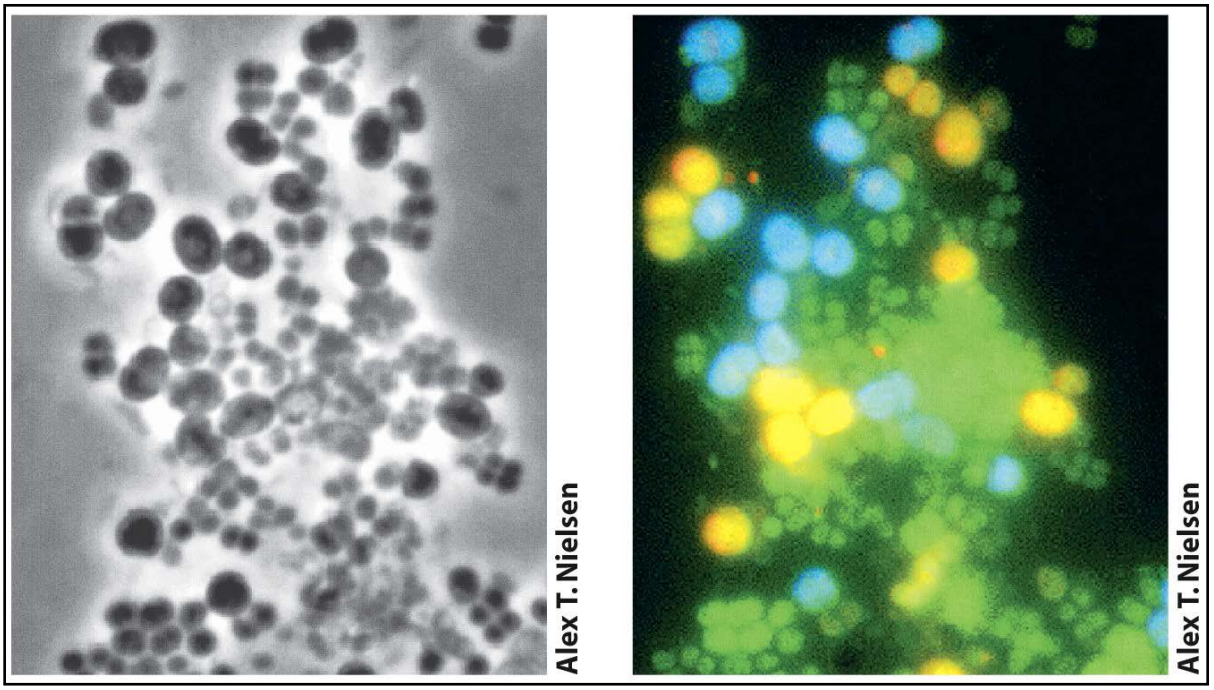
UNIVERSITÄT
DUISBURG
ESSEN

Environmental Microbiology Practical

Masters: Water Science



Laboratory Protocols
(Molecular Biology Part, Prof. B. Siebers)

Name: Matriculation number:

Summer semester 2016

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PRACTICAL COURSE RULES

YOU MUST BE PRESENT EVERY DAY OF THE PRACTICAL COURSE.

- YOU MUST BE PRESENT AT THE **START** OF THE DAY. IF YOU ARE LATE, THIS IS COUNTED AS YOU BEING ABSENT.
- YOU HAVE TO BE PRESENT **ALL** DAY. THE SUPERVISORS WILL TELL YOU WHEN YOU CAN TAKE A BREAK.

THE UNIVERSITY HAS MADE AVAILABLE ALL ITEMS OF EQUIPMENT REQUIRED BY YOU TO COMPLETE THIS PRACTICAL COURSE. THIS EQUIPMENT IS ON LOAN TO YOU FOR THE DURATION OF THE COURSE. ALL ITEMS OF EQUIPMENT MUST BE RETURNED TO COURSE SUPERVISORS IN THE SAME STATE AS IT WAS GIVEN TO YOU. IT IS THE LAW IN GERMANY THAT IF YOU BREAK THE PROPERTY OF ANOTHER PERSON YOU ARE LEGALLY OBLIGED TO PAY FOR THE BROKEN ITEM. ANY BREAKAGES OR DAMAGE MUST BE PAID BY THE RESPONSIBLE STUDENT BEFORE THE COURSE CERTIFICATE CAN BE OBTAINED.

DURING THE PRACTICAL COURSE, ANY STUDENT BEHAVING IN A MANNER WHICH PLACES THEMSELVES AND/OR THE OTHER STUDENTS AND SUPERVISORS IN A SITUATION WHICH ENDANGERS THEIR HEALTH AND SAFETY WILL BE EXPELLED IMMEDIATELY FROM THE COURSE.

IT IS YOUR RESPONSIBILITY TO READ THE PRACTICAL PROTOCOL IN ADVANCE AND KNOW WHICH EXPERIMENTS YOU HAVE TO COMPLETE EACH DAY. ANY GAPS IN YOUR KNOWLEDGE CAN BE FILLED BY YOU GOING TO THE LIBRARY AND READING A BOOK!

THE SUPERVISORS ARE PRESENT TO GIVE YOU THE GUIDANCE YOU REQUIRE. THE RESPONSIBILITY IS ON YOU TO BE PREPARED TO DO THE WORK REQUIRED, TO SHOW INITIATIVE AND TO BE DISCIPLINED!

CLEANING YOUR WORKPLACE: IT IS YOUR RESPONSIBILITY THAT YOUR WORKPLACE IS MAINTAINED IN A CLEAN AND ORDERLY MANNER FOR THE ENTIRE PRACTICAL COURSE. THIS WILL BE CHECKED REGULARLY BY THE COURSE SUPERVISORS.

CLEANING GLASSWARE: IT IS YOUR RESPONSIBILITY TO CLEAN ALL GLASSWARE DURING THE COURSE AND RETURN IT TO THE COURSE SUPERVISORS. IN THE CASE OF AUTOCLAVED GLASSWARE, IF YOU PUT A 1 L FLASK IN TO BE AUTOCLAVED THEN YOU HAVE TO CLEAN A 1 L FLASK AFTER AUTOCLAVING. WHETHER IT IS EXACTLY THE ONE YOU PUT IN IS IRRELEVANT!

PLAGIARISM: WHEN FILLING IN YOUR LABORATORY NOTEBOOKS IT IS IMPORTANT THAT YOU ALWAYS WRITE IN YOUR OWN WORDS. COPYING OFF THE INTERNET OR FROM OTHER STUDENTS WILL RESULT IN ALL STUDENTS CONCERNED HAVING TO REWRITE THEIR WORK. IF THEY PLAGIARISE A SECOND TIME, THEN THEY FAIL.

YOU MUST SIGN TO SAY THAT YOU WILL ABIDE BY THE RULES OF THE COURSE BEFORE YOU CAN TAKE PART IN THE MASTERS PRACTICAL COURSE.

GENERAL LABORATORY SAFETY

- S1 & L1 - LABORATORIES

- When in the laboratory, always wear a laboratory coat and safety glasses (if necessary), as well as appropriate closed shoes.

- To avoid accidental hand-mouth contamination with bacterial cultures, eating, drinking and chewing gum is strictly forbidden. For the same reason, the use of mobile phones and the application of cosmetics is forbidden.

- Do not operate any of the equipment unless you are told to do so by a supervisor.

- Always check the label on all solutions to inform yourself of the level of hazard. We will be working with **ETHIDIUM BROMIDE** which is hazardous material.

- Take care working with samples in the water baths as they will be set at 80°C and 65°C for some of the experiments. Also, take care lifting the molten agarose from the microwave!

- Always operate the fumehoods and sterile bench with the protective cover as closed as possible. No work with genetically modified organisms (gentechnisch veränderte Orgainsm, GVOs) is allowed in the fumehoods.

- Be aware of the possible routes of infection when working with microbiological materials: inhalation, ingestion, inoculation and skin contamination.

- If you spill a culture, inform the supervisor and they will disinfect the contaminated area. The same applies if you contaminate any of the equipment, for example, the centrifuge.

- If you contaminate your skin, inform the supervisor and they will disinfect the skin with the appropriate solution. If biological material gets into eyes, use the eyewash located at the sinks.

- **EVERY TIME** you leave the laboratory, disinfect your hands with Sterillium for 30 seconds. You can then wash off the sterilant with Baktolin soft soap. Both these solutions are available at the sink.

- Your laboratory coat must remain in the laboratory for the duration of the practical course. It will be disinfected by autoclaving at the end of the course and returned to you.

In certain experiments there will be specific waste containers for the hazardous materials, for example, ethidium bromide. They are clearly labelled. Use them!

PRACTICAL COURSE OVERVIEW

AIM

The aim of this practical course is to learn and apply a range of classical molecular biological techniques that are used in **molecular microbial ecology**. **Microbial ecology** deals with the interaction of microorganisms with one another and their biotic and abiotic environment. The use of classical culture-dependent analysis of microbial communities reveals limited information about the microorganisms present in the environment since only a small number of microorganisms (< 1%) can be cultivated. Furtheron, the cultivation and analysis of monocultures provides limited information about the actual role of that microorganism in its natural habitat. This limitation has been overcome by the development of **molecular, culture-independent analysis of microbial communities**. Beside staining methods (viability and quantification, genetic stains (fluorescent *in situ* hybridization, FISH)) the application of molecular biology methods to study microbial diversity overcomes the problem of cultivation by directly analysing the DNA and RNA present in the microbial community **"molecular microbial ecology"**. This molecular approach offers new possibilities to solve the problem of microbial taxonomy by creating a framework with which all microorganisms could be identified, differentiated and organised.

One approach used in molecular microbial ecology is to link specific genes to specific organisms –as the measure of biodiversity- using the polymerase chain reaction (PCR). The identification of a gene specific for an organism in an environmental sample implies that this organism is present. The respective genes/organisms can be identified by methods like denaturing gradient gel electrophoresis (DGGE) or molecular cloning (clone library), DNA sequencing and analysis.

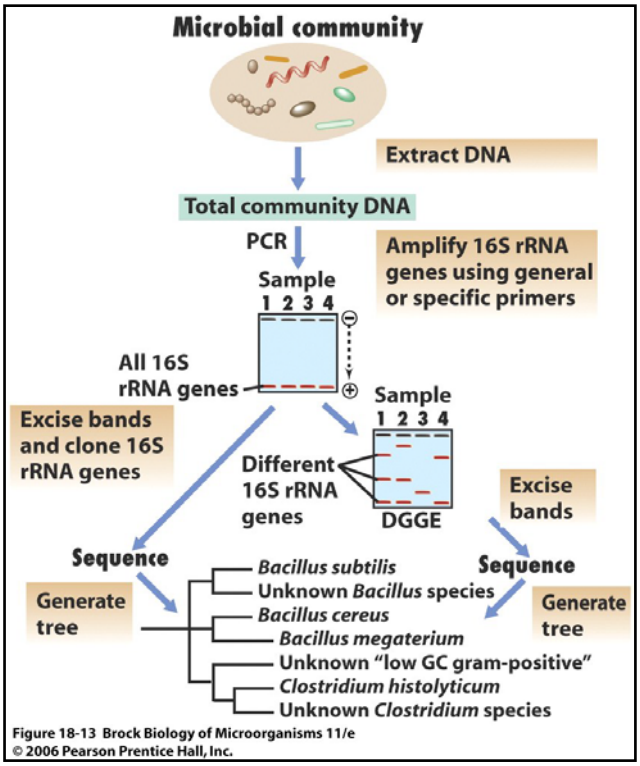


Fig. 1: Linking specific genes to specific organisms using PCR.

The most consistent, reliable and accurate approach to address microbial diversity was to base the phylogeny on the sequence of a single molecule. Ribosomal RNA (rRNA) was shown to be a good marker for phylogenetic studies of microorganisms for the following reasons:

- The ribosome and its rRNA are found in all organisms on Earth (translation machinery).
- rRNA is found in high copy number in active cells (cells producing proteins).
- rRNA is functionally conserved (loss of activity leads to loss of ability to produce proteins = cell death).
- 16S & 18S rRNA (small subunit) are sufficiently long and contain enough variation to provide a large amount of evolutionary information.
- rRNA based phylogenetic framework provides a means to comprehensively define the microbial composition of any given niche.

Noteworthy the sequencing and comparison of DNA encoding rRNA (16S rRNA prokaryotes & 18S rRNA eukaryotes) by Carl Woese and co-workers lead to our current understanding or the three domains of life: Eukarya, Bacteria and Archaea.

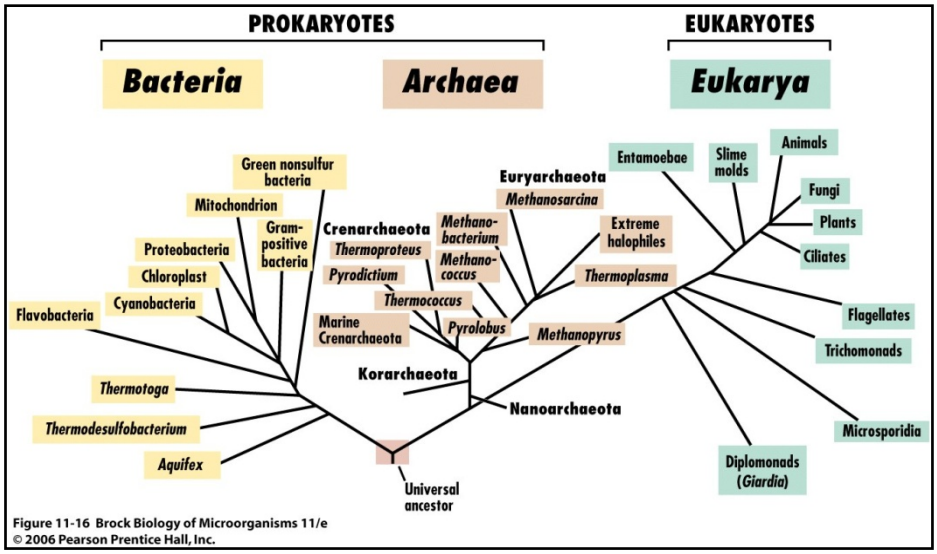


Fig. 2: The tree of life „Carl Woese“. Universal phylogenetic tree from comparative ribosomal RNA analysis.

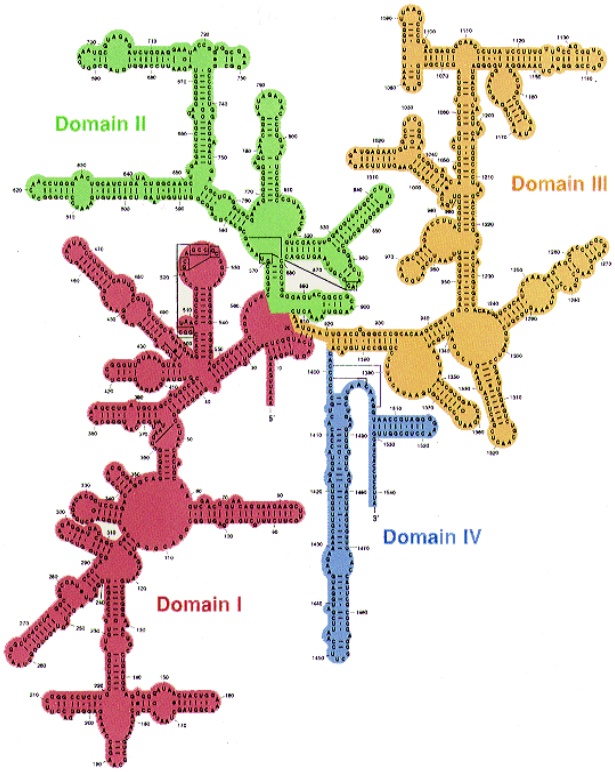


Fig. 3: 16S rRNA of *Escherichia coli*

Molecular Microbiology part:

In the course you will learn basic molecular biology techniques including genomic DNA isolation and PCR amplification of the 16S rRNA gene and ligation into a prepared vector.

You will carry out a transformation experiment into self-prepared competent *E. coli* cells and generate *E. coli* clones containing your recombinant plasmid. You will then re-isolate the plasmids from the positive clones and carry out colony PCR as well as restriction analysis to confirm the size of the cloned inserts.

ASSESSMENT – YOU ARE ASSESSED INDIVIDUALLY

Protocols (one per group) should be filled out as follows:

1. On the first page fill in your names, matriculation numbers and group number.
2. At the start of each page include the date and name of experiment.
3. Include all results (calculations) and observations.
4. Write the protocol for the whole experiment, not for each day.
5. You must write **legibly** and **in your own words – no copying!**

The protocols have to be completed in English.

How and why to write a protocol

In a protocol the already noted results from the labjournal are processed and displayed for the scientific educated readership. Many details mentioned in the labjournal are excluded whereas background information and conclusions are added. The use of literature and the according correct quotation is preferable.

Introduction

- Describes the intention of the experiment and shows its scientific background.
- Try to focus on a clear line and avoid skipping between topics.

Materials and Methods

- Address the scientific educated reader and avoid irrelevant details.
- Present the essential information in a way that it is possible to repeat the experiment only with your given description.

Results

- Is the part where you present your own results.
- Describe them in continual or chronological order.
- Calculated stats have to be explained and results are displayed best in tables or figures.
- Keep in mind that you have to label tables and figures correctly so that you can refer to them in the text (e.g. Fig.3.01).

Discussion

- The discussion of an experiment is an evaluation of the gained results, which are presented in scientific context to be able to draw further conclusions.
- You should present your results accordingly and if possible compare them with references from the literature and other groups of the practical course.
- The results have to be analyzed (do the results match the expectations?) and conclusions are to be made (changes in the experiments, mistakes, etc.).
- Concluding take the ideas from the introduction and carry them forward to finish the experiment.

General comments

Write in whole sentences and regard to use the right tense! When you describe general content present is used whereas for the presentation of results past tense is the better choice.

TIMETABLE 2016

ACCOMPANYING SEMINAR: TUESDAY 16^{00}- 18^{00} ROOM S05 T02 B02

FRIDAY 3RD JUNE	9.00 – 10.00	ROOM T03 R02 D81, PREL. DISCUSSION
	10.00 – 18.00	ROOM S05 T02 A32, PRACTICAL

(Vector DNA Isolation and Restriction, Genomic DNA Isolation)

FRIDAY 10TH JUNE	9.00 – 10.00	ROOM T03 R02 D81, PREL. DISCUSSION
	10.00 – 18.00	ROOM S05 T02 A32, PRACTICAL

(PCR, Gel Electrophoresis & Ligation)

FRIDAY 17TH JUNE	9.00 – 10.00	ROOM T03 R02 D81, PREL. DISCUSSION
	10.00 – 18.00	ROOM S05 T02 A32, PRACTICAL

(Preparation of Competent Cells & Transformation)

FRIDAY 24TH JUNE	9.00 – 10.00	ROOM T03 R02 D81, PREL. DISCUSSION
	10.00 – 18.00	ROOM S05 T02 A32, PRACTICAL

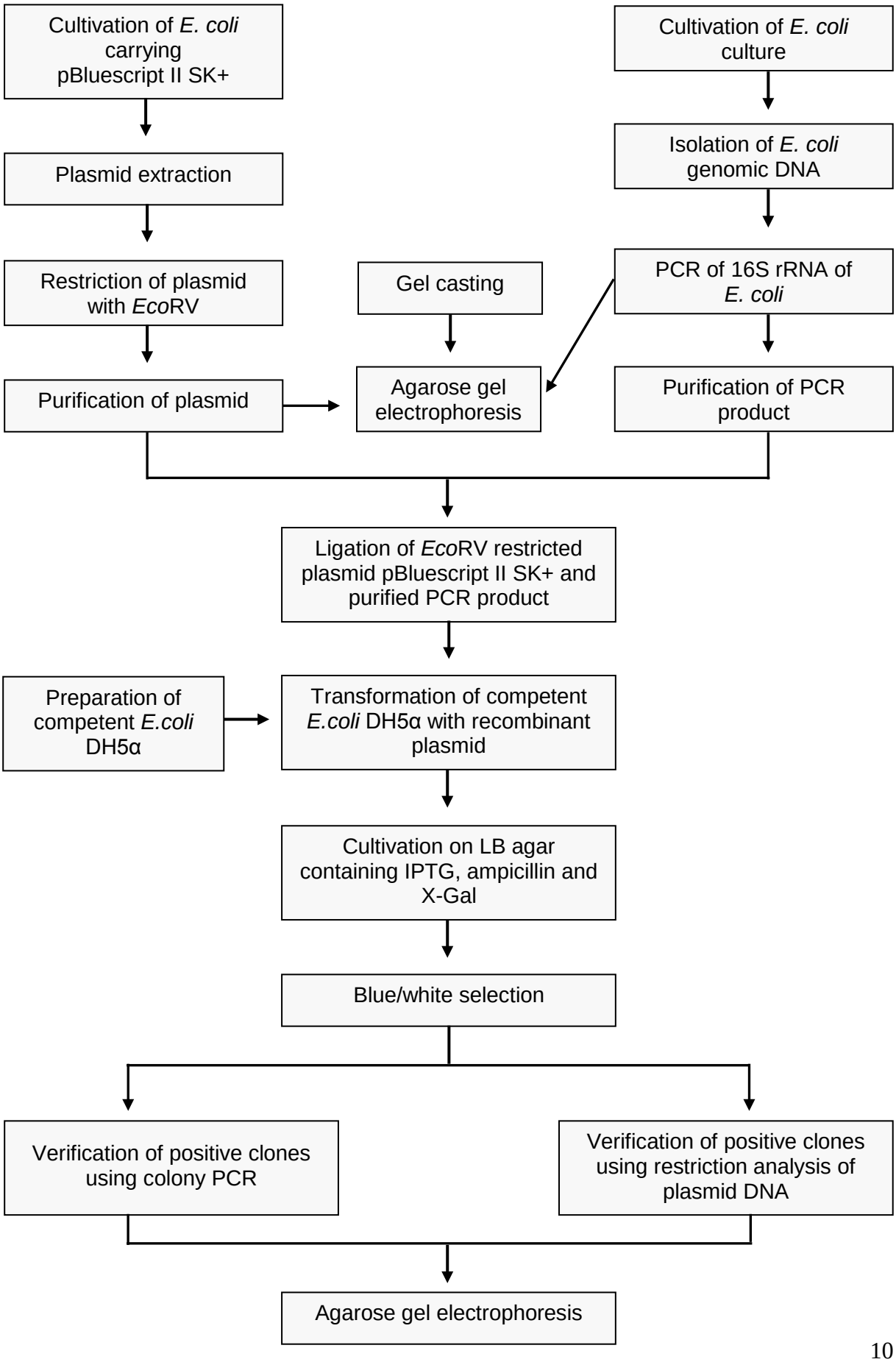
(Pick White Colonies, Check by Colony PCR and Gel Electrophoresis of Products, Inoculate Cultures for Plasmid Preparation)

FRIDAY 1TH JULY	9.00 – 10.00	ROOM T03 R02 D81, PREL. DISCUSSION
	10.00 – 18.00	ROOM S05 T02 A32, PRACTICAL

(Plasmid Isolation & Restriction, Gel Electrophoresis of Products)

[FRIDAY 8TH JULY	9.00 – 10.00	ROOM T03 R02 D81, PREL. DISCUSSION
	10.00 – 18.00	ROOM S05 T02 A32, PRACTICAL**]**

- OVERVIEW -


Cultivation of E. coli carrying pBluescript II SK+
Plasmid extraction
Restriction of plasmid with EcoRV
Purification of plasmid
Gel casting
Agarose gel electrophoresis
Cultivation of E. coli culture
Isolation of E. coli genomic DNA
PCR of 16S rRNA of E. coli
Purification of PCR product
Ligation of EcoRV restricted plasmid pBluescript II SK+ and purified PCR product
Preparation of competent E.coli DH5α
Transformation of competent E.coli DH5α with recombinant plasmid
Cultivation on LB agar containing IPTG, ampicillin and X-Gal
Blue/white selection
Verification of positive clones using colony PCR
Verification of positive clones using restriction analysis of plasmid DNA
Agarose gel electrophoresis

MOLECULAR BIOLOGY TECHNIQUES

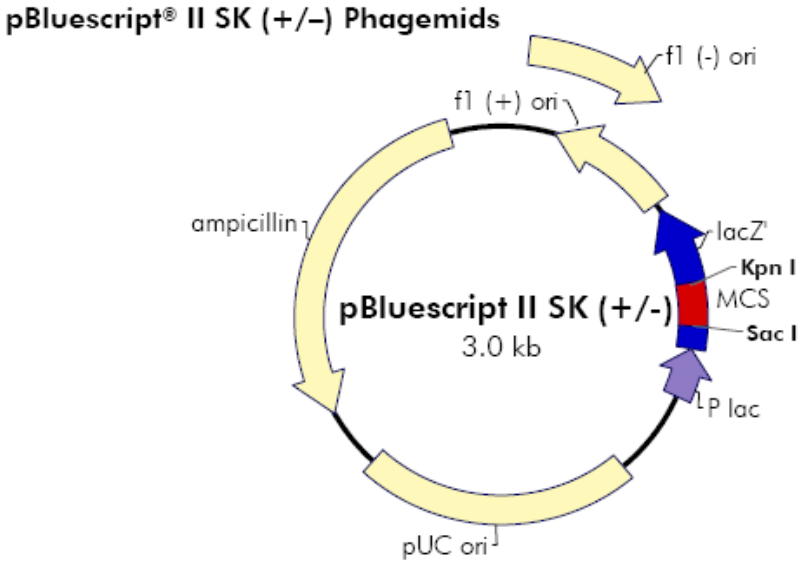
AIMS

The aim of this experiment is to gain experience of basic molecular biology techniques. You will learn how to isolate DNA from a pure culture and check the quality of the DNA by gel electrophoresis. Next you will PCR amplify the gene encoding 16S rRNA by the use of domain specific primers and clone it into a vector. The steps involved in cloning are listed below.

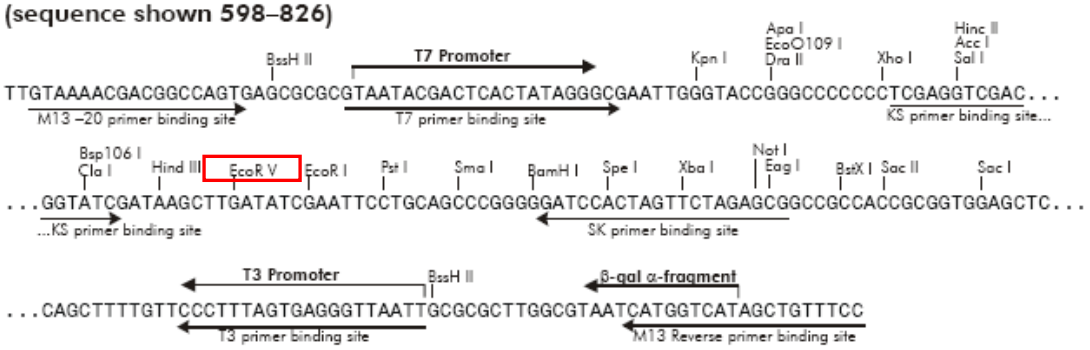
1. Isolation and purification of vector DNA.
2. Restriction and purification of vector DNA.
3. Isolation of genomic DNA from a Pure *E. coli* Culture.
4. PCR: Amplification of 16S rRNA gene from *E.coli* by domain specific primers.
5. Agarose gel electrophoresis.
6. Ligation of the PCR-DNA product into the vector.
7. Preparation of chemical competent *E. coli* cells.
8. Transformation.
9. Verification of positive clones by colony PCR.
10. Verification of positive clones by restriction analysis of the plasmid DNA.

VECTOR PREPARATION (BACKGROUND)

The vector you will use in this practical is pBluescript from Stratagene. This vector is a high copy number vector that is useful for cloning (blue white selection) and sequencing (provides great plasmid amounts). The vector is maintained in an *E. coli* host culture. In order to have sufficient quantity of vector DNA for the restriction and ligation you need to grow the *E. coli* overnight and then isolate the vector from the bacterial host.



pBluescript II SK (+/–) Multiple Cloning Site Region (sequence shown 598–826)



Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication [pBluescript SK (+) only]	135–441
f1 (–) origin of ss-DNA replication [pBluescript SK (–) only]	21–327
β-galactosidase α-fragment coding sequence (lacZ')	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (*bla*) ORF	1976–2833

FIGURE 1 The pBluescript® II SK (+/–) phagemid vectors. The complete sequence and list of restriction sites are available at *www.stratagene.com*. Genbank® #X52328 [SK(+)] and #X52330 [SK(–)].

Fig. 4: Vector pBluescript II SK+ (Stratagene).

The multiple cloning site (MCS) contains an *Eco*RV restriction site, which can be used to open the vector (linearize) providing a site for blunt ended ligation of the PCR product. The MCS is localised in the lacZ' gene, encoding β-galactosidase (see lactose operon for further information). If the cloning is successful the gene interrupts

the open reading frame encoding β-galactosidase (resulting in an inactive enzyme) and the cells are no longer able to degrade 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The transcription of the gene under control of the lac operator/promoter is induced by isopropyl-β-D-thiogalactopyranoside (IPTG), an artificial inducer of the lac operon that cannot be used as substrate. The ability to degrade X-Gal can be easily observed by the blue colour of the colony. Therefore, blue colonies are negative — they do not harbour an insert in the MCS/β-galactosidase — and white colonies are positive — they harbour an insert in the MCS/β-galactosidase. There is also the *Eco*RI restriction site, which can be used to linearize the recombinant vector to check the size of the plasmid and insert (on the last day).

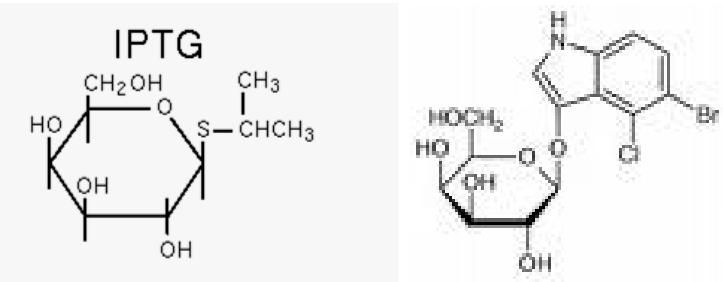


Fig. 5: Structure of IPTG and X-Gal.

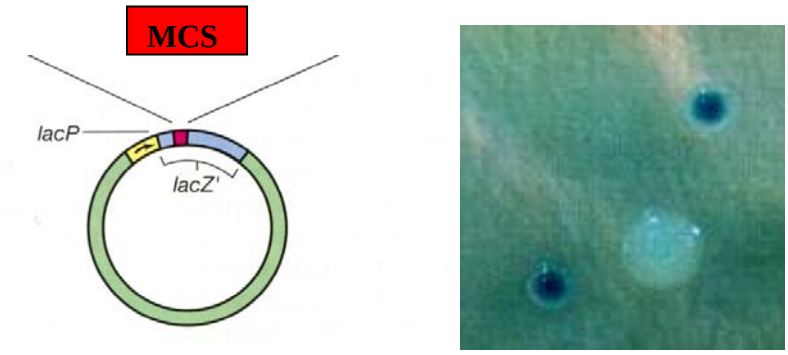


Fig. 6: Blue white selection via pBluescript II SK+.

WEEK 1

VECTOR DNA ISOLATION

VECTOR CULTURE PREPARATION IS DONE FOR YOU

Groups will be provided with a 5 ml *E. coli* overnight culture for vector DNA isolation.

For the preparation of the *E. coli* overnight culture you need a test-tube containing 5 ml LB medium + 100 µg/ml ampicillin. The antibiotic is added AFTER autoclaving and immediately prior to use.

- LB liquid medium — 1 test-tube per group of 2 students

  5 g NaCl
  10 g Tryptone
  5 g yeast extract

Make up to 1 L with bidestilled water; adjust to pH 7 and aliquot into test tubes (5 ml in each).

Autoclave & store at 4°C

The test-tube containing 5 ml LB medium + 100 µg/ml ampicillin (add **50 µl** from a 100x stock solution of ampicillin (10mg/ml)) is inoculated with the *E. coli* host (this is provided either as a glycerol stock where you need to take 20 µl for the inoculation or as an *E. coli* culture on an agar plate where you need to take one colony for the inoculation). Incubate at 37°C overnight with shaking. Label your test-tubes clearly so that you can find your culture the following day!

VECTOR DNA ISOLATION (PROTOCOL)

To isolate the plasmid from the *E. coli* host cell you will use the isolation kit from Fermentas (isolation based on the Alkaline Lysis method). Follow the protocol for microcentrifuges. Prepare one plasmid sample per group.

►centrifuge 1,5ml cell suspension (2min, 13.000rpm) and remove the supernatant
fill the reaction tube again with 1,5ml and repeat centrifugation and removement of the supernatant

1. Resuspend cells, lyse and neutralize
   1.1. Add to the pelleted cells:
      1.1.1. 250 µl of resuspension solution and resuspend by pipetting
      1.1.2. 250 µl of lysis solution and invert the tube 4-6 times

1.1.3. 350 µl of neutralization solution and invert the tube 4-6 times

1.2. Centrifuge 5 min (11000 rpm)

2. Bind DNA
   2.1. Load the supernatant to GeneJET™ spin column
   2.2. Centrifuge 1 min (11000 rpm)
3. Wash the column
   3.1. Add 500 µl of Wash solution and centrifuge (11000 rpm) for 1 min
   3.2. Discard the flow through
   } x 2 times
   3.3. Centrifuge the empty column for 1 min (11000 rpm)
   3.4. Transfer column into a clean 1.5 ml eppendorf tub
4. Elute purified DNA
   4.1. Add 30 µl of DNase and RNase-free water to the column and incubate 2 min.
   4.2. Centrifuge 2 min (11000rpm)
   4.3. Collect the flow through

Fig. 7: GeneJET™ Plasmid Miniprep Kit (Fermentas).

RESTRICTION AND PURIFICATION OF VECTOR DNA

VECTOR RESTRICTION (BACKGROUND)

Now, to be able to insert the PCR amplified 16S rRNA gene into the isolated vector, you have to digest the vector with a restriction enzyme, which has a restriction site within the multiple cloning site (MCS). In this practical we will use a small amount of vector DNA and an enzyme called ***Eco*RV**. This enzyme is a blunt-end cutter (no "sticky ends") and allows for blunt-end ligation.

***Eco*RV restriction site:** **5' GAT↓ATC 3'**
3' CTA↑TAG 5'

VECTOR RESTRICTION (PROTOCOL)

To digest the vector DNA you have to pipette the following into a clean 1.5 ml Eppendorf tube:

5 µl vector DNA
2 µl restriction buffer (10X concentration)
12 µl DNase- RNase free water
1 µl *Eco*RV fast digest (add at last component)

- Mix the sample carefully and centrifuge for 10 s.
- Incubate for 15 min at 37°C.
- Add 100 µl ice cold isopropanol p.A. and incubate on ice for 10 min.
- Centrifuge at 13,000 rpm for 20 minutes at 4°C. **Make sure to remember the orientation of the tube in the rotor so that you can locate your DNA pellet!**
- Remove the isopropanol carefully without touching the reaction tube with the tip
- Add 500 µl ice cold 70 % ethanol p.A. and resuspend pellet
- Centrifuge at 13.000 rpm for 10 minutes at 4°C and afterwards remove the ethanol
- Dry pellet at 70°C for approximately 10 min.
- Add 20 µl DNase and RNase free H_2O and (incubate at 70°C for 10 min).
- The restricted vector has to be stored at -20°C.

ISOLATION OF GENOMIC DNA FROM A PURE *E. COLI* CULTURE

ISOLATION OF GENOMIC DNA (BACKGROUND)

The crucial step in any PCR based analysis is the first step: DNA isolation. There are different techniques for DNA isolation depending on the source of the bacteria or environment. You will use the GeneJET™ Genomic DNA Purification Kit (Fermentas) for obtaining good quality DNA from a pure culture.

DNA isolation involves 3 basic steps:

(i) Breaking the cells,

(ii) Separating the DNA from the rest of the cellular components, and finally

(iii) Cleaning and concentrating the DNA.

ISOLATION OF GENOMIC DNA (PROTOCOL)

- An *E. coli DH5α* 5ml LB-medium overnight culture is provided for this experiment.
- Each group prepares **1 Eppendorf tube** of culture for DNA isolation

D. Gram-Negative Bacteria Genomic DNA Purification Protocol

Step	Procedure
1	Harvest up to 2x10⁹ bacterial cells in a 1.5 or 2 ml microcentrifuge tube by centrifugation for 10 min at 5000 x g. Discard the supernatant.
2	Resuspend the pellet in 180 µl of Digestion Solution. Add 20 µl of Proteinase K Solution and mix thoroughly by vortexing or pipetting to obtain a uniform suspension.
3	Incubate the sample at 56°C while vortexing occasionally or use a shaking water bath, rocking platform or thermomixer until the cells are completely lysed (~30 min).
4	Add 20 µl of RNase A Solution, mix by vortexing and incubate the mixture for 10 min at room temperature. -
5	Add 200 µl of Lysis Solution to the sample. Mix thoroughly by vortexing for about 15 s until a homogeneous mixture is obtained.
6	Add 400 µl of 50% ethanol and mix by pipetting or vortexing. -
7	Transfer the prepared lysate to a GeneJET™ Genomic DNA Purification Column inserted in a collection tube. Centrifuge the column for 1 min at 6000 x g. Discard the collection tube containing the flow-through solution. Place the GeneJET™ Genomic DNA Purification Column into a new 2 ml collection tube (included).
8	Add 500 µl of Wash Buffer I (with ethanol added). Centrifuge for 1 min at 8000 x g. Discard the flow-through and place the purification column back into the collection tube.
9	Add 500 µl of Wash Buffer II (with ethanol added) to the GeneJET™ Genomic DNA Purification Column. Centrifuge for 3 min at maximum speed (≥12000 x g). *Optional*. If residual solution is seen in the purification column, empty the collection tube and re-spin the column for 1 min. at maximum speed. Discard the collection tube containing the flow-through solution and transfer the GeneJET™ Genomic DNA Purification Column to a sterile 1.5 ml microcentrifuge tube (not included).
10	Add 200 µl of Elution Buffer to the center of the GeneJET™ Genomic DNA Purification Column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 1 min at 8000 x g. **Note** • - For maximum DNA yield, repeat the elution step with additional 200 µl of Elution Buffer. • - If more concentrated DNA is required or DNA is isolated from a small amount of starting material the volume of the Elution Buffer added to the column can be reduced to 50-100 µl. Please be aware that smaller volumes of Elution Buffer will result in smaller final quantity of eluted DNA.
11	Discard the purification column. Use the purified DNA immediately in downstream applications or store at -20°C.

Additional : Step 1 : do it 1 times with 2,0 ml cell suspension

Step 10 : take only 50 µl DNase-and RNase-free water

The quality of the DNA will be examined by gel electrophoresis on an agarose gel. This will be done **next FRIDAY.**

WEEK 2

PCR: AMPLIFICATION OF 16S rRNA GENE FROM *E. COLI* DNA BY DOMAIN SPECIFIC PRIMERS

PCR (BACKGROUND)

Polymerase Chain Reaction (PCR) is a technique for amplifying one specific section of DNA from a mixed template.

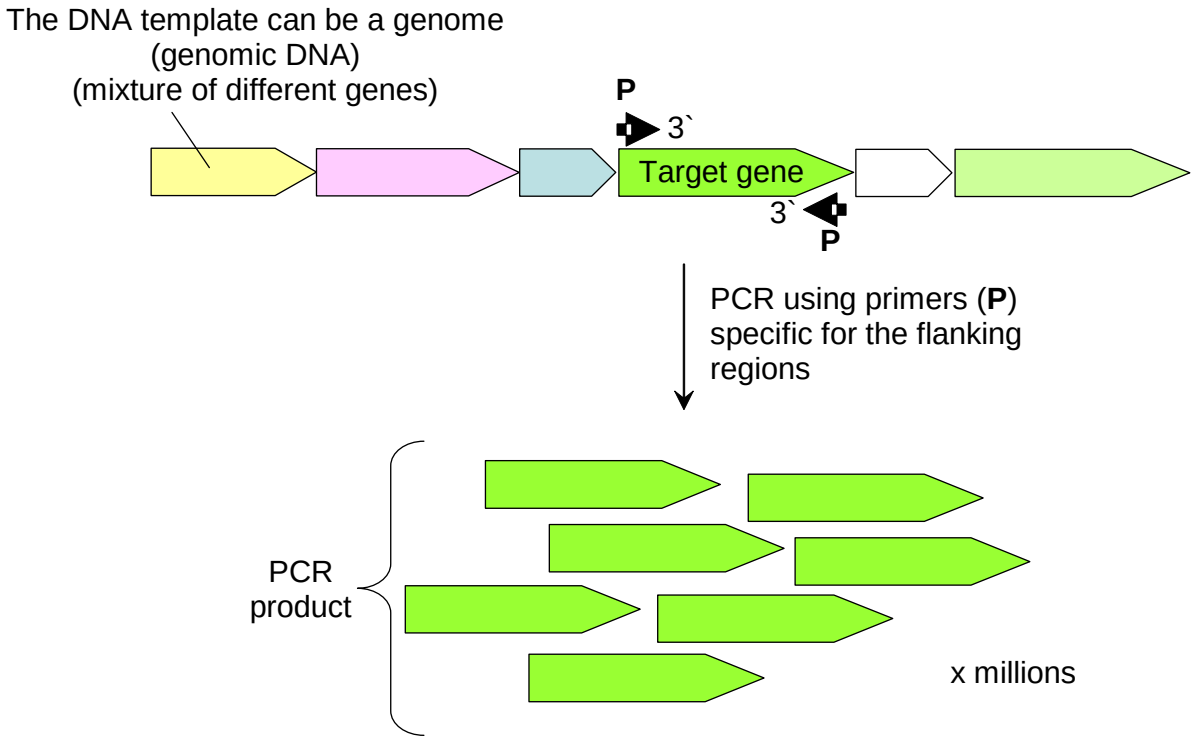


Fig. 8: PCR amplification of a gene from genomic DNA.

PCR is a method which includes 3 different temperature dependent steps:

(i) Denaturing

(ii) Annealing

(iii) Extension

In the denaturing step the double stranded DNA template is separated into 2 single strands. This takes place at temperatures around 98°C. In the annealing step the primers, oligonucleotides, which complement the flanking regions of the target gene, anneal to the flanking regions of the target gene. The temperature of this step is mainly dependent on the GC content of the primers. In the final step, extension, the DNA polymerase enzyme adds nucleotides to the 3' end of the primers (see Fig. 9). Like this a DNA strand complementary to the existing target strand is produced. The DNA polymerase used in this experiment is **Phusion DNA polymerase**, which generates a PCR product with **blunt ends.** This is essential for the cloning into the restricted pBluescript vector.

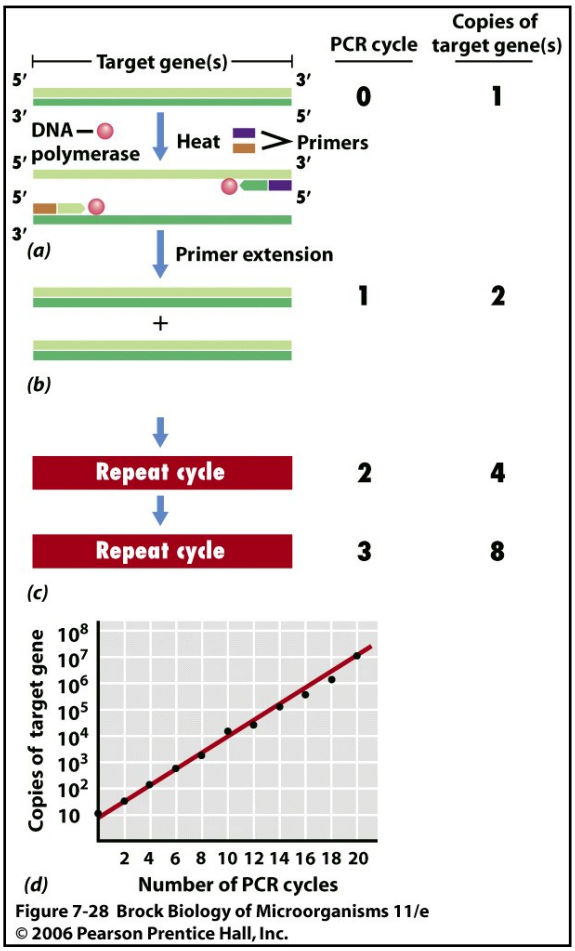


Fig. 9: The polymerase chain reaction (PCR).

The target DNA is double stranded with the strands being antiparallel, meaning they are oriented in opposite directions. One strand shows a 5' ("five prime")→ 3' ("three prime") direction, the other strand a 3' → 5' direction. The "prime" denotations refer to the carbon atom in the deoxyribose to which the next phosphate in the strand attaches. For each PCR two primers are designed. One primer is complementary to one strand of the target DNA at one end of the gene, and the other primer is complementary to the other strand of DNA at the other end of the gene (see Fig.7). The primers at both ends of the genes are orientated in such a way that the 3' end extends towards the other end of the gene. This is necessary as the DNA polymerase can only add nucleotides to a pre-existing 3' end resulting in the production of a new double stranded DNA molecule, which is complementary to the single strand of the target gene. PCR involves about 30 cycles of denaturing, annealing and extension, which results in a PCR product containing millions of molecules of the target gene.

As PCR is a highly sensitive technique, which will amplify DNA from any DNA present it is important to include a **negative control** in the experiment. This can be for example a reaction mix, which includes all the different constituents except the DNA template. If this results in a PCR product it indicates that one of the PCR constituents is contaminated with DNA, or the plastic PCR tube was contaminated with DNA. It also means that the PCR products from the reactions containing the genomic DNA as template most likely have the target gene from the genomic DNA, the required product, and/or genes from the contaminating DNA. Therefore, the product cannot be used for the further experiments. Only when the negative control contains no product it is possible to conclude that the PCR product in the reactions containing genomic DNA contain only the target gene from that genomic DNA.

In this class we will use two different primer sets. One primer set specific for Bacteria and the other for Archaea, which target the phylogenetic marker molecule 16S rRNA. **Only the Bacteria specific primers should result in a PCR product using *E. coli* DNA** as template (size 1,500 bp), whereas the Archaea specific primes should reveal no product. In addition a negative control without DNA addition will be performed (only with bacterial primers).

PCR (PROTOCOL)

For the PCR **three** different reaction mixtures are prepared:

1. PCR of 16S rRNA: 1 µl DNA template and bacterial primer set
2. Negative control: 1 µl DNA template and archaeal primer set
3. Negative control: 1 µl H_2O instead of DNA template and bacterial primer set

Calculate the volumes of PCR constituents required and fill them into the following table. Start with the H_2O. Add all the other constituents and mix thoroughly. The DNA polymerase is in a glycerol based liquid and is quite viscous. **Add it to the mixture last.** Be careful when pipetting the according primer sets into the 3 **pre-labelled tubes**! For the negative control you use 1 µl H_2O instead of 1 µl DNA template and the bacterial primer set! You have a final reaction volume of 50 µl.

When pipetting small volumes always observe the solution in the pipette tip and make sure that you add it to the solution in the tube.

Table 1: PCR-Reactionmix

Constituent	Concentration of stock soln.	Final conc.	Volume in 50 µl
PCR buffer	5 X	1 X	
§ dNTPs mix	2 mM	200 µM	
§ Forward-primer (f, A/B)	10 µM	0.4 µM	
§ Reverse-primer (rev, A/B)	10 µM	0.4 µM	
# Phusion DNA polymerase	2.5 U/µl	1 U	
DNA template			1 µl
H_2O (PCR clean)			add to 50 µl
		Total volume	50 µl

§ defrost and keep on ice in an ice-bucket.

keep DNA polymerase at -20°C in the freezer until needed. Keep it on ice and return it to the freezer immediately.

Primer sequences

Bacteria specific primers; (Hicks et al. 1992, Kane et al. 1993)

27f-primer: AGA GTT TGA TCC TGG CTC AG

1492r-primer: GGC TAC CTT GTT ACG ACT T

Archaea specific primers; (Banning et al. 2005)

109f-primer: ACA GCT CAG TAA CAC GT

915r-primer: GTG CTC CCC CGC CAA TTC CT

Table 2: PCR programme parameters

Cycle	1	2 - 30	31
Denaturation	98°C, 5 min	98°C, 30 sec	
Annealing		55°C, 30 sec	
Extension		72°C, 45 sec	72°C, 5 min

The PCR programme runs for approximately 2 hours.

The uncleaned PCR products are checked by gel electrophoresis.

PURIFICATION OF PCR PRODUCT

Before the PCR product can be ligated into the prepared vector it must be cleaned to remove the DNA polymerase enzyme, the primers and the remaining nucleotides. To clean a PCR product you can either precipitate it, clean it and resuspend it in 25 µl H_2O or electrophorate the PCR product and extract the product band from the agarose gel. In this practical course you will use the Promega SV Gel and PCR Clean-Up System.

Before you clean up the PCR product (DNA with bacterial primer set / one sample per group) a certain volume of each PCR reaction is applied on an agarose gel (see page 26). While the gel is running and stained by the supervisors you can clean the remaining PCR mixture (not the controls).

PURIFICATION OF PCR PRODUCT (PROTOCOL)

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

Binding of DNA

1. Insert SV Minicolumn into Collection Tube.
2. Transfer prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 13,000 rpm for 1 minute. Discard flow-through and reinsert Minicolumn into Collection Tube.

Washing

4. Add 700 µl Membrane Wash Solution (ethanol p.A.added). Centrifuge at 13,000 rpm for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500 µl Membrane Wash Solution. Centrifuge at 13,000 rpm for 5 minutes.
6. Empty the collection Tube and centrifuge the column assembly for 1 minute to allow evaporation of any residual ethanol.

Elution

7. Carefully transfer Minicolumn to a clean 1.5 ml microcentrifuge tube.
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 2 minutes. Centrifuge at 13,000 rpm for 1 minute.
9. Discard Minicolumn and store DNA at 4°C or –20°C.

AGAROSE GEL ELECTROPHORESIS: VISUALISATION OF ISOLATED DNA, PCR PRODUCTS AND RESTRICTED AND UNRESTRICTED VECTOR DNA

AGAROSE GEL ELECTROPHORESIS (BACKGROUND)

Agarose gel electrophoresis is a procedure for the analysis of DNA fragments. DNA molecules migrate in an electrical field dependent on their negative charge (negative phosphate groups in the desoxyribose-phophate backbone of DNA), size and shape (conformation) to the anode (+). **Agarose**, made of glycosidic linked D-galactose and 3,6-anhydro-galactose, forms a porous gel (matrix) and serves for the separation of the negatively charged DNA. The gel meshwork hinders the progress of DNA and small or more compact molecules migrate faster than large molecules. The migration velocity depends on different factors i.e. molecule size, conformation of DNA, agarose concentration (Tab.3) and applied voltage. Linear, double stranded DNA migrates in the agarose gel inversely proportional to the common logarithm of its molecular weight.

Using standard samples with fragments of known size (marker) one can determine the size of unknown samples. The band pattern of the marker used in this practical is shown in figure 10. The DNA can be stained with a compound that binds to DNA, such as **ethidium bromide**, which intercalates into DNA (carcinogen !!) and shows a strong orange fluorescence under UV light (Ex. max 532 nm, Em. max 585 nm). Up to 1 ng DNA can be detected; a nicely visible band contains 10-100 ng DNA.

Table 3: Which agarose concentration is useful for the separation of DNA fragments of different size?

Agarose concentration (%)	Size of DNA fragments (kb)
0,5	30 - 1,0
0,7	12 - 0,8
1,0	10 - 0,5
1,2	7 - 0,4
1,5	3 - 0,2

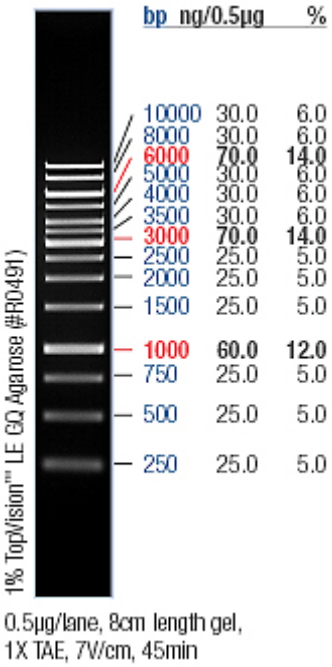


Fig. 10: Marker: GeneRuler™ 1 kb DNA Ladder (Fermentas); 14 fragments ranging from 250 – 10000 bp

AGAROSE GEL ELECTROPHORESIS (PROTOCOL)

Each group analyses the following samples:

1. DNA isolation
2. uncleaned PCR product (DNA template) bacterial primer set
3. uncleaned PCR product (DNA template) archaeal primer set
4. uncleaned PCR product (H_2O template, control)
5. unrestricted vector
6. restricted and cleaned vector
7. DNA size ladder (GeneRuler^{TM} 1 kb DNA Ladder, Fermentas)

For the gel electrophoresis you have to mix:

1. DNA isolation products

8 µl DNA

1 µl gel loading buffer

2.-4. PCR products

5 µl PCR product

1 µl gel loading buffer

5. Unrestricted vector

5 µl vector DNA

1 µl gel loading buffer

6. Restricted vector

10 µl vector DNA

1 µl gel loading buffer

Pipette the whole mix into the well in the gel.

7. DNA ladder

Provided by the supervisors (5-7 µl)

After electrophoresis the gel is stained with ethidium bromide, which binds the DNA and fluoresces under UV light. The gel is then exposed to UV light, so that the DNA becomes visible and can be photographed. A comparison of the PCR product or the restricted vector band with the DNA ladder bands will indicate the size. The DNA ladder is made up of linear pieces of DNA, which have a specific size. This can only be used to determine the size of linear DNA molecules (the PCR product and the restricted vector). You need to consider the physical state of the DNA you are analysing by electrophoresis as the unrestricted vector DNA is circular and can be present in different supercoiled forms.

Gel casting

Make sure the gel casting chamber and well combs are clean and dry. Place one well comb so that it is about 1 cm from the top of the gel, and place the second well comb half way down the casting chamber.

Add 100 ml TAE (40 mM Tris-HCl (pH 8.0), 20 mM acetic acid, 1 mM EDTA) and a teflon coated stirring bar to a 250 ml Erlenmeyer flask.

1. Add 1.0 g agarose (1.0 %) and stir.
2. Add a loose cap to the Erlenmeyer flask.
3. Boil in the microwave and stir until the agarose is fully dissolved.

   (! WEAR SAFETY GLASSES !)
4. Leave the solution stirring slowly (avoid bubbles) until it reaches 65°C.

Pour the agarose solution into the casting chamber and let it cool to room temperature for 30 min, or until solid.

Steps 1 to 4 are prepared for you. It is your job to pour the gels carefully, avoiding bubbles.

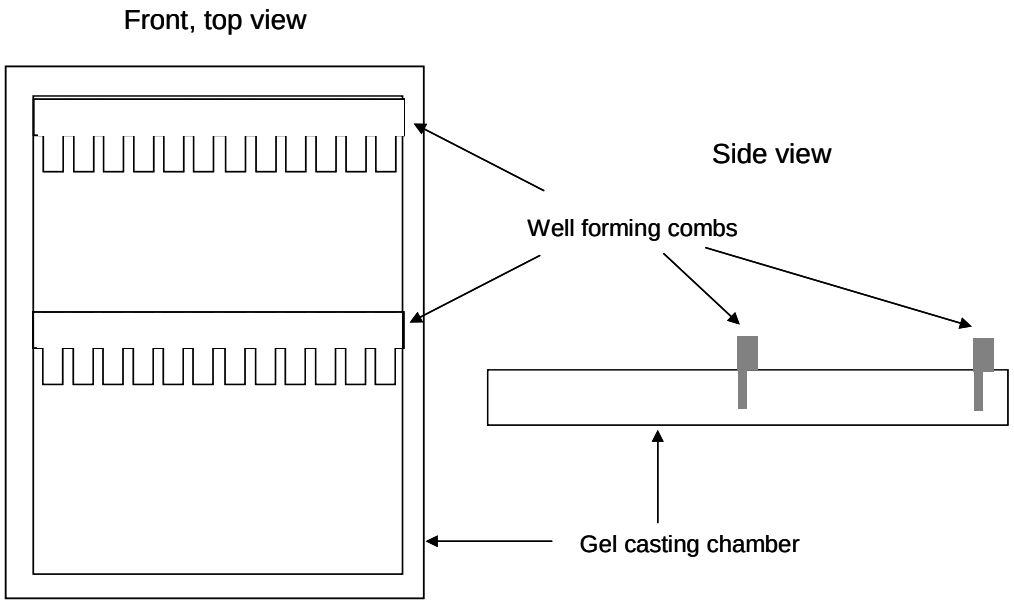


Fig. 11: The location of the well combs in the gel casting chamber.

Gel loading

Pipette the PCR product/gel loading buffer mixture carefully into the wells.

Gel electrophoresis

The DNA is electrophoresed at 100 V/400 mA for 35 min. Negatively charged DNA moves towards the positive electrode. Make sure the current is set up properly.

Gel staining ! PERFORMED BY Supervisors!

The agarose gel is lifted from the electrophoresis chamber and carefully set into the ethidium bromide staining bath. The gel is stained for 10 min and then transferred, using the scoop, into the destaining bath for 1 min.

! WEAR NITRILE GLOVES ! WHEN WORKING WITH ETHIDIUM BROMIDE! TOXIC & CARCINOGEN

Avoid splashing the ethidium bromide solution. Do not get ethidium bromide on your gloves. If you contaminate your gloves, remove them carefully and place them in the

ethidium bromide waste container. If you contaminate the work surface inform your supervisor.

Gel documentation ! PERFORMED BY Supervisors!

Detection of fluorescence is performed using the Geldoc. The camera is focused on the gel to take a photo, which will be printed for you.

! WEAR UV PROTECTION SHIELD ! UV LIGHT IS HARMFUL FOR YOUR EYES!

INSERT THE PHOTO OF YOUR GEL IN YOUR PROTOCOL.

RECORD IN YOUR PROTOCOL WHAT YOU GOT AND WHAT IT MEANS:

- Quality of the genomic DNA isolation. Were there additional bands or evidence of contamination with proteins and/or RNA?
- Size of PCR product and whether or not there was a product in both PCRs and the negative control.
- Vector preparation. How many product bands were present in the unrestricted vector preparation? What is the size of the restricted vector? Why does the unrestricted vector look smaller than the restricted vector?

By this stage you will be able to confirm whether you got a PCR product and whether you were able to purify and restrict plasmid DNA. These samples are now ready for ligation.

LIGATION

You will need to set up 1 ligation per group of 2 students. For the ligation of the PCR products into the prepared vector you need to pipette the following into a sterile 1.5 ml Eppendorf cup:

7.0 µl of the purified PCR product
2.0 µl ligase buffer (10 x concentrated)
2.0 µl 50 % PEG 400 solution (for blunt end ligation)
3.0 µl vector DNA (restricted vector!)
1.0 µl T4-Ligase (1 U/µl)
5.0 µl H_2O

- Mix gently and centrifuge for 30 s.
- Incubate overnight at 16°C.

WEEK 3

PREPARATION OF CHEMICAL COMPETENT *E. COLI* CELLS

Cells that have the ability to take up foreign DNA from a variety of sources are termed “competent” cells. In this practical course we prepare chemical competent cells of *E. coli* strain DH5α using the calcium chloride ($CaCl_2$) method (PromegaTechnical Manual, 1994).

SINCE YOU ARE NOW WORKING WITH A CULTURE AGAIN REMEMBER TO WORK STERILE!

1. Add 0.2 ml overnight culture to 20 ml fresh LB medium in 100 ml flask, and shake at 37°C until an OD_{600} of 0.3-0.5 is reached (approximately 90-120 min).
2. Transfer the culture to a sterile, 50 ml centrifuge tube (provided by the supervisors).
3. Incubate cell suspension on ice for 10 min.
4. Collect the cells by centrifugation at 4000 rpm for 7 min at 4°C (Centrifuge).
5. Discard the supernatant carefully. **IMPORTANT** : Always keep cells on ice!!!
6. Resuspend the cells gently in 1 ml ice-cold (4°C) **$CaCl_2$ Solution** (10 mM PIPES, 60 mM $CaCl_2$, 15% Glycerol) Collect the cells by centrifugation at 4000 rpm for 5 min at 4°C (Centrifuge).
7. Discard the supernatant carefully. Always keep cells on ice.
8. Resuspend the cells gently in 0.2 ml ice-cold (4°C) **$CaCl_2$ Solution** (10 mM PIPES, 60 mM $CaCl_2$, 15% Glycerol) with blue pipette tips (tip is cut off), to avoid shear forces, which would damage the sensitive competent cells.
9. Aliquote in two 100 µl portions

Store the competent cell on ice, until you use them for transformation. Alternatively competent cells can be frozen in liquid nitrogen in the presence of glycerol (15%) and can be stored at -70°C for months without loosing their competence.

TRANSFORMATION

Transformation refers to the uptake of DNA by competent cells. In this practical course *E. coli* DH5α will be transformed with the recombinant plasmid. For this part of the experiment each group will require 3 plates of LB agar media containing ampicillin,
X-Gal and IPTG.

- LB agar medium — 3 plates per group of 2 students

  5 g NaCl
  10 g Tryptone
  5 g yeast extract
  15 g of agar
  Make up to 1 L with tap water

  Autoclave

Antibiotics, X-Gal and IPTG are added once the agar has cooled down to **50°C**! Ampicillin, X-Gal and IPTG will be given to you.

Store at 4°C

Once you have prepared the competent cells do the following:

1. Make sure the heat block is set to 42°C.
2. Cells are stored on ice (100 µl, at least 5 min).
3. Add 10 µl of your ligation and mix carefully (tube 1) and 10 µl water to the control (tube 2).
4. Leave on ice for 10-15 min.
5. Heat shock the cells for **EXACTLY** 90 s at 42°C.
6. Transfer the cells back to ice for 2 min immediately.
7. Add 600 µl LB medium (RT).
8. Incubate at 37°C for 1 h.
9. Pipette 100 µl of the cell suspension (ligation mix) on plate 1. Carefully spread the cellular suspension over the surface of the plates using a **COOLED**, sterilised Drigalski spatula. Centrifuge the residual cell

suspension as well as the control (1 ml) at 4.000 rpm at room temperature for 2 min.

10. Remove supernatant leaving approx. 100 µl in the tube, Cut the top from a blue tip to enlarge the opening and resuspend cells with this tipPlate as described above on plate 2 (residual ligation mix) and plate 3 (control).
11. The plates are incubated overnight at 37°C.

WEEK 4

VERIFICATION OF POSITIVE CLONES (WITH INSERTS) BY COLONY PCR

If the transformation was successful you will have a few white and many blue colonies. Count the white ones and the blue ones. Calculate the ratio of the blue vs. white colonies and record your results in your protocol.

For this part of the experiment each group will need 1 fresh LB plates containing ampicillin, X-Gal and IPTG, 3 test-tubes containing 5 ml LB media + 100 µg/ml ampicillin (50 µl from a 10 mg/ml solution of ampicillin) and 4 Eppendorf tubes for colony PCR. Colony PCR is used as quick (growth-independent) method to analyze two clones for the presence of the insert (16S rRNA gene) via the bacterial domain specific primer set/vector primers and Phusion polymerase.

- LB agar medium — 1 plates per group of 2 students
  (see Part 8 for composition)
- LB liquid medium — 3 test-tubes per group of 2 students
  - 5 g NaCl
  - 10 g Tryptone
  - 5 g yeast extract
- 10 mM Tris-HCl, pH 7.0

Make up to 1 L with tap water and aliquot into test tubes (5 ml in each).

→ Autoclave & store at 4°C

Each group selects **one blue and two white** colonies that they use for colony PCR (today) and plasmid preparation (next Friday). Use the same sterile pipette tip for transferring the colony to:

- LB plates (using a grid)
- 1.5 ml reaction tube for colony PCR containing 50 µl Tris-buffer
- 5 ml LB medium + ampicillin for plasmid preparation.

Make sure the numbering on the test-tubes and LB medium matches the numbering on the LB plate grid.

- Transfer all the residual white colonies on the fresh LB plates using the grid and sterile yellow pipette tip.
- Incubate the agar plates and LB medium (shaker) at 37°C overnight.

Colony-PCR

- One blue and two white colonies were picked and transferred to labelled 1.5 ml reaction tubes containing Tris-buffer (50 µl 10 mM Tris/HCl, pH 7,0) (see above). As a negative control reaction use water instead of DNA.
- All three reaction tubes (except the water control) are incubated for 5 min at 94°C for cell lysis.
- Centrifuge the cell lysate at 13,000 rpm at room temperature for 1 min.
- Use 5 µl of the supernatant directly for PCR (as DNA template). The PCR will be performed with Phusion Polymerase.

Table 4: PCR-Reactionmix

Constituent	Concentration of stock soln.	Final conc.	Volume in 25 µl
PCR buffer	5 X	1 X	
§ dNTPs mix	2 mM	200 µM	
§ Forward-primer T7 Prom (vector specific)	10 µM	0.4 µM	
§ Reverse-primer M13	10 µM	0.4 µM	
# Phusion DNA polymerase	2.5 U/µl	1 U	
DNA template			5 µl
H_2O (PCR clean)			add to 25 µl
		Total volume	25 µl

- § defrost and keep on ice in an ice-bucket.
- # keep DNA polymerase at -20°C in freezer until needed. Keep on ice and return to freezer immediately.

Table 5: PCR programme parameters

Cycle	1	2 - 29	30
Denaturation	98°C, 5 min	98°C, 30 sec	
Annealing		48°C, 30 sec	
Extension		72°C, 45 sec	72°C, 5 min

The colony PCR products will be identified by agarose gel electrophoresis together with the DNA fragments of the restriction analysis (Part 5).

WEEK 5

VERIFICATION OF POSITIVE CLONES (WITH INSERTS) BY RESTRICTION ANALYSIS OF PLASMID DNA

CHECK WHITE CLONES

Check the colonies that you transferred to the fresh LB plate last Friday. Are they still white?

PLASMID ISOLATION

To confirm the size of the cloned inserts it is first necessary to isolate the recombined plasmid DNA from *E. coli* from one positive clone per group. This time you will use the following solutions

- **P1** for vector DNA isolation
  - 10 mM Tris-HCl
  - 1 mM EDTA
  - 20 µg/ml RNase

- **P2** for vector DNA isolation
  - 0.2 M NaOH
  - 1% SDS (w/v)

- **P3** (2.55 M KAc^-) for vector DNA isolation
  - 29.5 ml acetic acid
  - Make up to approx. 80 ml with H_2O
  - Alter to pH 4.8 by adding KOH pellets and mixing
  - Make up to final volume 100 ml

PROTOCOL

- Pipette 2 ml of the overnight culture into a 2 ml Eppendorf tube.
- Centrifuge for 30 s, discard the supernatant, add another 2ml and repeat
- Resuspend the pellet in 200 µl buffer P1
- Add 200 µl of buffer P2 and invert 5-6 times to mix
- Add 200 µl of buffer P3 and invert 5-6 times to mix

- Centrifuge for 5 min at 13.000 rpm
- Transfer the supernatant to a clean 1.5 ml Eppendorf tube
- Precipitate with 1 ml ice-cold isopropanol p.A. and incubate on ice for 5 min
- Centrifuge for 20 min at 4°C. Make sure to remember the orientation of the tube in the rotor so that you can locate your DNA pellet!
- Pipette off the isopropanol
- Add 1 ml 70% ethanol p.A. and incubate at room temperature for 10 min
- Centrifuge for 5 min and pipette off ethanol completely
- Dry the pellet at 60°C for approximately 10 min
- Re-dissolve your DNA in 50 µl DNase- and RNase-free H_2O (5-10 min 60 °C)

The isolated plasmid must now be restricted so that it is linear and you can confirm the size of the plasmid + insert by gel electrophoresis.

To digest the DNA pipette together the following:

- 17,0 µl DNA
- 2,0 µl restriction buffer
- 1 µl BamHI restriction enzyme

- Mix and centrifuge briefly
- Incubate at 37°C for 10 min

Visualisation of DNA fragments of colony PCR and restriction analysis using agarose gel electrophoresis

To confirm the size of the inserts the restricted plasmid will be analysed by gel electrophoresis as well as the colony PCR products. See above (Part 2) for gel preparation, electrophoresis and staining protocols.

10 µl PCR product/restricted plasmid
1 µl gel loading buffer

In your protocol include a picture of your gel. Determine the size of the linearised recombinant vector and record the size of the cloned insert (the size of the vector can be found on the vector map).

APPENDIX 1.

SOLUTIONS FOR MICROBIOLOGY

During this course you will be working with a variety of solutions. The concentrations of these solutions can be expressed as molarity, % composition (weight/volume, w/v) and % composition (volume/volume, v/v). In the following section you will learn how to calculate the concentrations of solutions and how to prepare them.

SOLUTIONS 1: MOLARITY

Normally, the concentration of the solution is given as molarity (M). Molarity, number of moles per litre, is an expression of concentration. Moles are an expression of the quantity.

$$\text{Molarity} = \frac{\text{moles of solute}}{\text{volume of solution (L)}}$$

Example: NaCl has a molar weight of 58.44 g.
If you want 1 mole of NaCl you would weigh out 58.44 g NaCl powder.
If you require a 1 Molar solution of NaCl you would then dissolve 1 mole (58.44 g) in 1 litre final volume

1.1 CALCULATION OF MOLARITY

If you know the volume of a solution and the number of grammes of solute dissolved in it, you can calculate the molarity.
i) calculation of moles of solute from grammes solute
5 g NaCl must be converted into moles before it can be used in the above equation.

$$5\text{ g} \quad X \quad \frac{1\text{ mole}}{\text{Formula weight}} = \text{moles}$$

$$= 0.085 \text{ moles}$$

If you have 300 ml of a solution containing 0.085 moles of NaCl you can calculate the molarity.

$$\text{Molarity} = \frac{\text{Moles of solute}}{\text{volume of solution (L)}}$$

$$0.28\ \text{M} = \frac{0.085}{0.3\ \text{L}}$$

1.2 Calculation of mass of solute corresponding to molarity

If you want to make 250 ml of a 0.1M NaCl solution, you need to calculate the number of grammes NaCl.

$$\frac{\text{Final volume (L)}}{1\ \text{L}} \text{ X molarity X molecular weight } = \text{g}$$

$$\frac{0.25}{1\ \text{L}} \text{ X } 0.1 \text{ X } 58.44 = 1.461\ \text{g}$$

Solutions 2: Percentage by weight % (w/V)

Sometimes solutions are described as having % concentration (weight/volume). This means that a certain mass of dried chemical is dissolved in a volume of solution. For example, 0.9% NaCl solution is commonly used to dilute bacterial cultures. To make a 0.9% solution of NaCl requires 0.9 g NaCl in a final volume of 100 ml H_2O:

$$\frac{\text{Mass of solute (g)}}{\text{Volume of liquid (ml)}} \text{ X } 100 = \%$$

If you require 2000 ml of 0.9% NaCl you would use the following formula.

$$\frac{\%}{100} \text{ X desired volume (ml)} = \text{g}$$

For example: To make 2L of a 0.9% solution of NaCl you would need 18 g NaCl.

$$\frac{0.9\%}{100} \text{ X } 2000 = 18\text{g NaCl}$$

When making 100 ml 0.9% NaCl solution you would first weigh out 0.9 g NaCl and dissolve it completely in 80 ml H_2O. Once the solute is completely dissolved, then make the final volume to 100 ml. If you were to start by adding 0.9 g NaCl to 100 ml H_2O then the final volume of the solution would be greater than 100 ml and the concentration would be wrong (too low).

SOLUTIONS 3: PERCENTAGE BY VOLUME % (V/V)

Sometimes solutions are described as having % concentration (v/v). In the DNA isolation protocol a 70% ethanol solution is required for cleaning the DNA pellet. This is made by diluting 96% ethanol. To make a 500 ml solution of 70% ethanol from a 96% solution:

$$C_1V_1 = C_2V_2$$

$C_1 = 70\%$, $V_1 = 500$ ml

$C_2 = 96\%$, $V_2 = ?$

$$V_2 = \frac{70 \text{ X } 500}{96}$$

$$V_2 = 364.6 \text{ ml}$$

The 70% solution is made by adding 364.6 ml 96% ethanol to a measuring cylinder and making it up to a final volume of 500 ml with H_2O.

SOLUTIONS 4: CONCENTRATION BY RATIO

Another way of expressing the concentration of a solution is by the ratio of the component solutions. For example: chloroform/isoamylalcohol (24:1). To make 10 ml of this solution requires 9.6 ml chloroform + 0.4 ml isoamylalcohol.

This is calculated by taking the final volume, 10 ml, and dividing it by 25 (24 +1) = 0.4. The volume of chloroform is calculated by 24 X 0.4 = 9.6 ml, and the volume of isoamylalcohol 1 X 0.4 = 0.4 ml.