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1. Growing bacteria

1.1. Cultivation of bacteria

For the isolation of chromosomal DNA, plasmid DNA, and for the preparation of competent cells of *E. coli* and *B. subtilis* the bacteria can be cultivated over night in LB liquid medium with agitation at 220 rpm and 37°C. To inoculate the culture tube, re-suspend bacteria either from a single colony that has been taken from a fresh agar plate or from a cryoculture. Cultivation of *M. pneumoniae* takes much more time because these bacteria are extremely slow-growing organisms (see specific methods). For growth of *L. monocytogenes* you should use BHI medium.

It is recommended to inoculate the pre-cultures containing minimal medium (for β-gal assays and growth curves) not too early and to incubate them over night at 28°C. Otherwise you will have many spores in your precultures that do no like to germinate in minimal medium.

1.2. Monitoring growth in a multi-well plate reader

For growth experiments with the Synergy MX II multi-well plate reader (Biotek) prepare pre-cultures and grow them over night at 28°C. Next day dilute these cultures (for instance 1:100, 1:500 and 1:1000) in 1-5 ml of the same medium that will be later on used for recording growth curves and incubate them at 37°C (or other temperatures) and 220 rpm. At an OD_{600} of about 0,2 – 0,5 use the cultures to inoculate a 96 well microtitre plate that has been supplemented with 100 µl medium per well. The outermost wells of the microtitre plate should contain 150 µl of distilled water. Incubate the plate for a maximum of 48 h at the intermediate shaking mode. The OD_{600} can be detected every 10 to 15 min. It is recommended to do at least three technical replicates per strain and condition on the same plate. Sometimes the detection of the OD can be disturbed by condensing water under the lid and your growth curve will look really strange.

1.3. Selection of resistant bacteria by antibiotics

Most of the antibiotics we are using in the lab are translation inhibitors (inhibition of ribosomes), only a few of them inhibit cell wall biosynthesis (please check the Table below).

Mode of action of antimicrobial agents

Antibiotic	Group of antibiotics	Mode of action
Ampicillin	β-Lactams	Inhibition of cell wall synthesis
Penicillin G	β-Lactams	Inhibition of cell wall synthesis
Kanamycin	Aminoglycosides	Inhibition of ribosome function (30S SU)
Gentamycin	Aminoglycosides	Inhibition of ribosome function (30S SU)
Spectinomycin	Aminoglycosides	Inhibition of ribosome function (30S SU)
Tetracycline	Polyketides	Inhibition of ribosome function (30S SU)
Erythromycin	Macrolides	Inhibitors of ribosome function (50S SU)
Chloramphenicol	Amphenicoles	Inhibitors of ribosome function (50S SU)
Lincomycin	Lincosamides	Inhibitors of ribosome function (50S SU)

All antibiotic stock solutions are usually filter-sterilized and stored of at -20°C. It is highly recommended to keep the tetracycline stock solution and also agar plates that were supplemented with tetracycline in the dark. A tetracycline stock solution changes its colour from yellow to red when it's aging.

Suitable solvents and selective concentrations

Suitable solvents			Selective concentrations (µg/ml)			
Antibiotic	**Solvent**	**Stock (mg/ml)**	***E. coli***	***B. subtilis***	***L. monocytogens***	***M. pneumoniae***
Ampicillin	Water	100	100	-	-	-
Penicillin G	Water		-	-	-	-
Kanamycin	Water	10	50	10	50	-
Gentamycin	Water	80	-	-	-	80 - 160
Spectinomycin	Water	100	100	100	-	-
Tetracycline	70% Ethanol	10	-	12.5	-	2
Erythromycin	70% Ethanol	2	5	2	5	-
Chloramphenicol	70% Ethanol	5	10 - 15	5	7.5	-
Lincomycin	Water	25	-	25	-	-

-

2. Working with DNA

2.1. Advices for cloning

What has to be considered for the construction of plasmids for complementation studies and for overexpression of proteins?

- The plasmid should contain a promoter (regulatable or constitutively active)!
- Make sure that the final construct will contain a ribosome-binding site (Shine-Dalgarno (SD) sequence)!

A)

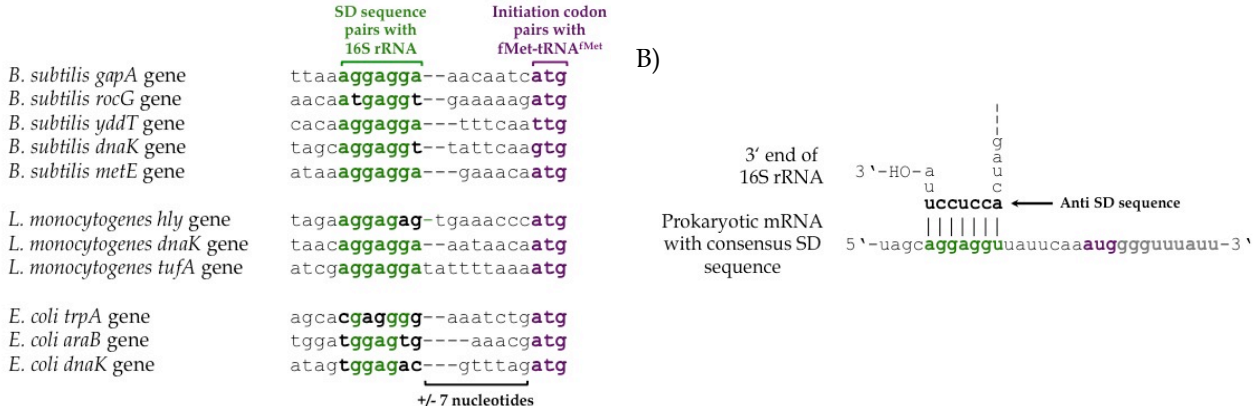


Figure 2. A) SD sequences from model organisms. Usually, the SDs of phage genes and chaperone-encoding genes are very strong. The optimal spacing between the SD sequence and the initiation codon is 7 nucleotides. B) The SD sequence is essential for translation of your gene because the anti-SD in the 16S rRNA of the ribosome binds to it.

- Don't forget to include at least one stop codon (TAA, TGA TAG) with the reverse primer during PCR!
- In case you want to express a fusion protein make sure that the ORF of the *goi* will be introduced *in frame* into the expression plasmid!

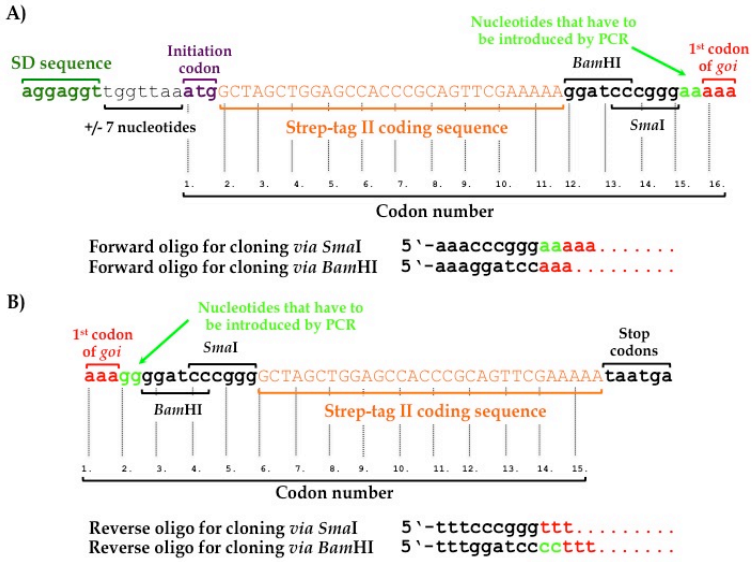


Figure 3. A) In case you use the *Sma*I restriction site you have to add two nucleotides between the *Sma*I site and the first codon of the DNA segment in the primer that hybridizes with the ORF of the *goi*. Rule of thumb: the number of the nucleotides

between the initiation codon and the first codon of the *goi* must be divisible by 3! B) Similar considerations have to be taken into account if you want to obtain a C-terminally tagged fusion protein.

What is the difference between a transcriptional and a translational promoter-reporter gene fusion?

- With a transcriptional promoter-reporter gene fusion you can measure the strength of a promoter. To create a transcriptional promoter-reporter gene fusion you have to fuse the promoter of interest to a *lacZ* gene that contains a SG sequence (see figure below).

- With a translational promoter-reporter gene fusion you measure the sum of transcriptional and translational regulation of a given gene. To create a translational promoter-reporter gene fusion you have to fuse the promoter of interest together with the SD sequence and a part of the ORF to a *lacZ* gene. Make sure that the ORF will be fused *in frame* to the ORF of the *lacZ* gene!

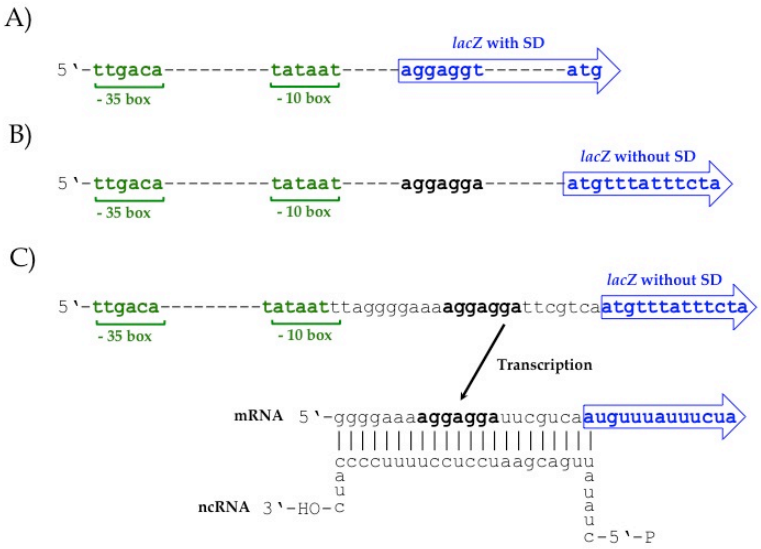


Figure 4. A) Schematic illustration for a transcriptional promoter-*lacZ* fusion. The plasmid pAC6 is suitable to create transcriptional promoter-*lacZ* fusions in *B. subtilis*. B) Schematic illustration for a translational promoter-*lacZ* fusion. The plasmids pAC5 and pAC7 are suitable to create translational promoter-*lacZ* fusions in *B. subtilis*. Translational promoter-reporter gene fusions can be uset to test the effect of small non-coding antisense RNAs that might bind to and prevent the access of the ribosome to the SD sequence.

2.2. Plasmids

Plasmids available in the Department of General Microbiology

Plasmid	Purpose	Resistance	Reference
	Promoter reporter gene fusions		
pAC5	Translational promoter-*lacZ* fusions in *B. subtilis*	Chloramphenicol	Martin-Verstraete *et al.*, 1992
pAC6	Transcriptional promoter-*lacZ* fusions in *B. subtilis*	Chloramphenicol	Stülke *et al.*, 1997
pAC7	Translational promoter-*lacZ* fusions in *B. subtilis*	Kanamycin	Weinrauch *et al.*, 1991
pBaSysBioII	transcriptional promoter-*gfp* fusions for high-throughput analysis of gene expression		Botella *et al.*, 2010
	Protein localization studies		
pBP43	Integrative plasmid to fuse monomeric GFP to the C terminus of a protein, keeping the control of the expression under its natural promoter; **this is the preferred plasmid for constructing GFP fusions!**	Spectinomycin	Unpublished
pBP1871	Integrative plasmid to fuse YFP to the C-terminus of a protein, keeping the control of the expression under its natural promoter		Rothe *et al.*, 2013
pBP1080	Integrative plasmid to fuse GFP to the C-terminus of a protein, keeping the control of the expression under its natural promoter; expression of the downstream gene is driven by the P_{spac} promoter, **ATTENTION: This plasmid encodes the dimeric GFP that tends to produce artifacts!!!**	Erythromycin/ Lincomycin	Unpublished
	Labelling for a protein with a triple FLAG tag		
pGP1331	Integrative plasmid for the fusion of 3X FLAG-tag to the C-terminus of a protein, keeping expression under the control of the natural promoter	Spectinomycin	Lehnik-Habrink *et al.*, 2010
pGP1087	Integrative plasmid for the fusion of 3X FLAG-tag to the C-terminus of a protein, keeping expression under the control of the natural promoter, allowing the expression of downstream genes under the control of the IPTG-inducible P_{spac} promoter	Erythromycin/ Lincomycin	Diethmaier *et al.*, 2011
	Expression and purification of proteins from *B. subtilis*		
pBQ200	Overexpression of proteins under control of a strong P_{degQ36} promoter	Erythromycin/ Lincomycin	Martin-Verstraete *et al.*, 1994
pGP380	Overexpression N-terminally Strep-tagged proteins	Erythromycin/ Lincomycin	Herzberg *et al.*, 2007
pGP382	Overexpression C-terminally Strep-tagged proteins	Erythromycin/ Lincomycin	Herzberg *et al.*, 2007
pGP886	Integration vector (integrates in *xkdE*); gene expression can be controlled by the xylose-dependent P_{xylA} promoter	Erythromycin/ Lincomycin	Unpublished
pGP888	Integration vector (integrates *ganA*); gene expression can be controlled by the xylose-dependent P_{xylA} promoter	Kanamycin	Diethmaier *et al.*, 2011
pGP1459	Overexpression N-terminally Strep-tagged proteins from the *ganA* locus	Kanamycin	Unpublished
pGP1460	Overexpression C-terminally Strep-tagged proteins from the *ganA* locus	Kanamycin	Unpublished
pGP1389	Integrative plasmid for the fusion of Strep-tag to the C-terminus of a protein, keeping expression under the control of the natural promoter	Spectinomycin	Lehnik-Habrink *et al.*, 2011a
	Expression and purification of proteins from *E. coli*		
pWH844	Expression of N-terminally His-tagged proteins	Ampicillin	Schirmer *et al.*, 1997
pGP570	Expression of N-terminally His-tagged proteins; the tag can be removed by thrombin protease	Ampicillin	Unpublished
pGP172	Expression of N-terminally Strep-tagged proteins	Ampicillin	Merzbacher *et al.*, 2004

pGP574	Expression of C-terminally Strep-tagged proteins	Ampicillin	Schilling *et al.*, 2006
	Transposon mutagenesis in *B. subtilis*		
pIC333	The *spc*^{R} resistance gene is located on the mini transposon Tn*10*	Erythromycin/ Lincomycin & Spectinomycin	Maguin *et al.*, 1992
	Analysis of protein-protein interactions by a bacterial two-hybrid system		
pUT18	For the fusion of the T18 domain of the *B. pertussis* adenylate cyclase to the C terminus of the protein; high-copy number plasmid	Ampicillin	Karimova *et al.*, 1998
pUT18C	For the fusion of the T18 domain of the *B. pertussis* adenylate cyclase to N terminus of the protein; high-copy number plasmid	Ampicillin	Karimova *et al.*, 1998
p25-N	For the fusion of the T25 domain of the *B. pertussis* adenylate cyclase to C terminus of the protein; **low-copy number plasmid!**	Kanamycin	Claessen *et al.*, 2008
pKT25	For the fusion of the T25 domain of the *B. pertussis* adenylate cyclase to N terminus of the protein; **low-copy number plasmid!**	Kanamycin	Karimova *et al.*, 1998
	Construction of markerless deletions		
pMAD	Construction of markerless deletions in Gram-positive bacteria	Erythromycin/ Lincomycin	Arnaud *et al.*, 2004

*) Please check the *Subti*Wiki database for more information (map & oligos for sequencing; http://subtiwiki.uni-goettingen.de/wiki/index.php/Plasmids).

2.3. Isolation of plasmid DNA from *E. coli*

Method 1 (dirty method for restriction analysis of a high number of plasmids)

The material

Liquid LB medium	
RNase solution	10 mg/ml RNase A
Lysozyme	
STET buffer	8% Sucrose (w/v) 5% Triton X-100 (w/v) 50 mM EDTA 50 mM Tris-HCl, pH 8.0 Filter sterilization, store at 4°C
STET-L buffer	10 ml STET 15 mg Lysozyme
i-Propanol	100%, Room temperature
Ethanol	70%, -20°C
TE buffer	10 ml Tris-HCl, pH 8.0 (1 M) 2 ml Na_2EDTA, pH 8.0 (0.5 M) Ad 1 l with deionised water
3 M Na-Acetate	408,24 g Na-Acetate $\cdot 3\ H_2O$ Add 800 ml deionised water Adjust the pH to 5.2 with glacial Acetic Acid Adjust the volume to 1 l with water

The procedure

1. Use single colonies from your transformation plates to inoculate 4 ml LB liquid medium supplemented with the appropriate antibiotic and grow the culture over night at 37°C with agitation.

2. Collect the cells from 1.5 ml of the over night cultures by centrifugation for 2 min at 13000 rpm. Resuspend the pellet in 300 µl of the STET-L solution and incubate the cell suspensions in boiling water for 40 sec.

3. Separate the cell debris by centrifugation for 10 – 15 min at 13000 rpm. Remove the pellet with a toothpick and precipitate the DNA by adding 40 µl of a 3 M Na-acetate solution and 420 µl *i*-propanol.

4. Centrifuge the samples for 15 min at 13000 rpm and wash the pellet with 70% ethanol. Remove the ethanol either by placing the Eppendorf reaction tubes under the sterile bench or by using the Speedvac.
5. Resuspend the DNA in 25 µl TE buffer. The DNA can be stored at -20°C for further analysis.

Method 2 (NucleoSpin® plasmid kit, Macherey & Nagel; for restriction analysis, sequencing)

The material

Liquid LB medium

NucleoSpin® plasmid kit, Macherey & Nagel

The procedure

1. Use single colonies from you transformation plates to inoculate 4 ml (15 – 20 ml for low-copy plasmids) LB liquid medium supplemented with the appropriate antibiotic and grow the culture over night at 37°C with agitation.

2. Collect the cells from 1,5 ml (15 ml for low-copy plasmids) of the over night cultures by centrifugation for 1 min at 13000 rpm. Resuspend the pellet in 250 µl of buffer A1, add 250 µl of buffer A2, mix gently 6 – 8 times and incubate the suspension for 5 min at room temperature. For the isolation of low-copy plasmids add 500 µl of buffers A1 and A2 to the cells.

3. Add 300 µl (0.6 ml for low-copy plasmids) of buffer A3, mix gently 6 – 8 times and centrifuge the lysate for 5 – 10 min at 13000 rpm.

4. Transfer the supernatant into NucleoSpin® plasmid column, centrifuge for 1 min at 13000 rpm and discard the flow-through.

   (Optional: for sequencing of low-copy plasmid DNA add 500 µl buffer AW to the column, centrifuge for 1 min at 13000 rpm and discard the flow-through.)

5. Add 600 µl of buffer A4, centrifuge for 1 min at 13000 rpm and discard the flow-through.

6. To dry the silica membrane, centrifuge again for 2 min at 13000 rpm.

7. Add 50 µl of sterile and deionized water on top of the silica membrane of the column, incubate the column for 1-5 min at room temperature and elute the DNA by centrifugation for 1 min at 13000 rpm.

8. The plasmid DNA is highly pure and can be stored at –20°C.

2.4. Agarose gel electrophoresis

Linear and double-stranded DNA molecules can move through a gel matrix with a velocity that is proportional to the logarithm of their molecular weight (MW). Therefore the MW of unknown DNA species can be determined by comparing their electrophoretic mobility with that of DNA molecules having known MWs. DNA molecules with lengths ranging from 0.5 kbp to 25 kbp can be separated using agarose gels.

Some important parameters for running an agarose gel

The agarose concentration: The agarose concentration is an important parameter for the separation of DNA species.

The optimal agarose concentration

Fragment size (kbps)	Agarose (%)	Bromphenol (kbps)	Xylencyanol (kbps)
1 – 30	0,5	1000	10
0,8 – 12	0,7	700	6
0,5 – 7	1,0	300	3
0,4 – 6	1,2	200	1,5
0,1 – 3	1,5	120	1
0,1 – 3	1,0	< 100	0,8

Voltage: The velocity with that DNA molecules move through a gel matrix depends on the applied voltage. It is important to know that the velocity of large DNA molecules increases more rapidly than that of small DNA molecules. As a consequence, large molecules can be separated from each other with a high accuracy at low voltages.

The electrophoresis buffer: Most gel systems are based on Tris-Acetate-EDTA (TAE) and Tris-Borat-EDTA (TBE) buffers. The electrophoretic mobility of DNA molecules is quite similar in both buffers. However, the buffering capacity of the TBE buffer is significantly higher. Therefore, it is recommended to use TBE buffer for mini-gels that run at a high voltage.

The DNA conformation: Supercoiled, nicked and linear DNA molecules, having the same molecular mass move with different velocities through the gel matrix. Because supercoiled DNA has the smallest hydrodynamic radius, these DNA species show the highest electrophoretic mobility.

The material

Agarose

50 X TAE buffer
2 M Tris
57.1 ml Acetic acid (100%)
100 ml EDTA (0.5 M, pH 8.0)

The procedure

1. Prepare a 100 ml mixture of 1% agarose in 1 X TAE by adding the appropriate amount of agarose to 100 ml of the 50-fold diluted TAE stock solution. Carefully heat the mixture in a microwave to dissolve the agarose and pour the gel in a small custom-made gel chamber. Don't forget the comb!

You can either store the agarose solution at 65°C or cool it down to room temperature and heat it again for the next gel.

2. While the gel is cooling down, prepare your DNA samples by mixing 2 – 5 µl of your DNA solution with 0.4 – 1 µl of loading dye, either in Eppendorf reaction tubes or in a multi-well microtitre plate (self-made or from Fermentas (Thermo scientific)).

3. Remove the comb from the cold gel, add 1-fold TAE buffer into the chamber and load the slots with your DNA samples. Don't forget the size standard (*Eco*RI-*Hind*III-digested DNA from the phage λ, or 1 kb DNA ruler from Fermentas (Thermo scientific)).

4. Connect the gel chamber with the power supply and switch it on. Adjust the voltage to 100 – 130 V and press the start button.

5. If the stain bromphenol blue has passed ¾ of the gel, you should switch off the power supply and stain the DNA molecules by ethidium bromide.

6. Put on one glove and transfer the gel into the staining solution (0.5 µg/ml of ethidium bromide in water). After 10 min you can destain the gel in the water bath. Please wear only one glove to avoid contamination of handlebars! One glove is sufficient to transfer your gel from the staining bath, to the water bath and to the imager.

7. After 10 – 30 minutes of destaining you may analyse your agarose gel, either on a UV table or with a gel documentation device (imager).

8. If possible, take a picture for the group seminar and your lab notebook.

Error sources

The DNA molecules were separated with low accuracy: Wrong agarose concentration; low voltage may cause diffusion of DNA molecules due to a long run.

Smearing bands: Too much DNA has been loaded to the slots; the applied voltage has been too high.

The gel was melting: Even old lab veterans sometimes use water instead of 1-fold TAE to dissolve agarose. You should also regularly refresh the TAE buffer inside the running chamber because this buffer is not as stable as TBE buffer.

2.5. Determination of DNA/RNA concentration

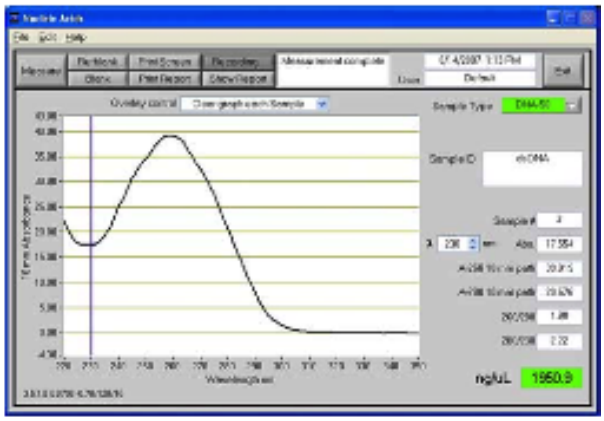
A reliable method for the quantification of DNA and RNA is essential for our work in the lab. The concentration of nucleic acids can be determined by fluorescence and absorption spectroscopy. The quantification of nucleic acids by fluorescence spectroscopy relies on binding to fluorescent dyes, e. g. ethidium bromide. This method is very sensitive (10 – 100 ng of DNA can be detected) but time-consuming. This is the reason that DNA is mainly quantified by absorption spectroscopy. DNA can be quantified by absorption spectroscopy because it absorbs ultra violet (UV) light with a maximum at the wavelength of 260 nm.

The purity of the DNA preparation

The quantification of DNA makes only sense if the sample is pure. The purity of the sample can be evaluated by recording a spectrum of your DNA solution in the range between 220 and 320 nm. Proteins in the sample absorb light at 280 nm and cause a reduction of the A_{260} nm/A_{280} nm ratio. If the DNA sample is pure, this ratio is in the range of 1.8 – 2.0. A typical absorption spectrum for double-stranded DNA is shown below.

The material

Nanodrop ND-1000 Spectrophotometer



The procedure

1. Clean the measurement pedestal (it contains the receiving fibre) with deionised water and pipette 1 µl of water on top of it.

2. Close the lid (sampling arm). Now the upper measurement pedestal (it contains the second fibre optic cable) is brought into contact with the liquid sample causing the liquid to bridge the gap between the two fibre optic ends. The gap is controlled to both 1 and 0.2 mm paths.

3. Initiate a spectral measurement using the operating software on the connected PC by pressing the "blank" button to blank the nanodrop. A pulsed xenon flash lamp will provide the light source and the spectrophotometer analyses the light after passing through the sample. After the measurement the data is logged in an archive file on the PC.

4. When the blanking is done, open the sampling arm and wipe the sample from both the upper and lower pedestals using a soft laboratory wipe. Wiping prevents sample carryover in successive measurements for samples varying by more than 1000-fold in concentration.

5. Pipette 1 µl your DNA sample and press the "Measure" button to determine the amount of DNA in your sample.

6. Remove the DNA solution from the measurement pedestal and clean the lid and the pedestal with deionised water.

2.6. The polymerase chain reaction (PCR)

Although the basic principle of the polymerase chain reaction (PCR) has been described already in 1985, this simple method is still very important in molecular biology (Sakai *et al.*, 1985). You just need a few things to set up a PCR: template DNA, oligonucleotides that hybridize to the template, a thermostable polymerase, buffer and nucleotides (see below).

A PCR pipetting scheme for a 100 µl sample

Volume (µl)	Compound
20	5 X PCR buffer (HF buffer, Thermo Scientific)
4	dNTP-Mix (12.5 mM each)
4	Forward oligo (5 pM)
4	Reverse oligo (5 pM)
1-2	Template DNA (chromosomal or other DNA)
1	DNA polymerase (for Phusion, PhuS)
67 – 66	Sterile H_2O

The simplest PCR program consists of a denaturation step to melt the template DNA, an annealing step to allow binding of the oligonucleotides to the melted DNA and an elongation step in which the DNA polymerase elongates the complementing DNA strand. Usually, these steps form a cycle that is repeated about 30 times. We added three additional steps to our PCR program: the initial denaturation step, which is helpful to melt up chromosomal DNA, the final elongation step, allowing the DNA polymerase to complete complementing DNA strands and the "hold" step to stop the reaction and to avoid DNA degradation.

The PCR program (repeat steps from 2. – 4. 30 times)

Step	Temperature, Time
1. Initial denaturation	98°C, 2 min
2. Denaturation	98°C, 20 sec
3. Annealing	54°C, 1 min
4. Elongation	72°C, variable (30 sec/kbp)
5. Final elongation	72°C, 10 min
6. Hold	15°C, ∞

The material

5 X HF buffer, Finnzymes
dNTPs (dATP, dTTP, dCTP, dGTP, 100 mM stocks), Fermentas
PhuS polymerase, purified in-house
Template DNA (chromosomal, plasmid DNA)

The inventor

Kary Bank Mullis, a passionate surfer, invented the PCR in 1983. Already in 1993 he received the Nobel price. Once he said that LSD might have stimulated his brain for the groundbreaking invention!

2.7. Combined-chain reaction (CCR) and multiple-mutation reaction (MMR)

Several methods for PCR-based site-directed mutagenesis have been developed. Among these, the combined-chain reaction method proved to be very rapid and reliable (Bi & Stambrook, 1997). The principle of this method is the use of mutagenic primers that hybridize more strongly to the template than the external primers. The mutagenic primers are phosphorylated at their 5′ ends, and these are ligated to the 3′-OH groups of the extended upstream primers by the action of a thermostable DNA ligase (see the Figure below for the principle). Moreover, the DNA polymerase employed must not exhibit 5′ 3′ exonuclease activity, to prevent the degradation of the extended primers. In our view, *Pfu* and *Pwo* polymerases are both well suited. The original protocol describes the introduction of two mutations simultaneously. In a previous study, we used a combined chain reaction to mutagenize four distant bases in a DNA fragment in a one-step reaction.

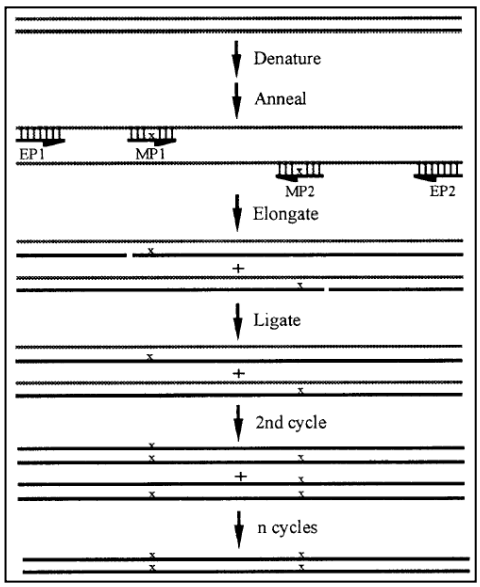


Figure 5. The principle of CCR and MMR. EP1 and EP2 are elongation primers. Oligos MP1 and MP2 are mutagenic primers.

The MMR is a further development of the CCR and allows the simultaneous introduction of up to nine mutations in a single PCR approach (more have yet to be proven; Hames *et al.*, 2005). The MMR was especially designed for the work with *Mycoplasma* genes in order to express recombinant genes in *E. coli*. *Mycoplasma* species differ in their genetic code and use the UGA opal codon to introduce tryptophan instead of terminating the translation. Hence, it is absolutely necessary to exchange the opal codons, if present, with UGG codons (TGG on DNA level). Meanwhile, the *in vitro* synthesis of oligonucleotides is getting more inexpensive and will replace this method in the future, especially, because the MMR can be very time consuming.

The material

10 X CCR/MMR buffer	200 mM	Tris-HCl (pH 8.5)
	30 mM	$MgCl_2$
	500 mM	KCl
	5 mM	NAD^+
	0.4 mg/ml	Bovine serum albumin (BSA)

Ampligase	5 U/ml Epicentre (Art.-Nr.: 111025 (A32250))
PhuS polymerase	Purified inhouse
5 X HF buffer	Thermoscientific

The procedure

Before you start your CCR or MMR you should amplify your gene of interest and clone it into a plasmid of your choice. The introduction of mutations is far more efficient when including this step.

Reaction set-up CCR	
2 µl	Forward primer (20 pmol)
2 µl	Reverse primer (20 pmol)
4 µl	Mutagenesis primer
1 µl	Plasmid DNA
5 µl	NAD^+ (50 mM)
10 µl	5 X HF buffer (Fermentas)
1 µl	PhuS polymerase (2.5 U μl^{-1}) or *Pfu* polymerase (Fermentas)
3 µl	Ampligase
1.5 µl	$MgCl_2$ (50 mM)
2 µl	dNTPs (12,5 µmol ml^{-1})
ad 50 µl	with dest. H_2O

Reaction set-up MMR	
2 µl	Forward primer (20 pmol)
2 µl	Reverse primer (20 pmol)
4 µl	Mutagenesis primer (each)
1 µl	Plasmid DNA
5 µl	10 X CCR buffer
1 µl	PhuS polymerase (2.5 U μl^{-1}) or *Pfu* polymerase (Fermentas)
3 µl	Ampligase
2 µl	dNTPs (12,5 µmol ml^{-1})
ad 50 µl	with dest. H_2O

NOTE that both protocols can be used *vice versa*; the only difference it the composition of the buffers. Don't forget to adjust the elongation time according to your polymerase and the length of the template.

The CCR/MMR program (repeat steps from 2. – 4. 30 times)

Step	Temperature, Time
1. Initial denaturation	95°C, 5 min
2. Denaturation	95°C, 1 min
3. Annealing	52°C, 1 min
4. Elongation	66-72°C, 4 min
5. Final elongation	66-72°C, 10 min
6. Hold	15°C, ∞

2.8. Purification of DNA fragments

We will use the QIAquick PCR purification kit to purify PCR products and linearized plasmids.

The material

QIAquick PCR purification kit, Qiagen

The procedure

1. Add 5 volumes of binding buffer PB to 1 volume of your DNA solution (it does not matter if you use 500 µl of buffer PB to purify a 50 µl PCR sample or 10 µl digested plasmid DNA)

2. Transfer the mixture to a spin column and centrifuge for 1 min at 13000 rpm. Discard the flow-through and add 750 µl buffer PE.

3. Centrifuge for 1 min at 13000 rpm, discard the flow-through and centrifuge again to dry the silica membrane.

4. Put the column into a labelled Eppendorf reaction tube, add 30 - 50 µl of water (not less than 30 µl) on top of the silica membrane, incubate for 5 min at room temperature and elute the DNA by centrifugation for 1 min at 13000 rpm. Please keep in mind that you have to add less water if you want to elute the DNA from a restriction analysis! Always check the purified DNA by agarose gel electrophoresis to make sure that you did not loose the DNA.

2.9. Digestion of DNA

Digestion of DNA by restriction endonucleases is an important method in molecular biology. Restriction enzymes recognize 4 – 10 bps-long palindromic DNA sequences in a highly specific manner. For instance, the enzyme *Eco*RI from the *Escherichia coli* strain R recognizes the sequence 5'-GAATTC-3'. In the early days, the reaction samples to digest DNA were incubated for up to 20 h in order to obtain a fully digested DNA. Nowadays, many biotech companies sell hyperactive variants of the restriction enzymes that are commonly used in the lab. We will use FastDigest enzymes from Thermo Scientific (for further information please check this web site: http://www.thermoscientificbio.com/restriction-and-modifying-enzymes/restriction-enzymes/fastdigest). According to the manual from the manufacturer, 1 µg of DNA (for instance λ phage DNA) are cleaved by 1 µl of the FastDigest enzyme within 5 – 15 min. With the same enzyme activity, 1/5 of a purified PCR product should be cleaved within 20 min.

The procedure

A typical reaction mixture to digest DNA

Volume (µl)	Compound
4	10 X FastDigest buffer
3	FastDigest enzyme
8	Plasmid DNA (125 ng/µl)
ad 40 µl	Sterile H_2O

We recommend to incubate the reaction mixtures for 30 min at 37°C to obtain fully digested DNA fragments. After digestion, you can use the PCR purification kit from Qiagen to purify the digested DNA. Please keep in mind that you have diluted the DNA solution in the reaction mixture. For eluting the DNA from the spin column you should use 28 – 30 µl of sterile water. To dephosphorylate your plasmid DNA, add 1 µl alkaline phosphatase to the reaction mixture and incubate the plasmid for additional 15 min at 37°C. Watch out for star activity of the restriction enzyme in your sample (e. g. *Eco*RI)! Star activity depends on the enzyme amount, the pH value, the presence of organic solvents, and the incubation time (Wei *et al.*, 2008).

There are different types of restriction enzymes:

The types of restriction enzymes (Roberts *et al.*, 2010)

Type	Characteristics	Example
I	3 subunits: S recognizes a specific sequence, M methylates and R cuts the DNA unspecifically	*Eco*KI M
II	2 subunits, a methylase and a restriction enzyme; both enzymes recognize the same DNA sequence	*Bam*HI
III	Several subunits; these enzymes cut a specific sequence 20-25 away from the recognition site	*Eco*PI

Some questions

While the reaction mixture is incubating you may try to find the answer to the following questions:

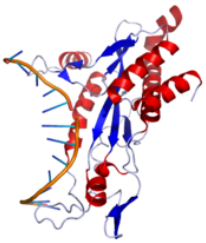
Have you ever heard about a "neoschizomer"?
What is an "isoschizomer"?
How often does *Eco*RI statistically cut the 4,2 Mbps-long chromosomal DNA of *B. subtilis*?

Useful online tools to

In silico digestion of your DNA: http://tools.neb.com/NEBcutter2/
The restriction enzyme database: http://rebase.neb.com/rebase/rebrev.html

The discoverers of the restriction enzymes

In 1978, the swiss microbiologist Werner Arber received together with Daniel Nathans who died in 1999 and Hamilton Othanel Smith the Nobel price for the discovery of the restriction enzymes. While Werner Arber has only a post box at the Biozentrum in Basel, Hamilton O. Smith is still active in research. He is leading the synthetic biology and the biological energy group at the J. Craig Venter Institute (http://www.jcvi.org/cms/about/bios/hsmith).



The *Eco*RI structure

2.10. Ligation of DNA

Ligation is the process where a digested plasmid is covalently connected to an insert (PCR product), which has been digested with the same restriction enzyme(s). If both, the insert and the plasmid, had been digested with only one enzyme, it is strongly recommended to dephosphorylate the plasmid prior to mix the ligation sample. Otherwise it takes your whole Master thesis or Ph. D. thesis until you find a plasmid containing the insert. We are using the T4 DNA ligase, which is a standard enzyme for ligation. In contrast to the NAD^+-dependent DNA ligase, which needs NAD^+ as a cofactor, the T4 DNA ligase uses ATP. Moreover, while the NAD^+-dependent DNA ligase can connect only DNA fragments with sticky ends, the T4 DNA ligase can ligate sticky and blunt ends.

The material

T4 DNA ligase (Fermentas)

10 X ligation buffer (Fermentas)

The procedure

1. Defreeze an aliquot of the ligation buffer on ice (it contains ATP) and mix the ligation and re-ligation samples in a 1,5 ml Eppendorf reaction tube:

Compound	Re-ligation sample	Ligation sample
T4 DNA ligase	1 µl	1 µl
10 X ligation buffer	2 µl	2 µl
Insert	-	3 µl (150 ng)
Plasmid	1 µl (50 ng)	1 µl (50 ng)
Water	16 µl	13 µl

2. Incubate the samples for at least 2 h at room temperature or over night at 16°C.

3. Use the complete ligation and re-ligation samples for transformation of competent *E. coli* cells. You should include a positive control (circular plasmid DNA) and a negative control (no DNA) to ensure that your *E. coli* cells were competent and not contaminated, respectively. Moreover, the ligation/re-ligation ratio will tell you whether it is worth to analyse your clones.

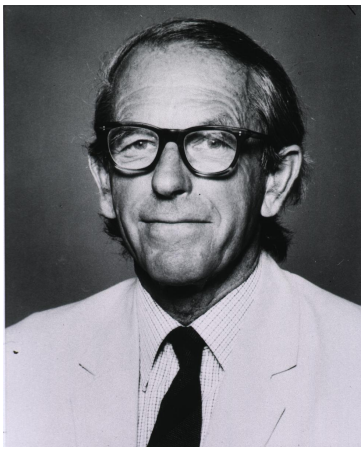
Error sources

No transformants at all on the plates: The ATP in the ligation buffer was hydrolysed (old buffer). Probably you forget a compound in the ligation mixture. The cells were not competent.

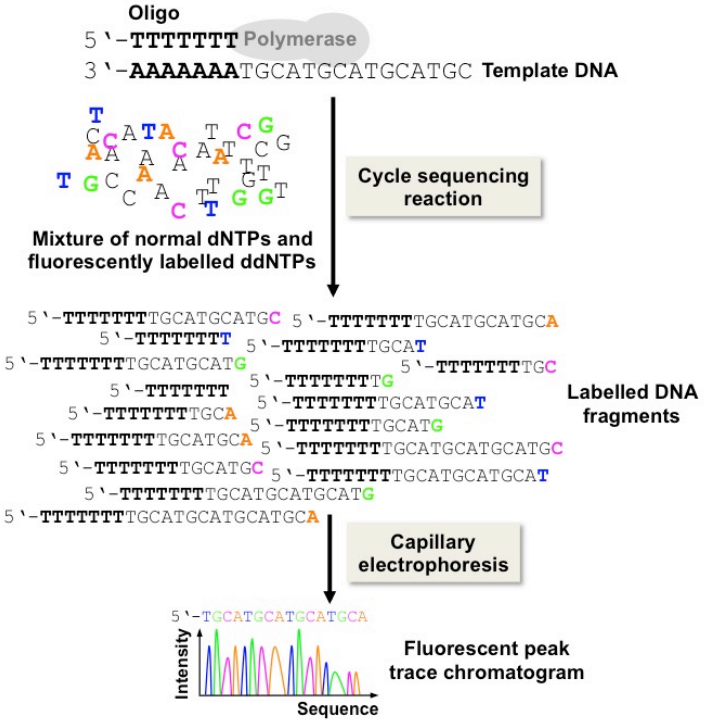
Same number of ligands and re-ligands on the plates: Probably your DNA was not completely digested. Therefore, it is very important to check you digested DNAs by agarose gel electrophoresis prior to the ligation. You can at a least see that your plasmid DNA was linearized.

2.11. DNA Sequencing

The correct sequences of PCR products or of newly constructed plasmids having the desired insert can be confirmed by the in-house sequencing facility, the G2L or by sending the DNA to the companies SeqLab (Göttingen) and LGC Genomics (Berlin). All facilities determine the DNA sequence by the chain-termination method that has been developed by Frederick Sanger in 1977. Sanger received in 1980, together with Paul Berg and Walter Gilbert, the Nobel price in chemistry. This became his second Nobel price because Sanger had been already awarded in 1958 for his work on protein sequencing. The Sanger method is based on the amplification of the template by a DNA polymerase, and the incorporation of normal deoxynucleotidetriphosphates (dNTPs) and modified dideoxynucleotidetriphosphates (ddNTPs). The incorporation of modified (fluorescently-labelled) ddNTPs results in termination of strand elongation. Sequencing with fluorescently-labelled ddNTPs permits DNA sequencing in a single reaction and the fast analysis of the DNA fragments by an optical read out system (see below).



Frederick Sanger



Sequencing service	G2L	SeqLab	LGC Genomics
DNA (ng/X µl)	200/4	600 – 700/6	100, 40 (plasmid, PCR product)/10
Oligo (µl, pM)	(1, 5)	(1, 20)	(4, 5)
Final volume (µl)	5	7	14
Reaction tube	1,5 ml	PCR	1,5 ml

Evaluation of your sequencing data

You can analyse your sequencing data using the DNA Star software package which is installed on a PC in our department.

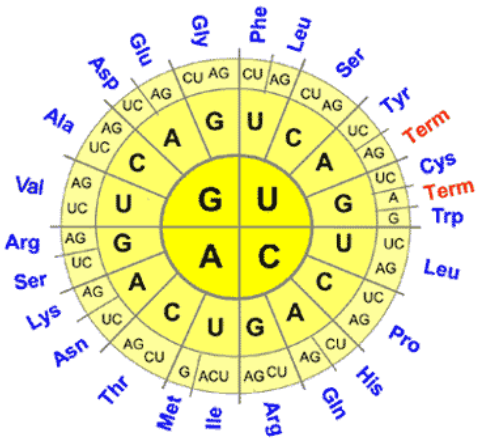


Figure 9. The genetic code. If you read the bases from inside to the outside of the codon sun you will get to the amino acid that is encoded by the codon (e. g. the triplet GCG encodes the amino acid alanine (Ala)).

3. Working with RNA

3.1. Isolation of RNA from *B. subtilis*

For the isolation of RNA from *B. subtilis* the Sartorius Mikro-Dismembrator and the Qiagen RNeasy Plus kit are used.

The material

Mikro-Dismembrator, Sartorius

RNeasy Plus kit, Qiagen

The procedure

1. Cell cultivation

1. Use a single colony of *B. subtilis* to inoculate 4 ml of LB medium supplemented with the appropriate antibiotics. Incubate the culture over the day at 37°C with agitation.

2. Use the LB preculture and prepare in a 100 ml shake flask a 10 ml overnight culture at 28°C with agitation in a defined medium.

3. At the next morning inoculate 100 ml of the medium in a 1 l shake flask to an approximate OD_{600} of 0.1. Incubate the shake flask at 37°C and 200 rpm until the OD_{600} is about 0.5 (or other growth phase).

4. To harvest the cells, transfer 25 ml of the culture into a 50 ml falcon tube with 15 ml frozen killing buffer. After the frozen killing buffer is melted centrifugation for 5 min at 8000 rpm and 4°C.

5. Discard the supernatant and freeze the pellet immediately in liquid nitrogen. The pellet can be store at -80°C.

Killing buffer

20 mM Tris-HCl, pH 7.5
5 mM $MgCl_2$
After autoclaving add 20 mM NaN_3 (be careful NaN_3 is toxic!)

3.2. RNA preparation

1. Prepare yourself and your bench to work with RNA! Wear gloves and clean everything with 70% EtOH.

2. Cool down the sample box and the steel ball (placed inside of the box) of the Mikro-Dismembrator in liquid nitrogen.

3. Resuspend the pellet in 200 µl RNase-free water. Take the sample box out of the liquid nitrogen so that some liquid nitrogen remains inside of the box. Fill in your resuspended sample directly into the liquid nitrogen of the box. The sample has to be frozen. Screw the box together and put it into the Mikro-Dismembrator.

4. Run the Mikro-Dismembrator for 3 min at 1800 rpm.

5. Resuspend your sample in 2 ml RLT Plus buffer, add 20 µl 2-mercaptoethanol and transfer the sample into a 2 ml Eppendorf cup.

6. Centrifuge your sample for 5 min at 13000 rpm and 4°C.

7. Start using the Qiagen RNeasy Plus kit. Transfer 700 µl of the supernatant to a G-column and centrifuge for 1 min at 10.000 rpm. The rest of the supernatant can be stored at -20°C.

8. Take 600 µl of the flow-through and mix it with 600 µl 70% EtOH. Transfer the half of it to an RNeasy spin column and centrifuge for 1 min at 10000 rpm. Repeat the step with the rest of the flow-through.

9. Add 700 µl RW1 buffer and centrifuge for 1 min at 10000 rpm. Discard the flow-through.

10. Add 500 µl RPE buffer and centrifuge for 1 min at 10000 rpm. Discard the flow-through.

11. Add again 500 µl RPE buffer and centrifuge for 1 min at 10000 rpm. Discard the flow-through.

12. Dry the column by centrifuging at full speed for 1 min.

13. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 150 µl RNase-free water directly to the spin column membrane. Centrifuge for 1 min at 10000 rpm to elute the RNA.

14. Store the RNA at -80°C.

3.3. *In vitro* transcription

In vitro transcription is defined as a RNA synthesis outside of a living cell. For the RNA synthesis the RNA-polymerase of the bacteriophage T7 (T7 polymerase) is used. With a DNA template and NTPs (not dNTPs, dNTPs are used for in a PCR) the polymerase synthesizes RNA. The T7 polymerase requires a specific start sequence (T7 promoter). Thus, during the synthesis of a DNA template for the *in vitro* transcription (by PCR) a T7 promoter has to be added. As the T7 promoter sequence is quite short it can be added to the primer sequence. In the absence of the T7 polymerase the level of basal transcription from the T7 promoter is very low (Martin *et al.*, 2005). Please keep in mind that you have to attach the T7 polymerase promoter sequence to the reverse primer if you want to detect a specific mRNA in your Nothern blot analysis! Otherwise the probe will not hybridize with the transcript.

5'-**CTAATACGACTCACTATAGGGAGA**cgttaacacccataacgattgtgatatc-3'

Figure. A typical reverse oligo to generate a DNA template by PCR for *in vitro* transcription using the DNA-dependent T7 polymerase. The DNA sequence that is bound by the T7 polymerase is shown in bold letters. The reverse complementary DNA sequence that hybridize to the *gapA* mRNA from *B. subtilis* is shown in small letters.

The procedure

1. Amplification of a DNA template by PCR

2. *In vitro* transcription (reaction volume 100 µl)

DNA-template with T7 Promoter (200 - 500 ng)	15 µl
NTP mix (each NTP 25 mM)	20 µl
10 Xtranscriptions buffer	10 µl
RNase Inhibitor (40 U)	1 µl
1 M DTT	2 µl
T7 RNA polymerase (80 U)	4 µl
RNase free water	48 µl

   Incubate the reaction mix over night at 37°C.

3. At the next day add 10 U RNase free DNase to digest the DNA template. Incubate the mixture at 37°C for 1 hour. To stop the reaction, add 4 µl of 0.5 M EDTA pH 8.0.

4. To purify the RNA the Qiagen RNeasy Plus kit can be used or the RNA can be precipitated with ethanol.

5. RNA precipitation with ethanol:

   a. Add 12.5 µl of 4 M LiCl and 350 µl of ice-cold 96% EtOH to the mixture and incubate at -70°C for 2 hours. Do you know why we use Li instead of other ions for RNA precipitation?

   b. Centrifuge the mixture for 30 min at 13000 rpm and 4°C. Discard the supernatant and wash the visible RNA pellet with 500 µl ice-cold 70 % EtOH. Don´t resuspend the pellet in 70% EtOH. Just add the EtOH centrifuge for a short time and discard the supernatant. After washing the pellet is only hardly visible!

   c. Remove the residual EtOH by evaporation at 60°C and resuspend the RNA in 50 µl of nuclease-free water. The RNA can be stored at -80°C.

3.4. Quantitative reverse transcriptase real-time PCR (qRT-PCR)

qRT-PCR is a method based on the classical PCR reaction that allows the detection and quantification of DNA transcripts (RNA). By using a fluorescent dye that binds DNA (SYBR® Green) you can monitor the amplification of DNA in real-time. For the quantification of your transcript amount the obtained C_t values are important. The C_t (cycle threshold) value gives you the number of cycles until enough DNA was amplified to reach a fluorescence threshold. This threshold is the same for all samples you want to compare and should be within the exponential phase of the amplification curve. Samples with a small transcript level need more cycles to reach the threshold fluorescence than samples with high amounts of transcript. Consequently, the C_t value of samples with small transcript amounts is higher than for samples with high amounts. To calculate differences in transcript level you can use the formula given at the end of this method. For further information you can also go to the respective Wikipedia site. ☺

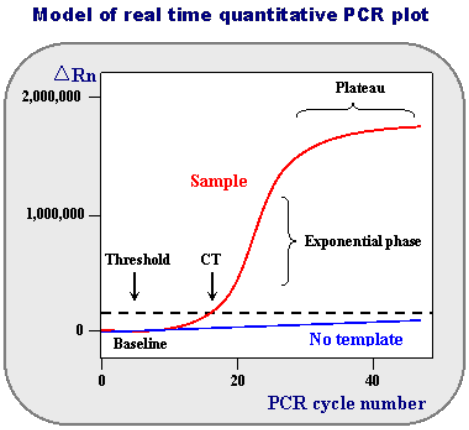


Figure 16. Quantification of transcript amounts by qRT-PCR.

The material

iScript™ One-Step RT-PCR kit with SYBR® Green (BioRad)
DNase I and EDTA (Thermo Scientific)
RNase-free water
Primer (KG42, KG43, KG44, KG45, CD76, CD77, & for gene of interest)
96-well qRT-PCR plate and sealing foil (BioRad)
RNase-free pipette tips
Gloves

Before you start

Wear gloves for all steps to avoid RNase contaminations and clean your bench, pipettes, etc. with ethanol (70%)!

Primer design

Design the primer so that they amplify a product of 150 bp. Also the primers should have a length of 20 bp and an annealing temperature of 60°C (program "TmCalc"). To simplify the primer design you can use the "Primer3" program (e.g. http://primer3.wi.mit.edu/).

The procedure

Start the computer, the qRT-PCR cycler (BioRad) and the software before you start pipetting your samples.

1. Turn on the cycler and the detection device (two switches)
2. Lock into windows by using "am_cycler" as user name and password
3. Open the program "Bio-Rad iQ5"

Protocol

The standard reaction protocol "One Step RT PCR + Melt" you can find in the folder Bio-Rad/iQ5/ sample files/

Plate layout

Create a new plate layout by clicking on the respective button. Then you can select the well you will pipette your samples/ reaction in. Via the "spreadsheet" you can name the single wells to have a better overview. If needed, you can print a summary as a pipetting scheme and for your lab book. Finally, save your plate layout and exit the editing.

DNase I digestion

Before the actual qRT-PCR possible DNA contaminations from the RNA isolation have to be removed. Therefore, you perform a DNase I digestion.

25 µl mix:
2.5 µg RNA
2.5 µl 10x Puffer (Thermo Scientific)
5 µl DNase I (Thermo Scientific)
x µl H_20 in a PCR tube
30 min at 37°C in a PCR cycler
Stop the reaction with 2.5 µl EDTA (Thermo Scientific) at 65°C for 10 min

After the DNase I digestion you have to test if the RNA is really DNA free. Thus, you perform a test PCR with 1 µl of the RNA as a "template" within a 50 µl mix. As test primers you take one of the pairs you use for the qRT-PCR later on. If the used RNA is DNA-free there should be no PCR product. Consequently, it is important to perform a positive control with chromosomal DNA as a template in parallel to check if the PCR works in general.
For the PCR use the *Pfu*S polymerase and determine the RNA concentration by NanoDrop (Peqlab).

The real-time PCR run

With a qRT-PCR you can monitor transcript amount of a gene of interest and thereby calculate the relative expression of the gene.
As reference genes you use the two ribosomal genes *rpsE* and *rpsJ* (KG44/KG45 (E), KG42/KG43 (J)). As an additional internal control you can use the gene *ptsH* (CD76/CD77). If you design new RT

primers it is useful to perform a no template control (NTC) with them. For the NTC you use the same mix as for the normal PCR but without any RNA template.
Please keep in mind that you dilute your RT primers 1:20 in DNase-free water. It is important to prepare a mix of the primer pairs for pre-pipetting.

Reaction mix with RNA for one reaction (prepare a master mix!):
10 µl 2 X SYBR® Green reaction mix (light sensitive!)
0.4 µl Reverse Transcriptase
x µl RNA (100 ng/20 µl)
x µl H_20
∑ 17.6 µl

In the PCR plates you pre-pipette 2.4 µl of your primer mix per well and then add 17.6 µl of the master mix with your RNA. Please leave the outer wells of the plate empty, because the foil for closing the plate sometimes comes off the plate. Finally, put the self-adhesive cover foil on top of the plate.
Now you can put the plate into the cycler and start the run. ☺

Quantification of the results

By the following formula you can calculate the relative transcript level of your strain/ condition of interest compared to the wild type strain/ standard condition. If the value for "fold change" is 1 there is no different in transcript level between the compared strains/ conditions. A value above 1 means an induction, a value below 1 means a reduced transcript level.

Fold changes = $2^{-\Delta\Delta ct}$
$\Delta\Delta Ct = (C_t - C_t^{constant})RNA2 - (C_t - C_t^{constant})RNA1$
C_t = transcript amount of the respective gene/cycles until threshold value is reached
$C_t^{constant}$ = median of the C_t values of the reference genes *rpsE* und *rpsJ* in the respective strain (e.g. RNA1)
RNA1 = RNA of the wild type/reference strain (e.g. *B. subtilis* 168)
RNA2 = RNA of the strain of interest (e.g. Δ*ymdB*)

4. Genetic modification of bacteria

4.1. Preparation of competent *E. coli* cells

Method 1 (low amount of competent cells)

The material

Buffer RF1 (150 ml):	1.44 g RbCl 1.16 g $MnCl_2 \cdot 4\ H_2O$ 3.6 ml Potassium Acetate (1 M) pH 7.5 with Acetic Acid
Buffer RF2 (80 ml):	1.6 ml MOPS (0.5 M) stock solution pH 6.8 with NaOH 0.096 g RbCl 0.88 g $CaCl_2 \cdot 2\ H_2O$

The procedure

1. Inoculate a 4 ml culture either with a single colony or with a cryoculture of the desired *E. coli* strain and incubate the culture with agitation over night at 37°C.

2. Inoculate a 300 ml shake flask containing 100 ml LB medium with the over night culture to an OD_{600} of 0.05 and grow the culture at 37°C until the OD_{600} is about 0.3.

3. Transfer the cells into two 50 ml Falcon tubes, incubate the cultures for 15 min on ice and harvest the cells by centrifugation for 15 min at 5000 rpm and 4°C. Discard the supernatants.

4. Resuspend the cells in 1/3 of the original volume (~ 16 ml/50 ml) of buffer RF1, incubate the cells again on ice and harvest the cells by centrifugation for 15 min at 5000 rpm and 4°C. Discard the supernatants.

5. Resuspend the cells in 4 ml of buffer RF2 and incubate the suspensions for 15 min on ice. Now it's time to label Eppendorf reaction tubes and go and get liquid nitrogen.

6. Put 0.4 ml of the cell suspension into the Eppendorf reaction tubes and freeze the cells by transferring them immediately to the liquid nitrogen. Store the competent cells at -70°C.

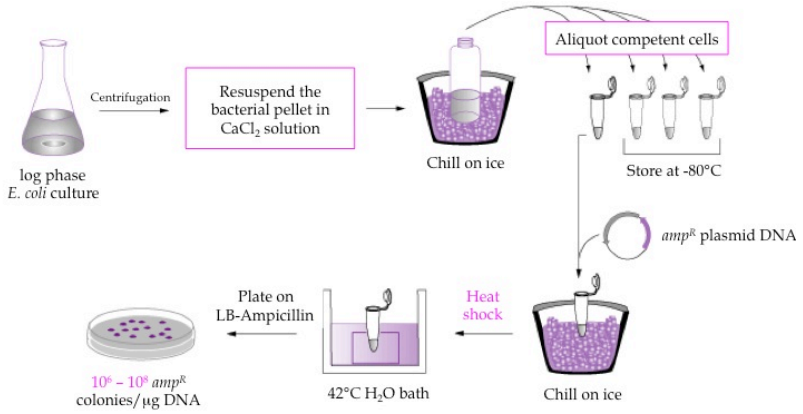


Figure 18. Preparation of competent cells by the $CaCl_2$ method.

Method 2 (fast method, cells cannot be stored)

The material

$CaCl_2$ solution	50 mM
LB liquid medium	

The procedure

1. Inoculate a 4 ml culture either with a single colony or with a cryoculture of the desired *E. coli* strain and incubate the culture with agitation over night at 37°C.

2. Inoculate a 100 ml shake flask containing 10 ml LB medium with the over night culture to an OD_{600} of 0.05 - 0.1 and grow the culture at 37°C until the OD_{600} is about 0.3.

3. Transfer the cells into 15 ml Falcon tubes, harvest the cells by centrifugation for 6 min at 5000 rpm and 4°C. Discard the supernatants and resuspend the cells in 5 ml of ice-cold $CaCl_2$ solution.

4. Incubate the cells for 30 min on ice and collect them again by centrifugation for 6 min at 5000 rpm and 4°C. Resuspend the pellet in 1 ml of the ice-cold $CaCl_2$ solution. Now the cells are ready to be transformed with your re-ligation and ligation samples (see page 66).

Method 3 (high amount of competent cells, time-consuming)

The material

TB	3,46 g Piperazine-N,N′-bis(2-ethanesulfonic Acid (Pipes, 11 mM) 2,2 g $CaCl_2 \cdot 2 H_2O$ (15 mM) 18,64 g KCl (250 mM) pH 6.7, autoclaving add 55 ml $MnCl_2$ (1 M, sterile) to a final volume of 1 l
SOB-Mg	20 g/l Tryptone (2%) 5 g/l Yeast extract (0,5%) 0.58 g/l NaCl (10 mM) 0.186 g/l KCl (2,5 mM)
SOB	SOB-Mg 10 mM $MgCl_2$ 10 mM $MgSO_4$
LB liquid medium	
DMSO (Dimethyl sulfoxide)	

The procedure

1. Inoculate a 20 ml culture either with a single colony or with a cryoculture of the desired *E. coli* strain and incubate the culture with agitation for 20 h at 28°C.

2. Inoculate* 250 ml SOB medium supplemented in a 2 l shake flask and grow the cells to an OD_{600} of 0,5 – 0,9 (20 – 24 h) at 18°C and 200 – 250 rpm.

3. Incubate the whole flask for 10 min on ice. Collect the cells by centrifugation for 10 min at 4°C and 5000 rpm. Resuspend the cells in 80 ml of ice-cold TB and incubate them for 10 min on ice. Collect the cells by centrifugation for 5 min at 5000 rpm.

4. Resuspend the cells in 20 ml of ice-cold TB. Add DMSO to a final concentration of 7% (1,4 ml) by gently shaking the Falcon tube.

5. Transfer 0,2 ml aliquots into labelled Eppendorf reaction tubes and freeze the cells in liquid nitrogen. Store the cells at -70°C.

*) The volume of the preculture strongly depends on the *E. coli* strain. Using DH5α it is recommended to use 4 ml preculture and inoculate at 8 o'clock in the morning. Next day around 10 o'clock this strain should have reached an OD_{600} between 0.5 and 0.6. If you use the strain XL1-Blue, you will need less cells and it is sufficient to inoculate the SOB medium around lunchtime.

4.2. Transformation of *E. coli*

The material

Liquid LB medium

LB medium agar plates supplemented with the appropriate antibiotics

The procedure

1. Put your ligation samples on ice, defreeze 100 µl of your competent *E. coli* cells on top of the ice and add the cells to your ligation samples. Mix it carefully!

2. Incubate the Eppendorf reaction tubes for 30 min on ice, transfer the tubes for 90 sec to 42°C (heat shock) and put them back on ice for 5 min.

3. Add 500 µl LB medium to the cells, transfer them to 15 ml Falcon tubes (or in sterile 1.5 ml Eppendorf reaction tubes) and incubate the cell for 1 h at 37°C with agitation.

4. Propagate 50 µl of the cells on LB medium agar plates supplemented with the appropriate antibiotics. The remaining cells are collected by centrifugation for 1 min at 13000 rpm and remove 400 µl of the supernatant. Resuspend the pellet in the remaining 50 µl of the supernatant propagate the cells on the same LB medium agar plates. It is highly recommended to do a negative control (only cells, no DNA).

5. Incubate the plates over night at 37°C. The plates should be stored after incubation over night at 4°C to avoid the emergence of satellite colonies (see figure below).

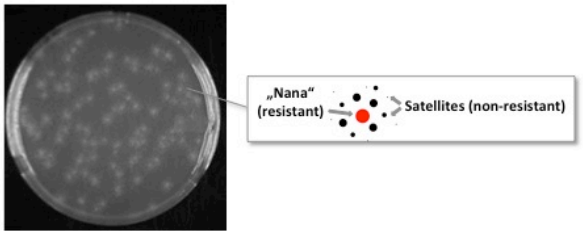


Figure 19. This is how a typical LB-Amp plate containing ampicillin-resistant *E. coli* transformants ("nanas") may look like after prolonged incubation of the plate and low ampicillin concentration. Ampicillin-resistant *E. coli* transformants excrete the β-lactamase, which cleaves the ampicillin and non-resistant clones, so-called satellite colonies that surround the transformants can emerge on the plate.

4.3. Preparation and transformation of competent *B. subtilis* cells

The transformation of bacteria, a programmed mechanism for genetic exchange has been original discovered in *Streptococcus pneumoniae*. However, many bacteria exchange genetic material by transformation (Claverys *et al.*, 2006). Most transformable bacteria use the same molecular machinery to acquire DNA from the environment. The assembly of this machinery occurs in cells that develop a special physiological state termed as competence. The process of natural transformation involves (i) DNA uptake, (ii) recombination of homologous DNA into the chromosome or reconstitution of circular plasmid DNA and (iii) eventually phenotypic expression of acquired genetic material. What could be the selective advantage for a bacterium to take up foreign DNA? There are three ideas that might explain the evolutionary reason d'être for competence: foreign DNA is useful to acquire novel genes, genetic information may serve as a template for DNA repair, and DNA may serve as food.

The Laboratory strains of *B. subtilis* are naturally competent and capable of taking up foreign DNA from the environment. The laboratory strain *B. subtilis* 168 is easily made competent as prolonged exposure to nutritional stress results in the development of competence (Hamoen *et al.*, 2003). Other *B. subtilis* strains such as NCIB 3610, which are ancestors of the laboratory strain 168 are not that easy to genetically manipulate. However, ko's can be transferred from the laboratory strain 168 to the so-called "wild" wild type strain NCIB 3610 by phage transduction (see page 80).

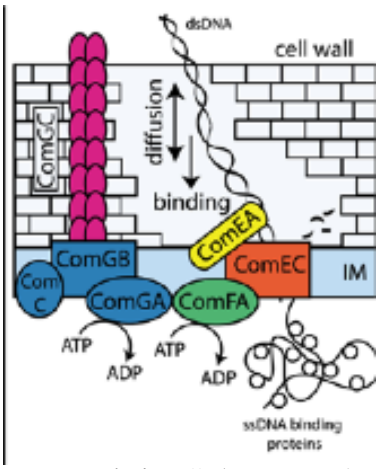


Figure 20. Model for DNA uptake by competent *B. subtilis* cells (Krüger & Stingl, 2011). A pseudopilus composed of ComG proteins (mainly ComGC, with contributions of comGD, GE, GG) spans the cell wall. Assembly of the pseudopilus is mediated by the integral membrane protein ComGB, a peptidase for processing of pilin subunits (ComC) and the assembly ATPase ComGA. Disassembly/retraction of the pseudopilus opens a cell wall hole that enables DNA to diffuse ~ 55 nm from the surface to the DNA receptor ComEA, which is anchored in the membrane. The originally double-stranded DNA (dsDNA) enters the cell as single-stranded DNA (ssDNA). IM = inner membrane.

The material

MN medium

MNGE medium

10% CAA, 1 M $MgSO_4$, 5% Yeast extract, 5 mg/ml Tryptophan, 20% Glucose, 40% Potassium Glutamate, 2.2 mg/ml CAF (see section "Media, buffers and solutions")

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 over night 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 (no CAA) and incubate the culture for another h at 37°C on a shaker.

4. Transfer 15 ml of the culture to 15 ml Falcon tubes and harvest the cells by centrifugation for 5 min at 5000 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% glycerine, mix the cell suspension and store the competent cell in 300 µl aliquots at -70°C.

6. **You can also decide to continue with the transformation of *B. subtilis* directly after the nutrional starvation step (step 3).** Take 400 µl of the competent bacteria from the flask, transfer them to a sterile reaction tube, add the DNA and incubate the reaction tube for 30 min at 37°C (continue with step 9).

Transformation of *B. subtilis*

7. Thaw 300 µl of the frozen, competent bacteria and mix them with 1.7 ml 1 X MN medium that has been supplemented with 43 µl glucose (20%) + 34 µl 1 M $MgSO_4$.

8. Add 0.1 - 1 µg DNA (2 µg plasmid DNA) to the cells and incubate the reaction tube for 30 min at 37°C.

9. Add 100 µl expression mix (500 µl yeast extract (5%), 250 µl CAA (10%), 250 µl sterile H_2O, 50 µl Tryptophan (5 mg/ml) and if required an inducer (IPTG, xylose…).

10. 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

Remarks: do not forget the negative control!!!

5. Working with proteins

5.1. Cell disruption by French press

Many experiments required the disruption of bacterial cells, e.g. to purify proteins for enzyme activity assays or protein-protein interaction studies. A widely used method in our lab for cell disruption is using a French pressure cell, or simply French press. In principle, a French press disrupts the cell wall and plasma membrane by passing the cells through a narrow valve under high pressure. The name has nothing to do with the machine some people prepare their coffee in, but was named after its inventor Charles Stacy French of the Carnegie Institution of Washington" (http://en.wikipedia.org/wiki/French_pressure_cell_press).

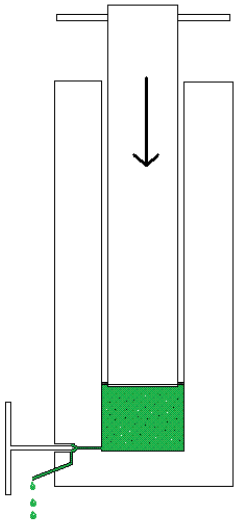


Figure 29. Principle of cell disruption using the French press. The arrow indicates the direction of the force exerted on the piston by a hydraulic ram. The fluid compressed by the piston is prevented from escape by a rubber O-ring at its lower end (source: http://en.wikipedia.org/wiki/French_pressure_cell_press)

Do not use the French press without being introduced by the responsible person Chris, AG Stülke!

The material

French press	Only use the French press after the safety instruction by Chris
Disruption cell (bomb)	Pre-cooled in a water bath or in the fridge
Resuspension buffer	Pre-cooled in the fridge or on ice
Cell pellets	Frozen cells within a 50 ml falcon tube.

The procedure

1. Resuspend your cells pellet in resuspension buffer (e.g. buffer W for SPINE) by vortexing or pipetting up and down. Usually we use a pellet from 500 ml of cells with an OD_{600} of 0.8 – 1.2 and resupend it in 15 ml cold buffer.

2. Turn on the French Press and select the pressure for cell disruption (**18000 psi** for *Bacillus* and *E. coli*).

3. Fill the resuspended cells in the disruption cell and close it tightly.

4. Put the closed disruption cell into the machine so that the piston is on the top. Make sure that the handles of the piston are turned in your direction so they cannot come in contact with the mounting while the cell gets compressed

5. Press start and follow the instructions on the display of the machine.

6. Open the valve slowly while you put a 50 ml falcon under it.

7. Collect the opened cells that flow out of the valve. Watch out that the cell suspension flows out slowly and the pressure remains almost constant until the whole suspension has flown out.

8. Reload the disruption cell. For Gram-positive bacteria, open up the cell **three** times. For Gram-negative cells one run should be enough.

9. Clean the Disruption cell with **ethanol (70%)** and distilled water. Then store it in the fridge until next use.

5.2. Purification of tagged proteins by affinity chromatography

For supporting and detailed information/manuals on Strep- and His-tag purification visit the www.iba-lifesciences.com website.

Purifcation of His-tagged proteins

The 6 X histidine-tag Ni-NTA interaction is based on the selectivity and high affinity of Ni-NTA (**Ni**ckel-**N**itrilo**t**riacetic **A**cid) resin for proteins containing an affinity tag of six consecutive Histidine residues. NTA, which has four chelating sites for Nickel ions, binds nickel more tightly than metal-chelating purification systems like IDA, which have only three sites available for interaction with metal ions. The extra chelating site prevents Nickel ion leaching, thus providing greater binding capacity and high-purity protein preparations. Under denaturing conditions this system is the system of choice because the 6 X Histidine tag/ Ni-NTA interaction tolerates high concentrations of urea and guanidine. Under physiological conditions host proteins with histidine stretches or host proteins containing metal ions may contaminate the protein preparation. Working with 20 mM imidazole can reduce this problem.

The material

Crude extracts of your sample cells harboring the desired protein

Ni-NTA® Sepharose 50% suspension; Iba Göttingen (2.5 ml per 1 l cell culture)

ZAP buffer W (50 mM Tris-HCl, pH 7.5, 200 mM NaCl)

500 mM Imidazol in ZAP buffer

1 M DTT stock solution in H_20 (for dialysis)

The procedure

Equilibration of the column

Remove the bottom cap from the column and apply the appropriate amount of Ni-NTA® matrix (2.5 ml of the 50% suspension per 1 l of cell culture). For all steps, allow the solutions on the column to drain off by gravity before adding the next one. Equilibrate the column by adding 10 CV (CV = column bed volume) of ZAP buffer.

Adsorption of His-tag proteins to the matrix and column washing

Load your crude extract on the column and keep 50-100 µl as a sample for later analysis (CE). Also, collect the flow through as a sample (FT). Wash the column by adding 10 ml buffer W and collect the wash fraction as "W".

Elution of His-tag proteins from the matrix

Use the 500 mM Imidazol solution to create appropriate dilutions in ZAP buffer. The following elution pattern is given as an example and is useful if the Imidazol concentration for optimal and clean elution of the protein of interest in unknown. Add indicated volumes on the column and collect every elution fraction:

8 ml	10	mM Imidazol
8 ml	50	mM
5 ml	100	mM
5 ml	200	mM
5 ml	500	mM

Analysis of the fractions

To analyze the purification result, perform a SDS PAGE. For the gel, choose the appropriate PAA %age according to the molecular weight of the purified protein. Load 5 µg of CE and FT fractions (determined by Bradford assay, see page 98) onto the gel and 15 µl of wash and elution fractions. Stain the gel with Coomassie brilliant blue.

Dialysis/buffer exchange

To get rid of the imidazol in the protein solution or to change the buffer perform dialysis with the protein fraction of interest. Dialyze o/n against 1000 X volume of buffer W or another buffer. The addition of 1 mM DTT to this buffer can be useful for many proteins to prevent oxidation. It's not necessary for every protein but it doesn't harm as well.

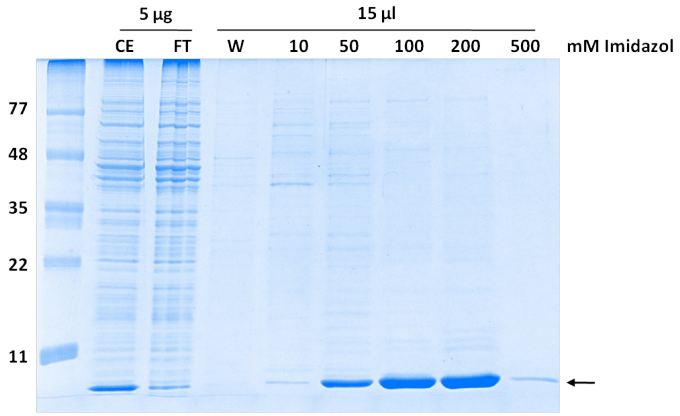


Figure 30. An example for the purification of a His-tagged protein (indicated by the arrow) from *E. coli*. 100 and 200 mM Imidazol fractions contain high yields of protein with good purity. To improve this result, elution of contaminating proteins with 50 mM imidazol and subsequent elution of target protein with 200 mM Imidazol would be best.

Purification of Strep-tagged proteins

The *Strep*-tag II is a short peptide (8 amino acids, WSHPQFEK), which binds with high selectivity to *Strep*-Tactin®, an engineered streptavidin. The binding affinity of *Strep*-tag II to *Strep*-Tactin® (K_D = 1 mM) is nearly 100 times higher than to streptavidin. This technology allows one-step purification of almost any recombinant protein under physiological conditions, thus preserving its bioactivity.

The material

Crude extracts of your sample cells harbouring the desired protein

Strep-Tactin® Sepharose 50% suspension; Iba Göttingen (1 ml per 1 l cell culture)

Buffer W (100 mM Tris-HCl pH 8.0, 150 mM NaCl; 1 mM EDTA)

Buffer E (buffer W + 2.5 mM D-Desthiobiotin)

1 M DTT stock solution in H_20 (for dialysis)

The procedure

Equilibration of the column

Remove the bottom cap from the column and apply the appropriate amount of *Strep*-Tactin® matrix (1 ml of the 50% suspension per 1 l of cell culture). For all steps, allow the solutions on the column to drain off by gravity before adding the next one. Equilibrate the column by adding 10 CV (CV = column bed volume) of buffer W.

Adsorption of Strep-tag proteins to the matrix and column washing

Load your crude extract on the column and keep 50-100 µl as a sample for later analysis (CE). Also, collect the flow through as a sample (FT). Wash the column by adding 5 times 2.5 CV of buffer W. Collect every fraction as "W1"-"W5".

Elution of Strep-tag proteins from the matrix

Add 4 times 0.5 CV of buffer E and collect all fractions as "E1" - "E4".

Analysis

To analyze the purification result, perform SDS-PAGE. For the gel, choose an appropriate PAA %age according to the molecular weight of the purified protein. Load 5 µg of CE and FT fractions (determined by the Bradford assay) onto the gel and 15 µl of wash and elution fractions. Stain the gel with Coomassie Brilliant blue.

Dialysis/buffer exchange

To get rid of the Desthiobiotin in the protein solution or to exchange the buffer perform dialysis with the protein fraction of interest. Dialyze o/n against 1000 X volume of buffer W or a desired buffer. The addition of 1 mM DTT to this buffer can be useful to prevent oxidation. Not every protein is subject to oxidation but DTT doesn't harm as well.

Removal of the His-tag from purified proteins by proteolysis

AcTEV Protease is an enhanced form of Tobacco Etch Virus (TEV) protease that is highly site-specific, active, and more stable than native TEVprotease. AcTEV Protease is purified from *E. coli* by affinity chromatography using the polyhistidinetag. It recognizes the seven-amino-acid sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly and cleaves between Gln and Gly with high specificity. The protease is used to cleave affinity tags from fusion proteins. The optimal temperature for cleavage is 30°C; however, the enzyme is active over wide ranges of temperature and pH (pH 6.0 - 8.5). Following digestion, AcTEV Protease is easily removed from the cleavage reaction by affinity chromatography using the polyhistidine tag at the N-terminus of the protease. **Therefore it is important to have no imidazol in your sample fusion protein**, e.g. dialyze prior to the cleavage reaction.

The material

AcTEV Protease 10 U/µl (Invitrogen, #12575-15)

20 X TEV buffer (1 M Tris-HCl, pH 8; 10 mM EDTA)

0.1 M DTT

SDS loading dye

The procedure

Example of a time course experiment with 10 units AcTEV Protease is shown below. If the protein of interest is heat-labile, incubate at 4°C with longer incubation times and/or more enzyme (see table on next page).

1. Add the following to a microcentrifuge tube:

Fusion Protein	20 µg
20 X TEV Buffer	7.5 µl
0.1 M DTT	1.5 µl
AcTEV Protease, (10 units)	1.0 µl
Add water to	150 µl

2. Incubate at 30°C. Remove 28 µl aliquots at 1, 2, 4, and 6 h.

3. Add 7 µl 5 X SDS loading dye. Keep the samples at -20°C until experiment is complete.

4. Analyze samples by SDS PAGE using a suitable gel. The percent of protein cleavage is determined by analyzing the amount of cleaved products formed and amount of uncleaved protein remaining after digestion. After evaluating the initial results, you may optimize the cleavage reaction for your specific protein by optimizing the amount of AcTEV Protease, incubation temperature, or reaction time.

Variing parameters for cleavage

The percent of **3 µg** control substrate hydrolyzed by **1 U** of AcTEV Protease at various temperatures.

Percentage hydrolyzed substrate

t [h]	4°C	16°C	21°C	30°C
0.5	36	62	72	85
1	58	85	99	99
2	77	99	99	99
3	88	99	99	99

More cleaved protein is formed with AcTEV by increasing the incubation time. If time is critical, add more AcTEV Protease to increase hydrolysis. *In many cases, reaction at 4°C or 16°C is sufficient (own experience).*

Removal of ACTEV Protease after cleavage

The AcTEV Protease contains a polyhistidine tag at the N-terminus. After cleavage of the fusion protein, remove AcTEV Protease and the cleaved His-tags from the cleavage reaction by Ni-NTA affinity chromatography. **The cleaved native protein will be in the flow-through fractions.**

5.3. Bradford assay

The Bradford reagent can be used to determine the concentration of proteins in solution (Bradford, 1976). The procedure is based on the formation of a complex between the dye Coomassie brilliant blue G-250 and proteins in solution. The protein-dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The absorption is proportional to the amount of protein present in the sample. The assay is suitable for a protein concentration in the range between 0.1 - 1.4 mg/ml of protein using bovine serum albumin (BSA) as the standard protein. Protein solutions or crude extracts with protein concentrations above 1.4 mg/ml have to be diluted to be in the linear range of the assay. The Bradford assay is advantageous because it is fast, the staining does not depend on the protein and the reagent is commercially available (Roth).

The material

Roti-Quant Bradford solution, 5-fold concentrate

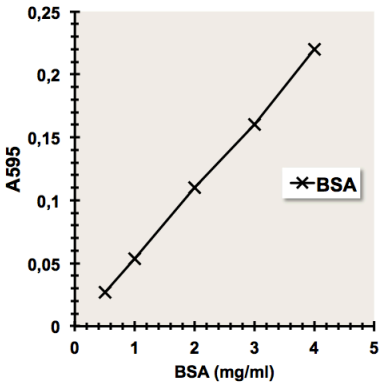
The procedure

For the preparation of samples for Western blotting & SDS PAGE

Sample	**Reference sample**
800 µl H_2O	800 µl H_2O
0.5, 1.0 & 2.0 µl crude extract	no protein but the same volume of buffer
200 µl Bradford solution	200 µl Bradford solution

If you have many samples to measure, we suggest to do a master mix and distribute 1 ml of the master mix to labeled Eppendorf reaction tubes. Using a master mix, you can measure more samples at the same time and it helps you to avoid errors.

After mixing the samples with the protein solutions, the mixtures have to be incubated for 5 minutes at room temperature. Transfer the mixtures and the reference sample to 1.5 ml cuvettes and measure the absorption at a wavelength of 595 nm.



The calibration curve (see above) shows the linear relation between the amount of protein (BSA) in the sample and the absorption at 595 nm. For calculating the protein concentration in your samples, subtract $A_{595\ nm}$ of the reference from the absorption of your samples. Next you can calculate the amount of protein in your sample using the following formula:

$$\mathbf{A_{595\ nm} = a\ (ml/mg)\ x\ c\ (mg/ml))}$$

The slope "a" of the calibration curve has been determined using the protein BSA. After several repetitions, this value turned out to be 0.0536 when 1 µl (with c = 1 mg/ml BSA) has been added to 1 ml of the five-fold diluted Bradford solution. Don't forget to substract the absorption of your blank from the absorption of your sample!

For the β-Gal assay, add 20 µl of your crude extract to the Bradford-water solution. Use 20 µl of the Z-buffer/LD-Mix as a reference for the measurement. The determined absorption can be directly used together with the formula on page 112 for the calculation of the Miller units.

5.4. SDS PAGE

Sodium dodecyl sulfate polyacrlyamide gel electrophoresis (SDS-PAGE) is a commonly used method to separate complex mixtures of proteins. In an electric field the negatively charged proteins migrate towards the anode. Moving through the small pores of the gel matrix, the proteins are separated according to their size. Using a marker, the relative molecular weights of proteins within the loaded sample can be determined. Denaturating gels are prepared as described by Laemmli *et al. (*1970). The gels consist of a stacking and a running gel and are poured to a thickness of 1 mm.

A B

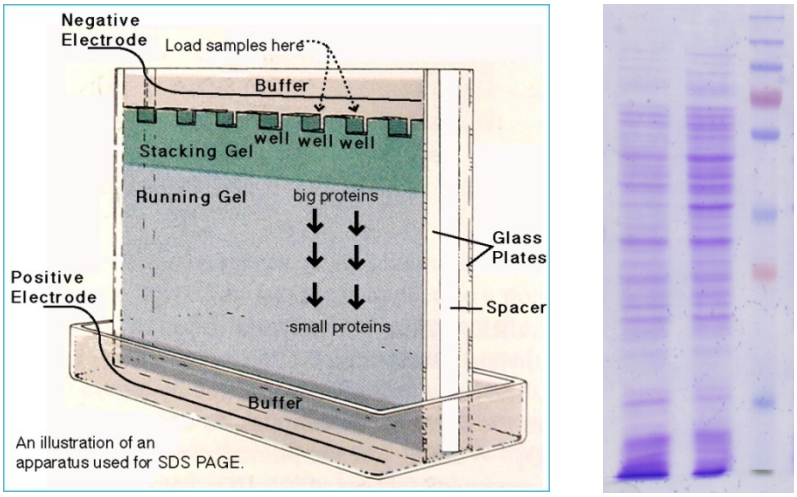


Figure 32. A) The principle of SDS PAGE. B) Commassie-stained SDS gel containing *Bacillus subtilis* 168 crude extract (http://ww2.chemistry.gatech.edu/~lw26/bCourse_Information/4581/techniques/gel_elect/page_protein.html).

The material

5 X SDS loading dye
1.4 ml Tris-HCl, pH 7.0
3 ml Glycerol (100%)
2 ml SDS (20%)
1.6 ml ß-Mercaptoethanol (100%)
0.01 g Bromophenol blue
2 ml H_2O_{deion}

10 X Running (PAGE) buffer
1.92 M Glycerine
0.5 M Tris
1% SDS

100% *i*-Propanol (*iso*-Propanol, 2-Propanol, or 1,1-Dimethyl-1-Hydroxy-Methan)

Power supply & SDS PAGE device

Running gel for denaturing SDS PAGE (enough for 2 gels)

Components for gel	**15 %**	**12 %**	**10 %**
H_2O	2.3 ml	3.3 ml	4.0 ml
30% Acryl-Bisacrylamide mix	5.0 ml	4.0 ml	3.3 ml
1.5 M Tris (pH 8.8)	2.5 ml	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml	0.1 ml
10% Ammonium Persulfate (APS)	0.1 ml	0.1 ml	0.1 ml
TEMED	4 µl	4 µl	4 µl

5 % stacking gel for denaturing SDS PAGE (enough for two gels)

Components for 5 % gel	**9 ml**
H_2O	6.83 ml
30 % Acryl-Bisacrylamide mix	1.3 ml
1.5 M Tris (pH 6.8)	0.87 ml
10% SDS	0.1 ml
10% Ammonium Persulfate (APS)	0.1 ml
TEMED	0.02 ml

The procedure

1. Clean the glass plates, combs and mats with ethanol and assemble the glass plate sandwich. Be sure that the plates are well aligned. Place the plates in the casting frame and tighten them. Place the casting frames into the casting stands.

2. Wear gloves for making the gels because acrylamide is a neurotoxin in its unpolymerized form!!!

   Mix the ingredients of the running gel and pour the solution quickly (gel starts to polymerize once TEMED has been added) into the gap between the glass plates. Leave enough room for the stacking gel – appr. 1 cm below the bottom of the comb´s teeth. After a minute you can overlay the solution with isopropanol.

3. After polymerization, pour off the isopropanol and rinse with distilled water. Mix the reagents for the stacking gel and pour it on top of the running gel till the space is completely full. Then insert the comb.

4. After polymerization clamp the gel into the electrophoresis apparatus and fill it with running buffer. Remove the comb carefully and use a pipette or syringe to wash the wells with running buffer.

5. The protein samples (up to 15 µl) are mixed with SDS loading buffer (2.5 µl) and incubated for 10 min at 95° C.

6. After heating briefly centrifuge the samples to spin down the condensed water.

7. Then you can apply the samples on a SDS PAGE with a pipette or Hamilton microliter syringe. Don´t forget to add molecular weight marker in one lane.

8. Run the gel at 80 V until the front has moved into the running gel. Then you can increase the voltage to 120 V. Run the gel until the running front has reached the bottom of the running gel.

9. Pry the plates apart by using a gel spacer. The gel can then be stained (Coomassie or Silver staining) to visualize the separated proteins or processed further (Western Blot).

5.5. Coomassie staining

After separation of proteins by SDS PAGE or native gel electrophoresis, the proteins can be fixed and visualized by different staining procedures. The Coomassie Brilliant blue-staining procedure is a fast and very easy method that is commonly used in the lab to detect proteins. Coomassie Brilliant blue is the name of two similar triphenylmethane dyes that were developed for use in the textile industry but are now commonly used for staining proteins in analytical biochemistry. Coomassie brilliant blue G-250 differs from Coomassie Brilliant blue R-250 by the addition of two methyl groups (see Figure below). The name "Coomassie" is a registered trademark of Imperial Chemical Industries.

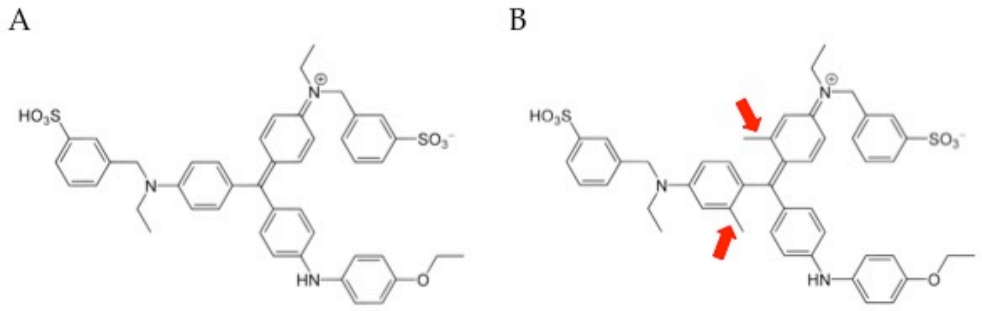


Figure 33. The two Coomassie stains R-250 A) and G-250 B). The stain R-250 is suitable to detect between 0.1 µg and 5 ng protein per lane. Stain G-250 is less sensitive (but faster!) because the detection limit is about 0.5 µg protein per lane.

The material	**Fixation solution**	10% Acetic Acid 45% Methanol (toxic!)
	Staining solution	0.5% Commassie Brillant blue 10% Acetic Acid 45% Methanol
	Destaining solution	10% Acetic Acid

The procedure

1. For staining (or the membrane after doing a Western Blot), place the gel in a plastic container and cover the gel with fixation solution. Shake the gel for 10 min at room temperature.

2. Remove the fixation solution and cover the gel with staining solution. Incubate the gel for 10 – 20 min at room termperature on a shaker.

3. Remove the staining solution (it can be re-used). Destain the gel over night with 10% acetic acid under rigorous shaking at room temperature. Adding a paper towel to the solution speeds up the destaining process. Boiling the gel in a microwave speeds up destaining.

5.6. Dialysis

In our lab proteins are purified from crude extracts by affinity purification. Usually, overexpressed proteins carry a His or Strep affinity tag that allows binding to and elution from respective matrices. Proteins that are bound to the matrix can be removed either with Imidazol or D-desthiobiotin (for more information see page 90). These small molecules might interfere with further applications.

Dialysis is a technique to remove those small, unwanted compounds from your sample by selective and passive diffusion through a semi-permeable membrane. The sample and the dialysate are placed on opposing sites of the membrane. Sample molecules that are larger than the membrane-pores are retained on the sample side of the membrane. On the other hand small molecules such as Imidazol and D-desthiobiotin as well as buffer salts pass freely through the membrane, thereby reducing the concentration of those molecules in the sample till they reach equilibrium. The buffer can be changed several times to reduce the amount of unwanted molecules to negligible concentrations. Dialysis is performed in ~1000 fold excess of dialysis buffer (for example: 5 l dialysis buffer for 5 ml sample volume) for two times between ~16 and 24 hours. For long-term storage of your sample, glycerol can be added to the second diaylsis step with a final concentration of 25% (v/v).

The material

Dialysis tube (MWCO [molecular-weight cutoff] 3.5 kDa)

Dialysis buffer I	10 mM Tris 100 mM KCl 1 mM DTT Adjust pH to 7.5 with HCl (37%) (The buffer can be prepared 10 X concentrated)
Dialysis buffer II	10 mM Tris 100 mM KCl 1 mM DTT Adjust pH to 7.5 with HCl (37%) Add glycerin to a final concentration of 25%
Dialysis buffer III	100 mM EDTA

5 l bottle

The procedure

1. Prepare all buffers and samples.
2. Incubate dialysis tube in buffer III and let it boil for 10 min.
3. Repeat step 2.
4. Place the dialysis tube into buffer I to cool it down to 4°C.
5. Load sample in the tube. Seal the tube with clips and place it in buffer I.
6. Remove the tube after 16-24 hours and place it in buffer II.
7. The sample is ready for further experiments at the following day.

Remarks: keep your samples on ice; touch the dialysis tube carefully (avoid sharp objects, wear gloves); constantly stirr buffers I and II (the sample should float within the buffer).

6. Miscellaneous methods

6.1. Fluorescence microscopy

Fluorescence microscopy is a versatile method to investigate protein localization and/or the activity of a promoter. Here we describe a method that is suitable to monitor the activity of a promoter-fluorophore gene fusion in single cells of *B. subtilis* (Diethmaier *et al.*, 2011).

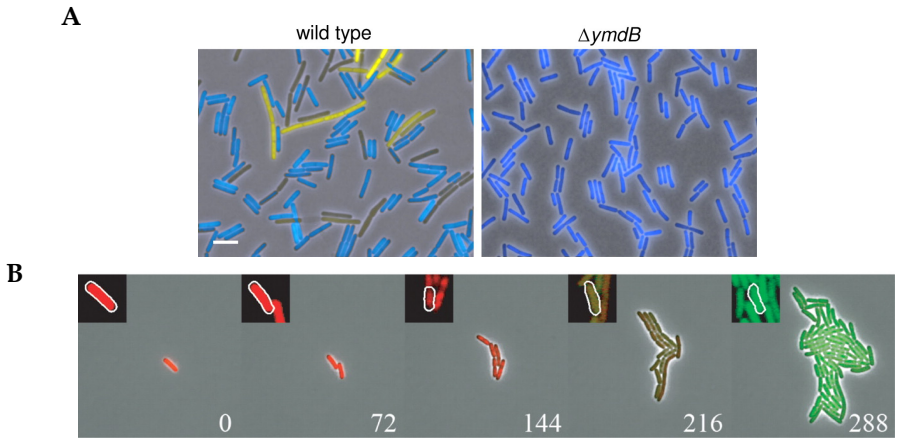


Figure 41. A) The YmdB protein affects the bistable expression of motility and biofilm genes in *B. subtilis*. Fluorescence microscopy of cells harboring both P_{hag}-*cfp* and P_{tapA}-*yfp* fusions in the wild-type strain or the isogenic *ymdB* mutant. Cells were observed using fluorescence microscopy. CFP was false-colored blue, and YFP is shown in yellow. Wild type and cells of the *ΔymdB* mutant were grown in LB medium and prepared for microscopy in the logarithmic phase of growth. Scale bar, 5 µm (Diethmaier *et al.*, 2011). B) Measuring a gene regulation function in individual *E. coli* cell lineages. Snapshots of a typical regulator dilution experimen. CI-YFP fusion protein is shown in red and CFP is shown in green. Times, in minutes, are indicated on snapshots. (Insets) A selected cell lineage is outlined in white (Rosenfeld *et al.*, 2005).

The material

Microscope, slides and cover slides

1% Agarose	0.5 g Agarose 50 ml Water
1 X PBS buffer, pH 7.5	(see section "Media, buffers & solutions")

The procedure

1. Use a single colony of *B. subtilis* to inoculate 4 ml of LB medium supplemented with the appropriate antibiotics. Incubate the culture over night at 37°C with agitation.

2. Next morning inoculate 10 ml LB liquid medium supplemented in a shake flask to an approximate OD_{600} of 0.05. Incubate the shake flask at 37°C and 200 rpm in the dark and collect the cells at the growth phase of interest.

3. To harvest the cells, transfer 1 ml into a 1.5 ml lightproof (brown) Eppendorf reaction tube and centrifuge the tube for 1 min at 13000 rpm. Discard the supernatant and resuspend the cells in 1 X PBS buffer (in a volume (ml) = OD_{600} X (1/10) (ml)). Keep the cell suspension on ice until microscopy.

4. Preparation of the agarose slide for microscopy: boil the agarose, put some agarose on the slide and place the cover slide on top of the agarose; remove the cover slide from the rigid agarose and pipet your cells on top of the agarose; finally, cover the cells with the cover slide.

5. Fluorescence images will be obtained with an Axioskop 40 FL fluorescence microscope, equipped with digital camera AxioCam MRm. The AxioVision Rel 4.8.2 software will be used for image processing (Carl Zeiss, Göttingen, Germany). We will use objective of the Neofluar series is used to produce a 100 X primary magnification. The applied filter sets is the EGFP HC-Filterset (BP: 472/30, FT 495, LP 520/35; AHF Analysentechnik, Tübingen, Germany) for GFP detection. Images can be taken with different exposure times (e.g. 2 sec)

6.2. Quantification of c-di-NMPs from *E. coli* and *B. subtilis* cultures

With this method the *in vivo* levels of c-di-AMP or c-di-GMP from bacteria can be quantified. The procedure describes the extraction of the nucleotides from the cells. The quantification is performed at the "Medizinische Hochschule Hannover" and combines the separation of the specific nucleotide (c-di-AMP or c-di-GMP) *via* HPLC with the subsequent and highly sensitive quantification *via* mass spectrometry (Spangler *et al*. 2010). For examples, see Mehne *et al*. 2013.

The material

Extraction mixture (acetonitrile/methanol/water 40/40/20 *v*/*v*/*v*)

Glass beads (0.1 mm)

0.1 N NaOH

Liquid nitrogen

The procedure

For the detection of c-di-nucleotides from supernatants of *E. coli* cultures

1. Grow *E. coli* cultures in 50 ml LB medium at 37°C and add IPTG at an OD_{600} of 0.5 - 0.7 to induce the expression of a c-di-nucleotide cyclase encoding gene.

2. Harvest 3 X 10 ml (technical duplicate & backup) of the culture by quick centrifugation for 5 minutes at 8500 rpm and 4°C. Take two additional samples (1 ml each) for the determination of total protein amount for later normalization purposes.

3. Resuspend the cell pellet in 300 µl extraction mixture (acetonitrile/methanol/water 40/40/20 *v*/*v*/*v*). Snap freeze the sample in liquid nitrogen, following a heating step for 10 min at 95°C and centrifugation for 10 min at 20800 g at 4°C.

4. Store the supernatant on ice. Use the resulting pellet to repeat the extraction procedure twice with 200 µl extraction mixture at 4°C but replace the heating step with incubation on ice for 15 min. This results in 3 supernatants – pool and store them at -20°C over night.

5. Spin sample for 10 min at 20800 g at 4°C and transfer the supernatant into a new tube. This is your sample which has to be dried in a speedvac (about 2 h at 40°C).

For the detection of c-di-nucleotides in *B. subtilis*

1. Grow *B. subtilis* cultures at desired conditions (i.e. CSE-Glc at 37°C). Harvest 10 ml of the culture by quick centrifugation at 4°C. Take two additional samples (1 ml each) for the determination of total protein amount for later normalization purposes.

2. Resuspend the cell pellet in 800 µl extraction mixture and transfer to a 2 ml tube with screw cap (previously filled with 700 mg glass beads 0.1 mm). Snap freeze the sample in liquid nitrogen, following a heating step for 10 min at 95°C.

3. Lyse the sample in a Tissue Lyser machine (Qiagen) 7.5 min at 30 Hz. Separate glass beads by centrifugation for 5 min at 17000 g at 4°C.

4. Remove the supernatant and store it on ice. Resuspend the remaining sample mixture in 600 µl extraction mixture, incubate on ice for 15 min and lyse again for 7.5 min at 30 rpm.

5. After centrifugation for 5 min at 17.000 g at 4°C remove the supernatant and pool with the previous one.

6. Once again, resuspend sample in 400 µl extraction mixture, incubated on ice for 15 min and centrifuge. Pool all supernatants and store at -20°C o/n.

7. Spin the sample for 10 min at 20800 g at 4°C and transfer the supernatant into a new 2 ml SafeSeal tube (Sarstedt). This is your sample which has to be dried in a speedvac (about 2 h at 40°C).

Remarks: The samples can be stored in the freezer. However, c-di-NMPs are stable at RT, thus no specific treatment/storage is necessary. For Quantification of c-di-NMPs by HPLC/MS send the samples to our collaboration partners in Hannover. Please ask for the procedure how to do this.

Normalization of data

Once you receive data from Hannover you have to normalize them. For this purpose use the two 1 ml cell pellets to quantify the amount of protein from the cells. For each pellet, add 800 µl 0.1 N NaOH and heat for 10 min at 95°C. Spin sample 5 min at 20800 g at RT and transfer the supernatant into a new tube. Resuspend the remaining pellet with another 800 µl 0.1 N NaOH, heat and spin again. Remove the supernatant and pool it with the previous one. Use this solution for Bradford quantification – two times; usually 50 and 75 µl (It is important to add the same amounts of extraction mixture to the blanks). Note that the calculated protein concentration is usually mg/ml. But since you later need *mg/ml cells*, multiply by 1.6 (because bradford quantification was done from 1.6 ml protein extracts which represent the whole amount of your original cell pellet).

Use the formula below to calculate cdiNMP concentrations from raw data normalized to protein levels.

$$\frac{c_{cdiNMP}\,[nM]\cdot 200}{cv\,[ml]\cdot c_{595}[\frac{\mu g}{ml\ cells}]} = \frac{cdiNMP\,[pmol]}{protein\,[mg]}$$

c_{cdiNMP}	raw data concentration Hannover
cv	culture volume; usually 10 ml
c_{595}	protein concentration Bradford; mean

M_W (c-di-AMP) = 658.1
M_W (c-di-GMP) = 690.0

Usually, values in ng c-di-NMP/mg protein are useful for graphs!

7. Media, buffers & solutions

Complex media

LB medium	10 g Tryptone 5 g Yeast extract 10 g NaCl Ad 1000 ml deionised H_2O
SP medium	8 g Nutrient broth 0.25 g $MgSO_4$ X 7 H_2O 1 g KCl Ad 1000 ml deionised H_2O After autoclaving don't forget to add 1 ml $CaCl_2$ (0.5 M) 1 ml $MnCl_2$ (10 mM) 2 ml Iron Ferric Ammonium Citrate (CAF; 2.2 mg/ml)
Starch plates	7.5 g Nutrient broth 5 g Starch Ad 1000 ml deionised H_2O
TBAB medium	33 g Tryptose Blood Agar Base (TBAB) Ad 1000 ml deionised H_2O
VY medium	25 g Veal infusion broth (VY) 5 g Yeast extract Ad 1000 ml deionised H_2O
TSA medium	40 g Tryptone Soy Agar (TSA) Ad 1000 ml deionised H_2O
BHI agar	52 g Brain Heart Infusion (BHI) agar Ad 1000 ml deionised H_2O
BHI broth	37 g Brain Heart Infusion (BHI) broth Ad 1000 ml deionised H_2O

Minimal media

1 X C minimal medium	20 ml 5 X C salts 1 ml Tryptophan (5 mg/ml) 1 ml Iron Ferric Ammonium Citrate (CAF; 2.2 mg/ml) 1 ml III' salts Fill up to 100 ml with deionised H_2O
1 X CSE minimal medium	20 ml 5 X C salts 1 ml Tryptophan (5 mg/ml) 1 ml Iron Ferric Ammonium Citrate (CAF; 2.2 mg/ml) 1 ml III' salts 2 ml Potassium Glutamate (40 %) 2 ml Sodium Succinate (30 %) Fill up to 100 ml with deionised H_2O
1 X CSE Glc minimal medium	is CSE minimal medium with 0.5% Glucose
1 X CS minimal medium	is CSE minimal medium without Glutamate
1 X CS Glc minimal medium	is CS minimal medium with 0.5% Glucose

1 X C Glc minimal medium
is C minimal medium with 0.5% Glucose

III′ salts
0.232 g $MnSO_4$ X 4 H_2O
12.3 g $MgSO_4$ X 7 H_2O
Fill up to 1000 ml with deionised H_2O

5 X C salts
20 g KH_2PO_4
80 g K_2HPO_4 X 3 H_2O
16.5 g $(NH_4)_2SO_4$
Fill up to 1000 ml with deionised H_2O

10 X MN medium
136 g K_2HPO_4 X 3 H_2O
60 g KH_2PO_4
10 g Sodium citrate X 2 H_2O

MNGE medium
9.2 ml 1 X minimal medium
1 ml Glucose (20 %)
50 µl Potassium Glutamate (40 %)
50 µl Iron Ferric Ammonium Citrate (CAF; 2.2 mg/ml)
100 µl Tryptophan (5 mg/ml)
30 µl $MgSO_4$ (1 M)
+/- 100 µl CAA (10 %)

10 X Spizizen minimal salts (SMS)
20 g $(NH_4)_2SO_4$
183.4 g K_2HPO_4 X 3 H_2O
60 g KH_2PO_4
10 g Na Citrate X 2 H_2O
2 g $MgSO_4$ X 7 H_2O

MSgg
2.5 ml 1 M K Phosphate buffer, pH 7
50 ml 1 M MOPS buffer, pH 7
5 ml 50% Glycerol
0.05 ml 20 mM Thiamine
6.25 ml K Glutamate (40 %)
2.5 ml L-Trytophan (10 mg/ml)
2.5 ml L-Phenylalanine (10 mg/ml)
0.5 ml $CaCl_2$ (0.7 M)
1 ml $MgCl_2$ (1 M)
0.5 ml $MnCl_2$ (50 mM)
0.5 ml $FeCl_3$ (50 mM)
0.5 ml $ZnCl_2$ (1 mM)
Ad 0.5 l with deionized water

M9 base medium (20 X, *E. coli*)
140 g Na_2HPO_4 X 2 H_2O
60 g KH_2PO_4
20 g NH_4Cl
ad 1 l with deionized water

M9 minimal medium
50 ml M9 (20 X)
1 ml $MgSO_4$ (1 M)
1 ml $CaCl_2$ (0.1 M)
0.5 ml $FeCl_3$ (1 mM)
1% (w/v) Carbon source
ad 1 l with deionized water

Supplements for M9	66 ml CAA (10%) 10 ml Proline (4 mg/ml) 1 ml Thiamin-di-Chlorid (1 mg/ml)
Agar plates	Agar is usually used at a final concentration of 1.5% (e. g., for 1 l SP medium agar you need 15 g agar)
2 X Agar solution (3%)	9 g Bacto agar Fill up to 300 ml with deionised H_2O
SMM agar plates	1 X SMS 0.5% Glucose 1.5% Agar
Buffer	
10 X PBS	Phosphate-buffered saline (PBS) 80 g NaCl 2 g KCl 26.8 g Na_2HPO_4 X 7 H_2O 2.4 g KH_2PO_4 Add 800 ml deionised H_2O Adjust the pH with HCl to 7.5 (or another pH) Ad 1000 ml with deionised H_2O
1 M K-phosphate buffer, pH 7	610 ml 2 M K_2HPO_4 390 ml 2 M KH_2PO_4
1 M MOPS	Sterilized by filtration and stored in the dark at 4°C
10 mg/ml Tryptophan	Sterilized by filtration and stored at 4°C
10 mg/ml Phenylalanine	Sterilized by filtration and stored at 4°C
50 mM $FeCl_3$	Prepare freshly in a 15 ml Greiner tube, do not sterilise
Chromogenic substrates	
X-Gal	80 mg/ml in N,N-Dimethylformamide (DMF)
TPTZ	40 mg/ml 2,3,5-Triphenyltetrazolium chloride (TPTZ) in water
Other solutions	
IPTG	2.38 g IPTG Ad 10 ml with deionised H_2O Sterilization by filtration, store at -20°C in aliquots
5 X DNA loading dye	5 ml Glycerol (100%) 4.5 ml deionised H_2O 0.2 ml 50 X TAE 0.01 g Bromphenol blue 0.01 g Xylene cyanol
DTT	1 M Dithioerythol (DTT) in water, store at 4°C

Lugol (Iodine/K Iodide) solution	100 g KJ 50 g J ad 1 l with water
50% Glycerol solution	50 ml 100% Glycerol add 50 ml deionized water
20 mM Thiamine (Vitamin B1)	Dissolve in deionized water, Storage after sterile filtration in the dark at -20°C
Egg Yolk Emulsion	30 ml Egg yolk (Freilandeier) 70 ml sterile Saline (0.9%)
Activate charcoal solution (10 X)	Add heat-sterilized charcoal (to water) to a final concentration of 5% (w/v)

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