iGEM Team Tübingen Experiments

Agarose gel electrophoresis

Introduction

Separation of DNA fragments.

Materials

- 1x TAE-buffer
- Agarose
- nitrile gloves
- loading dye: 6x purple loading dye from NEB
- DNA ladder: GenLadder 1kbp (250 bp-10000 bp)
- Diamond nucleic acid dye / Midori green
- agarose gel chamber
- power supply
- UV-desk

Method

- Weigh 2.5 g Agarose in a 250 ml screw-cap laboratory bottle.

- Fill up to 250 ml with 1x TAE-buffer.

- Heat up in microwave until agarose completely dissolves, take out of microwave and stir every 30 to 60 s. Store bottle in 60°C incubator or store at RT and heat up in microwave before using.

- Pour agarose into gel tray. (If Midori green is used, add 1-2 µL when pouring agarose into the tray.) Let the gel solidify, covered with paper towel.

- Put the gel into electrophoresis chamber, fill up with 1x TAE-buffer until gel is slightly covered.

- Add 5 µl 6x loading dye to PCR-reaction, mix by pipetting.

- Apply whole 25 µl of PCR-reaction supplemented with loading dye and 5 µl of DNA-ladder.

- Run gel electrophoresis at 120 V for about 30 min (until dye front has run far enough).
- If no Midori green is used, stain gel in Diamond nucleic acid dye (Promega) according to their protocol.

- Analyze gel on UV-desk and take a picture.

Control digest

Introduction

To monitor cloning success.

Materials

- Promega restriction enzymes: EcoRI (12 U/µl), BamHI (10 U/µl), HindIII (10 U/µl)
- Promega 10x restriction buffers: buffer H (EcoRI), buffer E (BamHI, HindIII)
- Promega acetylated BSA (10 mg/ml) as enzyme stabilizer
- autoclaved ddH_2O
- DNA sample
- heat block

Method

-In a sterile tube, assemble the following components in the order listed below:

Component	**Volume (µl)**
sterile ddH2O	to 20
10x restriction buffer (Promega)	2
acetylated BSA (Promega, 10 mg/ml)	0.2
DNA sample	0.2 - 1.5 µg
restriction enzyme (Promega)	0.5
total	**20**

- Mix gently by pipetting, close the tube and centrifuge for a few seconds in a microcentrifuge. Incubate at 37 °C for 1 - 4 hours.
- Proceed with agarose gel analysis protocol

E. coli DH5α transformation

Introduction

Transformation of NEB 5-α chemically competent *E. coli* with cloning products; derived from NEB instructions.

Materials

- C2987I NEB 5-α competent *E. coli* cells
- DNA or cloning reaction
- SOC medium (NEB)
- LB selection plates
- heat block
- shaker/rotator

Method

- Thaw a tube of NEB 5-α competent *E. coli* cells on ice. Mix gently and carefully pipette 50 µl of cells into a transformation tube on ice.
- Add 1 - 5 µl sample containing 1 pg – 100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4 - 5 times to mix cells and DNA. Do not vortex.
- Place the mixture on ice for 30 min.
- Heat shock at 42 °C for 30 s.
- Place on ice for 5 min.
- Pipette 950 µl of SOC into the mixture.
- Place at 37 °C for 60 min. Shake vigorously (250 rpm) or rotate.
- Warm selection plates to 37°C.
- Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in SOC.
- Spread 100 µl of each dilution onto a selection plate and incubate overnight at 37 °C.

GoldenGate BpiI (BbsI)

Introduction

Cloning level I and level III vectors with BpiI (BbsI).

Materials

- Nuclease free H_2O
- T4 DNA ligase buffer (10x)
- Plasmids/fragments
- T4 DNA ligase (NEB)
- BbsI-HF (NEB)
- Thermocycler

Method

- Set up 25 µl reaction in a PCR-tube by pipetting in the given order; pipet and store on ice:

Component	**Amount**
Nuclease free H_2O	fill up to 25 µl
T4 DNA ligase buffer (10x)	2.5 µl
Plasmids/fragments	75 ng
T4 DNA ligase (NEB)	0.5 µl (200 U)
BbsI-HF (NEB)	1.5 µl (30 U)

- Mix gently by pipetting up and down several times.
- Run the following program in a thermocycler:

Temperature (°C)	**Time (min)**	**Cycles**
37	5	} 30x
16	5	
37	5	1x
60	5	1x

- Transform 5 µl GoldenGate reaction into competent *E. coli*.

GoldenGate BsaI

Introduction

Cloning level II vectors with BsaI.

Materials

- Nuclease free H_2O
- T4 DNA ligase buffer (10x)
- Plasmids/fragments
- GoldenGate assembly mix (NEB)
- BbsI-HF (NEB)
- Thermocycler

Method

- Set up 20 µl reaction in a PCR-tube by pipetting in the given order; pipet and store on ice:

Component	**Amount**
Nuclease free H_2O	fill up to 20 µl
T4 DNA ligase buffer (10x)	2 µl
Plasmids/fragments	75 ng
GoldenGate assembly mix (NEB)	0.5 µl (200 U)
BbsI-HF (NEB)	1 µl (20,000 U/ml)

- Mix gently by pipetting up and down several times.
- Run the following program in a thermocycler:

Temperature (°C)	**Time (min)**	**Cycles**
37	1	} 30x
16	1	
37	5	1x
60	5	1x

- Transform 5 µl GoldenGate reaction into competent *E. coli*.

GoldenGate Esp3I

Introduction

Cloning cyclotides into level III vectors with Esp3I.

Materials

- Nuclease free H_2O
- T4 DNA ligase buffer (10x)
- Plasmids/fragments
- T4 DNA ligase (NEB)
- Esp3I (NEB)
- Thermocycler

Method

- Set up 25 µl reaction in a PCR-tube by pipetting in the given order; pipet and store on ice:

Component	**Amount**
Nuclease free H_2O	fill up to 25 µl
T4 DNA ligase buffer (10x)	2,5 µl
Plasmids/fragments	75 ng
T4 DNA ligase (NEB)	0.5 µl (200 U)
Esp3I (NEB)	1,5 µl (15 U)

- Mix gently by pipetting up and down several times.
- Run the following program in a thermocycler:

Temperature (°C)	**Time (min)**	**Cycles**
37	5	} 30x
16	5	
37	5	1x
60	5	1x

- Transform 5 µl GoldenGate reaction into competent *E. coli*.

Mini-/Midiprep

Introduction

Producing and multiplying plasmid DNA (e.g., after cloning) from *E. coli*.

Materials

- LB agar plates
- LB medium
- appropriate antibiotics for selection
- autoclaved glass test tubes
- preparation kit (NEB Monarch® Plasmid DNA Miniprep Kit)
- incubator
- nanodrop

Method

<u>Day 1:</u>

- Transform competent *E. coli* cells and plate them on LB agar plates supplemented with the appropriate antibiotics.

- Incubate plates overnight at 37 °C

<u>Day 2:</u>

- Add 5 ml of LB medium with appropriate antibiotics into sterile glass test tubes.

- Pick a colony from a plate with a sterile pipette tip or inoculation loop and stir in the 5 ml LB.

- Grow the bacteria overnight in shaker at 37 °C.

<u>Day 3:</u>

- Grow bacteria for 8 h at 37 °C and shaking by inoculation of 50 ml LB with 50 µl preculture.

- Spin down liquid cultures and proceed according to the preparation protocol by NEB.

- Measure DNA concentration yielded from the preparation, using a nanodrop.

Sequencing

Introduction

To sequence plasmid or other DNA by Sanger sequencing.

Materials

- Sequencing reaction
- 1.5 ml Eppendorf tubes
- Labels

Method

- Prepare plasmid according to protocol

- Prepare sequencing reaction in 1,5 ml Eppendorf tubes:
  15 µl, containing 40 - 100 ng/µl plasmid/DNA and 4 µM primer

Component	**Amount**
Plasmid/DNA	V (600-1500 ng)
20 µM primer	3 µl
Autoclaved ddH_2O	Fill up to 15 µl

- Label the 1.5 ml Eppendorf tubes and send it to sequencing (Mycrosynth).

Inhibition zone assay

Introduction

To test antimicrobial properties of samples against *E. coli* and *B. subtilis*.

Materials

- LB-agar plates
- crude extract / purified protein solution
- LB medium
- *E. coli* cells
- *B. subtilis* cells
- glass tubes
- filter paper discs

Method

Day 1:

- Inoculate cells in 10 ml LB media overnight (*E. coli* and *B. subtilis*) in glass tube.

Day 2:

- Dilute overnight culture 1:10.
- Inoculate new culture (OD_{600} = 0.1) and let it grow for 3 - 4 h until a OD_{600} = 0.8-1.0.
- Dilute culture 1:50.
- Prepare a series of dilutions of crude extract/protein solution:
  1:10, 1:50, 1:50, 1:100, 1:500 and 1:1000 (200 µl each)
- Spread 200 µl of 1:50 bacteria cultures onto LB-agar plates and let it dry.
- Put small filter paper discs onto the LB-agar plates and add 20 µl of protein dilutions, each to one filter paper disc.
- Incubate plates overnight at 37 °C.
- Take photos of the petri dishes on the next day.

10x TAE-buffer

Introduction

Standard protocol for making 10x TAE-buffer.

Materials

- Tris-base (M = 121.14 g/mol)
- Glacial acetic acid (17,4 M)
- EDTA (M = 372.23 g/mol)
- ddH_2O

Method

- Dilute 48.5 g Tris-base, 11.4 ml glacial acetic acid and 3.7 g EDTA in 800 ml ddH_2O.
- Fill up with ddH_2O to 1 l.

1x Tricine running buffer

Introduction

Standard protocol for making 1x tricine running buffer.

Materials

- 100 mM Tris-base (M = 121.14 g/mol)
- 100 mM Tricine (M = 179.172 g/mol)
- 0.1% (w/v) SDS

Method

- Weigh in the appropriate amount of chemicals.
- Dissolve in ddH_2O and set pH to 8.3.
- Fill up to desired volumed with ddH_2O.

Antibiotics

Introduction

Preparation and storage of antibiotics.

Materials

- Gentamicin
- Ampicillin
- Kanamycin
- Rifampicin
- Streptomycin
- Tetracycline

Method

- Make solutions of 10 mg/ml gentamicin, 50 mg/ml ampicillin, 50 mg/ml kanamycin, 100 mg/ml rifampicin, 100 mg/ml streptomycin or 5 mg/ml tetracycline with ddH_2O.

- Filter the antibiotic solutions with a 0.2 µM sterile filter under the hood and take 0.5 ml aliquots. Store them at - 20 °C.

Media

Introduction

Preparation of used media.

Materials

- Müller-Hinton Broth (MH)
- Brain Heart Infusion Broth (BHI)
- LB broth
- LB agar plates

Methods

MH broth

- Dissolve 21 g of MH broth in 1 l ddH_2O.
- Autoclave the solution at 121 °C for 15 min.

MH 1/5 broth

- Dilute 10 ml of autoclaved MH broth with 40 ml of autoclaved water to obtain a 1/5 dilution.

BHI broth

- Dissolve 37 g of BHI broth in 1 l ddH_2O.
- Autoclave the solution at 121 °C for 15 min.

LB media

- Dissolve 20 g of LB broth in 1 l ddH_2O.
- Autoclave the solution at 121 °C for 15 min.

TE buffer

Introduction

Standard protocol for making TE-buffer.

Materials

- Tris-base (M = 121.14 g/mol)
- EDTA (M = 292.25 g/mol)
- ddH_2O

Methods

- Dissolve 605.7 mg Tris and 14.6 mg EDTA in 400 ml ddH_2O.
- Adjust the pH to 8.0 using a pH-meter

- Fill up to 500 ml total volume with ddH_2O and autoclave.

Acetone protein precipitation

Introduction

To concentrate protein solutions, for SDS-PAGE or other methods not requiring properly folded proteins.

Materials

- Acetone
- Centrifuge

Methods

- Cool acetone to -20 °C.

- Pipet protein sample solution into an Eppendorf tube and add four times the sample volume of acetone.
- Vortex tube and incubate for 60 min at -20°C.
- Centrifuge 10 min at 13.000-15.000 × g.
- Decant and remove supernatant carefully.
- Allow the acetone to evaporate from the uncapped tube at RT for 30 min.

- Add buffer appropriate for the downstream process and vortex thoroughly to dissolve protein pellet.

Agrobacterium transformation

Introduction

Transformation of A*grobacterium tumefaciens* with our 2in1 and 3in1 vectors for transient expression in tobacco.

Materials

- *A. tumefaciens* strain AGL1
- Antibiotics: 100 mg/ml Rif, 100 mg/ml Strep, 5 mg/ml Tet, 25 mg/ml Kan
- Vector
- LB medium
- LB agar
- Shaker
- Incubator
- Centrifuge

Methods

- Thaw *Agrobacteria* on ice
- Add 3 µl plasmid DNA to 100 µl agrobacteria, mix by flicking the tube.
- Incubate 5 min on ice.
- Incubate 5 min in liquid nitrogen.
- Thaw in hand and incubate 5 min at 37 °C.
- Add 900 µl LB medium.
- Incubate 3 h at 28 °C.
- Centrifuge 2 min at 4000 rpm.
- Remove 850 µl supernatant, resuspend pellet in remaining liquid.
- Plate 100 µl of the suspension on LB agar plates containing 10 µg/ml Rif, 2.5 µg/ml Strep, 2.5 µg/ml Tet and 25 µg/ml Kan.
- Incubate plates 2 days at 28 °C.

His-tag affinity purification

Introduction

How to purify His6-tagged peptide from tobacco crude extract. Protocol adapted from Promega and *Potula et al., 2007*.

Materials

- HisLink™ Protein Purification Resin (Promega)
- Millipore filter (0.2 µm pore size)
- Syringes
- Column
- Dialysis chamber, 2 kDa MWCO (ThermoFisher Slide-A-Lyzer™ Dialysis Cassettes)
- Ultracentrifuge
- Magnetic stirrer
- Buffers for extraction, purification and dialysis (see table below)

Buffers	Extraction	Binding	Wash	Elution	Dialysis
Chemicals	150 mM NaCl	150 mM NaCl	150 mM NaCl	150 mM NaCl	150 mM NaCl
	3 mM KCl	3 mM KCl	3 mM KCl	3 mM KCl	3 mM KCl
	100 mM HEPES	100 mM HEPES	100 mM HEPES	100 mM HEPES	100 mM HEPES
	0.2-2 mM PMSF*	10 mM Imidazole	20 mM imidazole	500 mM Imidazole	
	0.05% (v/v) Tween20*				

*add freshly

Methods

- Cut off and weight infiltrated leaves.
- Grind the leaves with liquid nitrogen using mortar and pestle in 200% per weight ice-cold extraction buffer.
- Incubate 1 h on ice before proceeding. Invert every 5-10 min.
- Centrifuge crude extract at 20.000 g for 30 min at 4 °C
- Collect aliquot (200 µl) from the pellet.
- Filter supernatant with Millipore filter, using pre-cleaned syringe.
- Collect aliquot (200 µl) from the filtrate
- Fill column with 1 ml HisLink resin and wait until resin has settled. Allow the column to drain, and equilibrate the resin with 5 column volumes (= 5 ml) of binding buffer, allowing the buffer to completely enter the resin bed.

- Gently add the cleared, filtered lysate to the resin until the lysate has completely entered the column.

- Collect aliquot from the flow-through.

- Wash unbound proteins from the resin using 5 ml of binding buffer.

- Collect aliquot from the flow-through.

- Wash unbound proteins from the resin using 10 ml of wash buffer.

- Collect aliquot from the wash.

- Once the wash buffer has completely entered the resin bed, add elution buffer and start collecting 0.5 ml fractions.

Infiltration of tobacco using *Agrobacterium tumefaciens*

Introduction

Infect tobacco plants with genetically modified *A. tumefaciens* to produce our construct.

Materials

- LB medium
- Antibiotics (100 mg/ml Rif, 100 mg/ml Strep, 5 mg/ml Tet, 25 mg/ml Kan)
- AS medium (see table below)
- Tobacco plants
- P19 bacteria solution
- Syringe (1 ml)
- Fluorescence microscope
- Shaker

Chemicals	**Concentration**
MES-KOH buffer, pH 5.6	1 M
$MgCl_2$	3 M
Acetosyringone in DMSO	150 M

Methods

- Inoculate 2 ml of LB medium with corresponding antibiotics with a single *Agrobacterium* colony.

- Incubate over night at 28 °C while shaking.

- Centrifuge bacterial culture for 15 min at 4000 rpm and discard the supernatant. P19 culture is centrifuged as well.

- Resuspend the pellet in 2 ml of cold AS medium. Add AS medium until an OD_{600} of 0.8-1.0.

- Combine 100 µl of the bacterial solution and 100 µl of P19 bacteria solution in a 2 ml Eppendorf tubes and fill up with AS medium to 2 ml.

- Incubate on ice for at least 1 h.

- Infiltrate 2 - 3 young tobacco leaves per plant with the bacterial solution.

Figure 1: Picture of how to perform the *Agroinfiltration* procedure. A 1 ml syringe is used to infiltrate the young tobacco plants from the underside of the leaves.

Peptide purification according to Poon *et* al. 2018

Introduction

Protocol for extraction and purification of peptides from *Nicotiana benthamiana* adapted from Poon *et* al. 2018.

Materials

- Acetonitrile
- Formic acid
- Filter circles, 110 mm, 5-13 µm
- Drying cabinet
- Mortal and pestle
- Magnetic stirrer
- Round-bottomed flask
- Rotation evaporator
- RP-HPLC MS
- MALDI-TOF MS

Methods

- Dry leaves overnight at 40 °C.
- Grind leaves to fine powder with mortar and pestle.
- Add 20 ml/g (dry weight) extraction buffer (50% acetonitrile - 1% formic acid in ddH_2O).
- Incubate for 1 h at room temperature in a beaker glass on a magnetic stirrer.
- Filter with filter paper to remove insoluble extract into round-bottomed flask.
- Repeat incubation and filtering with insoluble extract with the same amount of extraction buffer.
- Dry soluble fraction in round-bottomed flask with rotation evaporator.

Tricine SDS-PAGE

Introduction

SDS-PAGE (with tricin instead of glycine) to get a clear separation of small protein bands (1 to 5 kDa peptides); gel casting according to Schägger 2006.

Materials

- 10x anode buffer
- 10x cathode buffer
- 2x Tricine sample buffer
- Prestained Protein Ladder - Extra broad molecular weight (5 – 245 kDa) (Abcam)
- 3x Gel buffer: 3 M Tris, 1 M HCl, pH 8.45, 0.3 % (w/v) SDS
- Acrylamide AB-3 stock solution: 49.5 % T, 3 % C mixture
- 10 % APS
- TEMED
- Isopropanol
- heat block
- SDS gel chamber
- Power source
- Tray for staining the gel
- Shaker
- Scanner

Buffer	**10x anode buffer pH 8.9**	**10x cathode buffer pH 8.25**	**2x Tricine sample buffer pH 6.8**	**3x Gel buffer pH 8.45**
Chemicals	1 M Tris 0.225 M HCl	1 M Tris 1 M Tricine 1 % SDS	200 mM Tris/HCl 2% (w/v) SDS 40 % (v/v) glycerol 0.04 % (w/v) Coomassie brilliant Blue R-250 2 % (w/v) β-mercaptoethanol	3 M Tris 1 M HCl 0.3 % (w/v) SDS

16 % Separation gel	**Component**	**Amount for 30 ml**
Chemicals	AB-3	10 ml
	3x Gel buffer	10 ml
	ddH_2O	10 ml
	10 % APS*	100 µl
	TEMED*	10 µl

* Add latest

4 % Stacking gel	**Component**	**Amount for 12 ml**
Chemicals	AB-3	1 ml
	3x gel buffer	3 ml
	ddH_2O	8 ml
	10 % APS*	90 µl
	TEMED*	9 µl

* Add latest

Methods

- Pour 16 % separation gel.
- Cover separation gel with isopropanol until it is fully polymerized.
- Pour 4 % stacking gel on top.
- Add comb and let gel polymerize.
- Mix 10 µl sample with 10 µl 2 x sample buffer by pipetting (For flowthroughs and crude extracts dilute 1:10 to prevent gel overload. For samples with known protein concentration use 15 - 20 µg protein).
- Heat samples at 95 °C for 10 min.
- Spin down samples shortly in centrifuge.
- Apply 15 µl of each sample onto the gel; apply 10 µl protein ladder.
- Run SDS-PAGE for about 100 min at 100 V.
- Stain gel for 20 min to 1 h in Coomassie blue staining solution, either PageBlue™ Protein Staining Solution (Thermo Fisher Scientific) or Der Blaue Jonas ultrafast protein stain (GRP), shaking at RT.
- Collect staining solution, wash gel in ddH_2O for 10 min.
- Take gel pictures using the scanner.

Western blot

Introduction

To specifically detect proteins separated by SDS-PAGE using antibodies.

Materials

- 1x Blotting buffer
- TBS-T
- Milk-powder
- 100 % Methanol (can be reused)
- 1x PVDF membrane (Immobilion)
- 4x Whatman-paper, 8.6 x 6.2 cm
- Mouse anti-His antibody
- Anti-mouse HRP-coupled antibody
- Luminescence reagents (Roche Lumi-Light Western Blotting substrate)
- Semi-dry blotter
- Rotator

Buffer	**1x blotting buffer**	**TBS-T pH 7.4**
	25 mM Tris	150 mM NaCl
Chemicals	19.2 mM glycerol	0.1 % Tween20
	20 % (v/v) MeOH	50 mM Tris/HCl

Methods

- Perform SDS-PAGE
- Activation of the PVDF-membrane in 100 % MeOH; afterwards equilibration in blotting buffer.
- Equilibration of Whatman-papers (4 X) and SDS-gel in blotting buffer.
- Assemble blotting sandwich:

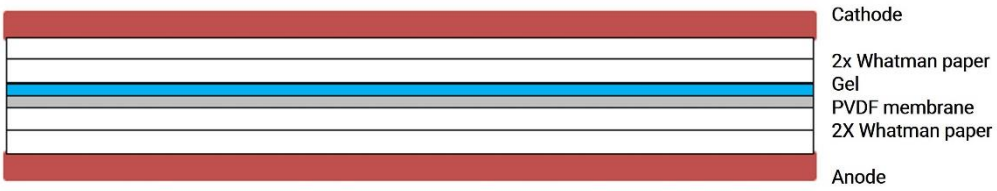

Figure 2: Set-up of the blotting sandwich for western blotting. We used a Semi-Dry blotting technique. Therefore, the PVDF membrane was activated in methanol and two layers of Whatman paper, preequilibrated in blotting buffer, were placed above and under gel or membrane, respectively.

- Run blot at 20 V (1000 mA) for 30 min.
- Block the membrane for 1-2 h at RT (or O.N. at 4 °C) on a rotator with 5% milk-powder/TBS-T
- Wash 3 times (each 10 - 15 min) with TBS-T.
- Transfer membrane in a clean 50 ml tube.

- Incubate with mouse anti-His antibody (diluted 1:1000 (10 µl AB in 10 ml TBS-T or 5% milk/TBS-T) rotating O.N. at 4 °C (or for 1 h rotating at RT).
- Wash 3 times (each 10 - 15 min) with TBS-T.
- Incubate with anti-mouse HRP-coupled antibody (diluted 1:5000 (2 µl in 10 ml TBS-T); wrap the 50 ml tube in aluminum foil to protect the HRP-coupled antibody from light damage) for 1 h rotating at RT.
- Wash 3 times (each 10 - 15 min) with TBS-T.
- Mix detecting reagent A and B in a 1:1 ratio (300 µl A and 300 µl B, for 6.2 x 8.6 cm membrane). Mix the reagents shortly before use in an Eppendorf tube wrapped in aluminum foil and store it on ice.
- Put the membrane on a plastic foil, distribute detection reagent on the membrane, cover the membrane with another plastic foil and remove air bubbles. Incubate for 5 min at room temperature (covered from light).
- Detect luminescence with increasing exposure time step-wise (e. g. 5 s, 1 min, 5 min, 10 min). Take a picture of the ladder with an exposure time of 0.1 s.

Activity Testing – microdilution assay (Koehbach *et* al. 2021 / Wiegand *et* al. 2008)

Introduction

Purified construct or crude extract will be used to test the antimicrobial activity. We will use several different methods for testing since the antimicrobial effects of AMPs can be hidden by different factors of an assay. To overcome this problem, we will do many different assays.

Materials

- 96-well plates (sterile, polypropylene, U-bottom (Nunclon Delta polystyrene))
- Mueller-Hinton I Broth (MH)
- Brain Heart Infusion Broth (BHI)
- *E. coli* K12
- *Bacillus* subtilis

Methods

Assay with MH Broth

- Twofold serial dilution of the antibiotics and peptides or of crude extracts using the respective media (MH, 1/5 MH, BHI)**.**

- For sterility control use media without bacterial solution.

- For growth control use media with bacteria but without peptide/antibiotics.

- Prepare 10 ml of a $1x10^6$ CFU/ml (bacterial cultures should be in log-phase):
  For this, take 3 - 5 colonies of a plate and resuspend in 500 µl saline (0.9 % NaCl).
  Pipette 100 µl of this bacterial suspension into a cuvette and add 900 µl saline (1:10 dilution).
  Measure OD600 for CFU/ml determination.
  Dilute cells depending on needed concentration (OD600 = 0.1 equals *E. coli* BW25113: $1.4x10^8$ CFU/ml; *B. subtilis* 168: $2x10^7$ CFU/ml.)

- Add 50µL bacteria suspension with the needed concentration to the wells.

- Incubate plates at 37 °C O.N.

- Measure OD600.

SDS-extraction

Introduction

We could not extract our peptide with the former extraction buffer (100 mM HEPES, pH 7.5, 150 mM NaCl, 3 mM KCl), but with Lämmli buffer containing SDS. Therefore, extraction might be successful with a new extraction buffer containing SDS.

Materials

- extraction buffer
- precipitation buffer
- Mortar and pestle
- centrifuge
- dialysis chamber
- exicator

Buffer	**extraction buffer pH 8.8**	**Precipitation buffer**
	125 mM Tris/HCl	90 % (v/v) acetone
Chemicals	10 % (v/v) glycerol	5 % (v/v) triethylamine
	1 % (w/v) SDS	5 % (v/v) acetic acid

Methods

- Cut off and weight infiltrated leaves.
- Grind the leaves with liquid nitrogen using mortar and pestle.
- Add 200 % (v/w) extraction buffer
- Centrifuge shortly to move down plant remnants, mix briefly afterwards.
- Incubate on ice for 1 h.
- Centrifuge 2 x 10 min with 4200 rpm, at 4 °C.
- Take supernatant into dialysis chamber, dialysis at 4 °C O.N.
- Take sample out of the dialysis chambers.
- Add 19 x volume of precipitation buffer, mix gently, incubate on ice for 1 h.
- Centrifuge 10 min at 4 °C with 4200 rpm, discard supernatant.
- Wash pellet 2 x with 1 ml precipitation buffer.
- Wash pellet 2 x with 1 ml acetone.
- Dry pellet in the exicator.
- Dissolve pellet in destination buffer.

Mass spectrometry of whole proteins eluted from sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels

Introduction

Protein bands are excised from SDS-PAGE gels, destained, and extracted for subsequent mass spectrometric analysis of whole proteins.

Materials

- Acetonitrile
- Ammonium bicarbonate
- Trifluoroacetic acid
- HEPES extraction buffer for re-solubilisation (10 mM HEPES, 15 mM NaCl, 0.3 mM KCl, pH 7.5)

Buffer	**destaining buffer**	**extraction buffer**
Chemicals	50 % (v/v) acetonitrile 50 % (v/v) ddH_2O (+ 100 mM $(NH_4)HCO_3$)	50 % (v/v) acetonitrile 45 % (v/v) ddH_2O 5 % (v/v) TFA

Methods

- The respective protein bands were excised from the Coomassie-stained SDS-PAGE gels with a scalpel and transferred into 1.5 ml tubes.
- Destaining of the gel slices was performed two times in 1 ml destaining buffer 1 (AcN:ddH_2O 1:1) and two times in 1 ml destaining buffer 2 (buffer 1 + 100 mM $(NH_4)HCO_3$). The tubes were placed on a heat block (40 °C) for about 15 minutes and rigorously vortexed in between until the blue color faded.
- Afterwards the buffer was removed and the gel slices were crushed using tweezers. The crushed gel slices were then incubated in 1 ml extraction buffer at 30 °C for about two hours on a rotator.
- Thereafter the solvent was evaporated on a vacuum concentrator O.N. and the extract was re-solubilized in 10 µl of 1:10 diluted HEPES extraction buffer.
- After the first measurements the samples were additionally concentrated and desalted using C18 resin.
- The samples were analyzed via MALDI-TOF MS (Bruker) and LC-MS.