

# Methodic Protocols & Materials

---



*For everybody's guidance*

*iGEM Team TU Darmstadt 2018*

Updated: 13.10.2018

# Content

A. In vitro.....	1
I. Cloning .....	1
a) Polymerase Chain Reaction (PCR) .....	1
b) Restriction digest.....	3
c) Dephosphorylation .....	3
d) DNA Ligation .....	3
II. Analysis and Purification.....	5
a) Agarose gel electrophoresis .....	5
b) Gel and PCR Clean-Up System (Wizard® SV).....	5
c) Plasmid Miniprep (PureYield™ System) .....	6
d) DNA Quantification with NanoDrop .....	7
e) Sequencing of DNA (Eurofins Genomics).....	7
f) SDS PAGE.....	8
g) Western Blot.....	10
h) HPLC .....	10
B. In vivo.....	11
I. E. coli.....	11
a) Chemically competent cells .....	11
b) Bacterial cell culture .....	12
c) Cell counting/plating .....	12
d) Heat Shock Transformation.....	13
e) Colony PCR .....	13
f) Glycerol stock.....	14
g) Protein Expression (T7-promoter system) .....	14
II. Yeast .....	14
a) Chemically competent cells .....	14
b) Transformation .....	14
c) Deletion.....	14
d) Cell disruption .....	14
C. Protein purification .....	15
I. His-tag purification (E. coli) .....	15
II. His-tag purification (Yeast).....	15
D. Assays.....	16
I. NADPH-dependent Assay .....	16
II. Phenylhydrazine-dependent Assay.....	16

E. Chemistry .....	17
F. Materials.....	18
I. Buffers .....	18
a) ?_? .....	18
e) Protein purification.....	18
f) _?_?_ .....	19
II. Media.....	20
g) 1.7-DYT Media (recipe for 500 ml) .....	20
a) LB Media (recipe for 1000 ml) .....	20
b) SOB Media (recipe for 1000 ml) .....	20
c) SOC Media (recipe for 1000 ml).....	20
III. Stock solutions.....	21
a) Antibiotics.....	21
b) Induction chemicals.....	21
IV. Recipes.....	22
a) 9:25 Pfu MM (450 $\mu$ l).....	22
b) 2 x Taq MM (1250 $\mu$ l) .....	22
c) 10 x Pfu buffer (100 ml).....	22
d) 10 x Taq buffer (100 ml) .....	22

## 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.

#### OneTaq® DNA Polymerase

Component	20 µl Reaction	50 µl Reaction	Final [Conc]:
5X OneTaq Standard Reaction Buffer		10 µl	1X
10 mM dNTPs		1.0 µl	200 µM
10 µM Forward Primer		1.0 µl	0.2 µM
10 µM Reverse Primer		1.0 µl	0.2 µM
Template DNA			< 1,000 ng
Nuclease-free water (MQ)		to 50 µl	
OneTaq® DNA Polymerase		0.25 µl	1.25 units/50 µl

Step	Temperature	Time	Number of Cycles
Initial Denaturation	94 °C	0:30 min.	1 cycle
Denaturation	94 °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 $\mu$ M Reverse Primer	1.0 $\mu$ l	2.5 $\mu$ l	0.5 $\mu$ M
Template DNA	variable	variable	< 250 ng
DMSO (optional)	(0.6 $\mu$ l)	(1.5 $\mu$ l)	3 %
Nuclease-free water (MQ)	to 20 $\mu$ l	to 50 $\mu$ l	
Phusion	0.2 $\mu$ l	0.5 $\mu$ l	1.0 units/50 $\mu$ l

Step	Temperature	Time	Number of Cycles
Initial Denaturation	98 °C	0:30 min.	1 cycle
Denaturation	98 °C	0: 50 – 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 PCR

Component:	20 $\mu$ l Reaction	50 $\mu$ l Reaction	Final [Conc]:
Q5 Reaction Buffer 5x		10 $\mu$ l	1x
dNTPs (10mM)		1 $\mu$ l	200 $\mu$ M
Forward Primer (10 $\mu$ M)		2.5 $\mu$ l	0.5 $\mu$ M
Reverse Primer (10 $\mu$ M)		2.5 $\mu$ l	0.5 $\mu$ M
Template DNA		...	1 ng/ $\mu$ L
(Q5 High GC Enhancer)		(10 $\mu$ l)	(1x)
Nuclease-free water (MQ)		...	(to 50 $\mu$ l)
Q5 DNA Polymerase		0.5 $\mu$ l	0.02 U/ $\mu$ l
<b>Total:</b>	20 $\mu$ l	50 $\mu$ l	

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:
  - 500 ng DNA
  - 5  $\mu$ l 10 x appropriate NEBuffer
  - 1  $\mu$ l restriction enzyme (10 U)
  - Fill up to 50  $\mu$ l with nuclease free H<sub>2</sub>O
2. Incubate for 30 minutes at 37°C
3. Heat inactivation: Incubate at 80 °C for 20 minutes

Larger or smaller scale DNA digestions can be accomplished by scaling this basic reaction proportionately.

### Multiple Restriction Enzyme Digests

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, BioBrick Assembly: Buffer 2.1). Follow the single restriction enzyme digestion by using 1  $\mu$ l of the additional enzyme and take off 1  $\mu$ l from the nuclease-free water.

## c) Dephosphorylation

Antarctic 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 10 x Antarctic Phosphatase Reaction Buffer
  - 1  $\mu$ l of Antarctic Phosphatase
2. Incubate at 37 °C for 30 minutes
3. Heat inactivation: Incubate at 70 °C for 5 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 (20 $\mu$ l batch)

1. Reaction mix

- T4 Ligase Buffer 2  $\mu$ l
  - T4 Ligase 1  $\mu$ l
  - Digested Insert 6  $\mu$ l
  - Digested Backbone 2  $\mu$ l  
Molar ratio: 3 : 1 (Insert : Vector)  
For calculation: [www.php.rsdnerf.com](http://www.php.rsdnerf.com)
  - add ddH<sub>2</sub>O to 20  $\mu$ l end volume
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

## 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 1 x 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  
Mix 2 µl Nancy-520 or HDGreen plus with 50 ml of agarose gel
4. Fill mixture into gel chamber and let it cool down (do not forget the well combs)
5. Fill-up chamber with 1 x 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 6 x loading dye (5 µl sample with 1 µl loading dye) and pipette into pockets
9. Run electrophoresis with 120 V for 45 minutes.

### b) Gel and PCR Clean-Up System (Wizard® SV)

Gel and PCR Clean-Up Systems are used to remove unincorporated primers, salts, and left-over dNTP's from generated amplicons after PCR. This System also provides purification of amplicons from preparative agarose gel.

#### Procedure

Reference Wizard® SV Gel and PCR Clean-Up System Quick Protocol from Promega

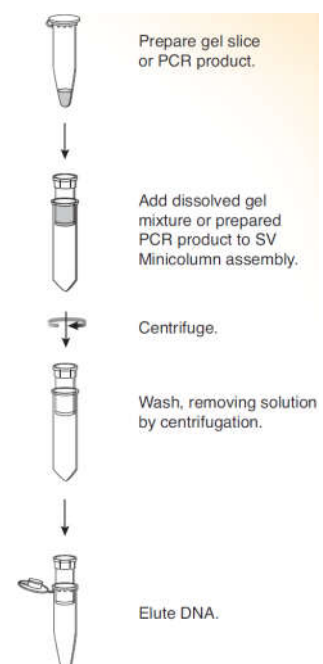
#### Gel Slice and PCR Product Preparation

##### A. Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5 ml microcentrifuge tube.
2. Add 10 µl Membrane Binding Solution per 10 mg of gel slice. Vortex and incubate at 50 – 65 °C until gel slice is completely dissolved.

##### B. Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.





## Binding of DNA

1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at  $16,000 \times g$  for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

## Washing

4. Add 700  $\mu\text{l}$  Membrane Wash Solution (ethanol added). Centrifuge at  $16,000 \times g$  for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500  $\mu\text{l}$  Membrane Wash Solution. Centrifuge at  $16,000 \times g$  for 5 minutes.
6. Empty the Collection Tube and re-centrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

## Elution

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
8. Add 50 $\mu\text{l}$  of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at  $16,000 \times g$  for 1 minute.
9. Discard Minicolumn and store DNA at  $4^\circ\text{C}$  or  $-20^\circ\text{C}$ .

Source: <https://www.promega.com/~media/files/resources/protcards/wizard%20sv%20gel%20and%20pcr%20clean-up%20system%20quick%20protocol.pdf>

### c) Plasmid Miniprep (PureYield™ System)

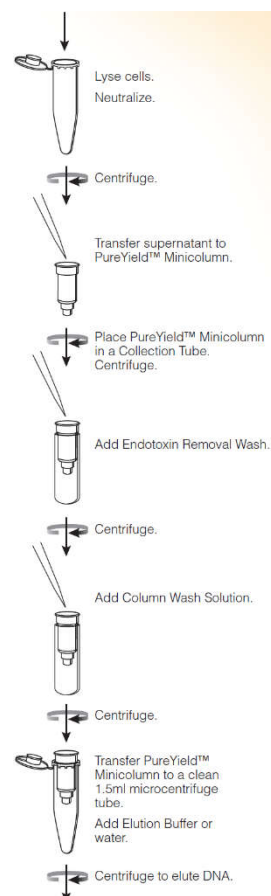
To get the plasmid out of the cells, we use the PureYield Plasmid Miniprep System Kit from Promega. It is a fast way to purificate plasmid DNA out of a bacteria culture by cetrifugation.

#### Procedure

The whole purification was done according to the "Quick Protocol" of the PureYield Plasmid Miniprep System from Promega.

#### Prepare Lysate

1. Add 600  $\mu\text{l}$  of bacterial culture to a 1.5 ml microcentrifuge tube.  
Note: For higher yields and purity use the alternative protocol below to harvest and process up to 3 ml of bacterial culture.
2. Add 100  $\mu\text{l}$  of Cell Lysis Buffer (Blue), and mix by inverting the tube 6 times.
3. Add 350  $\mu\text{l}$  of cold ( $4 - 8^\circ\text{C}$ ) Neutralization Solution, and mix thoroughly by inverting.
4. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
5. Transfer the supernatant ( $\sim 900 \mu\text{l}$ ) 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.

### **Wash**

8. Add 200  $\mu$ l of Endotoxin Removal Wash (ERB) to the Minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds.
9. Add 400  $\mu$ l 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 1.5 ml microcentrifuge tube, then add 30  $\mu$ l 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, and store eluted plasmid DNA at  $-20^{\circ}\text{C}$ .

Source: <https://www.promega.com/~media/files/resources/protcards/pureyield%20plasmid%20miniprep%20system%20quick%20protocol.pdf>

### **d) 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  $\mu$ l.

#### **Procedure**

1. Start the program for the NanoDrop and choose "Nucleic Acids"
2. Pipette 1  $\mu$ l 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/ $\mu$ l, the 260/280 value and the 230/260 value.

### **e) Sequencing of DNA (Eurofins Genomics)**

Sequencing is a method to control if you have your construct of interest in your plasmid. With or without mutations.

#### **Procedure**

1. Pipette 15  $\mu$ l of template into 1.5 ml reaction tube, DNA concentration needs to be between 50 & 100 ng/ $\mu$ l. Dilute if necessary.
2. Add 2  $\mu$ l primer (10  $\mu$ M) of choice (do only use one primer!).

#### **Premixed Samples (a mixture of template and primer)**

- Templates should consist of 15  $\mu$ l purified DNA with the concentration given above.

- Add 2 µl of primer with a concentration of 10 pmol/µl (10 µM).
- Ensure that the total volume of your premixed sample is 17 µl.

### Optimum Primer Conditions

- Primers must not contain phosphorylation or fluorescent dyes
- The optimum primer length is between 16 – 25 bases
- The primer melting temperature (T<sub>m</sub>) should be 50 – 62 °C
- The GC content of the primer should be 35 – 60 %
- Ideally one G or C should be located at the 3' primer end
- The number of 3' Gs or Cs should not exceed 2 Gs or Cs
- If possible, avoid >3 identical bases in a row in the sequence

### Primer Concentration and Volume

- Exactly **10 pmol/µl** primer concentration is required per sequencing reaction
- Each primer must have a total **volume of 15 µl** (double distilled water or 5mM Tris-HCl); **5 µl of primer volume** is required for every additional sequencing reaction
- Concentration of primers with wobble bases must be calculated according to the following formula: **n<sup>x</sup> x ConcPrimer**

**n** = number of bases within a wobble according to IUPC code, **X** = number of wobbles within the primer sequence. [e.g. 1 V (AGC) = 31 x 10 pmol/µl; 2 V (AGC) (AGC) = 32 x 10 pmol/µl]

### Source:

[http://www.eurofinsgenomics.eu/media/892645/samplesubmissionguide\\_valuereadtube.pdf](http://www.eurofinsgenomics.eu/media/892645/samplesubmissionguide_valuereadtube.pdf)

### Address:

Eurofins Genomics | Anzinger Str. 7a | 85560 Ebersberg | Germany

## f) SDS PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) is a widely used method to separate different proteins by mass.

### Gels

	Separation gel (10 %)	Stacking gel (4 %)
MilliQ	1.975 µl	2.065 µl
Acrylamid/Bisacrylamid 37.5 : 1	1.670 µl	506 µl
Separation gel buffer	1.250 µl	
Stacking gel buffer		375 µl
SDS (10 %)	50 µl	30 µl
APS (10 %)	25 µl	15 µl
TEMED	2.5 µl	1.5 µl

### Buffers

- Separation gel buffer (0.5 M Tris, 0.4% SDS, pH = 8.8)
- Stacking gel buffer (0.5 M Tris, 0.4% SDS, pH = 6.6)
- Running buffer (0.25 M Tris, 2 M glycine, 1% SDS , pH = 8.3)
- 2 x sample buffer “Laemmli-Buffer” (5 ml)

	Volume	Final concentration
Tris-HCL (1 M, pH 5.8)	650 µl	130 mM
SDS (10 %)	2000 µl	4 %
Glycerol (80 %)	1250 µl	20 %
β-Mercaptoethanol	500 µl	10 %
Bromphenolblue (pH = 6.75)	spatula tip	

- Staining buffer

	Volume/Amount
Coomassie brilliant blue G250	0.5 g
Coomassie brilliant blue R250	0.5 g
Methanol	100 ml
Acetate	20 ml
Aqua dest.	100 ml

## Procedure

### Load & run

1. Prepare separating gel and fill it into the chamber
2. Pour 1 ml isopropyl alcohol on the top of the gel to destroy air bubbles and prevent dehydration
3. Discard the isopropyl alcohol and pour the prepared stacking gel after separating gel is polymerized
4. Stick in the comb
5. If not used immediately, store the gel in wet cloth (to prevent dehydration) at 4°C
6. If used immediately, remove comb when the gel is fully polymerized and place it into SDS-PAGE chamber
7. Fill up chamber with running buffer
8. Heat up samples with 2 x sample buffer at 95 °C for 5 minutes and apply 20 µl to each pocket
9. Load one pocket with a commercial protein marker
10. Start the PAGE by applying 20 mA while stacking
11. Apply 40 mA while separation

### Staining & washing of gel

1. Disconnect glass plates containing the already run gel
2. Cut off stacking gel
3. Put separation gel into staining buffer and shake at room temperature for at least one hour or heat it in the microwave
4. Put stained separation gel into distilled water and let shake for 10 minutes
5. Repeat previous step at least twice again with fresh distilled water

#### g) Western Blot

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<sub>2</sub>O).
4. Use ponceau staining to determine whether the protein transfer was successful.
5. Block the membrane with 5% skimmed milk in TBS-T buffer (20 mM Tris, 150 mM NaCl and 0.05 % Tween 20) overnight.
6. Wash the membrane 3 times for 10 minutes.
7. Add primary antibodies (anti-6X His tag rabbit polyclonal antibody, Santa Cruz Biotechnology)
8. Wash the membrane again 3 times for 10 minutes
9. Incubate with secondary antibodies (rabbit- anti mouse IgG, HRP, Santa Cruz Biotechnology).
10. visualize the blot by using a detection reagent (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare).
11. generate image using ImageLab (Bio-Rad Laboratories, Inc.)

#### h) HPLC

## 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 OD<sub>600</sub> 0.4 – 0.6 (usually 2 – 3 h)
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 x g for 10 minutes
6. Gently resuspend each pellet with 15 ml of cold Mg<sup>2+</sup>/Ca<sup>2+</sup> solution (Do not vortex!).
7. Incubate in an ice bath for 30 minutes
8. Centrifuge the culture at 4 °C and 3000 x g for 10 minutes
9. Resuspend each pellet with 1.6 ml of cold 100 mM CaCl<sub>2</sub> 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 as 100 µl aliquots
14. Store at - 80 °C.

#### Mixtures

- Mg<sup>2+</sup>/Ca<sup>2+</sup>
    - 3.25 g MgCl<sub>2</sub>·6 H<sub>2</sub>O
    - 0.6 g CaCl<sub>2</sub>·2 H<sub>2</sub>O
    - 200 ml ddH<sub>2</sub>O
    - Autoclave
  - CaCl<sub>2</sub> (100 mM)
    - 2.95 g CaCl<sub>2</sub>·2H<sub>2</sub>O
    - 200 ml dH<sub>2</sub>O
    - Autoclave
- Note: You can also make a 1:10 dilution of the 1 M stock

Source: [http://openwetware.org/wiki/Griffitts:Chemocompetent\\_Cells](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](http://openwetware.org/wiki/Bacterial_cell_culture)

## c) Cell counting/plating

Cell counting/plating is a procedure to calculate the number of cells in a sample.

### Procedure

1. Fill each tube in the dilution with 90 µl of LB.
2. Add 10 µl of the sample to the first tube and mix.
3. From the first tube, remove 10 µl and mix it in a second tube.
4. Repeat for the number of dilutions you wish to do (8 should be more than enough) [1].
5. Take 10 µl from each dilution and spread it out on an agar plate.
6. Allow droplet to dry and incubate.

The first dilutions will contain a thick lawn of cells and the last dilutions will contain no cells. There should be one drop which contains countable single colonies. From this, you can calculate the number of cells in the original sample. For example, if there 4 colonies on dilution 5, there are  $4^4$  cells/µl.

Source: [http://openwetware.org/wiki/Bacterial\\_cell\\_culture](http://openwetware.org/wiki/Bacterial_cell_culture)

#### d) 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 15 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 an agar plate

#### e) 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.

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<sub>2</sub>O 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 1 x mix.
4. Run a agarose gel to determine the product length

##### Procedure

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



### f) Glycerol stock

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

#### Procedure

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

### g) Protein Expression (T7-promoter system)

(If working with different promotor system, use appropriate inducing chemical and take care of using appropriate strain)

**Procedure** (Choose volume appropriate to your desired amount of expressed protein)

1. Inoculation of 50 ml LB medium in a 100 ml flask with E. coli BL21 (DE3) strain transformed with desired plasmid
2. Incubation at 180 rpm per minute (rpm) at 30 °C to an  $OD_{600} = 4$
3. Transfer starter culture into 1 L LB medium in a 3 L flask resulting in an  $OD_{600} = 0.2$
4. Incubation to an  $OD_{600} = 0.6$  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 1 M).
7. Incubation of the cell suspension over night at 180 rpm at 30 °C.

## II. Yeast

### a) Chemically competent cells

### b) Transformation

### c) Deletion

### d) Cell disruption

## C. Protein purification

### I. His-tag purification (E. coli)

For ycdW (Glyoxylate reductase), aceA (Isocitrate lyase), ADH (alcohol dehydrogenases) and CHMO (cyclohexanone monooxygenase)

Cell resuspension and disruption

1. Inoculation of cell culture in 1 L LB media by an  $OD_{600} = 0.1$ ,
2. Induction of cell culture with 0.5 mM IPTG by an  $OD_{600} = 0.5$ , growing over night
3. Centrifugation of cells (15 min, 8000 rpm, 4 °C) after 19 hours inducing
4. Thawing the cell pellet on ice, resuspend pellet in 50 ml ice-cold Resuspension buffer
5. Wash EmulsiFlex with water and ice-cold Resuspension buffer while adjusting the pressure to about 1500 bar
6. Add resuspended cells in EmulsiFlex
7. Collect disrupted cells in a 50 ml Flacon tube on ice
8. To ensure that all cells are crushed repeat the disruption step twice
9. Fill cell lysate into clean ultracentrifuge tubes and centrifuge cells (60 min, 25.000 rpm, 4 °C)
10. Collect the supernatant into a new tube on ice. Perform this step carefully
11. If the supernatant is still cloudy, sonicate it for 30 s and then filter it through a 0.2  $\mu$ m filter

Purification via ÄKTA

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. Calibrate 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 80 ml of the sample, put flow through in waste.
3. Wash column with 10 column volume water to get rid of the remaining sample
4. Elute the protein with an increasing concentration of imidazole starting at 5 % and reach the final concentration of 100 % imidazole in 7 ml
5. Collect fractions of 0.5 ml
6. Wash apparatus with water and EtOH

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

### II. His-tag purification (Yeast)

## **D. Assays**

### **I. NADPH-dependent Assay**

### **II. Phenylhydrazine-dependent Assay**

## E. Chemistry

## F. Materials

### I. Buffers

#### a) ?\_?

Tris (1M, pH 7.5)

- 60.5 g Tris base
- adjust pH to 7.5 using 5 M HCl
- add ddH<sub>2</sub>O to 500 ml
- store at room temperature

1 x PBS

- 8.18 g NaCl (140 mM)
- 0.2 g KCl (2.7 mM)
- 1.77 g Na<sub>2</sub>HPO<sub>4</sub> (10 mM)
- 0.24 g KH<sub>2</sub>PO<sub>4</sub> (1.8 mM)
- Add. 1 l ddH<sub>2</sub>O
- Adjust to pH 7.4 using HCl
- store at room temperature

TE buffer

- 10 mM Tris, adjust to pH 8.0 using HCl
- 1 mM EDTA (Ethylenediaminetetraacetic acid)
- store at room temperature

50 x TAE

- 242 g Tris base
- add 57.1ml glacial acetic acid
- 100 ml of 500 mM EDTA (pH 8.0) solution
- add up to 1 L with H<sub>2</sub>O
- mix Tris with stir bar to dissolve in about 600 ml of ddH<sub>2</sub>O
- add EDTA and Acetic Acid
- bring final volume to 1 L with ddH<sub>2</sub>O
- store at room temperature

#### e) Protein purification

Resuspension/Equilibration buffer

- 500 mM NaCl
- 20 mM NaPO<sub>4</sub>, pH = 7,4
- 20 mM Imidazol

- 1 mM PMSF (only in Resuspension buffer, if you don't work with proteases later)

#### Elution buffer

- 500 mM NaCl
- 20 mM NaPO<sub>4</sub>, pH = 7,4
- 350 mM Imidazol

f) \_?\_?\_

## II. Media

### g) 1.7-DYT Media (recipe for 500 ml)

- 2.5 g NaCl
- 8.0 g tryptone
- 5.0 g yeast extract
- add 5 M NaOH to adjust the pH at 7.0 (few drops will be enough)

### a) LB Media (recipe for 1000 ml)

- 10 g tryptone
- 5 g yeast extract
- 10 g NaCl
- add 5 M NaOH to adjust the pH at 7.0 (few drops will be enough)
- for plates: add 15 g agar

### b) SOB Media (recipe for 1000 ml)

- 0.186 g KCl
- 2.4 g MgSO<sub>4</sub>
- 0.584 g NaCl
- 20 g tryptone
- 5 g yeast extract
- add 5 M NaOH to adjust the pH at 7.0 (few drops will be enough)

### c) SOC Media (recipe for 1000 ml)

- 980 ml SOB
- 20 ml 1M glucose

**Autoclave all media after mixing!**

### III. Stock solutions

#### a) Antibiotics

##### ➤ Ampicillin

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

##### ➤ Tetracycline

#### 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

##### ➤ Arabinose



## IV. Recipes

### a) 9:25 Pfu MM (450 µl)

- 10x Pfu buffer (BSA)..... 250 µl
- Pfu polymerase..... 50 µl
- dNTPs (10 mM) ..... 50 µl
- MgCl<sub>2</sub> (50 mM)..... 50 µl
- DMSO..... 50 µl

### b) 2 x Taq MM (1250 µl)

- 10 x Taq buffer..... 250 µl
- Taq polymerase..... 25 µl
- 50 mM MgCl<sub>2</sub> ..... 50 µl
- 10 mM dNTPs..... 50 µl
- DMSO..... 75 µl
- ddH<sub>2</sub>O ..... 800 µl

### c) 10 x Pfu buffer (100 ml)

- 200 mM Tris-HCl (pH 8.8 at 25 °C)..... 2.42 g
- 100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ..... 1.32 g
- 100 mM KCl..... 0.75 g
- 20 mM MgSO<sub>4</sub> x 7 H<sub>2</sub>O ..... 0.49 g
- 1 % Triton X-100..... 1 ml
- ddH<sub>2</sub>O.....up to 100 ml
- Autoclaving/sterile filtration after mixing.
- add 1 ml 100 mg/ml BSA (-> end conc. 1 mg/ml)

### d) 10 x Taq buffer (100 ml)

- 500 mM KCl..... 3.7 g
- 0.8 % Triton X-100..... 800 µl
- 100 mM Tris-HCl (pH 8.8 at 25 °C) .....up to 100 ml
- Autoclaving/sterile filtration after mixing.