METHODS

iGEM TU DARMSTADT
2019

Content

A. In vitro

I. Cloning

a) Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a precise method for exponentially amplifying a fragment of DNA (from a mixture of DNA molecules) in vitro.

Standard PCR is a method that is used to amplify DNA sequences of various lengths with a thermostable polymerase (Taq-Polymerase) with proofreading function.

Touchdown PCR is variation of Standard PCR. The annealing temperature for the primers is not constant during the PCR but decreases by 1 degree per cycle in the first cycles in order to avoid unspecific primer binding. (Phusion-Polymerase)

Mutagenesis PCR is used in order to introduce point mutations, insertions or deletions into DNA sequences of interest. For this purpose, specific primers are designed. (Q5-Polymerase)

Taq DNA Polymerase

Component	25 µl Reaction	50 µl Reaction	Final [Conc]:
10X Taq Reaction Buffer	2.5 µl	5.0 µl	1X
10 mM dNTPs	0.5 µl	1.0 µl	200 µM
10 µM Forward Primer	0.5 µl	1.0 µl	0.2 µM
10 µM Reverse Primer	0.5 µl	1.0 µl	0.2 µM
Template DNA	variable	variable	< 1,000 ng
Nuclease-free water (MQ)	to 25 µl	to 50 µl	
OneTaq® DNA Polymerase	0.5 µl	1.0 µl	1.25 units/50 µl

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95 °C	0:30 min.	1 cycle
Denaturation	95 °C	0:15 – 0:30 min.	
Annealing*	45 – 68 °C	0:15 – 1:00 min.	30 cycles
Extension	68 °C	1:00 min. / kb	
Final Extension	68 °C	5:00 min.	1 cycle
Hold	4 – 10 °C	Indefinite	1 cycle

Phusion High-Fidelity DNA Polymerase

Component	20 µl Reaction	50 µl Reaction	Final [Conc]:
5X Phusion HF or GC Buffer	4.0 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1.0 µl	200 µM
10 µM Forward Primer	1.0 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.0 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 250 ng
DMSO (optional)	(0.6 µl)	(1.5 µl)	3 %
Nuclease-free water (MQ)	to 20 µl	to 50 µl	
Phusion	0.2 µl	0.5 µl	1.0 units/50 µl

Step	Temperature	Time	Number of Cycles
Initial Denaturation	98 °C	0:30 min.	1 cycle
Denaturation	98 °C	0: 05 – 0:10 min.	
Annealing*	45 – 72 °C	0:10 – 0:30 min.	25 – 35 cycles
Extension	72 °C	0:15 – 0:30 min. / kb	
Final Extension	72 °C	5:00 – 10:00 min.	1 cycle
Hold	4 – 10 °C	Indefinite	1 cycle

Q5 High-Fidelity PCR

Component:	25 µl Reaction	50 µl Reaction	Final [Conc]:
Q5 Reaction Buffer 5x	5.0 µl	10 µl	1x
dNTPs (10mM)	0.5 µl	1 µl	200 µM
Forward Primer (10µM)	1.25 µl	2.5 µl	0.5 µM
Reverse Primer (10µM)	1.25 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	1 ng/µL
(Q5 High GC Enhancer)	(5.0 µl)	(10 µl)	(1x)
Nuclease-free water (MQ)	to 25 µl	to 50 µl	(to 50 µl)
Q5 DNA Polymerase	0.25 µl	0.5 µl	0.02 U/µl

Step	Temperature	Time	Number of Cycles
Initial Denaturation	98 °C	0:30 min.	1 cycle
Denaturation	98 °C	0: 50 – 0:10 min.	
Annealing*	50 – 72 °C	0:10 – 0:30 min.	25 – 35 cycles
Extension	72 °C	0:20 – 0:30 min. /kb	
Final Extension	72 °C	2:00 min.	1 cycle
Hold	12 °C	Indefinite	1 cycle

* The annealing temperature for a specific amplification reaction will depend on the sequences of the two primers.

b) Restriction digest

In order to insert DNA fragments into plasmids via ligation, it is necessary to digest both components with restriction enzymes.

Single DNA Digestion

The following is an example of a typical analytical single restriction enzyme digestion:

1. Add up the following:

Plasmid DNA..... 500 ng
10x appropriate NEB-Buffer..... 5 µl
Restriction enzyme (10U)……...1 µl
ddH_2O.....to 50 µl

2. Incubate for at least 30 minutes (depends on the restriction enzyme) at 37 °C
3. Heat inactivation: Incubate at 80 °C for 20 minutes

Multiple Restriction Enzyme Digest

Use the optimal Buffer supplied with one enzyme if the activity of the second enzyme is acceptable in that same Buffer (check table supplied by NEB). Follow the single restriction enzyme digestion by using 1 µl of the additional enzyme and take off 1 µl from the nuclease-free water.

c) Dephosphorylation

Antartic Phosphatase catalyzes the removal of 5'-phosphate groups of DNA/RNA and thus prevents re-ligation of cut vectors. It is used before ligation.

Procedure

1. Reaction Mix
- Restriction product
- 1/10 of reaction end volume 10x Antarctic Phosphatase Reaction Buffer
- 1 µl of Antarctic Phosphatase

2. Incubate at 37 °C for 30 minutes
3. Heat inactivation: Incubate at 80 °C for 2 minutes
4. Continue with ligation

d) DNA Ligation

DNA ligation is necessary to assemble digested DNA parts into a vector. The cut ends generated by restriction enzymes are put together by DNA ligase.

Procedure

1. Reaction Mix
- T4 Ligase Buffer..... 2 µl
- T4 Ligase..... 1 µl
- Digested Insert..... 6 µl
- Digested Backbone..... 2 µl
- ddH_2O.....to 20 µl

The ratio between Insert and Vector should be 3:1

2. Incubate at 16 °C overnight or at room temperature for 30 minutes (results might be worse)
3. Inactivate at 65 °C for 10 minutes

e) Deletion

- Plasmid (250 ng) ..... 0,5 µl
- fw Primer..... 2,5 µl
- rv Primer..... 2,5 µl
- Q5 Master Mix..... 25 µl
- ddH_2O.....to 50 µl

Step	Temperature	Time	Number of Cycles
Initial Denaturation	98 °C	0:30 min.	1 cycle
Denaturation	98 °C	0:10 min.	
Annealing*	55 °C	0:30 min.	30 cycles
Extension	72 °C	0:30 min. /kb	
Final Extension	72 °C	2:00 min.	1 cycle
Hold	12 °C	Indefinite	1 cycle

f) Ligase Cycling Reaction (LCR) [1] [2]

The Ligase Cycling reaction (LCR) is a cloning method used in synthetic biology. The method combines standard PCR and a variation of ligation processes by utilizing non-overlapping DNA parts that are ligated together by using so called bridging oligos (SOCs).

1. Primer Phosphorylation

Mix suitable primers according to the following table. Final reaction volume is 50 µl.

Component	Amount (µl)	Final concentration
100 µM fw Primer	1,5	3 µM
100 µM rv Primer	1,5	3 µM
10 U/µl T4 Polynucleotide Kinase	1	0.2 U
10x T4 Ligase Buffer	5	1x
ddH_2O	41	
Total	50	

Incubation: 37 °C for 30 minutes

2. PCR

Use following table for upscaling PCRs with phosphorylated primers. The top row is the number of reactions and all units are in µl. Final reaction volume is 50 µl.

	1	**2**	**4**	**6**
DNA-Template (Plasmid [20ng])	1	2	4	6
Phosphorylated primer mix	8	16	32	48
dNTPs	1	2	4	6
HF or GC Buffer	10	20	40	60
H_2O	29,5	59	118	177
Phusion	0,5	1	2	3

3. Gel Extraction

4. Dilution

The PCR products need to get diluted to 12 nM by using the following formula:

$$molar\ concentration\ [nM] = (c\ [ng/\mu l] * 10^6) / (650 * DNA\ length\ in\ bp)$$

5. Producing Scaffold oligonucleotide connector (SOC)-Mix (each SOC should be present with a concentration of 100 nM)

6. Cycled ligation reaction (LCR) set-up

According to the following table. Final reaction volume is 20 µl.

Component	Amount (µl)	Final concentration
12 nM DNA-Parts (backbone, insert)	4	3 nM
100nM SOC-Mix	6	30 nM
10x Taq Ligase Buffer	2	1x
40 U/µl Taq DNA Ligase	2	80U
H_2O	6	
Total	20	

7. Cycler program

De KOK-PROTOCOL:

Time	Temperature [°C]	Cycles
2 min	94	1
10 sec	94	50
30 sec	55	
60 sec	66	
Incubation	4	hold

8. Dpn1-Digest

Use following table. Final reaction volume is 30 µl.

LCR product	20 µl
Cutsmart Buffer	3 µl
Dpn1	1 µl
H_2O	6 µl

Incubation: 37 °C for 60 minutes

g) Gibson Assembly [3]

Gibson Assembly has been one of our methods of choice in order to insert DNA fragments into a variety of vectors. The purified insert(s) were diluted and then mixed to fit the molar concentrations and ratios recommend. Common volumes used were 4 µl of DNA solution which were then added to an identical volume of Gibson Assembly Master Mix. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Store at -20 °C.

h) Cloning Buffer

Tris (1 M, pH 7.5)

- Tris base .....60.5 g
- ddH_2O .....to 500 ml

Adjust pH to 7.5 add the needed amount of 5 M HCl. Store at room temperature.

Tris Buffer (1000ml)

- Tris HCl.....100 mM
- NaCl .....150 mM
- EDTA .....1 mM
- ddH_2O .....to 1000 ml

Adjust to pH 8, add the needed amount of HCl.

1 x PBS

- NaCl (140 mM) .....8.18 g
- KCl (2.7 mM).....0.2 g
- Na_2HPO_4 (10 mM) ....1.77 g
- KH_2PO_4 (1.8 mM).....0.24 g
- ddH_2O .....to 1000 ml

Adjust to pH 7.4, add the needed amount of HCl. tore at room temperature

TE Buffer

- Tris HCl.....10 mM
- EDTA .....1 mM
  (Ethylenediaminetetraacetic acid)

Adjust to pH 8.0, add the needed amount of HCl. Store at room temperature.

50 x TAE

- Tris base.....242 g
  mix Tris with stir bar to dissolve in about 600 ml ddH_2O
- glacial acetic acid..... 57.1 ml
- EDTA solution (500 mM, pH 8.0)..100 ml
  add EDTA and Acetic Acid
- ddH_2O ..... to 1000 ml

Store at room temperature.

II. Analysis and Purification

a) Agarose gel electrophoresis

Agarose gel electrophoresis is the most common used method to separate nucleic acids. Due to their negative charge DNA and RNA molecules can be moved through an agarose gel by an electric field (electrophoresis). Longer molecules move slower through the agarose matrix while short DNA fragments move faster and migrate further.

Procedure

In common we used 0.8 - 2.0 % agarose gels. Low concentrated gels lead to better results for large DNA fragments (2-6 kbp), while high concentrated gels lead to better results for small DNA fragments (0.3 – 0.7 kbp).

1. Mix desired amount of agarose with 1x TAE-Buffer.
2. Heat up liquid in microwave until whole agarose is dissolved.
3. Let liquid cool down until you can touch the bottle with your hands. Add a certain amount of Gel Stain (the tube normally contains information about the exact amount).
4. Fill mixture into gel chamber and let it cool down (!!do not forget the well combs!!).
5. Fill-up chamber with 1x TAE-Buffer.
6. Take off well comb.
7. Pipette 3-4 µl DNA ladder of choice into first pocket.
8. Mix samples 5:1 with 6x loading dye (5 µl sample with 1 µl loading dye) and pipette Into pockets.
9. Run electrophoresis with 120 V for 45-60 minutes.

b) Gel extraction Clean-UP

Gel Clean-Up Systems are used to remove unincorporated primers, salts and leftover dNTP's from generated amplicons after PCR.

Protocol: GeneJET Gel Extraction Kit (Thermo Scientific)

1. Excise gel slice containing the DNA fragment using a clean scalpel or razor blade. Cut as close to the DNA as possible to minimize the gel volume. Place the gel slice into a pre-weighed 1.5 mL tube and weigh. Record the weight of the gel slice.

Note: If the purified fragment will be used for cloning reactions, avoid damaging the DNA through UV light exposure. Minimize UV exposure to a few seconds or keep the gel slice on a glass or plastic plate during UV illumination.

2. Add 1:1 volume of Binding Buffer to the gel slice (volume: weight) (e.g., add 100 µL of Binding Buffer for every 100 mg of agarose gel).
3. Incubate the gel mixture at 50-60 °C for 10 minutes or until the gel slice is completely

dissolved. Mix the tube by inversion every few minutes to facilitate the melting process. Ensure that the gel is completely dissolved. Vortex the gel mixture briefly before loading on the column. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.

4. Transfer up to 800 µL of the solubilized gel solution to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.

Note:

 If the total volume exceeds 800 µL, the solution can be added to the column in stages. After each application, centrifuge the column for 30-60 s and discard the flow-through after each spin. Repeat until the entire volume has been applied to the column membrane. Do not exceed 1 g of total agarose gel per column.

 Close the bag with GeneJET Purification Columns tightly after each use!

5. *Optional:* Use this additional binding step only if the purified DNA will be used for sequencing. Add 100 µL of Binding Buffer to the GeneJET purification column. Centrifuge for 1 minute. Discard the flow-through and place the column back into the same collection tube.
6. Add 700 µL of Wash Buffer (diluted with ethanol!) to the GeneJET purification column. Centrifuge for 1 minute. Discard the flow-through and place the column back into the same collection tube.
7. Centrifuge the empty GeneJET purification column for an additional 1 minute to completely remove residual Wash Buffer.
8. Transfer the GeneJET purification column into a clean 1.5 mL microcentrifuge tube (not included). Add 50 µL of Elution Buffer to the center of the purification column membrane. Centrifuge for 1 minute.

Note:

 For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended.

 If DNA fragment is >10 kb, prewarm Elution Buffer to 65 °C before applying to column.

 If the elution volume is 10 µL and DNA amount is ≤ 5 µg, incubate column for 1 min at room temperature before centrifugation.

9. Discard the GeneJET purification column and store the purified DNA at -20 °C.

c) PCR Clean-UP

PCR Clean-Up Systems are used to remove unincorporated primers, salts and leftover dNTP's from generated amplicons after PCR.

Protocol: GeneJET PCR Purification Kit (Thermo Scientific)

1. Add a 1:1 volume of Binding Buffer to completed PCR mixture (e.g. for every 100 µL of reaction mixture, add 100 µL of Binding Buffer). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.

2. Transfer up to 800 µL of the solution from step 1 to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through.

Notes: If the total volume exceeds 800 µL, the solution can be added to the column in stages. After the addition of 800 µL of solution, centrifuge the column for 30-60 s and discard flowthrough. Repeat until the entire solution has been added to the column membrane.

3. Add 700 µL of Wash Buffer (diluted with the ethanol!) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through and place the purification column back into the collection tube.
4. Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any residual Wash Buffer.
5. Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube (not included). Add 50 µL of Elution Buffer to the center of the GeneJET purification column membrane and centrifuge for 1 min.

Note:

 For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended.

 If DNA fragment is >10 kb, prewarm Elution Buffer to 65 °C before applying to column.

 If the elution volume is 10 µL and DNA amount is ≥5 µg, incubate column for 1 min at room temperature before centrifugation.

6. Discard the GeneJET purification column and store the purified DNA at -20 °C.

d) Plasmid Miniprep

Follow the steps shown on the right side.

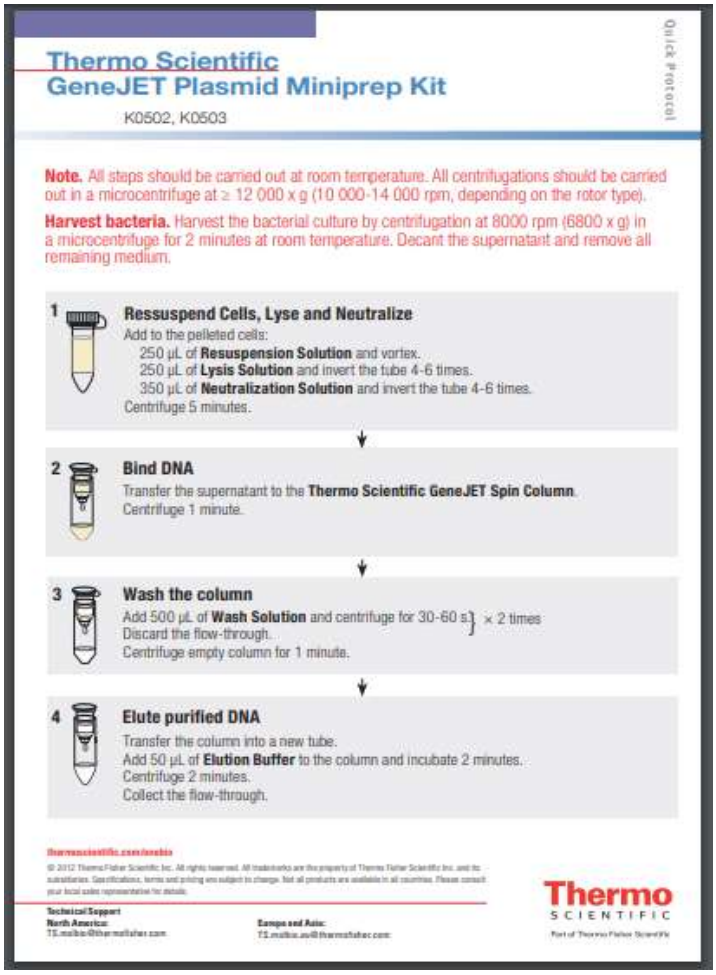

Abb. 1: Plasmid Miniprep Protocol [1]

[1] https://assets.thermofisher.com/TFSAssets/LSG/manuals/MAN0013117_GeneJET_Plasmid_Miniprep_UG.pdf

e) DNA Quantification with NanoDrop

NanoDrop is as UV-Vis spectrophotometer which can be used to measure quantity and purity of DNA in a sample using only 1-2 µl.

Procedure

1. Start the program for the NanoDrop and choose "Nucleic Acids"
2. Pipette 1 µl of a water sample onto the lower measurement and click "OK" for initializing
3. Load your blank and click "Blank"
4. Load your DNA samples and click "Measure"

The system calculates automatically the concentration in ng/µl, the 260/280 value and the 230/260 value.

f) Sequencing

Microsynth (Sanger-Sequencing)

DNA-Sequencing is a method to determine the order of nucleotides in DNA. With this method you can check if your construct of interest is in your plasmid and whether there are mutations or not.

Procedure

1. Pipette µl of DNA into a 1.5 ml reaction tube; for plasmids the concentration of DNA should be between 40 and 100 ng/µl. For PCR products the concentration should be 1.5 ng/µl per 100 bp.
2. Add 3 µl Primer (10 µM). Do only use one Primer per Tube!
3. Each reaction tube is labeled with a microsynth-barcode.
4. Now the reaction tubes are put into a transparent plastic bag for transportation. In our case you can toss it into the specific mailbox on our campus.
5. After all, fill in your order form on *www.microsynth.ch*

g) SDS-Page

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) is an analytic method to separate proteins by their molecular mass.

Preparations:

Lower Tris (Separation Buffer):
- 1,5 M Tris
- 0,4 % SDS
- Adjust pH-value to 8.8

Upper Tris (Stacking Buffer):
- 0,5 M Tris
- 0,4 % SDS
- Adjust pH-value to 6.6

Running Buffer (1 L 10x):

- 30.2 g Tris
- 144 g Glycine
- 1% SDS → 10 g

Buffer W:

- 150 mM NaCl
- 100 mM Tris adjust pH-value to 8
- optional: 1 mM EDTA

Procedure:

Prepare gel:

- Insert Shortplate and Longplate in bracket
- Put bracket in clamping device
- Prepare Separation Buffer and Stacking Buffer
- Prepare two 15 mL Falcons

Mixture for 12 % Separation gel and 2 gels in total (should get adjusted to protein size):

	Separation gel	**Stacking gel**
ddH_2O	3,94 mL	2,65 mL
Upper Tris	2,79 mL	-
Lower Tris	-	1,25 mL
Acrylamide	4,6 mL	0,83 mL
TEMED	7 µL	10 µL
APS	170 µL	200 µL

- First prepare separation gel
- Add TEMED and APS last
- Invert 3 times
- Fill in Separation gel
- Pour over with isopropanol
- Wait until separation gel is polymerized
- Discard isopropanol
- Prepare stacking gel
- Add TEMED and APS last
- Invert 3 times
- Fill in stacking gel up to the rim
- Stick in the comb
- Wait until stacking gel is polymerized
- If not used immediately, store gel in wet cloth (to prevent dehydration) at 4°C
- If used immediately, remove comb and place gel into the SDS PAGE chamber
- Fill up chamber with running Buffer

Prepare samples:

- Dilute samples with Buffer W (5 µL sample + 5 µL Buffer W)
- Dilute samples with Laemmli Buffer (2x Laemmli) 10 µL
- Heat up samples at 95°C for 5 min
- Apply 20 µL of each sample into the pockets
- Load one pocket with commercial protein marker

Run gel:

- Stacking gel: 90 V
- Separation gel: 120 V

Staining and washing of gel:

- Get gel out of the glass plates
- Cut off stacking gel
- Put separation gel into Staining Buffer and shake at room temperature for at least one hour or heat it in the microwave
- Put stained separation gel into ddH2O and let shake for 10 min
- Repeat last step at least two times with fresh ddH2O
- Take a picture of the gel at an imager

h) Western Blot

The “Blotting” is a widely used method in analytics to transfer proteins to a support membrane. Afterwards they can get verified via different kind of reactions.

1. Place nitrocellulose membrane in a sandwich consisting of sponges, Whatman paper and the SDS Page.
2. Align the sandwich with the membrane facing the anode and the gel facing the cathode.
3. Blot the membrane for 1.5 hours with 200 mA in Western Blot transfer Buffer (25 mM Tris/HCL, 200 mM glycine, 3.5 mM SDS, 20% (v/v) EtOH and 70% H_2O).
4. Use ponceau staining to determine whether the protein transfer was successful.
5. Remove ponceau staining by washing with purified water.
6. Block the membrane with PBS-blocking Buffer (PBS Buffer with 3% BSA and 0.05% v/v Tween 20) for one hour or overnight.
7. Wash the membrane 3 times for 5 minutes using PBS-tween Buffer (PBS Buffer with 0.1% v/v Tween 20).
8. Add 10 ml PBS-tween Buffer to the membrane
9. Pre-dilute Strep-Tactin horse radish peroxidase conjugate (rom Iba) 1:100 in Enzyme dilution Buffer (PBS with 0.2% BSA and 0.1% Tween) and add 10 µl to 10 ml PBS-tween.
10. Incubate 60 minutes at room temperature, gentle shaking.
11. Wash the membrane once for 15 minutes and 3 times for 5 minutes using PBS-tween.
12. Visualize the blot by using a detection reagent (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare).
13. Generate image using ImageLab (Bio-Rad Laboratories, Inc.).

i) HPLC

High-performance liquid chromatography is method in analytics (chemistry) to characterize a sample regarding size and quantity.

1. Put 100µl in a vile.
2. Insert the vile into the HPLC detector.
3. Adjust injected volume and measure for 30 minutes having a gradient from 0-100% azetonitril.

j) VLP *in vitro*/*in vivo* assembly

In vitro

1. Purified coat protein (CP) and purified scaffold protein (SP) is defrosted on ice.
2. SP and CP are combined in a molar ration of 1:2.8 (reaction volume up to 500 µl worked well in ultracentrifugation).
3. Incubation for 20 h at 20°C.

In vivo

1. Grow VLP producing *E. coli* cells in LB Media (at least 2 L for 4 h).
2. Harvest cells at minimum 6000g for 20 minutes.
3. Resuspend in as little as possible lysis Buffer (50mM Tris, 150mM NaCl, pH 7,4) → ideally less than 5 mL.
4. Lyse cells by Sonication (use Eppi for efficiency; Settings: 10:10:50 % to 700 J per Eppi).
5. Clear from debris by centrifugation at 12.000 x g 45min at 4°C.
6. Repeat 5. with supernatant.
7. Carefully put supernatant on 35% (w/v) Sucrose solution (4mL on 20mL works out).
8. Ultracentrifugation in Beckman L7-65 Type 50 TI Rotor at 41.000rpm (→ 150.000 x g).

k) Ultracentrifugation

An ultracentrifuge is basically a very fast spinning centrifuge that can accelerate speeds up to 1000000 g. In our case we used the Sucrose Cushion method to separate the assembled VLPs from the cell lysate.

1. Carefully place sample on 20 mL 35% (w/v) Sucrose solution (sample sizes should not exceed 4 mL otherwise use more tubes).
2. Ultracentrifugation in Beckman L7-65 Ultracentrifuge Type 50 TI Rotor at 41.000rpm (→ 150.000g)
3. Carefully remove some of the supernatant
4. Collect sediment with as little sucrose as possible
5. Carry out Size-exclusion Chromatography

l) Size-exclusion Chromatography (SEC)

Size exclusion chromatography (SEC) is a kind of gel filtration. It separates molecules by differences in size as they pass through a SEC resin packed in a column.

1. Apply ~ 60 µl sample onto the column.

2. Add a few milliliters of PBS Buffer to elute the column.

Note: Bigger molecules will elute first. Small molecules will elute last.

m) Transmission electron microscopy (TEM) [4]

It is possible to enlarge images up to 150.000 times using a Transmission Electron Microscope (TEM), resulting in a resolution of a few nanometers. An electron beam is focused on the sample trough an electromagnetic lens which creates a high-resolution image. A diffraction image is also created through this process, which is especially interesting for crystallographic applications. The beam is diffracted trough the coulomb interactions at the core of the atom. Because of this, a larger atomic mass (mass contrast) or thicker (thick contrast) samples result in darker image since there is a higher diffraction probability, less electrons are detected. These two factors are considered amplitude contrast.

To create a TEM image special sample preparations must be undertaken. The sample must be very thin to avoid too much diffraction, which muddles the resolution. If needed a coating can be applied to protect organic compounds from being destroyed by the intensity of the electron beam.

Thanks for the special help from the Thiel lab and Brigitte Hertel who explained and showed us everything regarding TEM!

Procedure

1. 10µL of the sample were placed on a "formwar" coated copper grid and after 5 min of drying the remaining fluid was removed with filter paper.
2. The sample was covered with 10µL of 1% Uranyl Acetate solution for 10 minutes before removing excess fluid with filter paper.
3. The sample was dried over several hours.
4. The sample was installed on the sample holder.
5. The Sample holder was inserted into the microscope.
6. Restore vacuum by pressing button “pump“.
7. The electron beam is being activated.
8. Sample can be investigated by focusing the electron beam with the buttons “Beam XY“.
9. Find suitable area on sample grid in low magnification (3000x).
10. Gradually increase magnification to 140.000x.
11. Insert camera by clicking „start view“ on the computer.
12. Search the area for particles and take nice pictures.

B. In vivo

I. *E.coli*

a) Chemically competent cells

The transformation of *E.coli* with plasmid DNA via heat shock transformation requires chemically competent cells.

Procedure

Day 1:

1. Grow Top10/BL21 (DE3) overnight in 5 ml LB at 37° C

Day 2:

1. Inoculate 100 ml LB with 1 ml of saturated overnight culture of *E.coli* cells
2. Incubate at 37° C and 150 rpm until an OD600 0.4 – 0.6 (usually 2-3h)
3. Incubate cells on ice for 5 minutes

Note: After this point the cells should never touch anything that is warm – chill solutions, pipets, tubes, etc. beforehand.

4. Divide culture into 2 tubes with ~ 40 ml each
5. Centrifuge the culture at 4° C and 3000 xg for 10 minutes
6. Gently resuspend each pellet with 15 ml of cold Mg^{2+}/Ca^{2+} solution (Do not vortex!)
7. Incubate in an ice bath for 30 minutes
8. Centrifuge the culture at 4° C and 3000 xg for 10 minutes
9. Resuspend each pellet with 1.6 ml of cold 100 mM $CaCl_2$ solution
10. Incubate in an ice bath for 20 minutes
11. Combine cells to one tube
12. Add 0.5 ml cold 80% glycerol and swirl to mix
13. Flash freeze in liquid nitrogen a 100 µl aliquots
14. Store at -80° C

Mixtures

- Mg^{2+}/Ca^{2+}
  - 3.25 g $MgCl_2 * 6 H_2O$
  - 0.6 g $CaCl_2 * 2 H_2O$
  - 200 ml ddH_2O
  - Autoclave

- $CaCl_2$ (100 mM)
  - 2.95 g $CaCl_2 * H_2O$
  - 200 ml ddH_2O
  - Autoclave

Note: You can also make a 1:10 dilution of the 1 M stock.

Source: http://openwetware.org/wiki/Griffitts:Chemocompetent_Cells

b) Bacterial cell culture

Bacterial cell culture is a method by which bacterial cells are cultivated under controlled conditions to multiply the number of cells.

Procedure

Starting culture: Under sterile conditions add about 5 ml of medium to a culture tube and insert the picked colony.

1. Cultivate the stock on agar plate e.g. until colonies grow (incubation usually at 37° C).
2. Flame a glass pipette, open the bottle of medium and flame the mouth measure out the amount you need to fill your tubes, flame the cap and recap the bottle as quickly as possible.
3. Remove the tube cap, flame the top of the culture tube, pipette in 5 ml, flame the top of the tube, and cap it. Pick a single colony (to assure the cells are from the same single clonal population) and transfer it to the medium by tapping a small (0.1 µl) pipette tip (held on a pipette) on the surface of the plate. Uncap a tube, flame the top, tip the tube so as to transfer cells from the pipette tip to the surface of the media without touching the inside of the tube with the non-sterile portion of the pipette, flame, cap.
4. Pipette the desired amount of antibiotic into each tube along the wall. Do not put the non-sterile part of the pipette inside the tube and use a new tip for each tube.
5. Vortex each tube for 1-2 seconds to mix well.
6. Take the tubes to incubate (usually at 37° C) in an incubator or warm room.
7. Wait overnight or until your cells have reached the desired concentration.

Source: http://openwetware.org/wiki/Bacterial_cell_culture

c) Heat Shock Transformation

Heat Shock Transformation is a widely used technique to insert foreign plasmid DNA into chemically competent bacteria cells.

Procedure

1. Defrost stocks of competent cells (100 µl in 1.5 ml reaction tube) on ice.
2. Add DNA (2-6 µl) and incubate the suspension for 30 minutes on ice.
3. Heat shock is done by incubating the cells for 45 seconds at 42° C.
4. Put samples back on ice for 2 minutes.
5. Add 1 ml of LB medium and incubate for 1 hour at 37° C in order to obtain antibiotic resistance.
6. It might be useful to spin down cells at 5000 rpm for 5 minutes. Resuspend pellet in 100 µl LB.
7. Spread out cells on agar plate.

d) Colony PCR

E.coli

Colony PCR is used to analyze whether a sequence of interest is present on a plasmid in *E.coli.* Flanking primers are used to amplify DNA in between the primer binding sites that are located on the plasmid backbone.

Procedure

The colony PCR is a modified PCR program employed to verify transformation success by amplifying the insert or the vector construct used for transformation. This is necessary due to the fact that a transformation with the empty vector may lead to antibiotic resistance.

1. Reaction mixture 1x – (25 µl)

- 12.5 µl 2x Taq Master Mix
- 0.5 µl VF2 (10 µM)
- 0.5 µl VR (10 µM)
- ddH_2O to 25 µl

2. Pick one colony with a sterile tip and suspend in reaction mixture.
3. Start the PCR using the following program and 1x mix.
4. Run an agarose gel to determine the product length.

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95 °C	5:00 min.	1 cycle
Denaturation	95 °C	0:10 min.	
Annealing	55 °C	0:15 min	30 cycles
Extension	72 °C	1 min/kbp	
Final Extension	72 °C	1.5 min/kbp	1 cycle
Hold	4 – 8 °C	Indefinite	1 cycle

e) Glycerol stock

In order to have a permanent culture of cells glycerol stocks can be made.

Procedure

1. Add 200 µl of sterilized glycerol or DMSO to 800 µl cell culture and mix well.
2. Shock freeze with liquid-nitrogen.
3. Store the stock at -80° C.

f) Protein Expression

Throughout the project we worked with different promotor systems. The basics of the procedure won't change in general only make sure to use appropriate inducing chemicals and take care of using appropriate strain.

Procedure (T7-promotor system)

1. Inoculation of 50 ml LB medium in a 100 ml flask with *E.coli* BL21 strain transformed with desired plasmid.
2. Incubation at 180 repulsion per minute (rpm) at 30° C to an OD600 = 0.4.
3. Transfer starter culture into 1 L LB medium in a 3 L flask resulting in an OD600 = 0.2.
4. Incubation to an OD600 = 0.6 at 180 rpm and 30° C.
5. Incubation for 15 minutes on ice.
6. Induction of protein expression with 20 ml of IPTG (stock concentration 0.5 mM).
7. Incubation of the cell's suspension over night at 180 rpm at 30° C.

C. Protein-Purification

I. Cell culture and disruption

1. Inoculation of 1L LB medium with transformed BL21 from pre culture to reach OD600 = 0.1
2. Induction of cell culture at OD600 = 0.6 with 5mM IPTG
    a. Expression of enzymes for 4h at 30°C
    b. Expression of proteins over night at 30°C
3. Put cell culture on ice to inactivate the expression
4. Harvest of cells through centrifugation (15min, 8.000 rpm. 4°C)
5. Resuspend cell pellet with cold resuspension Buffer (40-50mL)

II. EmulsiFlex-C3 (by Avestin)

1. Put metal piece of tube in ice
2. Discard Ethanol which is stored in the system
3. Wash system with water and cold resuspension Buffer
4. Start applying pressure to reach about 1500 bar (~22000 psi)
5. Add cell suspension to system
6. Run cell suspension 4x through system
7. Prepare a fresh falcon prior to running the cell lysate a third time through the system
8. Centrifuge the cell lysate in ultracentrifuge tubes (60min, 4°C, 20000 x g)
9. Collect supernatant as soon as possible after centrifugation (to prevent cell lysate from resuspending)
10. Filter supernatant using a syringe and 0.45 µm filter tips
11. Clean EmulsiFlex-C3 in this order: 0.1M NaOH, ddH2O, EtOH 80%, EtOH 20%

III. ÄKTA (purification)

His-Tag

<u>Buffer/Solutions</u>

Resuspension/Binding Buffer

- 20mM Imidazol
- 500mM NaCl
- 20mM NaPO4 pH 7,4

Elution Buffer
- 500mM NaCl
- 20mM NaPO4 pH 7,4
- 350mM Imidazol

Buffer W
- 150mM NaCl
- 100mM Tris

For SrtA7M (Sortase A7M), turboGFP-LPETGG

Supernatant of cell disruption is removed and purified with the ÄKTApure purifier with the software Unicorn and a HisTrap column with a volume of 1 ml by GE Healthcare. Elution is elaborated with a linear increasing concentration of imidazole by elution Buffer.

1. Wash ÄKTA system with 7 ml distilled water at a flow rate of 1 ml/min at a pressure of 0.5 MPa to remove remaining ethanol.

2. Apply all of the sample using the sample pump, put flow through in waste or collect it for SDS-PAGE analysis.

3. Wash column with 10 CV[2] Equilibration Buffer to get rid of the remaining sample.

4. Elute the protein with an imidazole gradient starting at 5 % and reach the final concentration of 100 % Elution Buffer.

5. Collect fractions of 0.5 ml.

6. Wash apparatus with water and EtOH.

After sample collection use a PD-10 column for Buffer exchange to Buffer W.

PD-10 protocol [5]

1. PD-10 Desalting column preparation
   • Remove the top cap and pour off the column storage solution.
   • Cut the sealed end of the column at notch

2. Column equilibration
   • Fill up the column with equilibration Buffer and allow the equilibration Buffer to

[2] **1 CV** = 1 column volume → in one 1 ml column ~ 1 ml

enter the packed bed completely.
• Repeat 4 times.
• Discard the flow-through.

Note: About 25 ml equilibration Buffer should be used in total for all three steps.

3. Sample application
   • Add maximum 2.5 ml of sample to the column.
   • For sample volumes less than 2.5 ml, add equilibration Buffer to adjust the volume up to 2.5 ml after the sample has entered the packed bed completely.
   • Let the sample or equilibration Buffer enter the packed bed completely.
   • Discard the flow-through.

4. Elution
   • Place a test tube for sample collection under the column.
   • Elute with 3.5 ml Buffer and collect the eluate.

The respective protein was collected in several fractions and an SDS-PAGE was performed to show if the purification has been successful.

Strep-Tag

Buffer/Solutions

Resuspension Buffer/Equilibration Buffer/ Buffer W
- 150 mM NaCl
- 100 mM Tris

Elution Buffer
- 150 mM NaCl
- 100 mM Tris
- 26,7 mg/50mL Desthiobiotin

SrtA5M (Sortase A5M), mCherry-LPETGG, mCherry-poly G, SP (P22 Scaffold-Protein), sfGFP-SP (superfolder GFP-Scaffold Protein), CP (P22 Coat Protein), CP-LPETGG (P22 Coat-Protein-LPETGG), Sortase A from Stockholm (BBA_K2144007), sfGFP-poly-G with TEV cleavage site, mCherry with TEV cleavage site.

Supernatant of cell disruption is collected and purified via the ÄKTApure purifier with the software Unicorn and a Strep-Tactin column with a volume of 1 ml.

1. Wash ÄKTA system with ~ 7 mL distilled water at a flow rate of 1 mL/min at a pressure of 0.5 MPa to remove remaining ethanol.

2. The column is equilibrated with 6 CV of Buffer W.

3. Apply all of the sample using the sample pump, put direct flow through in waste or collect it for SDS-PAGE analysis.

4. Wash column with 7 CV equilibration Buffer

5. Elute the protein with 4 CV elution Buffer

6. Collect fractions of 0.5 mL

7. To regenerate the column, fill at first 15 CV of HABA into ÄKTA system. This is followed by 30 CV of equilibration Buffer and 3 CV of distilled water.

8. 3 CV of EtOH equilibrate in the apparatus.

The protein was collected in several fractions and an SDS-PAGE was performed to show if the purification has been successful.

V. Anion Exchange Chromatography

Buffer/Solutions

Start/Resuspension/Binding Buffer:
- 50mM NaCl
- 100mM Tris pH 8

Elution Buffer
- 1M NaCl
- 100mM Tris pH 8

Wash Buffer
- 2M NaCl
- 100mM Tris pH 8

- Always clean hoses with some ddH2O before putting them into new solution!
- Clean hoses with water
  → Manuel → Pumps → Pumpwash → (select the right one) → insert → execute (small macro), depending on your program it may not be necessary

- Clean system with water to get rid of EtOH
- After cleaning put each hose in designated liquid (notes in computer programme) and make sure that there is no air in the system

- Put column in, first the top and then the bottom, in this case anion exchange column (DROP TO DROP CONNECTION) → check everything is closed
- Add falcon to system to collect flow through (outlet 1, has pink tag on it)
- Fill up 15 mL Falcons in collection apparatus, if needed and make sure the arm is in right position
- **Run binding Buffer through sample loop FIRST, and then put hose into sample**
- Start system
- Clean everything in water after programme has finished

- Nanodrop your results
- Clean ÄKTA, first with water and then put in EtOH

D. Assays

I. Copper click reaction with Fluorescein and Azidopropanamin

Reagents: Fluorescein, Phosphate Buffer (pH 7), Sodium Ascorbate, Azidopropanamin, $CuSO_4$

Reaction solution

- 5 mM Azidopropanamin
- 50 mM Fluorescein
- 20 mM $CuSO_4$
- 100mM Potassium Phosphate
- 100mM Sodium Ascorbate

Final concentrations

- 100 µM Azidopropanamin
- 560 µM Fluorescein in phosphate Buffer
- 0,25 mM $CuSO_4$
- 5 mM Sodium Ascorbate

Prepare Buffers

1. Weight in 0.68 g Potassium Phosphate and dissolve in 50 mL Milli-Q water for 100 mM Buffer, measure pH and calibrate to pH 7 with NaOH
2. Weight in 0.198 g Sodium Ascorbate and dissolve in 50 mL Milli-Q water for 100mM Buffer
3. Weight in 0.0252 g $CuSO_4$ and dissolve in 20 mL Milli-Q water for 20 mM

Prepare Reactants

1. Mix 0.5 µL Azidopropanamin (1M) with 99,5 µL Milli-Q water for 5 mM
2. Mix 29.5 µL Fluorescein (9.5mM) with 470.5 µl of the Phosphate Buffer (100 mM)

Mix in reaction solution (for 500 µL)

1. 446 µL Fluorescein in Phosphate Buffer
2. 10 µL Azidopropanamin
3. 6,3 µL $CuSO_4$
4. 25 µL Sodium Ascorbate
5. 12,5 µL Milli-Q water

Process: Let the reaction incubate for 3h at room temperature. After 3h add 13 µL EDTA (20 mM) to complex the copper.

II. Sortase reaction with Fluorescein and TAMRA

1. Take 297 µL of the Fluorescein of the copper click reaction and add 1µL Sortase A7M (560 µM) and 2 µL TAMRA-LPETG (10mM).
2. For negative control leave out Sortase A7M and add water instead.
3. Let incubate at 37 °C for 1,5 h.
4. Put reaction solution in Vivaspin with cut off 10000 and centrifuge for 20 min.
5. Check product in ESI-MS

ESI-MS [6]

Electrospray ionization mass spectrometry (ESI-MS) is a method to determine the molecular mass of molecules after their conversion to ions. First the molecules of interest are ionized and transferred from solution into the gaseous phase. Then the ions travel through the analyzer and arrive according to their mass/charge (m/z) ratio at different detector-parts. The detected signals are recorded by a computer and displayed as a mass spectrum.

III. Sortase reaction with TAMRA and Azidopropanamin

Final concentrations in reaction solution

- 0,5 mM TAMRA-LPETG (stock 10 mM)
- 10 mM Azidopropanamin (stock 1 M)
- 100 mM Ammonium Bicarbonate (stock 1 M)
- 20 µL Sortase A7M (stock 560µM)

Prepare:

1. Dilute Sortase A7M 1:12.8

Mix:

1. 2 µL Azipropanamin
2. 10 µL TAMRA-LPETG
3. 20 µL Ammonium Bicarbonate
4. 10 µL of diluted Sortase A7M
5. 148 µL Milli-Q water

Process:

1. Let incubate for 2h at 37 °C.
2. Put solution in Vivaspin with cut off 10000 and centrifuge for 20 min.

Check product in ESI-MS.

IV. Sortase Assay: Peptid GGGGGRWSSG with TAMRA-LPETG

Peptid synthesis

1. Weight in the needed amino acids as given by the synthesizer.
2. Dissolve in given volume DMF.
3. Synthesize via liberty blue.

4. Wash with DMF, DCM and Diethylether.
5. Let dry in Ekksikator overnight.
6. Add a solution of 90% TFA, 2% Anisol, 2% TES and 6% water.
7. Let shake for 2h.
8. Wash with 50 mL Diethylether and centrifuge.
9. Wash with 2x 25 mL Diethylether and centrifuge each time.
10. Let pellet dry on 37°C block.
11. Weigh pellet.
12. Dissolve pellet in 2 mL Water and analyze via HPLC and ESI-MS.

Sortase reaction

1. Put in 30µM TAMRA-LPETG, 30µM Poly G Peptide and 10µM Sortase A7M and fill with Sortase reaction Buffer to desired volume.
2. Incubate at 30 °C for 1h.
3. Stop reaction with same volume 2% formic acid.
4. Centrifuge with Vivaspin with cut off 10000 to separate from Sortase.
5. Analyze with HPLC and ESI-MS.

V. Expression Assay

Before the experiment *E.coli* BL21 (DE3) were transformed with the desired dual expression plasmid and selected overnight at 37° C on an agar plate with fitting antibiotic. The plate could be stored up to three days in the cooler.

At the day before the measurement, three 5 mL overnight cultures per plasmid variant were inoculated with a single colony. After incubation in a rotation shaker, the OD_{600} was measured, so that the 1.5 mL of LB media in every well of the 24 well plate could be inoculated at OD_{600} = 0.1. Following, the 24 well plate was incubated at 37°Cand 200 rpm until OD_{600} = 0.4 was reached. The moment this value has been reached was defined as O h (t=0) and the following incubation period was performed at 30° C. The wells were induced with 37.5 µL of a specifically concentrated inducer stock which is dissolved in ddH_2O, depending on the experiment. At 6 h, 100 µL samples were collected from each well and the OD_{600} was measured as well. This process was repeated after the 24 well plated had been incubated overnight (22 h) at 30°C and 200 rpm.

The 100 µL samples were centrifuged at 11000 rpm for a minute and the supernatant was discarded. At this point the samples were snap frozen in liquid nitrogen for storage. Then each cell pellet was resuspended in a 200 mM Na_2HPO_4 (Disodium hydrogen phosphite) solution, so that OD_{600} = 5 was reached. The suspension was mixed 1:1 with 2x Rotiload Buffer. 20 µL of the samples were used to perform an SDS-PAGE at 120 V for 100 minutes. After the SDS-PAGE, fluorescent images were taken using an Amersham™ Imager 600.

VI. Spectrophotometric Measurement using a TECAN Reader

Before the experiment *E.coli* BL21 (DE3) were transformed with the desired dual expression plasmid and selected overnight at 37° C on an agar plate with fitting antibiotic. The plate could be stored up to three days in the cooler.

At the day before the measurement, three 5 mL overnight cultures per plasmid variant were

inoculated with a single colony. After incubation in a rotation shaker, the OD_{600} was measured, so that the 1.5 mL of LB media in every well of the 24 well plate could be inoculated at OD_{600} = 0.1. Following, the 24 well plate was incubated at 37 °C and 200 rpm for 30 minutes. After the incubation the spectrophotometric measurement at 30° C was started and measured for 1 h without induction. Later, the wells were induced with 37.5 µL of a specifically concentrated inducer stock which is dissolved in ddH_2O, depending on the experiment. The measurement was ended 6 h after the first induction. At the end of the measurement, 100 µL samples were collected from each well and the OD_{600} was measured as well. This process was repeated after the 24 well plated has been incubated overnight (22h) at 30° C and 200 rpm.

The 100 µL samples were centrifuged at 11000 rpm for a minute and the supernatant was discarded. At this point the samples were snap frozen in liquid nitrogen for storage. Then each cell pellet was resuspended in a 200 mM Na_2HPO_4 (Disodium hydrogen phosphite) solution, so that OD_{600} = 5 was reached. The suspension was mixed 1:1 with 2x Rotiload Buffer. 20 µL of the samples were used to perform and SDS-PAGE at 120 V for 100 minutes. After the SDS-PAGE, fluorescent images were taken using an Amersham™ Imager 600.

VI. Spectrophotometric Measurement using a Spectramax M5e

Before the experiment *E.coli* BL21 (DE3) were transformed with the desired dual expression plasmid and selected overnight at 37° C on an agar plate with fitting antibiotic. The plate could be stored up to three days in the cooler.

At the day before measurement, three 5 mL overnight cultures per plasmid variant were inoculated with a single colony. After incubation in a rotation shaker, the OD_{600} was measured, so that the 1.5 mL of LB media in every well in the 96 well plate could be inoculated at OD_{600} = 0.1. Following, the 96 well plate was incubated at 37° C at 400 rpm for 30 minutes. After the incubation the spectrophotometric measurement at 30° C was started and measured for 90 minutes without induction. Later, the wells were induced with 37.5 µL of a specifically concentrated inducer stock which is dissolved in ddH_2O, depending on the experiment. The measurement was ended after 5 – 7 h of measuring time.

Abb. 2:
Platereader “SpectraMax” M5

VII. LAL(/Endotoxin)-test [7] [8]

The LAL-test is based on the reaction between an amebocyte of the horsehoe crab (*Limulus Polyphemus)* and bacterial endotoxins. The endotoxin concentration is determined by a highly sensitive photometric measurement of a color change which is proportionate to the amount of endotoxin in the sample. Beforehand the sample was mixed with Limulus-Amoebocyten-Lysat (LAL) and a coloring substrate.

Procedure

1. Dissolve your product in endotoxin-free water (Dilution 1:1000; 1:100000).
2. Measurement of the endotoxin amount in the extract.
3. Calculation of the endotoxin-bearing per protein (EU/mL).

E. Materials

I. Media

LB Media (1000 mL)

- Tryptone……10 g
- Yeast extract……5 g
- NaCl……10 g
- Agar (for plates)……15 g

Add 5 M of NaOH to adjust the pH at 7.0 (a few drops will be enough).

II. Stock solutions

a) Antibiotics

Ampicillin

1. Mix
   - 4 g ampicillin (100 mg/ml)
   - add 40 ml ddH2O
2. Sterile filtration
3. Aliquot in 1 ml stocks and store at -20°C
4. Use 1 µl per 1 ml medium

Chloramphenicol

1. Mix
   - 1 g chloramphenicol
   - add 40 ml ethanol
2. Aliquot in 1 ml stocks and store at -20°C
3. Use 1 µl per 1 ml medium

Kanamycin

1. 1.Mix
   - 3 g kanamycin (75 mg/ml)
   - add 40ml ddH2O
2. Sterile filtration
3. Aliquot in 1 ml stocks and store at -20°C

4. Use 1 µl per 1 ml medium

b) Induction chemicals

IPTG (Isopropyl-beta-D-thiogalactopyranoside)

1. Dissolve 238 mg IPTG in 10 ml water.
2. Store in 1 ml aliquots at -20° C.

AHT (Anhydrotetracycline hydrochloride)

1. Dissolve 2 mg AHT in 10 ml water.
2. Store in 1 ml aliquots at -20° C.

G. References

[1] De Kok (2014), https://www.ncbi.nlm.nih.gov/pubmed/24932563

[2] Roth (2014), https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0107329

[3] NEB (13.10.2019), https://www.neb.com/protocols/2012/12/11/gibson-assembly-protocol-e5510

[4] Dr. K. Hofmann, Elektronenmikroskopie 1 und 2, Kurs Spezielle Instrumentelle Analytik, TU Darmstadt, Sommersemester 2019

[5] GE Healthcare (2007), http://wwwuser.gwdg.de/~jgrossh/protocols/protein-purification/PD10.pdf

[6] Ho, C S et al. “Electrospray ionisation mass spectrometry: principles and clinical applications.” *The Clinical biochemist. Reviews* vol. 24,1 (2003): 3-12., https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1853331/

[7] https://www.cleancontrolling.com/medical/produkte-leistungen/laboranalytik/endotoxintest?gclid=EAIaIQobChMIofXrhvai5QIVhOJ3Ch1dDQm9EAAYASAAEgLrMvD BwE, (17.10.2019)

[8] https://www.antikoerper-online.de/news/2/1149/limulus-amoebozyten-lysat-test-lal-test/, (17.10.2019)