Forschung hautnah:

Wissenschaftliches Schülerpraktikum vergeben durch den Förderverein der BiologieOlympiade e.V.

Institute for Molecular Biology
Mainz
Group Leader: Dr. Falk Butter
Supervisor: Vivien Schoonenberg
Quantitative Proteomics

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Gymnasium Calvarienberg Ahrweiler

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Table of Contents:

1. Introduction

1.1 Personal

I am an 18-years-old high-school graduate, whose interest in biology started in the senior classes at school. Especially the field of biochemistry, I have learned about in the 11th grade, fascinated me as well as genetics in 13th grade. So I participated in the German competition for the 29th *International Biology Olympiade (IBO)* and reached the third qualifying round, which took place in Kiel in February 2018. There the *Förderverein der Internationalen Biologieolympiade e.V.* offered me the chance to do an internship at the *Institute of Molecular Biology (IMB)* in Mainz from May 28 - June 22, 2018. There I have been able to earn some more practical experience how working in a lab is going to be like and what researchers are doing exactly. This great and interesting time confirmed my decision to start studying Molecular Life Sciences at the University of Hamburg in October.

1.2 About the IMB

The IMB was founded in 2011 by the State of Rhineland-Palatinate and the *Boehringer Ingelheim Foundation (BIF)*, which is a non-profit organisation funding research in the fields of medicine, biology, chemistry and pharmacy. IMB‘s Founding Director is Christof Niehrs, but currently the institute is headed by the Executive Director Helle Ulrich. The building is shaped in a very modern architecture, which fits the purpose, but offers areas for scientific exchange and socialising as well. Furthermore it is located on the campus of the University Mainz.
I spent my time in the group of Dr. Falk Butter, which focuses on quantitative proteomics, trying to identify the proteins binding to specific parts of DNA or RNA, which is done by the use of mass spectrometry often. In the Butter group there are many PhD students from different countries, so it is a very multi-cultural and welcoming atmosphere.

Figure 1.2.1: IMB

1.3 Abstract

During my time at the IMB I worked at three smaller projects with my supervisor Vivien Schoonenberg, a PhD student from the Netherlands. These projects were:

1. Identification of DNA-binding proteins
   In this main project we tried to identify proteins binding as possible transcription factors to Single Nucleotide Polymorphisms (SNPs). So we had to polymerize the SNPs to increase the protein binding sites while doing a pulldown and incubating it with nuclear extract (proteins). After that we monitored our results by Western Blot with antibodies or we used the Mass Spectrometer. There the desired result is, that through a change in the nucleotides, the proteins binding to the SNP or their binding affinities differ as well.
2. Cloning SNP into V19
   In the second project we tried to clone different SNPs, which we also used in project 1, into the luciferase vector (V19) with a minimal promoter. Then, after transferring the vector into bacteria, it should be possible to measure differences in the expression of the luciferase gene, if the different SNP-binding proteins are affecting the minimal promotor in up- or downregulation.
3. Cloning DHRS2 into V10 and V11
   In this project we cloned the gene DHRS2, which is coding for the enzyme dehydrogenase/reductase SDR family member 2, into the vectors V10 and V11 by doing a gene exchange with another vector. Afterwards we transferred it into bacteria and through the GFP tag in the vector, we were able to discover the location of our protein.

2. About the Project

2.1 Proteomics

Proteomics in general is a discipline that deals with the proteome, the entirety of proteins in a living system. To identify different proteins a mass spectrometer can be used. This instrument is able to differentiate between lighter and heavier as well as between more and less positively charged peptides because their behavior differs, when they are exposed to a magnetic field. These two factors together are called the mass / charge ratio, which is used to deduce the sequence of amino acids and to quantify the proteins in a sample. Figure 2.1.1 shows this most important part of the mass spectrometer.

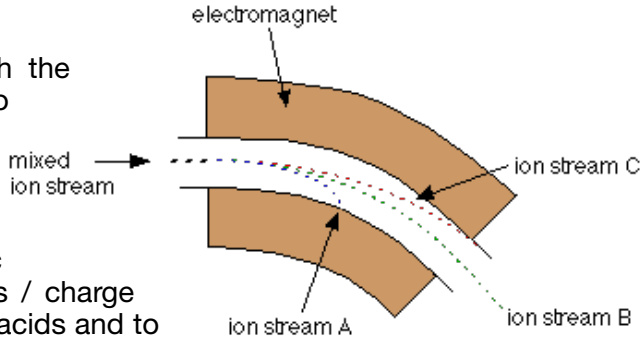

Figure 2.1.1: Mass Spectrometer

2.2 Transcription factors

Transcription factors are proteins binding to DNA, which can affect the transcription of nearby genes. This results in a higher or lower copy number of transcripts and, consequently, a higher or lower protein concentration in the cell. Most TFs recognize specific DNA-motives, so that they can regulate several genes separately. This explains, why SNPs may change the binding of TFs.

Figure 2.2.1: function of transcription factors

2.3 SNPs

Single Nucleotide Polymorphisms (SNPs) are DNA-variations between two alleles regarding only one nucleotide (e.g. figure 2.3.1). They can occur in coding regions as well as in non-coding regions of the DNA. In coding regions they might change the coded sequence, but in the non-coding regions they can be able to influence the regulation of genes leading to diseases like cancer.

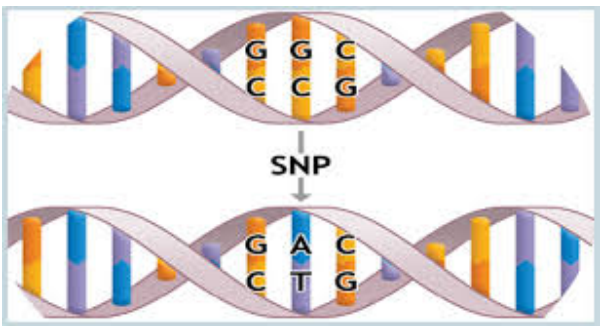

Figure 2.3.1: SNP

2.4 Antibodies

Antibodies are an important part of the immune system. They are a highly diverse class of Y-shaped molecules, each with its own binding specifities. This leads to agglomeration as well as to labeling of harmful proteins or cells, so that the other parts of the immune system (B- or T-cells) can react accordingly.
In the molecular biology it is possible to benefit from the specificity of the antibodies. For example they are used in Western Blots for protein detection and quantification. If you want to monitor a certain protein mixed in a sample with other proteins, you can load it in a gel and run it for a while to differentiate the proteins by their different mass. Then you use a primary antibody, which binds exactly to the wanted protein. After that you use a second one, which is labeled fluorescently and binds to the primarily one. With this marker the location of the protein becomes visible.

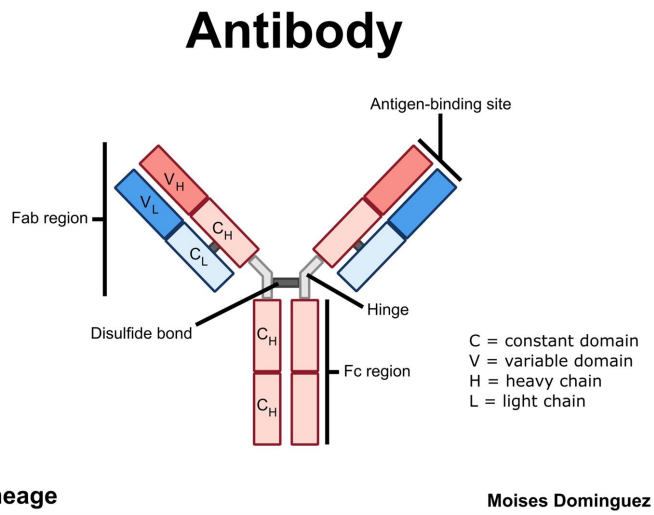

Figure 2.4.1: Antibody

2.5 Vectors

In bacteria there is the bacterial genome consisting of one big, ring-shaped chromosome. But there can be smaller DNA-rings as well, which are called plasmids, and they are able to contain resistance-genes for antibiotics for example. Plasmids are a frequently used tool in molecular biology. They can be cloned into bacteria, where they are multiplied, when the bacteria reproduce and from where they can be extracted easily. They can also be used to express certain genes in the bacteria.

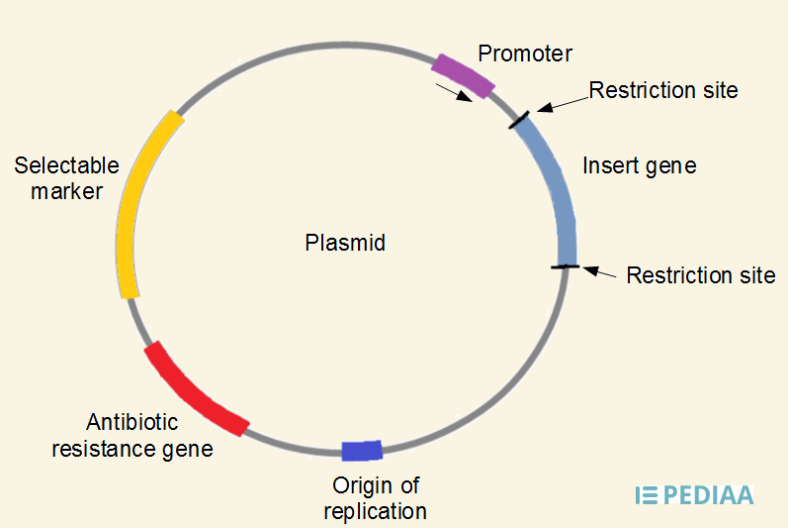

Figure 2.5.1: A vector plasmid

2.6 Luciferase vector (V19)

The luciferase gene in V19 is used as a reporter gene. Because of its bioluminescence it is able to show whether and where the luciferase gene is expressed.

2.7 GFP-tag

In molecular biology the GFP-gene (green fluorescent protein) is frequently used as another reporter of expression besides the luciferase gene, which means, that it is also used to show the gene‘s localization. Through the translational fusion with the target protein, it gets a fluorescent tag to monitor the temporal and special localization in the cell.

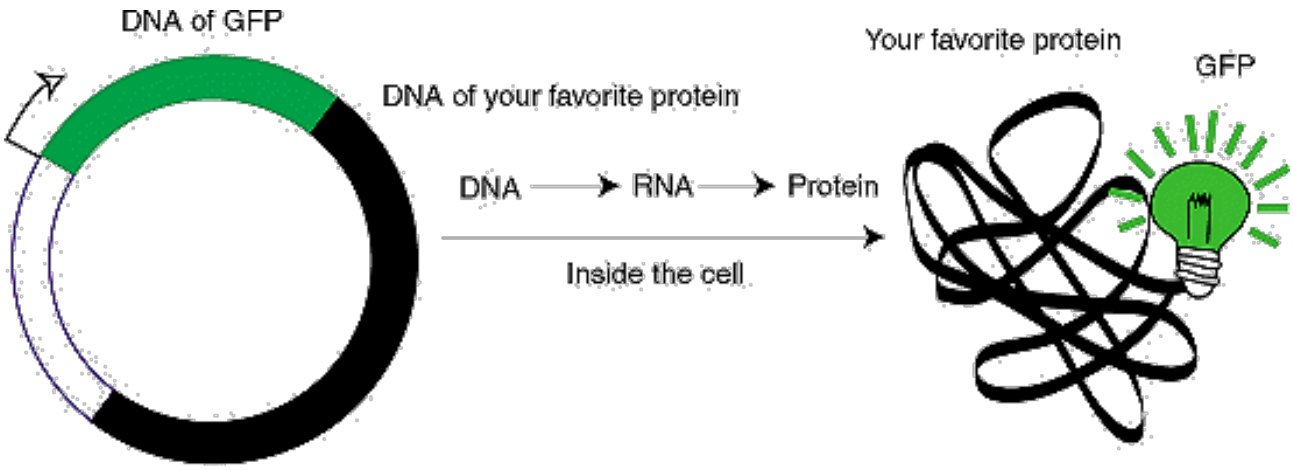

Figure 2.7.1: Function of a GFP-tag

3. Material and Methods

3.1 Identification of DNA-binding proteins

3.1.1 Preparation of the biotinylated target DNA

Annealing (30min):

- 25 µL forward DNA-strand (100 pmol/µL)
- 25 µL reverse DNA-strand (100 pmol/µL)
- 10 µL annealing buffer (200 mM Tris-HCl, pH 8.0; 100mM MgCl2; 1M KCl)

Fill up to 100 µL with DI water (deionized water).
Heat mixture up for 5 minutes at 80°C and let cool it down to Room Temperature (RT).

Sequences used:
- Rs509813 V1 forward and reverse
- Rs509813 V2 forward and reverse
- Rs80347459 V1 forward and reverse
- Rs80347459 V2 forward and reverse
- Rs11703929 V1 forward and reverse
- Rs11702929 V2 forward and reverse

Phosphorylation and Polymerisation (2 hours and over night):

Preparation of mastermix (MM) x7:
- 55 µL DI water
- 20 µL 10x T4 DNA ligase buffer (ThermoScientific)
- 10 µL PEG 6000 (24% (m/v) Polyethylenglycol)
- 2 µL 1M DTT
- 10 µL 100 mM ATP (self made)
- 5 µL T4 Polynucleotide Kinase (10U/µL) f.c. 350 units

Mix 102 µL MM with 100 µL annealed oligos.
Incubate 2 hours at 37°C and let it cool down to RT.

Add 4 µL T4 DNA ligase to each sample.
Ligate / polymerize oligos at room temperature over night.

Monitor the progress of ligation by agarose gel electrophoresis:

Preparation of 1.5% agarose gel:
- 50 mL TBE
- 0.75 g agarose

Mix and heat up in microwave.
Add 5 µL SYBR safe.
Let it cool down in gel shape for about 30 min.

1. Pocket: 5 µL 100 bp DNA ladder
2. Pocket: 1.5 µL 509 V1 + orange loading dye
3. Pocket: 1.5 µL 509 V2 + orange loading dye
4. Pocket: 1.5 µL 803 V1 + orange loading dye
5. Pocket: 1.5 µL 803 V2 + orange loading dye
6. Pocket: 1.5 µL 1170 V1 + orange loading dye
7. Pocket: 1.5 µL 1170 V2 + orange loading dye

Run gel electrophoresis at 120 Volts for 30 minutes.

Phenol/chloroform-extraction (2 hours):

Add to each polymerized sample:
• 200 µL of DI water
• 200 µL Phenol/Chloroform/IAA (25:24:1) pH 8.0

Vortex and centrifuge 2 min at 16,000 xg.
Transfer 320 µL of aqueous phase to new tube and mix with 1 mL EtOH 100%.
Precipitate for 30 min at -20ºC.

Centrifuge at 16,000 xg for 45 min at 4ºC.
Resuspend pellet in 74 µL DI water.

Biotinylation (over night):

Prepare mastermix x6.5:
• 10 µL 10x Polymerase buffer (Reaction buffer for Klenow fragment)
• 10 µL 0.4 mM Biotin-7-dATP (Jena Bioscience, NU-835)
• 6 µL DNA polymerase 30 units (Klenow fragment exo- 5 u/µL)
Add 26 µL MM to each sample.
Incubate at 37ºC over night.

Size-exclusion chromatography (5 min):

Open Sephadex G-50 column and loose lid.
Centrifuge at 2,000 rpm for 1 min to remove storage solution.
Add max. 100 µL of biotinylation reaction to the column and centrifuge at 2,000 rpm for 1 min into 1.5 mL Eppendorf tube.
Store the samples at -20ºC.

3.1.2 Bradford measurement protein concentration

• 26.4 mL DI water
• 6.6 mL 5x protein assay reagent

Total: 33 mL Bradford solution
6 samples x4 for the standard curve
+ 2 samples x3 protein extract lot N GM12878
A 21.03.18 8 µL + 72 µL DI water
B 11.04.18 8 µL + 72 µL DI water
Always mix 20 µL sample with 1 µL Bradford solution.
Measure absorption at 595 nm wavelength.

3.1.3 Pulldown using Desthio-DNA

Adding beads to the DNA:

1. Vortex beads to mix. Use 15 µL streptavidin sepharose high performance, GE Health Care, beads per oligo. Wash to remove ethanol with 10 volumes PBB+DTT (150 µL).
2. Centrifuge at 1,500xg for 1 minute, remove supernatant.
3. Resuspend oligos: 20 µL (flow through) in 200 µL PBB+DTT.
4. Add resuspended oligos to the beads (total ca. 235 µL), incubate on a rotating wheel at RT for 1 hour.
5. Centrifuge beads at 1,500xg for 1 minute, remove supernatant and store at -20°C for reuse (up to 3 times).

6. Resuspend with 200 µL PBB+DTT.
7. Transfer beads to a 96-well filter plate. Wash beads two times with 200 µL PBB+DTT.
8. Centrifuge plate slowly att 100xg for 30 seconds to remove supernatant.
9. Add 150 µL PBB+DTT and add 1.5 µL salmon sperm DNA as a competitor to the beads.
10. Add nuclear proteins to the beads (400 µg total protein).
11. Incubate for 2 hours at 4°C, slowly shaking.
12. After incubation of NE: spin at 100xg for 30 seconds (did not flow through), so spin at 300xg for 5 minutes.
13. Add to the beads 30 µL of 1x LDS sample buffer + 0.1 M DTT.
14. Heat sample for 10 minutes at 70°C (1,000 rpm shaking).

Western Blot:

Loading in Running buffer (MES):
1. ladder
2. Input (21.03.18 NE extract 10% = 20 µL)
3. SNP 509 V1
4. SNP 509 V2

Run gel at 180 Volts for 55 minutes.

Transfer (in transfer/blotting buffer):
Put membrane in DI water before building blot.
1. Black part
2. Sponge
3. Whatman paper x2
4. Gel
5. Membrane
6. Whatman paper x2
7. Sponge
8. White part

Run at 300 mA for 1 hour + coolpack.
(First picture)

Ponceau staining, wash with water + TBS-T.
Shake membrane in 40 mL TBS-T 0.1% + 2 g BSA (4°C) for 1 hour.

Roll membrane into tube with primary antibody against SP1.
Incubate for 1 hour (dilution 1 : 8,000).
Wash it 3 times (always 5 minutes shaking).
Put secondary antibody (anti-rabbit) onto glass plate ~600 µL (dilution 1 : 3,000) + 5% skim milk TBS-T.
(Second picture)

3.1.4 Preperation of MassSpec-measurement for SNP-binding proteins

In Gel digestion on 96-well plates:

Do Pulldown using Desthio-DNA (3.1.3) for the SNPs 509 V1, 509 V2, 803 V1, 803 V2, 1170 V1 and 1170 V2 (without Western Blot) x2.

Load the gel with samples + LDS and a ladder in running buffer.
Run at 180 Volts for 10 minutes.

Use the staining kit (coomassie):
Fixation with Methanol (10 min).
Staining Solution with 16.5 mL DI water, 6 mL Ethanol and 6 mL Stainer A (10 min).
Staining Solution with 1.5 mL Stainer B (15 min).
Destaining with DI water.

1. Place gel on a clean glass plate and rinse with DI water.
2. Excise bands with a clean scalpel (cut as close to the edge of the band as possible).
3. Chop each band into pieces and transfer to the respective well of a 96-well plate, filled with destaining buffer.
4. Destain gel pieces for 15 minutes at 37°C shaking at 300 rpm as many times as until they are destaind or slightly blue; vacuum liquid out every time.
5. If gel pieces turn white (precipitation), add digestion buffer (50mM ABC), let them swell for 10 minutes at 37°C, vacuum liquid out and go on with destaining buffer until gel pieces are destained.
6. Dehydrate gel pieces in 100% ACN for 10 minutes 25°C, shaking. Repeat twice until gel pieces are hard and white, vacuum liquid out.
7. Cover the gel pieces in reduction buffer and incubate for 1 hour at 56°C (without the deep well plate, put the lid on top) to rehydrate and reduce them. Vacuum out all the liquid afterwards.
8. Cover the gel pieces in alkylation buffer and incubate the gel pieces in the dark for 45 minutes at RT. Vacuum out all the liquid.
9. Wash gel pieces once with 50 mM TEAB buffer pH 8 (for the dimethyl labeling afterwards) for 20 minutes at 300 rpm and 25°C. Vacuum out all the liquid.
10. Dehydrate gel pieces twice in 100% ACN for 10 minutes at 25°C, shaking until the gel pieces are hard and white, spin down liquid.
11. Dry the samples without the lid at 37°C until the membrane turns white.
12. Rehydrate the gel pieces in 50 mM TEAB buffer pH 8 (for the dimethyl labeling afterwards). Put the filter-plate onto the deep-well-plate you will extract in the next day, put sealing foil on it, put lid on and incubate the plate overnight at 37°C.

Extraction of peptides:

13. Recover supernatant of trypsin digest by centrifugation at 300 XX for 2 minutes into the deep well plate (Pipette a Tara plate for the following steps!).
14. Extract peptides twice with extraction buffer for 15 minutes at 25°C, shaking at 300 rpm. After each extraction cycle spin down supernatant.
15. Dehydrate gel pieces twice with 100% ACN for 10 minutes at 25°C, shaking at 300 rpm. Spin down supernatant.
16. Transfer peptides into labeled Eppendorf tubes.
17. Dry sample in a speed-vac (mode V-AQ) until 10-20% original volume (100 - 200 µL) to remove acetonitrile (1.5 hours, heat up to 60°C if it takes longer). Speed-vac usually takes up to 2 hours.

Digestion Buffer	50 mM ammonium bi-carbonate (NH4HCO3, ABC) pH 8.0	Dissolve 200 mg ABC in 50 mL water. Store at RM.
TEAB Buffer	50 mM TEAB pH 8.0	1 : 20 dilution of 1 M stock solution. Store at 4°C.
Destaining Buffer	25 mM NH4HCO3 / 50% ethanol (EtOH)	Combine equal volumes of digestion buffer and 100% EtOH. Store at RT.
Reduction Buffer	10 mM DTT (dithiothreitol) in 50 mM ABC	10 µL of 1 M DTT + 990 µL digestion buffer.
Alkylation Buffer	50 mM IAA (iodoacetamide) in 50 mM ABC	Dilute 50 µL of 500 mM IAA aliquot with 450 µL of digestion buffer. Keep in the dark.
Trypsin Buffer	1,000 ng / 160 µL trypsin in 50 mM ABC per 10 µL protein concentration	Prepare immediately before use and keep it always on ice to minimize autocatalysis. Use 1,000 ng/µL stock concentration at -80°C.
Extraction Solution	30% acetonitrile (ACN)	Dilute 20 mL ACN in 70 mL water. Store at RT.

Dimethyl labeling:

1. After extraction of the peptides from the gel reduce the volume down to ~100 µL in the speed-vac. pH should be between 5 - 8.5.
2. Add 4 µL of 4% formaldehyde to each light sample.
3. Add 4 µL of 4% formaldehyde-d2 to each medium sample.
4. Add 4 µL of 4% formaldehyde-C13-d2 to each heavy sample optionally.
   Pipette in a fume hood!
5. Mix briefly, spin down and immediately
6. Add 4 µL of 0.6 M NaBH3CN to each of the light and medium samples and 4 µL of 0.6 M NaBD3CN to the heavy sample, mix briefly and spin down.
7. Incubate 1 hour at 20°C in the fume hood.
8. Quench the labeling reaction with 16 µL of 1% ammonia solution.
9. Mix briefly and spin down. Put on ice.
10. Add 4 µL Formica acid to further quench the reaction and acidify the sample. Perform in a fume hood.
11. Mix the different labels according to your scheme (light + medium + heavy = 1 : 1 : 1).

Light	4 µL of 4% formaldehyde	4 µL of 0.6 M NaBH3CN
Medium	4 µL of 4% formaldehyde-d2	4 µL of 0.6 M NaBH3CN
Heavy	4 µL of 4% formaldehyde-13C-d2	4 µL of 0.6 M NaBD3CN

Material:

50 mM TEAB pH 8.0	1 : 20 dilution of triethylammonium bicarbonate buffer volatile buffer 1 M in LC grade water	Fluka, 17902
4% CH2O	1 : 9.25 dilution 37% formaldehyde solution in LC grade water	Sigma-Aldrich, F8775-4X25ML
4% CD2O	1 : 5 dilution of formaldehyde-d2 solution (~20 wt. % in D2O), in LC grade water	Sigma-Aldrich, 492620-20G
4% C13D2O	1 : 5 dilution of formaldehyde-13C-d2 solution (~20 wt. % in D2O), in LC grade water	Sigma-Aldrich, 596388-1G
0.6 M NaBH3CN	Dilute 10 mg sodium cyanoborohydride in 280 µL LC grade water. Toxic, wear protection and discard in the liquid waste disposal.	Sigma-Aldrich,156159-10G Keep on ice for max. 24 hours.
0.6 M NaBD3CN	Dilute 10mg sodium cyanoborodeuteride in in 280 µL LC grade water, Toxic, wear protection and discard in the liquid waste disposal.	Sigma-Aldrich, 190020-1G Keep on ice for max. 24 hours.
1% ammonia solution	1 : 25 dilution ammonium hydroxide solution (~25%) in LC grade water. Use thin tip from gel loading for easier pipetting.	Sigma-Aldrich, 30501-1L

Stage Tip Purification:

1. Prepare desalting tips using Empore C18 material 2 layers.
2. Activate with 50 µL methanol: shortly spin to 500 xg to see if material is dense, spin for 5 minutes at 500 xg, check if through.
3. Wash with 50 µL buffer B (80% ACN, 0.1% formic acid), 5 minutes at 500 xg, check if through.
4. Wash with 50 µL buffer A (0.1% formic acid), 5 minutes at 500 xg, check if through.
5. Add sample and centrifuge 5 -10 minutes at 500 xg (until no liquid is left).
6. Wash column with 50 µL buffer A, 2 minutes at 500 xg.

At this point samples can be stored.

7. Elute the peptide in 30 µL buffer B using a syringe directly in 24-well-plate fitting the auto sampler or centrifuge using the tip adapter.
8. Speed-vac the plate for 10 minutes to evaporate the ACN (setting V-AQ).
9. Add 8 µL buffer A to end up with 14 µL total volume.
10. Pipette half of the volume to another „back-up“ plate and freeze at -20°C.
11. Measure on the MassSpec.

3.2 Cloning SNP into V19

Digest Primers with Nhe1 and Xho1:

- 25 µL annealed oligos
- 0.5 µL Nhe1
- 0.5 µL Xho1
- 5 µL cutsmart buffer
- 19 µL DI water

Incubate 1 hour at 37°C.

Add to each sample:
- 500 µL Ethanol
- 0.5 µL Glycoblue

Store 10 minutes at -80°C.
Centrifugate for 45 minutes at highest speed.
Remove supernatant and resuspend with 25 µL DI water.
Freeze at -20*C.

Digest luciferase/minP vector:

- ~2.5 ng plasmid (8.27 µL)
- 1 µL Nhe1
- 1 µL Xho1
- 5 µL cutsmart
- 34.73 µL DI water

Incubate 1 hour at 37°C.

Add to each sample:
- 500 µL Ethanol
- 0.5 µL Glycoblue

Store 10 minutes at -80°C.
Centrifugate for 45 minutes at highest speed.
Remove supernatant and resuspend with 30 µL DI water.

Measure the concentration of primers and vector.

Phosphorylation:

- 10 µL plasmid
- 2 µL Antarctic Phosphatase buffer
- 1 µL Antarctic Phosphatase (5 units)
- 7 µL DI water

Incubate for 30 minutes at 37°C.
Heatshock for 2 minutes at 80°C.

Add to each sample:
- 500 µL Ethanol
- 0.5 µL Glycoblue

Store 10 minutes at -80°C.
Centrifugate for 45 minutes at highest speed.
Remove supernatant and resuspend with DI water.

Ligation:

- ~ 100 ng insert
- ~ 50 ng vector
- 0.5 µL 10x T4 Ligase buffer
- 0.5 µL T4 Ligase
- Fill up with DI water to 5 µL.

Incubate at RT over night.

Transformation:

- 10 µL cells
- 5 µL sample

Incubate 30 minutes on ice.
Heatshock at 42°C for 45 seconds.
Incubate 1 - 2 minutes on ice.

Add 250 µL SOC.
Shake at 37°C for 30 minutes.

Use beads to spread on heated agar-plates.
Incubate over night.

Second try:
Use other oligos (with 3, 6 and 9 added basepairs).

3.3 Cloning DHRS2 into V10 and V11

3.3.1 Preparation of the target-plasmid

Creation of Amp-resistant bacteria:

1. Prepare 3 tubes with 5 mL LB solution with Ampicillin.

2. Add vector with ccdB and Amp-resistance-gene (V10 and V11).
3. Let them grow over night shaking at 37°C.
4. Control tube without bacteria should be clear, tubes with V10 and V11 should be cloudy.
5. Throw out control tube.

MiniPrep:

1. Centrifugate for some minutes at very high speed and throw out supernatant.
2. Resuspend pellet with 250 µL Buffer P1, invert 4 - 6 times and transfer into 2 mL Eppi.
3. Add 250 µL Buffer P2 and 350 µL Buffer N3 and centrifugate for 5 minutes.
4. Transfer 800 µL supernatant into column and centrifugate.
5. Wash with 500 µL Buffer PB.
6. Transfer column into 1.5 mL Eppi.
7. Elute DNA into Eppi with 750 µL Buffer EB.
8. Measure the concentration.

Recombination Reaction:

- 0.2 µL entry clone
- 0.2 µL destination vector (V10 and V11)
- 3.5 µL DI water

Vortex Gateway LR Clonase II enzyme mix briefly.
Add 1 µL to the components above.
Vortex and spin down.

Incubate reaction at 25°C (RT) for 1 hour.

Add 0.5 µL Proteinase K solution and incubate at 37°C for 10 minutes.

Transforming competend cells:

- 25 E.coli cells on ice
- 2.5 µL vector solution

Heatshock at 42°C for 45 seconds.
Incubate 1 - 2 minutes on ice.
Add 1,000 µL SOC and let E.coli grow for 30 - 45 minutes at 37°C.

Transfer on agar-plates with Ampicillin using glasbeads and incubate over night.

Replication of bacteria:

1. Prepare 5 tubes with 5 mL LB solution (with Ampicillin).
2. Add colonies (2 colonies per vector in 2 tubes + 1 control).
3. Let bacteria grow over night at 37°C shaking.

MiniPrep:

Throw out control tube.
Safe 100 µL bacteria of each tube.
Follow MiniPrep-instruction above.

Digestion:

- 1 µL enzyme PstI
- 1 µL plasmid (200 ng at least)
- 2 µL cutsmart
- 16 µL DI water

Incubate 1 hour at 37°C.

Plasmids used:
- V10 + ccdB
- V10 + DHRS2 1
- V10 + DHRS2 2
- V11 + ccdB
- V11 + DHRS2 1
- V11 + DHRS2 2

Agarose Gel electrophoresis to control cloning:

Prepare 1.2% Agarose Gel:
- 50 mL DI water
- 0.6 g agarose

Mix and heat up in microwave.
Add 5 µL SYBR safe.
Let cool down in gel shape for 30 minutes.

Load the gel:
1. 5 µL 100bp DNA ladder
2. 12 µL V10 + ccdB + orange loading dye
3. 12 µL V10 + DHRS2 1 + orange loading dye
4. 12 µL V10 2 + DHRS2 + orange loading dye
5. 12 µL V11 + ccdB + orange loading dye
6. 12 µL V11 + DHRS2 1 + orange loading dye
7. 12 µL V11 + DHRS2 2 + orange loading dye

Let it run for 20 minutes at 120 V.

Replication of bacteria:

1. Prepare 2 bottles with ca. 120 mL LB solution with Ampicillin.
2. Add plasmid V10 1 and V11 1 (ca. 100 µL)
3. Let bacteria grow over night at 37°C shaking.

MidiPrep:

Follow instructions of the Sigma-Aldrich-kit. Use 15 mL tubes.
Measure the concentration.

3.3.2 Transfection in cell culture

Prepare growing medium:

DMEM
+ FBS 10%
+ Glutamine 1%

Prepare cell culture:

1. Remove supernatant of the cell culture.
2. Wash the plate.
3. Add trypsin to make cells loose and store it for 1 minute at 37°C.
4. Add about 4 mL growing medium.
5. Centrifugate at 300 rpm for 5 minutes.
6. Remove supernatant and resuspend pellet with 3 mL growing medium.
7. Measure the number of cells.
8. Put 9 - 10 mL growing medium onto a new plate and add the desired number of cells.
9. Store it at 37°C.

Prepare the plasmids and transfect:

Use V10 (because of its GFP-tag) and a positive cotrol vector with GFP-tag.

1. Fill 4 Eppis with 480 µL DMEM.
2. Add 48 µL PEI to two of them.
3. Add 12 µg V10 to the third and 12 µg positive control vector to the last Eppi.
4. Incubate them for 5 minutes.
5. Add Eppis with PEI to Eppis with the plasmid.
6. Incubate for 15 - 20 minutes.
7. Transfer solution dropwise to cell culture plate.
8. Incubate over night.

Localization of proteins:

Take pictures of the cells under a microscope in brightfield and with laser. Try to take the pictures as sharp as possible to get more details.

4. Results

4.1 Agarose Gel to control DNA length

Loading from left to right:
1. 100 bp DNA ladder
2. 509 V1
3. 509 V2
4. 803 V1
5. 803 V2
6. 1170 V1
7. 1170 V2

A ladder-shaped pattern is visible, which means, that the polymerization worked. The SNPs ligated to each other, building up strands with different lengths.

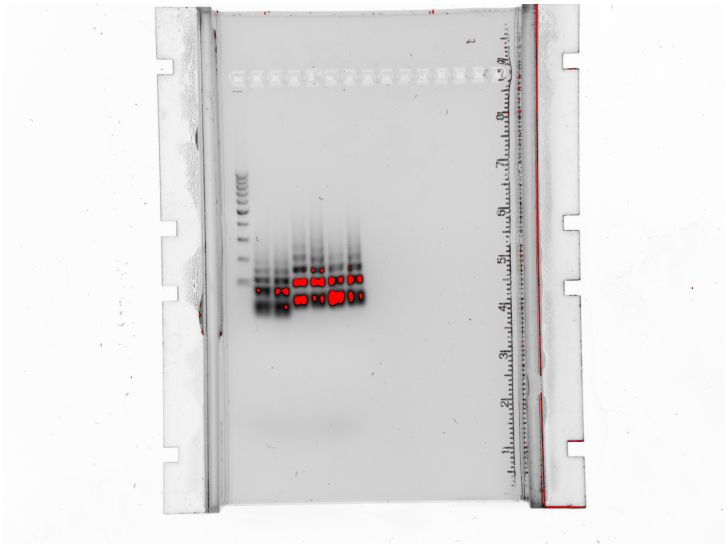

Figure 4.1.1: agarose gel

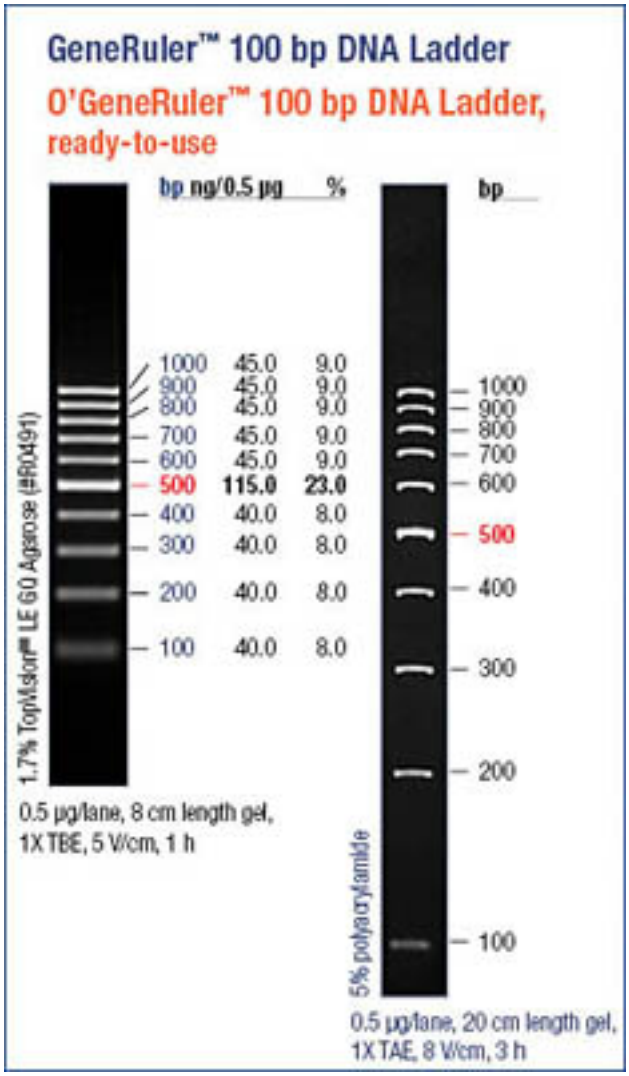

Figure 4.1.2: GeneRuler™ 100bp DNA Ladder

4.2 Bradford measurement protein concentration

standard curve	concentration ug/ul	d1	d2	average	
s1		0	0	0	0
s2		0,125	0,143	0,151	0,147
s3		0,25	0,274	0,271	0,2725
s4		0,5	0,548	0,549	0,5485
s5		0,75	0,695	0,723	0,709
s6		1	1,016	1,042	1,029

	A	B
NE1	0,199	0,11
NE2	0,178	0,111
NE3	0,172	0,107
average	0,183	0,1093333
m	1,0163	1,0163
concentration dil 10	0,180064941	0,1075798
final concentration	1,800649415	1,0757978

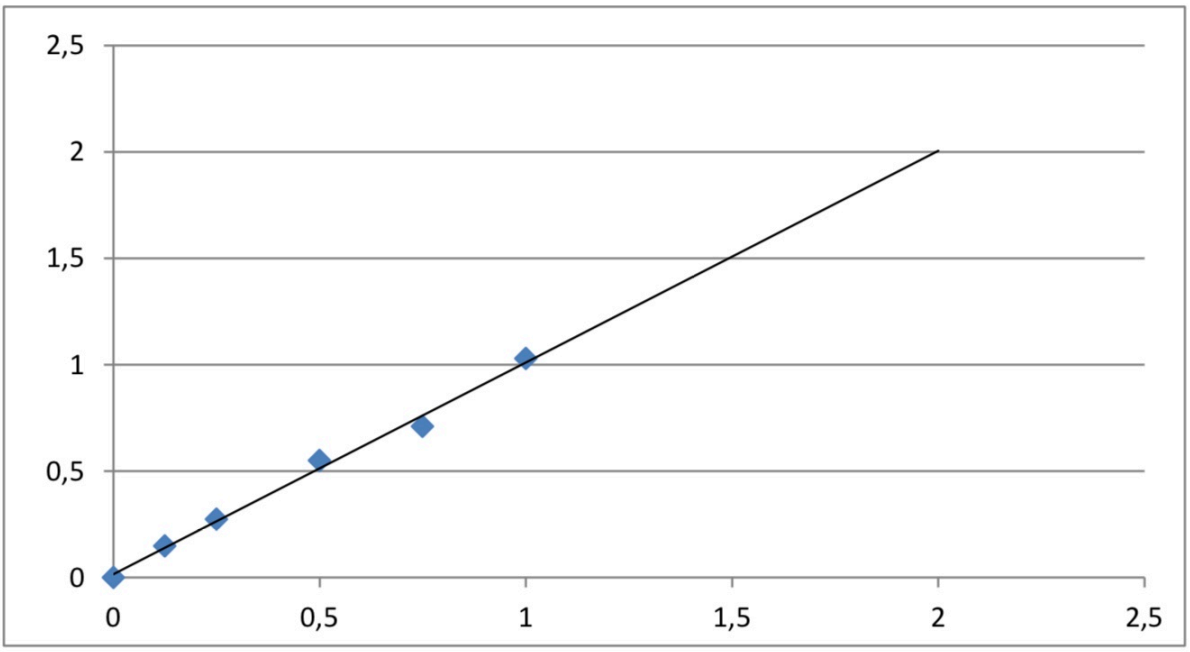

Figure 4.2.1: Regression line

By measuring the absorption of different protein concentrations, it was possible to create a regression line. Using this line we were able to deduce the unknown concentration of our sample from measuring the absorption of them as well.
a = absorption
c = concentration

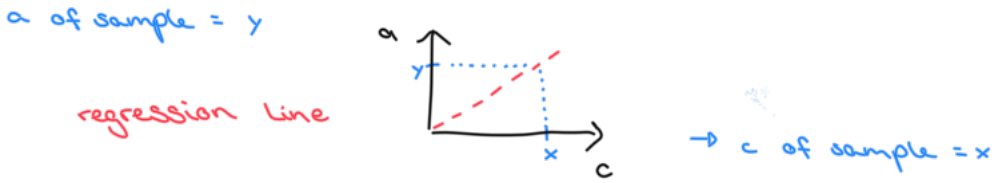

Figure 4.2.2: Function of Bradford measurement

4.3 Western Blot

Loading from left to right:
1. ladder
2. Input (21.03.18 Ne extract 10% = 20 µL)
3. SNP 509 V1
4. SNP 509 V2

In Figure 4.3.1 all the proteins stained with ponceau are visible. Because protein SP1, which we desire to see, is about 81 kDa, it becomes clear, that it is not visible in the first picture we took. In Figure 4.3.2 on the other hand making SP1 visible with the help of antibodies worked out well. You can see it in the input line, which means, that SP1 is existing in the used cell line in general. Also it is clearly visible in the SNP 509 V1 line, but almost completely not in the SNP 509 V2 line. So we can conclude, that the binding of SP1 to the SNPs 509 V1 and V2 differs. The next step would be now, to measure the impact of SP1 as a transcription factor.

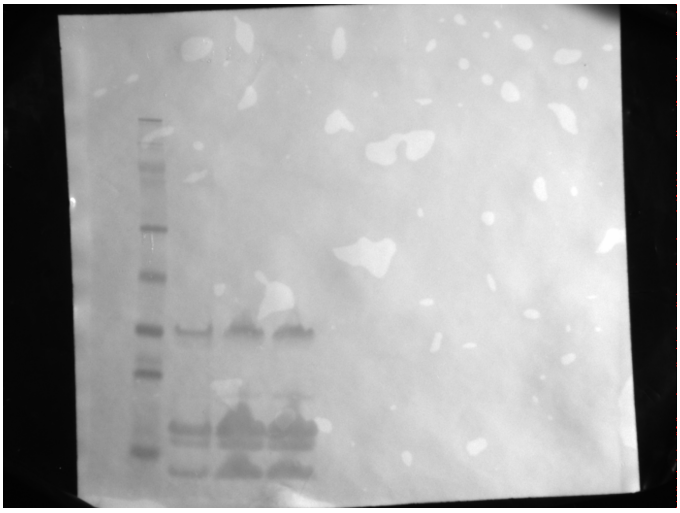

Figure 4.3.1: Western Blot membrane

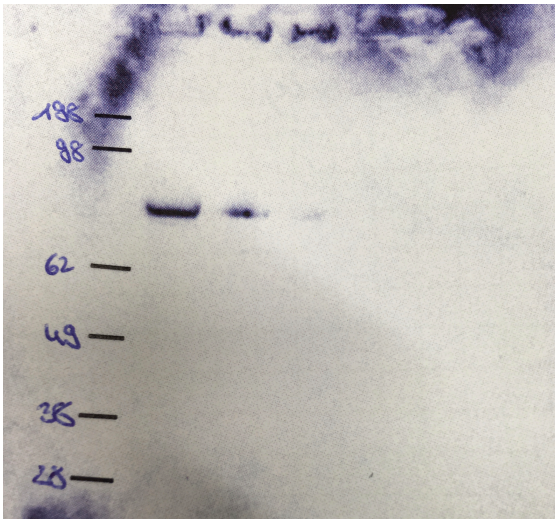

Figure 4.3.2: Western Blot + SP1 antibodies and ladder

4.4 In Gel digestion

Figure 4.4.1:
1. SNP 509 V1
2. SNP 509 V2
3. Ladder
4. SNP 803 V1
5. SNP 803 V2
6. SNP 1170 V1
7. SNP 1170 V2

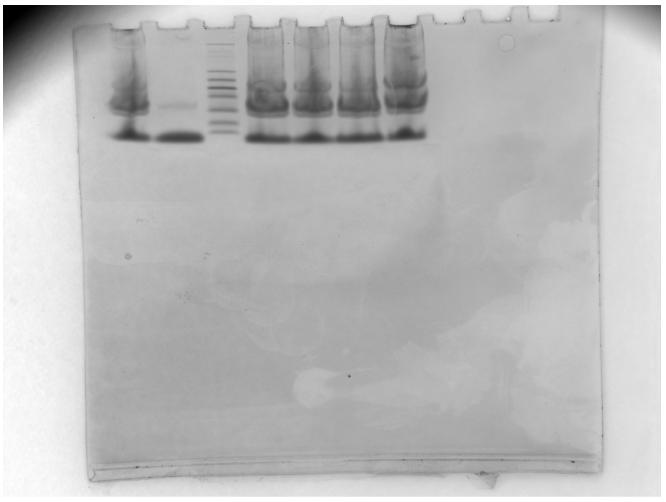

Figure 4.4.1: In Gel digestion - 1

Figure 4.4.2:
1. Ladder
2. SNP 509 V1
3. SNP 509 V2
4. SNP 803 V1
5. SNP 803 V2
6. SNP 1170 V1
7. SNP 1170 V2

In Figure 4.4.1 and 4.4.2 all proteins, which bound to the SNPs during the pulldown and are stained with coomassie, are visible. We did it two times, in case there might be a problem with one sample. Through the pictures it becomes clear, that there are proteins of different sizes binding to them.

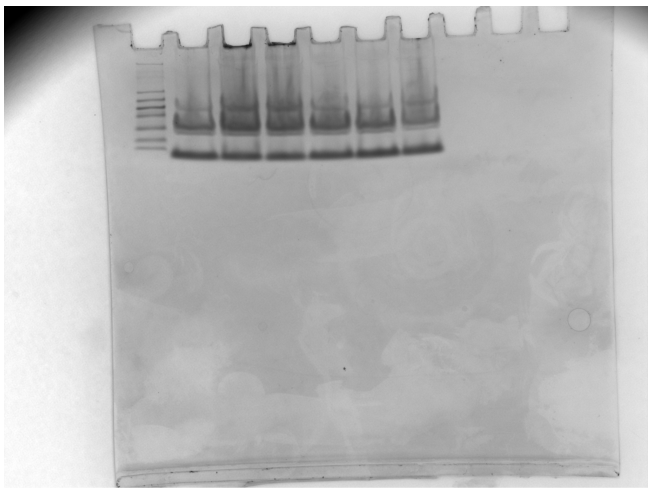

Figure 4.4.2: In Gel digestion - 2

4.5 Results of Cloning SNP into V19

First try:
No colonies visible.

Second try:
No colonies visible.

Because there have been no colonies on our plates in the first try, it meant that the cloning did not work properly. So we had to figure out, which step caused the problem. Our first idea was, that the enzymes may not have enough space to bind to the SNPs to cut them. So for the second try we ordered three new SNPs of 509 V1, which had 3, 6 and 9 basepairs additionally added at their ends. But unfortunately you could not find any colonies growing as well. Only on the plate with the digested vector, our positive control, some colonies showed up. Our next thought was now, that the enzyme XhoI has been too old and was not working anymore, so it had to be replaced with a new one. Because my internship was over at this point, it was not possible for me to tell, whether it worked out with that or not. If it worked, they will be trying to figure out, whether the SNP-binding protein (for example SP1) leads to up- or downregulation of the luciferase-gene.

4.6 Results of Cloning DHRS2 into V10 and V11

Agarose gel electrophoresis:

Loading from left to right:
1. 100 bp DNA ladder
2. V10 + ccdB
3. V10 + DHRS2 1
4. V10 + DHRS2 2
5. V11 + ccdB
6. V11 + DHRS2 1
7. V11 + DHRS2 2

For the digestion of the cloned vectors, we chose an enzyme cutting only a few times. With the gel electrophoresis (Figure 4.6.1) we have been able to control, whether our cloning worked properly by comparing the length of the cutted out DNA-strands. So there is an obvious difference between V10 + ccdB and V10 + DHRS2 1, which confirms the assumption, that there is actually DHRS2 cloned in. If not, the pattern should look the same, which is unfortunately the case at V10 + DHRS2 2. There the cloning did not work and it should not be used for next steps. For V11 it becomes clear, that cloning did work in both cases.

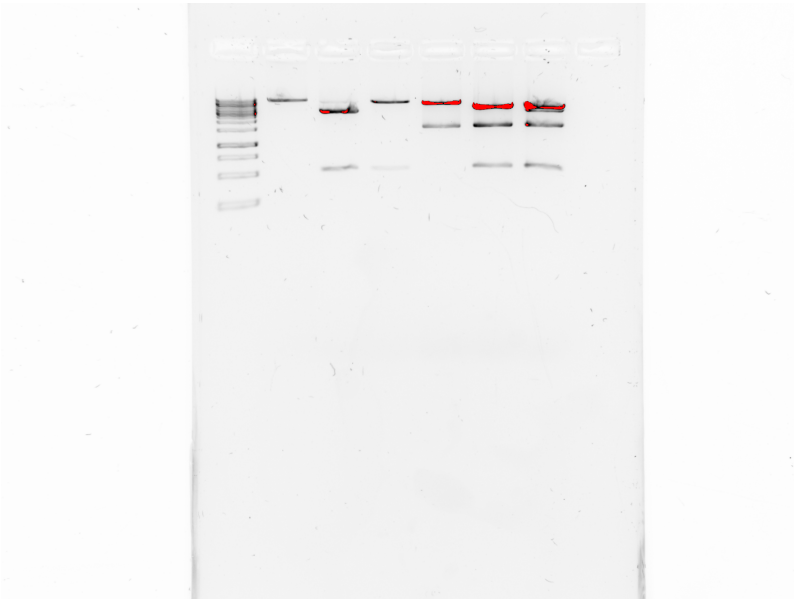

Figure 4.6.1: Agarose gel electrophoresis with V10 & V11

Localization of proteins:

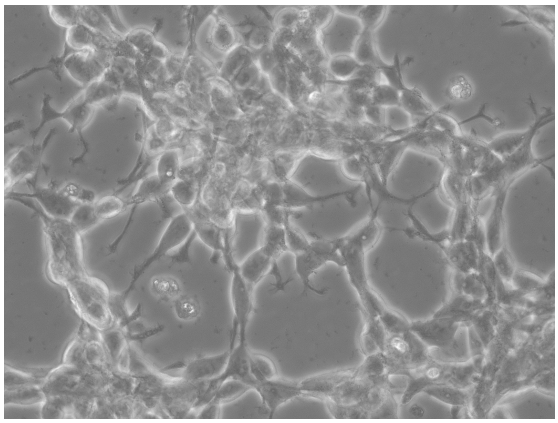

Figure 4.6.2a: HEK DHRS2 Brightfield

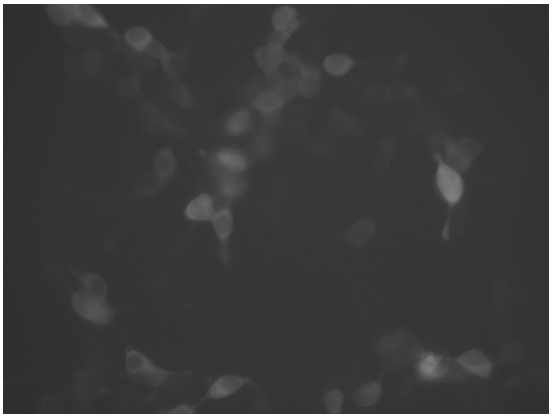

Figure 4.6.2b: HEK DHRS2 GFP laser

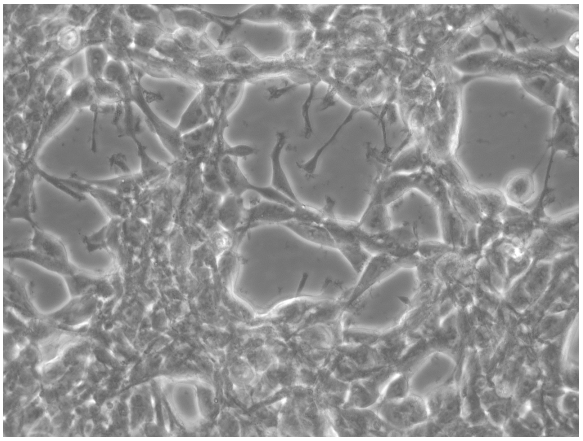

Figure 4.6.3a: HEK Control Brightfield

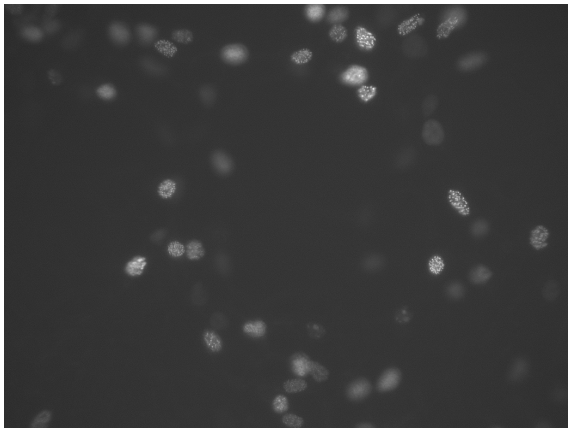

Figure 4.6.3b: HEK Control GFP laser

In figure 4.6.2a HEK cells with DHRS2 are visible in brightfield. In figure 4.6.2b we see the localization of the DHRS2 protein in the same cells through GFP-tag and laser. There it is noticeable that the protein is mostly located in the cell core, but also in some other parts of the cell as well.
As a comparison we took pictures of a control protein. We know, that it is located in the cell core, so it is comprehensible, that figure 4.6.3b looks quite similar to the DHRS2 laser picture. But at the same time it is spread more punctually within the core and not at all outside of it.

5. Discussion

1. Identification of DNA-binding proteins
   Though all results are preliminary and the project is to be continued, you can already see that the polymerization of the SNPs and the Western Blot worked. Also there is the desired binding difference of the protein SP1 between SNP 509 V1 and V2. The impact of SP1 as a transcription factor has to be discovered in future research.
   Furthermore we found out that there are a lot of proteins binding to the three SNPs we used. These proteins are going to be identified by mass spectrometry.
2. Cloning SNP into V19
   In our second project we have not been able to get positive results to discuss. The next step would be to try the digestion with new enzymes to check, if that has been the problem.
3. Cloning DHRS2 into V10 and V11
   In the results we have seen that the gene exchange worked in three out of four cases. So we could verify the localization of the DHRS2 protein.

6. Conclusion

In these four weeks during my internship I was able to learn a lot of practical things in a laboratory. I really enjoyed to get from the theory we learned in school to the application in real research. It was great to learn new techniques and to be allowed to use them for parts of the project autonomously. Also I got an insight of the work of a PhD student, which I would like to do as well after studying.
I am really thankful, that Vivien gave me so much of her time to answer my question, explain the theoretical background of our experiments and show me how to perform them. Also everyone else in the Butter group was so welcoming and did not hesitate to introduce me to their work, which I am really grateful for. Of course I also want to thank the *Förderverein der Internationalen BiologieOlympiade e.V.* for enabling me to do this internship. Now I can tell, that biochemistry will be the right direction for me.

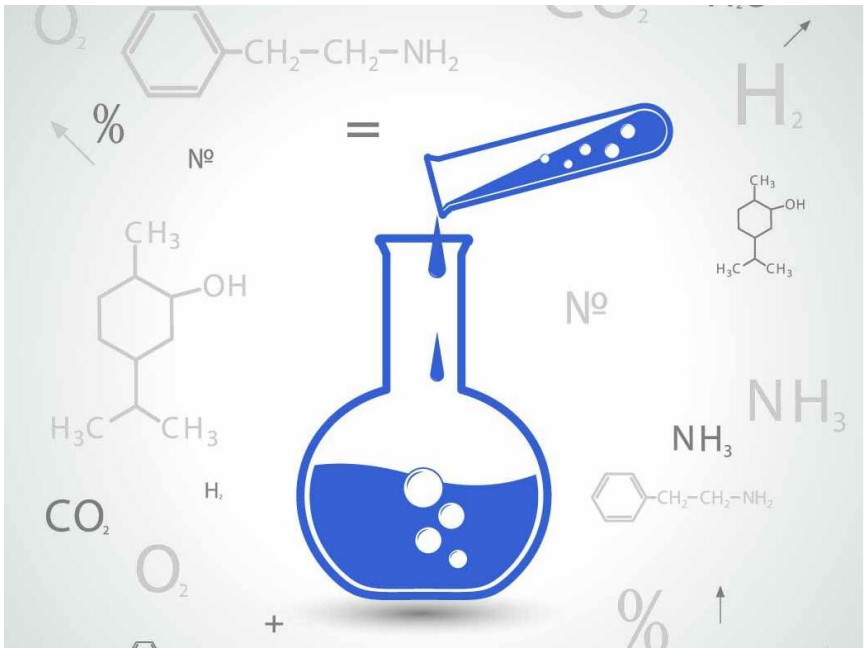

Figure 6.1: biochemistry

7. References

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