NTNU

Kunnskap for en bedre verden

Using the QuickChange method to introduce mutations into *algL* whilst adding a restriction site for *Bpm*I

TBT4146 Molecular Genetics

(Department of Biotechnology and Food Science)

Norwegian University of Science and Technology

Trondheim, November 22, 2021

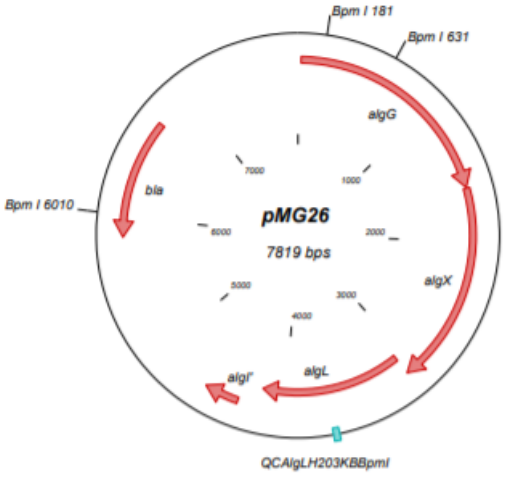

Figure 1: Plasmid map of pMG26. The resistance marker bla (Amp^r), the algL gene that is going to be mutagenized, and the location of primer pair QCAlgLH203KBBpmI and QCAlgLH203KBpmI2 are depicted (1).

Authors:
Lukas Meyvisch,
Robynn Vancompernolle,
Anke Vanden Berghe,
Marie Verdonck,
Haroon Zafar

Group 2 - Week 45

Abstract

This experiment focused on site-directed mutagenesis using the QuickChange method, after which the bacterium *Escherichia coli* (*E. coli*) was transformed. Mutations that replace a histidine (H203) with a lysine (K203) were introduced into the *algL* gene from *Pseudomonas fluorescens*, contained in the pMG26 plasmid. This gene codes for the enzyme alginate lyase (AlgL) that cleaves alginate, a polysaccharide.

Firstly, plasmid DNA pMG26 was isolated from *E. coli* with SV Miniprep. Afterwards, the QuickChange method, a temperature cycling technique, was performed using primers not entirely complementary to the plasmid DNA. The aim was to introduce site-directed mutations. This technique results in the creation of a large amount of single stranded DNA (ssDNA) with mutations combined with wild type strands. Because *E. coli* methylates its DNA, the wild type and mutated strands could be differentiated and the wild type strands removed using an enzyme (*Dnp*I) that digests methylated DNA.

Then, these plasmids were transformed into supercompetent *E. coli* DH5α. These cells repair the nicks and replicate the mutated plasmids. The bacteria were plated out on two plates with ampicillin and then incubated overnight. On both plates, growth was observed as was expected.

The plasmids in the cell cultures were then isolated using SV Miniprep and the *Bpm*I restriction enzyme. The restriction mix was then incubated overnight before running it on an agarose gel. The aim was to determine which of the samples had the desired mutation. A new restriction site was successfully introduced into the biggest fragment.

The results of this experiment overall were good. This methodology taught us some very useful information to apply in future experiments.

Index

1. Theory/Principles

Site-Directed mutagenesis

Site-directed mutagenesis (SDM) created one or several mutations in a specific target site of a double-stranded DNA molecule. This *in vitro* procedure uses customized primers that are designed to confer specific mutations. Different methods are available. The Phusion SDM and GeneArt system require modifications or unique strains to introduce mutations. Other methods use inverse PCR with standard primers. In QuikChange, the primer is designed to have an overlapping orientation while Q5 SDM uses back-to-back orientation (2).

Site-directed mutagenesis has been proven very useful in genetic studies and gene technology. It can be used to perform precise experiments with one nucleobase precision (3). These experiments are used to determine gene structure and function. A good understanding of these gene properties is essential in the development of, for example, a cure for genetic diseases. Site-directed mutagenesis is also used in protein engineering. Proteins, including enzymes and antigens, with specific properties can be designed.

algL gene in the pMG26 plasmid

During the experiment a mutation was introduced in the *algL* gene, found in plasmid pMG26 in the bacterium *Pseudomonas fluorescens.* This gene, *algL*, encodes for an enzyme called alginate lyase which catalyzes the cleavage of alginate polysaccharide. Each *algL* gene contains a conserved amino acid sequence. Through site-directed mutation, the first mutation is introduced in the histidine codon in the conserved domain which changes to lysine whereas the second mutation accounts for the insertion of guanine in place of adenine which introduces a new restriction site for *Bpm*I-restriction site. The mutations can be seen in Figure 5 of the Appendix. Because of the introduction of new restriction sites it makes it easier to check whether or not the mutagenesis was successful. This is done by samperating fragments on gel electrophoresis as the introduction of a new restriction site produces more and smaller sized fragments as compared to the unmutated control.

QuickChange Method

QuickChange Method is a way of introducing one or more-point mutations into the sequence of target DNA. This method follows the same principle as Polymerase Chain Reaction (PCR)

with the only difference of not using a fully complementary primer. Hence, when DNA polymerase elongates, it creates a piece of DNA that contains one original strand and one strand with mutation. PCR is a common laboratory technique that is used to make millions or billions of copies of a particular piece of DNA. There are 3 basic steps of PCR reaction (4):

1. **Denaturation**: For the separation of the double-stranded DNA into a single-stranded product, high reaction temperatures are needed. The single-stranded DNA is used as a template in the next step, annealing.
2. **Annealing**: In this step, primers bind to the complementary sequences in the single-stranded template DNA. The reaction temperature has to be lowered for annealing to occur.
3. **Extension**: In this step, the temperature is increased, as the enzyme *Taq* polymerase has a high optimal working temperature. This enzyme extends the primers and synthesizes the new strands of DNA.

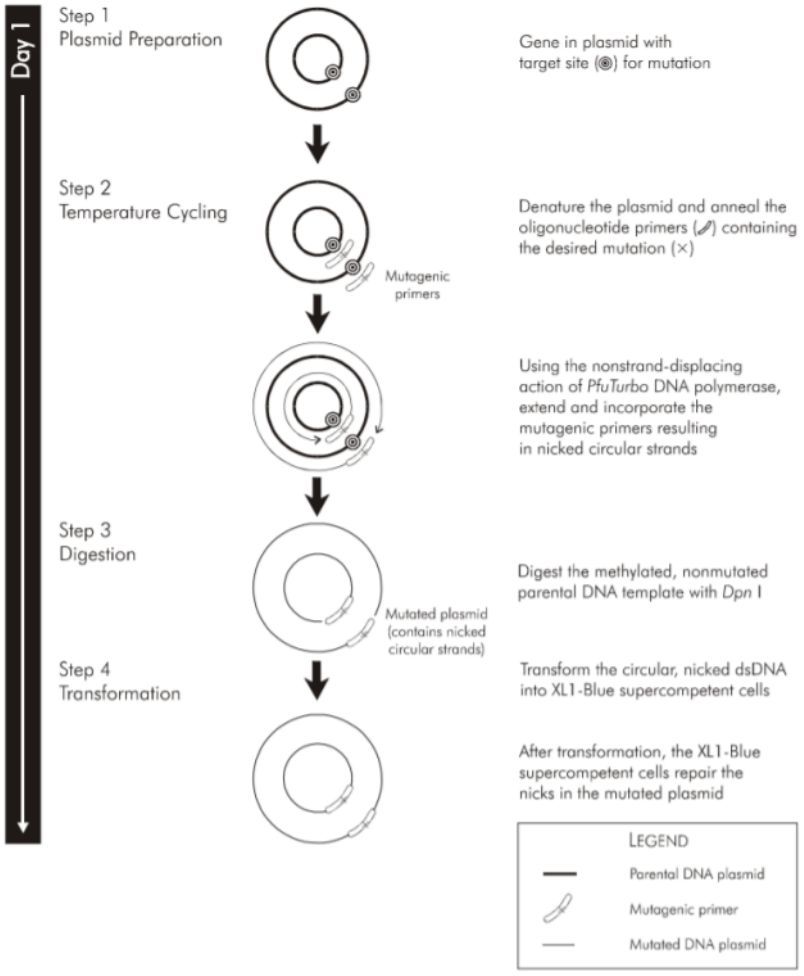

Figure 2: Overview of site-directed mutagenesis by the QuikChange method (1).

This process continues in a cyclic manner until a reasonable amount of DNA with the desired mutation is achieved. Unmutated DNA is separated from mutated DNA by treating it with the restriction enzymes that specifically cleaves the unmutated methylated DNA, thus leaving the desired mutated DNA intact.

Transformation

Transformation refers to the process by which foreign DNA is introduced into a cell (5). Transformation of bacterial cells with plasmid is important not only for studies in bacteria but also because bacteria are used as the means for both storing and replicating plasmids. Through genetic modifications, researchers have created several bacterial strains that can be more easily transformed and that will help to maintain the plasmid without rearrangement of the plasmid DNA. Additionally, specific treatments have been discovered that increase the transformation efficiency and make bacteria more susceptible to either chemical or electrical-based transformation, generating what is commonly referred to as 'competent cells'.

It is generally believed that after transformation the bacteria that make colonies should all contain a plasmid (which provides antibiotic resistance). However, it's not necessarily the case that all of the plasmid-containing colonies will have the *same* plasmid. While cutting and pasting DNA, it is often possible to get the formation of side products. For example, while inserting a gene into a plasmid using a particular restriction enzyme, we may get some cases where the plasmid closes back up (without taking in the gene), and other cases where the gene goes in backward. In some cases, it doesn't matter where the genes are adding up on the plasmid. However, if we want the inserted gene to express or to make proteins, it is important that genes must be inserted in the right direction relative to the promoter, or the control region which drives the gene expression. On the contrary, if the genes are inserted backward, a wrong strand of the DNA would be transcribed and there would be no expression of a desirable protein, therefore, it is important to collect plasmid DNA from each colony and check to see if it matches the plasmid we were trying to build. Restriction digests, PCR, and DNA sequencing are commonly used to analyze plasmid DNA from bacterial colonies.

Gel Electrophoresis

Gel electrophoresis is a technique used to separate the DNA fragments and other macromolecules like RNA and proteins on the basis of their size and charge (6). In principle, an electric pulse is transmitted through the gel which is loaded with the molecule of interest (DNA). On the basis of their size and charge, these molecules will travel through the gel in different directions at different speeds which allow them to get separated from one another. However, in the case of DNA, all molecules have the same amount of charge per mass, therefore, the separation of different fragments of DNA happens only on the basis of the differences in their sizes. For example, circular pieces of DNA run differently on the gel as compared to linear pieces of DNA. For the separation of DNA fragments, gels are often made of agarose. Smaller molecules travel a longer distance.

When a gel is stained with a DNA-binding dye and placed under UV light, the DNA fragments glow which allows detection of DNA present at different locations in the gel.

As plasmid pMG26 has three *Bpm*I-restriction sites, we expect three bands on the gel. Each band should appear at a different height as the fragments have different molecular sizes (Appendix). For the wild-type plasmid one band around 0.5 kb, one around 2 kb, and 5 kb.

2. Materials and methods

The materials and methods were used and carried out as described in the lab compendium (1). With the exception that on day 4, to isolate the plasmid DNA we spinned down the three cell cultures before following the instructions on pages 7-8 of the lab compendium.

3. Results

The QuickChange method was used to introduce a point mutation in pMG26 plasmid. This mutated plasmid was then transformed into *E. coli* cells. Two cell suspensions were plated out on an LA+Amp plate. The first cell suspension resulted in bigger but fewer colonies than the second suspension. The difference between the suspensions is that for the second plate, the cells were centrifuged again and the supernatant was removed before plating out the cells. This result was expected as the cell concentration was increased.

Both wild-type plasmids and mutant plasmids were digested by restriction enzyme *Bpm*I after which the fragments were loaded onto an agarose gel to separate the DNA fragments by molecular size (Figure 3).

As plasmid pMG26 has three restriction sites, we expect three bands on the gel. Each band should appear at a different height as the fragments have different molecular sizes (Appendix). For the wild-type plasmid one band around 0.5 kb, one around 2 kb, and 5 kb. In Figure 3, three bands appear for the wild-type plasmid. One band is visible around 8 kb, one at 2 kb, and one at around 0.5 kb. The latter, however, is barely visible on the figure.

For the colonies with mutant plasmids, four bands appear on the gel. Out of the four bands, three bands appear between 2 and 5.5 kb while the fourth fragment has a much smaller size of around 500 bp. This confirms that the new restriction site was introduced in the biggest fragment.

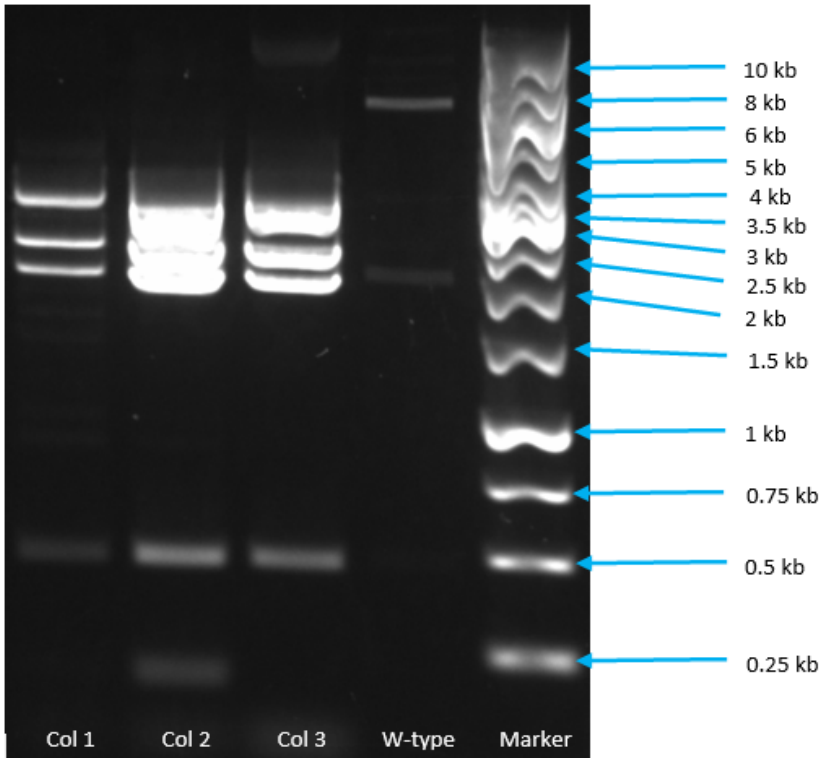

Figure 3: Picture of the gel containing the DNA Ladder (Right), Wild-type plasmid digestion mix (2nd from right) and mutant digestion mix of 3 colonies. In Col 1,2 and 3, first the point change mutations were inserted in the pMG26 plasmid which was later used to transform E. coli cells. Successfully transformed cells were digested by restriction enzyme Bpml and reaction mixture was loaded on agarose gel to confirm the success of mutation by comparing it with wild type.'GeneRuler™ 1kb DNA ladder' was used to assign correct values to the ladder.

4. Discussion

Using the QuickChange method, we introduced a point mutation in the conserved region of the *algL* gene as well as a new restriction site for *Bpm*I enzyme through mutation in the *algL* gene of the plasmid pMG26. The sole purpose of this additional restriction site is to allow the detection of mutated DNA. In the wild-type strain, plasmid pMG26 contains three restriction sites. Thus, digestion by restriction enzyme *Bmp*I gives three fragments. One of 0.5 kb, 2 kb, and 5 kb in length.

In our experiment, we observed three fragments in the wild type ladder that are roughly around the sizes of the 8 kb and 2 kb, and 0.5 kb. This last one was barely visible. A reason for this could be that the amount of fragments of that particular size just wasn't large enough to have a good visibility on the gel. The highest band is visible at 8 kb instead of the expected value of 5 kb. The DNA probably occurred in the nicked form, which is the slowest migrating form, instead of the linear form (7).

In the mutated plasmid, the bigger fragment of 5 kb is cut into two smaller fragments. Four bands are visible in the gel. This is in accordance with the expectations and confirms the mutations were effectively introduced in the plasmid DNA.

The procedure of introducing point mutations is useful for introducing a new restriction site. Sequences that are close to palindromic can be changed into restriction sites by applying minor changes.

Conclusion

Through the QuickChange method, point mutations were successfully introduced in the *algL* gene in the pMG26 plasmid originating from *Pseudomonas fluorescens*. The results of the gel electrophoresis confirm the introduction of the desired mutations. In the control, the unmutated plasmid, three bands appear. In the transformed plasmids, four bands appear. This not only proves the successful mutagenesis of *algL* genes but also shows that the transformation of *E.coli* cells was successful/effective.

In general, this experiment yielded good results. Almost everything went as expected. The only exception was an unanticipated size of one fragment we ran through the gel. We expected a band at 5 kb for the wild-type plasmid. This band however appeared way higher on the gel, at 8 kb. The DNA, therefore, likely occurred in the nicked form, instead of the linear form. By performing this experiment, we gained more experience using the QuickChange method to introduce site-directed mutations. This technique proves to be very useful in future experiments where we want to, for example, generate restriction sites.

References

1. Course compendium (2021), *Laboratory course in TBT4146 Molecular genetics,* NTNU, Trondheim

2. Kunkel, T.A., Roberts, J.D., Zakour, R.A. (1989), Rapid and Efficient Site-Specific Mutagenesis without Phenotypic Selection, *Recombinant DNA Methodology*, p.587–601.

3. Sutipatanasomboon, A. (2021), *Site-Directed Mutagenesis: Methods and Applications* [online]. Source: Conduct Science. Available at: https://conductscience.com/site-directed-mutagenesis-methods-and-applications/ [Accessed 22 Nov 2021]

4. s.n. (s.d.), *Polymerase chain reaction (PCR)* [online]. Source: Khan Academy. Available at: https://www.khanacademy.org/science/ap-biology/gene-expression-and-regulation/biotechnology/a/polymerase-chain-reaction-pcr [Accessed 20 2021 November 2021]

5. s.n. (s.d.), *Bacterial transformation & selection* [online]. Source: Khan Academy. Available at: https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-cloning-tutorial/a/bacterial-transformation-selection [Accessed 20 November 2021]

6. s.n. (s.d.), *Gel electrophoresis* [online]. Source: Khan Academy. Available at: https://www.khanacademy.org/science/ap-biology/gene-expression-and-regulation/biotechnology/a/gel-electrophoresis [Accessed 20 November 2021]

7. Tirabassi, R. (2014), *How to Identify Supercoils, Nicks and Circles in DNA Plasmid Preps* [online]. Source: BiteSizeBio. Available at: https://bitesizebio.com/13524/how-to-identify-supercoils-nicks-and-circles-in-plasmid-preps/ [Accessed 20 November 2021]

Appendix

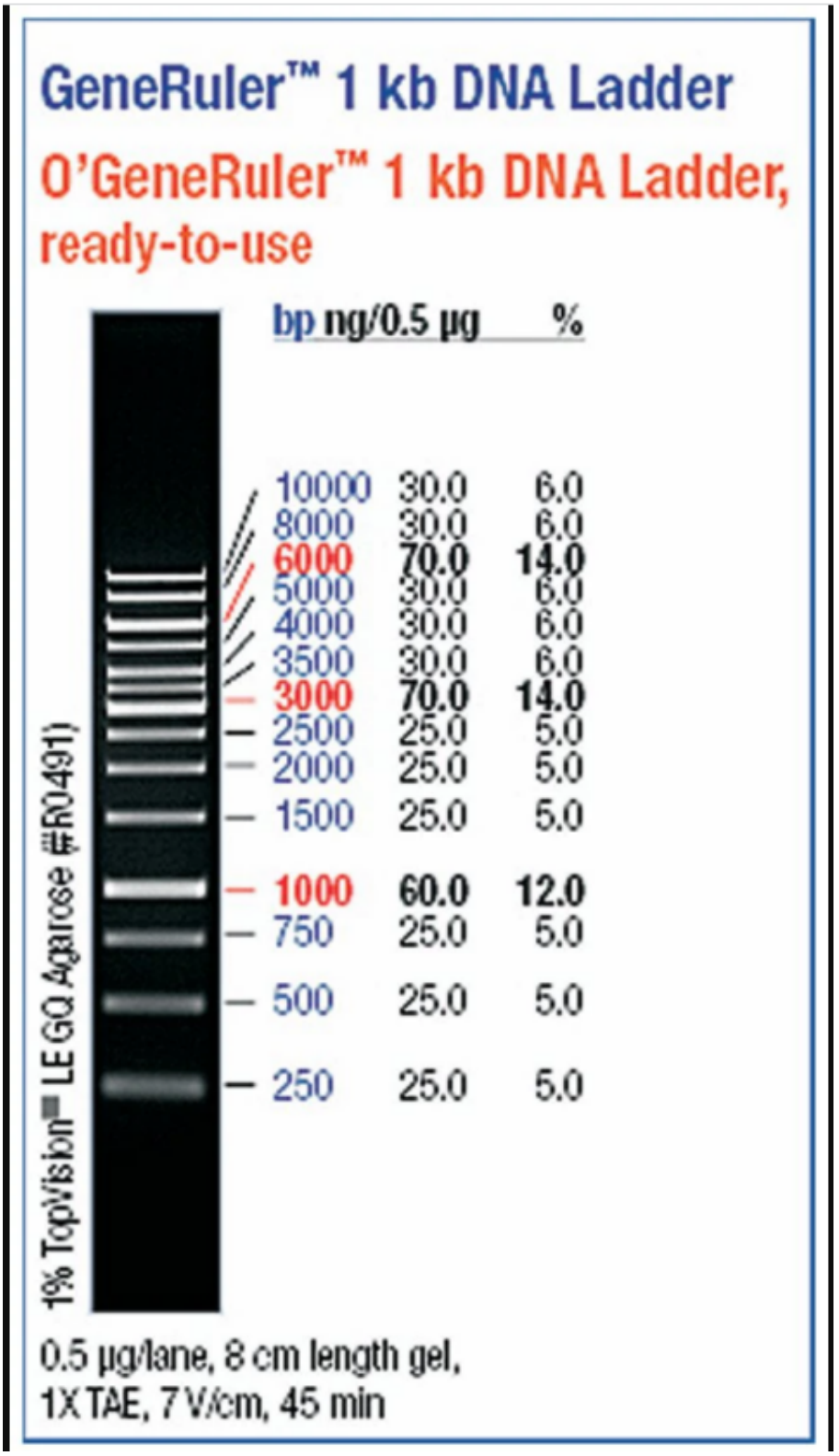

Figure 4: The 'GeneRulerTM 1kb DNA ladder' used to assign correct values to the ladder in Figure 3

Benchling

Translation for the two different *algL*

As can be seen in Figure 5, the NNHSYW-motif of the pMG26 plasmid changed to the NNKSYW-motif in pMG26mutations because of the mutation of two bases in amino acid 203. Histidine (CAC) becomes Lysine (AAG). There is also a mutation in amino acid 198, but this is a silent mutation.

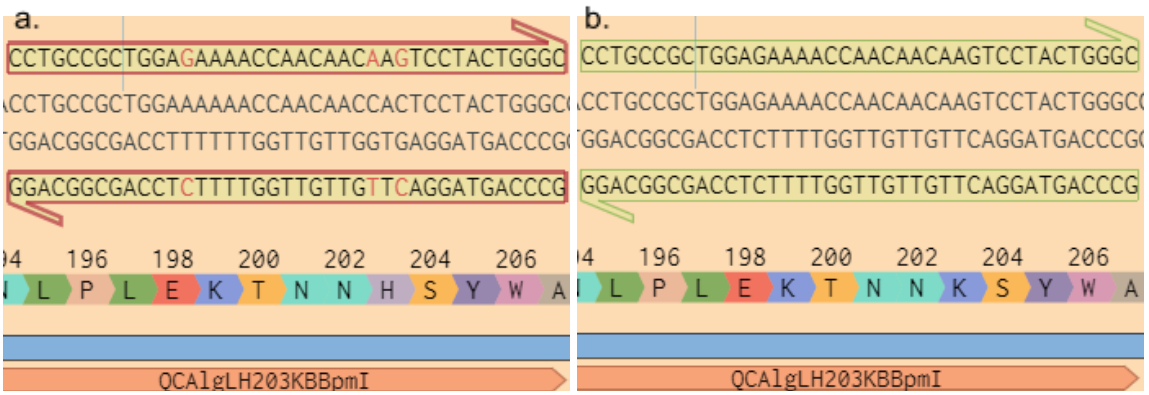

Figure 5: a. Translation for the algL gene in the pMG26 plasmid, the NNHSYW-motif can be observed; b. Translation for the algL gene in the pMG26mutations plasmid, the NNKSYW-motif can be observed

Digestion

In Figure 6 and 7 the different expected digestion pattern sizes can be observed. These are obtained by doing a virtual digestion with *Bpm*I in Benchling. pMG26 has three restriction sites for *Bpm*I. One of the mutations in pMG26mutations is a silent mutation, but it created a new restriction site for *Bpm*I. This explains why there is one more fragment for pMG26mutations.

Enzymes	Cuts	Temp.	1.1	2.1	3.1	4/CS
BpmI	3	37°C	75	100	100	100*

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
167	652	486	BpmI	3'	BpmI	3'
653	5995	5343	BpmI	3'	BpmI	3'
5996	166	1990	BpmI	3'	BpmI	3'

Figure 6: Expected digestion pattern sizes for pMG26 after virtual digestion with BpmI in Benchling

Enzymes	Cuts	Temp.	1.1	2.1	3.1	4/CS
BpmI	4	37°C	75	100	100	100*

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
167	652	486	BpmI	3'	BpmI	3'
653	3678	3026	BpmI	3'	BpmI	3'
3679	5995	2317	BpmI	3'	BpmI	3'
5996	166	1990	BpmI	3'	BpmI	3'

Figure 7: Expected digestion pattern sizes for pMG26mutations after virtual digestion with BpmI in Benchling

Own primer pair

To encode for the NNASYW-motif instead of the NNHSYW-motif in pMG26, the following primers can be used. They will introduce mutations to encode for alanine instead of histidine and the mutated DNA sequence will contain a new restriction site for identification of the mutation by restriction enzyme AloI. The mutated bases are indicated in blue. Also the open reading frame encoding for the NNASYW-motif and the introduced restriction site are indicated.