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Research project (14 ECTS) in biology for foreign students (LÍF039L)

HÁSKÓLI ÍSLANDS

Construction of knock-out vectors for *nifD* and *vnfDG* in *Nostoc 210A*, *232* and *213* and *N6*

Report

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1. Introduction

This is the report of a seven weeks participation in the project **Alternative nitrogenases in cyanobacteria of Arctic and Subarctic ecosystems** which is supervised by *Ólafur S. Andrésson*.

The aim of the project is to investigate what drives the selection of the alternate Vnf nitrogenase under certain conditions in cyanobacteria in lichens, mosses and streams. Another objective is to characterize the nitrogenase activities and Vnf clusters i.e. gene expression, acetylene reduction, discrimination of the isotopes ^{15}N and ^{13}C under different conditions of Molybdenum (Mo) and Vanadium (V) availability, temperature and light. Also it is being evaluated how much the Vnf nitrogenase is involved in the nitrogen budget of subarctic and arctic ecosystems. It is assumed that $\Delta^{15}N$ values can be used as a proxy of V nitrogenase contribution.

Another aim is to characterize the Mo and V nitrogenase and their regulation in sequenced cyanobacterial strains (*N210A, N213, N232* and *N6*). Knock out (KO) constructions will be utilized for this. They are made by inserting antibiotic resistance cassettes into key genes of each system. *nifD* is a gene transcribed in the presence of molybdenum and *vnfDG* is transcribed in the absence of molybdenum. During the seven weeks course Research project in biology for foreign students (LÍF039L) essential pieces for the KO vectors have been constructed by PCR amplification of sequences flanking the insertion points of *nifD* and *vnfDG*. The PCR constructs also have adaptor sequences for facile a vector construction using the Gibson assembly procedure. [1, 2, 3] This report will explain what has been done and for what reason and finally sum up the results.

2. Materials and Methods

To amplify the needed genes, polymerase chain reactions (PCR) were performed. For this a thermocycler (MJ Tedrad) was used. For the reactions different polymerases were tried. The manufacturers provide ready-to-use buffers (that already contain magnesium cations). Every manufacturer has a protocol of how to prepare the reaction mixtures. For example for *Q5 High-Fidelity DNA Polymerase* a final dNTP concentration of 200 µM is recommended and for that an adequate dilution from dNTP stocks was made. [4] As tool to calculate how much of which ingredient is needed a simple excel table was made. The templates were already ready to use, made by colleagues who also participated in this project.

2.1 Getting PCR to work - PCR of *rbcLX* with CW and CX primers

In the beginning it was necessary to find out whether the polymerase together with the provided buffer and with the primers and templates works and also determine the parameters for the thermocycler.

Table 1 shows the recipe from which the reaction mix had been made. Ideally, the ingredients were thawed and assembled together on ice. With the small amounts added (1 µl polymerase) the reaction needed to be gently mixed and then all the liquid collected to the bottom by spinning the tubes briefly in a centrifuge. The mix then had to be transferred to the thermocycler as soon as possible. The general parameters for this polymerase are shown in table 2. [4] To amplify the gene *rbcLX*, which encodes *Ribulose-1,5-bisphosphate carboxylase/oxygenase*, the primers called CW and CX were used. The annealing temperature is dependent on the melting temperature of the primers and it should be considered that the melting point of the forward and the reverse primers should not be very different. The used primers CW (5' -CGTAGCTTCCGGTGGTATCCACGT-3') and CX (5'-GGGGCAGGTAAGAAAGGGTTTCGT- 3') have melting points of 62.9°C and 61.2°C, so a an annealing temperature of 58°C was chosen. The expected product length was around 850 b so an extension time of 25 seconds was chosen (20-30 seconds / 1000 b). In addition, one tube was prepared without template and a 3 kb ladder was added before running on a gel.

Component	1 x 25 µl Reaction	2 x 25 µl Reactions	3 x 25 µl Reactions	4 x 25 µl Reactions
5X Q5 Reaction Buffer	5	10	15	20
2 mM dNTPs	2,5	5	7,5	10
10 µM Forward Primer	1,25	2,5	3,75	5
10 µM Reverse Primer	1,25	2,5	3,75	5
Template DNA	1	2	3	4
Q5 High-Fidelity DNA Polymerase	0,25	0,5	0,75	1
Nuclease-Free Water	13,75	27,5	41,25	55
	25µl	50µl	75µl	100µl

Table 1: Reaction setup for Q5 High-Fidelity DNA Polymerase

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C	5–10 seconds
	*50–72°C	10–30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

Table 2: Thermocycling Conditions for a Routine PCR with Q5 High-Fidelity DNA Polymerase

To check if the PCR was successful, (submarine) gel electrophoresis was performed. This electrophoresis consists of an Agarose gel with Tris/Acetate/EDTA buffer and Ethidium bromide. Table 3 [5] lists how much Agarose is ideal for which DNA length. More Agarose result in dense agarose gives smaller pores and thus separation of smaller molecules. In this case, 1% agarose was chosen because it is suitable for lengths of 850 b. To pour the gel it has

to be heated up ("melted") first in the microwave and then cooled down to a "hand warm" temperature. After its polymerization the gel is put into an electrophoresis chamber. 5 µl of each sample were mixed with 5 µl loading dye and then pipetted into a well. The electrophoresis is then performed at a voltage of 100 volts for approximately 30 minutes.

Agarosegelelektrophorese

Zonenelektrophorese

- DNA ist immer negativ geladen
- Quotient Masse / Ladung = konstant

Trennung nach
- Größe
- Form

Agarose-konzentration	Trennbereich
0.3 %	5 – 60 kb
0.6 %	1 – 20 kb
0.7 %	0.8 – 10 kb
0.9 %	0.5 – 7 kb
1.2 %	0.4 – 6 kb
1.5 %	0.2 – 3 kb
2.0 %	0.1 – 2 kb

Nachweis durch
- Ethidiumbromid
- andere, nicht interkalierende Fluoreszenz-farbstoffe

Table 3: separation ranges in different Agarose gel concentrations

Ethidium bromide is a dye which binds DNA and fluoresces making it visible under UV. After the electrophoresis the gel is taken to an UV lamp and photographed with a fluorescence filter.

Figure 1 is a gel photo and shows the result of PCR reactions and a DNA ladder. Besides the strains *Nostoc 210*, *213*, *232* and *N6*, the closely related *Anabaena variabilis* was also used. Strains *210* and *232* appear two times since two different templates of the same strains were tried out. A black bar shows positive result, a missing bar negative. Weak bars at the bottom are leftover primers that a relatively short und thus run so fast.

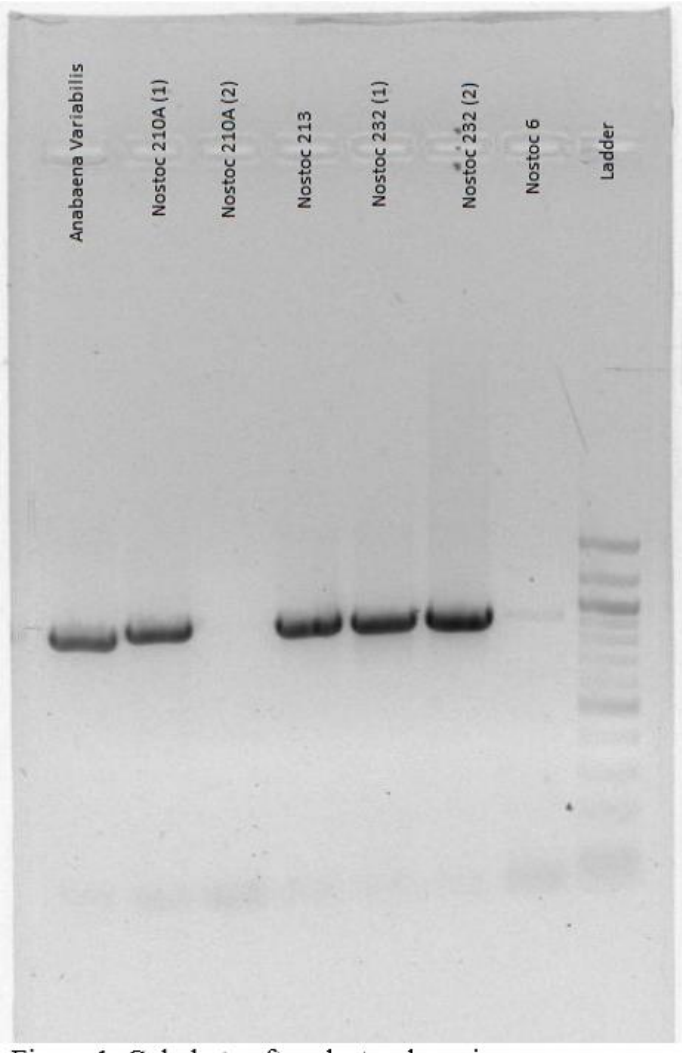


Figure 1: Gel photo after electrophoresis

2.2 PCR of flanking sequences *nifD* and *vnfDG*

The insertion points of *nifD* and *vnfDG* have flanking sequences and for them primers were devised. In this strategy for constructing a knock-out vector use is made of overlapping GC adaptor sequences as illustrated in Figure 2. [3]

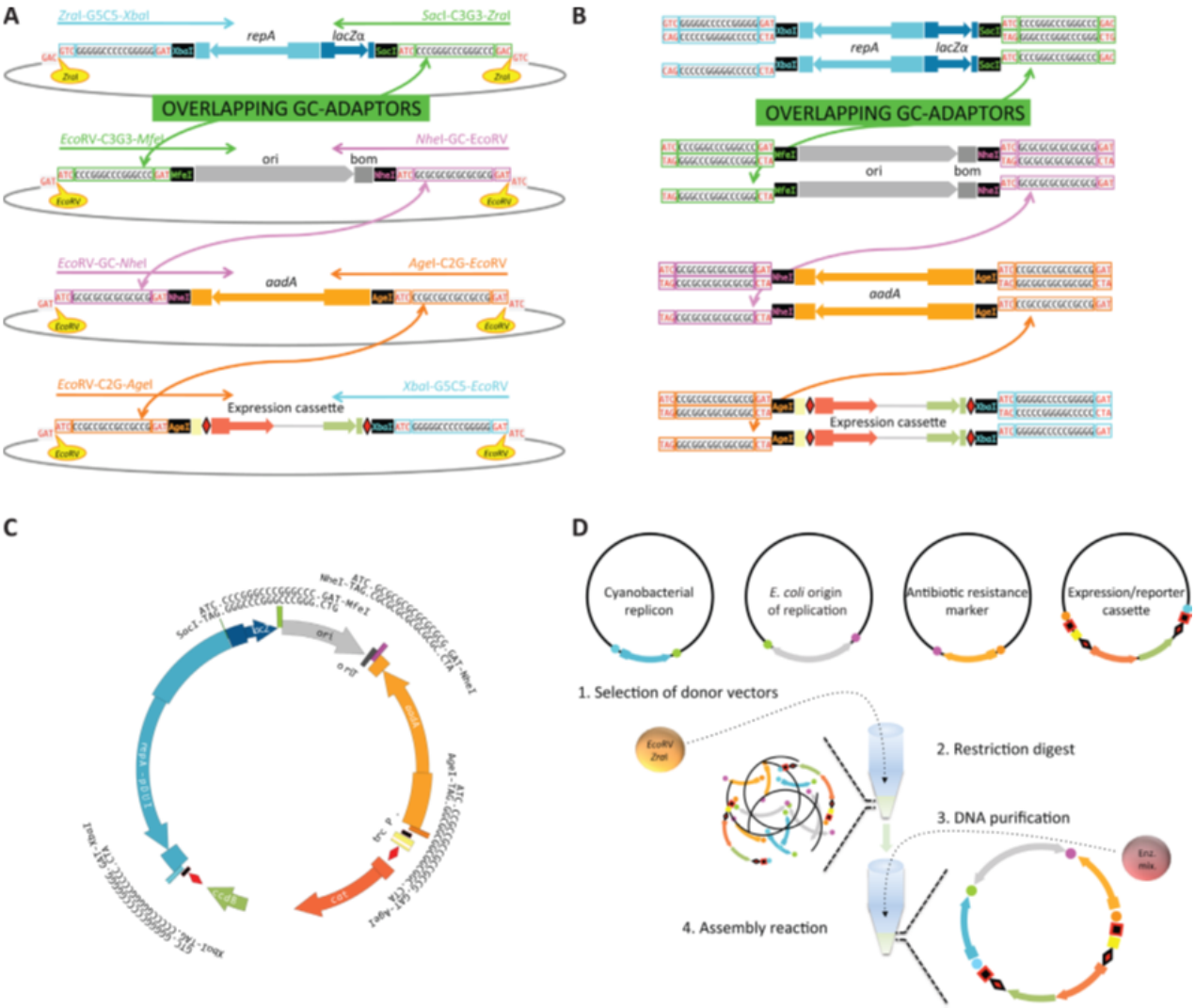


Figure 2: Strategy and method for combinatorial construction of plasmids, using a shuttle vector for *A*. PCC7120 as an example. (**A**) Four different donor plasmids harboring the devices required to build a destination vector for *A*. PCC7120 are shown. Each device is flanked by GC-adaptors that overlap with the GC-adaptors of the previous and following devices. The devices are released from the donor plasmid using the restriction enzyme *Eco*RV or *Zra*I. (**B**) During the assembly reaction, single-stranded overhangs are generated and anneal with the complementary sequences of adjacent fragments. The one-base-pair mismatch (G/T) in the overhangs that result from devices released from the donor plasmids using *Eco*RV and *Zra*I does not affect efficient assembly of the shuttle vectors. (**C**) Assembled shuttle vector for *A*. PCC7120 that contains four modules. (**D**) Overview of the steps required for the construction of a typical shuttle vector

To devise that, different primers for the flanking sides had been designed that have these GC sequences on one of their ends. Also, the GC adaptors are different so they can only be pieced together in a certain order. During this seven weeks course these primers were tested. In Figure 3 a scheme shows which primers finally worked to amplify the flanking sequences of *nifD*/*vnfDG* of *N210A*. It is representative of the strains *213* and *232*. *N6* doesn't have *vnfDG*. The right side of *vnfDG* and the left side of the four strains could not get amplified initially. Seemingly they are too complicated but they could be amplified with the help of internal primers which overlap. The internal primers are additional forward/reverse primers and their products can be joined together in a second PCR.

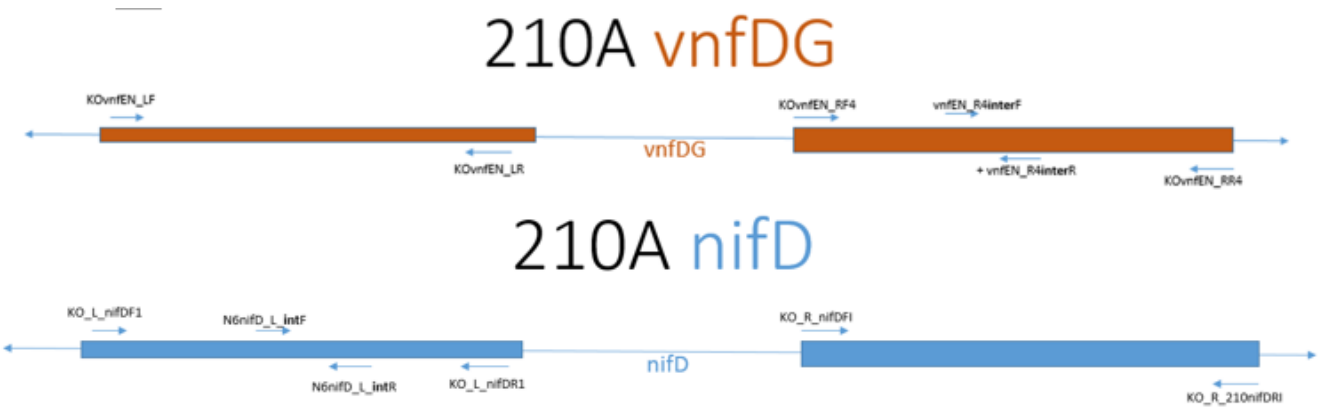


Figure 3: Flanking sides of 210A vnfDG/nifD with their primers. Right side of vnfDG and left side of 210A needed internal primers which overlap so they can be joined in a second step.

To facilitate joining of these internal pieces they had to be purified. For this, a purification kit (QlAcquick PCR Purification Kit) was used. The kit consists of a column to which the DNA binds first, washed free of impurities and then eluted in a small volume. At first, 1 volume of PCR reaction is mixed with 5 volumes of PB buffer (5M Guanidinium chloride in 30% isopropanol). The provided columns are put into 2 ml receptacle tubes and then the samples are applied to the columns. Next, the columns are centrifuged at 18,000 x G (13,000 rpm) for 1 minute. Then, the flow-through has to be discarded and 750 µl of washing buffer PE (10 mM Tris-HCl (pH 5) in 80% ethanol) added. The washing buffer is passed through the column, again by centrifugation. To make sure that all buffer goes through, the tubes should be centrifuged a second time. The columns are placed into new 1.5 ml tubes and the bound DNA is eluted by adding 50 µl of 0.1 x TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8) to the column and centrifuging for 2 minutes. [6]

After the purification the samples were tested for purity and for the amount auf nucleic acids by using a Nanodrop device. It measures the UV absorbance and displays the result as a graph. DNA has a maximum absorbance at 260 nm. A smooth curve shape with a peak at 260 nm indicates a pure sample and from the height of that peak the software calculates the nucleic acid content (Figure 4). The curves should not have a peak at around 230 nm since that is where proteins have their maximum.

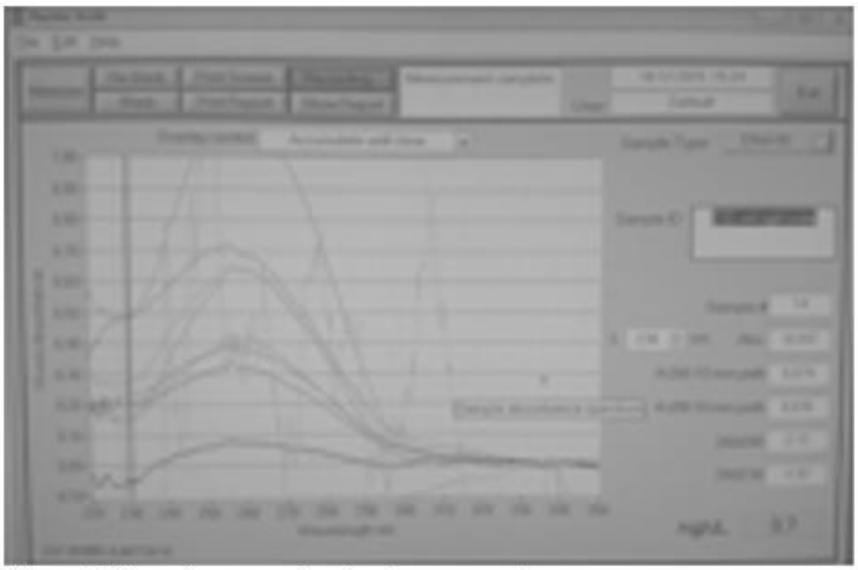


Figure 4: Nanodrop sample absorbance spectra

When the purification was successful the internal parts can be joined together by using the Knock out primers. For this the annealing step can be skipped and instead a longer extension time. Also, since there is already plenty of DNA, less cycles are needed. In this case, OneTaq polymerase were used,

the extension time was 75 seconds and the reaction was repeated for 20 cycles.

In the attempt to yield the right flanking side of nifD from *N6* and *213* new primers were designed. The National Center for Biotechnology Information (NCBI) offers a primer designing tool which were used to design them. [7] They were then ordered from Microsynth and after they arrived they were tested but they did not work either.

2.3 Determining sensitivity to streptomycin/spectinomycin and chloramphenicol

In order to verify a successful insertion of the KO vector, the modified bacteria will be grown on antibiotics so it must be ensured that they are not resistant in the first place. Agar plates with BG11 agar and various antibiotics concentrations were made. The chosen concentrations were based on the paper "Broad-host-range vector system for synthetic biology and biotechnology in cyanobacteria" (Arnaud Taton et al.).With streptomycin (Sm) and spectinomycin (Sp) a concentration range from 10 to 50 µg/ml and for chloramphenicol (Cm) a range from 20 to 100 µg/ml were made. For the inoculation preexisting cultures were used. They were relatively old and that is why they were cloaked together to chains. Ideally the bacteria should be single cells before streaking them out. To singularize them, the fluid (with the colonies) were sucked up repeatedly with a pipette. Then 1 ml were taken and the current state were controlled under a microscope. After that, the tube were put into a clean and disinfected sonificator. The sonification separates the cells when a gentle force is chosen and destroys them when the force is too strong. To find the right sonification time and force, the sample were checked frequently under a microscope. When there were enough single cells, each sample was filled up to 1.5 ml to improve the comparability between the growths of the strains. Then, the plates were labeled properly and 100 µl of a sample spread evenly with a sterilized Drigalski spatula over half of a plate to save resources. Eventually, the plates were incubated at room temperature under GRO-LUX plant lights (Sylvania GRO-LUX F18W) with a light intensity of circa 100 lux. The four strains were also streaked out onto a control plate without antibiotics. After three weeks the inhibition was evaluated. It should be noted that *N232* grew at a lower rate than *N6*, *N210*, and *N213* on the control plate. The results of the inhibition are shown in table 4.

N6

	Antibiotic concentration (µg/ml)				
antibiotic	10	20	30	50	100
Sp	-	(+)	(+)	++	/
Sm	++	++	++	++	/
Cm	/	-	-	+	++

210

	Antibiotic concentration (µg/ml)				
antibiotic	10	20	30	50	100
Sp	-	-	-	(+)	/
Sm	++	++	++	++	/
Cm	/	++	++	++	++

213

	Antibiotic concentration (µg/ml)				
antibiotic	10	20	30	50	100
Sp	-	(+)	++	++	/
Sm	++	++	++	++	/
Cm	/	-	(+)	++	++

232

	Antibiotic concentration (µg/ml)				
antibiotic	10	20	30	50	100
Sp	-	(+)	++	++	/
Sm	++	++	++	++	/
Cm	/	++	++	++	++

Table 4: Inhibition of the Nostoc strains N6, 210, 213 and 232.

N6 could be inhibited by Streptomycin (30-50 µg/ml) and chloramphenicol (50 µg/ml). *N210* could be inhibited by a high concentration of streptomycin (<50 µg/ml) and chloramphenicol. *N123* could be inhibited by streptomycin (30-50 µg/ml) and

chloramphenicol (50 µg/ml). *N232* could be inhibited by streptomycin (20 µg/ml) and chloramphenicol. Additionally, all strains could be inhibited well by streptomycin. Figure 5 shows a pictures of the plates.

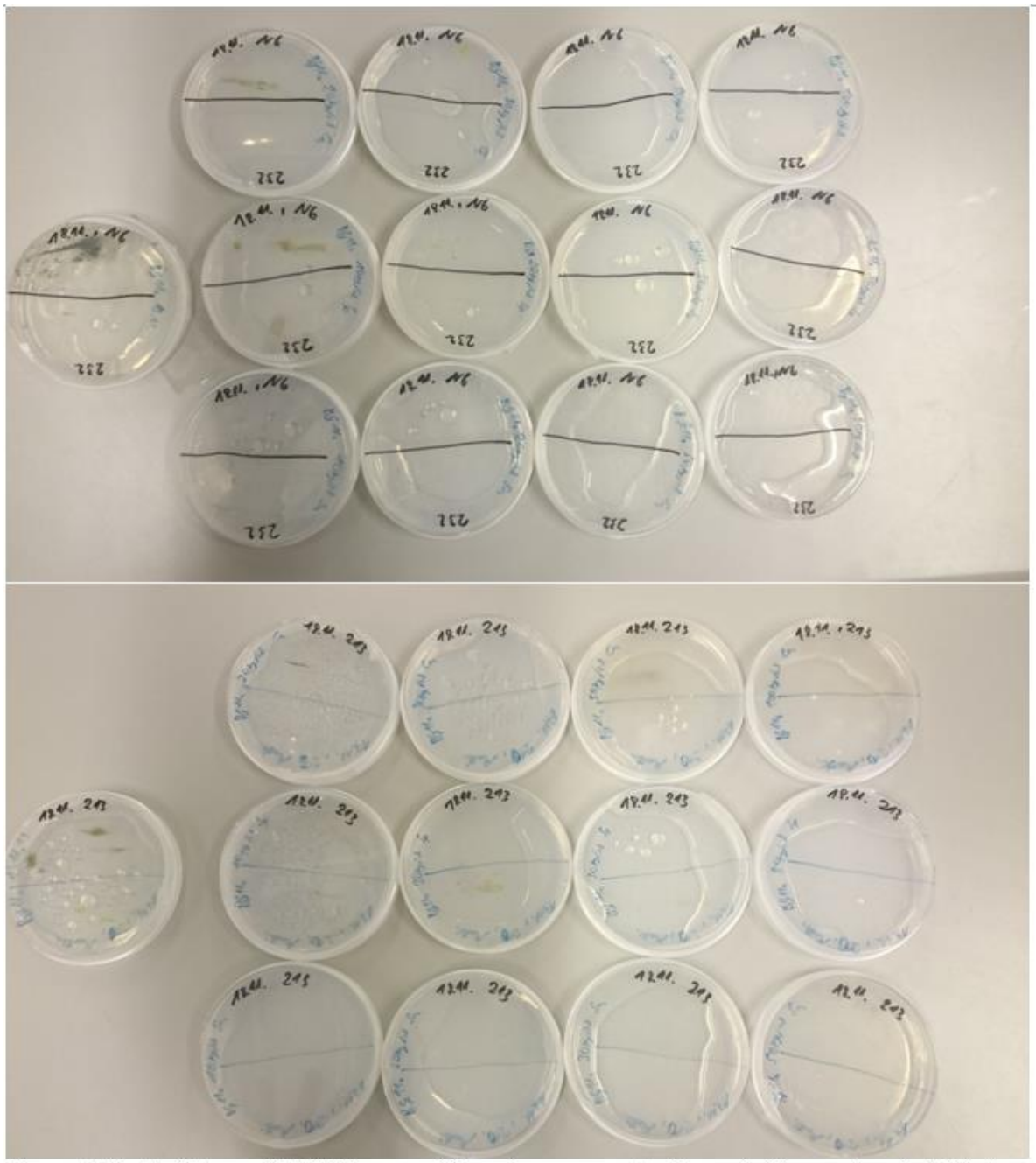


Figure 5: Petri plates with BG11 agar with various concentrations of chloramphenicol (first row; 20, 30, 50 and 100 µg/ml), streptomycin (second row; 10, 20, 30 and 50 µg/ml) and spectinomycin (third row; 10, 20, 30 and 50 µg/ml). Strains *N6* & *232* in the top and *213* & *210* in the bottom. Positive control leftmost.

3. Conclusion

Various polymerases were used with different results. In the beginning the only aim was to make the PCR working and for that *Q5* polymerase was used. After the right primers were determined, *Q5* ran low and *FastTaq* [8] was used, which worked worse. After that, *OneTaq* [9] polymerase was tried and that eventually worked, except for the right flanking sided of nifD of *N6* and *N213*. In the approach to amplify them, the *Phusion* [10] polymerase was tried but it did not work either. Despite the greatest efforts the right flanking sides of nifD from *N6* and *213* could not be yielded. In table 5 the (formerly) working primers are summarized and table 6 gives their sequences.

N210

Template	Primer
rbcLX	Cw + Cx
nifD → right flanking side	KO_R_nifDFI + KO_R_210nifDRI
nifD → left flanking side	KO_L_nifDF1 + N6nifD_L_**int**R KO_L_nifDR1 + N6nifD_L_**int**F
vnfDG → right flanking side	KOvnfEN_RF4 + vnfEN_R4**inter**R KOvnfEN_RR4 + vnfEN_R4**inter**F
vnfDG → left flanking side	KOvnfEN_LF + KOvnfEN_LR

N213

Template	Primer
rbcLX	Cw + Cx
nifD → right flanking side	KO_R_nifDFI + KO_R_210nifDRI
nifD → left flanking side	KO_L_nifDF1 + N6nifD_L_**int**R KO_L_nifDR1 + N6nifD_L_**int**F
vnfDG → right flanking side	KOvnfEN_RF4 + vnfEN_R4**inter**R KOvnfEN_RR4 + vnfEN_R4**inter**F
vnfDG → left flanking side	KOvnfEN_LF + KOvnfEN_LR

N232

Template	Primer
rbcLX	Cw + Cx
nifD → right flanking side	KO_R_nifDFI + KO_R_210nifDRI
nifD → left flanking side	KO_L_nifDF1 + N6nifD_L_**int**R KO_L_nifDR1 + N6nifD_L_**int**F
vnfDG → right flanking side	KOvnfEN_RF4 + vnfEN_R4**inter**R KOvnfEN_RR4 + vnfEN_R4**inter**F
vnfDG → left flanking side	KOvnfEN_LF + KOvnfEN_LR

N6

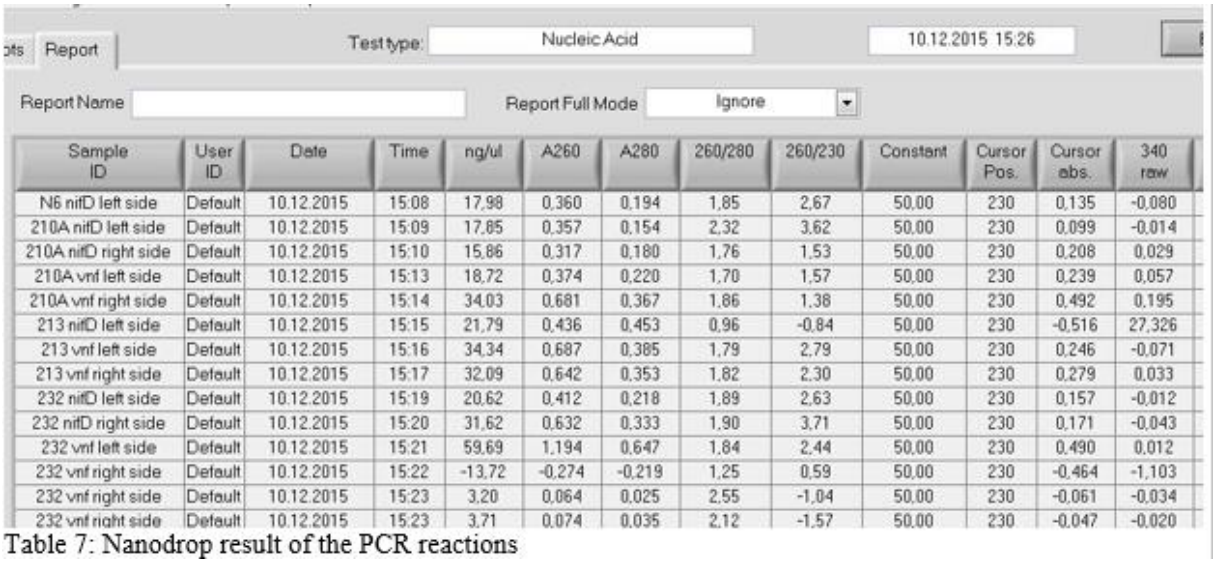
Template	Primer
rbcLX	/
nifD → right flanking side	KO_R_nifDFI + KO_R_210nifDRI
nifD → left flanking side	KO_L_nifDF1 + N6nifD_L_**int**R KO_L_nifDR1 + N6nifD_L_**int**F
vnfDG → right flanking side	KOvnfEN_RF4 + vnfEN_R4**inter**R KOvnfEN_RR4 + vnfEN_R4**inter**F
vnfDG → left flanking side	KOvnfEN_LF + KOvnfEN_LR

Table 5: Conclusion of the primers that worked

After all amplifications were done, every sample was purified as described previously and their nucleic acids content measured as well. The results are shown in tables 7 and 8.

Primer	Sequence
Cw	5'-CGT AGC TTC CGG TGG TAT CCA CGT- 3'
Cx	5'-GGG GCA GGT AAG AAA GGG TTT CGT- 3'
KO_R_nifDFI	5´-**ATC CCG CCG CCG CCG CCG GAT** GCC ATC ATG GTT GGT GGT CTA- 3´
KO_R_210nifDRI	5´-**ATC CCC CCG GGG GCC CCC GAC** AGC AGA TCG GTT TCC GTG TC- 3´
KO_L_nifDF1	5'-**ATC CCC GGG CCC GGG CCC GAC** GTT AAC GAG TAC GCA CCA GA – 3´
N6nifD_L_intR	5'-TAG GAC CCC AAA CCA CAC CT-3´
KO_L_nifDR1	5´-**ATC CGC GCG CGC GCG CGC GAT** CAA GCA TCA CCA CCG ATG TT-3´
N6nifD_L_intF	5'-ATG ACC GCT CGT GGT TGT G-3´
KOvnfEN_RF4	5´-**ATC CCG CCG CCG CCG CCG GAT** TGT CGC AGC TGG GTA TGA TG-3´
vnfEN_R4interR	5´-AGA AGA CTG TCT GTA CTT TAA TCC T-3´
KOvnfEN_RR4	5'-**ATC CCC CCG GGG GCC CCC GAC** GGG AAC TAA GAC CGA GGC TG-3´
vnfEN_R4interF	5´-TGG GCT GAT TTG GAA TGG GT-3´
KOvnfEN_LF	5´- **ATC CCC GGG CCC GGG CCC GAC** GGC GCC ATT CGA GAA GTA GA-3´
KOvnfEN_LR	5´- **ATC CGC GCG CGC GCG CGC GAT** CCG GGT CTA ACC CTG TTT CC-3´

Table 6: List of Primer sequences. The GC parts of the KO primers are marked in bold letters



Report | Test type: Nucleic Acid | 10.12.2015 15:26

Report Name | Report Full Mode: Ignore

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
N6 nifD left side	Default	10.12.2015	15:08	17,98	0,360	0,194	1,85	2,67	50,00	230	0,135	-0,080
210A nifD left side	Default	10.12.2015	15:09	17,85	0,357	0,154	2,32	3,62	50,00	230	0,099	-0,014
210A nifD right side	Default	10.12.2015	15:10	15,86	0,317	0,180	1,76	1,53	50,00	230	0,208	0,029
210A vnf left side	Default	10.12.2015	15:13	18,72	0,374	0,220	1,70	1,57	50,00	230	0,239	0,057
210A vnf right side	Default	10.12.2015	15:14	34,03	0,681	0,367	1,86	1,38	50,00	230	0,492	0,195
213 nifD left side	Default	10.12.2015	15:15	21,79	0,436	0,453	0,96	-0,84	50,00	230	-0,516	27,326
213 vnf left side	Default	10.12.2015	15:16	34,34	0,687	0,385	1,79	2,79	50,00	230	0,246	-0,071
213 vnf right side	Default	10.12.2015	15:17	32,09	0,642	0,353	1,82	2,30	50,00	230	0,279	0,033
232 nifD left side	Default	10.12.2015	15:19	20,62	0,412	0,218	1,89	2,63	50,00	230	0,157	-0,012
232 nifD right side	Default	10.12.2015	15:20	31,62	0,632	0,333	1,90	3,71	50,00	230	0,171	-0,043
232 vnf left side	Default	10.12.2015	15:21	59,69	1,194	0,647	1,84	2,44	50,00	230	0,490	0,012
232 vnf right side	Default	10.12.2015	15:22	-13,72	-0,274	-0,219	1,25	0,59	50,00	230	-0,464	-1,103
232 vnf right side	Default	10.12.2015	15:23	3,20	0,064	0,025	2,55	-1,04	50,00	230	-0,061	-0,034
232 vnf right side	Default	10.12.2015	15:23	3,71	0,074	0,035	2,12	-1,57	50,00	230	-0,047	-0,020

Table 7: Nanodrop result of the PCR reactions

Strain	Gene	Amount	Nucleotic conc.
N6	nifD left side	45 µl	18 ng/µl
N6	nifD right side	0	
210A	nifD left side	45 µl	17,9 ng/µl
210A	nifD right side	45 µl	15,9 ng/µl
210A	vnf left side	45 µl	18,7 ng/µl
210A	vnf right side	45 µl	34 ng/µl
213	nifD left side	45 µl	21,8 ng/µl
213	nifD right side	0	
213	vnf left side	45 µl	34,3 ng/µl
213	vnf right side	45 µl	23,1 ng/µl
232	nifD left side	45 µl	20,6 ng/µl
232	nifD right side	45 µl	31,6 ng/µl
232	vnf left side	45 µl	59,7 ng/µl
232	vnf right side	45 µl	3,7 ng/µl

Table 8: Conclusion of how many product has been yielded

4. Acknowledgements

I acknowledge, with gratitude, my thanks to Professor *Ólafur S. Andrésson* for accepting me as his student and giving me the opportunity of getting an insight in scientific research work.

5. References

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[8] https://pim-eservices.roche.com/LifeScience/Document/8212cc35-06ee-e311-98a1-00215a9b0ba8

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[10] https://www.neb.com/~/media/Catalog/All-Products/8C333ACDF094414B909058E6ED55AE35/Datacards%20or%20Manuals/M0530Datasheet-Lot0031206.pdf