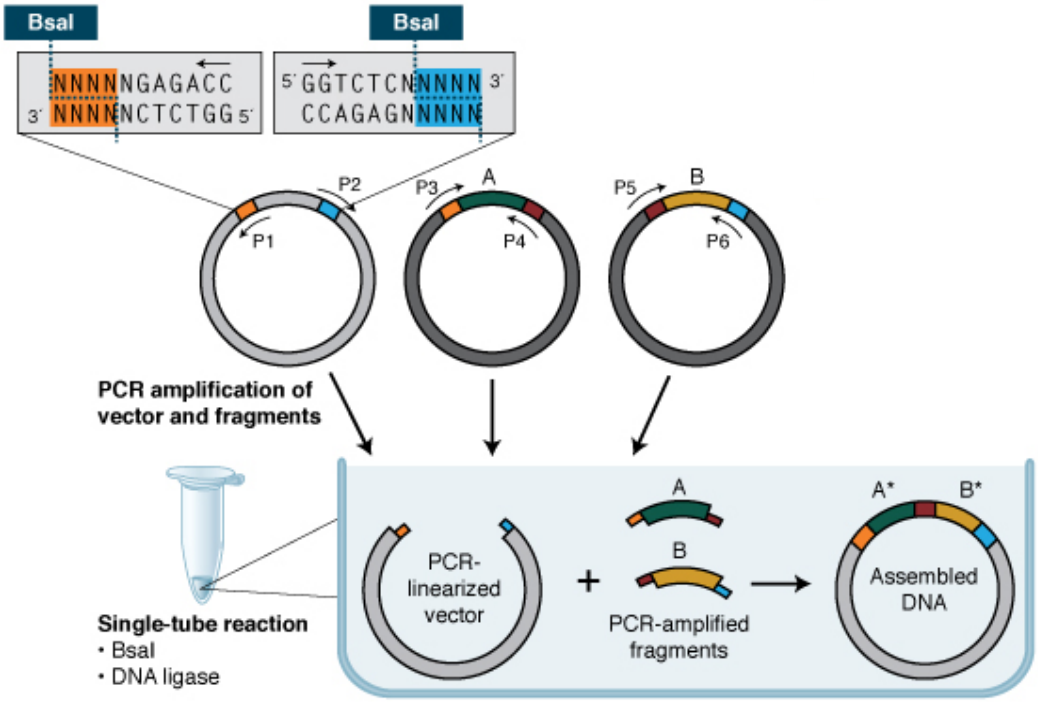
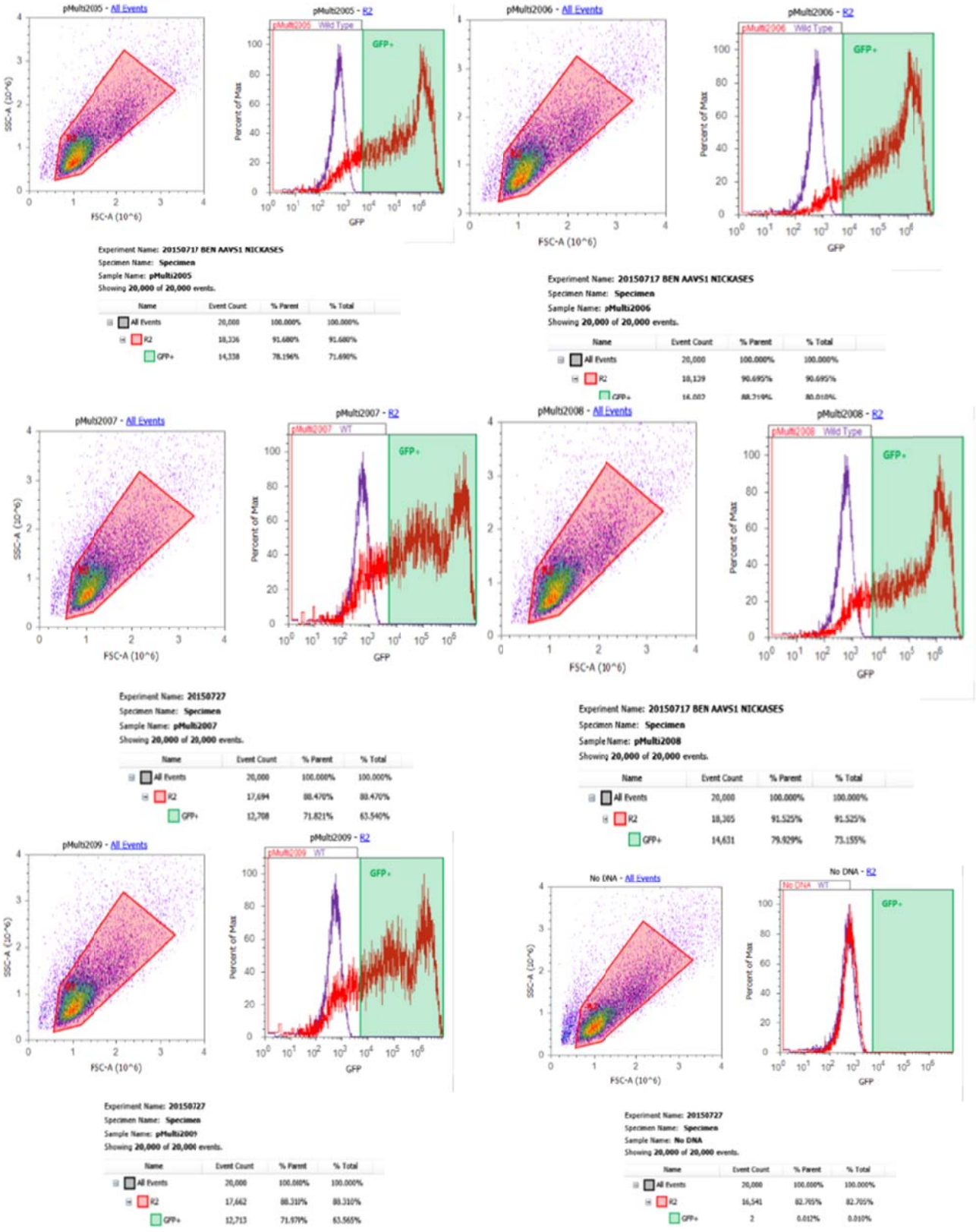


Figure 2: New England Biolab's (NEB) graphic overview of the golden gate assembly strategy. Desired fragments are isolated using specific restriction endonucleases and PCR amplified. The PCR products are then assembled in tandem in a linearised vector and ligated simultaneously. Golden gate assembly allows for the generation of multigene constructs.



Flow cytometry values for the five nickase pairs pMulti2005-pMulti2009. The GFP+ value denotes the transfection efficiency. It can be seen that the lowest transfection efficiency of the nickase pairs was ~71%, whereas the highest value was ~88%. High amounts of cell health and viability is denoted by an intense scatter in the lower right of the graph, which all transfections show. As well as this, the no DNA control shows no GFP+ which proves that proper aseptic technique was used. pMulti2008 and 2006 show a possibility of two distinct transfected populations co-existing within the transfection media, due to the existence of two defined peaks in the GFP+ column.

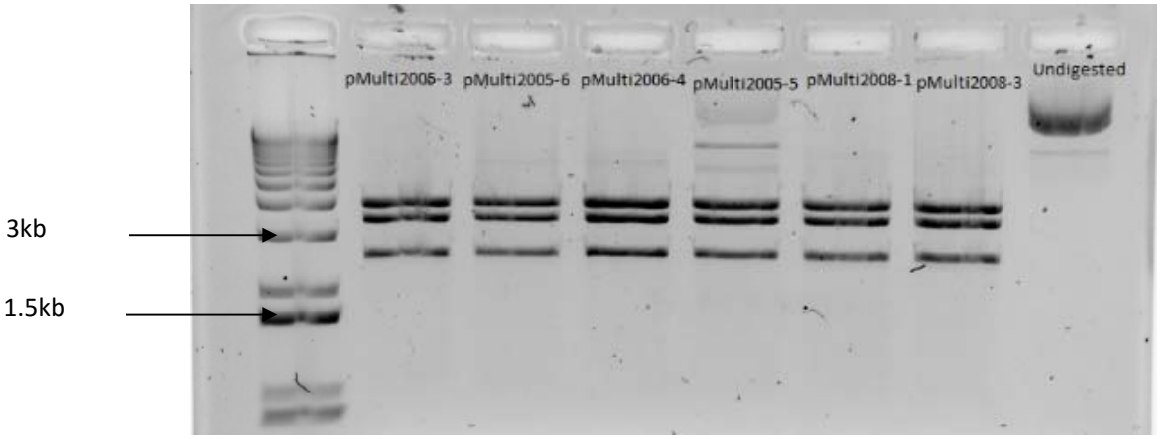


Figure 3: Triple restriction digest of pMulti Nickase pairs with a 1KB+ ladder on a 0.8% agarose gel to verify assembly. Presence of three identical bands at the same size shows that each golden gate assembly with DH5α cells was successful. pMulti2005-5 shows some undigested plasmid. The presence of these three bands shows that golden gate assembly can be used with cells which are prone to recombination.

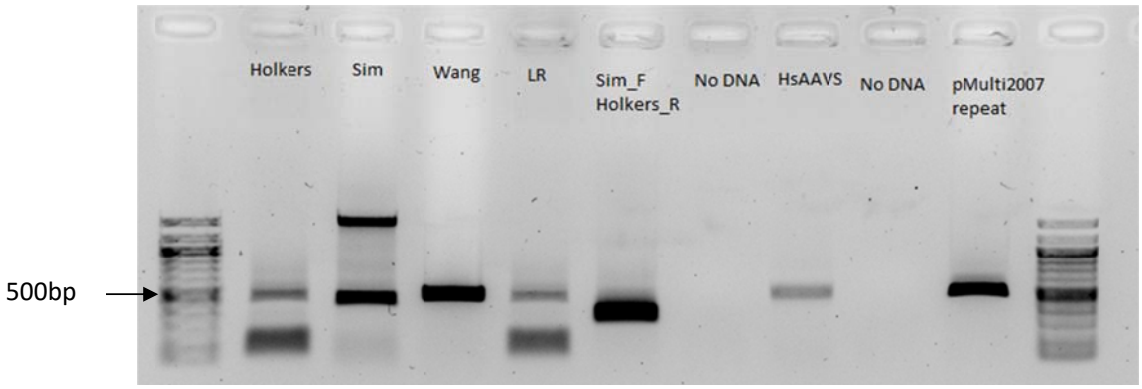


Figure 4: Various primer sets after genomic PCR of WT DNA on a 1% agarose gel using a 100bp ladder. The primers anneal to specific sequences and amplify the region in-between. The pMulti2007 repeat shows nickase pair 3 which used the Hockemeyer set. The image shows that Hockemeyer and Wang both worked very well, meaning the experiment could use the Hockemeyer or Wang set.

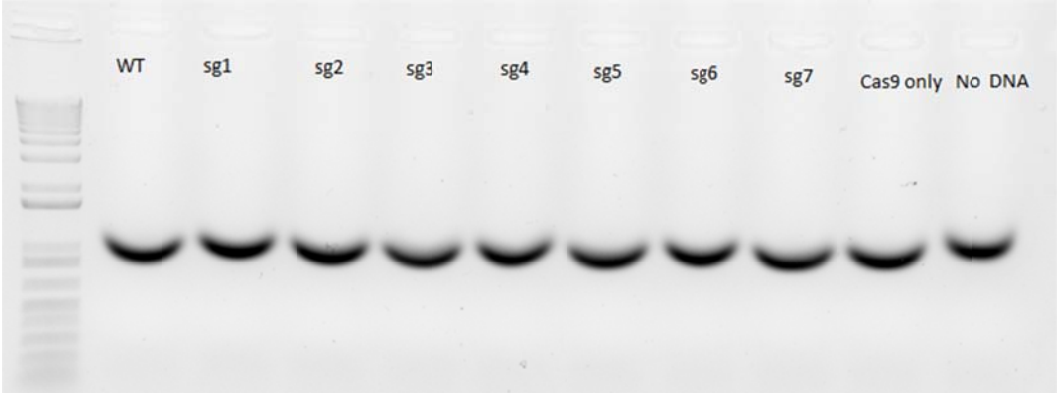


Figure 5: An example of the "smiling" band present when using purple loading dye and performing a genomic PCR on a 1% agarose gel. This gel shows a genomic PCR of single guides 1-7, with a Cas9 only and DNA only control, with a 1kb ladder. The fact that the band appears in the no DNA control implies that contamination is the most likely explanation.

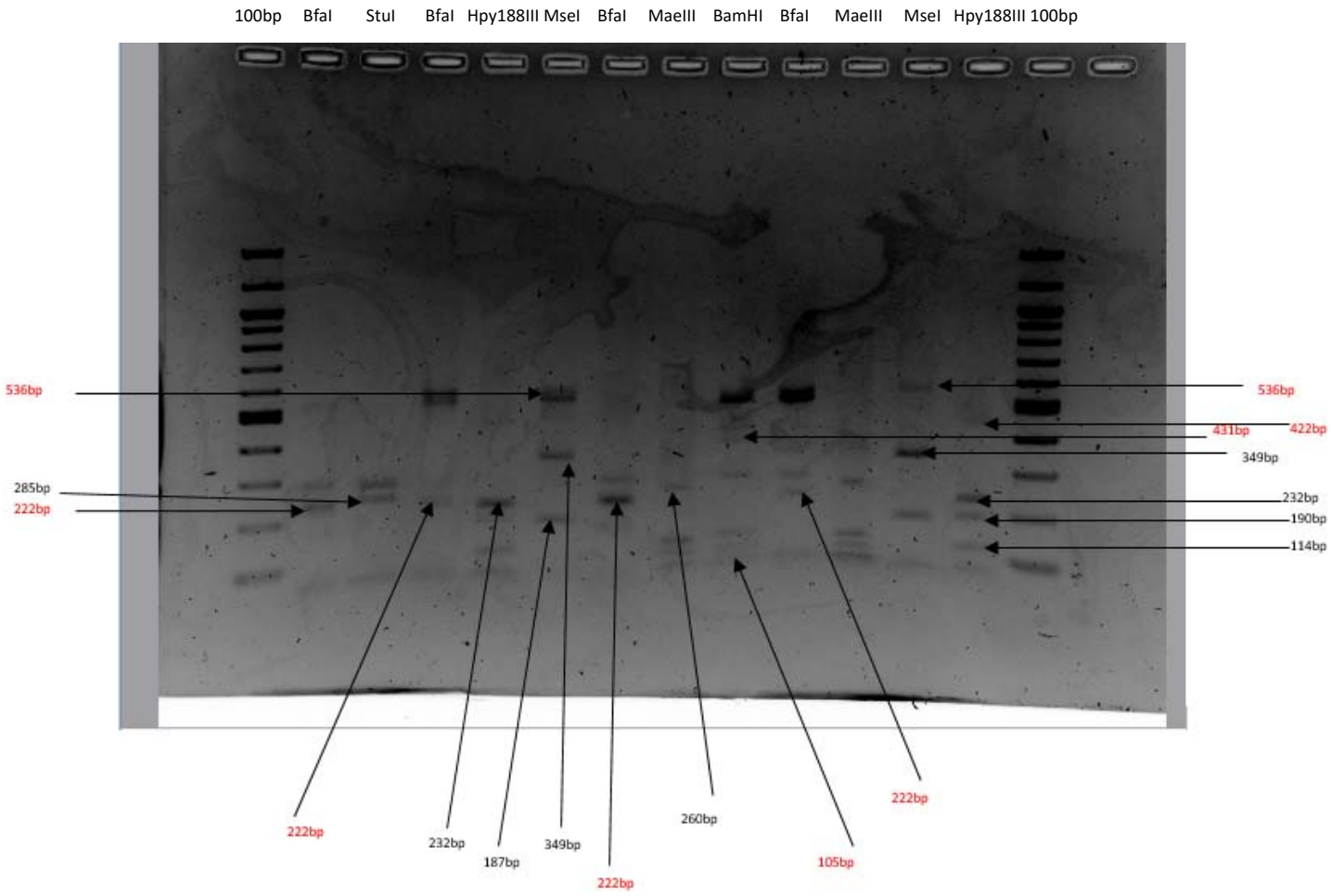


Figure 6: Restriction fragment length polymorphism (RFLP) analysis of pMulti nickase pairs. Red annotations show bands which are expected when mutagenesis has occurred and the DNA has been digested with a specific enzyme. Black annotations show bands which are expected in the wild type and no mutagenesis has occurred. The image shows that there has been a varying amount of mutagenesis occurring for each nickase pair. Presence of mutant bands shows that the dual nickase D10A proteins have simultaneously cut and induced a double strand break at the target location. Band intensity roughly corresponds to mutation efficiency. As well as this, transfection efficiency was ~70%, so there will always be some wild type banding present.

The image shows that pMulti2005 shows a mutagenesis rate of ~50%. pMulti2006 only shows one very faint mutant band when digested with BfaI. This shows that some mutagenesis has occurred, and either the Hpy188III site has been knocked out or the enzyme hasn't cut.

pMulti2007 shows an intense mutant band of 222bp when digested with BfaI. This shows a high level of dual nickase cutting. There is high amounts of the mutant 536pb band when digested with MseI. There is no band present when digested with MaeIII, suggesting that, like pMulti2006, the restriction site may have been knocked out.

pMulti2008 shows very faint mutant bands when digested with BamHI and BfaI. Similarly to pMulti2007, there are no mutant bands present when digested with MaeIII suggesting an enzyme issue.

pMulti2009 shows a faint mutant band of 536bp when digested with MseI, and a very faint band when digested with Hpy188III of 422bp.

Overall, all nickase pairs show some level of mutagenesis which is a very good result.

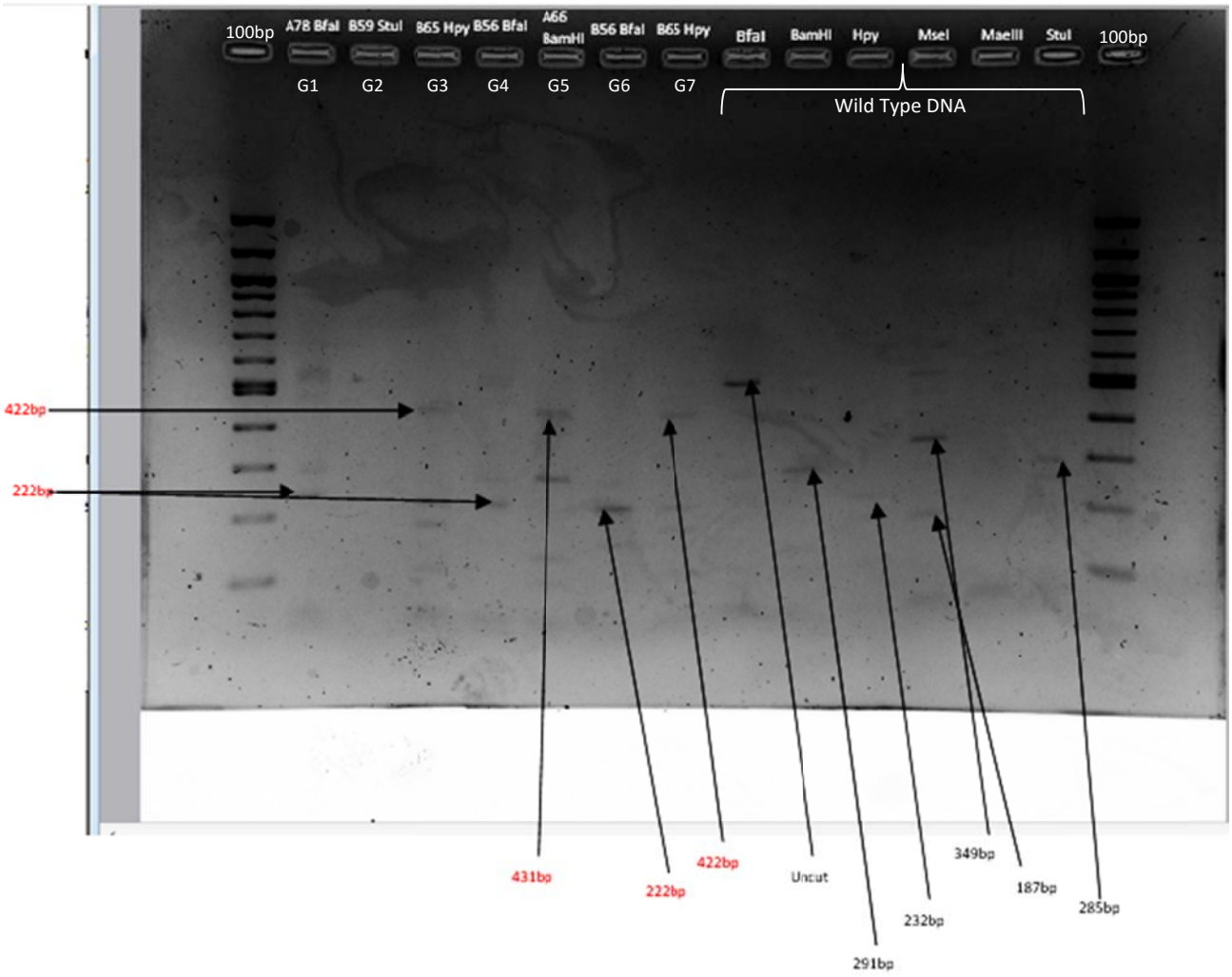


Figure 7: RFLP analysis of single guides on a 2% agarose gel with a 100bp ladder. Red annotations again show mutant bands and black show wild type bands. Single guides 1-7 were digested after transfection. WT DNA was also digested with each enzyme. The image shows that all single guides induced mutagenesis, although the bands are faint. Guide 2, digested with StuI however shows no bands. This is due human error when applying the DNA to the well. Not enough loading dye was used, and the DNA floated out of the well. The same situation occurred in the MaeIII WT lane unfortunately.

Oligonucleotides

- HsAAVS-A78-mU6T: TTGTTTGTCACCAATCCTGTCCCTAG
- HsAAVS-A78-mU6B: AAACCTAGGGACAGGATTGGTGACAA
- HsAAVS-B59-hU6T: CACCGAAACCCCCACCACGG
- HsAAVS-B59-hU6B: AAACCCGTGGTGGGGGTTTTC
- HsAAVS-B65-hU6T: CACCGCTTCCTAGTCTCCTGATATT
- HsAAVS-B65-hU6B: AAACAATATCAGGAGACTAGGAAGC
- HsAAVS-A64-hH1T: TCCCAGAACCAGAGCCACATTAAC
- HsAAVS-A64-hH1B: AAACGTTAATGTGGCTCTGGTTCT
- HsAAVS-B56-h7SKT: CCTCGGGGCCACTAGGGACAGGAT
- HsAAVS-B56-h7SKB: AAACATCCTGTCCCTAGTGGCCCC
- HsAAVS-A66-mU6T: TTGTTTGGTCCCAGCTCGGGGACAC
- HsAAVS-A66-mU6B: AAACGTGTCCCCGAGCTGGGACCAA
- HsAAVS-B56-hU6T: CACCGGGGCCACTAGGGACAGGAT
- HsAAVS-B56-hU6B: AAACATCCTGTCCCTAGTGGCCCC
- HsAAVS-B65-h7SKT: CCTCGCTTCCTAGTCTCCTGATATT
- HsAAVS-B65-h7SKB: AAACAATATCAGGAGACTAGGAAGC

References

- Biolabs, N. (2015). *Golden Gate Assembly | NEB*. [online] Neb.com. Available at: https://www.neb.com/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/golden-gate-assembly [Accessed 9 Sep. 2015].
- Ran, F., Hsu, P., Lin, C., Gootenberg, J., Konermann, S., Trevino, A., Scott, D., Inoue, A., Matoba, S., Zhang, Y. and Zhang, F. (2013). Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. *Cell*, 155(2), pp.479-480.