TECHNISCHE UNIVERSITÄT MÜNCHEN

PHYSIK DEPARTMENT

E14 - Physik Synthetischer Biosysteme

Advanced Lab Course in Physics

Cloning

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ZNN room 2.012

Contents

General Guidelines and Recommendations

- You are not allowed to enter the lab without long trousers and closed shoes.
- You are not allowed to drink or eat in the lab.
- You have to wear gloves (and a lab coat in the gel lab).

Rules of Handling Enzymes

- Remove enzymes from -20°C only prior to usage.
- Do not leave enzymes at room temperature.
- Use an icebox or cooling device while working with enzymes on the bench.
- Always add enzymes as the last component to the reaction mix.

List of Terms

amino acids	building blocks of peptides and proteins
cloning	process used to insert DNA fragments into cloning vectors
cloning vector	DNA molecule that can be stably maintained in a host organism and which can be modified to carry the desired DNA insert
competent cells	cells which are able to take up exogenous genetic material
DH5α	*E. coli* strain optimized for cloning and plasmid purification
DNA	desoxyribonucleic acid, carrier of genetic information
DNA polymerase	enzyme that synthesizes a copy of a DNA molecule. DNA polymerases need a short RNA or DNA molecule (primer) paired to the template strand to initialize the synthesis;
electroporation	transformation method: A high voltage pulse is applied to a mixture of competent cells and a purified plasmid or ligation product. Since the cell membrane is not able to pass current (except in ion channels), it acts as an electrical capacitor. Subjecting membranes to a high-voltage electric field results in their temporary breakdown, creating pores that are large enough to allow macromolecules (such as DNA) to enter or leave the cell [1].
gene	a region on a DNA strand which codes for a molecule that has a function e.g. a protein
gene expression	process in which the information of a gene is used for the synthesis of a gene product e.g. a protein
GMO	genetically modified organism
heat shock transformation	transformation method: competent cells are mixed with a purified plasmid or a ligation product and heated to 42°C for 30-45 s. In this step the uptake of the plasmid from the solution takes place. After this step the bacteria are cultured on a agar plate.
ligase	enzyme that can seal nicks in DNA strands by creating a phosphodiesther bond between the 3' OH and the 5' phosphate group of adjacent nucleotides
nfH_2O	nuclease free water
nucleotide	building block of nucleic acids composed of a base (A,T or U,G,C), a sugar (desoxyribose or ribose) and a phosphate group
OD_{600}	optical densitiy = absorbance measurement at a wave length of 600 nm
operon	functioning unit of DNA containing a cluster of genes under the control of a single promoter
ori = origin of replication	DNA sequence that allows for the replication of genetic material
PBS	phospate buffered saline: a buffer solution used in biological research
PCR	polymerase chain reaction: process to amplify DNA fragments
phosphatase	removes the 5' phosphate of DNA strands
primer	short RNA or DNA molecule needed by DNA polymerases to initialize replication
promoter	a region on a DNA molecule which is recognized by a RNA polymerase and where transcription is initiated

replication	process of producing two identical copies of DNA from one original DNA molecule; this process is essential to maintain the genetic material in the cells during cell division
restriction enzyme	enzyme that recognizes a short DNA sequence and cuts the DNA double helix in a specific way around its recognition site
RNA	ribonucleic acid (mRNA: messenger RNA)
RNA polymerase	enzyme that synthesizes the RNA strand during transcription
sticky ends	single stranded overhangs on DNA double helices
Taq polymerase	DNA polymerase which was originally isolated from the thermophilic bacterium *Thermus aquaticus*
transcription	a particular part of the DNA is copied into RNA
transformation	process of transferring genetic material through the cell membranes
translation	the mRNA is decoded in a ribosome to produce a specific amino acid chain

1 Introduction

The process of inserting DNA fragments (like genes coding for proteins) into cloning vectors (see Section 3.3 for details) is referred to as cloning, resulting in an genetic alteration of organisms. If the cloning was successful, the genetically modified organisms (GMOs) can be used for the recombinant expression of proteins and their subsequent purification. Also regulatory parts like inducible promoters [2] or genetic networks [3] can be studied within GMOs. In addition, GMOs are used for large scale productions of pharmaceuticals (e.g. insulin) or other applications in biotechnology [4].

Objectives of the Lab Course
In the lab course you will perform main steps of a typical cloning protocol. You will analyze the products of these steps in a gel electrophoresis. In the last part you will culture *E. coli* and study the growth phases.

2 Theoretical Background and Methods

2.1 *E. coli* growth phases and OD measurement

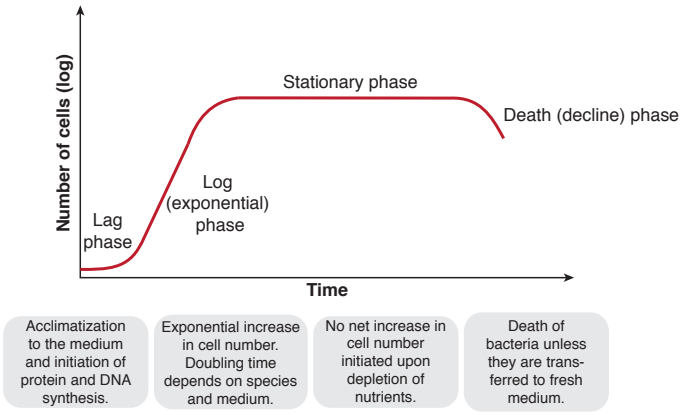

Figure 1: Bacterial growth phases.

One of the most common host organisms is the bacterium *Escherichia coli* (*E. coli*) which has a maximum reproduction rate of about 20 minutes and can be cultured in inexpensive culture media (LB medium).
During the cultivation of *E. coli* in liquid culture media, the bacteria undergo different growth phases. The bacteria have to acclimate to the culture medium in the lag phase. Then the cell number increases exponentially in the Log or exponential phase. Nutrients are depleted in the stationary phase so the cell number does not increase anymore. The bacteria die, if they are not transferred into fresh medium in the death phase (see Figure 1) [5].
The growth state of such a culture can be read-out by the optical density (see Table 2). The optical density is measured with a photometer at a wavelength of 600 nm (OD_{600}). The mathe-

matical relationship between the transmitted light intensity (I), the incident light intensity (I_0) and the optical density (OD_{600}) is:

$$OD_{600} = -\log\left(\frac{I}{I_0}\right) \tag{1}$$

The incident light can lose intensity in the sample due to two processes: absorption and scattering, but in a cell suspension the dominant effect is scattering. The combined effect of absorption and scattering is called extinction.

$$\text{Extinction} = \text{Scattering} + \text{Absorption} \tag{2}$$

The optical density increases proportionately to the number of cells in the culture. For OD values > 0.5 individual cells shade each other and the linear dependency between OD and number of cells disappears. To get reliable results in this regime, the sample has to be diluted prior to the measurement [6].

OD_{600}	growth phase
<0.2	lag phase
0.2 - 1	exponential phase
>1	stationary phase

Table 2: Optical density ranges and the corresponding growth state of an *E. coli* culture. These ranges depend on the strains and the culture conditions. The given values are rough estimates.

2.2 DNA

Deoxyribonucleic acid (DNA) is the carrier of genetic information. The molecular structure of DNA was first identified by Watson and Crick in 1953 [7]. Basically DNA can be described as a polymer of four different subunits, so-called nucleotides, each consisting of a base linked to a sugar and a phosphate. The nucleotides are joined to one another in a polymer by covalent bonds between the sugar of one nucleotide and the phosphate of the next, resulting in an alternating sugar-phosphate backbone (and in an orientation of the DNA strand (5' end and 3' end)).The four different nucleotides differ only in the type of base, adenine (A), cytosine (C), guanine (G) or thymine (T). A main feature of DNA is given by the Watson-Crick base pairing, i.e. the preferred binding of the complementary bases adenine-thymine and cytosine-guanine. It is the basis for the formation of the double helical structure out of two antiparallel DNA strands with complementary sequence [8][9].

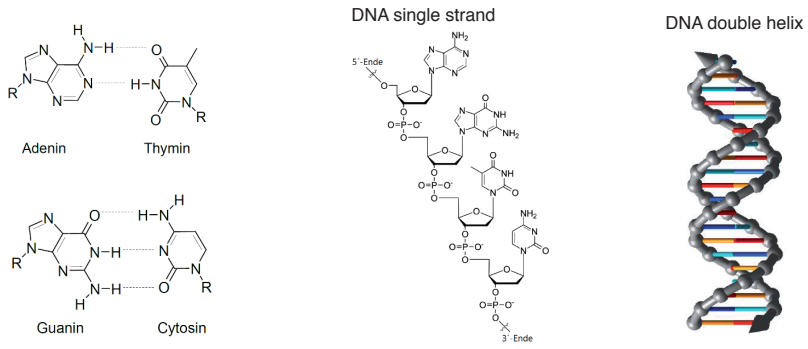

Figure 2: (a) Adenine, thymine guanine and cytosine. (b) A DNA single strand. (c) A DNA double helix.[9] [10]

2.3 Polymerase Chain Reaction (PCR)

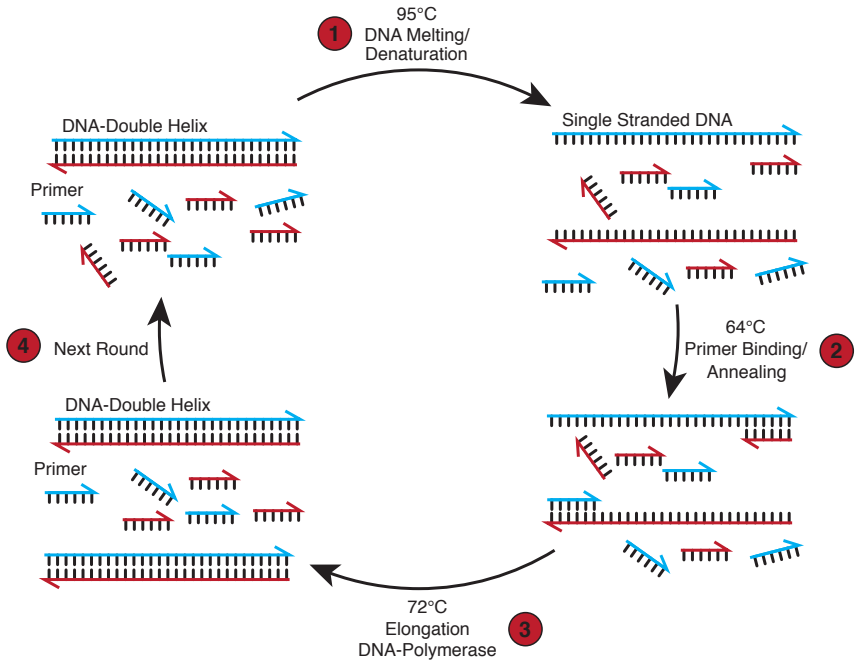

Figure 3: Polymerase chain reaction steps. (1) The DNA template is melted in the first step at a temperature of 95°C. (2) Then the reaction mixture is cooled down to the primer melting temperature (T_m=64°C). (3) In the third step new DNA strands are produced by a DNA polymerase at 72°C. (4) These steps are repeated several times to amplify the amount of the desired DNA fragment.

Polymerase chain reaction (Figure 3) is a technique used in molecular biology to exponentially enrich few copies of a desired DNA fragment. For an initial amount of 1 ng/µl of DNA template an amplification to up to 100-150 ng/µl is possible. The method is based on thermal cycling which involves exposing the reactants to cycles of repeated heating and cooling, permitting different temperature dependent reactions.
The reaction takes place at defined buffer and salt conditions and the mixture consists of a small amount of the DNA template, a thermostable DNA polymerase (Taq polymerase) and nucleotides. In addition, short (20 bp) DNA oligonucleotides which are complementary to the flanking region of the desired DNA sequence called "primers" have to be added. The DNA polymerase starts the synthesis of a new DNA strand at the 3' end of these primers as soon as they are bound to the DNA template strand [11].
A typical PCR round starts by melting the DNA double helix (denaturation) at 95°C for 5 s. Then the mixture (which contains now single stranded DNA) is cooled down to the primer melting temperature (T_M=64°C, 15 s). The primers bind at their binding site (annealing) and the mixture is heated to 72°C which is the optimal elongation temperature for the Taq polymerase to produce the new DNA strand (elongation). After about 25 cycling rounds, there is a 5 min step at 72°C to ensure that any remaining single-stranded DNA is fully elongated.

In a slightly modified protocol, amplification of nonspecific sequences is avoided by decreasing the annealing temperature in defined limits during the PCR (touchdown PCR). The annealing temperature during a PCR determines the specificity of primer annealing and the efficiency of the reaction.
The specificity of primer binding means the number of primers that bind to their primer binding site (= complementary sequence without base pair mismatches) divided by the total number of primers that bind during the annealing step to the DNA template (primer binding sites + mismatch regions).

$$\text{specificity} = \frac{\text{number of primer bound to primer binding sites}}{\text{total number of primer bound to the DNA template}} \tag{3}$$

However a PCR cycle with primers bound to the wrong sequence can continue and amplify a non desired fragment. Increasing the annealing temperature of the PCR solves this problem presuming that the spurious primer interactions are sufficiently less stable than the specific (correct) ones due to degrees of sequence mismatch [12]. In other words the specificity of primer binding can be increased if the annealing temperature is higher than the primer melting temperature (T_M).
The efficiency of the reaction means the amplification yield in each round. The maximum amplification is a doubling of the desired fragment concentration every cycle.

$$\text{efficiency} = \text{amplification yield} = 2^{\text{number of cycles}} \text{ (maximum)} \tag{4}$$

The amplification yield decreases for increasing annealing temperatures.

In summary this means:

$$T_{\text{annealing}} > T_M \rightarrow \text{specificity high, efficiency low} \tag{5}$$

$$T_{\text{annealing}} < T_M \rightarrow \text{specificity low, efficiency high} \tag{6}$$

Thus amplification of undesired fragments can be avoided (at a simultaneously good efficiency), by choosing the highest annealing temperature (T_M+5°C) for the first cycle (highest specifity).Then the annealing temperature is decreased in 1°C increments for every subsequent cycle until the annealing temperature is T_M-1°C (specifity is reduced in each cycle, but efficiency is inreased in each cycle). In the following 20 cycles this annealing temperature is kept constant (see Table 3).[11][12] [13][14]

Item		Repeats	Temp. (°C)	Time (s)	Goal
Cycle 1		1x	98	30	DNA melting
Cycle 2		6x	98	5	DNA melting
	2nd Step		69-63	10	Primer binding
	3rd Step		72	20	Elongation
Cycle 3		20x	98	5	DNA melting
	2nd Step		63	10	Primer binding
	3rd Step		72	20	Elongation
Cycle 4		1x	72	5 min	Elongation
Cylce 5		1x	4	Inf	Storage

Table 3: Thermal cycling program for a TD PCR with a primer melting temperature of 64°C.

2.4 Cloning - Restriction/Ligation Protocol

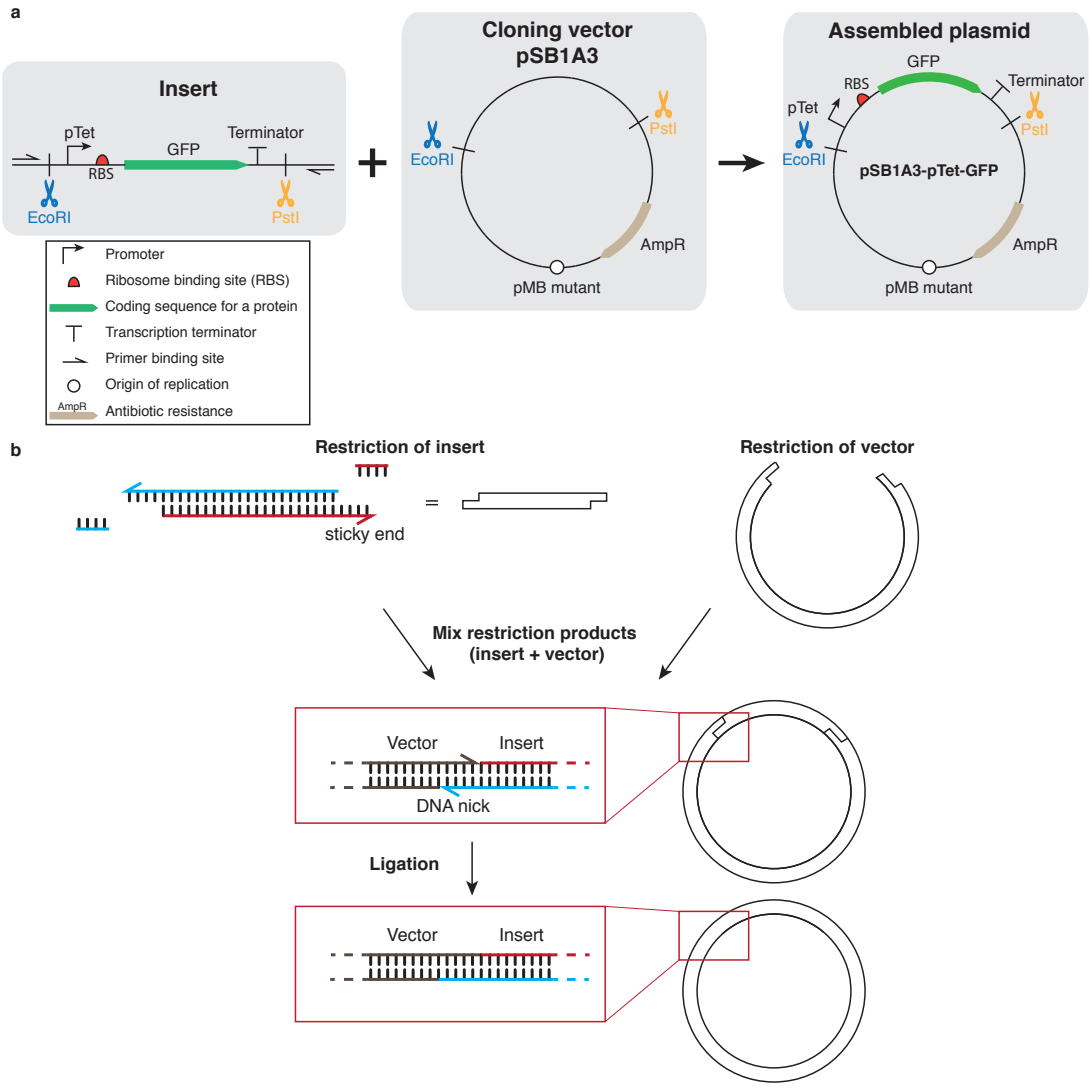

Figure 4: (a) The insert carries the desired gene including a promoter (RNA polymerase starts transcription), ribosome binding site (ribosome recognizes this sequence on the mRNA and starts translation) and a transcription terminator (RNA polymerase stops transcription). A plasmid serves as the cloning vector. This plasmid already contains the corresponding restriction sites, an antibiotic resistance and the origin of replication (pMB mutant). To assemble the plasmid with the desired insert, both the insert and the cloning vector are treated with the same pair of restriction enzymes. (b) The assembly is done with a pair of restriction enzymes that produce single stranded overhangs and a subsequent ligation (the nicks in the DNA double helix are sealed).

Vectors and Plasmids
Linear DNA fragments are degraded by *E. coli*. Therefore the desired DNA fragments have to be inserted in circular DNA molecules known as cloning vectors:

A cloning vector is a DNA molecule that can be stably maintained in a host organism and which can be modified to carry the desired DNA insert like a gene. There are many cloning vectors, but the most commonly used ones are plasmids.
A plasmid is a small circular DNA molecule (2-10 kbp) which is replicated independently from the genome of the bacterium since it codes for its own origin of replication.[13]

Foreign DNA fragments can be inserted into plasmids following a cloning protocol. Here we use a restriction/ligation protocol (see Figure 4). The plasmid with the desired insert is assembled using an empty plasmid as the cloning vector and a linear insert. This plasmid contains the corresponding restriction sites, an antibiotic resistance and the origin of replication. The linear insert carries the gene of interest. Both the insert and the cloning vector are treated with the same pair of restriction enzymes (= enzymes that recognize a short DNA sequence and cut the DNA double helix in a specific way around its recognition site) in separate tubes. After this step there are compatible single stranded overhangs (= sticky ends) at the ends of the plasmid and the insert. In the subsequent ligation step the plasmid and the insert can be joined together by a ligase (= enzyme that can seal nicks in DNA strands by creating a phosphodiesther bond between the 3' OH and the 5' phosphate group of adjacent nucleotids). The ligation product can be transformed to *E. coli*. Cloning protocols do not have a 100% yield so the plasmid with the correct insert has to be selected in subsequent steps. One selection marker is the antibiotic resistance. All bacteria which did not incorporate the plasmid during transformation will die in a culture containing the corresponding antibiotic.[13]

Restriction Ligation Protocol Flow Chart

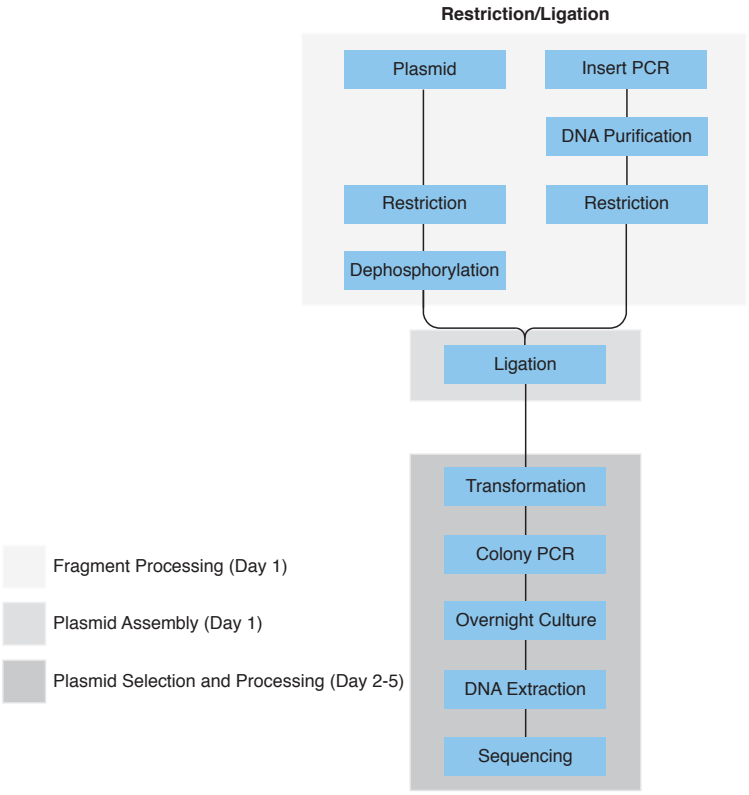

Figure 5: Standard procedure for plasmid construction. In the restriction/ligation protocol the plasmid and the insert are cutted separately, then the fragments are mixed and ligated. The plasmids with the correct insertion are selected in five steps.

The restriction/ligation protocol is shown in more detail in Figure 5. The protocol starts with an insert PCR (to amplify the amount of insert, see Section 3.4) and a PCR cleanup kit which removes the primers and the enzymes from the reaction mixture. Both the plasmid and the insert are restricted in parallel (in separate tubes) with the same pair of restriction enzymes. Thereby complementary sticky ends on the insert and the cloning vector are created which enable the assembly. The plasmid backbone is dephosphorylated using a phosphatase (A phosphatase removes the 5' phosphate of DNA strands. The ligase needs this phosphate to join the backbone nick between two fragments. Therefore the original fragment of the plasmid can not be inserted again. The new insert is not treated with the phosphatase so the ligase can join the nick.) The plasmid can not religate after this step, so the false-positive background is reduced in the subsequent cloning procedure. After heat inactivation of the restriction enzymes and over night ligation at 16°C the sample can be transformed.

Plasmid Selection and Processing
Five steps of plasmid selection and processing are necessary, which take four days. The subsequent steps are performed to identify clones which carry a plasmid with the correct insert without any mutations (e.g. deletions and insertations).The plasmid sample is transformed to DH5α by heat shock transformation or electroporation. Then the bacteria are cultured on a agar plate over night. On the next day samples of single colonies are added to a PCR reaction to determine which clone carries the correct plasmid. The plasmid is released from the cell during the first heating step, so it can serve as a template for a PCR. Primers targeting vector DNA flanking the insert can be used to determine whether or not the insert has the correct molecular size. This method is known as colony PCR. The desired colonies are cultured over night (5ml) and the DNA is extracted on the 4th day. Purified plasmids can be sequenced.

The whole cloning procedure usually takes 5 days. In our lab course, only two steps of the cloning are performed: PCR and restriction. In addition one step of the plasmid selection and processing part is performed: the plasmid extraction. These samples are analyzed in an agarose gel electrophoreses.

2.5 Plasmid Extraction

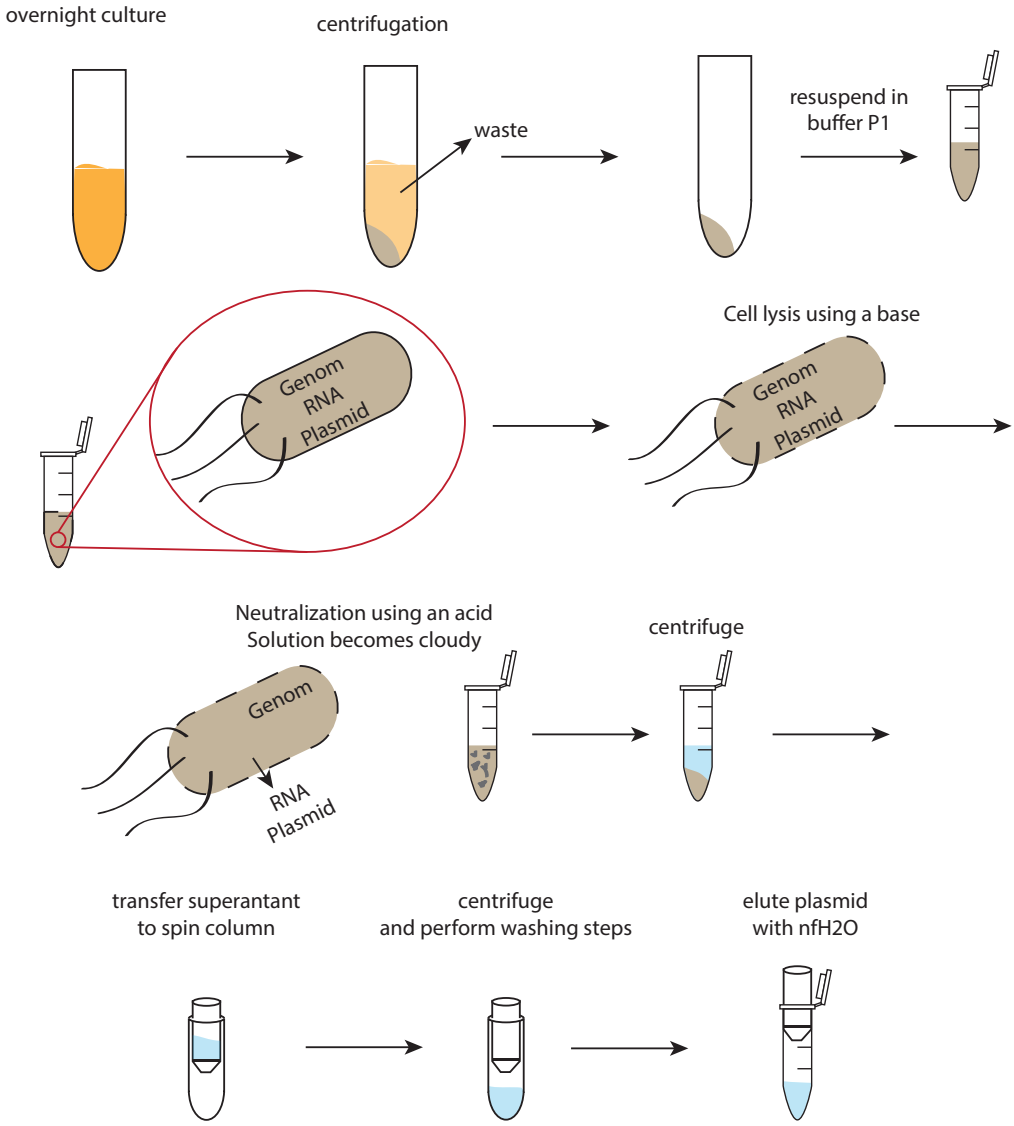

Figure 6: Plasmid extraction.

In this step we want to extract the cloned plasmid from an overnight culture. In the first step the overnight culture is centrifuged. You can observe a pellet containing the bacteria and a clear supernatant. The supernatant is removed and the pellet is resuspended in a small volume of buffer and transferred to a fresh reaction tube. Now the cells are broken down by adding a base. The reaction is stopped after a short incubation time (neutralization using an acid) and the solution becomes cloudy. By doing this the created holes in the cell membrane are small

enough to keep the large bacterial genome in the cell. The plasmid and RNA can go out. The RNA is degraded by an enzyme (RNAase). The cell fragments are separated from the plasmid in solution by centrifugation. The supernatant which contains the plasmid is transferred to a spin column. The plasmid binds to the silica membrane in the column in presence of high salt concentrations. Washing steps are performed to remove residuals of the cells. Then nuclease-free water is added to the spin column. After a short incubation time the column is centrifuged and the eluted plasmid is collected in a reaction tube.

2.6 Agarose Gel Electrophoresis

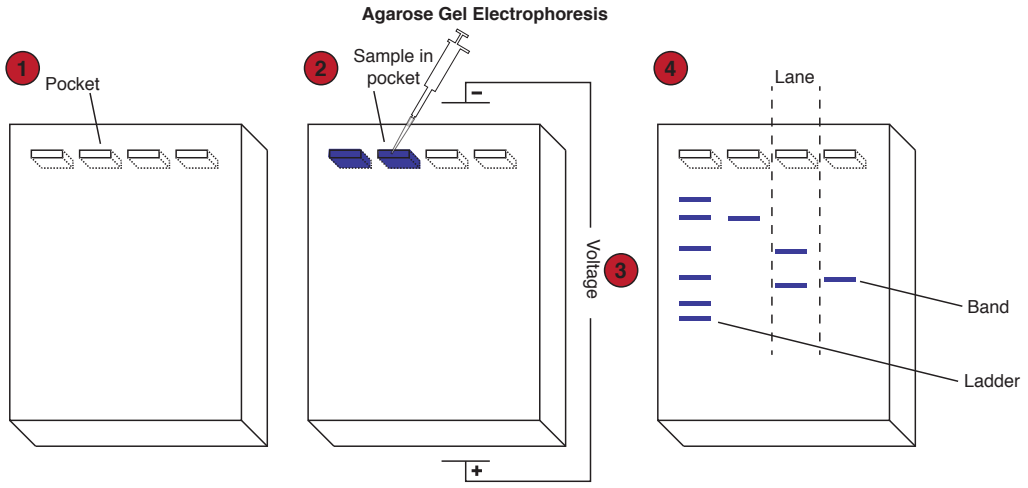

Figure 7: Preparation, loading and running of an agarose gel electrophoresis.

Agarose gel electrophoresis is a method to separate biomolecules by size and charge in a gel. If the biomolecules have a constant mass to charge ratio (as DNA), the molecules can be separated by size in a pattern such that the distance traveled is inversely proportional to the log of its molecular weight.
Agarose forms a physically cross-linked gel (the agarose polysaccharide chains are entangled and form a mesh with pores and channels). If a voltage is applied the negatively charged DNA molecules are forced to migrate towards the anode in the gel. The rate of migration of a DNA molecule through a gel is determined by the several parameters e.g. size of DNA molecule, agarose concentration, DNA conformation, voltage applied, type of agarose and electrophoresis buffer. [15][11]
An agarose gel electrophoresis is performed in few steps (see Figure 7): First the gel is prepared by heating a solution of agarose and pouring it into the corresponding chamber. After 10 min waiting time, the gelation is finished and the chambers can be prepared. Therefore running buffer is filled into the chamber, the samples are pipetted into the gel pockets and an electric field is applied for 30-40 min. Finally the gel can be analyzed under UV light (detailed protocol in the experimental part).

3 Experimental Part

3.1 *E. coli* Culture

The lab course starts in the morning with a 1:100 dilution of an overnight *E. coli* culture:

- Prepare a culture flask with 15 ml of LB Medium and 1:1000 dilution of the antibiotic carbenicilin.
- Inoculate the culture with a volume corresponding to a 1:100 dilution of the overnight culture.
- Cover the flask with aluminium foil and put it back into the 37°C incubator.
- Check the OD_{600} with the nanophotometer in 30 min intervals. Plot the OD_{600} over time afterwards and determine the exponential phase.

3.2 PCR

- Calculate the pipetting volumes (Table 4).
- Thaw a Taq polymerase master mix aliquot on ice.
- Prepare your sample (pipette nfH_2O as the first component and the Taq Master mix as the last component)
- Mix your sample by pipetting carefully up and down without creating air bubbles in your sample.
- Put your sample in the thermal cycler and start the program.

Component	c(stock)	c(sample)	volume
Plasmid		1 ng/µl	
Forward-Primer	5 µM	0.5 µM	
Reverse-Primer	5 µM	0.5 µM	
Taq polym. Master Mix	2x	1x	
nf H_2O	fill to final Volume = 10 µl		

Table 4: Pipette scheme for the PCR. Calculate the pipetting volumes.

3.3 Restriction

Use the provided plasmid to set up a restriction reaction.

Component	c(stock)	c(sample)	volume
Plasmid		20 ng/µl	
Cutsmart	10x	1x	
Restriction enzyme 1			0.4 µl
Restriction enzyme 2			0.4 µl
nf H_2O	fill to final volume = 10 µl		

Table 5: Pipetting scheme for a standard restriction protocol. Calculate the pipetting volumes.

Put the samples in the thermal cycler and start the program: The samples are incubated at 37°C for 30 min and heat inactivated afterwards at 80°C for 20 min.

3.4 Plasmid extraction

We use a commercial kit (Qiaprep Spin Miniprep Kit) to extract plasmids:

- Harvest the bacteria (centrifugation at 6000 rcf, 5min, 4°C) and decant the supernatant.
- Resuspend the pellet in 250 µl buffer P1 which contains RNase to degrade RNA which is released from the cell during the subsequent cell lysis step.
- Transfer the cell suspension into a fresh 1.5 ml tube.
- Add 250 µl buffer P2 to lyse the cells. Mix gently by inverting the tube. Do not allow the lysis reaction to proceed for more than 5 min.
- Stop the lysis reaction using 350 µl buffer N3 (mix gently by inverting the tube). Proteins are degraded and the solution becomes turbid.
- Centrifuge for 10 min at 16000 rcf at room temperature. A compact white pellet will form.
- Apply the supernatants from the previous step to the Qiaprep spin column by decanting and pipetting.
- Centrifuge for 1 min. DNA binds to the silica membrane in presence of a high salt concentration.
- Discard the flow-through.
- Wash the column by adding 500 µl buffer PB and centrifuge for 1 min. Discard the flow-through.
- Wash the column by adding 750 µl PE buffer and centrifuge for 1 min.
- Discard the flow-through and centrifuge again 1 min to remove residual wash buffer.
- Place the column in a clean 1.5 ml tube and add 37 µl nfH_2O. Wait for 3 min. Centrifuge for 1 min.
- Measure DNA concentration.

3.5 Gel Electrophoresis

- Prepare 30 ml 1x TAE buffer (use ddH_2O and 50x TAE stock).
- Add 300 mg Agarose.
- Heat the mixture in the microwave (200 W, 1.5 min).
- Set up a gel chamber with a gel tray and a gel comb.
- Cool the solution and fill it into the gel tray. Wait 10 min.
- Prepare your samples: Add 2 µl of gel loading buffer and 2 µl prestain (Gel Red) to 10 µl of your samples (plasmid, PCR product and restriction product)
- After 10 min put the tray out, rotate it until the sample pockets are at the cathode and fill the chamber with 1x TAE buffer. Remove air bubbles under the tray.
- load the gel with your samples and a reference ladder (1 kb plus DNA Ladder (NEB)).
- The gel runs 45 min at 150 V.
- Analyze the gel in a UV box.
- Clean the chamber and the UV box.

4 Information for your Report

The report should contain the following:

- short introduction
- growth curve, exponential fit, determine doubling time
- determine the length of the samples from the gelelectrophoresis
- answer the question: Physics in Focus 1

Physics in Focus I

Charged biomolecules are surrounded by counter ions in solutions which screen the charge of the molecule (stern layer, diffuse layer). Explain in a few words how agarose gel electrophoresis can work!

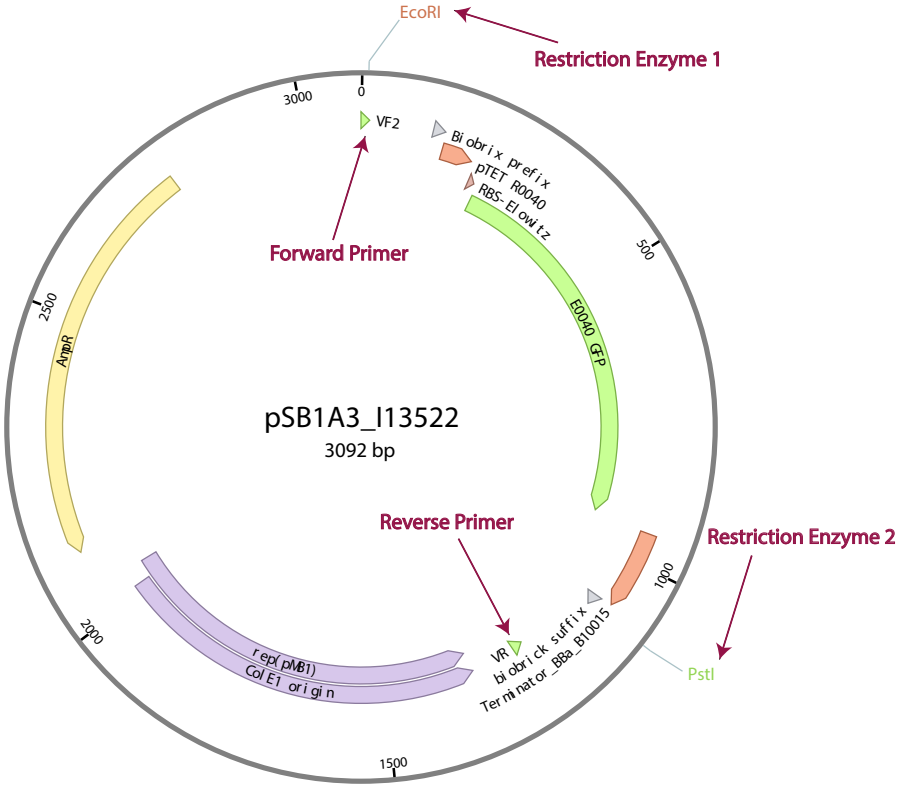

Figure 8: Plasmids map of the used plasmid. The used forward and reverse primers for the PCR are highlighted, as well as the used restriction enzymes for the restriction.

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