Methodic Protocols and Material

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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 with proofreading function.

Touchdown PCR is a 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.

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.

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
10 mM dNTPs	0.5 µl	1.0 µl	200 µM
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	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	
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	8°C	Indefinite	1 cycle

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

b) Quick change PCR

Optional site directed mutagenesis can be applied to enhance laccase activity. Mutation sites are taken from studies for laccase mutagenesis.

PCR Quick Change or site directed mutagenesis is used to change DNA bases on a sequence of interest (maximum 5 bases). The most important step in this experiment is the primer design. They must have a length between 25 – 35 bp and a GC-content of 40 to 60 %, with the desired bases subjected to change sitting in the center of the sequence. After PCR amplification, the DNA strands undergo DpnI digestion to remove methylated strands.

PCR-Amplification (i. e. Phusion High-Fidelity DNA Polymerase, see above)

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

DpnI digestion

After the PCR, collect the mixtures:

1. Add 1 µL of DnpI
2. Place the tube in the PCR machine

Step	Temperature	Time
Activation	37°C	60min.
Inactivation	80°C	20min.
Denaturation	90 °C	15min.

After this cycle, let the mixture cool at room temperature. This step is very important, it allows a slow hybridization of the DNA strands.

Possible mutations for SDM: CotA[1]

- 5E29 mutant (T232P/Q367R) rationally designed CotA-mutant (5E29) | 1,21-fold catalytic activity
- Met502 (weakly coordinating to the T1 copper) Exchange to Leucine/Isoleucine (not coordinating) keeps geometry of copper-coordinating center, increases redox-potential (100mv)

Possible mutations for SDM: CueO[2]

- Mutations at 5th copper binding site

c) 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
   ddH2O.....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.

d) 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

e) 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
    - ddH2O.....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

f) Deletion

- Plasmid (250 ng) ..... 0.5 µl
- fw Primer..... 2.5 µl
- rv Primer..... 2.5 µl
- Q5 Master Mix..... 25 µl
- ddH2O.....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

g) Gibson Assembly [3]

Gibson Assembly has been one of our methods of choice to insert DNA fragments into a variety of vectors.

1. To generate the required overhangs a PCR has to be performed with either the plasmid or the insert. Use special designed overhang primer.
2. After successful PCR a PCR Cleanup is required. DNA concentration is measured via Nanodrop.
3. Then a Dpn1 digest is used, to remove the wildtype DNA (the starting material). The endonuclease Dpn1 digest all DNA that is methylated.

   Procedure:

   Mix:
   20µL PCR Cleanup product
   3µL CutSmart Buffer
   1µL Dpn1
   6 µL H_2O
   Incubation: 37 °C for 60 minutes
   Inactivation: 80°C for 20 minutes
   For the Gibson Assembly calculator: take the in 2. measured concentration *2/3

4. **Gibson Assembly:**
   Approach: 8 µL reaction volume in total
   (Take the Gibson Assembly calculator to calculate the required amounts)
   4µL Mastermix
   1 µl H_2O
   ? µL Insert
   ? µL Vector

Note: Add the master mix last and thaw on ice, if frozen. Freeze directly after use.
Incubation: DIRECTLY at 50°C for 1h (no long waiting times / work quickly)
Store product at -20°C
Variation: Dpn1 digest can also be carried out after the Gibson Assembly

h) Golden Gate Cloning

Mix:

- pGGA selected destination plasmid (75 ng/µL) .....1 µL
- Insert .....ng

The molar ratio of insert to vector should be about 5:1. For calculation, we used the NEBioCalculator.

- T4 DNA Ligase Buffer (10×) .....1 µL
- T4 DNA Ligase (10 U/µL) .....1 µL
- BsaI (10 u/µL) .....1 µL
- sterile ddH2O .....to 10 µL

1. T4 DNA ligase and the restriction enzyme will be added as the last components. Mix by gently pipetting up and down.
2. Keep the mixture on ice until it can be placed into a Thermocycler.
3. The reaction product can be stored at 4°C (or -20°C for long-term storage).

Step	Temperature	Time	Number of Cycles
Restriction	37°C	2 min.	
Ligation	16°C	5 min.	50 cycles
Denaturation	80°C	20 min.	1 cycle
Hold	4°C	Indefinite	1 cycle

i) Golden Braid Cloning

GoldenBraid2.0 uses type IIS restriction enzymes for DNA assambly in synthetic plant biology. GoldenBraid destination vectors (pDGBs) contain a GB cassette (the LacZ selection gene flanked by two restriction/recognition sites corresponding to two different type IIS enzymes). The arrangement of restriction enzymes defines two levels of pDGBs, the alpha-level plasmids containing a kanamycin resistance and the omega-level plasmids containing a spectinomycin resistance). [4]

Procedure:

Mix:

α-level

- pDGB3_α (destination vector) .....75 ng
- Insert .....ng

The molar ratio of insert to vector should be about 3:1 to 2:1.

- T4 DNA Ligase Buffer (10×) 1µL
- T4 DNA Ligase (10 u/µL) 1µL
- **BsaI** (10 u/µL) .....1 µL
- sterile ddH2.....to 10 µL

Ω-level

- pDGB3_ Ω1 (destination vector) .....75 ng
- pDGB3_α_insert .....75 ng

- T4 DNA Ligase Buffer (10×) 1µL
- T4 DNA Ligase (10 u/µL) 1µL
- **Esp3I** (10 u/µL) .....1 µL
- sterile ddH2.....to 10 µL

1. T4 DNA ligase and the restriction enzyme will be added as the last components. Mix by gently pipetting up and down.
2. Keep the mixture on ice until it can be placed into a Thermocycler.
3. The reaction product can be stored at 4˚C (or -20˚C for long-term storage).

Step	Temperature	Time	Number of Cycles
Restriction	37˚C	2 min.	
Ligation	16˚C	5 min.	50 cycles
Denaturation	80˚C	20 min.	1 cycle
Hold	4˚C	Indefinite	1 cycle

II. Analysis and Purification

a) Plasmid Miniprep

Monarch® Plasmid DNA Miniprep Kit Protocol (NEB #T1010)[5]

- All centrifugation steps should be carried out at 16,000 x g (~13,000 rpm).
- If precipitate has formed in Lysis Buffer (B2), incubate at 30–37˚C, inverting periodically to dissolve.
- Store Plasmid Neutralization Buffer (B3) at 4˚C after opening, as it contains RNase A.

  Note: unlike other commercial kits, all wash steps are required.

1. Pellet 1–5 ml bacterial culture (not to exceed 15 OD units) by centrifugation for 30 seconds. Discard supernatant.

   Note: For a standard miniprep to prepare DNA for restriction digestion or PCR, we recommend 1.5 ml of culture, as this is sufficient for most applications. Ensure cultures are not overgrown (12-16 hours is ideal). Resuspend pellet in 200 µl Plasmid Resuspension Buffer (B1) (pink). Vortex or pipet to ensure cells are completely resuspended. There should be no visible clumps.
2. Lyse cells by adding 200 µl Plasmid Lysis Buffer (B2) (blue/green). Invert tube immediately and gently 5–6 times until color changes to dark pink and the solution is clear and viscous. Do not vortex! Incubate for one minute.

   Note: Care should be taken not to handle the sample roughly and risk shearing chromosomal DNA, which will co-purify as a contaminant. Avoid incubating longer than one minute to prevent irreversible plasmid denaturation.
3. Neutralize the lysate by adding 400 µl of Plasmid Neutralization Buffer (B3) (yellow). Gently invert tube until color is uniformly yellow and a precipitate forms. Do not vortex! Incubate for 2 minutes.

   Note: Be careful not to shear chromosomal DNA by vortexing or vigorous shaking. Firmly inverting the tube promotes good mixing, important for full neutralization.
4. Clarify the lysate by spinning for 2–5 minutes at 16,000 x g.

   Note: Spin time should not be less than 2 minutes. Careful handling of the tube will ensure no debris is transferred and the 2 minute recommended spin can be successfully employed to save valuable time. For culture volumes > 1 ml, we recommend a 5 minute spin to ensure efficient RNA removal by RNase A. Also, longer spin times will result in a more compact

pellet that lower the risk of clogging the column.

To save time, spin for two minutes only.

5. Carefully transfer supernatant to the spin column and centrifuge for 1 minute. Discard flow-through.

   Note: To save time, spin for 30 seconds, instead of 1 minute.

6. Re-insert column in the collection tube and add 200 µl of Plasmid Wash Buffer 1. Plasmid Wash Buffer 1 removes RNA, protein and endotoxin. (Add a 5 minute incubation step before centrifugation if the DNA will be used in transfection.) Centrifuge for 1 minute. Discarding the flow-through is optional.

   Note: The collection tube is designed to hold 800 µl of flow-through fluid and still allow the tip of the column to be safely above the top of the liquid. Empty the tube whenever necessary to ensure the column tip and flow-though do not make contact. To save time, spin for 30 seconds, instead of 1 minute.

7. Add 400 µl of Plasmid Wash Buffer 2 and centrifuge for 1 minute.
8. Transfer column to a clean 1.5 ml microfuge tube. Use care to ensure that the tip of the column has not come into contact with the flow-through. If there is any doubt, re-spin the column for 1 minute before inserting it into the clean microfuge tube.
9. Add ≥ 30 µl DNA Elution Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.

   Note: Nuclease-free water (pH 7–8.5) can also be used to elute the DNA. Delivery of the Monarch DNA Elution Buffer should be made directly to the center of the column to ensure the matrix is completely covered for maximal efficiency of elution. Additionally, yield may slightly increase if a larger volume of DNA Elution Buffer is used, but the DNA will be less concentrated as a result of dilution. For larger plasmids (≥ 10 kb), heating the DNA Elution Buffer to 50°C prior to eluting and extending the incubation time after buffer addition to 5 minutes can improve yield.

ZymoPURE™ Plasmid Miniprep Kit [6]

1. Centrifuge 0.5-5 ml of bacterial culture in a clear 1.5 ml tube at full speed for 15- 20 seconds in a microcentrifuge. Discard supernatant.
2. Add 250 µl of ZymoPURE™ P1 (Red) to the bacterial cell pellet and resuspend completely by vortexing or pipetting.
3. Add 250 µl of ZymoPURE™ P2 (Green) and immediately mix by gently inverting the tube 6-8 times. Do not vortex! Let sit at room temperature for 2-3 minutes. Cells are completely lysed when the solution appears clear, purple, and viscous.
4. Add 250 µl of ice cold ZymoPURE™ P3 (Yellow) and mix thoroughly by inversion. Do not vortex! Invert the tube an additional 3-4 times after the sample turns completely yellow. The sample will turn yellow when the neutralization is complete and a yellowish precipitate will form.
5. Incubate the neutralized lysate on ice for 5 minutes.
6. Centrifuge the neutralized lysate for 5 minutes at 16,000 x g.
7. Transfer 600 µl of supernatant from step 6 into a clean 1.5 ml microcentrifuge tube. Be careful not to disturb the yellow pellet and avoid transferring any cellular debris to the new tube.
8. Add 275 µl of ZymoPURE™ Binding Buffer to the cleared lysate from step 7 and mix thoroughly by inverting the capped tube 8 times.
9. Place a Zymo-Spin™ II-P Column in a Collection Tube and transfer the entire mixture from step 8 into the Zymo-Spin™ II-P Column.

10. Incubate the Zymo-Spin™ II-P/Collection Tube assembly at room temperature for 2 minutes and then centrifuge at 5,000 x g for 1 min. Discard the flow through.
11. Add 800 µl of ZymoPURE™ Wash 1 to the Zymo-Spin™ II-P Column and centrifuge at 5,000 x g for 1 min. Discard the flow through.
12. Add 800 µl of ZymoPURE™ Wash 2 to the Zymo-Spin™ II-P Column and centrifuge at 5,000 x g for 1 min. Discard the flow through.
13. Add 200 µl of ZymoPURE™ Wash 2 to the Zymo-Spin™ II-P Column and centrifuge at 5,000 x g for 1 min. Discard the flow through.
14. Centrifuge the Zymo-Spin™ II-P Column at ≥ 10,000 x g for 1 minute in order to remove any residual wash buffer.
15. Transfer the Zymo-Spin™ II-P Column into a clean 1.5 ml tube and add 25 µl of ZymoPURE™ Elution Buffer2,3 directly to the column matrix. Incubate at room temperature for 2 minutes, and then centrifuge at ≥ 10,000 x g for 1 minute in a microcentrifuge.

Store the eluted plasmid DNA at ≤ -20˚C.

PureYield™ Plasmid Miniprep System[7]

Before lysing cells and purifying DNA, prepare the Column Wash Solution by adding ethanol.

1. Add 600µl of bacterial culture to a 1.5ml microcentrifuge tube.

   Note: For higher yields and purity to harvest and process up to 3ml of bacterial culture, centrifuge 1.5ml of bacterial culture for 30 seconds at maximum speed in a microcentrifuge. Discard the supernatant and add an additional 1.5ml of bacterial culture to the same tube. Centrifuge again and discard the supernant. Add 600µl of TE buffer or water to the cell pellet, and resuspend completely.

2. Add 100µl of Cell Lysis Buffer (Blue), and mix by inverting the tube 6 times.
3. Add 350µl of cold (4–8˚C) Neutralization Solution, and mix thoroughly by inverting.
4. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
5. Transfer the supernatant (~900µl) to a PureYield™ Minicolumn without disturbing the cell debris pellet.
6. Place the minicolumn into a Collection Tube, and centrifuge at maximum speed in a microcentrifuge for 15 seconds.
7. Discard the flowthrough, and place the minicolumn into the same Collection Tube.
8. Add 200µl of Endotoxin Removal Wash (ERB) to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds.
9. Add 400µl of Column Wash Solution (CWC) to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 seconds. Elute
10. Transfer the minicolumn to a clean 1.5ml microcentrifuge tube, then add 30µl of Elution Buffer or nuclease-free water directly to the minicolumn matrix. Let stand for 1 minute at room temperature.
11. Centrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube.

Store eluted plasmid DNA at –20˚C.

b) 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.

c) Gel extraction Clean-UP

Gel Clean-Up Systems are used to remove unincorporated primers, salts, and leftover dNTPs 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 previously 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.

d) PCR Clean-UP

PCR Clean-Up Systems are used to remove unincorporated primers, salts, and leftover dNTPs 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.
   - 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.

e) DNA Quantification with NanoDrop

NanoDrop is a 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 NanoDrop and choose the program''Nucleic Acids''
2. Pipette 1 µl of a water sample (or buffer in which the DNA is dissolved) 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 automatically calculates the concentration in ng/µl, the 260/280 value and the 230/260 value.

f) 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.

Microsynth (Sanger-Sequencing)

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

Eurofins-Genomics (Sanger-Sequencing)

Procedure

1. Pipette 15 µl of DNA into a 1.5 ml reaction tube; for plasmids the concentration of DNA should be between 50 and 100 ng/µl.
2. Add 2 µl Primer (10 µM). Do only use one Primer per Tube!
3. Each reaction tube is labeled with a Eurofins-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.eurofinsgenomics.eu/

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 % (w/v) SDS
- Adjust pH-value to 8.8

Upper Tris (Stacking Buffer):

- 0.5 M Tris
- 0.4 % (w/v) 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
- Clean pockets with syringe before loading samples
- 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

B. *In vivo*

I. *E. coli*

a) Chemically competent cells[8]

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 OD_{600} 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

- CaCl2 (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.

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.

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

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.

- Solubilize a small amount of a bacterial colony (too much biomass will further contaminate your reaction mix) in 20µl ddH_2O by pipetting
- Incubate for 5 minutes at 95°C (water baths provide optimal heat dissipation)
- Centrifuge at max. speed for 3 minutes
- Carefully pipet 1 µl of the supernatant into your PCR mix

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 (100%) to 800 µl cell culture and mix well.
2. Store the stock at -80° C.

Note: Pipetting glycerin works better if the tip is cut off beforehand

II. Bacillus subtilis

a) Preparation and transformation of competent *B. subtilis* cells [9]

The Material

LB medium	10 g Tryptone
	5 g Yeast extract
	10 g NaCl
	Ad to 1,000 ml with deionized H_2O
SP medium	8 g Nutrient Broth
	0.25 g $MgSO_4 \cdot 7\ H_2O$
	1 g KCl
	Ad to 1,000 ml with deionized H2O
	After autoclaving do not forget to add
	1 ml 0.5 M $CaCl_2$
	1 ml 10 mM $MnCl_2$
	2 ml Iron Ferric Ammonium Citrate (CAF; 2.2 mg/ml)
10x MN	0.596 M $K_2HPO_4 \cdot 3\ H_2O$ (136 g)
	0.441 M KH_2PO_4 (60 g)
	34 mM Sodium citrate $\cdot 2\ H_2O$ (10 g)
	Ad to 1,000 ml with deionized H_2O
1x MNGE	10 % MN medium (1 ml 10x)
	2 % Glucose (400 µl 50 %)
	0.2 % Potassium Glutamate (50 µl 40 %)
	42 µM Iron Ferric Ammonium Citrate (50 µl CAF; 2.2 mg/ml)
	0.24 mM Tryptophan (100 µl 5 mg/ml)
	3 mM $MgSO_4$ (30 µl 1 M)
	8.37 ml deionized H_2O
	+/- 0.1 % Casaminoacids (100 µl 10 % CAA)
Expression mix	500 µl Yeast extract (5 %)
	250 µl CAA (10 %)
	50 µl Tryptophan (5 mg/ml)
	250 µl sterile H_2O

Suitable for 10 samples

The Procedure

Preparation of competent cells

1. Inoculate 4 ml LB liquid medium with a single colony of a *B. subtilis* strain and incubate the culture over night at 28˚C with agitation.
2. Use the overnight culture to inoculate 10 ml MNGE medium supplemented with 0.1 % CAA in a 100 ml shake flask to an approximate OD_{600} of 0.1. Incubate the culture at 37˚C and 220 rpm until an OD_{600} of about 1.3. This may take up to 5 h, depending on the strain.
3. Dilute the culture 1:1 with pre-warmed MNGE (w/o CAA) and incubate the culture for 1 h at 37˚C on a shaker.

   You can continue with the transformation of *B. subtilis* directly after the nutritional starvation step (see Transformation of *B. subtilis*, step 7) or continue for long term storage.

4. Transfer 15 ml of the culture to 15 ml Falcon tubes and harvest the cells by centrifugation for 5 min at 5,000 rpm. Transfer the supernatant into a sterile Falcon tube.
5. Re-suspend the cells in 1.8 ml of the supernatant, add 1.2 ml 50 % glycerin, mix the cell suspension and store the competent cell in 300 µl aliquots at -80˚C after freezing the aliqouts in liquid nitrogen.

Transformation of *B. subtilis*

6. Thaw an aliquot of the frozen, competent bacteria and mix 300 µl of them with 1.7 ml 1x MN medium that has been supplemented with 43 µl glucose (20 %) + 34 µl 1 M $MgSO_4$.
7. Add 0.1 - 1 µg DNA (2 µg plasmid DNA) to 400 µl of the competent cells and incubate the reaction tube for 30 min at 37˚C.
8. Add 100 µl expression mix and if required an inducer (IPTG, Xylose,…).
9. Incubate the bacteria for 1 h at 37˚C with agitation and propagate the cells on SP medium agar plates supplemented with the appropriate antibiotics. It is recommended to plate 150 µl and the remaining culture on two plates.

   <u>Remarks: Do not forget the negative control!!!</u>

b) Biofilm formation[10]

1. Determine the OD of the overnight culture
2. Dilute the culture with Msgg-medium to OD=0,05
3. Add 200 µL of the diluted overnight culture into a well
4. Cover the 96 wellplate with parafilm
5. The 96-well plate is incubated under static conditions at 37˚C for up to 48 h or at room temperature for up to 72h

c) Coculture-Biofilm formation[10]

Preparation of the overnight culture

1. Dilute 30 µL of one and 30 µL of the other monoculture in 6 mL LB5 medium.
2. Incubate inoculated cell culture at 37˚C in a shaker

Biofilm formation

1. Dilute 20 µL of the coculture in 180 µL Msgg media
2. Cover the 96 wellplate with parafilm
3. The 96-well plate is incubated under static conditions at 37˚C for up to 48 h or at room temperature for up to 72h

d) Flow chamber

1. For sample preparation it is mandatory to grow them on solid media with a height of 4 – 5 mm. 25 – 30 mL of media are to be used for a petri dish with a diameter of 90 mm.
2. The overnight culture used for inoculation should have an OD_{600} of 2.0.
3. When the sample has grown, a 2x3 cm rectangle is cut out to be used in the assay.
4. To create a reference, prepare a second flask containing the same volume of water as the flow chamber assay waste-water container.
5. Then inoculate the reference with the same volume of a fresh overnight culture (with an OD_{600} of 2.0) that was used for biofilm creation.
6. Gently stir the two vessels to ensure that the contents are mixed well.
7. Take four culture tubes and create the following cultures:
   a. 3 ml of media and 10 µL of the water used for the flow chamber assay
   b. 3 ml of media and 10 µL of the wastewater container
   c. 3 ml of media and 10 µL of your reference sample
   d. 3 ml of media and 10 µL of the overnight culture with an OD_{600} of 2.0
8. These will ensure a positive (4) and negative (1) control, as well as the sample (2) and a reference (3).
9. The tubes are incubated over night at 37˚C.
10. After incubation the OD_{600} of all four cultures is measured.
11. The negative control should ideally be clear, the positive control should have the OD600 expected from a normal overnight culture.

12. The OD_{600} of the reference culture should show how a normal inoculation with the same dilution would have grown.
13. The OD_{600} of the sample culture is supposed to show how many living cells were removed from the biofilm during the flow chamber assay.

A statement about the relative number of living cells separated from the biofilm can be made by calculating the quotient of sample to reference OD_{600}.

e) Diclofenac sorption measurement[11]

Diclofenac sorption measurement:

1. Calibrate HPLC with Diclofenac-Solution
2. Put carrier for suspended beds (with biofilm) into a beaker with 100 mL Diclofenac-Solution (in phosphate-buffer) (concentrations: 1 / 2.5 / 5 / 7.5 / 10 / 20 µg/mL in wastewater)
3. Control: concentration of 0 µg/L
4. Leave for 0/15/30/45 minutes.

HPLC - Analysis with solution samples:

5. Collect samples for micropollutant analysis (solution) (200 µL) and transfer to an Eppi
6. Add 800 µL Methanol and vortex
7. Centrifuge for 5 min at 12000 rpm
8. Take supernate and transfer to a new HPLC vial/Eppi
9. Dilute with 1/5 volume of MilliQ (The solution must not be murky and / or contain undissolved particles)
10. Analyze via direct injection (injected volume of 10 µL) in HPLC

HPLC – Analysis with samples from biofilm on carrier:

5. Collect cells from biofilm on carrier and resuspend in 1 mL ddWater
6. Repeat 2-3 times: centrifuge 5 min at 12000 rpm, wash cell pellet with water
7. Resuspend cell pellet in MilliQ
8. Disrupt cell samples by using sonication with 20 kHz for 15 min
9. Take 200 µL of the supernate to an Eppi
10. Add 800 µL MeOH and vortex
11. Centrifuge for 5 min at 12000 rpm
12. Take supernate and transfer to a new HPLC vial/Eppi
13. Dilute with 1/5 volume of MilliQ if solution is murky (The solution must not be murky and / or contain undissolved particles)
14. Analyze via direct injection (injected volume of 10 µL) in HPLC (see HPLC in Assays)

First high diclofenac concentration to see anything at all then go further down

f) GFP-Production and immobilization on TasA[12]

1. Solution-based biofilm culture.

- Strains were inoculated from frozen glycerol stocks and grown in LB medium supplemented with 5 μ g/mL chloramphenicol.
- Seed cultures were first grown in 5 ml LB medium overnight at 37 °C.
- The cell pellets collected from the initial seed cultures were then grown for 2–3 d at 30 °C in MSgg medium at an initial cell density of 5 × 107 cells/mL, supplemented with 5 μ g/mL chloramphenicol and 0.5 mM IPTG when necessary

2.

- Biofilms containing TasA - GFP nanofibers from liquid culture were collected by centrifugation at 5,000 g for 10 min.
- 10 μ L of biofilm solution dripped on a glass slide was then imaged under Nikon confocal microscope A1R.

 if fluorescence is visible then GFP-TasA fusion protein was expressed and incorporated into the matrix

LB medium: 1% tryptone (Difco), 0.5% yeast extract (Difco), and 1% NaCl
MSgg medium (45,46): pH = 7.0, 5 mM potassium phosphate,
100 mM morpholino propanesulfonic acid (MOPS), 2 mM $MgCl_2$, 700 μ M $CaCl_2$, 50 μ M $MnCl_2$, 50 μ M $FeCl_3$, 1 μ M $ZnCl_2$, 2 μ M thiamine, 0.5% glycerol, 0.5% glutamate, 50 μ g/ml tryptophan, and 50 μ g/ml phenylalanine

g) Atomic Force Microscopy (AFM)[13]

Preparation of the bacteria and the surface

See page 21 for *B. subtilis* preparation

Measurement with AFM

For the first attempt we would use the non-contact mode, where it is not necessary to prepare the cantilever. But if there were no useful results obtained, we would also try the contact mode which is described below:

1. Attach bacterium (immobilized)/biofilm and cantilever to the "centerless" AFM (cantilever with spring constant 0.05 N / m was used
2. Cantilever cleaned with ddH20, ethanol, acetone, ddH20 for 5 minutes and then air-dried
3. Immerse the cantilever in a 0.01% (w / v) poly-L-lysine (MW 70,000 ~ 150,000, Sigma) for 1 minute
4. Immerse cantilever in bacterium substrate for 1 minute and then use immediately
5. AFM: PicoForce, Contact Mode, scan rate 0.5 Hz, ramp size 1 μm, threshold 1nM
6. Measure the adhesion force by dragging the tipless cantilever over the surface of the probe

C. Protein-Purification

I. Cell culture and disruption (T7-promotor system)

1. After successful transformation of BL21 cells
2. Inoculate 50-100mL LB with the transformed cell (300 mL Erlenmeyer flask)
3. Inoculation of 1L LB (3 L Flask) medium with transformed BL21 from pre culture to reach OD600 = 0.1
4. 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
5. Put cell culture on ice to inactivate the expression
6. Harvest of cells through centrifugation (15 min, 8.000 rpm. 4°C)
7. Resuspend cell pellet with cold resuspension Buffer (40-50mL)

II. EmulsiFlex-C3 (by Avestin)

1. Put metal spiral 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 (60 min, 4°C, 20000 x g)
9. Collect supernatant as soon as possible after centrifugation (to prevent cell lysate from resuspending)
10. Clean EmulsiFlex-C3 in this order: 0.1M NaOH, ddH2O, EtOH 80%, EtOH 20%
11. Filter supernatant using a syringe and 0.45 µm filter tips

III. ÄKTA (purification)

His-Tag

Buffer/Solutions

Resuspension/Binding Buffer

- 20 mM Imidazole
- 500 mM NaCl
- 20 mM NaPO4 pH 7.4

Elution Buffer

- 500 mM NaCl
- 20 mM NaPO4 pH 7.4
- 350 mM Imidazole

Supernatant of cell disruption is removed and purified with the ÄKTApure purifier with the software

Unicorn and a His-Trap 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˙ 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 your respective storage or reaction buffer.

PD-10 protocol [14]

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 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.

˙**1 CV =** 1 column volume → in one 1 ml column ~ 1 ml

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

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. Fluorescence Assay – *E. coli*

Before starting the experiment, make sure the plate reader is correctly adjusted with the pipetting scheme and the wavelength to be measured.

1. Determine the OD_{600} of the overnight culture and dilute it to OD_{600}=0,1
2. Pippett the culture in 96-well plates, so that after adding the induction substance a total volume of 200 µL is reached
3. Incubate the well plate at 37 °C and at 160 rpm until the OD_{600} is approximately 0,5
4. Add the induction substance

   Note: In our case, we used 0,5 mM IPTG for induction.

5. Measure the fluorescence signal in a plate reader every 30 min for at least 3h

II. Proving laccase activity using ABTS[15]

a) Qualitative:

1. Soak standard 6mm paper disks for 2 min in a beaker with a 2mM ABTS in 100mM phosphate-citrate-buffer (pH:4.0)
2. dry them in an oven at 60°C
3. after cooling down, store the disks sealed in the refrigerator until use
4. to test, give ca. 10 µl aliquot on the disk
5. incubate at 30°C for 10 Minutes

➔ if the aliquot has laccase activity, it should have a green-blueish colour after about 10 Minutes

b) Quantitative:

1. Assay-mixture: 2mM ABTS in 100mM phosphate-citrate-buffer and a suitable amount of Laccase
2. Measuring of the absorbance increase at 420nm against time (extcoeff: $3,6*10^4$ $M^{-1}*cm^{-1}$)
3. Enzyme activity is expressed in units (U=mmol of ABTS oxidized per minute per enzyme) .

III. HPLC kinetic activity assay

a) Determination of an effective laccase concentration

Solutions:
Phosphate citrate buffer (PCB) 0.15 M, pH 5.0
100 mM $NaHPO_4$
50 mM citric acid
40 µg / mL substance (Diclofenac, Carbamazepine, Sulfamethoxazole) in MilliQ
Methanol
Before use, the laccase may have to be desalinated using a miniGPC with Bio-Gel P-6 polyacrylamide beads.

Reaction mixture
67 µL PCB (50 mM), 40 µg/mL substance, variable concentration Laccase, end volume 200 µL Incubation 24 h, 30 °C, 200 rpm

Place the substance to be converted and PCB in a 1.5 mL Eppi. Use different protein concentrations (determination with Nanodrop or BCA) and make up to 200 µL final volume. In addition, controls without enzyme and substance as well as only without enzyme should be carried out. Incubation at 30 ° C for 24 hours.
After reaction add 800 µL methanol, vortex well and centrifuge for 5 min at 12000 rpm. Carefully pull off the supernatant without touching the precipitate (attention, the precipitate is usually poor or not visible). Dilute the supernatant obtained with 1/5 volume of MilliQ and analyze it using HPLC; the solution must not be cloudy and / or contain undissolved particles.

b) Transformation of pharmaceutical solutions

Solutions
as above

Reaction approach
267 µL PCB (50 mM), 40 µg / mL substance, laccase (previously determined concentration), final volume 800 µL

Incubation for 24 h, 30 ° C, 300 rpm, samples taken after 0.5, 1, 2, 3, 4, 6, 8 and 24 hours.

Preparation

Place the substance to be converted and PCB in a 1.5 mL Eppi. Use the previously determined protein concentration and make up to 800 µL final volume. Carry out controls without enzyme and substance as well as without enzyme (= t0). Incubation at 30 ° C for 24 hours. After 0.5, 1, 2, 3, 4, 6, 8 and 24 hours, 100 µL samples are taken. 400 µL methanol is added directly to the samples, vortex well and centrifuge for 5 min at 12000 rpm. Carefully peel off the supernatant with 1/5 volume

c) HPLC Analytics [16][17]

1. **Materials & Solutions**
   - Hypersil™ ODS C18-column, 5 µM, 250 x 4.6 mm
   - MilliQ + 0.1 % FA or TFA (eluent A)
   - ACN + 0.1 % FA or TFA (eluent B)
   - Drug (Diclofenac, Carbamazepine, Sulfamethoxazole) stock solution 1 mg/mL in ACN or MeOH

2. **Procedure**

The following method was created for the analysis of Diclofenac, HPLC Methods depend on the analyte and need to be modified accordingly.

1. HPLC Method
   Flow Rate: 1 mL/min
   Injection Vol.: 10 µL
   Detection: UV 225 nm
   Gradient Method:

Time (min.)	Eluent A (%)	Eluent B (%)
2	80	20
12	30	70
15	30	70
16	90	10
20	90	10
21	80	20
25	80	20

Incorporation of a standard

Prepare 10 µg/mL and 1 µg/mL solutions of Diclofenac in 80/20 H2O:ACN from the stock solution. Determine the retention time (Rt) and appropriate concentration range based on measured intensity of absorbance (mAU, milli-Absorbance Units). If no signal is obtained increase the concentration by small steps. If Diclofenac is not detectable in the range of 1 – 10 µg/mL the used concentrations for the detection of transformation products also need to be adjusted.

Standard curve for quantification

Prepare solutions of equal distributed concentrations within the determined concentration range e.g. 0, 1, 2, 4, 6, 8, 10 µg/mL. Integrate the measured peaks and plot the area and concentration as standard curve.

Sample measurement

Measure the samples prepared as described. Analyze the decreasing signal of Diclofenac by peak integration. Are one or more new peaks at a slightly lower Rt observable and are those new peaks not detectable in the control samples? If so, these signals could possibly be transformation products of Diclofenac. Calculate the concentration of Diclofenac using the standard curve.

IV. Zebrafish Embryo acute toxicity Assay Protocol [18][19][20][21][22]

Aim & Introduction

This method would have been used to determine the acute fish toxicity as well as the developmental and reproductive toxicity (DART) of the enzymatic transformation products from wastewater polluting drugs such as Diclofenac. The comparison of the transformation products with the original substance provides an evaluation of whether the Laccase reduces the toxic impact of these drugs on the aquatic environment. For this purpose, embryos of the zebrafish (*Danio rerio*) are used, which are an established model in ecotoxicology and a good alternative to animal experiments on adult fish species.

This assay is based on the OECD Test Guideline 236 for the Fish Embryo Acute Toxicity (FET) test and is used to determine the lethal concentration LC_{50} value of Diclofenac and its transformation products.

Materials

- Fish tanks made of chemically inert material (e.g., glass)
- Inverted microscope and/or binocular with a capacity of at least 80-fold magnification
- Test chambers, standard 24-well plates with a depth of approx. 20 mm
- Self-adhesive foil to cover the 24-well plates
- Incubator allowing to maintain 26 ± 1 °C
- pH-meter
- Oxygen meter
- Equipment for determination of hardness of water and conductivity
- Spawn trap: instrument trays of glass, stainless steel or other inert materials; wire mesh (grid size 2 ± 0.5 mm) of stainless steel or other inert material to protect the eggs once laid; spawning substrate (e.g., plant imitates of inert material) (OECD 229, Annex 4a)
- Pipettes with widened openings to collect eggs
- Stock solutions of Diclofenac and 3,4-dichloroaniline

Procedure

Principle

Freshly spawned and fertilized zebrafish eggs are exposed to the substance for a period of 96 hours. Up to four apical observations are recorded every 24 hours as indicators of lethality: coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac and the lack of heartbeat. At the end of treatment, the acute toxicity is determined based on a positive outcome in any of the four apical observations recorded and the LC_{50} is calculated.

Controls

- Water control as negative and internal plate controls. If more than one dead embryo is observed in the internal plate control, the plate is not considered for evaluation.
- Positive control at a fixed concentration of 4 mg/L 3,4-dichloroaniline.
- Solvent control with the used solvent for the stock solutions. The solvent should not have significant effects on time to hatch, survival nor produce any other adverse effects on the embryos.

Treatment

- Test Chambers should be conditioned at least for 24 hours with the test solutions prior to treatment, care should be taken to minimize any stress to the embryos.
- Twenty fertilized embryos per concentration are treated with the substance.
- ± 20% of the initial substance concentration should be maintained throughout the test.
- At least five concentrations of the test substance diluted by a constant factor, not exceeding two, should be used. The highest concentration should result in 100% lethality and the lowest concentration should give no observable effect.
- The test is initiated as soon as possible after fertilization of the eggs and terminated after 96 hours of exposure. The embryos should be immersed in the test solutions before cleavage of the blastodisc commences.
- To start exposure with minimum delay, at least twice the number of eggs needed per treatment group are randomly selected and transferred into the respective concentrations and controls, not later than 90 minutes post fertilization.
- Viable fertilized eggs should be separated from unfertilized eggs and be transferred to 24-well plates pre-conditioned for 24 hours and refilled with 2 ml/well freshly prepared test solutions within 180 minutes post fertilization. By means of stereomicroscopy (preferably ≥30-fold magnification), fertilized eggs undergoing cleavage and showing no obvious irregularities during cleavage (e.g., asymmetry, vesicle formation) or injuries of the chorion are selected for observation.

The apical observations performed are listed in Table 1. A scheme of the test procedure is summarized in Figure 1.

Tabelle 1: Apical observations of acute toxicity in zebrafish embryos 24 - 96 hrs post fertilization.

	Exposure times			
	24 hours	48 hours	72 hours	96 hours
Coagulated embryos	+	+	+	+
Lack of somite formations	+	+	+	+
Non-detachment of the tail	+	+	+	+
Lack of heartbeat		+	+	+

If mortality exceeds 10% in the negative control (or solvent control), the test becomes invalid and should be repeated.

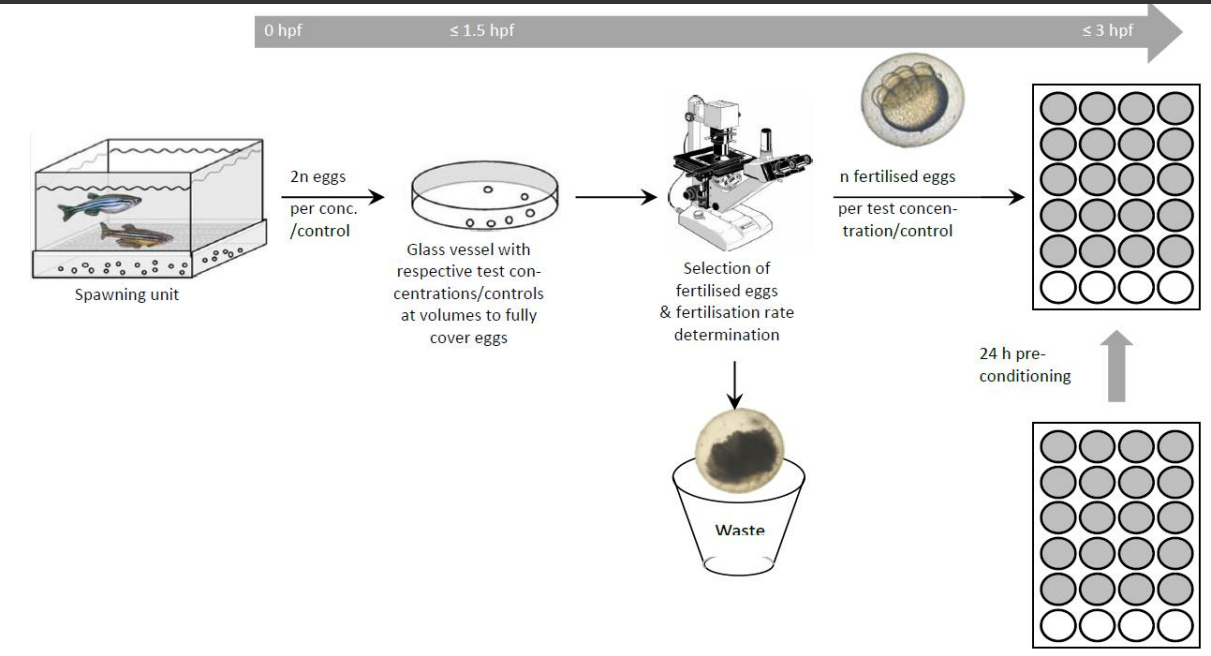

Figure 1: Scheme of the zebrafish embryo acute toxicity test procedure (from left to right): production of eggs, collection of the eggs, pre-exposure immediately after fertilisation in glass vessels, selection of fertilized eggs with an inverted microscope or binocular and distribution of fertilised eggs into 24-well plates prepared with the respective test concentrations/controls, n = number of eggs required per test concentration/control (here 20), hpf = hours post-fertilisation. Figure according to OECD Guideline 236.

The individual wells of the test chamber are considered independent replicates for statistical analysis. The percentages of embryos for which at least one of the apical observations is positive at 48 and/or 96 hours are plotted against test concentrations. For calculation of the slopes of the curve, LC_{50} values and the confidence limits (95%), appropriate statistical methods should be applied.

V. Kirby Bauer Assay of E. coli on Erythromycin / Azithromycin

Source: iGEM Munich 2013: Reaction mixture for Kirby-Bauer assay

Substance	Volume	Stock solution
EreB recombinant protein 40 nM	0.18 µl	11 µM EreB in PBS with 10 mM ß-mercaptoethanol, 2% glycerol (v/v) and 300 mM NaCl
Erythromycin 0.36 mM OR AZITHROMYCIN 0.36 mM	3 µl / ? µL	Erythromycin/Azithromycin in ethanol, 6 mM / ? mM
Tris-HCl buffer pH 7.5 100 mM	10 µl	Tris-HCl buffer pH 7.5, 500 mM
NaCl 0.08 M	4 µl	NaCl in water, 1 M
ddH20	32.82 µl	ddH20
TOTAL:	50 µl	

Modified after http://2013.igem.org/Team:TU-Munich/Results/Recombinant

- Incubate purified EreB with Erythromycin/Azithromycin for different times (t = 5 min, 15 min , …) and apply to 6 mm filter disk
- Reaction is stopped with methanol, heating optional
- Dependent on the enzymatic activity of EreB the filter plates will have varying concentrations of Erythromycin /Azithromycin
- Apply to media growing an E. coli colony and measure the inhibitory zone around the plates after time t (radial length in cm or px (image))
- E. coli can be replaced with erythromycin sensitive *Micrococcus luteus*

In this experiment, we only use the selected MO as a reporter (inhibitory zone). This must be done with an E. coli NOT containing ereB!

- graphically illustrate cm/px inhibitory zone as f(t)

VI. Site saturation mutagenesis

Primers with up to 3 AS randomized AS are ordered and amplified in PCR.
Via restriction/ligation cloning the variants are generated. Linearized plasmid transformat ion with the mixed variants are performed.

The generated plasmids are transformed into *chemically competent* BL21 *E. coli* using heat shock transformation.

The generated variants are selected on varied concentrations of azithromycin. (Neutral gene drift with Erythromycin selection would only make sense if we do more than one mutagenesis + selection cycle).

Agar plates Plates:

LB Media (**1000 mL**)

• Tryptone……..10 g • Yeast extract……5 g • NaCl……10g • **Agar (for plates)… … ..15 g** • **Varying Azithromycin concentrations (for 1L medium):** **Stock Volume: Concentration:** – **0 µL --> 0 µg/mL** – **10 µL --> 1 µg/mL** – **100 µL --> 10 µg/mL** – **1000 µL --> 100 µg/mL** – **2500 µL --> 250 µg/mL**	Add 5 M of NaOH to adjust the pH at 7.0 (a few drops will be enough).

Azithromycin (Stock solution)

5. Mix
   - 4g Azithromycin (100 mg/ml)
   - add 40ml ddH2O
6. Sterile filtration
7. Aliquot in 1 ml stocks and store at -20˚C

For 4 Plates (25 mL each):

LB Media (**100 mL**)

• Tryptone……..10 g • Yeast extract……5 g • NaCl……10g • **Agar (for plates)… … ..15 g** • **Varying Azithromycin concentrations for (100 mL Medium):** **Stock Volume: Concentration:** – **0 µL --> 0 µg/mL** – **1 µL --> 1 µg/mL** – **10 µL --> 10 µg/mL** – **100 µL --> 100 µg/mL** – **250 µL --> 250 µg/mL**	Add 5 M of NaOH to adjust the pH at 7.0 (a few drops will be enough).

The colonies that grow on the highest Azithromycin concentration are selected, grown in a ÜNK
and cultivated / harvested /(glycerol stocked) the next day. ÜNK should be selective for azithromycin resistance.

Then the plasmid is isolated (Miniprep). Measure purity of plasmid isolate via nanodrop. Determine DNA-Sequence with Sanger-Sequencing.

VII. LC-MS of Azithromycin degradation

Data (Molar mass):

M(Azi) = 748.98 g/mol
M(AziC1) = ~ 768.98 g/mol (product of hydrolysis, 20 g/mol for H2O, paper says ~ 18 g/mol)
M(AziC2) = 748.98 g/mol

M(Ery) = 733.93 g/mol
M(EryC1) = 751.5 g/mol (product of hydrolysis)
M(EryC2) = 733.93 g/mol (final dehydration)

Purified enzyme (in vitro test):[23]

- Incubate Ery for 0 min and 5 min with EreB
- Stop reaction using three volumes acetonitrile (and freezing in liquid nitrogen)
- Run LCMS (model name/column used see link above)
- A: 0.1 % formic acid in water,
- B: 0.1 % formic acid in ACN,
- linear gradient of 99% A at 0.5 min to 99% B at 6 min
- Column temp 40 °C
- Flux 0.4 ml/min
- Max p = 703 bar
- Tuning using pure educt (explains huge Ery wash peak)

Find solubility for erythromycin and azithromycin -> possibly drive a flatter gradient

E. Materials

I. Media

LB 10 Media (1000 mL)

- Tryptone……10 g
- Yeast extract……5 g
- NaCl……5 g

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

- For plates: Agar ……15 g

Autoclave the medium.

LB 5 Media (1000 mL)

- Tryptone……10 g
- Yeast extract……5 g
- NaCl……5 g

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

- For plates: Agar ……15 g

Autoclave the medium.

Minimal medium - MSgg (100 mL): [10]

Base

- 5mM - potassium phosphate
  KH_2PO_4……0,026 g
  K_2HPO_4……0,061 g
- 100 mM - MOPS ……2,09 g
  2 mM- $MgCl_2$……0,04 g

Add 5 M of NaOH to adjust the pH at 7.0 and autoclave.

Supplements

- 700 µM - $CaCl_2$ (0,7 M) ……0,1 mL
- 100 µM - $MnCl_2$ (0,1 M) ……0,1 mL
- 50 µM - $FeCl_3$ (50 mM) ……0,1 mL
- 1 µM - $ZnCl_2$ (1 mM) ……0,1 mL
- 2 µM - thiamine (2 mM) ……0,1 mL
- 0.5% - glycerol (86%) ……0,57 mL
- 0.5% - glutamate (5%) ……0,1 mL
- 50 µM - tryptophan (5mM) ……1 mL

Blue indicated stocks are filter sterilized, others are autoclaved.

Note: Stock solutions of supplements can be prepared in big volume.

Solified medium:

- MSgg medium
- +1.5% Bacto agar (Difco)

Dry at 25 ˚C for 16h before use

II. Buffer

Tris (1 M, pH 7.5)

- Tris base .....60.5 g
- ddH2O ..... 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
- ddH2O ..... 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
- Na2HPO4 (10 mM) ..... 1.77 g
- KH2PO4 (1.8 mM) ..... 0.24 g
- ddH2O ..... to 1000 ml

Adjust to pH 7.4, add the needed amount of HCl. Store 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.

0,5M EDTA

- EDTA .....18,61 g
- ddH2O .....80 ml

Adjust to pH 8.0, add the needed amount of NaOH-plates and fill up to 100 ml.

50 x TAE

- Tris base .....242 g

Mix Tris with stir bar to dissolve in about 700 mL ddH_2O

- glacial acetic acid .....57.1 ml
- EDTA solution (0,5m , pH 8.0) ....100 ml
- ddH2O .....to 1000 ml

Store at room temperature.

III. 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. 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

Spectinomycin

1. Mix
   - 4 g Spectinomycin (100 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.

$3CO_{12}$-HSL (*N*-(3-oxododecanoyl)-homoserine lactone) - $1*10^{-1}$M

1. Dissolve 3,6 mg $3CO_{12}$-HSL in 120 µl DSMO
2. Store at -20° C.

F. References

[1] Fengiu Ouyang and Min Zhao. 2019. Enhanced catalytic efficiency of CotA-laccase by DNA shuffeling. Bioengineered. [12.10.21]; 10(1): 182-189. https://www.tandfonline.com/doi/full/10.1080/21655979.2019.1621134

[2] L. Zhang, H. Cui, G.V. Dhoke, Z.Zou, D.F.Sauer, M.D.Davari, U. Schwaneberg 2020. Engineering of Laccase CueO for Improved Electron Transfer in Bioelectrocatalysis by Semi-Rational Design. Chem. Eur. J. [12.10.21]; 26: 4974 – 4979. doi.org/10.1002/chem.201905598

[3] New England Biolabs Inc. Gibson Assembly® Protocol (E5510). [13.10.19]. https://www.neb.com/protocols/2012/12/11/gibson-assembly-protocol-e5510

[4] A. Sarrion-Perdigones, M. Vazquez-Vilar, J. Palací, B. Castelijns, J. Forment, P. Ziarsolo, J. Blanca, A. Granell, D. Orzaez. 2013. GoldenBraid 2.0: A Comprehensive DNA Assembly Framework for Plant Synthetic Biology. Plant Physiology. [12.10.21]. 162(3) 3:1618–1631 https://doi.org/10.1104/pp.113.217661

[5] New England Biolabs Inc. Monarch® Plasmid DNA Miniprep Kit Protocol (NEB #T1010). [12.10.21]. https://www.neb.com/protocols/2015/11/20/monarch-plasmid-dna-miniprep-kit-protocol-t1010

[6] Zymo Research. ZymoPURE™ Plasmid Miniprep Kit. [12.10.21] https://files.zymoresearch.com/protocols/_d4208t_d4209_d4210_d4211_d4212_zymopure_plasmid_miniprep.pdf

[7] Promega Corporation. PureYield™ Plasmid Miniprep System. [12.10.21]. https://www.promega.de/-/media/files/resources/protcards/pureyield-plasmid-miniprep-system-quick-protocol.pdf?rev=592591947f9c47efb5a576173f4f77f6&sc_lang=de-de

[8] Griffitts Lab. 2009. Griffitts: Chimically competent cells. Brigham Young University. [28.09.2020]. http://openwetware.org/wiki/Griffitts:Chemocompetent_Cells

[9] Protocol on the preperation and transformation of competent B. subtilis cells we recieved from Prof. Dr. Jörg Stülke from the Technical University Darmstadt in 2020.

[10] Protocol on the cultivation of B. subtilis biofilms we received from Prof. Dr. Ákos T. Kovács from the Technical University Denmark in 2021.

[11] S.S. Branda, J.E. González-Pastor, S. Ben-Yehuda, R. Losick, R. Kolter. 2001. Fruiting body formation by *Bacillus subtilis*. PNAS. [12.10.21]; 98(20): 11621–11626. https://www.pnas.org/content/98/20/11621

[12] S. Naveed, F. Qamar. 2014. UV spectrophotometric assay of Diclofenac sodium available brands. Journal of Innovations in Pharmaceuticals and Biological Sciences. 1: 92-96 www.pnas.orgycgiydoiy10.1073ypnas.191384198

[13] Huang, J. *et al*. 2018. Programmable and printable Bacillus subtilis biofilms as engineered living materials. Nat Chem Biol. [12.10.21]; 15: 34–41. https://doi.org/10.1038/s41589-018-0169-2

[14] Q. Huang, H. Wu, P. Cai, J.B. Fein W. Chen. 2015. Atomic force microscopy measurements of bacterial adhesion and biofilm formation onto clay-sized particles. Scientific Reports. [12.10.21]; 5: 16857. https://doi.org/10.1038/srep16857

[15] GE Healthcare Bio-Science AB. 2007. PD-10 Desalting Columns. [12.10.21]. http://wwwuser.gwdg.de/~jgrossh/protocols/protein-purification/PD10.pdf

[16] Katarzyna Litwińska et. Al. 2019 Characterization of recombinant laccase from Trametes versicolor synthesized by *Arxula adeninivorans* and its application in the degradation of pharmaceuticals. ABM Express. 9(1):102. doi: 10.1186/s13568-019-0832-3

[17] Sultan K. et al. 2019. Degradation of diclofenac, trimethoprim, carbamazepine, and sulfamethoxazole by laccase from Trametes versicolor: Transformation products and toxicity of treated effluent. Biocatalysis and Biotransformation. 37(6):399-408. https://doi.org/10.1080/10242422.2019.1580268

[18] Test No. 236: Fish Embryo Acute Toxicity (FET) Test. 2013. Paris: OECD Publishing. https://doi.org/10.1787/20745761

[19] International Standard ISO 15088:2007(E). 2007. Water quality - Determination of the acute toxicity of wastewater to zebrafish eggs (*Danio rerio*). International Organization for Standardization.

[20] Thomas Braunbeck and Eva Lammer. 2006. Background paper on Fish embryo toxicity assays. German Federal Environment Agency. https://www.oecd.org/chemicalsafety/testing/36817242.pdf

[21] Patricia McGrath. 2012. Zebrafish: Methods for Assessing Drug Safety and Toxicity, John Wiley & Sons.

[22] S. SchmidtW. Busch, R. Altenburger, E. Küster. 2016. Mixture toxicity of water contaminants-effect analysis using the zebrafish embryo assay (Danio rerio). Chemosphere. [12.10.21]; 152:503- 512. https://doi.org/10.1016/j.chemosphere.2016.03.006

[23] iGEM Team TU-Munich. 2013. The PhyscoFilter for Erythromycin. [10.10.2020]. http://2013.igem.org/Team:TU-Munich/Results/GM-Moss