Macromolecule design and manipulation - practical textbook

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Table of contents

Learning outcomes

This book and the associated practice are focusing on to provide the following general leaning outcomes. (More specific learning outcomes are listed in each chapter.) Students who complete this course will acquire the following learning outcomes:

Knowledge:

1. They know the structure, function and regulation of biological systems
2. They know how to generate competent bacterial cells in the laboratory.
3. They understand the concept of plasmid preparation.
4. They know the theoretical background of agarose gelelectrophoresis and bacterial transformation.
5. They are aware of the type of inducing agents used for heterologue protein expression.
6. They are aware of the theoretical basis of sonication.
7. They know the theoretical background of PCR and also the materials and equipment required to perform such an experiment.
8. They know the differences between agarose- and polyacrylamide gel electrophoresis and the theoretical basis of them.
9. They know the function of molecular weight markers.
10. They understand the function of the protein purification process based on heat treatment.
11. They know how to do the protein purification with salting out.
12. They know how the dialysis works.

Skill:

1. They can use the principles of central dogma.
2. They are capable of generating competent cells and the genetic modification of them.
3. They can perform plasmid DNA preparation from bacterial cells.
4. They can prepare the agarose gel and prove their experience in bacterial transformation.
5. They can use the pET expression system for heterologous protein production in Rosetta cells.
6. They can apply sonication-based cell lysis method.
7. They can measure a PCR reaction and program the PCR machine.
8. They are capable of preparing their samples for the electrophoresis.
9. They can purify the thermostable proteins from bacteria.
10. They are capable of distinguishing the aim of salting out during purification and concentration steps.
11. They can use the salting out method and they can desalt their samples by using dialysis.

Attitude:

1. They are motivated for acquiring new information.
2. They pay attention on the importance of sterile work.
3. They make an effort to do the plasmid preparation without chromosomal DNA contamination.
4. They are willing to evaluate the results of agarose gel electrophoresis and perform the transformation on their own.
5. They keep in mind the importance of collecting the samples at the appropriate time point.
6. They make an effort to lyse the cells efficiently and then to take samples precisely.
7. They realize the importance of the use of positive and negative controls.
8. They make an effort to precisely document and present the results of the agarose gel photo.
9. They are critical during the evaluation of the gel photos.
10. They make an effort to work in groups.
11. They evaluate their experiments and are willing to learn their promiscuous mistakes.

Responsibility and autonomy:

1. They are responsible for overview the process of the practise based on the information.
2. They can follow the protocol with the help of technical direction.
3. They comply with the appropriate directions to prepare a pure plasmid without any contaminations.
4. They phrase independent suggestions about how to precisely and efficiently evaluate the results from agarose gel electrophoresis.
5. They comply with the accurate time-points of sampling.
6. They lyse the cells and collect the samples with the help of technical direction.
7. They can follow the protocol on their own.
8. They comply with the safety rules, which are necessary for the lab work.
9. They can evaluate the results of the agarose and polyacrylamide gels with the help of technical direction.
10. They can work in contribution to the others.
11. They can evaluate the results of the polyacrylamide gels on their own at the end of the practice.

Introduction

This book is written to facilitate the understanding and implementation of the subject area thought in the Biology MSc practical course *Macromolecule design and manipulation* at the University of Szeged.

We recommend this book to those students, who would like to extend their knowledge in particular by familiarising themselves with methods used in modern molecular biology and biochemistry. This book provides useful information on basic methods through a detailed coverage of experiments by expression and purification of a thermostable DNA polymerase, which will be performed by the participating students. Basic English language knowledge is required for the successful achievement of the course, which also gives the opportunity for the students to get familiar with some of the basic terminologies used in a molecular biology laboratory.

In the last quarter of the 20th century, the field of molecular biology underwent a rapid and huge development that produced great achievements in several fields, which made the easy and affordable genetic modification of bacteria and yeast possible. The toolkit developed in molecular biology laboratories all over the word made the transfer and expression of foreign genes into bacteria possible, which in turn promote to obtain that the desired proteins can be achieved in unlimited quantities for biochemical studies. Proteins that was hard or nearly impossible to be obtained earlier, such as some proteins of human origin, are nowadays easily accessible for research usage. Besides basic

research, healthcare and other industries have also gained great benefits from these developments. For instance, before the revolution of the molecular biology cloning, insulin was purified from *Sus scrofa domestica* (pig) for the treatment of patients suffering from diabetes. Following the development of these new approaches human insulin could be produced in yeast or bacteria. Nowadays, much larger proteins, such as coagulation factors and antibodies are also produced by heterologous or homologous protein expression systems.

During this course, you are going to learn basic techniques based on protein expression in bacteria. This will be done by performing experiments intended to produce and purify a thermostable protein from *Escherichia coli*. Our protein of interest is the Pfu DNA polymerase frequently used in polymerase chain reaction (PCR), which is another basic technique of molecular biology. The enzyme encoding the Pfu gene is derived from the archeon *Pyrococcus furiosus*, which lives in hot springs. Therefore, it is a thermostable DNA polymerase, it can withstand surprisingly high temperatures (95 °C), but its highest activity is at 72 °C.

This practical description will guide you through the techniques required for expression and purification of the Pfu protein over 10 chapters, each of them corresponds to a laboratory practical session (Chapter 2-11). The workflow of the practices is summarized on the next figure (Figure 0.1).

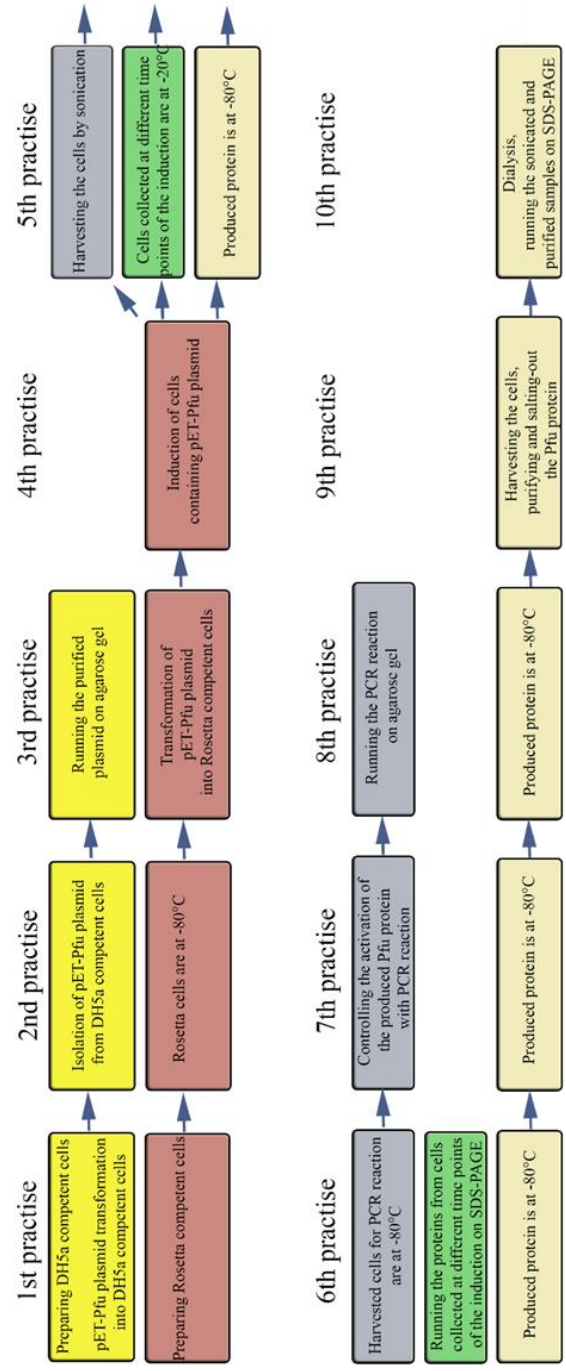


Figure 1 Thematics

Chapter 1

Before entering the lab

Basics of pipetting

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will be familiar with the correct utilisation of pipettes.

Skill:

2. They will be capable of using a pipette.

Attitude:

3. They are willing to follow the rules of pipetting to avoid contaminations or any damages of the instrument.
4. They are willing to work with the pipettes as precisely as they can.

Responsibility and autonomy:

5. They will be able to perform the correct pipetting steps on their own.

Basics of pipetting

In the laboratory you will come to know several different types of equipment, some of them are rather simple, while others are very sophisticated and extremely expensive. However, we will not overview the proper use of all of them, whichever you want to use the same basic rules should be applied: **always make sure that you know what you are actually doing on and that what the proper way is to do it!** If you have any doubts, make sure to collect the proper information by reading your practical guide, your notes or by asking your laboratory supervisor. You are responsible for every step you take in the laboratory. With irresponsible behaviour you can endanger your own or the other fellow's health and you cannot risk the chance of destruction of any equipment, reagents or experiments!

In a molecular biology laboratory, volumes are measured in microliters and quantities in micrograms or might be only fractions of these. Therefore, the correct utilisation of the laboratory equipment by which these small quantities of materials can be handled is crucial. This is the reason that from many tools you are about to use, here we consider only one type, the so-called automatic pipettes, which are used to measure small quantities of liquid in the laboratory. The correct usage of pipettes is important because even small changes in the desired volumes could alter the results of experiments and compromise the reproducibility of your work. The inappropriate usage of pipettes also shortens the life-span of these expensive instruments, therefore learning how to use them

correctly is one of the first and extremely important steps in the process of learning basic laboratory work techniques.

Theoretical background

Automatic pipettes make the lab work much more comfortable and working with them increases the reproducibility of the experiments. By using automatic pipettes, we can measure fractions of millilitres with great precision allowing the reduction of volumes and by this decreasing the cost of biochemical reactions.

Important notes: Being familiar with the International System of Units and with the meaning of prefixes are also essential for lab work.

nl = nanolitre	ul = microlitre	ml= millilitre
1 nl = 10^{-9} l	1 ul = 10^{-6} l	1 ml = 10^{-3} l
1/1,000,000.000 l	1/1,000,000 l	1/1,000 l
1,000 nl = 1 ul	1,000 ul = 1 ml	1,000 ml = 1 l

In a standard molecular biology lab, automatic pipettes are used to measure volumes in the range from 0.5 ul to 1 ml. For this, usually a set of three pipettes are needed. For the smallest volumes we use a pipette that can be adjusted from 0.5 to 10 ul. For a little larger volume, we use the pipette, which can measure from 10 to 100 ul. For even larger volumes, we use a pipette that can be set from 100 ul to 1 ml (Figure 1.1). There are also other kinds of pipettes measuring different volumes, although the exact pipetting range is always indicated. Never force a

pipette to set volumes outside this indicated range since this can result in either inaccuracy or failure of the pipette!

Pipette tips are small disposable plastic cones that contain the liquid during pipetting. Different tips should be used for pipettes measuring at different ranges. Some tips (depending on the manufacturer) are colour-coded in order to help to identify the correct one. 0.5-10 ul ranged pipette tips are transparent white, 10-100 ul (or 20-200 ul) tips are yellow and 100-1,000 ul tips are blue coloured (Figure 1.1).

Important notes:

- Pipette tips have to be sterile and clean in order to avoid contamination of the sample being pipetted.
- Never touch tips with your naked hand because lipids, proteins and other contaminations from the human skin will attach to the surface of the tip. Later, during pipetting, this contamination could get into the sample and it will ruin the subsequent experiment.

Figure 1.1 Example of a set of micropipettes. Each pipette serves to measure in different volume range. For precise measurement, each pipette should be used with the compatible tip.

Parts of a pipette are shown on Figure 1.2. With the control button at the top of the pipette, the piston located inside the pipette can be moved. When the piston is moved upwards, vacuum is generated, which makes liquid transfer possible by sucking up the desired volume into the tip. The amount of liquid is proportional with the dislocation of the piston. The liquid is released by moving the piston downwards. The movement range of the piston could be regulated by the volume adjustment screw. The actual volume for what the pipette is set, is indicated on the volume display. The correct tip for the pipette fits perfectly on the tip cone. The removal of a used tip is done with the ejection button.

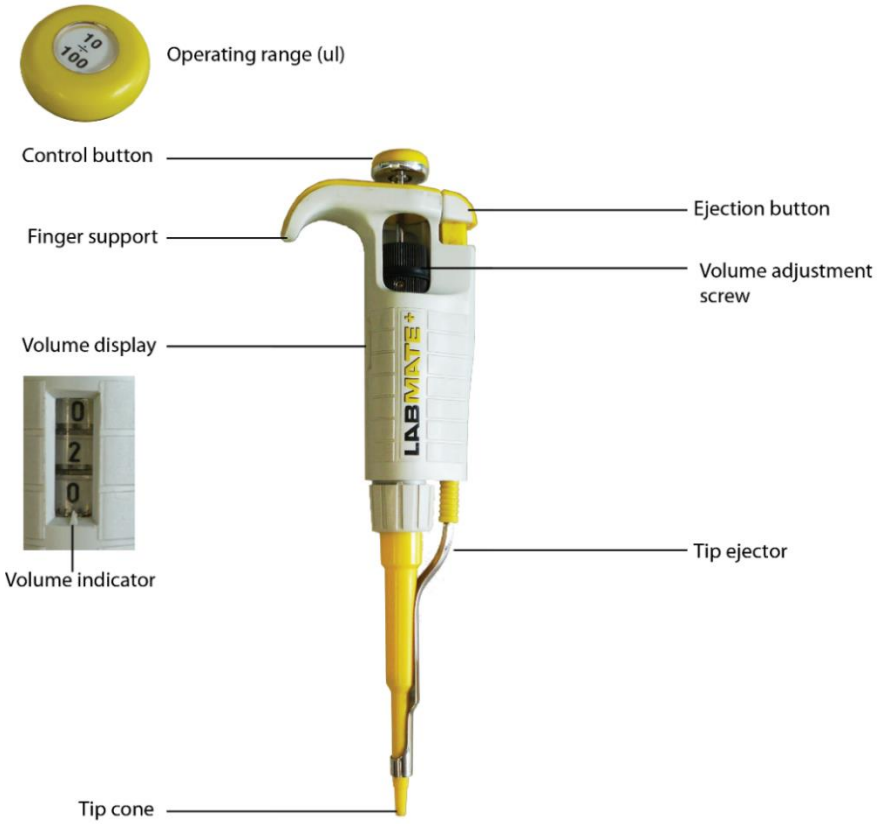


Figure 1.2 Parts of a pipette

Steps of pipetting

1. Setting the volume

Setting the correct volume starts with the pipette selection. Choose the pipette, which range fits to the desired volume. Set the volume with the adjustment screw, while watching the changes on the volume display of the pipette. NEVER adjust the pipette below or above the measuring range of it! The volume display usually shows three digits, but in case of different pipettes, these numbers represent different actual values. For

instance, in case of a 100-1,000 ul pipette, when the first digit is set to 1, it means that it is 1,000 ul in volume. However, in case of a 0.5-10 ul pipette, the first digit represents only 10 ul. Shifts in the scale are usually indicated with red colour as shown on Figure 1.3.

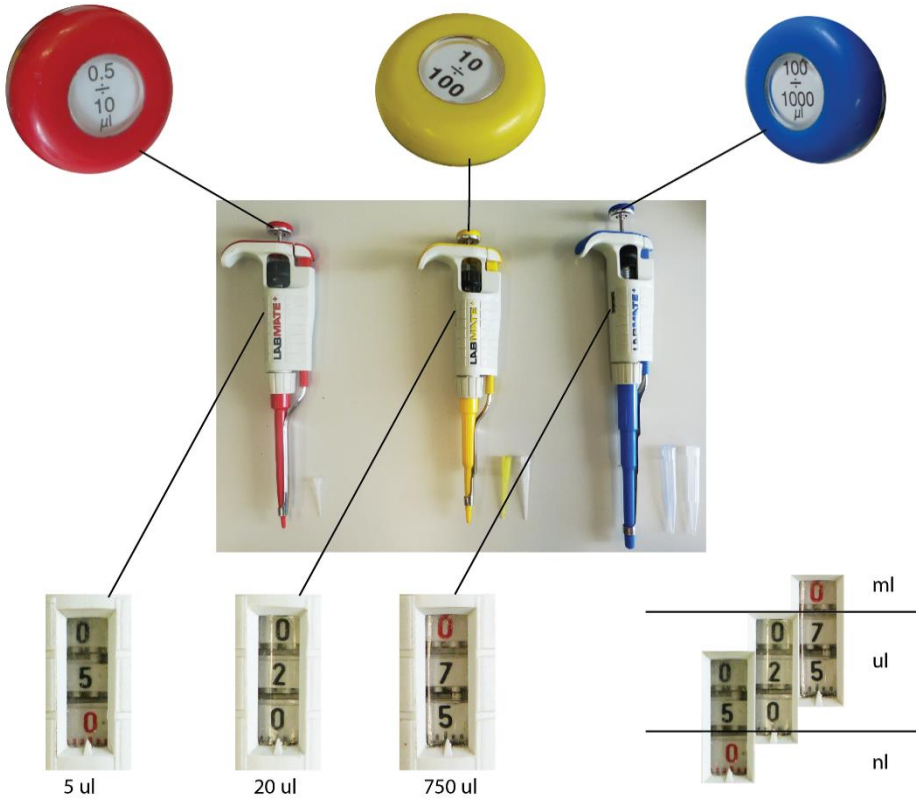


Figure 1.3 Examples of setting volume indicators on pipettes used in different volume ranges. On the left there is a 0.5-10 ul pipette on which the last digit (the lower one) is red coloured. If we set 0, 0, 5 on this pipette (downwards from top to bottom), it means 0.5 ul or in another dimension, it is 500 nl. In case of the 100-1,000 ul pipette, if we set 1, 0, 0, (downwards from top to bottom) it means 1,000 ul or in other word 1 ml. Other examples are indicated on the figure.

2. Applying the tip on a pipette

When applying the tip onto a pipette, you should be careful and avoid damaging the tip cone. It must not be bended or scratched. If the tip does not fit perfectly on the cone the volume will change or liquid will be lost during pipetting. To avoid bending the tip cone, always keep the pipette vertically straight as shown on Figure 1.4.

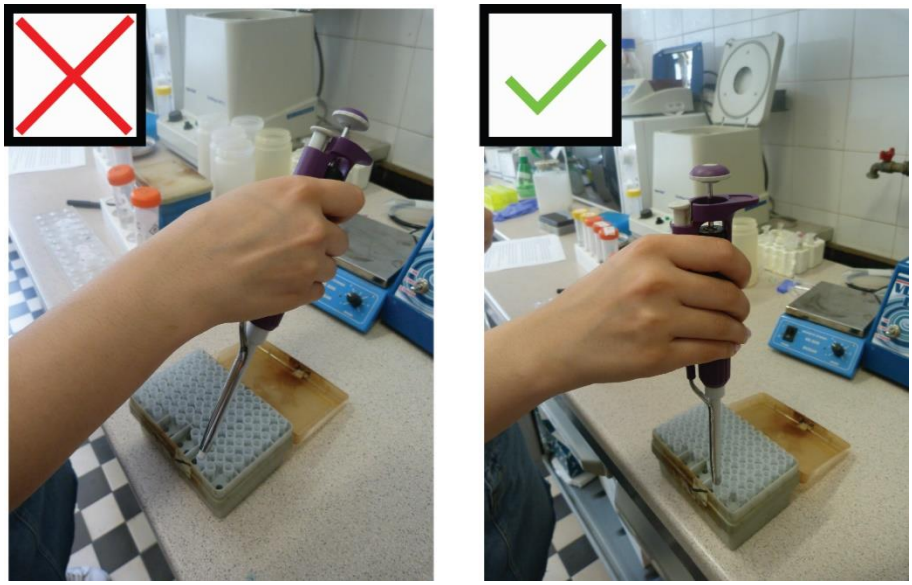


Figure 1.4 Applying tips on the pipette. Hold the pipette vertically and fix the tip on the tip cone.

3. Measuring

After selecting the right pipette, setting the desired volume and applying the tip, pipetting could be performed by using the control button. This button has two stopping points. By pushing the button until the first point of contact, air will be squeezed out from the pistone and the

volume of air leaving the pipette will be proportional with the selected volume. The second point of contact is a fixed distance from the first one, which makes possible to eject some more air from the pipette. This is used to remove any residual liquid that may remain in the tip.

According to the above described directions, pipetting should be carried out as the following steps:

1. push the control button until it reaches the first point of contact
2. submerge the end of the tip into the liquid, while holding down the control button
3. slowly release the control button upwards, which will fill the tip with liquid
4. position the tip into the other container (e.g. Eppendorf tube), gentle touching the wall of it
5. slowly push the button downwards, until it reaches the first point of contact to empty the tip
6. slowly push the button, until it reaches the second point of contact to empty the residual droplets that may remain in the tip due to adhesion-cohesion.

The steps of the pipetting are also shown on Figure 1.5.

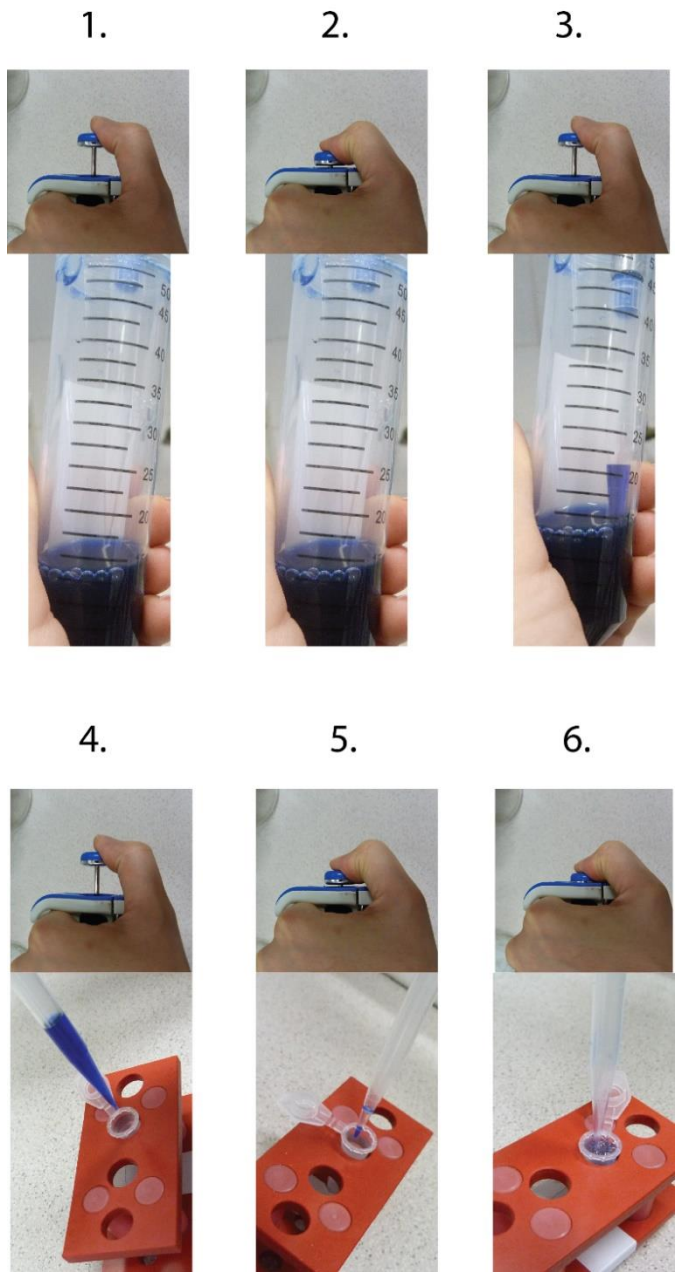


Figure 1.5 The steps of pipetting. The control button can be pushed with your thumb.

In addition to the proper choice of the pipette and its setting there are several further rules, which should be followed during pipetting of liquids. The most important is that do not move the control button too fast, since it could get the liquid to splash. If the liquid splashes inside the tip towards the pipette, it can contaminate the inner side of the pipette, which can result in corrosion and wrecking of the pipette. A contaminated pipette can contaminate every liquid that is pipetted with it. During pipetting, always hold the pipette vertically to avoid flowing of the liquid into the pipette, thereby preventing corrosion and contamination.

Always push your tip only into the surface area of the liquid you are pipetting and avoid submerging a large part or the whole tip into the solution (Figure 1.6.). A considerable amount of liquid could be attached to the outside surface of the tip, which will result in an inaccurate volume transfer. Be careful to submerge only the end of the tip.

Figure 1.6 Do not submerge large segment or the whole tip into the liquid you are pipetting. Only the end of the tip should be immersed into the liquid.

4. Removal of the tip

Use the ejection button to remove the tip. Always change the tip if you pipette different solutions. Remove the tip if you accidentally touch something with it (e.g. the outside wall of a container, workbench surface, tip holder box etc.) to avoid further contaminations.

When you are working in the lab, always keep in mind that you are working with cells and molecules that are from living organisms, therefore biological samples are as sensitive as the organisms themselves, from which they originate. Therefore, in order to slow down disintegration, keep your samples on ice. Additionally, do not forget that the quantities you are handling are often a fraction of a microgram or just several millionth of a litre. Unnoticed and hardly detectable contaminations being present in the environment and on your hands could easily destroy these small quantities of material. Consequently, be careful and keep the rules outlined above, while you are pipetting samples in the laboratory.

Questions

List the steps of the pipetting!

_____

_____

What should be avoided during pipetting?

_____

_____

_____

Which are the most often used pipettes in a molecular biology lab?

_____

_____

Why is it important to hold the pipette vertically when taking the tip?

_____

_____

Why is it important to hold the pipette vertically when pipetting?

_____

_____

Chapter 2

Research project in molecular biology

1st practice

Preparation of competent E. coli cells and transformation of pET-Pfu plasmid into DH5alpha E. coli cells

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will learn the basic experiments which lead to the discovery that DNA is the genetic material.
2. They will understand the method of transformation.
3. They will learn what transformation efficiency means.

Skill:

4. They will be able to use more basic equipment in the laboratory.
5. They will be able to generate competent bacterial cells.
6. They will be able to transform bacterial cells.
7. They will be capable of spreading bacterial cells avoiding any contaminations.
8. They will be able to calculate transformation efficiency.

Attitude:

9. They will be aware of the sterile work in the laboratory.

Responsibility and autonomy:

10. They will learn how to work in small groups during the practice.
11. They will be able to use automatic pipettes on their own.
12. They will learn how to write their lab reports.
13. They respect the deadlines.

Preparation of competent *E. coli* cells and transformation of pET-Pfu plasmid into DH5alpha *E. coli* cells

The aim of this chapter is to give a brief summary on some of the basics of recombinant DNA technology.

We will give a short theoretical overview here on:

- about the types of the most frequently used vectors in gene technology,
- how Escherichia coli (E. coli) cells are made competent to take up plasmid DNA,
- how DNA molecules are introduced to cells.

Hands on practice will be:

- preparation of competent cells,
- transformation of bacterium cells with a recombinant plasmid

Theoretical background

Recombinant DNA technology development started in the 1970's and since then it has been continuously evolving. The efficiency of the developed methods has resulted in the revolution of the modern biology and it makes the generation of genetically modified organisms and generation of a continuous flow of theoretical and practical results possible. One important step in recombinant DNA technology development was the utilization of plasmids as convenient carriers of foreign DNA fragments. Plasmids are small, circular, extrachromosomal DNA molecules that are used to develop vectors that replicate independently of the host chromosome, therefore are present in multiple copies within a bacterium cell. Plasmids can be used as tools for genetic modification of an organism since they can be easily extracted from or introduced into cells and manipulated in test tube (*in vitro*). Plasmid vectors contain several sequences with well-defined function. One of these is the region responsible for replication, which ensures that the plasmid can be multiplied independently of the DNA replication of the host cell and can be propagated to the daughter cells during cell division. Plasmids should also contain selection markers. Most frequently these are antibiotic resistance genes, which make possible to follow the presence of the plasmid in the host organism and to select only those cells which contain the plasmid. (Only those bacterium cells can grow on an antibiotic containing selective media that carry the plasmid with the appropriate antibiotic resistance gene.) In plasmids, many specific marker genes, such as resistance, lethal or reporter genes can be present in order to help the researcher to recognise

the presence of the inserted foreign DNA and to control its function. Additionally, plasmids should contain unique restriction sites, which permit to open the circular molecule with an endonuclease cleavage and insertion a piece of foreign DNA into it. In advanced plasmids a collection of unique restriction enzyme recognition sites is localized to a specific region, called multi cloning site (MSC). Restriction enzymes are sequence-specific and many of them produce 'sticky ends' with 2-4 nucleotides of complementary overhangs and others generate 'blunt ends' with no overhangs at the end of the DNA fragment. Upon digestion with endonucleases, a circular plasmid molecule can be opened up and easily linked with another DNA fragment (insert) that was treated with the same enzyme resulting in the generation of a recombinant plasmid. However, the conveniently inserted fragment size is limited, since molecules larger than 10 kb are hard to be inserted into plasmids and it is also difficult to maintain large recombinant plasmids in bacteria. Nonetheless, there are special types of plasmid vectors (e.g. plasmids based on the F plasmid of *E. coli*) that have been especially developed to take up large fragments (even Mb sized) and to maintain them. In the nature, plasmids are present in their specific host organisms, which can be different types of bacteria or yeast cells. However, in recombinant DNA technology specific plasmid vectors, called shuttle vectors, replicating in more than one cell types can be produced. These types of vector contain at least two types of replication regions specific for each host cell, which allow the plasmid to replicate in both of them; (usually in prokaryotic and eukaryotic cells e.g. bacteria and yeast). They also contain at least two types of selection markers (e.g. both

antibiotic resistance and auxotrophic complementation genes), which allow the selection in both hosts.
Most of the plasmid vectors used in DNA technology can replicate in the host cell independently of the genome. This phenomenon is called relaxed replication, which can result in many copies of the plasmid replicating as extrachromosomal elements. In addition, other plasmids might be present in the cell only in a single copy and some of these can integrate into the chromosomal DNA of the host cell.

Over the last 50 years molecular biology has produced a large number of plasmid types promoting several specific purposes in molecular biology experiments or in industrial usage. For instance, these plasmid types allow the production of specific proteins, regulation of gene functions, testing the activity of regulatory elements. In this course we do not extensively deal with these, except the so-called expression vectors, which can be used to produce foreign proteins in bacterial cells. More description on these will be given in a later chapter.

In addition to plasmids, other vector types are also frequently used to introduce foreign DNA into bacterial cells. A large group of these have been developed from bacteriophages. The two most common versions of these types of vectors are based on the lambda and M13 phages. The main advantage of lambda-based vectors is that larger DNA fragments can be easily inserted into them. Lambda phage vectors are suitable for 20-25 kb DNA insertions. On the other hand, shorter fragments (1-5 kb) can be incorporated into M13 phage vectors, but they have the unique advantage that they make possible to recover the inserted DNA both in single and in double stranded form. This can be realized because M13

phage contains ssDNA in the phage particle, but during its replication, double stranded intermediers are generated in the cells (replicative form). Thus, M13-based vectors can be used both as a phage and as a plasmid and are suitable for ssDNA expression that is often required for specific experiments (e.g. performing *in vitro* mutagenesis, preparing single stranded hybridization probe, determining nucleotide sequence). In addition to these two types of vector, cosmids have been generated from the combination of genetic materials of plasmids and lambda phages. Therefore, in cosmids certain advantages of plasmids and bacteriophages are combined. The former feature is that they can be propagated in and extracted from cells as a plasmid, the latter is that they can be introduced to cells similar to a bacteriophage, which is a very effective way of genetic modification. This is important when only a small amount of DNA is available for cloning experiment. A further advantage of cosmid vectors is that these can accept twice larger DNA fragments than lambda-based vectors (approximately 50,000 bp DNA can be inserted into a cosmid).

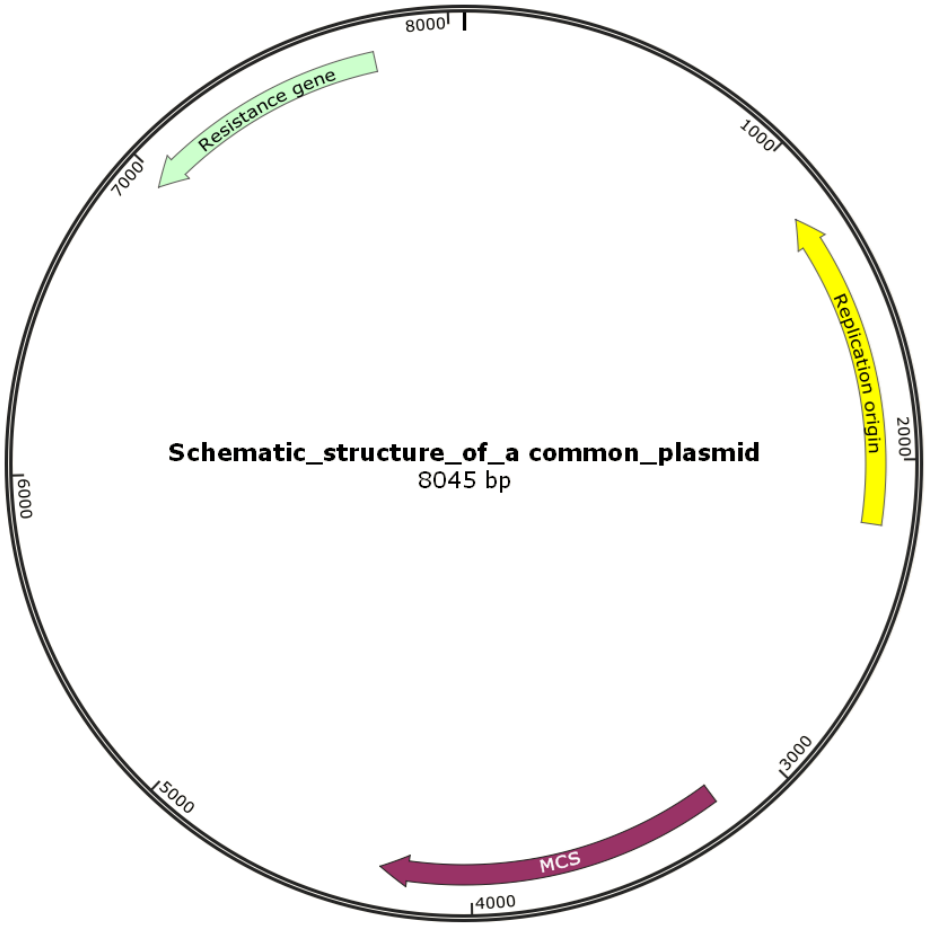


Figure 2.1 Schematic structure of a common plasmid vector. A plasmid vector must contain sequences required for the start of replication, encode selection marker genes and unique restriction sites (multicloning site (MCS)). These three features are the minimal essential elements required for a plasmid vector.

Introduction of DNA into living cells: transformation

There are many different types of techniques by which DNA can be introduced into cells. From these, the most prevalent method is transformation, by which *in vitro* generated recombinant plasmid DNA can be introduced into bacterium cells. The term transformation is frequently used for slightly different methods in which DNA can be also introduced into other types of cells (fungi, plant, animal). However, in the case of mammalian cells, we use the word "transfection" instead of "transformation". To make the nomenclature even more confusing, many other techniques have developed for the same purpose – to ingest DNA into cells – are known and designated by different names (injection, electroporation, lipofection).

Transformation is the most frequently used method to enter DNA into bacterial cells and it is based on the natural phenomenon that was discovered by Frederick Griffith in 1928. It has been known as a key experiment proving that DNA is the carrier of genetic information. In this famous experiment, virulent, type III-S (smooth) and non-virulent, type II-R (rough) strains of *Streptococcus pneumoniae* were used. The type III-S strain is covered with a polysaccharide capsule, which protects the bacteria from the immune system of the host cell. Therefore, mice treated with the non-virulent strain survived, while mice infected with the virulent strain were killed. When the virulent strain was heat-inactivated, the mice survived. Combined treatment of non-virulent *Streptococcus pneumoniae* with the heat-inactivated form of the virulent bacterial strain were also performed and it was found that the harmless form of *Streptococcus pneumoniae* became transformed into a virulent

strain, which infected and killed the experimental animals. This phenomenon was called 'transformation' in which – as later it was proved - the 'transforming principle' was the DNA.

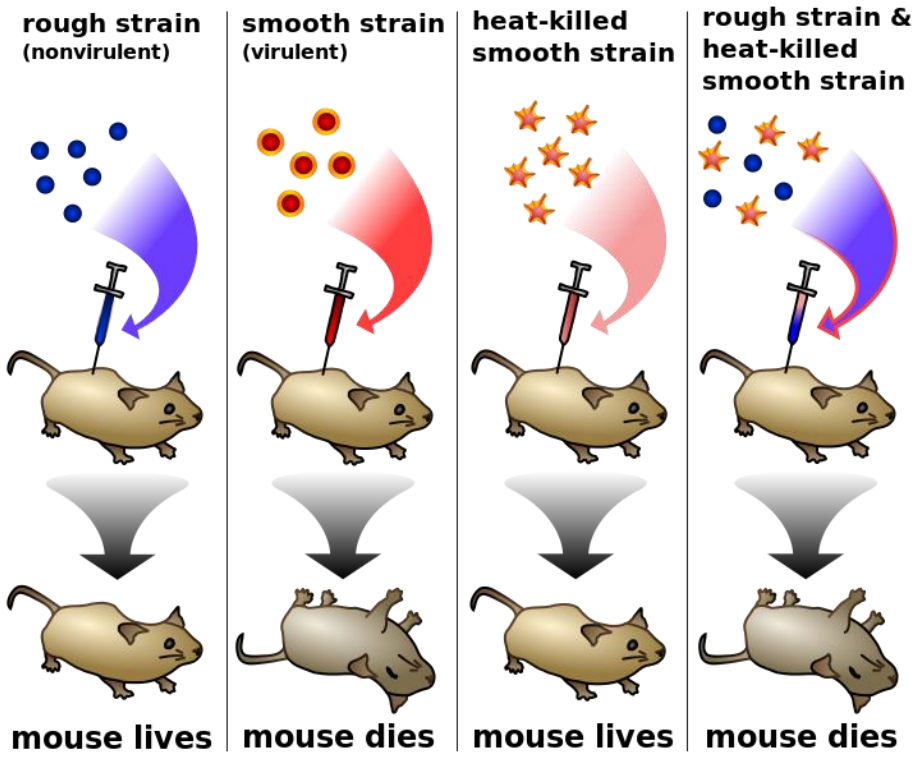


Figure 2.2. A summary of the Griffith experiment

In the experiment two types of *Streptococcus pneumoniae* strains were used to infect mice. The rough strain was non-virulent, while the smooth strain was virulent. After heat treatment the smooth strain lost its virulency. On the other hand, when a mixture of cells from heat-killed smooth strain and untreated rough strain was injected, the mice died. The only explanation could be that the smooth strain transferred its genetic material to the rough strain.

https://en.wikipedia.org/wiki/Griffith%27s_experiment

In 1944, Avery, MacLeod and McCarty proved by a further elaboration of Griffith's experiment that the DNA must be the carrier of the genetic information since solely the DNA molecules were able to transform the harmless *Pneumococcus* strain into a virulent strain.

In 1952, another famous experiment performed by Alfred Hershey and Martha Chase confirmed that the genetic material was indeed the DNA. During their experiment, they infected bacteria with T2 phages and studied whether the DNA or the protein was responsible for the transfer of the genetic information. T2 phage consists of only an external protein shell and an internal DNA content. They used radioactive ^{35}S to label the proteins and radioactive ^{32}P to label the DNA. When they infected bacteria with these labelled bacteriophage samples, they found that the ^{35}S localized in the protein coat remained outside of the bacteria, while the ^{32}P found in the DNA entered the bacterial cells. Since the infected cells produced new bacteriophage particles with protein coat and DNA, the only interpretation of this result could be that the material – the DNA – taken up by bacteria contained all the information required to generate new protein and DNA molecules.

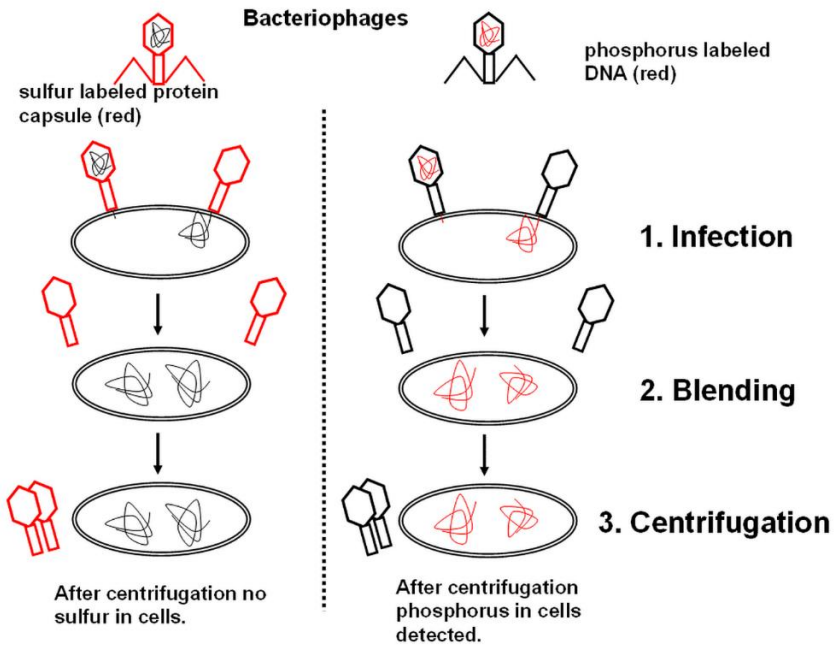


Figure 2.3. Schematic draft of the Hershey-Chase experiment. Bacteria were infected with either ^{32}P or ^{35}S radiolabeled bacteriophages. The result of the experiment confirmed that the genetic material is DNA.

https://commons.wikimedia.org/wiki/File:Hersheychaseexperiment

In summary, two of the most famous experiments that proved the role of DNA are the bases of the techniques by which DNA is introduced into cells in recombinant DNA experiments, in other word by genetic engineering. Transformation is the process by which bacterial cells can take up foreign DNA from their environment. Under natural conditions, transformation is important since taking up foreign genetic material can increase the survival efficiency of a cell (e.g. a plasmid that encodes antibiotic resistance gene helps to protect its host). In the laboratory, we use transformation to introduce *in vitro* constructed recombinant

plasmids into cells. Bacteriophage infection is another effective way by which foreign DNA can enter the cells under natural conditions (however viral infection is not beneficial for the host cell). In the laboratory, we mimic this process by assembling bacteriophage particles *in vitro* by applying recombinant DNA molecules. In that way recombinant cosmids can be introduced into cells.

Transformation in the laboratory

In recombinant DNA technology bacterial transformation is a widely used process. Bacterial cells can take up exogenous DNA molecules when they are in the competent status. Since DNA can be absorbed to the bacterial cell surface in the presence of bivalent cations, e.g. Ca^{2+}, we treat the cells with them to increase the competency of bacterial cells. Both the bacterial cell wall and the DNA are negatively charged therefore it is important to shade the negative charges of the cell wall, thereby dissipating the electrostatic repulsion to increase the efficiency of DNA entrance into the cells. By exposing the cells to a sudden temperature increase (heat shock) or a pressure difference between outside and inside of the cell, pores will be formed in the cell wall, which allow the plasmid DNA to enter the cell. After returning the cells to a favourable temperature (for *E. coli* it is 37 °C), the cell wall will be recovered and by spreading the transformed bacterial cells on agar plates complemented with the proper antibiotics, only the ones containing the resistance gene will grow and form colonies. This process of DNA introduction into the cells is called chemical transformation. Several modified version of this procedure have been developed, such as the

method based on the studies of Inoue et al. in 1990 (Gene 96(1): 23-28), which will be used in this course. We can use competent cells that can be either prepared freshly before transformation or previously prepared and freezed. The previously prepared cells were supplemented with an antifreeze agent (e.g. DMSO) then freezed in liquid nitrogen and stored at -80 °C for later usage.

Electroporation is another type of method by which DNA can be introduced to cells. During this technique a sudden change in the potential of the electric field is applied to form pores on the cell wall and by this higher transformation efficiency could be achieved. The electric shock (12.5-15 kV/cm) makes the bacterial cells competent for taking up DNA from the outside environment. Electroporation is more efficient than classical chemical transformation, but it requires more equipment than chemical transformation does.

Whichever protocol is used, the efficiency of the transformation can be determined by calculating the number of plasmid-containing cells obtained from the transformation of 1 ug of DNA (to the same amount of cells). To determine the transformation efficiency, bacterial colonies growing on antibiotic complemented agar plate should be counted. This is generally performed following an overnight incubation of the bacteria containing agar plates in a 37 °C incubator. During this short-time, every single transformed bacterium cell will divide several times and give rise to colonies consisting of millions of cells which all contain the same transformed recombinant plasmid. In other words, clones of the transformed unique cells grow up – this is reflected in the name used occasionally for recombinant DNA technology as DNA cloning. Depending of the circumstances, the transformation efficiency can reach

10^8, which means obtaining of 100 million of recombinant cells from 1 microgram of DNA.

Transformation methods of different cell types

1. **Bacteria:**

   Generally, they can be easily transformed, since well-developed transformation protocols and suitable selection markers (antibiotic resistance) are available. The most important vectors are: non-integrating plasmids, bacteriophage-based vectors, cosmids, bacterial artificial chromosome (BAC). Methods for DNA introduction into cells are: transformation, electroporation, phage infection.

2. **Yeast:**

   Since yeast cells possess thick cell wall, transformation of them requires specific steps to deal with this. On the other hand, similar to bacteria, yeast cells can be cultured easily and there are good markers available for selection (auxotrophic complementation). The most important vectors are: non-integrating or integrating plasmids, yeast artificial chromosome (YAC). Method for DNA introduction into cells is: transformation (intact cell or protoplast).

3. **Plant cells:**

   Plant cells also have thick cell wall therefore it is hard to transform them. The choice of selection markers is less diverse, nonetheless there are only a few markers which can be used in case of plants. The most important vectors are integrating plasmids. Methods for DNA introduction into cells are: transformation, infection, gene gun.

4. **Animal (mammalian) cells:**

   Animal cells are very diverse in types and without exception they are very sensitive, therefore handling and culturing them require expensive media and equipment. On the other hand, since many cell cultures are derived from human and other mammalian species, they are the most preferred models in experiments studying the structure and function of human genes. In recombinant DNA experiments, the most important vectors used for human cell transfection are plasmids, transposons, DNA, RNA viruses and artificial chromosomes. Methods for DNA introduction into mammalian cells are: transfection, lipofection, viral infection, electroporation and microinjection.

Bacterial growth curve

As we discussed above bacteria form colonies on the surface of solid media. However, bacteria can be cultured in liquid media as well. In a liquid culture the cells can access all the nutrients more effectively than from a surface of a gel, which increases the yield of the cells. The liquid culture is being continuously shaked, which provides equal distribution of nutrients and bacteria. As the cells are multiplying in the culture the optical density (OD) of the liquid is increasing and at a certain point it becomes turbid.

Bacterial growth curve - that is obtained by determining the change of optical density of a growing bacterial culture and plotting it as the function of time - can be separated to four phases. The graph on Figure 2.4 represents the growth phases of bacterial culture in a test tube, where the nutrients and the space are limited (batch cell culture). Lag phase occurs immediately after inoculation of the bacterial culture and represents a period of time when bacteria have to adapt to a new environment. During the logarithmic/exponential phase, cell divisions occur at a steady-state rate and the optical density of the culture is continuously increasing. When the cell density reaches a critical point and the nutrients become depleted in the media, the culture enters the stationer phase, when the cell division halts. After that the cells are starving and they will die, the metabolic processes will slow down and there will be more and more dead cells.

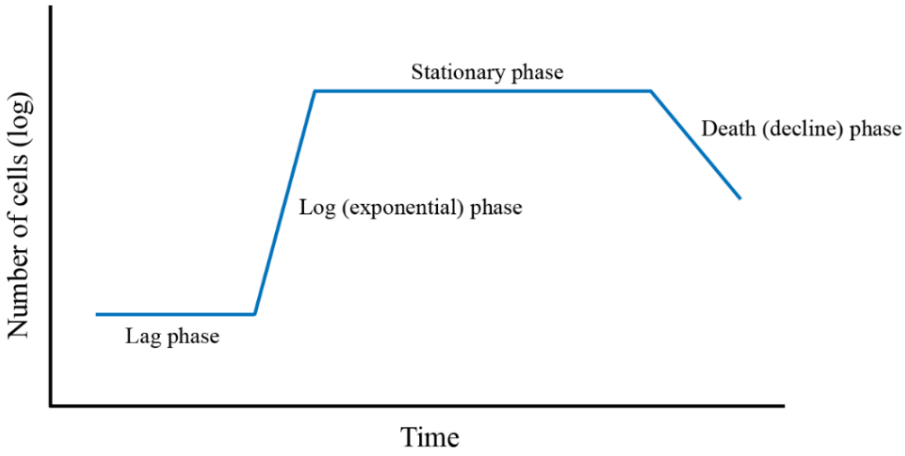


Figure 2.4 Bacterial growth curve. Bacterial growth can be separated into four phases: 1. Lag phase, 2. Log (exponential) phase, 3. Stationary phase, 4. Death (decline) phase. The optical density (OD) of a cell culture is directly proportional with the number of cells.

In the “log phase” of the cell culture, we can observe exponential bacterial growth, although it is often represented on a logarithmic scale, therefore it is referred to log phase, which is mathematically incorrect.

Growing conditions and the type of the media are important factors in determining when the bacterial culture reaches the maximal optical density. To determine the actual state of the culture, we use spectrophotometer. For instance, an *Escherichia coli* cell culture grown in a rich media at 37 °C is surely in the exponential phase of growth, if its optical density is between 0.4 and 0.8 measured at 600 nm. Cells

originated from cultures in the exponential phase, are the most suitable for transformation. It is important to note here that when bacterial cultures are used for other purposes, for example for protein production, as we will do in this course, induction of the protein expression should be done in that phase, too.

Practical workflow and protocol

1. Preparation E. coli competent cells

100 ml DH5α *E. coli* and Rosetta cells were grown overnight at 25 °C until they reached their optimal optical density for transformation. These bacterial cells are the most suitable for transformation at OD=0.5-0.8.

1. The optical density of the bacterial cultures is measured at OD_{600} by the lab assistant.
2. Each pair gets 15 ml from one culture in a 50 ml tube, which should be incubated on ice for 10 min.
3. Collect the cells by centrifugation (3,000 rpm, 10 min, 4 °C) and discard the supernatant!
4. Resuspend the cells in 2.4 ml ice cold TB buffer and incubate the suspension on ice for 10 min!
5. Centrifuge the samples for 10 min at 3,000 rpm, 4 ºC!
6. Discard supernatant and resuspend the cells in 1.2 ml ice cold TB buffer and incubate them on ice for 10 min. These steps made the bacterial cells competent for taking up the foreign DNA.
7. Transfer 200 ul Rosetta cells to an Eppendorf tube and add 14 ul DMSO to it to protect the cells from disruption during freezing. For fast freezing, put the Eppendorf tube containing Rosetta cells into liquid nitrogen for a few minutes, then store at -80 °C.

Competent cells are sensitive to temperature changes, so inappropriate handling will reduce the competency of the cells for DNA uptake. Therefore, it is important to always work on ice during handling competent cells.

2. Transformation of DNA into competent cells

1. Transfer 200 ul **DH5α** competent cells into a sterile Eppendorf tube and add 1 ul pET-Pfu plasmid DNA to the cells.
2. Incubate the samples on ice for 20 min!
3. Heat shock your sample at 42 °C for 90 sec.
4. Immediately after heat shocking, place the tube on ice for 2 min and then add 900 ul LB media.
5. Let the cells recover by incubating them at 37 °C for 30 min. During this incubation, the plasmid starts replicating and the antibiotic resistance genes are expressed.
6. Collect the cells with centrifugation (3,000 rpm, 3 min, RT) remove 1,100 ul supernatant and resuspend the cells in the remaining 100 ul LB.

7. Spread the 100 ul resuspended cells on LB-agar plates complemented with ampicillin antibiotic. After pipetting the cells in the middle of the agar plate, spread the cells with a sterilized glass rod.

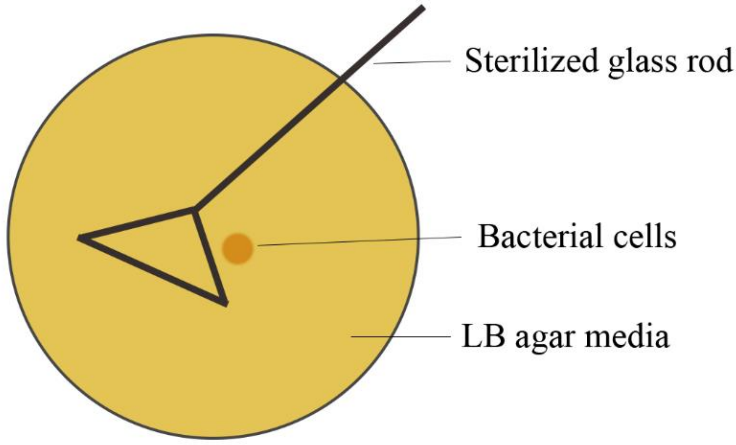


Figure 2.5. Spreading bacterial cells on LB plate complemented with antibiotic

8. Incubate the plates at 37 °C, overnight!

9. In the following day, calculate the transformation efficiency. The concentration of the transformed pET-Pfu plasmid DNA is 1500 ng/ul.

Materials

15 ml DH5α and Rosetta E. coli bacterial suspension (OD = 0.5-0.8)

Inoue-transformation buffer (TB)

- 55 mM $MnCl_2$ x 4 H_2O
- 15 mM $CaCl_2$ x 2 H_2O
- 250 mM KCl
- 10 mM PIPES-KOH pH 6.7

SOB medium

LB medium

1 piece of LB+ampicillin agar plate

Figure 2.6 Waterbath

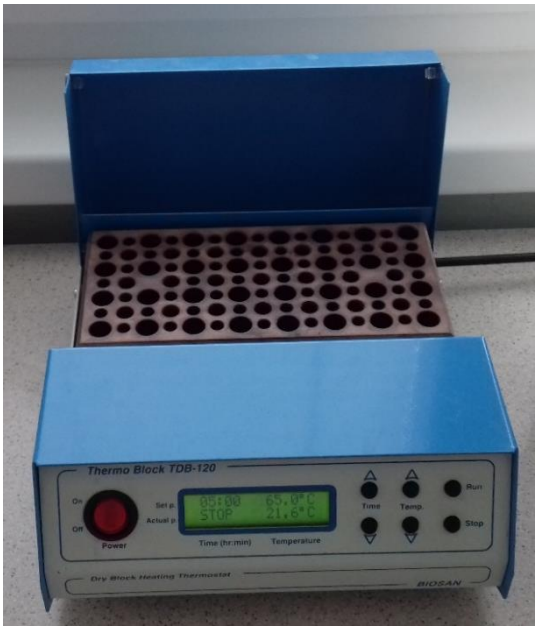


Figure 2.7 Thermoblock

Figure 2.8 Centrifuge

Figure 2.9 Refrigerated centrifuge for falcon tubes

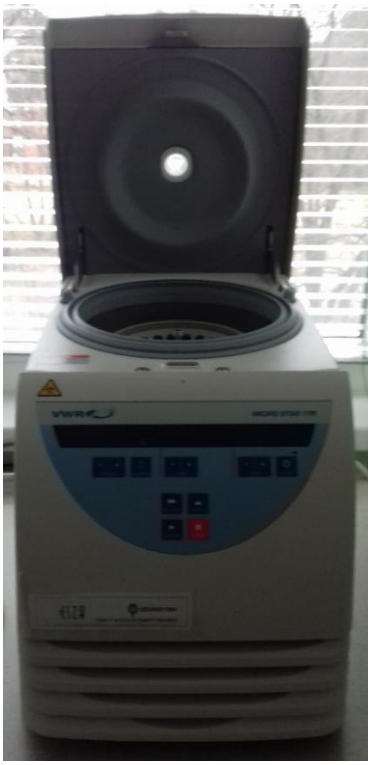


Figure 2.10 Refrigerated centrifuge for Eppendorf tubes

Figure 2.11 Vortex

Figure 2.12 Beaker

I. Lab report

Date: _____

Name: _____

Title: Generation of competent *E. coli* bacterial cells and transformation of pET-Pfu plasmid into DH5alpha *E. coli* cells

Aim:

_____

_____

Short summary (2-3 sentences about the experimental setup): We have prepared competent bacterial cells, which are suitable for taking up the foreign DNA. Then we transformed them according to Inoue's protocol.

Used materials (We should check all the solutions and materials before we start the experiment. We should know for what and why we use them during the experimental process.)**:**

- LB: liquid solution for growing E. coli bacteria
- LB-agar:

  _____
- SOB:

  _____
- TB:

  _____

  $MnCl_2$

(TB):

_____

$CaCl_2$ (TB):

_____

KCl (TB): ensures the appropriate ion concentration

Pipes pH 6.7 (TB): buffer, which ensures the appropriate pH

- Ice: We should take the samples on ice because room temperature decreases the efficiency of the transformation.
- pET-Pfu:

  _____

- DH5-alpha: a type of *E. coli* strain optimized for DNA transformation
- Rosetta: a type of *E. coli* strain optimized for protein expression
- ampicillin:

  _____

  _____

  _____

  _____

- liquid nitrogen:

  _____

- DMSO:

  _____

Changes in the protocol: (the experiment could be repeated only, if we write down everything):

_____

_____

Observations and Conclusion:

The OD of DH5alpha and Rosetta cells at 600 nm in the beginning of the experiment:

Calculation of transformation efficiency:

Questions

What are the minimal essential elements required for the function of a plasmid vector?

_____

_____

At which stage are the bacterial cells the most suitable for transformation?

_____

What is the difference between DH5 alpha and Rosetta cells?

_____

_____

_____

_____

How could you make bacterial cells competent for DNA uptake?

_____

What kind of transformation did we use in the practice?

_____

Why did we apply DMSO in case of freezing Rosetta cells?

_____

Chapter 3

Research project in molecular biology

2nd practice

Purification of pET-Pfu plasmid from transformed E. coli bacterial cells

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will be aware of the theoretical background of different DNA isolation and purification methods.

Skill:

2. They will know how to inoculate a bacterium colony into liquid LB media.
3. They will learn how to prepare plasmid from bacterial cells.
4. They will be able to calculate on their own the necessary volume of each component.

Attitude:

5. They will consciously inoculate the bacterial colony and prepare the plasmid DNA from the cells.

Responsibility and autonomy:

6. They will overview the role of each component necessary for plasmid DNA isolation.
7. They will be capable for transforming bacterial cells alone.

Purification of pET-Pfu plasmid from transformed *E. coli* bacterial cells

In the previous chapter plasmids have been introduced as tools of recombinant DNA technology that are frequently used as vectors, which can carry pieces of foreign DNA molecules. In order to use plasmids in the laboratory, as a first step they should be extracted from cells and separated from the other macromolecules of the cell.

In this chapter basic techniques suitable for this will be reviewed briefly.

For this we will give background information on:

- Methods used for cell lysis
- Methods applied for DNA purification

Hand on practice to these will be:

- Plasmid isolation from E. coli cells

Theoretical background

To start DNA isolation from biological sources, it is important to disrupt the cells to allow access for the desired DNA located inside the cells. Then all the small and large molecules should be removed since these will not be utilized but might interfere with further procedures. In practice it means that when it comes to plasmid isolation first the cells should be lysed in order to release the DNA then the plasmid molecules should be separated from the chromosomal DNA, RNA molecules and proteins. Many plasmid DNA isolation techniques have been developed, some of these uses specific material with secret composition, while other techniques are much simpler using only common reagents and can be completed in a short-time. Whichever technique is used, some basic rules should be followed and kept in mind.

These are the followings:

(1) The very large linear DNA molecules are sensitive to mechanical forces and can be broken into smaller fragments in case of vigorous handling. Since plasmids are relatively short and circular molecules, these can be hardly broken upon robust handling, but instead of that, the chromosomal DNA will be fragmented, thereby contaminating the plasmid DNA. Based on this, appropriate handling is indispensable during plasmid preparation.

(2) Nucleic acids remain in solution until water molecules surround them and form hydrate shell around them. If the water molecules are removed from this shell, the large polymers of nucleic acids will aggregate and form precipitates that can be sedimented from the solution by centrifugation. In the presence of sodium ions, 96 %

cold ethanol will disrupt the hydrate shell and results in the extraction of nucleic acids from the solution.

(3) Macromolecules can be degraded by specific digestive enzymes: proteins by proteases, DNA by DNases and RNA by RNases. These enzymes might get into samples as contamination from our hand, pipettes, reaction tubes or solutions. This should be avoided by using protease- or nuclease-free tools and reagents. On the other hand, some of these enzymes can be used during purification steps to remove the non-wanted types of macromolecules. In case of DNA preparation, generally Proteinase K is used to digest proteins and pancreatic RNase is used to destroy RNAs. Whenever any of these enzymes is used, it is important to make sure that it does not contain even a trace amount of contamination from the other one (for example DNase) since this might collapse the experiment.

(4) DNA molecules extracted from the cell can be stored in solution at -20 °C for an extended time. Since DNases require Mg^{2+} ions for their activity the buffer in which DNA is stored should always contain a chelating agent (EDTA) in a small concentration in order to prevent the activation of DNases that may remain in the sample.

Methods used for cell lysis

1. Digestion with enzymes, which degrade the cell wall (e.g. lysozyme, lyticase, proteinase K, pronase, digestive enzymes from the gut of snails for yeast cell disruption)
2. Treatment with detergents (e.g. SDS, Triton X-100, NP-40), which destroy the lipid membrane structure

3. Freezing-thawing cycles (e.g. liquid nitrogen – 37 °C), which produce ice crystals that destroy the cell membrane
4. Alkaline solution and organic solvent, which destroy the cell membrane
5. Osmotic pressure (e.g. high glucose concentration)
6. Mechanical disruption of cells (e.g. ultrasound, French press) – unlike all the above described ones, these techniques are not suitable for DNA preparation

The optimal method for cell lysis depends on the cell type and the purpose of the experiment. It is important to choose the right technique to the particular experiment. For instance, by changing the osmotic pressure mammalian cells can be lysed, but it has no effect on bacterial cells.

During cell lysis, it is important to use chelating agents (e.g. EDTA), which binds bivalent cations by this preventing the activity of the nucleases.

Removal of RNAs and proteins from DNA samples:

1. Several protein degrading enzymes (proteinase K, pronase) and RNA degrading enzymes (pancreatic RNase) can be used to remove these non-wanted macromolecule types from DNA preparations. To ensure their optimal activity the conditions of digestion (buffer, pH, ionic strength and temperature) should be set as required for the specific enzyme.

2. Extractions by a 25/24/1 ratio of phenol/chloroform/isoamylalcohol mixture: This method is based on the diverse solubility of proteins and nucleic acids or partially on the fact that this solution cannot mix with water, therefore it can be easily separated from the DNA containing aqueous solution. Proteins denatured by phenol and chloroform remain in the lower organic phase or form a precipitate in the so-called interphase, while DNA will be present in the upper aqueous phase. The distribution of the DNA between the two phases depends on the pH: under neutral conditions (pH 7), DNA is present in the aqueous phase, while under acidic conditions (pH 4.8), large amount of the DNA is found in the organic phase. On the contrary, RNA is dissolved in the aqueous phase in both cases. Phenol is generally used in combination with chloroform, because protein elimination is more efficient, when it is performed by two different organic solvents. Isoamylalcohol is added to the mixture of these organic solvents to improve separation of the upper aqueous and the lower organic phase. After phenol/chloroform/isoamylalcohol extraction a second extraction using only chloroform is recommended, since by this the traces of phenol remained in the aqueous phases can be completely removed. It should be noted here that both phenol and chloroform are highly dangerous solutions, which can cause serious damages if they get contact with the skin. Therefore, whenever it is possible, this type of extraction should be replaced by other much user-friendlier technique.

3. SDS extraction in the presence of univalent positive ions: Sodium dodecil-sulphate is a strong ionic detergent that can be easily dissolved but in case of low temperature, in the presence of potassium ions, it forms precipitate. After the addition of SDS to a cell lysate, in the presence of K^+ ions, large molecules of chromosomal DNA and proteins will be precipitated. On the other hand, the smaller plasmids remain in solution.

Precipitation of nucleic acids:

Precipitation with ethanol is the most commonly used method for collecting DNA (or RNA) from an aqueous solution. The precipitation is performed in the presence of monovalent cations, e.g. Na^+ or NH_3^+. Cations neutralize the negative charges of the DNA backbone and ethanol removes the hydrate shell of DNA molecules, which will be therefore aggregated and fallen out of solution. To set the optimal ion concentration for nucleic acid precipitation by ethanol the use of high concentration of ammonium-acetate is preferred, since it helps avoiding precipitation of dNTPs together with the DNA.

In addition, isopropanol can also be used to precipitate nucleic acids. In practice it is used mostly for RNA and for washing already precipitated DNA samples.

Polyethylene glycol (PEG) in a 10 % solution in the presence of 0.5-1 M NaCl can be also used to precipitate DNA from the aqueous solution.

The above described techniques are based on the removal of water hydrate shell, which promotes the precipitation of the DNA. However, it does not denature or damage the DNA or RNA structure, therefore after the precipitate has been collected by centrifugation, it can be dried and re-dissolved in the appropriate solution.

Techniques for obtaining highly purified DNA preparations

1. Using chromatographic matrix to bind DNA: Hydroxyapatite (a special form of calcium-phosphate) silica gel and several other types of material can be used to prepare matrixes to which DNA binds with high affinity. In fact, these types of matrixes are commonly used in commercially available DNA purifying kits. During purification, DNA is bound to the matrix that is generally placed into a small column while other contaminating molecules can be washed away. Then DNA can be released from the matrix and eluted by altering the salt concentration or the pH.

2. DNA purification by centrifugation based on CsCl-ethidium bromide density gradient: CsCl salt contains a high molecular mass metal atom (Cs) and can be dissolved in very high concentration. As a result of this similar density with CsCl solution can be prepared to that of DNA. Ethidium bromide is a fluorescent dye, which can intercalate between the DNA bases. Different amount of ethidium bromide can intercalate into linear, circular or superspiralised (supercoiled) DNA. Based on the the different density of the three forms of DNA molecules, they can be easily separated. During a

very high-speed centrifugation of a CsCl solution (60,0000 rpm or more), a density gradient is formed in the solution and if DNA with intercalated ethidium bromide is present in this, it will take the position that equals to its density. Traditionally this was the one and only way to obtain highly purified DNA preparations. However, this is a very time consuming and expensive technique, which has several other disadvantages, as well. Therefore, it is hardly used nowadays.

Plasmid preparation from bacterial cells by alkaline lysis:

A simple and quick plasmid preparation method applicable in case of bacterial cells, is the alkaline lysis method. One of the advantages of this method is that by this, a relatively high quantity of plasmid DNA (few micrograms) can be obtained from only a small amount of bacterial cell culture (1-2 ml). The purified plasmid DNA is clean enough to perform basic experiments (restriction digestion, transformation) with it. During this method, bacterial cells are lysed under alkaline conditions (by NaOH and SDS). In case of alkaline lysis, NaOH denatures both the chromosomal and the plasmid DNA, while SDS denatures the proteins. Then by using an acidic solution (e.g. acetic acid containing buffer) in a high concentration, the pH of the solution is restored to almost neutral. At this step, the small, supercoiled plasmid DNA molecules are quickly renatured. On the contrary, the large-sized chromosomal DNA forms precipitates together with the proteins denatured by SDS and K^+. Then the white precipitate can be easily separated from the plasmid containing supernatant by a centrifugation step. The plasmid DNA remains in the supernatant from which it can be precipitated by ethanol or isopropanol

and then can be collected by centrifugation. After careful removal of the alcohol, the pelleted DNA (that can be hardly visible) should be air-dried to completely get rid of the remaining ethanol, since it would interfere with further downstream reactions. Finally, the dried plasmid DNA can be re-dissolved in a small amount of buffer that has a neutral pH (7.0) and contains a small amount of chelating agent (e.g. EDTA).

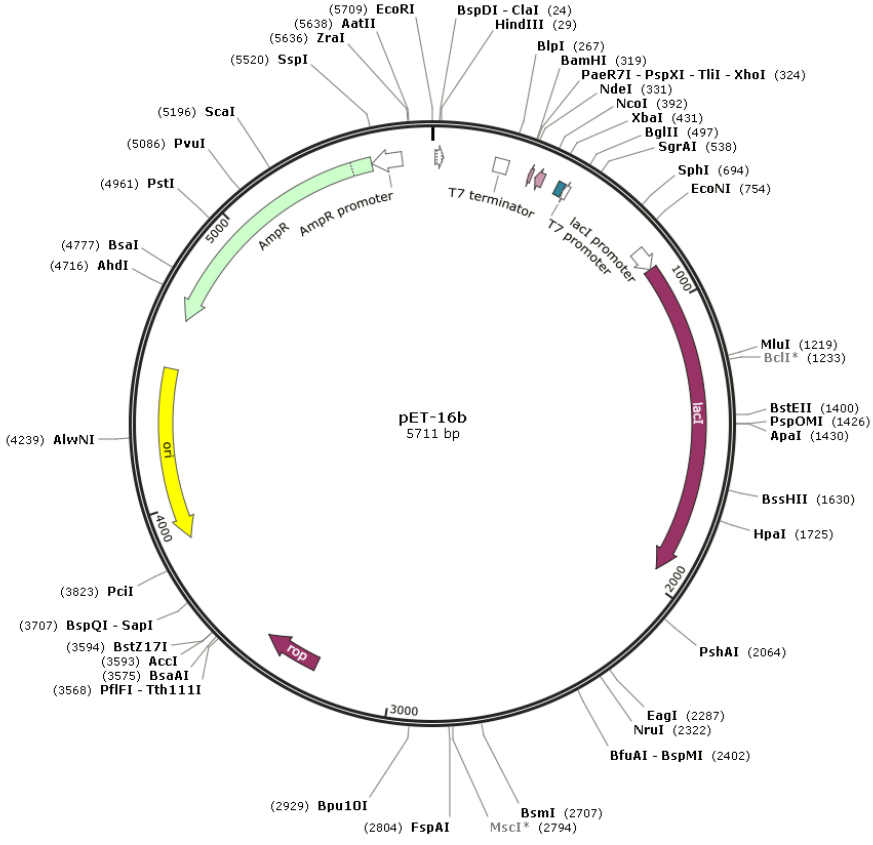


Figure 3.1 Restriction map of the pET-16b vector

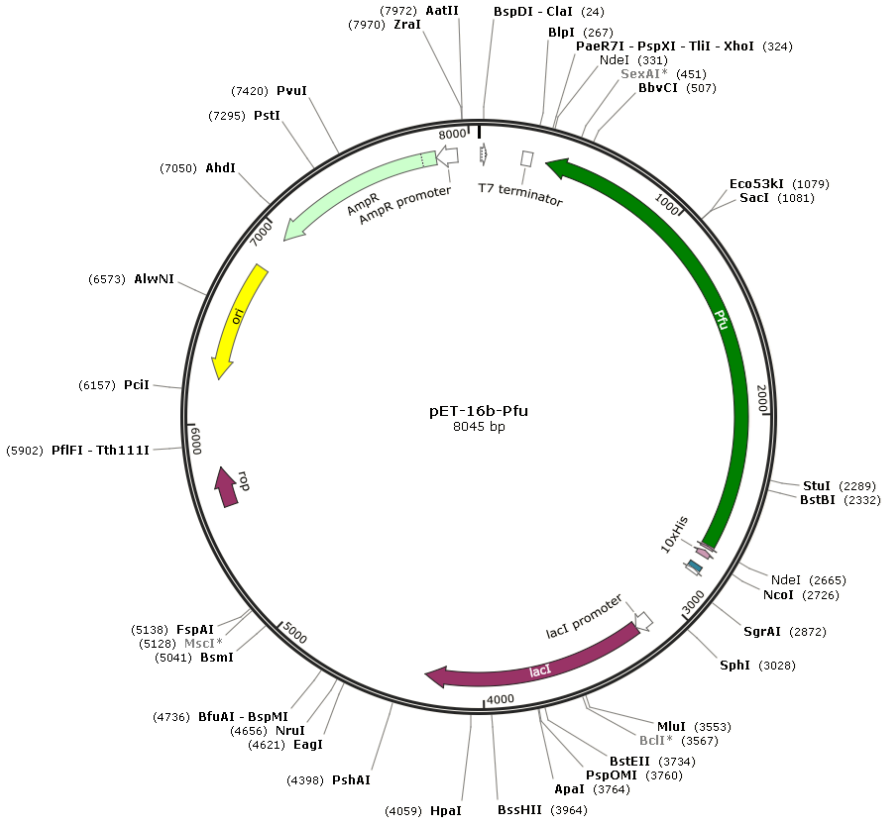


Figure 3.2 Restriction map of the pET-16b-Pfu plasmid

Following purification, the quality of the obtained plasmid preparation can be tested and the quantity of the plasmid DNA can be estimated by agarose gel electrophoresis. The same plasmid molecules can be present in different topological conformations (linear, relaxed circular and supercoiled circular forms). In the cells plasmids are present mostly in supercoiled form but during the preparation some of the plasmid will take the other two conformations, as well. This could be resulted by the breakage of a single phosphodiester bond, which leads to the occurrence

of the relaxed circular form, while breakage appeared in both polynucleotide chains results in the linearized plasmid form. These different topological isomers of a plasmid DNA can be easily separated by agarose gel electrophoresis (detailed description of agarose gel electrophoresis will be given in Chapter 4). On a gel the upper, most slowly migrating band represents the relaxed circular form of the plasmid DNA, while the lowest, most quickly migrating band corresponds to the supercoiled circular form of the plasmid (also called CCC from – covalently closed circular form). The supercoiled conformation is a compacted structure, which makes it capable to move faster in the agarose gel matrix than the relaxed, circular form. In the electric field, the linear form of the same plasmid migrates between the afore described two forms. In case of a plasmid preparation this form of the plasmid can be observed only in some cases when upon strong physical impact or digestion by contaminating enzyme(s), the linear form of the plasmid appears between the relaxed and circular forms (Figure 3.3 A). However, the position of the three topological isoforms can vary greatly relative to each other depending on the properties of the gel and the plasmid size. The actual size of a plasmid can be determined from the migration of the linear form since it has no higher order structure. During plasmid preparation obtained by the described alkaline lysis method a strong band can be usually detected at the bottom of the gel. This represents RNA contamination in the plasmid DNA preparation (Figure 3.3 B). By using RNase enzyme, it is possible to get rid of the RNA contamination from the purified plasmid DNA preparation. It is an important step, since RNA can disturb the further downstream reactions.

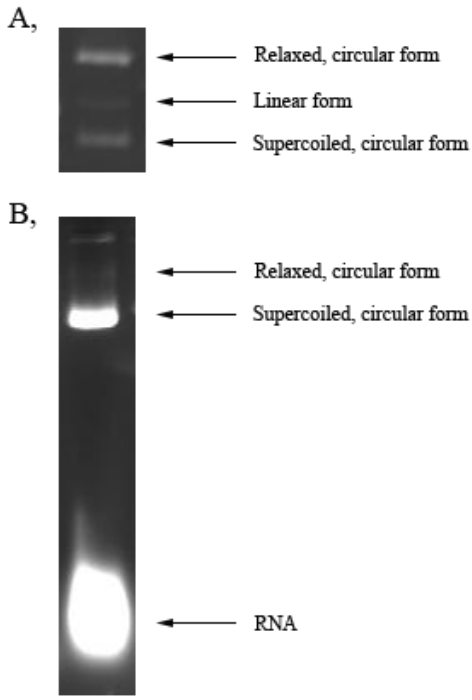


Figure 3.3 Photos of plasmids separated by agarose gel electrophoresis. A: The three topological isoforms of a plasmid. The supercoiled circular form is the fastest in the gel, since it has the most compacted form. The linear form occurs only upon vigorous mechanical forces or digestion and can be observed between the relaxed and the supercoiled circular forms. The relaxed circular form is the slowest migrating upper band. B: Agarose gel electrophoresis of a plasmid preparation before RNase digestion. At the bottom of the gel the strong band corresponds to RNA contamination. Note that in this preparation the linear and relaxed plasmid forms migrate very close to each other and an additional band can be seen at the very top of the gel. The latest consists of large fragments of chromosomal DNA that contaminate the plasmid preparation.

Practical workflow and protocol

During the practice you will purify pET-Pfu plasmid from 3 ml bacterial cell culture.

For this, the day before the practice you have to inoculate a colony from the LB agar plate obtained in the previous practice into 3 ml liquid LB media complemented with ampicillin. Let the cell grow in a shaker at 37°C, overnight.

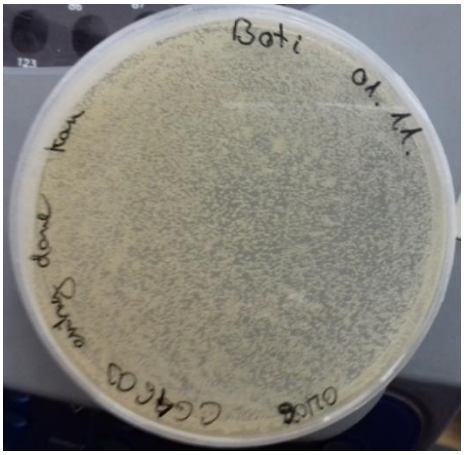


Figure 3.4 *E. coli* bacterial colonies

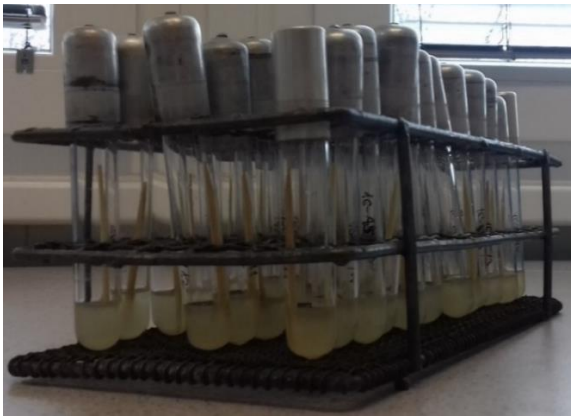


Figure 3.5 Overnight grown *E. coli* bacterial cultures

Protocol for plasmid preparation

Before starting the preparation, prepare the reagents and calculate the appropriate amount of each solution that you will need for the preparation!

I. Bacterial resuspension solution (BRS)

Required volume per sample = 150 ul

Components	c_{stock}	c_{final}	Dilution range	Volume
Glucose	500 mM	50 mM		
Tris-HCl pH 8.0	1 M	50 mM		
EDTA	500 mM	10 mM		
dH_2O				

II. Lysis Buffer (LS)

Required volume per sample = 250 ul

Components	c_{stock}	c_{final}	Dilution range	Volume
NaOH	2 M	200 mM		
SDS	10 %	1 %		
dH_2O				

III. K-acetate solution

Required volume per sample = 200 ul

Components	c_{stock}	c_{final}	Dilution range	Volume
K-acetate	5 M	3 M		
Acetic acid	100 %	11.5 %		
dH_2O				

IV. Tris-EDTA (TE)

Required volume per sample = 500 ul

Components	c_{stock}	c_{final}	Dilution range	Volume
Tris-HCl pH 8.0	1 M	10 mM		
EDTA	500 mM	1 mM		
dH_2O				

Steps of the plasmid preparation:

1. Transfer 1.5 ml bacterium suspension into an Eppendorf tube and centrifuge it at 13,000 rpm for 1 minute. Discard the supernatant and transfer the remaining bacterial culture into the same Eppendorf tube. Centrifuge it again at 13,000 rpm for 1 minute, then remove the supernatant completely by using a pipette.
2. Resuspend the bacterium cell pellet in 100 ul BRS. Mix it by vortexing to homogenize the cell suspension and to detect no clumps in it. Keep the sample on ice.
3. Add 200 ul LS buffer to the sample. Mix the solution gently by inverting the tube for three times and incubate it on ice for 5 min, until it turns to be transparent. This indicates that the cells have become lysed. DO NOT VORTEX the tube, since by that the chromosomal DNA could be fragmented and it will contaminate your plasmid DNA.
4. Add 150 ul of ice-cold K-acetate solution to your sample. Close the caps of the tube and mix the solutions by rapidly inverting the tube for a few times and let the sample incubate on ice for 5 minutes. A white precipitate will be formed, which contains the chromosomal DNA and the proteins.
6. Centrifuge the tubes for 10 min at 13,000 rpm.
7. Transfer the supernatant into a new Eppendorf tube. Avoid transferring of any precipitates from the bottom of the tube.
8. Repeat step 6 and 7 in order to remove all the precipitate.
9. Add 1 ml absolute ethanol to the clear supernatant to precipitate the plasmid DNA. Vortex gently and let the sample incubate on ice for 10 min. Collect the precipitated plasmid DNA by centrifugation (13,000 rpm, 10 min) and discard the supernatant. Remove the residual ethanol

and air-dry the DNA for a few minutes by opening the caps of the tubes or by using a vacuum concentrator.

10. Dissolve the plasmid DNA in 20 ul TE solution supplemented with RNase. Incubate the sample for 20 min at 37 ºC to let the RNase enzyme digest the RNAs being present in the sample.

11. Store the sample at -20 ºC until the next practice.

Figure 3.6 Vacuum concentrator

Materials

Stock solutions

10 mg/ml RNase

1 M Tris-HCl pH 8.0

absolute ethanol

500 mM EDTA

10 % SDS

500 mM glucose

2 M NaOH

5 M K-acetate

100 % acetic acid

Working solutions

1. BRS (bacterial resuspending solution)

- 50 mM glucose
- 50 mM Tris-HCl pH 8.0
- 10 mM EDTA

2. TE buffer

- 10 mM Tris-HCl pH 8.0
- 1 mM EDTA

3. Lysis solution

- 200 mM NaOH
- 1 % SDS

4. K-acetate solution

- 3 M K-acetate
- 11.5 % acetic acid

II. Lab report

Date: _____

Name: _____

Title:

_____

_____

Aim:

_____

_____

Short summary (2-3 sentences about the experimental setup):

_____
_____
_____
_____

Used materials (You should check all the solutions and materials before you start the experiment. You should know for what and why we use them during the experimental process.)**:**

- BRS:_____
  - glucose:_____
    _____
  - Tris-HCl:_____

_____

- EDTA:_____

_____

- LS:_____
  - NaOH:_____

_____

  - SDS:_____

_____

- K-acetate solution:_____
  - K-acetate:_____

_____

  - Acetic acid:_____

_____

- Ethanol:_____

_____

- TE:

  Tris-HCl:

  _____

  _____

  EDTA:_____

  _____

- RNaseA:_____

_____

Changes in the protocol: (the experiment could be repeated only, if we write down everything):

Observations and Conclusion:

Questions

List at least three methods suitable for cell lysis!

_____

Which type of lysis method did we use during the practice?

_____

Which desired gene is encoded on the pET-16b vector in our case?

_____

Why did we inoculate the bacterium colony into ampicillin complemented LB media?

_____

How did we get rid of the chromosomal DNA and proteins during plasmid preparation?

_____

_____

How did we precipitate the plasmid DNA?

_____

_____

Chapter 4

Research project in molecular biology

3rd practice

Agarose gel electrophoresis of pET-Pfu plasmid and Transformation of Rosetta cells

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will be able to understand the background of agarose gel electrophoresis.
2. They will understand how it is possible to visualize the plasmid DNA on agarose gel.
3. They will learn the basics of cloning procedure.
4. They will understand the differences between DH5alpha and Rosetta cells and why Rosetta cells are suitable for protein production.
5. They will be aware of the operation of Lac operon system in details.

Skill:

6. They will be able to recognize and distinguish the different forms of plasmid on agarose gel.
7. They will be capable for analyzing an agarose gel photo on their own.

Attitude:

8. They will inquire about the analysis of gel photos and transformation of bacterial cells.

Responsibility and autonomy:

9. They will be capable for transforming Rosetta cells on their own, following the protocol.
10. They will keep the safety instruction for gel casting.

Agarose gel electrophoresis

In this chapter agarose gel electrophoresis will be introduced that is one of the foremost frequently used separation technic in laboratories working with nucleic acids.

The basics of the technology dealing with foreign protein expression in bacteria are also discussed in this chapter.

For these, background information can be found here on:

- Agarose gel electrophoresis
- Gene expression regulation in bacteria
- Bacterial expression systems

Hands on practice will be:

- Preparation and running of agarose gel
- Analysis of plasmid quality by gel electrophoresis
- Transformation of expression plasmid to specific host cells

Part I: Agarose gel electrophoresis

Theoretical background

Agarose gel electrophoresis is suitable for the separation and size determination of DNA fragments. This method is suitable for the analysis, identification and isolation of DNA fragments. By standard gel electrophoresis, 0.2-50 kb sized DNA fragments can be easily separated although different agarose concentrations (0.8 to 3 %) are optimal for different ranges. By dissolving agarose in a specific buffer upon heating, linear molecules of polysaccharide polymer form a molecular mesh, which has comparable pore sizes to that of DNA fragments. When this gel is placed into electric field, charged molecules will move towards the oppositely charged pole. In practice agarose gels are placed in a horizontal arrangement by submerging the gel in a slightly alkaline buffer. DNA fragments (generally a mixture of those) are loaded into wells created close to the negative pole and when the electricity turned on the negatively charged DNA fragments migrate (run) towards the positively charged anode. In order to detect the position of fragments, fluorescent intercalating dye, most frequently ethidium bromide, is added to the gel. It is important to know that ethidium bromide (EtBr) is a mutagenic agent, since it can intercalate between the base pairs of the DNA! *You should be careful with handling those solutions and gels, which contain this dye, therefore always use gloves and lab-coat, and pay attention to avoid contamination of work bench, pipettes and other laboratory equipment with EtBr.* Due to the fluorescence of the EtBr under UV light, even a few ng of DNA could be detected. Gel images

can be recorded by using UV-light camera assemble for the analysis of results obtained from gel electrophoresis. The sizes of DNA fragments can be determined based on their mobility on the gel by comparing that to the mobilities of fragments with known sizes, called DNA ladder. DNA can also be isolated from the agarose gel and used for further experiments. In fact, this is the only way by which specific fragments from a mixture of fragments can be recovered for the usage of further genetic engineering.

Preparation of an agarose gel

First, the appropriate amount of agarose should be dissolved in electrophoresis buffer. For this, the required amount of agarose should be placed in a flask and after that the appropriate buffer should be added to it (the order is important!). Then the suspension should be heated until the agarose is completely dissolved (until the solution is clean and transparent). Since the inhalation of evaporated EtBr is very dangerous, the solution should be cooled down until it is hand-warm before the addition of it. After that the appropriate amount of EtBr should be added to the solution, then it should be mixed well but carefully to avoid bubble formation. As a next step, the EtBr containing solution should be poured into a gel tray with a comb placed in the correct position. When the agarose is cooled down, the gel is whitish-coloured. Gel can be kept for several hours wrapped in folia or overlapped with buffer or it can be used immediately.

Factors influencing the migration of DNA fragments in agarose gel:

1. *Electric field*

   DNA fragments are moved within the gel according to the electric potential. In slightly alkaline or neutral buffer the negatively charged DNA fragments migrate towards the positive pole. Electrophoresis can be performed at a wide-range of voltage gradient (0.25-7 V/cm). The higher voltage naturally results in quicker migration, but it also decreases the resolution of the gel and very high current could melt and destroy the gel. In practice 5 V/cm is the most frequently used voltage.

2. *DNA size:*

   In gels, larger DNA molecules migrate slower then shorter ones. The explanation of this is that for larger molecules it is more difficult to get through the pores of the gel. Note, that the driving force of the movement, such as the voltage difference between the poles of the power supply, exerts the same dragging force to a unique size of DNA fragment independently of the size of it, since the density of charges uniformly distributes in the polymer.

3. *DNA structure:*

   Relaxed circular, linear and superhelical circular DNA molecules consisting of the same nucleotides in identical order (such as topological isomers of the same DNA) migrate in the agarose matrix with different rates (see in Chapter 3).

4. *Electrophoretic buffer:*

   The mobility of the DNA also depends on the compounds and the ion concentration of the electrophoretic buffer. If the ion concentration is too high (e.g. if somebody uses 50x buffer instead of 1x), the current will be too high, thereby leading to high amount of heat generation. In the worst case the gel could melt, therefore the DNA could be denatured.

5. *Agarose concentration:*

   The density of the agarose gel determines the sizes of the pores. Gels with different concentrations have different separation ranges:

Amount of agarose	Separation range (to linear dsDNA)
0.7 %	0.8-12 kb
1.0 %	0.5-10 kb
1.5 %	0.2-3 kb
2.0 %	0.1-2 kb

Table 4.1 Relationship between agarose concentration and the separation range of agarose gel

Practical workflow and protocol - Part I - Agarose gel electrophoresis

1. Close the two edges of the plastic gel tray with the rubber gasket ends and put the casting tray on a horizontal surface. Place the comb into the tray. The comb serves to form the wells into which the samples will be loaded.
2. Prepare 100 ml of 1 % agarose gel: measure the appropriate amount of agarose and put it in a glass suitable for heating in a microwave oven, then add the appropriate amount of diluted TAE buffer to it (1x TAE).
3. Heat the suspension in a microwave oven until the agarose particles are dissolved. Swirl the glass gently time after time. *Be careful, since the agarose solution can be easily overheated. It means that the solution can be heated above its boiling point without triggering gas formation inside the liquid. However, upon physical impact burst like boiling of the hot agarose solution happens, which can cause an accident.* When the agarose has been completely dissolved, cool the solution to 40-50 ºC.
4. Add EtBr to reach 0.5 ug/ml final concentration to the agarose solution and swirl it gently. *Be careful because EtBr is a strong mutagen and carcinogen! Wear gloves at each time you work with a solution, which contains this dye!*
5. Pour the EtBr containing agarose solution slowly into the casting tray. The gel should be 5-6 mm thick. Be careful to avoid and if necessary remove air bubbles from the gel, because those will disturb the migration of DNA fragments.

6. Let the gel solidify. It takes about 20-30 minutes depended on the concentration of the agarose gel and the air temperature of the laboratory. Then carefully remove the comb from the gel and the rubbers from the two edges of the casting tray. Put the tray with the gel into an electrophoresis tank. Pay attention to the right direction: the wells for the samples should be located close to the connection attached to the negatively charged cathode.

7. Pour 1xTAE buffer into the electrophoresis tank to cover the gel with to 2-4 mm liquid.

8. To prepare your sample for loading, mix 3 ul isolated plasmid DNA + 7 ul dH_2O + 2 ul 6x loading buffer in an Eppendorf tube. Then load your sample into a well of the gel.

9. Put the cover onto the electrophoresis tank and set the power supply to 100 V. Let the gel run until the bromophenol blue dye migrates to about the 2/3 part of the gel.

10. Switch off the power supply, take out the gel and look at it under UV light. Record the image by taking a photo from the gel. Do not forget to wear gloves whenever you handle the gel, electrophoresis chamber or buffer, to avoid contamination with EtBr.

Figure 4.1 Electrophoresis tank for agarose gel electrophoresis

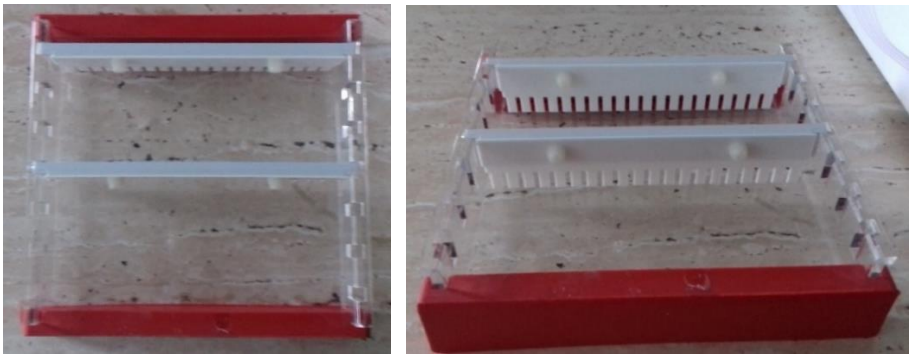


Figure 4.2 Casting tray with the inserted comb

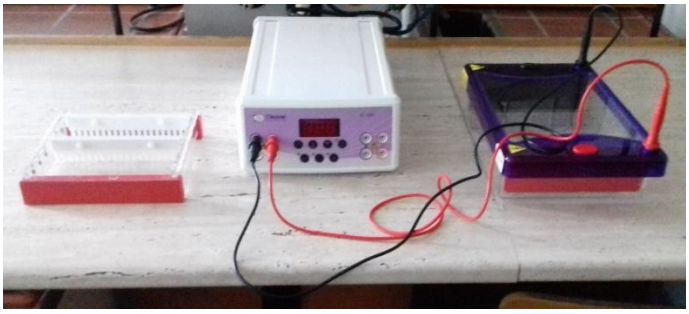


Figure 4.3 Equipment required for horizontal agarose gel electrophoresis: From left to right: casting tray with comb, power supply, electrophoresis tank connected to the power supply.

Figure 4.4 Gel documentation system

https://commons.wikimedia.org/wiki/File:OMLW_Gel_doc.jpg

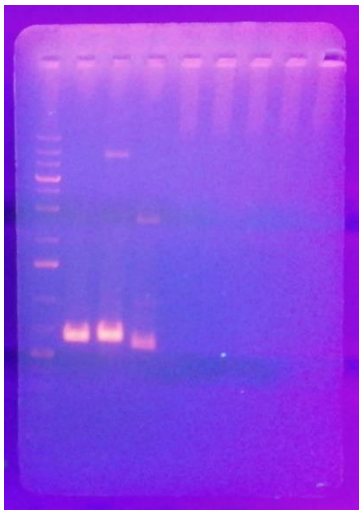


Figure 4.5 Image of an agarose gel under UV light

Analysis of plasmid preparation quality and plasmid structure by gel electrophoresis

In order to test the quality of the purified plasmid preparation we analyse the sample by agarose gel. By this we will get information on the amount of the obtained plasmid and also on the ratio of different forms of the plasmid in the sample and chromosomal DNA contamination, which might be present in the sample.

By electrophoresis we can also obtain information on the structure of the plasmid by using restriction endonucleases. On Figure 4.6 schematic representation of the structures of two plasmids is shown. One of them is an empty vector, while the other one contains an inserted region (indicated with red). To show that the plasmid contains the insert we can cleave it by a restriction endonuclease, which in this case the NheI enzyme. As a result of the digestion, different sizes of DNA fragments will be generated, which could be visualized on an agarose gel. Note that knowing the position of the cleavage sites of this enzyme both in the vector and in the insert, a simple digestion and electrophoresis can provide information about whether the insert is present in the plasmid, the size of the insert is correct (such as, the insert is what we expected) or the insert is localized in the plasmid in the right orientation.

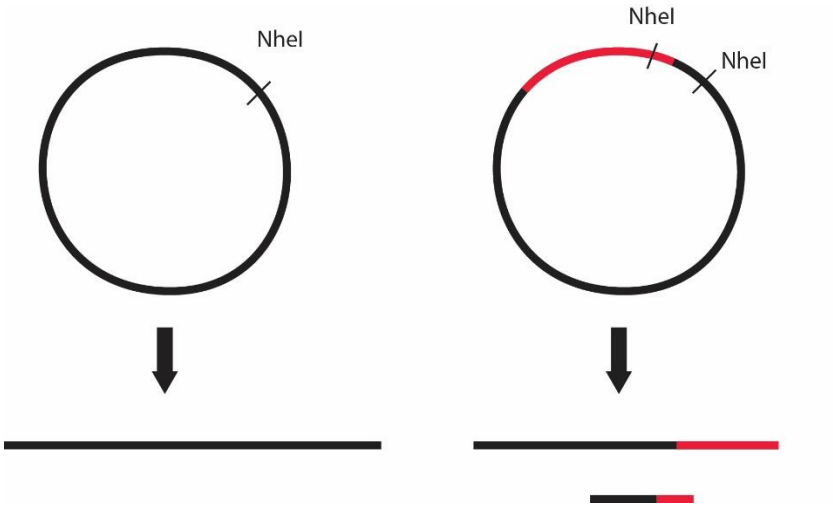


Figure 4.6 A plasmid containing only one site of a restriction endonuclease (e.g. NheI) is linearized upon restriction digestion (left), while a plasmid in which there is an insert with a restriction site of the same enzyme will give rise to two fragments (right). The sizes of the fragments give valuable information about the structures of the plasmids.

A practical example:

In a cloning experiment, three transformed bacterium colonies were selected and plasmid DNA was isolated from each of them. To determine whether the cloning process was successful (i.e. the plasmid contains an inserted region), restriction endonuclease digestion was performed.

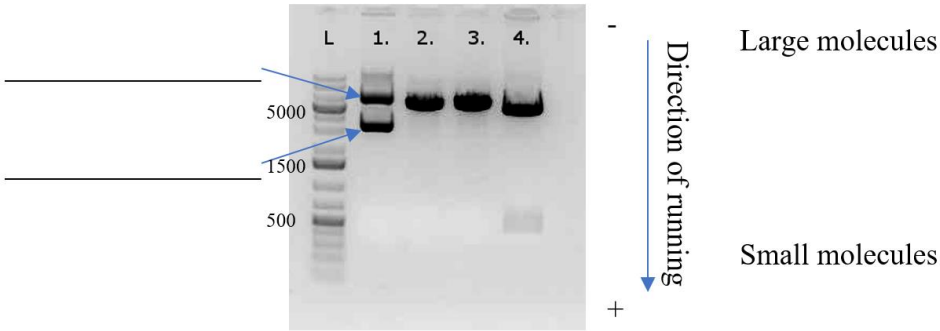


Figure 4.7 Electrophoretic separation of plasmids from a cloning experiment: L: ladder (molecular weight marker), 1: plasmid non-treated with restriction enzyme 2-4. plasmids digested with NheI enzyme.

Results from an agarose gel electrophoresis is represented on Figure 4.7. "L" stands for "Ladder", which is a mix of known sized linear DNA molecules. Sample "1" is a non-treated plasmid: we can observe the supercoiled and the circular forms. **(Identify and label them on the figure!)**

Samples from "2" to "4" were digested with NheI. We can observe that in samples "2" and "3" molecules with the same size are visible, but in sample "4" two bands can be detected.

When the goal of a cloning experiment is to obtain a specific fragment, the sizes of the expected DNA fragments can be calculated. The size of the vector is known from the sequence. The observed fragment sizes can be determined by comparing the signals to the ladder. If the calculated and observed results are in accord with each other, the cloning process was successful. In the given example, it was known that the desired

plasmid with the insert has two NheI restriction sites in 500 bp distance from each other, while the empty plasmid contains only one. Furthermore, the size of the empty plasmid is 5,500 bp.

Which one is the positive clone? Why?

Part II: Transformation of Rosetta cells
Basics of gene expression regulation in bacteria, and systems for foreign protein expression in bacteria

Basics of gene regulation in bacteria – the Lac operon

The basics of gene regulatory mechanisms characteristic for bacteria were discovered by Francois Jacob and Jacques Monod in 1961 by their studies on the regulation of lac operon. They showed that in bacteria the transcription of genes involved in the same metabolic pathways is tightly coordinated. This is achieved by the arrangement of these genes into a common transcription unit, called operon from which a polycistronic mRNA containing the coding sequences of the structure genes is transcribed. The intensity of prokaryotic transcription is depended on the interactions among different types of molecule. Some of these are proteins, such as the RNA polymerase, repressor, catabolite activator protein (CAP/CRP), while others are specific parts of the DNA, such as promoter, operator, CAP binding site. The third type of these molecules is designated as inducers (allolactose or isopropyl β-D-1-thiogalactopyranoside (IPTG)).

The products of the lac operon ensure the metabolism of lactose, which is a disaccharide catabolite. If glucose is present in the media, bacterial cells will metabolize it first, since glucose is an easier metabolizable catabolite than lactose. Therefore, the regulation of the lac operon ensures that the genes involved in it are not expressed if lactose

metabolism is not essential (in the absence of lactose and in the presence of glucose). However, in the absence of glucose and in the presence of lactose, the lac operon is switched on and the genes encoded the enzymes responsible for the lactose uptake and conversion are produced in the cells. This simple mechanism gives the possibility to the cells to choose the easier metabolizable carbon source from the environment and produce proteins only when they are necessary.

Note that this regulation also means that two types of regulations act on the lac operon:

1, Negative regulation, which is mediated by a repressor protein (LacI). This turns off the operon in the absence of lactose.

2, Positive regulation, which is mediated by an activator protein (CAP/CRP). This turns on the operon in the absence of glucose.

The two types of regulation can simultaneously act on the operon resulting in the fine-coordination of the lac operon.

Components of the lac operon:

Structure genes:	1, lacZ (β-galactosidase)
	2, lacY (permease)
	3, lacA (thiogalactoside-transacetylase)
Regulatory regions:	1, Promoter (lacP)
	2, Operator (lacO)
	3, CAP binding site

The lacI gene encodes the repressor protein of the lac operon. The repressor binds to the operator region and inhibits the transcription of

the operon. The repressor protein can also bind to allolactose. In fact, the affinity to this small molecule is higher than it is to the DNA. Therefore, if allolactose is present, the lac repressor binds to it, which results in a conformational change in its structure. This conformational change inhibits the binding to the lac operator and the repressor protein dissociates from the regulatory region. When the repressor protein has dissociated, the RNA polymerase can transcribe the genes of the operon. Due to this, allolactose is an inducer of the lac operon. Instead of allolactose, other inducer molecules, such as isopropyl β-D-1-thiogalactopyranoside (IPTG), can be used in the laboratory, which acts similarly, since it was designed to mimic the properties of allolactose, but it cannot be digested by the bacteria.

The repressor protein is always present in the cells in a few, approximately 10 copies. Its synthesis is regulated by its own promoter region ($lacP_I$), separately from the structure genes. The tetrameric repressor protein constitutively binds to a specific site of the regulatory region. This is the operator region (lacO), which overlaps with the transcription start site (-5…+21) and with the binding site of the RNA polymerase.

Structure genes:

The three structure genes of the lac operon (lacZ, lacY, lacA) form one transcription unit with a shared promoter region (lacP). This enables the simultaneous transcription of the three genes into one polycistronic mRNA, which serves as information for the parallel translation of the

three proteins. The amount of the newly synthesized mRNA depends on the available carbon sources.

The lacZ gene encodes the β-galactosidase enzyme, which cleaves the lactose into glucose and galactose. The active form of the β-galactosidase is a 500 kDa tetramer. In the cells, a low amount of β-galactosidase enzyme is always present, which first converts the lactose into allolactose. The lacY gene encodes the permease enzyme, which is a membrane-bound protein and is responsible for the lactose uptake through the cell wall. Similar to the β-galactosidase, a low amount of permease is always necessary for the lactose uptake.

The lacA gene encodes the thiogalactoside-transacetylase, which exact role is still unknown.

DNA Regulatory regions (cis elements) of the lac operon

LacP is the promoter region, which is the binding site of the RNA polymerase. LacP is a weak promoter, since it does not have strong -35 and -10 consensus sequences. For its high-level transcriptional activity, the presence of an activator protein is necessary.

LacO is the operator region that is the binding site of the repressor protein. It is located between the structure genes and the RNA polymerase binding site (lacP). The presence of the repressor protein does not inhibit the binding of the RNA polymerase to the promoter region, but the transcription cannot be started, because LacI creates a block in the way of the polymerase.

The CAP binding site is the DNA region where the activator protein binds to. The CAP (catabolit activator protein)/CRP (cAMP receptor protein) binds to this site, if the cAMP level is high in the cell. The cAMP level changes in opposite to glucose concentration: if the glucose level is low, the cAMP level is high. The CAP-binding site is located in the close proximity of the promoter region and when the CAP-cAMP complex binds to it, it helps for the binding of the RNA polymerase to the lacP region. Therefore, this site has a main role in the positive regulation, which depends on the presence of glucose.

		Lactose	
		+	-
Glucose	+	Lac repressor does not bind CAP does not bind Low level of TC	Lac repressor binds CAP does not bind No TC
	-	Lac repressor does not bind CAP binds High level of TC	Lac repressor binds CAP binds No TC

Table 4.2 Summary of the regulation of the Lac operon. TC = transcription

The activity of the Lac operon under different conditions

1, In the presence of lactose:

Allolactose binds to the repressor tetramer, which results in a conformational change in the structure of the repressor protein, thereby inhibiting its binding to the operator region. RNA polymerase can start the transcription from the promoter region. Since the RNA polymerase has been already bound to the promoter region, transcription can start immediately.

2, In the presence of glucose:

In the presence of glucose lactose is not necessary for the cells, the CAP activator protein does not bind to the CAP binding site and the transcription of the lac operon is not activated. Thus, if there is a low level of lactose in the media, the lac operon can operate only in a low basal level.

3, In the absence of glucose:

When the glucose level is low, the cells are starving and the cAMP level increases. Under such conditions if lactose is present the transcription of the lac operon can be strongly induced. As a result of high cAMP concentration, cAMP binds to its receptor, which is activated by the CAP protein, thereby inducing its binding to the CAP binding site upstream from the RNA polymerase binding site. The binding of the

CAP-cAMP results in a conformational change in the DNA, which activates the transcription of the lac operon.

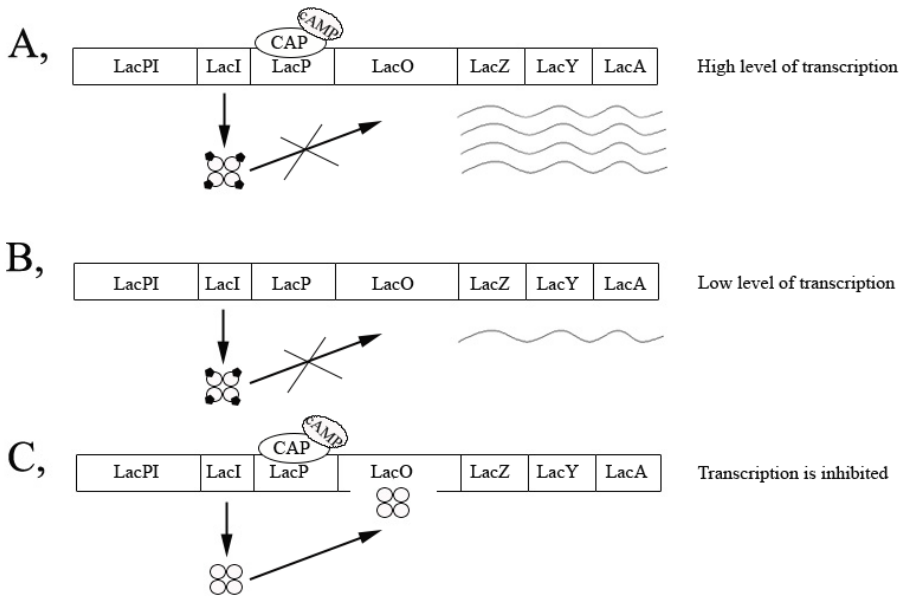


Figure 4.8 The schematic representation of function of the Lac operon. The activity of the operon is shown (A): in the presence of lactose, but in the absence of glucose; (B): in the presence of both glucose and lactose, (C): in the absence of lactose, but in the presence of glucose

pET expression system

Being aware of the regulation of the basic mechanisms of gene expression in bacteria made it possible to exploit this system and use bacterium cells to produce the desired proteins. By *in vitro* DNA recombination, several systems suitable for this have been developed.

These expression systems generally consist of specifically modified bacterial host cells and so-called expression plasmid vectors.

The vectors serve to accept the desired protein coding gene and ensure its high expression level in the cell. The specific host cells contribute to the high expression level and make possible the easy production of the protein.

During the practice, we will use the pET (**p**lasmid for **E**xpression by **T**7 RNA polymerase) protein expression system. This was developed by Studier and his co-workers in 1986. The pET system consists of bacteriophage (T7) elements to produce high-level of foreign protein in *E. coli* cells.

The principles of the expressing system are the followings:

- T7 bacteriophage RNA polymerase is highly selective to its own promoters, which means that it does not recognize the promoters of the host *E. coli* cells,
- T7 polymerase works approximately 5 times faster than the *E. coli* RNA polymerase, which allows the synthesis of a higher amount of mRNA,
- the promoter region recognized by the T7 RNA polymerase can be fused with elements of the lac operon, by this making the regulation of the heterologous gene expression possible.

If the *E. coli* cells are transformed with an expression vector in which the expression of the gene is controlled by a T7 specific promoter, only the T7 polymerase is capable for transcribing the gene. The first generation of these plasmids are called pET vectors.

For using these vectors, specific cells are required in which the T7 polymerase can be found. In the BL21 (DE3) *E. coli* cells the gene that

encodes the T7 polymerase is incorporated into the genome. In this bacterial strain, the expression of T7 RNA polymerase is inducible by the addition of lactose or the lactose analogue IPTG. In this system, the expression of the T7 RNA polymerase is regulated by the inducible promoter of the lac operon (lacUV), which allows the inducible induction of the T7 RNA polymerase. Under normal circumstances, the lacI repressor protein monomers encoded by the host genome form a tetramer and bind to the operator region of the Lac operon inhibiting the transcription of the T7 RNA polymerase. During induction, the exogenously added lactose or IPTG binds specifically to the repressor protein, which results in the release of it from the lac operator region. This will lead to the synthesis of the T7 RNA polymerase, which will bind specifically to its own promoter region being present on the pET vector. The expression of the heterologous protein is also regulated by elements of the lactose operon on the plasmid, making heterologous protein expression even more well-regulated.

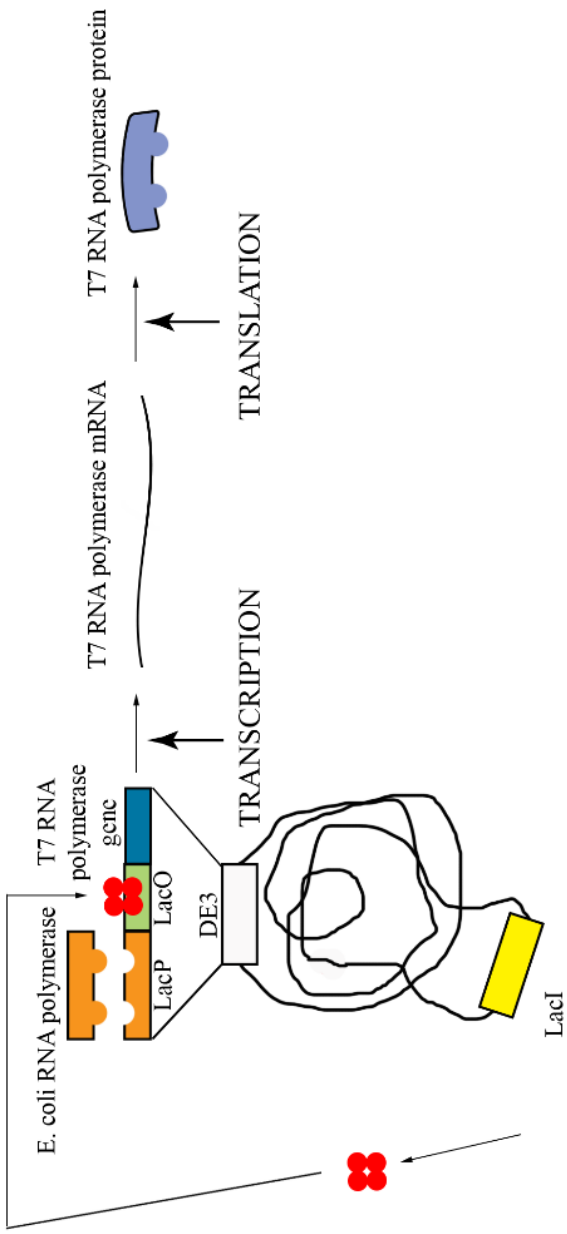


Figure 4.9 The schematic structure of T7 expression system

Eukaryotic protein expression in Rosetta cells

Rosetta cells were developed from the BL21 *E. coli* strain. These cells utilize the above described properties of BL21 - such as encoding T7 polymerase with the regulatory elements of the lac operon system in their genome - for efficient production of foreign proteins in bacteria, but in addition they have further features that make them useful for the expression of eukaryotic genes, as well.

During translation, tRNAs carry the amino acids and by recognizing the codon triplets direct their incorporation into the protein chain according to the mRNA sequence. Since there are 61 amino acid carrying tRNAs and only 20 amino acids, most of them belong to more than one tRNA. On the other hand, specific amino acids are encoded by more than one triplet (codon) and the different triplets are used with different frequencies. The term of codon usage describes what the frequency of given codons is in a particular organism. The distribution of different kinds of tRNA in a cell is proportional to the frequency of the codon usage: low number of tRNA belongs to rare codons, while tRNAs belonged to frequent codons occur the most often in the cells. However, different codon usage is observed between prokaryotes and eukaryotes, which can influence protein production. Eukaryotic genes contain many codons, which might be rare in bacteria and therefore high amount of these proteins cannot be produced in bacterial cells.

To bypass this problem, the number of the tRNAs, which are rare in bacterial cells, should be increased. In the case of Rosetta cells, the solution for this is that they contain pRARE plasmids, which encode

many rare tRNAs. As a result of that, the translation of eukaryotic proteins can be easily achieved in this cell line.

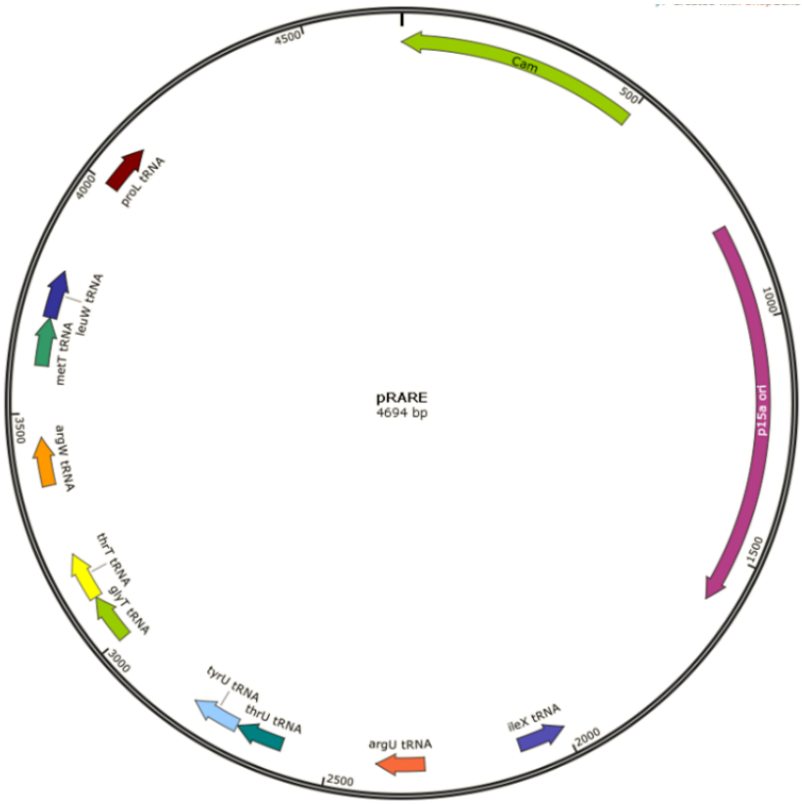


Figure 4.10 Restriction map of pRARE plasmid

Practical workflow and protocol - Part II – Transformation of Rosetta cells

1. Let the competent Rosetta cells thaw on ice and add 5 ul plasmid DNA to 200 ul of cells.
2. Incubate the sample on ice for 20 minutes.
3. Heat shock the sample for 35 seconds in a 42 °C water bath.
4. Put the sample back on ice and add 1 ml LB medium to it.
5. Incubate the sample in a 37 °C water bath for 30-60 min.
6. Collect cells by centrifugation (3,000 rpm 3 min, RT) and discard 1 ml from the supernatant and resuspend the cells in the remaining LB.
7. Spread the sample onto a LB + Amp (ampicillin) + Cam (chloramphenicol) plate!

In this case, we should spread the cells onto LB-agar medium complemented with two types of antibiotic – ampicillin and chloramphenicol. The ampicillin resistance gene is encoded on the pET-Pfu plasmid, while chloramphenicol resistance gene is present in the pRARE plasmid. Therefore, we should use both antibiotics: ampicillin for selecting only those cells, which carry the pET-Pfu plasmid, chloramphenicol for the selection of Rosetta cells, since only these cells could express the pRARE plasmid. At the previous transformation experiment we used the DH5α cells, which did not contain pRARE plasmid, but carried the pET-Pfu plasmid. Therefore, in that case ampicillin alone was required for the selection.

Materials

50xTAE buffer:

- 2 M Tris-acetate
- 50 mM EDTA (pH 8.0)

6x loading buffer:

- 60 % glycerol
- 60 mM EDTA
- 10 mM Tris-HCl (pH 7.7)
- 0.06 % bromophenolblue

Agarose

Ethidium bromide (0.5 mg/ml)

DNA 1 kb plus molecule ladder

Inoue-transformation buffer (TB)

- 55 mM $MnCl_2$ x 4 H_2O
- 15 mM $CaCl_2$ x 2 H_2O
- 250 mM KCl
- 10 mM PIPES-KOH pH 6.7

LB medium

1 piece of LB plate complemented with Amp and Cam

III. Lab report

Date: _____

Name: _____

Title:

_____

_____

Aim:

_____

_____

Short summary (2-3 sentences about the experimental setup):

_____

_____

_____

_____

Used materials (You should check all the solutions and materials before you start the experiment. You should know for what and why we use them during the experimental process.)**:**

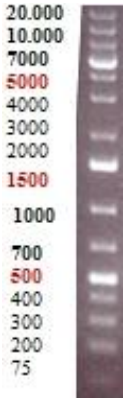
- agarose: _____
- ethidium bromide: _____
- TAE: _____

_____

- 6x loading buffer
  - glycerol: _____
  - bromophenol blue: _____
  - Tris:_____
    _____
  - EDTA: _____
- LB:_____
  _____
- LB+agar:_____
  _____
- Ice:_____
  _____
- ampicillin:_____
  _____
- chloramphenicol:_____
  _____
- 42 ºC:
  _____
  _____
- 37 ºC:
  _____
  _____

Changes in the protocol: (the experiment could be repeated only, if we write down everything):

Observations and Conclusion:



Gel photo

Don’t forget to label every sample!

(Ladder shows the sizes of DNA in base pairs - bp)

Questions

Why do we use ethidium bromide in case of agarose gel electrophoresis?

_____

List at least three factors, which could influence the migration of DNA in the gel!

_____

Which gene is encoded in the genome of Rosetta and BL21 cells? Why is it important in our case?

_____

Draw the schematic structure of the lac operon! Mark the binding site of the repressor protein on it!

Why did we select the cells for both chloramphenicol and ampicillin?

_____

Chapter 5

Research project in molecular biology

4th practice

Inoculation of Rosetta (DE3) colonies transformed with pET-Pfu plasmid and induction of protein expression in Rosetta (DE3) cells transformed with pET-Pfu with IPTG or lactose

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will learn the theoretical background of pET vector-based protein expression system.
2. They will understand the basis of lactose and IPTG induction of Rosetta cells.

Skill:

3. They will be capable for inducing Rosetta cells with lactose and IPTG.

Attitude:

4. They will inquire about the differences between lactose and IPTG induction.

Responsibility and autonomy:

5. They will be able to perform the inoculation of bacterial colony alone.
6. They are willing to take each sample in time.

Inoculation of Rosetta (DE3) colonies transformed with pET-Pfu plasmid – Part I

This part of the protocol covers the topics of inoculating and growing bacterial cultures and induction of protein expression in recombinant plasmid containing bacteria.

For these, background information is provided on:

- handling bacterium cultures
- induction of protein production by turning on the lac regulatory circuit

Hand on practice will be:

- inoculation of bacterium culture from a single colony of recombinant clone
- IPTG induction of the lac operon

Theoretical background - Part I.

Working with *Escherichia coli* in the laboratory

In molecular biology laboratories *Escherichia coli* is generally used for cloning experiments. Indeed, for decades, this bacterium strain, which has many variants, has been used for all over the word to study basic biological functions.

E. coli is a Gram-negative bacterium that lives in our intestine. Under normal conditions, *E. coli* is not pathogenic, although under specific conditions certain variants of *E. coli* can cause very serious health problems. Keep this in mind whenever you work with *E. coli*! Handling *E. coli* – and in fact any other microbes - requires sterile working conditions. The details of this practice have been already taught in microbiology course, therefore now we just emphasize a few basic things.

Bacterial cells can rapidly duplicate (*E. coli* in every 20 minutes under optimal conditions) and as a consequence a huge number of cells are generated from a single cell in a relatively short time. For their rapid growth, *E. coli* cells require optimal conditions including media containing nutrient and other essential substances, such as controlled temperature (37 °C for *E. coli*) and oxygen. These conditions are provided in the laboratory by culturing *E. coli* cells either on the surface of agar plates or in liquid media usually in thermo-regulating incubators with aeration. Bacterium cells spread on the surface of an agar plate form colonies in 12-18 hours, which diameter is approximately 2-3 min.

Each colony represents the offspring of a single cell that was spread on the plate. From these colonies unique ones can be inoculated into 2-10 ml of liquid media in order to produce higher amount of cells. It will give rise to a dense bacterium suspension that contains millions of bacterium cells per millilitre within a day. (You can find more information about the growth phases and density measurement of bacterial cultures in Chapter 2). In the growing bacterial culture, the quantity of plasmids is also increasing.

The above described things are the major power and advantage of genetic engineering: from a single cell, unlimited quantities of DNA can be obtained. Furthermore, if a protein coding DNA is inserted into a plasmid in a specific way, the transcription of it and based on the mRNA the protein translation can be also ensured. Naturally, since the genetic code is universal this protein will have the same amino acid composition as it is in the original host cell. However, it is very important in the laboratory to work carefully with recombinant DNA containing bacterium cells and prevent contamination of specific plasmid containing cells by mixing them with other ones.

Sterile work, careful attention and labelling the samples can help to precisely carry out the intended experiment.

A simple way to inoculate unique colonies into liquid LB media is shown on the following images:

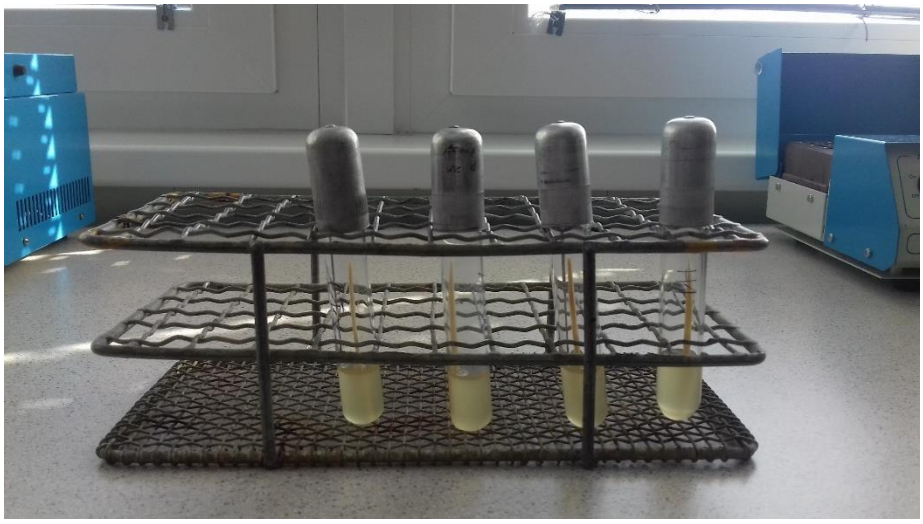


Figure 5.1 Grown bacterial starter cultures

Figure 5.2 Antibiotic addition to LB medium

Figure 5.3 Transfer the starter culture into higher amount of LB medium complemented with antibiotic

Induction of protein expression in Rosetta (DE3) cells transformed with pET-Pfu with IPTG or lactose – Part II

Theoretical background - Part II

Induction of pET vector-based prokaryotic expression system with IPTG or lactose

The basics of gene expression in bacteria were described in the previous chapter by introducing the regulation of the lac operon. Here we give some more information about bacterial gene expression regulation.

In prokaryotes, transcription is catalysed by the RNA polymerase enzyme, which does not require primer for transcription initiation, but it recognises specific sequences close to the site of transcription initiation and can start the RNA synthesis only from this site. During transcription, the RNA polymerase synthesises the RNA chain in a 5' to 3' direction according to the DNA template. At the end of the transcription unit, the enzyme releases from the DNA and dissociates from the newly synthesized RNA to be able to start a new synthesis again.

The promoter region, to which the RNA polymerase binds, is located upstream from the transcription start site (TSS). In *E. coli*, similar promoter structure could be observed for most of the genes, which can be characterised by the presence of two short sequences at the -10 and -35 positions upstream of the TSS. The more these sequences resemble

to an ideal consensus promoter, the stronger the promoter is, which means that it can more frequently direct the initiation of the transcription. The lac promoter region possesses neither strong -10 nor -35 consensus sequences, therefore it is a relatively weak promoter with low transcription efficiency. However, this does not cause problems in the cell, since as it was described in the previous chapter, a positive regulatory circuit involving cAMP and the catabolite activator protein could positively affect the promoter activity.

In case of bacteriophage infection, bacterial cells are reprogrammed in order to induce the formation of the components of new phage particles. In case of T7 phage infection, the transcription of bacterial genes is switched off by permitting the transcription of only the bacteriophage genes. It can be easily realised, since T7 phage possesses a special polymerase, called T7 RNA polymerase, which can only bind to its own promoter region (T7 RNA polymerase promoter) but not to the bacterial promoter region. The expression system used in the practice utilises the T7 RNA polymerase and induces the foreign gene expression from a T7 polymerase promoter. In addition, some elements of the lac operon are combined with the T7 promoter in order to make its inducible regulation possible by lactose or lactose analogue molecules, such as IPTG. If lactose or IPTG is added to BL21 (DE3) *E. coli* cells bearing pET vector, the T7 RNA polymerase gene is released from the inhibition of lac repressor, which results in the expression of the T7 RNA polymerase in the cells. Promoter region specific for the T7 RNA polymerase can only be found in the transformed pET vector, thereby T7 RNA polymerase can selectively transcribe only the protein coding gene we would like to produce. The added IPTG inhibits the binding of the lac repressor

protein to the lac operator region in both the genome and the pET plasmid, hence our protein is allowed to be produced.

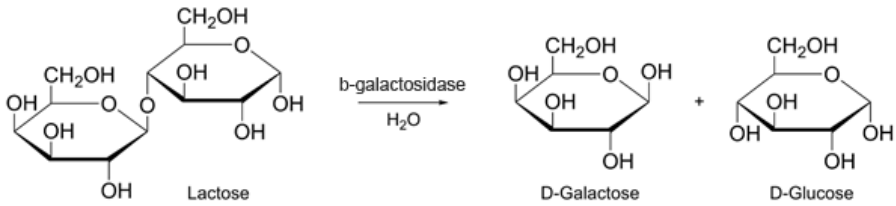


Figure 5.4 Structure of lactose

https://commons.wikimedia.org/wiki/File:Lactose_hydrolysis

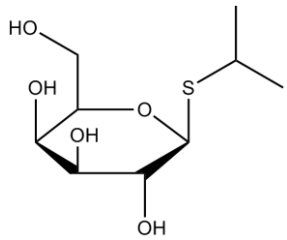


Figure 5.5 Structure of IPTG

https://commons.wikimedia.org/wiki/File:IPTG_(CBD)

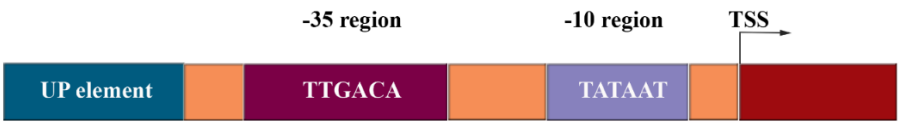


Figure 5.6. Prokaryotic promoter structure

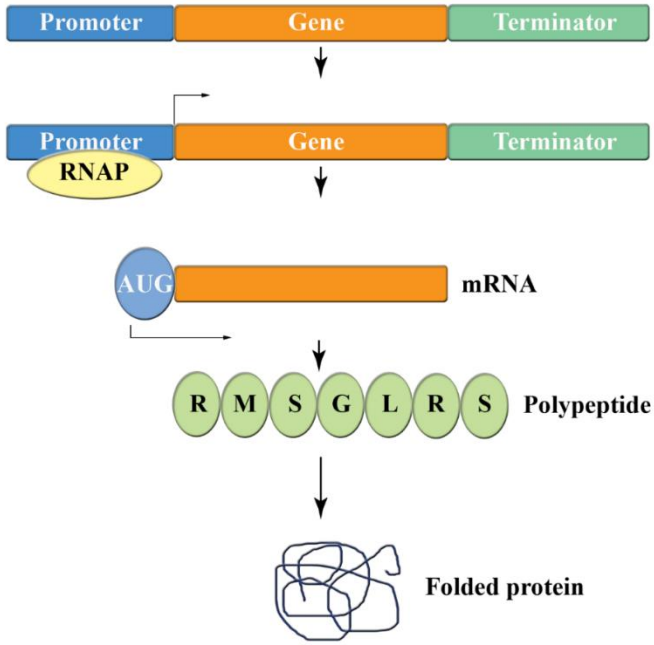


Figure 5.7 From RNA to protein synthesis

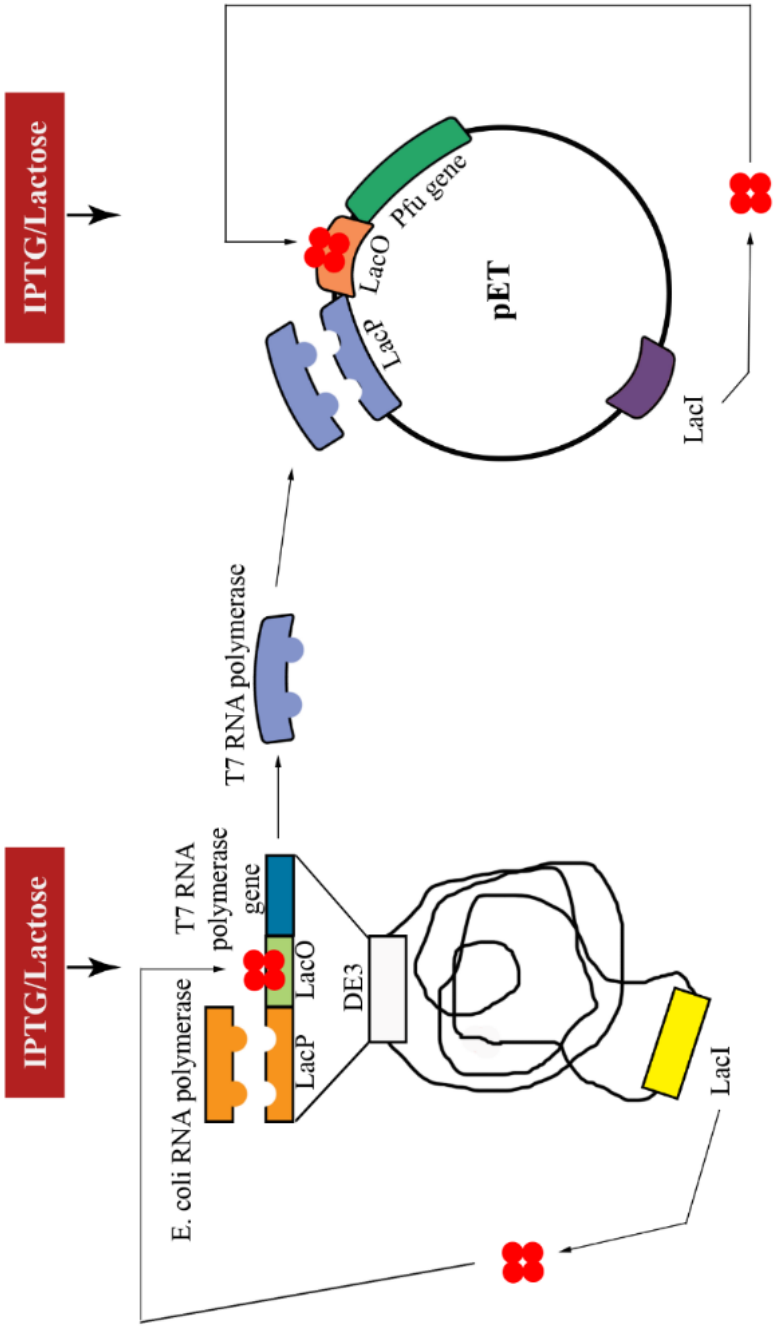


Figure 5.8 Inducible T7 expression system

Figure 5.9 Lab shaker

Practical workflow and protocol - Part II

Inoculate the overnight grown up starter culture into 60 ml LB+Amp+Cam-containing medium, grow it up until OD = 0.7 (at least for 1.5-2 h). This step is done by the lab assistant.

Induction:

- Work group (pair) 1: 1 mM IPTG (200 mM stock)
- Work group (pair) 2: 0.2 % lactose (20 % stock)

Don't forget to record the time point of the induction in order to be able to do the accurate sampling!

Order of the sampling:

- **1. Sampling 0 h** (at IPTG/lactose addition): 1 ml
- **2. Sampling 45 min:** 1 ml
- **3. Sampling 90 min**: 1 ml, 5 ml, 40 ml

Centrifuge 1ml samples at 13,000 rpm for 1 min. Discard the supernatants and store the pellets at -20 °C.

Centrifuge the 5 ml and 40 ml samples at 4,000 rpm for 10 min. Discard the supernatants and store the pellets at -20 °C.

Accordingly, every pair has **3 pieces of 1 ml** centrifuged samples (**0 h, 45 min, 90 min**) and **2 additional samples** (**90 min 5 ml, 90 min 40 ml**).

Materials - Part II

200 mM IPTG

20 % lactose

LB medium

IV. Lab report

Date:_____

Name: _____

Title:

_____

_____

Aim:

_____

_____

Short summary (2-3 sentences about the experimental setup):

_____

_____

_____

_____

_____

Used materials (We should check all the solutions and materials before we start the experiment. We should know for what and why we use them during the experimental process.)**:**

- Lactose:_____

  _____
- IPTG:_____

  _____

- Ice:_____

  _____

What is your sample number?____

What did you use for the induction? _____

Introduce the operation of the pET vector-based system in Rosetta cells used for protein production!

_____

_____

_____

_____

_____

_____

<u>Draw here:</u>

Changes in the protocol: (the experiment could be repeated only, if we write down everything):

_____

_____

_____

_____

_____

_____

_____

_____

Observations and Conclusion:

_____

_____

_____

_____

_____

_____

Questions

Which are the two inducing agents we used in this practice for inducing the lac operon?

_____

What is the main difference between these inducing agents?

_____

_____

_____

Delineate in detail how we can induce the protein expression in Rosetta cells!

_____

_____

_____

_____

_____

_____

_____

_____

_____

Chapter 6

Research project in molecular biology

5th practice

Lysation of Rosetta (DE3) cells by sonication

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will learn the theoretical background of cell lysis by sonication.
2. They will understand the main point of the workflow of the practice.

Skill:

3. They will capable to take the appropriate samples at each point of the protocol.

Attitude:

4. They inquire about the operation of the sonicator equipment.

Responsibility and autonomy:

5. They will be able to do the workflow on their own.

Lysation of Rosetta (DE3) cells by sonication

The aim of this practice is to prepare cell lysates from the recombinant plasmid containing E. coli cells in order to test the expression of the Pfu gene in a further practice.

For this background information is given about:

- the general rules of handling biological samples and the technique of cell disruption by sonication.

Hand on practice will be:

- sonication of E. coli cells induced for the production of foreign protein.

Handling biological samples

In molecular biology laboratories, the most frequently studied macromolecules are different types of DNA, RNA and protein molecules. The specific goals of working with them and the techniques required to achieve these goals can greatly vary, although there are a few basic rules which should be kept in case of performing experiments with these types of materials. However, some of these have been already mentioned in the previous chapters, it is important to overview the background of these in this chapter, too.

In general, we want to study protein molecules in a form when they keep their biological activity. For this, their complex higher order structure should be preserved and those effects and reagents, which might destroy these structural features, should be kept away from the solution. For this, we keep the samples on ice during the experiment and protease inhibitors should also be applied. By this, we inhibit reactions that result in spontaneous structure reorganisation and the activation of proteases that might contaminate and degrade our samples. For long term storage, we preserve the samples at -80 °C. However, RNA and DNA samples can be frozen without causing destruction in their structure, proteins might be destroyed by repeated freeze-thaw cycles. Therefore, to store proteins at -80 °C, an antifreeze reagent should be added to the samples (e.g. glycerol) and they should be stored in small aliquots.

Since we generally work with very small quantities of material, we should pay attention for the appropriate handling not to lose them as a result of digestion by contaminating enzymes. Enzymes that degrade

DNA, RNA and protein are generally present in our samples since these can be found among enzymes in the cell and are also present on our skin, on laboratory equipment, in the water or on the work bench. To prevent their activity, we frequently use inhibitors of proteases and chelators (e.g. EDTA) that prevent nuclease activity. It is very important to make all precautions to avoid contaminating our samples. For this, use gloves and never touch your pipette tips to avoid the cross-contamination among samples.

Most of the reagents used in molecular biology laboratories are very specific, prepared and purified with great care and consequently are very expensive. Without exaggeration, many of them are more expensive than the same amount of gold. Therefore, be careful, when you use these reagents. You should be aware of the proper quantity of the current reagent you want to use and the proper handling to avoid disruption of the stock solution by contamination and by improper treatment.

Bacterial cell lysis by sonication:

In the laboratory, one of the most frequently used method for bacterial cell lysis is sonication, when cell suspension is exposed to burst of ultrasonic sound. During this technique, cell lysis is caused by cavitation effects (bubbles are formed and collapsed in the liquid due to the ultrasound and the percussion waves raised by the cracking up bubbles generate viscous, dissipative swirls and cell disruption is caused by the shearing stress of them). The sonicator equipment developed for this, excites electrical waves, which is converted to mechanical oscillation,

which induces cavitation in an aqueous environment. The device is composed of an oscillator, an amplifier and a wave-converter. In the course of cell disruption by sonication, a lot of heat is generated, therefore the vessel, which contains the cell suspension, should be cooled down by ice or circulating refrigerant. In addition, to prevent overheating of the sample, sonication is most frequently done by alternating cycles of treatment and pauses between them.

Sonication can be used to disrupt cell membranes to release the cellular content. Therefore, sonication is an effective way to release the cellular content but it is not suitable for isolating higher molecular weight DNA, since it results in double stranded breaks. On the other hand, sonication is frequently used to shear chromatin or DNA molecules into smaller fragments, which can be used for chromatin immunoprecipitation experiments or for next generation sequencing.

A sonicator is a relatively simple equipment, since only the intensity, time and frequency should be set on it. The appropriate frequency used for the ultrasonic disruption of bacterial cells is between 15 and 25 kHz. The user should protect her/his eyes and ears during sonication.

This method has several advantages and disadvantages. The most important advantage is that the treatment can also be used in the liquid-flow procedure. The disadvantages are that it is not appropriate for larger-scale usage, since it is hard to solve the adequate refrigeration and overheating might cause enzyme degradation.

Figure 6.1 Sonicator with probe for cell lysis

Figure 6.2 Sonicator with a tank for chromatin fragmentation

Practical workflow and protocol

In this practice, we will use the pellet collected from the 5 ml lactose/IPTG induced cell culture.

1. Suspend the cell pellet in 500 ul Buffer I (5 ul protease inhibitor cocktail (PIC) should be added to Buffer I only before usage).
2. Sonicate the samples for 3 x 20 seconds (10 sec break between steps) with 45 % amplitude
2. Centrifuge the samples at 13,000 rpm, 4 °C for 20 min.
3. Transfer the supernatant into a new Eppendorf tube. **DO NOT DISCARD THE PRECIPITATE!!**

   We will also investigate how much protein is present in the precipitate by comparing it to the supernatant samples. Proteins from those cells, which are not lysed, can be found in the pellet therefore we can determine the efficiency of sonication based on this comparison.
4. Suspend the precipitate in 50 ul Buffer I and add 50 ul 2X SDS loading buffer to it.
5. Transfer 20 ul from the supernatant into a new tube and add 20 ul 2X SDS loading buffer to it.
6. Boil these samples for 5 minutes at 100 °C, then keep them at -20 °C until the next practice.
7. Incubate the SUPERNATANT (~450 ul) at 75 °C for 5 minutes, then on ice for 5 minutes.
8. Centrifuge the samples at 13,000 rpm, 4 °C for 20 min

Non-thermostable proteins are precipitated and removed therefore in the supernatant only the thermostable proteins (in this case, the Pfu) will be present.

9. Add 20 ul 2X SDS loading to 20 ul SUPERNATANT in a new Eppendorf tube.
10. Boil the samples from step 6. for 5 minutes at 100 °C. Store at -20 °C.
11. Store the residual supernatant at -20 °C to measure enzyme activity later.

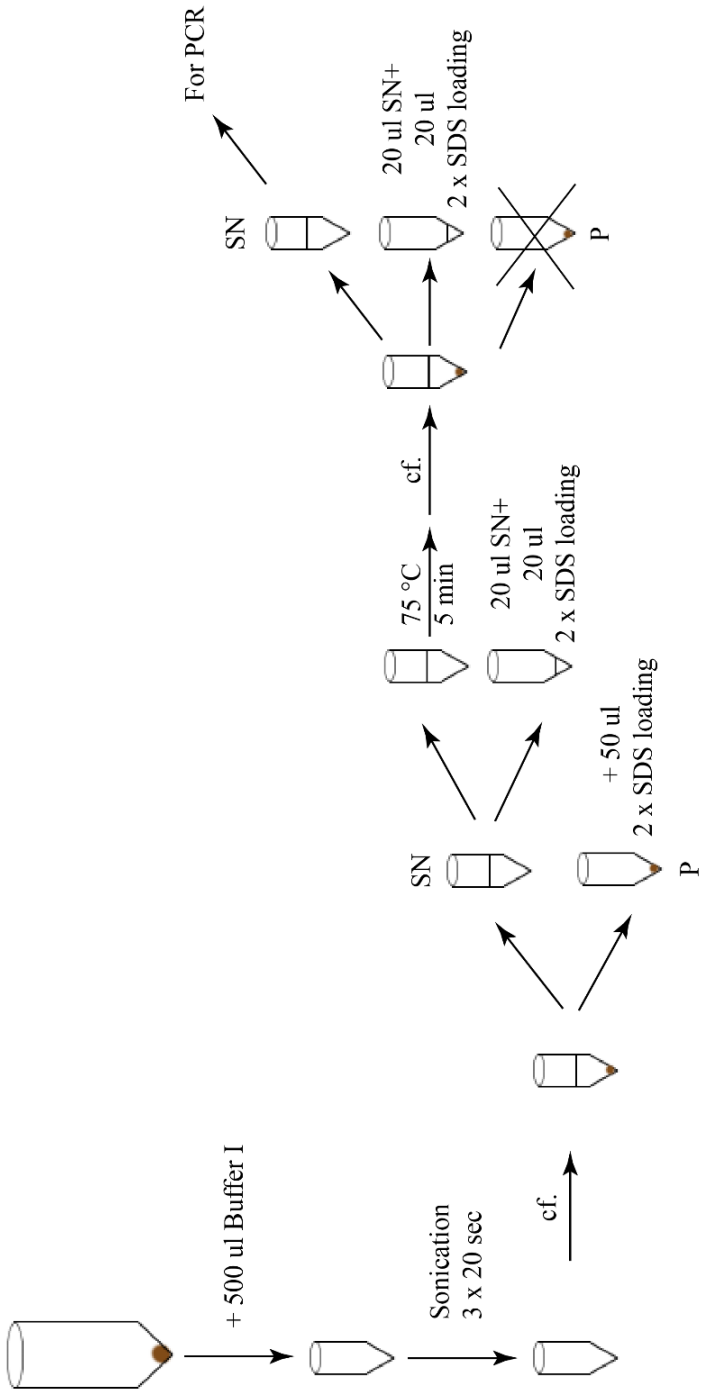


Figure 6.3 Working procedure during and after sonication

Materials

Buffer I:

- 100 mM Tris pH 7.5
- 2 mM EDTA

2X SDS loading buffer:

- 100 mM Tris pH 6.8
- 200 mM DTT
- 4 % SDS
- 0.2 % bromophenol blue
- 20 % glycerol

V. Lab report

Date:_____

Name: _____

Title:

_____

_____

Aim:

_____

_____

Short summary (2-3 sentences about the experimental setup):

_____

_____

_____

_____

Used materials (We should check all the solutions and materials before we start the experiment. We should know for what and why we use them during the experimental process.)**:**

- Buffer

  I:_____

  _____
- DTT:_____

  _____

- SDS:_____
  _____
- Bromophenol blue:_____
  _____
- Glycerol:_____
  _____

Changes in the protocol: (the experiment could be repeated only, if we write down everything):

_____

_____

_____

_____

_____

_____

_____

_____

Observations and Conclusion:

_____

_____

_____

_____

Questions

What kind of cell lysis method did we use in this practice?

_____

How does this method work?

_____

_____

Why should you generally keep your samples on ice (or in ice supplemented water bath) during this method? Why isn't it so important in our case?

_____

_____

_____

What is the function of bromophenol blue in the loading buffer?

_____

_____

What is the function of glycerol in the loading buffer?

_____

_____

Chapter 7

Research project in molecular biology

6th practice

Following the Pfu expression by SDS polyacrylamide gel electrophoresis

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will be familiar with the principles of SDS-PAGE.
2. They will understand the concept of concentrated stock solutions.

Skill:

3. They will be able to prepare and run their samples on SDS-PAGE.
4. They will be able to dilute the stock solutions to the working concentration.

Attitude:

5. They will inquire about performing an SDS-PAGE and evaluate their results.

Responsibility and autonomy:

6. They will be able to handle toxic materials with care.
7. They will learn how to write their lab reports and evaluate polyacrylamide gels on their own.

Following the Pfu expression by SDS polyacrylamide gel electrophoresis

Foreign protein expression in an organism is a complex experiment, since the success of it is affected by many factors. Accordingly, it is important to set the optimal conditions for protein production. Each protein has different amino acid composition, biochemical characteristics and folding, therefore *E. coli* cells respond differently to the heterologous expression of various proteins. Certain proteins could aggregate and form inclusion bodies within the bacteria, while others could be harmful for the host cells or even lethal and there are proteins, which have no major effects on the host organism. Nonetheless, production of an exogenous protein in a high quantity is demanding for the cell and requires optimal utilization of several components of the cellular synthesizing machinery. Changing the conditions of protein expression could alter the effect of the protein in *E. coli* cells, which could either increase or decrease the yield or the solubility of the heterologous protein. To determine how our protein behaves and to find the optimal conditions for its production, several factors should be tested. Among these factors could be the time of induction (the density of bacterial culture when the inducer is added and the extension of its use), the temperature, the type of inducing agent, host, plasmid vector, promoter, etc. During the practice, we will test two factors: the time period of induction and the type of inducing agent. To observe how these factors influence the heterologous protein expression, we should make the expressed proteins visible and identify the desired one among the several hundred proteins of *E. coli* host cells. For this, we use SDS

polyacrylamide gel electrophoresis (in short: SDS-PAGE or Laemmli gel) and Coomassie Brilliant Blue staining.

Theoretical background

Gel-electrophoresis is a commonly used method to separate macromolecules by their size. Separating proteins by electrophoresis according to their mass is much more complicated than separating DNA or RNA. Nucleic acids are uniformly charged and they have relatively simple structure (note: single stranded nucleic acids can have quite diverse structures), while proteins are differently charged molecules with different shapes (tertiary structures) and they can form oligomers (quaternary structures). In case of proteins, the size, charge and state of oligomerisation are three major properties that could influence the 'run' (migration) of these macromolecules during electrophoresis. For instance, under specific electrophoretic condition: proteins that have more positive charges will move towards the cathode, while mostly negatively charged proteins will run towards the anode. However, under a different condition, the net charge of the same proteins alters and they might migrate differently.

In order to eliminate the migration differences resulting from different charges and shapes, we utilise sodium-dodecyl-sulphate during electrophoresis (SDS-PAGE). The method is based on the effects of the strong anionic detergent, sodium-dodecyl-sulphate (SDS). SDS with heat treatment denatures the proteins and SDS binds to the proteins roughly in proportion to their size (an average every mass unit of protein binds 1.4 dodecyl-sulphate molecule). The binding of SDS unfolds the

proteins (this denaturation is further facilitated by heating of the sample before electrophoresis), ‘linearizes’ the molecules and provides a uniform negative charge to them, regardless to the types of initial charges. As a result of that, SDS-PAGE separates proteins by their size regardless their charge, structure or state of oligomerisation. However, covalently bound oligomers could not be separated by a simple SDS treatment. The covalent bindings between polypeptide chains are usually sulphur bridges, which can be destroyed by strong reducing agents, such as DTT or beta-mercaptoethanol. Therefore, the protein samples should be treated with SDS, reducing agent and heat before applying them to the gel matrix.

Polyacrylamide gel is composed of polymers formed by acrylamide and N, N bisacrylamide molecules. The polymer formation takes place only in the presence of free radicals. In the laboratory, ammonium persulphate (APS) provides the free radicals and tetramethyl-ethylene-diamine (TEMED) stabilizes them (Figure 7.1).

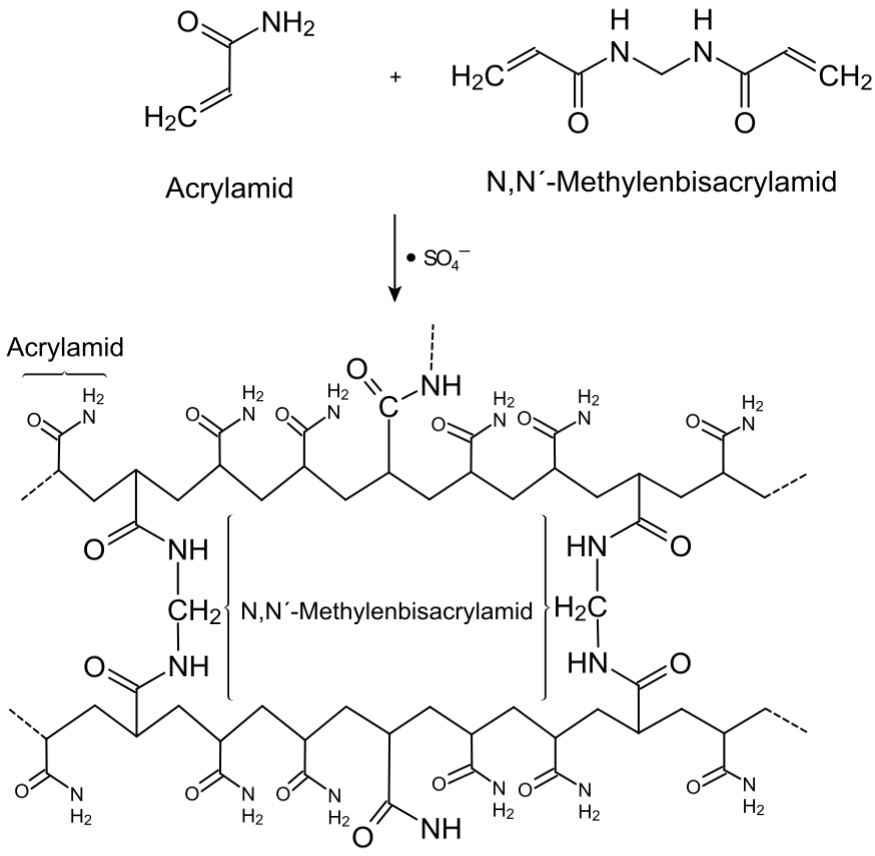


Figure 7.1 Acrylamide forms linear polymers, while N, N bisacrylamide cross-links these linear molecules forming the gel matrix.

https://commons.wikimedia.org/wiki/File:Acrylamide_gel.svg

Unlike agarose gel, SDS-polyacrylamide gel is casted vertically, between two glass plates. The wells for the samples are formed in the top of the gel. Nowadays, the most often used gel electrophoresis technique for protein analysis is the “Laemmli gel” named after the person who developed it. These types of gel consist of two parts, which differ from each other in concentration and buffer composition. The purpose of the smaller top part of the gel is to concentrate the sample,

while the larger bottom part of the gel is suitable for the separation of different proteins.

The Laemmli gel is very popular because the resolution of a vertical gel composed of only the separating part would be greatly influenced by the volume of the sample because the height of the loaded sample would be proportional with the height of the protein bands in the gel. To solve this problem, two types of gels are used: the upper one is the stacking part and the lower one is the separating part. In order to obtain sharp bands, we should start the separation of the proteins with concentrating them into a thin layer. The upper stacking gel 'stacks' the proteins into a thin line, therefore the height of the sample won't be a problem anymore. The thickness of stacking gel is only a few mm. It contains the pockets or wells, where the samples are loaded into. Its pH is 6.8 (TRIS-HCl) and the acrylamide content is around 3.5 %. (Figure 7.2). The larger, bottom separating gel part is responsible for the separation of the proteins according to their mass. It has pH 8.8 (TRIS-HCl) and its acrylamide content is between 6-15 % depending on the mass of the separated proteins (6 % for larger, 15 % for smaller proteins). As a first step, the separating gel part is poured between the glass plates and after it has been polymerised, the stacking gel can be casted on the top of it. The other important component of the SDS-PAGE is the running buffer, which is a TRIS-glycine pH 8.3 buffer. As mentioned above, the stacking gel has a different TRIS buffer component with lower pH (pH 6.8). For the proper working of the stacking gel and the entire process of PAGE, these pH values must be precisely set for each component.

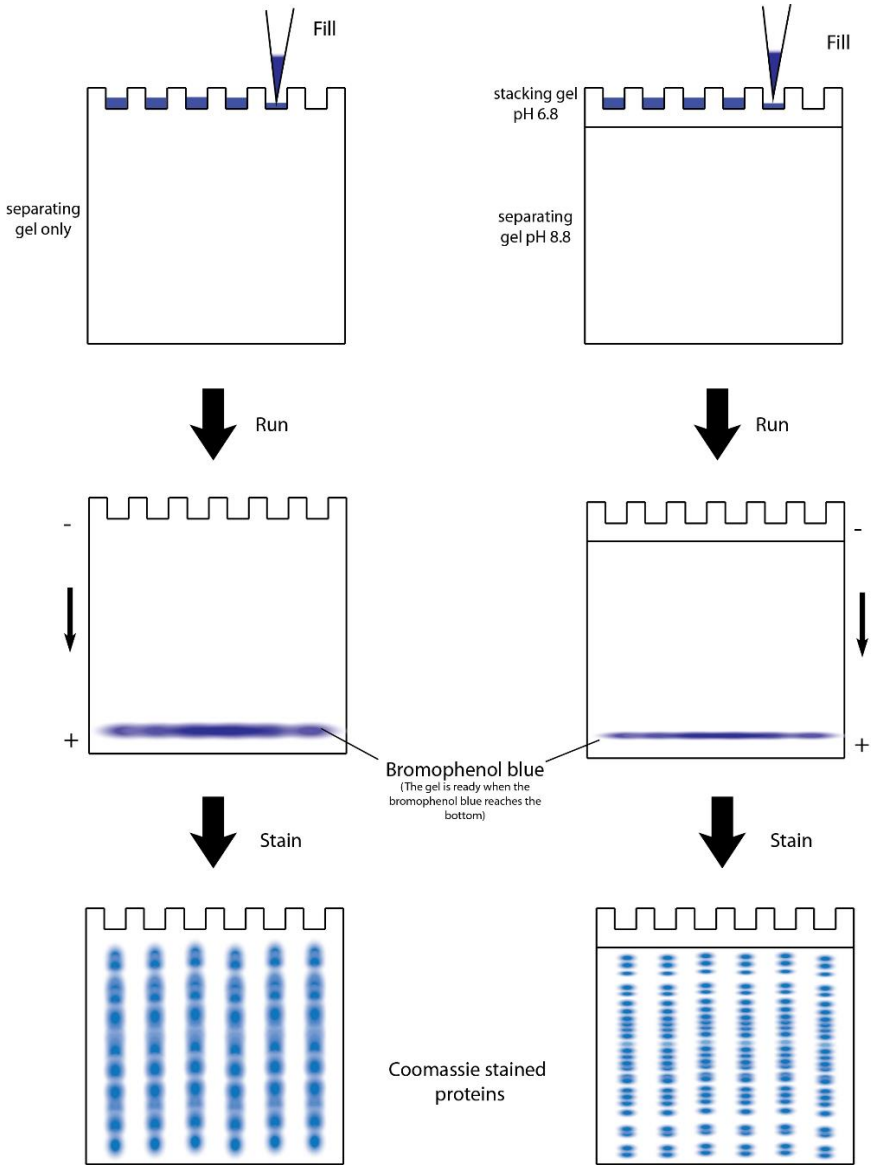


Figure 7.2 The effect of the stacking gel on the gel resolution

The stacking gel concentrates the proteins into a thin layer and when the proteins reach the separation gel, they remain concentrated and run in the gel as thin bands. The height of the sample that was loaded (the volume) does not matter in that case.

The glycine molecules are the key components of the running buffer. Understanding their behaviour helps us to realise how the stacking gel works. Glycine at higher pH acts as an anion, at neutral pH it works as a zwitter ion and at low pH it functions as a cation (Figure 7.3).

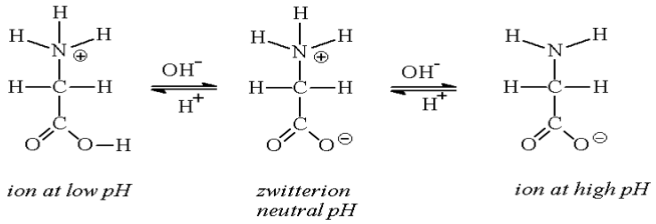


Figure 7.3 Charge of the glycine is influenced by the pH

During SDS-PAGE there are three types of negative ions in the gel: glycine (from the running buffer), protein-dodecyl sulphate (protein-DS) complex and chloride ion. All of these are negatively charged, so they migrate towards the anode. When the three ions enter the stacking gel, the chloride ions run in the front, since they are small in size and can migrate easily in the gel. After the chloride ions, the protein-DS complexes migrate, since they are also negatively charged. Glycine molecules at pH 6.8 behave as zwitterions, so these molecules migrate much slower and fall behind. Since less charged particles will be present, the field strength will affect these molecules more. Therefore, the running of the DS-protein complexes will be accelerated, and the molecules line up behind the chloride ions. At the border of the stacking and separating gel, the proteins reach the front formed by the chloride ions. However, in the separating gel more particles participate in the

conduction of electricity (at the higher pH glycine becomes negatively charged), which results in slowing down the proteins. This leads to a formation of a thin layer of proteins that could enter the separating gel (Figure 7.4).

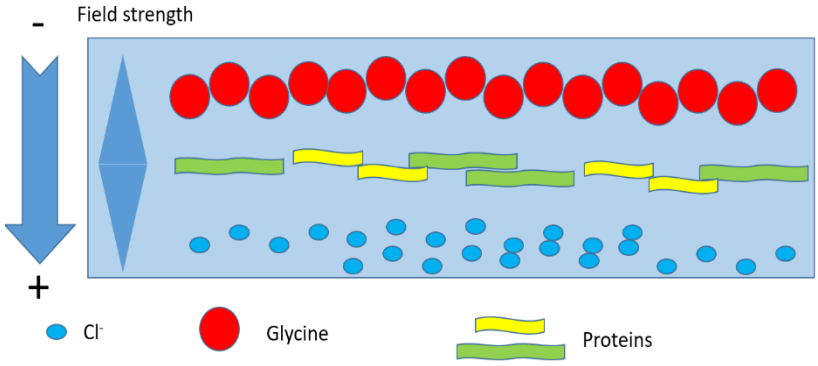


Figure 7.4 The Protein-DS complexes form a thin layer in the stacking gel

In the separating gel, the small chloride ions run forward but the glycine ions become anions again at pH 8.8 and run behind the chloride ions. In the homogenous field strength, the protein-DS complexes migrate with a constant speed that is mainly determined by the size of the molecules (Figure 7.5).

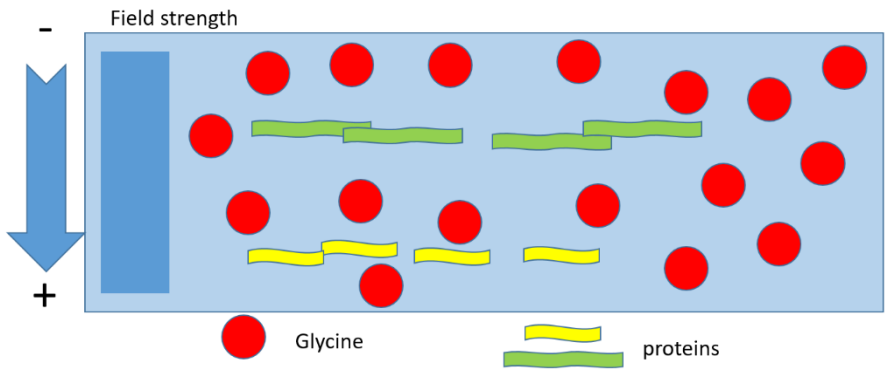


Figure 7.5 Proteins are separated by their mass in the separating gel

Beyond the pH, the different pore size between the stacking and the separating gel is also an important factor. (The stacking gel with its 3.5 % acrylamide content forms larger pores, while the separating gel with its 6-15 % acrylamide content results in smaller pore sizes.) In the stacking gel the molecules can move easier.

Practical workflow and protocol

A few useful practical comments:

- When casting the gel, after the solution of the separating gel has been poured between the glass plates, a thin layer of isopropanol or hydrated butanol should be placed on the top of the polymerizing gel. It will ensure anaerobe environment that promotes polymerisation of the gel. On the other hand, a more important function of this is that it flattens the meniscus of the gel. Without the alcohol layer, the meniscus would be U-shaped because of the capillary forces, which would decrease the resolution of the gel.

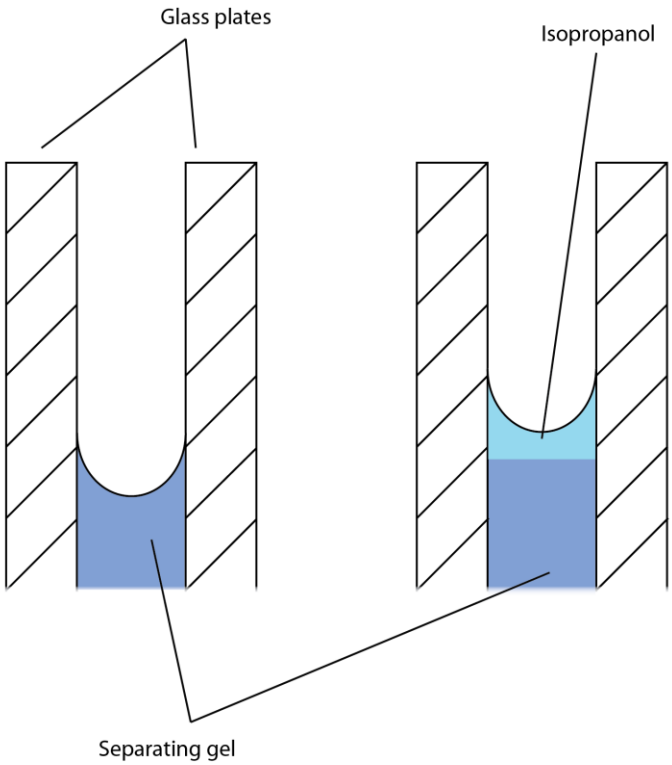


Figure 7.6. Isopropanol layer makes the gel surface flat

- Besides SDS and beta-mercaptoethanol, the sample buffer contains further ingredients. Glycerol makes the samples denser than the running buffer, therefore the samples are sedimented to the bottom of the wells of the gel. The blue colour of the sample loading buffer is from its bromophenol blue dye component, which is a small molecule that runs in the gel before the proteins, thereby showing the front. This dye does not stain the proteins, it is used to follow the progress of the electrophoresis: when the bromophenol blue reaches the bottom of the gel, the electrophoresis should be stopped.

- For convenience, the solutions used for gel preparation and electrophoresis are kept in the laboratories in concentrated stock solutions. From these solutions, the necessary volumes for the specific gels can easily be prepared. Our gel buffer stock solution is four times concentrated and it should be diluted during the gel mixing. The concentration of our acrylamide stock solution is 30 %. The running buffer stock is five times concentrated.

- NOTE that acrylamide is neurotoxic and carcinogenic! Be careful with handling it!

- Similarly, the methanol used during gel staining is toxic!

- After the bromophenol blue has reached the bottom of the gel, the electrophoresis is stopped. The gel is removed by lifting carefully one

of the glass plates, then by moving the gel from the other glass. Then it should be placed into a fixing solution, which contains 50 % methanol and 10 % acetic acid. In this solution, Coomassie Brilliant Blue (CBB) R250 dye (common textile paint) is added to stain the proteins. CBB diffuses into the gel and irreversibly binds to the proteins. After staining for a given time (10-20 minutes) CBB should be washed away from the gel, by soaking it in a differentiating solution. Silver-nitrate could also be used to detect proteins. Silver-nitrate staining is more sensitive, but it is a more complicated method than CBB staining.

- The stained gels could be preserved by drying them between stretched cellophane layers. The gels could be scanned or a photo could be taken from them, then the image could be preserved in the lab report.

Protocol:

The samples collected last time will be run on SDS-PAGE and will be visualized by Coomassie Brilliant Blue staining.

1. Sample preparation:

There are two types of sample:

- Rosetta (DE3) transformed with pET-Pfu and induced with 1 mM IPTG
- Rosetta (DE3) transformed with pET-Pfu and induced with 0.2 % lactose

In both cases, 3 samples were collected at different time points of the induction:

1. 0 min
2. 45 min
3. 90 min

Note, that we only monitor a short phase of protein production. It is usually recommended to follow the expression and take samples during a longer period of time. For instance, sampling after 1 h, 4 h, 6 h, 8 h and 24 h would provide a more detailed picture from the dynamics of protein production.

Two more samples have been taken to study the solubility of the induced protein, both are derived from the sonicated and centrifuged cell lysates, and a further sample is the purified protein:

4. Supernatant
5. Pellet
6. Boiled supernatant

In summary, there are 6 samples/workgroups that will be loaded on polyacrylamide gel.

Preparing the protein samples for PAGE:

1. Resuspend the cell pellet in 100 ul Buffer I.
2. Add 100 ul 2X SDS loading buffer to the solution.
3. Boil for 10 minutes at 95 °C

The other three samples (Supernatant, Pellet, Boiled supernatant) have been already prepared, just melt them at room temperature.

2. Gel casting and running

In this practice, we use a 10 % polyacrylamide gel.

Assembly of the gel casting apparatus:

Wash the two glass plates with alcohol to remove all the stains! (Stains can serve as nucleation points for the polymerizing acrylamide, which could result in uneven gel matrix.)

Assemble the two glasses and put them into the frame!

Stabilize the glasses in the frame by using the white hand-screws. (It will form a tank for the buffer.)

Lock the frame to its silicon base! (It will seal the bottom of the mould that prevents gel leaking.)

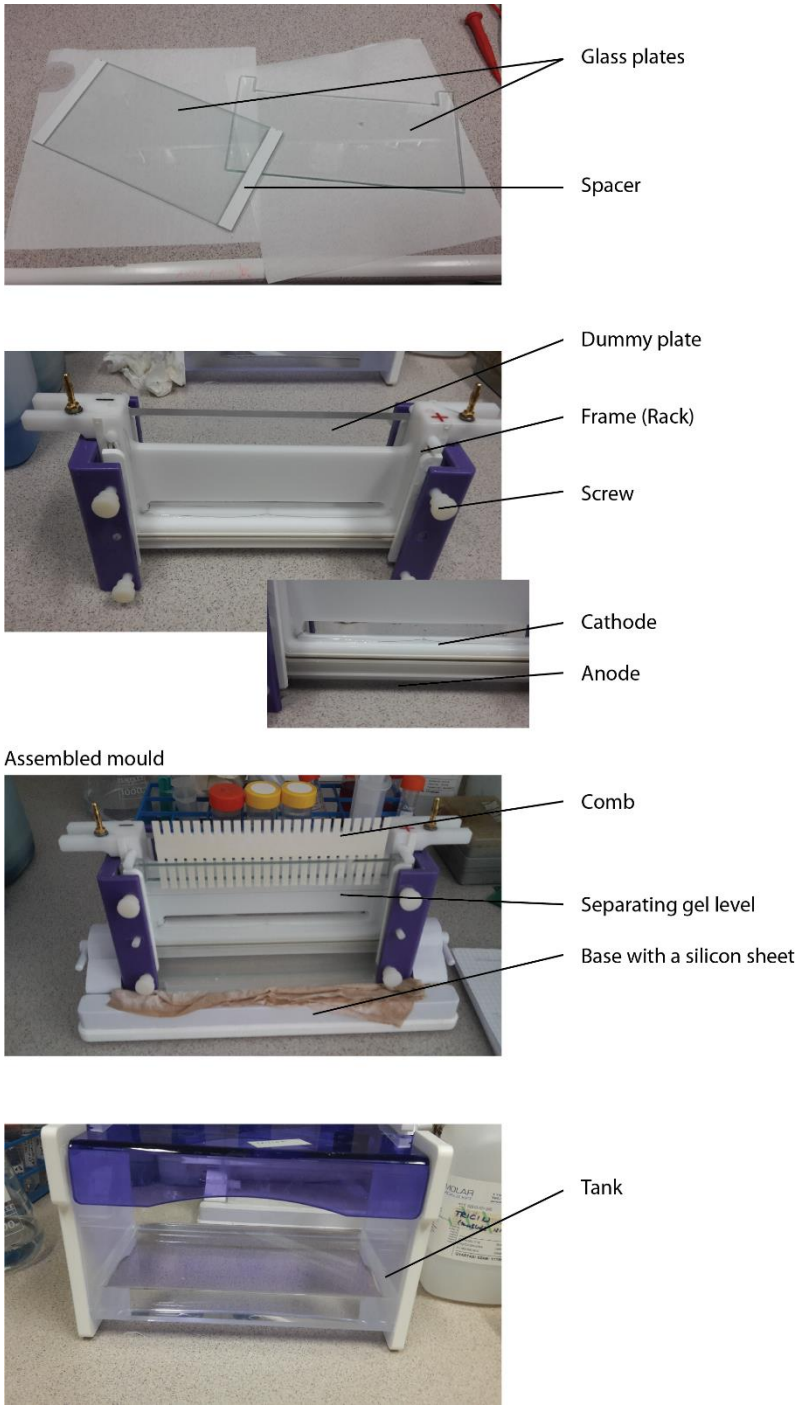


Figure 7.7 Assembly of the SDS gel casting apparatus

Gel casting:

Prepare the solution for the gel to 30 ml final volume. The volume of the mould is approximately 15 ml. The residual mixed gel solution will be used to follow the polymerisation.

Before casting the gel, it is recommended to mark the intended height of the separating gel. Put the comb between the glasses. Mark the glass 1-2 mm below the bottom of the comb with a marker.

Recipe for preparing the separating gel:

- 10 ml Acrylamide-bis-acrylamide (30:0.8 %)
- 7.5 ml 4x separating buffer (1.5 M Tris pH 8.8, 0.4 % SDS)
- 12.5 ml H_2O

The polymerisation will start after the addition of APS and TEMED.

- 120 ul 10 % APS
- 60 ul TEMED (the polymerisation starts. You have to complete the gel preparation in the following steps, you must not stop or take a break here.)

Pour the gel between the two glasses until the liquid reaches the mark.

Layer some isopropanol onto the polymerizing gel. During polymerisation, the gel shrinkes that could deform the glasses. To avoid this, put the comb between the glasses. The comb should not reach the isopropanol layer.

While the gel is polymerizing, mix the solution for the stacking gel.

Recipe for the stacking gel:

- 3 ml H_2O
- 1.25 ml 4x stacking buffer (0.5 M Tris pH 6.8)
- 0.6 ml acrylamide

APS and TEMED are also needed to start the polymerisation.

- 40 ul APS
- 20 ul TEMED (Do not add it until the separating gel polymerizes and the isopropanol is removed!)

Polymerisation of the residual separating gel in the falcon tube is an indication that the gel has also polymerised. Discard the isopropanol from the gel surface and wash it under tap water. Remove all the water by blotting it.

Put the comb between the glasses, add TEMED to the stacking gel mix and pour the solution into the mould.

While the stacking gel is polymerizing, dilute the SDS-PAGE electrophoresis buffer from the five times concentrated stock:

- 200 ml 5X SDS-PAGE electrophoresis buffer
- 800 ml H_2O

After the gel has polymerized, remove the frame from the base and put it into the electrophoresis tank. Fill the upper and the lower tank with 1x electrophoresis buffer.

Gently remove the comb, just by pulling it vertically with a constant speed. Dye the wells with 2x SDS loading buffer to check whether the wells are uniform. If the edges of the wells are tilted, use a needle to

make them straight. Wash the wells with a syringe. This step removes the buffer and the residual gel contamination.

Load 15 ul of each sample onto the gel. Pay attention that your different samples correspond to different dilutions of protein content. The sample prepared from the pellet is 5 times concentrated compared to the cell lysate and ten times concentrated compared to the supernatant, which was supplemented with the 2x SDS loading buffer. From this, 1.5 ul will be equivalent to the others. Supplement this volume with 1x loading buffer in order to get equivalent volumes to load.

The order of the samples should be the following:

Control (0h), 45 min, 90 min, Supernatant (S), Pellet (P), Boiled supernatant

Do not forget to load the protein ladder! Write down the order of the samples!

Turn on the power supply and run the gel at 120 V for 1.5-2 hours.

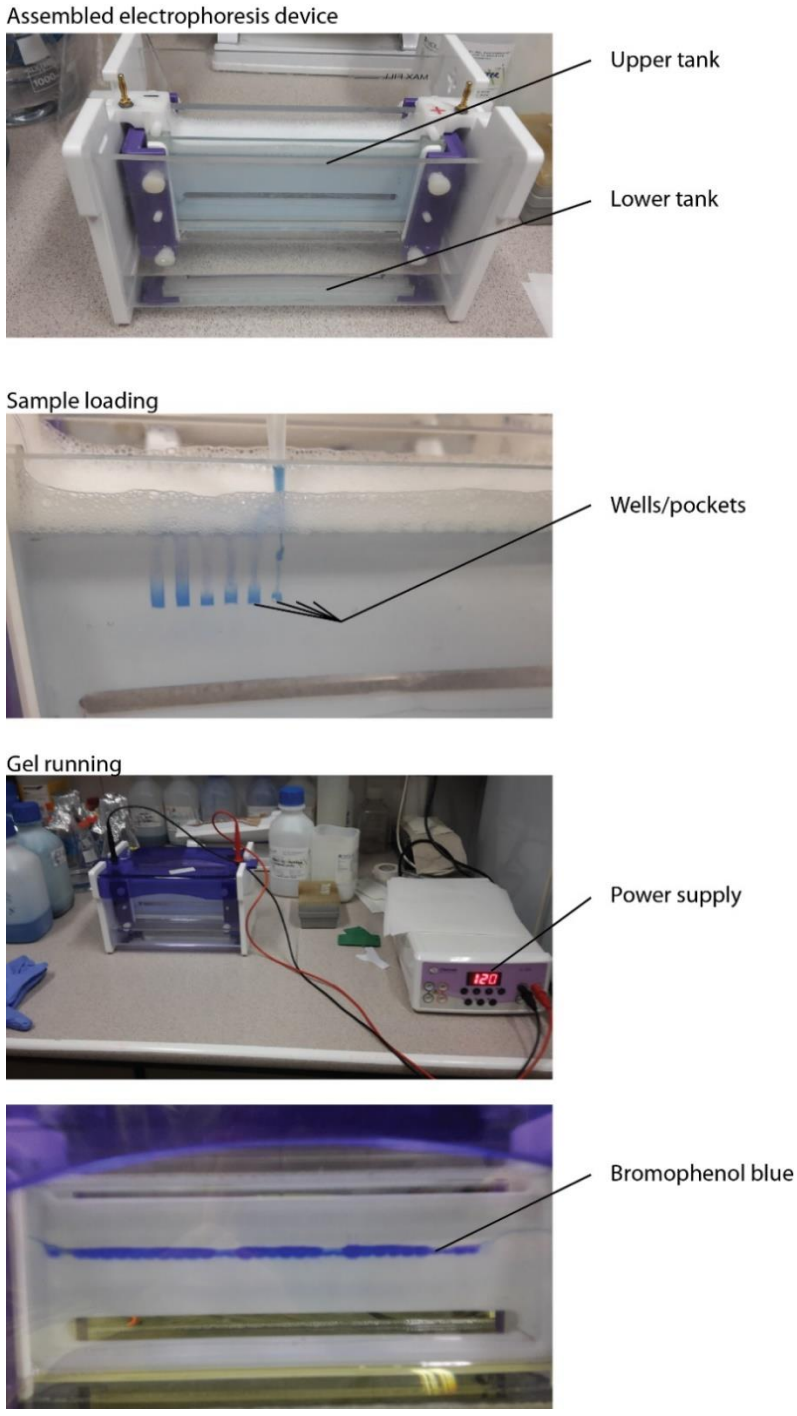


Figure 7.8 Loading and running the gel

3. Gel staining with Coomassie Brilliant Blue

- Disassemble the apparatus.
- Put the gel into the fixing solution (50 % methanol, 10 % acetic acid) and put it on a shaker for 15 min.
- Exchange the fixing solution to the Coomassie Brilliant Blue solution. Put it on a shaker for 30-120 min.
- Exchange the dye solution to differentiating solution (10 % acetic acid) and shake it at least 60-120 min. Some additional alcohol could facilitate the process.

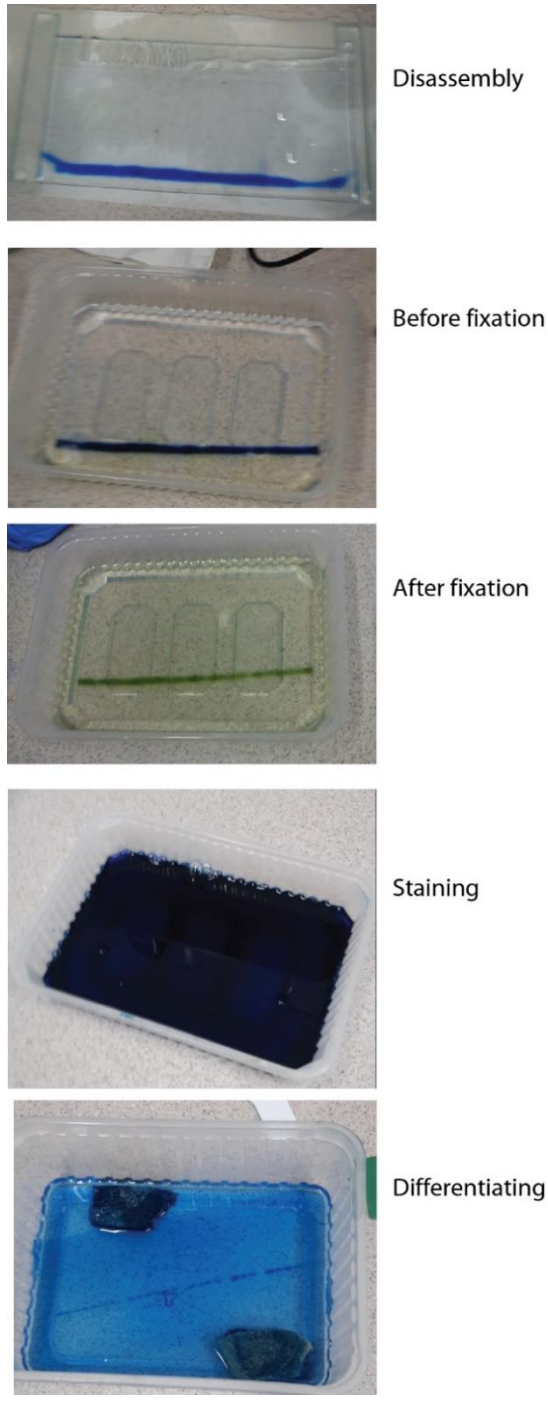


Figure 7.9 Staining the proteins by Coomassie staining

Materials

2X SDS loading buffer:

- 100 mM Tris pH 6.8
- 200 mM DTT
- 4 % SDS
- 0.2 % Bromophenol blue
- 20 % Glycerol

Buffer I:

- 100 mM Tris pH 7.5
- 2 mM EDTA

30-0.8% acrylamide-bisacrylamide

4x separating gel buffer

- 1.5 M Tris pH 8.8
- 0.4 % SDS

4x stacking gel buffer

- 0.5 M Tris pH 6.8
- 0.4 % SDS

10 % APS

TEMED

5 x SDS ELFO buffer (2 l)

- 20 g SDS
- 30.2 g Tris
- 188 g Glycine
- H_2O
- pH should be 8.3

Fixing solution

- 50 % methanol
- 10 % acetic acid

Coomassie Brilliant Blue

- 50 % methanol
- 10 % acetic acid
- 0,05 % Coomassie Brilliant Blue R-250

Differentiating solution

- 10 % acetic acid
- (5% methanol)

VI. Lab report

Date:_____

Name:_____

Title:_____

Aim: _____

_____

Short summary (2-3 sentences about the experimental setup):

_____

_____

_____

_____

_____

Used materials (You should check all the solutions and materials before starting the experiment. You should know the purposes/reasons of using them during the experiment.)**:**

acrylamide, bisacrylamide:_____

_____

APS, TEMED: _____

separating gel buffer (pH?): _____

stacking gel buffer (pH?):_____

SDS ELFO buffer: _____

_____

- SDS: _____
- Tris:_____
- glycine: _____

2x SDS loading buffer:

- glycerol: _____
- DTT or beta-mercaptoethanol: _____

  _____
- SDS: _____

  _____
- bromophenol blue: _____

  _____
- Tris:_____

Coomassie Brilliant Blue:_____

Changes in the protocol: (the experiment could be repeated only, if we write down everything):

Observations and Conclusion (Picture of the gel should be printed, labelled – ladder and samples as well – and attached):

Gel picture

Don’t forget to label every sample.

•Was the Pfu DNA polymerase expressed? At which size could we see it (kDa)?

_____

_____

_____

When should be the cells collected to reach optimal heterologous protein yield?

_____

_____

_____

•Did IPTG or lactose result in a stronger induction? Was there a significant difference?

_____

_____

_____

•Did the sonication work efficiently? (comparison of sonicated supernatant and pellet)

_____

_____

_____

•Was the Pfu protein present in the soluble fraction? (comparison of sonicated supernatant and pellet)

_____

_____

_____

•Was the purification successful? (comparison of sonicated supernatant and boiled supernatant)

_____

_____

•What modifications should be taken to improve the protocol?

_____

_____

_____

Additional information

Evaluating gels

The evaluation of an SDS-PAGE is simple, although polyacrylamide gels could be much more complex than agarose gels. In a situation similar to those that we used, the gel separates the full protein pool of the biological sample, therefore hundreds of proteins are visible on a gel after staining. Proteins that are expressed at a low level or are identical or very similar in their size to other proteins might not be visible or identifiable on the gel. For these cases, other methods with higher resolution (gradient gel or two-dimensional gels) or specific detection of the desired protein (for example Western blot) can be used. See more information about the theoretical background of Western blot in Chapter 12.

Despite the complexity of total bacterial cell lysates, if proper controls were applied and the level of protein expression is not too low, generally an overexpressed heterologous protein could be easily identified in the cell lysate. We can calculate the expected molecular weight of our heterologous protein by using its amino acid sequence or we can find it in a proper database on the internet. For a good estimation you can multiply the number of amino acids of your protein of intrest with 110 to get the approximate molecular mass of the protein. The overexpressed protein will appear as an extra band at the calculated position on the gel compared to the negative control.

By using SDS-PAGE, we can answer the most important questions that emerge during heterologous protein production and purification, such as:

- were the proteins induced?
- did the cell disruption work?
- was our protein soluble?
- was the purification successful?

Protein induction

As described above, the evaluation of polyacrylamide gels could be tricky. To identify our protein, we should know its molecular weight and we should use proper controls. For instance, by comparing the non-induced sample with an induced one, we could identify the induced protein.

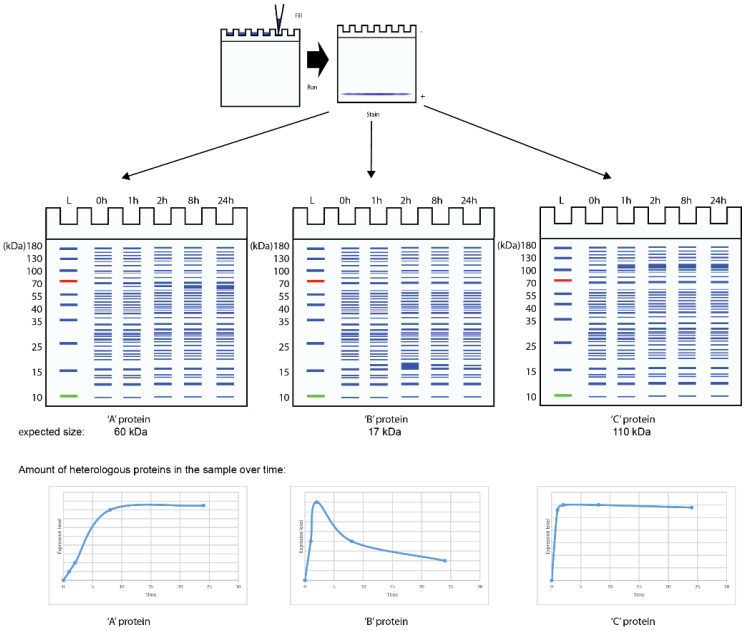


Figure 7.10 The dynamics of protein production could be different among different proteins

Try to identify the heterologous proteins at the given mass!

Real-life example:

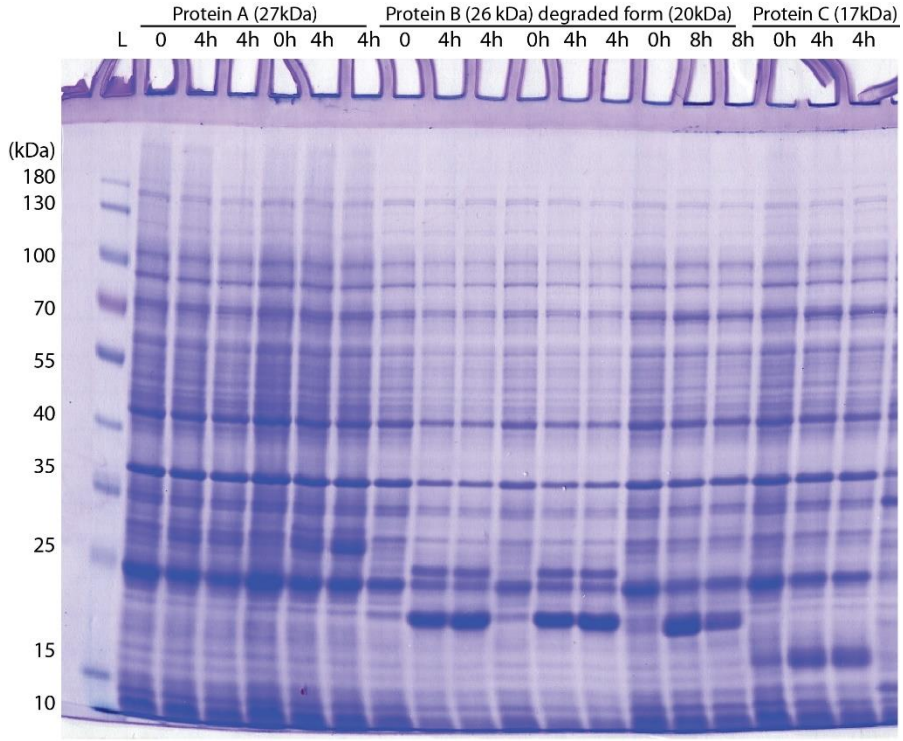


Figure 7.11 Identify the heterologous proteins! (Sample order: Ladder, non-induced sample, induced sample 4 h, induced sample 4 h - repeated in 6 cases - Protein B has 8 hours of induction in one case.)

Is Protein B a stable protein?

Effect of cell disruption

Proteins produced in *Escherichia coli* usually are not secreted, therefore we have to lysate the cells to get the cytoplasm, where our desired protein is localised. To test whether the cell disruption was successful, our samples should be centrifuged after sonication. After this step, we will get two fractions: the pellet and the supernatant. The intact cells are sedimented to the bottom of the tube with the cell debris forming a pellet, while the soluble components of the cytoplasm remain in the supernatant. Therefore, comparing the protein pool of the supernatant and the pellet provides information about the efficiency of the sonication.

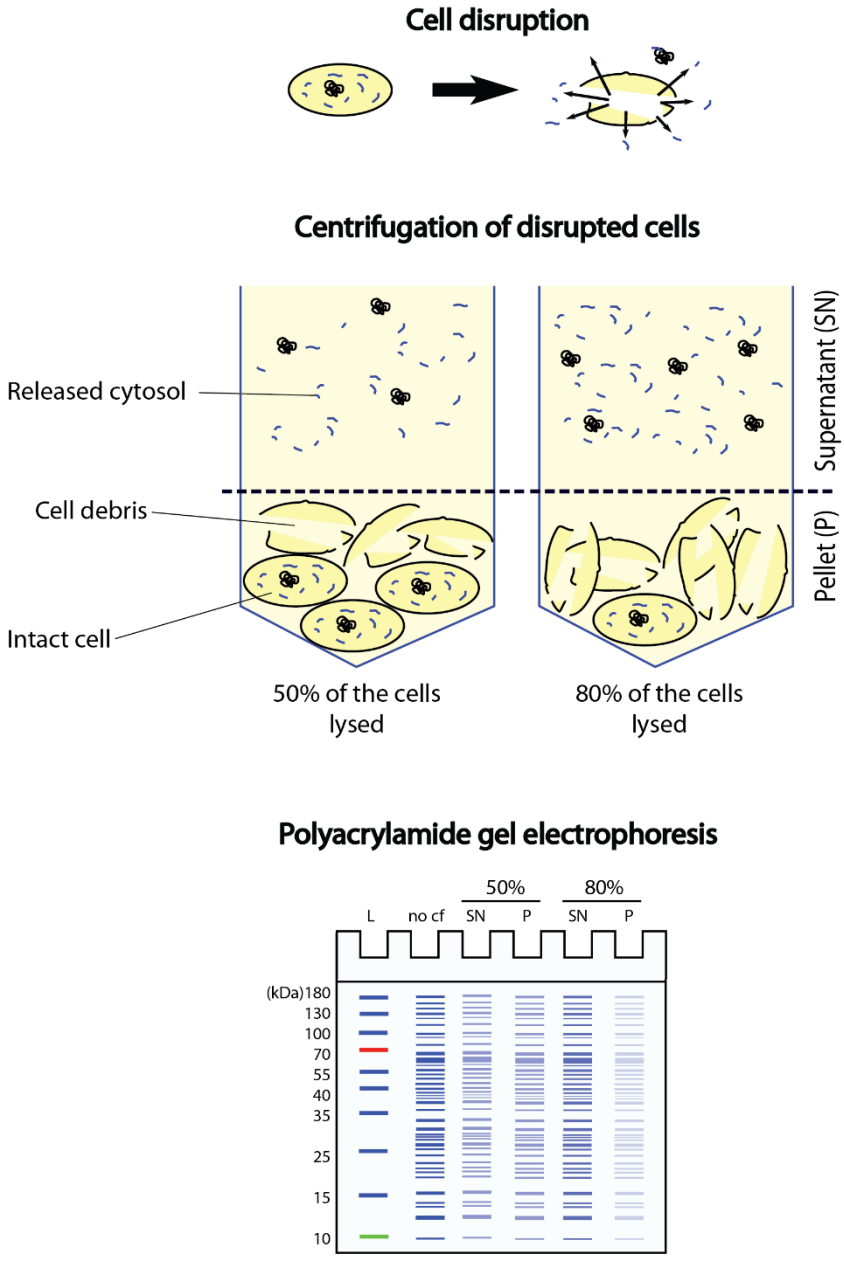


Figure 7.12 The efficiency of cell disruption. (SN - supernatant, P - pellet)

Real-life example:

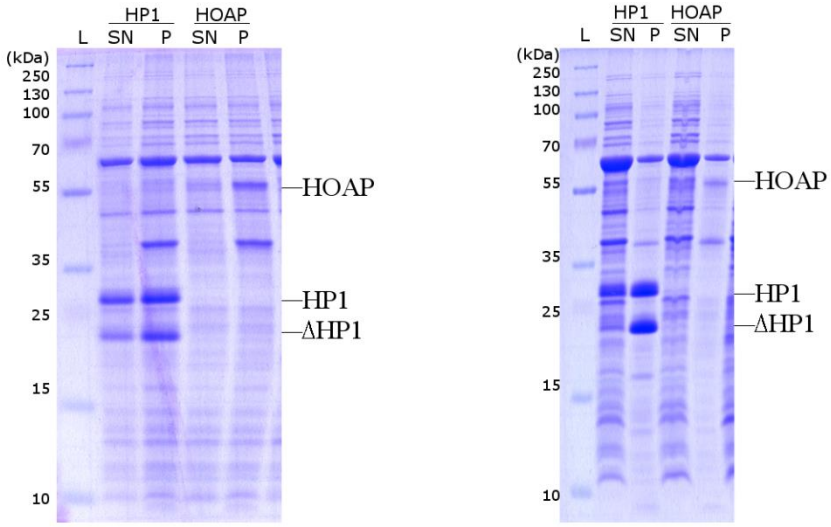


Figure 7.13 The efficiency of cell disruption. The example shows two proteins: HP1 and HOAP. (SN - supernatant, P - pellet)

At which case was the sonication more effective – in samples seen on gels on the left or on the right side?

Protein solubility

Eukaryotic proteins overexpressed in bacteria often form an insoluble precipitate, known as an inclusion body. By centrifugation, inclusion bodies can be also pelleted. If a protein has low solubility (forms an inclusion body), after cell disruption this protein appears only in the pellet independently from the efficiency of lysation. In the previous example, HP1 and HOAP formed inclusion bodies.

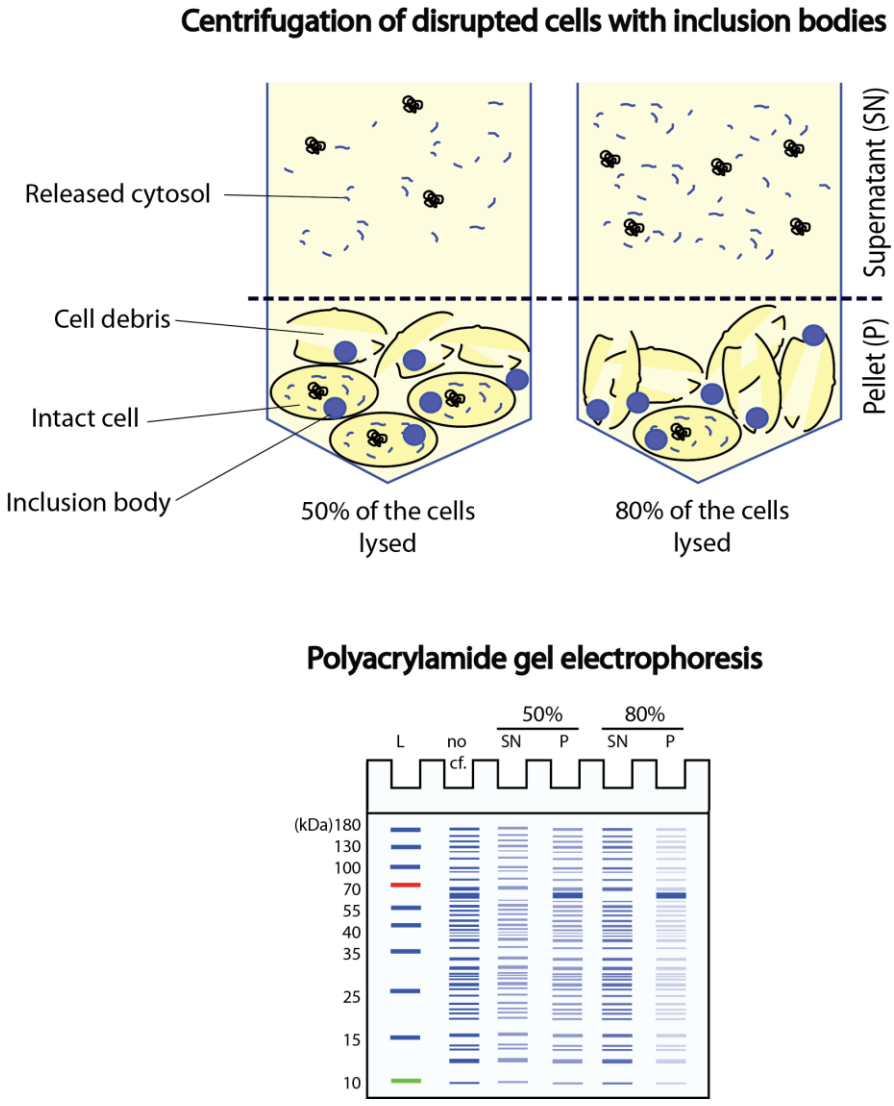


Figure 7.14 Inclusion bodies could be found in the pellet

Real-life example:

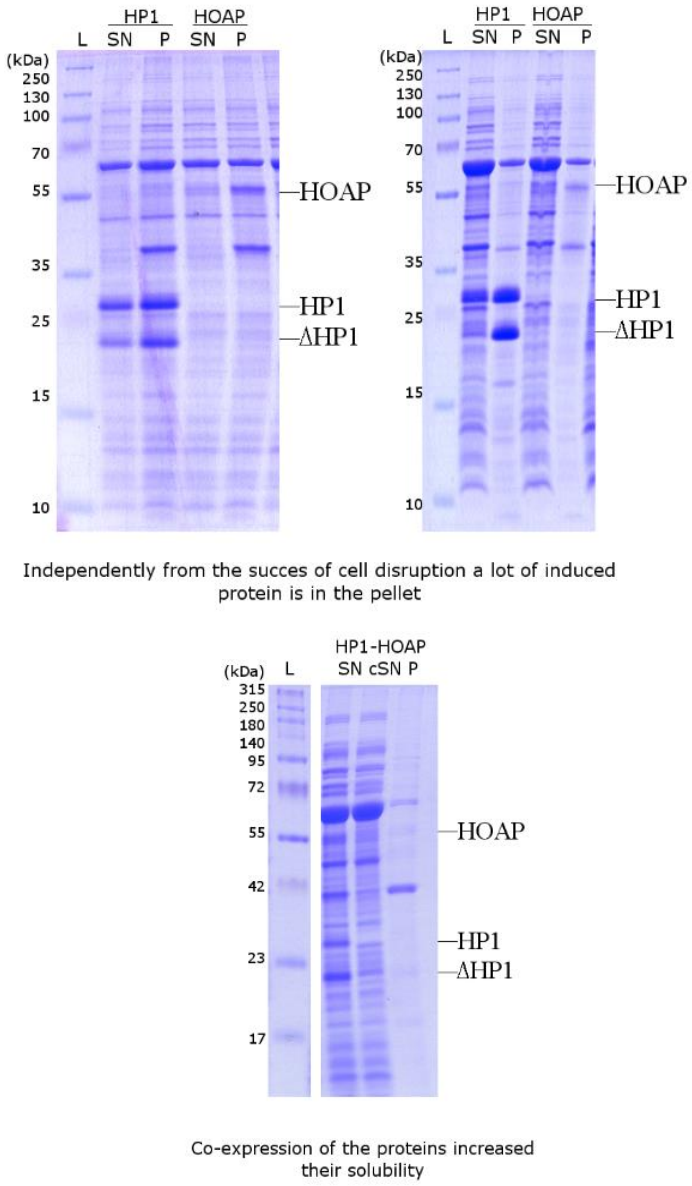


Figure 7.15 The solubility of a protein could be increased

Success of purification

We could also monitor the purification steps with SDS-PAGE, but this will be detailed in Chapter 11.

Laemmli's article

Protein electrophoresis under denaturing conditions as described above is also referred to Laemmli gel electrophoresis named after Ulrich K. Laemmli, who efficiently improved the gel-based protein separation methods. His protocol has become a standard in almost every laboratory. His article, in which he introduced his method, belongs to one of the most cited articles ever, with more than two hundred thousand citations.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Web of Science® Times Cited: 205649 (2018.01.02.)

Questions

Who did develop the SDS-PAGE?

_____

Why is electrophoresis of proteins more challenging than electrophoresis of DNA?

_____

_____

Why is casting a stacking gel important?

_____

_____

_____

Which are the negative ions that run in the gel?

_____

Why is setting the correct pH important during SDS-PAGE?

_____

_____

_____

Chapter 8

Research project in molecular biology

7th practice

Testing the polymerase activity of the extracted Pfu

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will be familiar with the principles of PCR.
2. They will be familiar with the ingredients and the steps of the PCR.
3. They will know the applications of PCR.

Skill:

4. They will be able to prepare a PCR reaction.

Attitude:

5. They will inquire about the background of the PCR reaction.

Responsibility and autonomy:

6. They will be able to make and use master mixes on their own.

Testing the polymerase activity of the extracted Pfu

During the previous practices, we transformed the pET-Pfu plasmid into Rosetta cells, then we applied IPTG and lactose to induce the protein production in these cells. As a next step, we disrupted the cells by sonication and heat treated the cell extract to precipitate the non-thermostable proteins. We pelleted the non-thermostable host proteins to separate them from the induced heterologous protein by centrifugation.

We determined whether the purification of the Pfu DNA polymerase was successful by analysing our samples on polyacrylamide gel. The aim of this practice is to test the activity of the purified Pfu DNA polymerase.

Theoretical background

For testing the biological activity of the Pfu DNA polymerase we are going to use the polymerase chain reaction (PCR). PCR makes the quick amplification of relatively short DNA fragments *in vitro* (in test tubes) possible. The invention of this technique in the early eighties by Kary B Mullis had a huge impact on life sciences, and nowadays PCR is used in many-many research- and biotechnology-related applications.

PCR is similar to the replication process in living cells, in which DNA template is used to generate new copies of the DNA by a polymerase enzyme. During PCR reaction, the double stranded DNA is getting single stranded (denaturation), then primers attach to it (annealing) and a DNA polymerase is used to extend the attached primers while reforming the double stranded DNA molecules again (polymerisation/extension). However, instead of using helicases (as in nature), during PCR heat is responsible for the denaturation of the double stranded DNA and instead of RNA primers (synthesized by the primases during replication), during PCR single stranded DNA oligomers are added to the reaction to make the start of DNA synthesis possible. For PCR, specific thermostable DNA polymerase is used, and the steps of this reaction are repeated in a cyclic program.

The required materials for a PCR

Six basic components are needed for a PCR.

First of all, we need a **template**, which can be a mixture of DNA molecules containing the DNA region, which needs to be amplified.

Generally, by a PCR reaction, we can copy a relatively short fragment of DNA. (Fortunately, we almost never need to or want to amplify very long template molecules. For example, handling a whole chromosome would be rather challenging.)

We also need **primers** because DNA polymerases cannot start DNA synthesis, but they can extend existing DNA molecules by adding new nucleotides to their 3' ends. Primers used in PCR are short single stranded DNA oligonucleotides (15-25 nucleotides) that are complementary with regions of the template molecules. Therefore, primers could hybridize to the template molecule and provide a docking surface for DNA polymerase. During PCR, two primers are used. These determine the borders, between which the DNA synthesis is performed. One is called forward and the other is called reverse primer. They are complementary to different strands of the template molecule. When the primers hybridise to the template strands, their 3' ends face towards each other, therefore the PCR product will be the region, which is located between the attachment surfaces of the primers on the template strand (see more at the end of this chapter).

For PCR reactions we also need the "building blocks" of the DNA molecule, the nucleotides: these are incorporated by the polymerise enzyme from nucleoside-triphosphates - dATP (d: deoxiribo; A: adenosine; T:tri; P: phosphate), dGTP (G: guanosine), dCTP (C: cytidine), and dTTP (T: thymidine). These molecules serve not only as building blocks but also provide the energy required for DNA synthesis: the cleavage of pyrophosphate group (PPi) from them provides the energy for phosphodiester bond formations. These precursor molecules are added to the reaction as a mixture labelled as **dNTP** (N: nucleoside)

mix. During synthesis, the 3’ OH of the growing chain of DNA attacks the α-phosphate on the next dNTP to be incorporated, resulting in a phosphodiester linkage and the release of a pyrophosphate (PPi).

DNA polymerase is the enzyme that performs the synthesis reactions. Since during PCR, denaturation (the separation of the DNA strands) is performed at a high temperature, thermostable enzymes should be used since it can preserve its activity even under these conditions. Such enzymes could be isolated from extreme thermophile organisms, mainly from archaea. The most well-known polymerase used for PCR was isolated from the archeon *Thermus aquaticus*, therefore it was named Taq DNA polymerase. Taq polymerase is a low fidelity polymerase, since it has no proofreading activity (3’-5’ exonuclease activity). The Pfu polymerase, which was isolated from *Pyrococcus furiosus*, has higher fidelity than Taq polymerase, because it has a 3’-5’ exonuclease activity. By this activity, nucleotide incorporation is double checked by the enzyme and in case of a wrong nucleotide incorporation into the growing DNA strand, the enzyme can immediately remove it. This increases the fidelity of DNA synthesis. Consequently, Taq-related DNA synthesis results in more errors in the DNA, than Pfu-related synthesis does. In addition to these two enzymes, many other polymerases are used for PCR and among them, there are some engineered versions produced by recombinant DNA technology (for example, DreamTaq and Phusion have been made from Taq and Pfu, respectively).

(The polymerase gene with which we work during this practical course was cloned by us from DNA isolated from *Pyrococcus furiosus* cells obtained from hot springs of the Yellowstone National Park US.)

The forth important requirement for PCR reactions is to provide the ideal conditions for the enzyme. This means mimicking the conditions that are present in a living cell concerning pH, types and concentration of ions and other circumstances. These are provided by the use of a proper **buffer**. Reaction buffers for Pfu polymerase contain Tris-HCl (pH 8.8) to set the optimal pH. Ions are also included in the solution to avoid precipitation of the proteins and to provide the correct ionic strength, which keeps the protein properly folded and catalytically active. Magnesium ions are needed as co-factors for the polymerisation. In addition, the buffer also contains some BSA (Bovine Serum Albumin) and Triton X-100 to prevent protein precipitation.

To avoid contaminations and to maintain a well-controlled environment for the reaction, the **water** used for the reaction must be very pure. Since PCR is an extremely sensitive technique, by which single molecules of DNA can be multiplied to hundreds of millions copies, it is very important to avoid any contaminations of the reactions with some traces of DNA from the environment.

Sometimes PCR reactions need to be supplemented with further additives to work more efficiently. For example, DMSO might be used to decrease the melting temperature of DNA, which might be important when somebody works with DNA that has high GC-content.

Specific equipment is also required to perform a PCR reaction. It should be capable for quickly warm up and cool down samples and precisely keep temperatures and time intervals that has been set for the reaction. The instruments developed for this are called thermocyclers, which will be introduced later in this chapter.

The steps of a PCR reaction:

I. Pre-Denaturation – Enzyme activation

Before starting the cycling steps of PCR, a short incubation (1-5 minutes) is generally performed at 95-98 °C. This step is required to completely melt the template DNA before the beginning of the reaction. Some of the commercially available polymerases require this step to be heat-activated before the PCR.

II. Cycling steps:

PCR is called a chain reaction, since the following three steps (denaturation, annealing and polymerisation) are repeated for 25 to 35 cycles.

1. Denaturation

The two strands of the template have to be separated from each other allowing the primers to find and hybridize to the single stranded DNA regions. To achieve this, we heat the sample to 95-98 °C. At this point, the H-bonds cease between the two strands, the DNA melts and becomes single stranded. This step takes 15 to 30 seconds.

In general, the term denaturation is used for the process, in which a form or a state of a molecule becomes different from its natural form or state. In biology we can speak about denaturation of nucleic acids and proteins, as well. Denaturation could be either reversible or irreversible. Melting the DNA by heating is a reversible denaturation since the single stranded DNA strands formed by denaturation can hybridize with each other, thereby double stranded DNA molecules will be formed again. This phenomenon is generally called renaturation. However, a similar

mechanism during PCR, the binding of primers is referred to as annealing. Denaturation of the proteins by heating is usually irreversible because the structure of the protein couldn't be recovered upon cooling.

2. Annealing or hybridization

The second step of the cycle is called annealing or hybridization, in which the primers bind to their complementary sequence.

It is worth to note here that nucleic acid renaturation, hybridisation and annealing are very similar processes, since each is determined by the H-bond formation between complementary strands of single stranded (ss) polynucleotide chains. We speak about renaturation when the two strands of a previously denatured DNA sample reform the double strands again. We speak about hybridisation, when single stranded nucleic acid molecules from different sources form double strands. Annealing is basically the same, but in this case one of the DNA strands is a short oligonucleotide.

To allow the formation of H-bonds between the primers and the single stranded template, the temperature must be reduced. In PCR reactions the primers are present in much higher concentrations than the template, therefore it is more likely that the template will anneal to the primers instead of its sister strand. The annealing temperature is determined by the length and base composition of the primer and by the buffer conditions. Generally, temperatures between 55-62 ºC are used during PCR. If the annealing temperature is too low, the primers can hybridize to sequences, which are not perfectly complementary with them. In that case, the DNA polymerase will elongate these primers, which leads to the generation of non-specific products. By increasing the annealing

temperature, the frequency of non-specific priming will be decreased, but too high temperature could inhibit the annealing to specific sites, as well. The annealing step takes 15 to 30 seconds.

3. Polymerisation or elongation

The third cycling step of PCR reaction is the elongation or DNA synthesis. In this step, the polymerase starts the polymerisation from the 3' end of the primers by 'copying' the template and incorporating dNTPs into the growing strand. The optimal temperature for thermostable polymerases is usually at around 72 °C. The duration of this step depends on the size of the PCR product (the distance between the primers) and on the speed of the polymerase, which is between 1,000-2,000 nucleotide incorporation/min. Polymerases without proofreading activity are usually faster.

III. Final elongation

After the cycling steps of PCR reaction, an additional step is applied as a final elongation. This step serves to allow completing the polymerisation of all newly synthesized DNA strands that have not been copied in full length during the cycling reaction for some reason. This step takes 2-7 minutes at the optimal working temperature of the polymerase (usually at 72 °C).

Figure 8.1 A basic thermocycler

Further information

However, PCR is a simple and reliable technique, some issues should be considered in order to perform it successfully.

First of all, as it has been mentioned, PCR is very sensitive for contaminations. If a PCR reaction becomes contaminated with foreign genetic material, we can amplify DNA products from the contamination instead of our sample. This could result in serious consequences by generating false positive results in a diagnostic application or making

the experimental outcomes difficult to evaluate and interpret by amplifying irrelevant sequences.

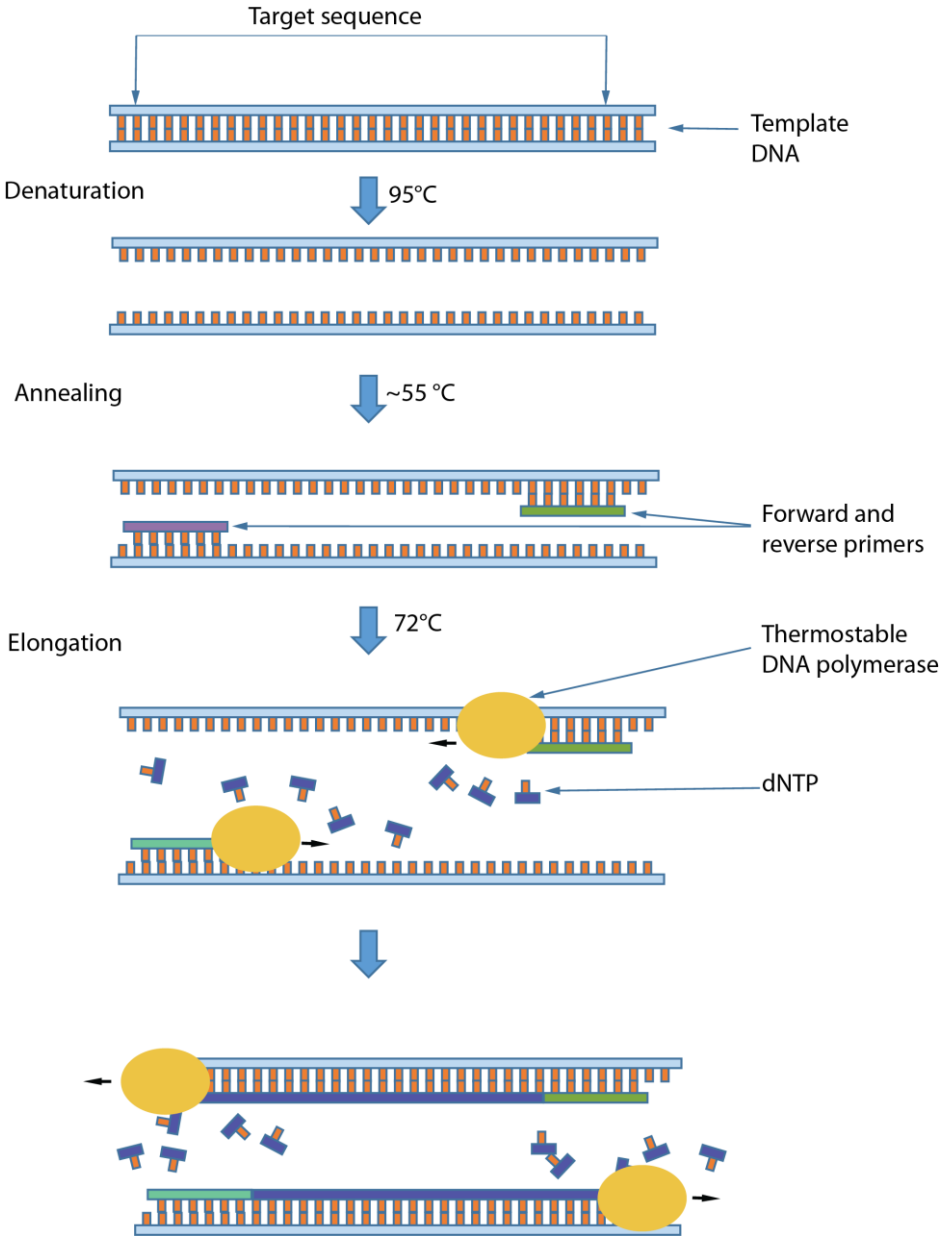


Figure 8.2 The steps of a PCR reaction: Denaturation, annealing and elongation

For a PCR, sequence information about the target sequence should be available in order to design the proper primers. We can get this information from databases expanding with high speed as an advent of next generation sequencing. Nowadays, several computer programs are available for choosing the optimal primer sequences for our application. By these the primers can be designed with consideration of the proper melting temperature, GC content, avoiding the formation of primer dimers or hairpin structures and also their non-specific binding to the template.

Theoretically, in a PCR reaction, the amount of the target DNA is doubled in each cycle. This means that the amount of the products is exponentially increasing: the base is 2 with the power of the cycle number (2^n, n = cycle number).

e.g. after 3 cycles – 2^3=8,

after 20 cycles - 2^{20}~1 million

after 35 cycles - 2^{35}~ 3.4 x10^{10}

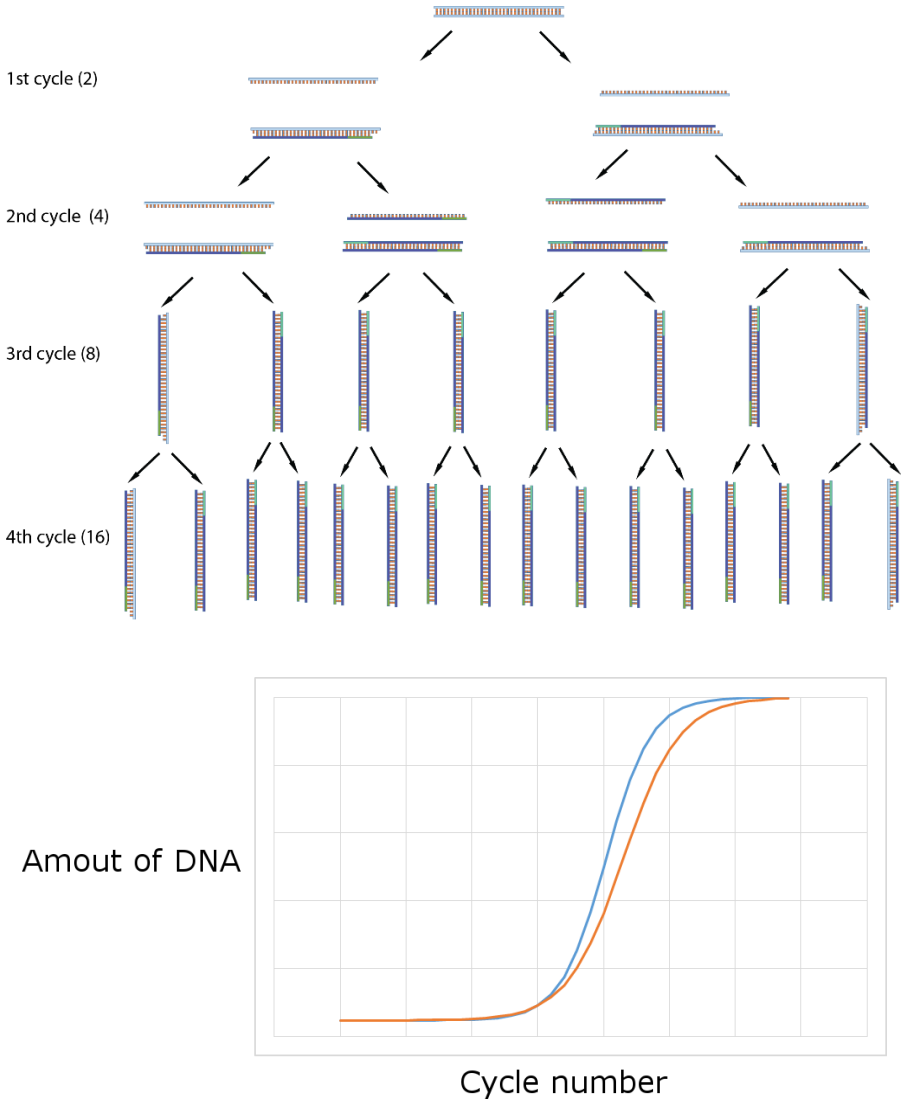


Figure 8.3 The number of PCR products is exponentially increasing until the reaction is saturated

In practice, the amount of PCR products cannot be indefinitely increased, since the reaction is saturated after a while. This can be

explained by the decreasing amount of dNTP and the increasing pyrophosphate levels.

Applications

PCR has a huge impact on medicine, basic science, forensic science and quality control.

In medical applications, PCR has become a basic tool to identify pathogens much faster than classical microbiological methods. The classification of viruses, bacteria and fungi is also possible with PCR. Furthermore, already known mutations could be detected by PCR, therefore this technique could be used for genotyping and prenatal screening. Forensic science also utilizes PCR for paternity tests and identifying offender culprits.

In quality control, PCR is used to identify pests, faked foods and GMO products.

PCR machine has become a basic equipment of a laboratory, such as a centrifuge is.

Several variations and types of PCR have been developed for specific applications. A list of these is: high fidelity PCR, nested PCR, inverse PCR, qPCR, RT-qPCR, digital PCR, multiplex PCR, colony PCR, asymmetric PCR, KASP PCR, touchdown PCR, solid phase PCR, methylation sensitive PCR, etc.

*"PCR has transformed molecular biology through vastly extending the capacity to identify, manipulate and reproduce DNA. It makes abundant what was once scarce – the genetic material required for experimentations. /*Paul Rabinow/

(Making PCR, A Story of Biotechnology, University of Chicago Press, 1996)

Practical workflow and protocol

Our goal is to test the activity of the purified Pfu DNA polymerase. For this, we perform a PCR reaction. Originally this PCR reaction was designed to show whether a promoter fragment of a gene we are interested in (Ada2b) was successfully inserted into a cloning vector (pGL3). (Note that since we want to test the polymerase activity of Pfu, any PCR reactions that has been shown to work could be used for this.)

Sample: Rosetta (DE3) cells induced with 1 mM IPTG or 0.2 % lactose were lysed, and the cell extract was heat-treated. From this, the non-thermostable proteins were precipitated and removed by centrifugation. The remaining supernatant contains the thermostable protein(s) among them there is the Pfu DNA polymerase. (The host *E. coli* cells might also have proteins that survived the heat treatment. However, as long as these do not interfere with our reactions we do not have to take them into consideration).

Protocol:

1. Prepare the PCR reaction according to this description:

Essential components of the PCR reaction:

1. Thermostable polymerase (e.g. Taq, Vent, **Pfu**): in our case, the source of the polymerase will be the protein that we purified from the Rosetta cells.
2. dNTP: mix of precursors of DNA synthesis (substrates of the polymerase).
3. PCR buffer: solution that ensures optimal conditions for the polymerase function by providing pH 8.8, ion concentration and other co-factors.
4. primers: single stranded oligonucleotides. Used in pairs: one forward and one reverse primer. The forward and reverse primers hybridize to different strands of the DNA template.
5. DNA template: in our case it is a plasmid molecule.
6. Water: milieu for biochemical reactions, such as DNA polymerisation. We add the components of the PCR reactions from stock solutions to determine the volume of water to achieve the desired concentrations and ensure equal final reaction volumes.

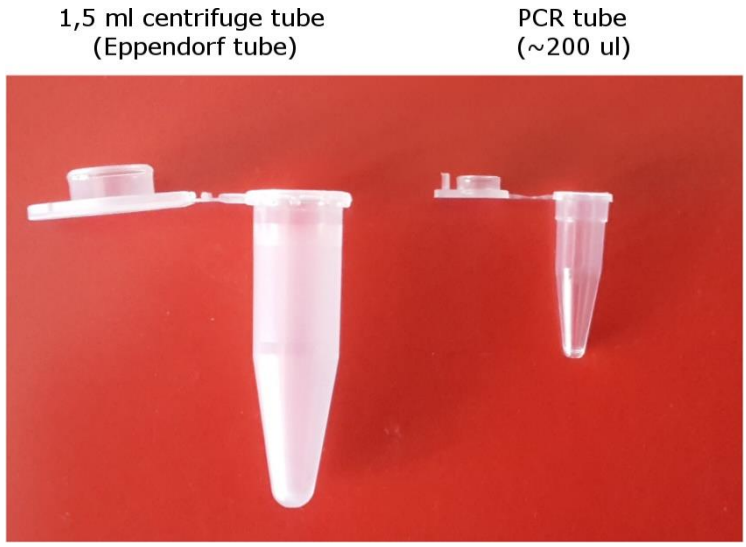


Figure 8.4 A 1.5 ml test tube (on the left) and a PCR tube (on the right). PCR tube has thinner wall to conduct heat more efficiently.

For performing a PCR, we use specific test tubes, designed for this experiment. These tubes have thinner wall that makes fast temperature adjustment possible and they also have stronger lid to avoid sample evaporation.

Recipe for one reaction

- 2 ul of boiled supernatant (different amount could be used)
- 1 ul dNTP mix (10 mM)
- 5 ul 10x Pfu polymerase buffer+$MgSO_4$
- 1 ul primer 1 Ada2b P Fwd (10 uM)
- 1 ul primer 2 Ada2b P Rev (10 uM)
- 1 ul template DNA (pGL3-p550)
- 39 ul distilled water

Tip:

✓ The PCR reaction has a lot of ingredients, if something is added write a check mark next to it.

Controls:

(One positive and one negative control will be enough for the whole group.)

Positive control

Since PCR is a quite complex method with several steps in which a mistake or an error could occur (pipetting error, inactivation of reagents, etc.), we need to use controls. As a positive control we set up a reaction with the same reagents that we use to test our enzyme, except that instead of our purified protein, we will use a commercial Pfu DNA polymerase. The result of this reaction will tell us if the conditions we used and the manipulations we did were correct.

- 0.5 ul commercially available Pfu DNA polymerase
- 1 ul dNTP mix (10 mM)
- 5 ul 10x Pfu polymerase buffer+$MgSO_4$
- 1 ul primer 1 Ada2b P Fwd (10 uM)
- 1 ul primer 2 Ada2b P Rev (10 uM)
- 1 ul template DNA (pGL3-p550)
- 40.5 ul distilled water

Negative control

We also need a negative control to monitor what happens if the polymerase is not active. In this case, add water instead of polymerase.

- 1 ul dNTP mix (10 mM)
- 5 ul 10x Pfu polymerase buffer+$MgSO_4$
- 1 ul primer 1 Ada2b P Fwd (10 uM)
- 1 ul primer 2 Ada2b P Rev (10 uM)
- 1 ul template DNA (pGL3-p550)
- 41 ul distilled water

In this case, we prepare a control to test Pfu activity. **Note:** in general, the negative control is used to control whether the reaction is contaminated with exogenous genetic material. In that case, instead of the polymerase, the DNA template is left out from the reaction.

2. Start the PCR machine according to the instructions

The parameters of the PCR reaction:

1. **Cycle number:** Generally, more cycle number means that more PCR products are generated, but the amount of synthesized DNA is exponentially increasing only in the first few cycles. The amount of the products starts to diverge from the theoretically amount at around 17^{th} cycle and it reaches a saturation after the 30^{th} cycle.
2. **Temperature:** The denaturation step is performed at 95 °C, when the double stranded DNA molecules become single stranded. Annealing

temperature is calculated according to the melting temperature (T_m) of the primers. Commonly, we use the temperature, which is 1-2 °C below the T_m of the primers. Be aware of that the buffer composition could influence the melting temperature. In order to calculate the correct value, several computer programs are available. Polymerisation should take place at the optimal working temperature of the polymerase, which is in general 72 °C.

3. **Duration:** Denaturation and annealing are very fast processes, although we use 30 seconds to ensure that the whole sample is reached the same temperature. Reducing the annealing time sometimes increases the specificity of the PCR. The time-span of the polymerisation step depends on the product length and the processivity of the applied enzyme (e.g. Taq – 1,000 bp/min, Pfu – 500 bp/min).

The PCR program:

95 °C 3 min

35 cycles:

95 °C 30 s

53 °C 30 s

72 °C 1 min 30 s

72 °C 5 min

4 °C ∞

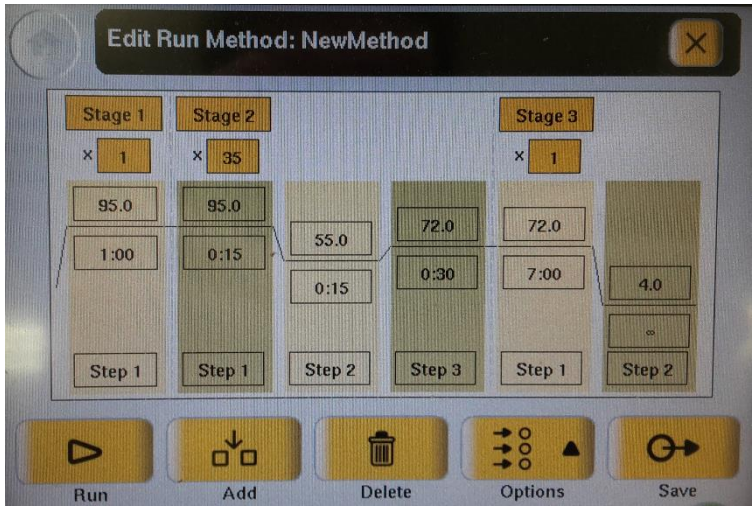


Figure 8.5 Example of a basic PCR program on a thermocycler. This is an example, we use a different protocol in the practice

Materials

DNA template (pGL3-p550)

dNTP mix(10mM)

Forward and Reverse primers (Ada2b P Fwd, Ada2b P Rev) (10 uM)

10x Pfu Buffer

Pfu DNA polymerase

H_2O

VII. Lab Report

Date: _____

Name: _____

Title: _____

_____

Aim: _____

_____

Short summary (2-3 sentences about the experimental setup):

_____

_____

_____

_____

_____

Used materials (You should check all the solutions and materials before you start the experiment. You should know for what and why we use them during the experimental process.)**:**

Pfu polymerase: _____

_____

dNTP: _____

_____

PCR buffer:_____

_____

primers:_____

_____

template: _____

_____

d.water: _____

_____

Changes in the protocol: (the experiment could be repeated only, if we write down everything, ex. changes in the template/primers/T_m/cycle number etc…):

_____

_____

_____

_____

_____

_____

_____

_____

_____

Observations and Conclusion:

Additional information about PCR

Using a master mix

In practice, it is rare that we have only one sample to test. In case of more samples, we could mix some of the reagents in the same tube, preparing a mixture of reagents, which is referred to as a master mix. This mix contains everything that is required for a PCR except those ingredients, which have to be tested in each case.

A practical example if we have two Pfu containing supernatants (e.g. induced with lactose or IPTG), which will be tested in 1x, 10x and 100x fold dilutions in PCR reactions, it means 6 samples. We also have to prepare a positive and a negative control to reveal whether our PCR reaction works, which means two more, altogether 8 samples. Since the liquids could stick to the plastic ware, we calculate the master mix to 8.2 samples.

Recipe for:

1 reaction	8.2 reactions
1 ul primer 1 Ada2b P Fwd (10 uM stock)	8.2 ul
1 ul primer 2 Ada2b P Rev (10 uM stock)	8.2 ul
1 ul dNTP mix (10 mM)	8.2 ul
5 ul 10X Pfu polymerase buffer+$MgSO_4$	41 ul
39 ul dH_2O +288 ul	319.8 ul
1 ul template DNA (pGL3-p550)	8.2 ul

Divide the mix into eight PCR tubes- each tube should contain 48 ul - and add the 6 supernatants and also the commercially available Pfu as a positive and water as a negative control to the appropriate reaction. (The commercially available Pfu should be diluted following the manufacturer's instructions.)

Normally, we do not want to test the activity of the polymerase enzyme, but we need information about the templates. In this case, the template should be left out from the master mix. The appropriate controls are also important: choose a template that works in the reaction as a positive control and a template that does not work (usually water) as a negative control.

The invention of the PCR

The idea of PCR is derived from Kary Banks Mullis. According to his recollection the idea occurred to him in 1983 when he drove on US 101 route in California. This route has two lanes for both directions separated by a large gap in the middle of it, which is similar to the two strands of a DNA. If we imagine the cars as DNA polymerases, we can realize that they can go to both directions. In that time, DNA polymerases were used in reactions, but these reactions contained only one primer. The road gave Kary Banks Mullis the idea of utilizing two primers in a chain reaction and he had developed the PCR method, which he presented in 1985. For his work, he was awarded the Nobel Prize in 1993.

The first PCR reactions were very expensive and they had low efficiency. The thermal cycling was performed manually by using water baths with different temperatures. In addition, the DNA polymerase

(Klenow fragment from *E. coli*) was denatured with the DNA at 95 °C and lost its activity, so it had to be supplemented at the end of each cycle. The optimal working temperature of this polymerase was 37 °C, therefore a lot of non-specific PCR products were amplified.

The first thermostable DNA polymerase was isolated from *Thermus aquaticus*, an archea living in hot springs. The enzymes of this species are stable at high temperatures and can even withstand boiling without denaturation. The discovery of Taq polymerase was awarded with Molecule of the Year 1989 by the *Science* journal. Pfu DNA polymerase was discovered later and was first used for PCR in 1991. (Lundberg et al., "A High Fidelity Thermostable DNA Polymerase Isolated from *Pyrococcus Furiosus*," Strategies 4:34-35 (1991))

It is important to mention that not only the development of enzymes but the development of the equipment was also necessary for the success of the PCR. The water baths were changed to Peltier plates, which allow much faster heating and cooling and most importantly, automation.

Nowadays, one of the fastest PCR machines are based on the principle of capillary flow in a tube system, which is an interesting solution worth mentioning (Figure 8.6).

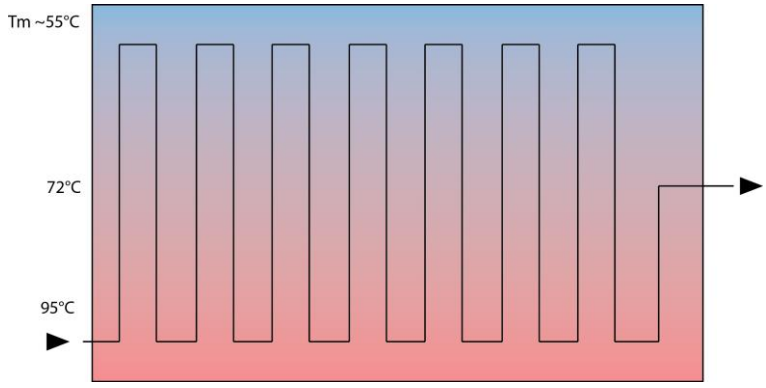


Figure 8.6 Capillary flow PCR

Primer design

It is important to plan the primers properly, since the success of the PCR depends on it. Therefore, we list some principles, which should be followed.

- The primer length is generally between 17-30 base-pairs.
- The complementarity between forward and reverse primers should be avoided because it can lead to the formation of primer dimers.
- Upon primer designing, primers that have strong secondary structure should be also avoided, since they can create hairpin structures, thereby inhibiting the reaction.
- The too high GC content and high melting temperature could also result in failure of the reaction.
- Differences in the melting temperature between the forward and reverse primers should not be more than 5 °C.
- More preferably primers should contain C or G at their 3’ end.

Questions

Who was rewarded with the Nobel Prize for the development of PCR in 1993?

_____

What are the ingredients of the PCR?

_____

_____

What are the steps of a PCR reaction?

_____

_____

_____

Why is PCR extremely important?

_____

_____

What are the practical applications of PCR?

_____

_____

_____

List at least five PCR-based techniques!

_____

_____

Chapter 9

Research project in molecular biology

8th practice

Detection of Pfu DNA polymerase activity – Running PCR products on agarose gel

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will be familiar with agarose gel electrophoresis.

Skill:

2. They will be able to prepare an agarose gel.

Attitude:

3. They will inquire about the theoretical background of agarose gel electrophoresis and gel evaluation.
4. They are willing to try basic troubleshooting to improve their experiments.

Responsibility and autonomy:

5. They will be able to evaluate agarose gels on their own.
6. They will handle toxic materials with care.

Detection of Pfu DNA polymerase activity – Running PCR products on agarose gel

So far, we have successfully purified the Pfu DNA polymerase in a small scale. We have also performed PCR reactions to test whether the Pfu DNA polymerase was active. In this practice, we should analyse the PCR results by visualising the DNA molecules. For this, we will cast an agarose gel and run our samples on it.

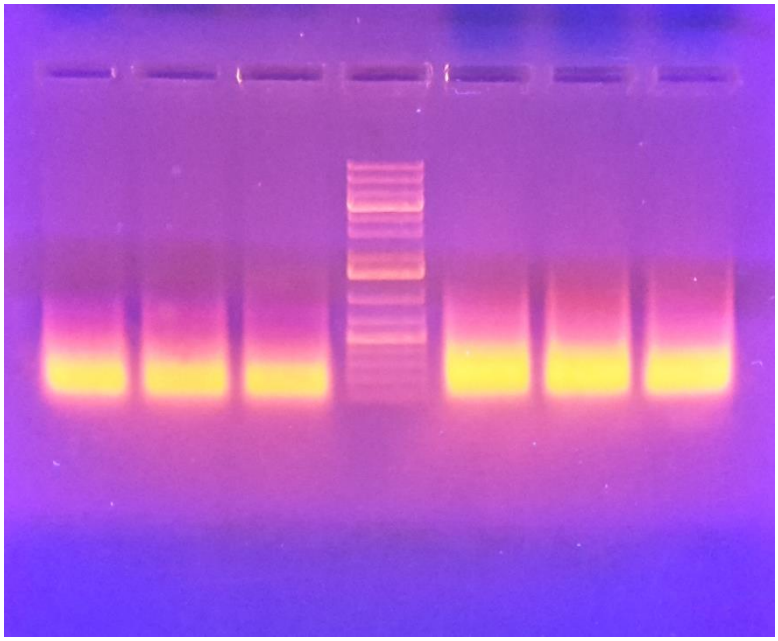


Figure 9.1 Short PCR products stained with ethidium bromide and illuminated with UV light

Theoretical background

Agarose gel electrophoresis is a method, which is suitable to separate DNA molecules according to their size. All DNA molecules are negatively charged at neutral or slightly alkaline pH, therefore in an electric field they behave as anions and move towards the positive electrode, the anode. If we force the migration of DNA molecules in an agarose gel by electric field, their movement will be limited by the agarose matrix and will be inversely proportional with the size of the DNA. Large molecules will run slower than small molecules because they have more physical interactions with the gel.

DNA is invisible when it is dissolved. To visualise the DNA fragments, we use ethidium bromide (but other less toxic reagents are also commercially available). Ethidium bromide molecules intercalate between the bases of the DNA and emit an orange light upon UV irradiation. (Read ‘Chapter 4’ for more.)

Practical workflow and protocol

Warning! Ethidium bromide is toxic, therefore protective equipment is required!

Running the agarose gel:

1. Defrost the PCR products.
2. Assemble the gel casting equipment following the lecturer's guidance (as described in 'Chapter 4').
3. Weight 0.5 g agarose and 50 ml of TAE buffer to prepare a 1 % gel. Boil the mixture in the microwave oven. **Attention!** Do not seal the lid hermetically, because the pressure generated upon heating could break the glass. In addition, the solution could be overheated - upon physical impact the overheated gel could rapidly start boiling.
4. Cool the solution to 40-50 °C under tap water and then add ethidium bromide in 1000x dilution from the stock solution. Cast the gel.
5. While the gel is solidifying, add 6x loading buffer to the defrosted PCR products and mix them.
6. After the gel has solidified, carefully pull out the comb from the gel, remove the unnecessary parts of the casting mould and put the gel into the electrophoresis chamber. The level of the TAE buffer should cover the gel for 2-4 mm.

7. Load the PCR products supplemented with loading buffer into the pockets of the gel. Write down the order of the samples. Don’t forget to load the ladder!
8. Run the gel at 120 V for 30-40 minutes. Take a photo of the gel, then evaluate the results.

Materials

50xTAE buffer:

- 2 M Tris-acetate
- 50 mM EDTA (pH 8.0)

6x loading buffer:

- 60 % glycerol
- 60 mM EDTA
- 10 mM Tris-HCl (pH 7.7)
- 0.06 % bromophenolblue

Agarose

Ethidium bromide (0.5 mg/ml)

DNA 1 kb plus molecule ladder

VIII. Lab report

Date: _____

Name: _____

Title:_____

Aim: _____

_____

Short summary (2-3 sentences about the experimental setup): _____

_____

_____

_____

_____

Used materials (You should check all the solutions and materials before you start the experiment. You should know for what and why we use them during the experimental process.)**:**

agarose:_____

ethidium bromide: _____

TAE: _____

_____

6x loading buffer-

- glycerol: _____

  _____
- bromophenol blue: _____

  _____
- Tris:_____

  _____
- EDTA: _____

Changes in the protocol: (the experiment could be repeated only, if we write down everything):

_____

_____

_____

_____

_____

Observations and Conclusion (Photos of the gel should be printed, labelled, evaluated and attached):

Additional information about PCR and gels

Evaluation of a gel photo after a PCR reaction seems to be an easy task, since we expect only one type of product after the reaction. But in practice, many surprises could happen. We discuss here the most common problems that makes gel evaluation difficult in order to be well-prepared and to avoid unpleasant surprises. The factors we have to consider are the formation of primer dimers and the appearance of smears and non-specific PCR products. To introduce these, we use "real-life examples", such as gel images obtained in our laboratory when we work on different research projects. The first example is taken from an experiment in which PCR was used to identify recombinant plasmid containing bacterium colonies, which is often called "colony PCR" in the laboratory. For colony PCR, we take bacterial cells from an agar plate and boil them to release the DNA, which will be used as a template to determine whether our DNA of interest is present in the cells. If a colony contains the specific DNA, which could be recognised by the specific primers, the region of interest will be amplified by PCR reaction, otherwise we see no PCR product on the gel.

Real-life example 1 – Primer dimers:

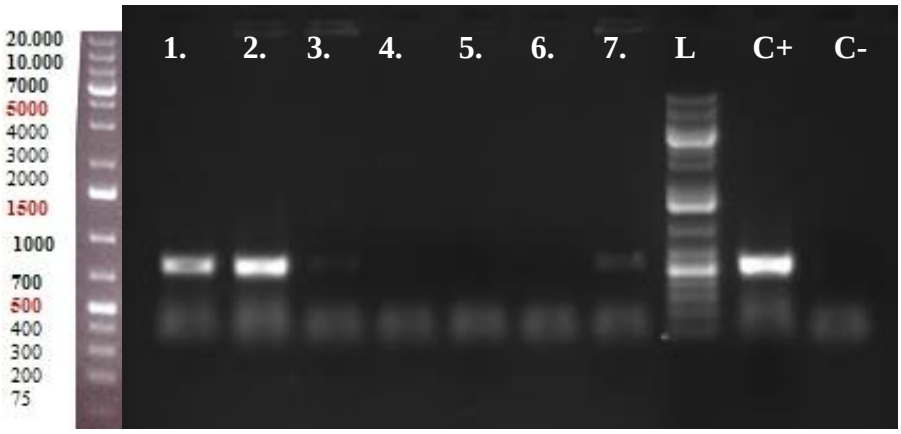


Figure 9.2 Example for primer-dimer formation

During gel evaluation, start with the controls to see whether the PCR worked or not. PCR performed from positive colonies should look like the positive control (C+), while PCR from negative colonies should be similar to the negative control (C-). If the positive and the negative control look alike either the PCR did not work or the reaction was contaminated. In the example represented on Figure 9.2 the PCR worked and we can detect the expected 550 bp length PCR product in the positive control and no product at around 550 bp in case of the negative control. PCR reactions from colonies '1.' and '2.' are similar to the positive control, therefore they must be positive colonies, while the others are negative ones.

In all samples, including the negative control, a band around 75 bp is also visible. These bands refer to primer dimers formed by dimerization

of the used primers. These dimers could be formed in the absence of template, therefore they are visible in the negative control, as well.

There are faint signals in samples ‘3.’ and ‘7.’ These are most probably false positive results, which might be the consequences of cross-contamination. PCR is very sensitive and we can detect even a few molecules, therefore we should take care of the appropriate handling to avoid any kind of contamination.

Real-life example 2 - Smears:

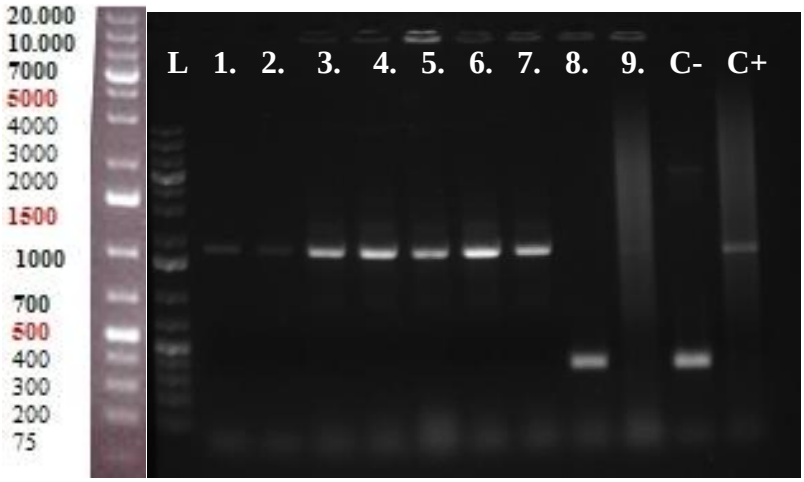


Figure 9.3 Example for smears

Figure 9.3 also represents a colony PCR. In this case we wanted to reveal whether a short piece of DNA was inserted into the coding region of our gene of interest, which has been already cloned into a recombinant plasmid. To decide this, we used a primer pair designed to the borders

of the coding region. If the gene contained the insertion, we would get a 1703 bp product, but if there was no insertion, we would get a product of 392 bp.

In colonies from 1 to 7, the insertion is present, while colony ‘8.’ has no insertion (such as the negative control).

In the samples from colony ‘9.’ and in the positive control, we can observe smears. Several reasons could explain the occurrence of smears, such as the non-specific binding of the primers, the high GC content of the primers, the too low annealing temperature during PCR or the complexity of the template. In case of the positive control we used the ligation reaction as template. It contains the correct plasmid and other DNA fragments ligated together. These result in the appearance of smear.

Smear caused by high GC content:

Smear caused by weak DNA degradation:

Figure 9.4 DNA Smears

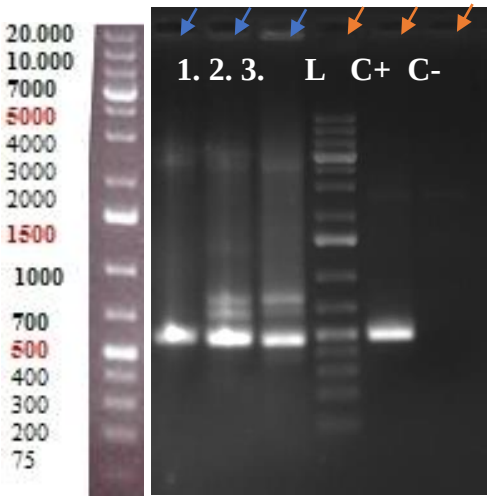


Figure 9.5 Smears caused by sample contamination

In Figure 9.5 the sample was probably contaminated with some kind of denatured proteins. We know that because we can see some DNA at the bottom of the wells in samples 1 to 3 (blue arrows), but no DNA

remained in the controls (orange arrows). The contamination made a barrier for the DNA, which restricted the migration of the molecules, which created smears. Non-specific products are also present in these samples.

Real-life example 3 – Non-specific bands

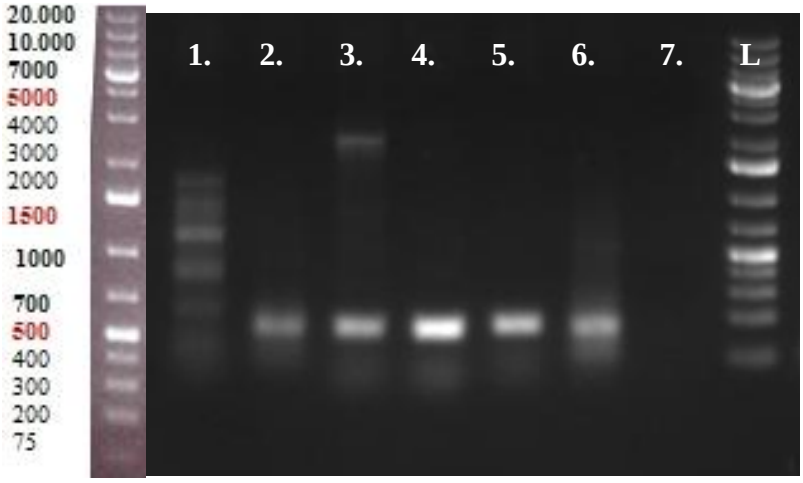


Figure 9.6 Example for non-specific PCR products

PCR shown on Figure 9.6 was performed to determine whether a new set of primers worked in a reaction. The different primer pairs used in each PCR reaction are indicated with numbers. Here, genomic DNA was used as template. The expected sizes of the PCR products are between 120 and 150 bp. The bands below 75 bp are primer dimers. We can detect some smears in sample '3.' and '6.'. Primer pair '7.' did not work at all.

In the case of ‘1.’ and ‘3.’ there are bands, which can be observed beside the expected 120-150 bp product, which are non-specific bands. The genomic DNA is huge and some regions of it could show partial homology with our primers by chance. If the 3’ end of the primers could attach to a somewhat homologous region of the template DNA, then non-specific PCR product would be able to be generated. Using higher annealing temperature or reducing the time for annealing usually solves this problem. If not, new primers should be designed.

Questions

How would you visualize your PCR products?

_____

If more non-specific bands are visible on an agarose gel, what would you do to reduce their numbers in the following experiments?

_____

_____

_____

_____

_____

What are primer dimers?

_____

How are non-specific PCR products formed?

_____

_____

Chapter 10
Research project in molecular biology
9th practice

Purification of the Pfu protein

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will be familiar with the ‘salting out’ method and with its principles.
2. They will learn how to monitor a protein purification process.

Skill:

3. They will be able to purify thermostable proteins from bacteria.

Attitude:

4. They will inquire about the theoretical background of salting out proteins.
5. They will respect their colleagues (other students) and willing to help to perform the experiment.

Responsibility and autonomy:

6. They will be able to cooperate and work in larger groups.

Purification of the Pfu protein

During previous practices Pfu DNA polymerase was expressed in an active form, and it also kept its activity during the purification steps. In this practice our aim is to purify the Pfu from the rest of the Rosetta cells in a larger scale. For this, we will use sonication for cell disruption and heat treatment and centrifugation as purification steps and we will use the “salting out” method for the concentration of the sample.

Theoretical background

For either chemical or biochemical analysis, the purification of the studied molecule is usually unavoidable. The diverse techniques for purification of biological macromolecules (nucleic acids, proteins, etc.) have accelerated the development of modern biology.

There is no general protocol for protein purification. Even in the most fortunate case, several attempts are necessary for finding the optimal protocol to purify a specific protein. However, based on many experiments, a few empirical rules could be lied down.

As a first step of protein purification, it is important to determine the source of our protein. Usually it can be found within certain cell types (bacteria, yeast and other eukaryotic cells), tissues or sometimes in serum or in supernatant. If the protein is present in the cell, as a first step cells should be disrupted. As it was introduced in a previous chapter, cells can be lysed by physical, chemical or biological methods. The appropriate method of disruption depends on the cell type. The most popular physical methods for *E. coli* are sonication and French press. The efficiency of these methods can be increased by treating the cells simultaneously with lysozyme. The lysozyme enzyme hydrolyses the the bacterial cell wall making bacterial cells extremely sensitive against osmotic changes and physical impacts. Combining physical methods, such as sonication with biochemical methods, such as lysozyme treatment, is a widely used protocol. (If sonicator or French press is not available in the lab, the principles of French press – utilizing pressure difference – could be mimicked by using syringes and needles for cell

disruption.) The efficiency of the cell lysis hardly ever reaches the 100 %.

During the purification process, proteases could degrade our protein of interest, therefore proteinase inhibitors are also recommended components of the lysis buffers. To preserve the function of the protein, purification steps are performed at low temperature. (Except if our protein is stable at room temperature, such as the Pfu DNA polymerase, although low temperatures can also inhibit protease activity.)

After lysis, the still intact cells and the cell debris are unwanted contaminants that can be removed by filtration or centrifugation. In addition, the nucleic acid content of the sample could cause complications by increasing the viscosity of the solution. (E.g. it is impossible to pipette a highly viscous solution.) To overcome this problem nucleic acids are usually degraded by nucleases or the sample could be treated with polyethylenimine to precipitate DNA, which could be removed by centrifugation. (During sonication, the genomic DNA is broken into small pieces, therefore it does not influence the viscosity of the solution.)

Different compartments of eukaryotic cells can be fractionated by sedimentation after gentle cell lysis. Isolating cell compartments could increase the efficiency of the purification process, since specific cell compartments could be better sources of a specific protein than the whole cell or cell lysate.

The steps of purification basically follow a simple law: keeping as many proteins of interest as it is possible, while disposing other components. The first step is usually a robust method that could handle the relatively

large volume of the extract. The methods used for this usually have low efficiency. In general, the consecutive purification steps result in a smaller sample volume and higher abundance of the protein of interest. The following steps are usually less robust but more selective methods, which are most frequently some kind of chromatographic procedures. However, chromatography is a very frequently used technique in protein purification protocols it is not essential.

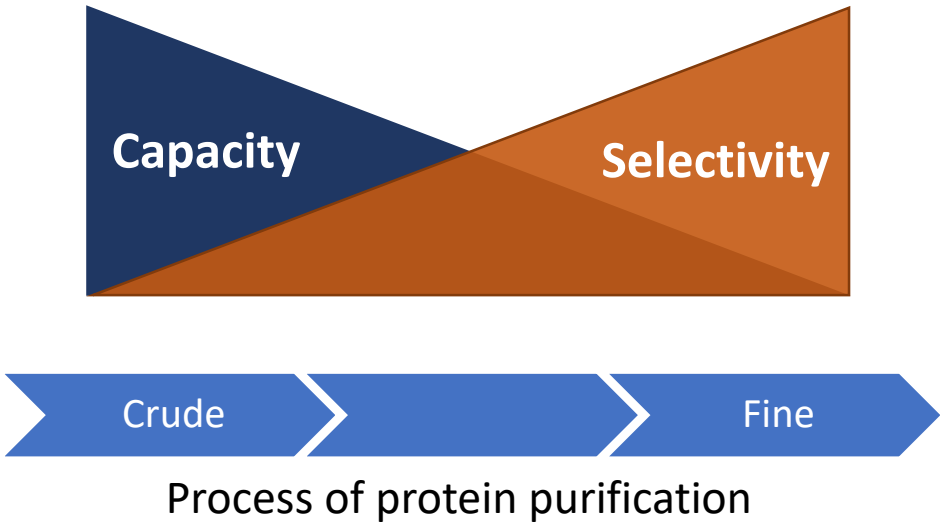


Figure 10.1 During protein purification, the capacity of the used methods is decreasing, while their selectivity is increasing

Some proteins could be purified with simple physical or chemical treatment, because they significantly differ from other components of the extract. A good example for this situation is the case of Pfu polymerase, which protein is derived from *Pyrococcus furiosus*, a thermofil archeon species that lives in extreme hot habitats. Therefore, the Pfu DNA polymerase must be a thermostable enzyme. *E. coli* is viable at 37 °C in the gut, therefore its proteins are not expected to be thermostable. If we express a protein from *Pyrococcus furiosus* in *E.*

coli, one of the main differences between the protein of interest and the other components of the extract is expected to be the heat stability. Therefore, heat treatment will precipitate the non-thermostable proteins (similarly as boiling of an egg results in precipitation of egg white proteins), while it will not affect the solubility of the Pfu DNA polymerase. The denatured proteins form aggregates upon heating and could be removed by centrifugation.

Another frequent requirement during purification that the protein should be concentrated and the buffer that contains it should be changed either to permit for further purification steps or for long-term storage. (In the storage buffer, the protein could be preserved for years at -20 °C by avoiding the freezing of the protein.)

In order to concentrate the protein we use the ‘salting out’ method. This is a very simple method that is frequently used in the laboratory. The mechanism is based on the observation that low or high salt concentrations influence protein solubility. If proteins are present in a solvent with low ion concentration, the differently charged side chains of the proteins interact with each other by forming a protein aggregate. If we add ions to the solution, the proteins would rather interact with the ions than with other proteins and become soluble. This phenomenon is called ‘salting in’. (That is why maintaining the ionic strength in buffers is important.) If we add salt to the solution, the ions will attract the water molecules and form a hydrate shell. Salt at lower concentration attracts free water molecules, but if we increase the salt concentration the free water molecules will be depleted and the ions ‘steal’ the water molecules from the surface (the hydrate shell) of the proteins. Since the hydrate

shell has a stabilizing effect, loosing it will partially denature the protein and the hydrophobic parts of it will be exposed. Through these exposed surfaces proteins interact with each other by hydrophobic interactions. Finally, the proteins form precipitates, which could be collected by centrifugation. This effect is the ‘salting out’. Since proteins differ in their shape, strength of the hydrate shell and hydrophobicity, salting out will differently precipitate each protein depending on the salt concentration.

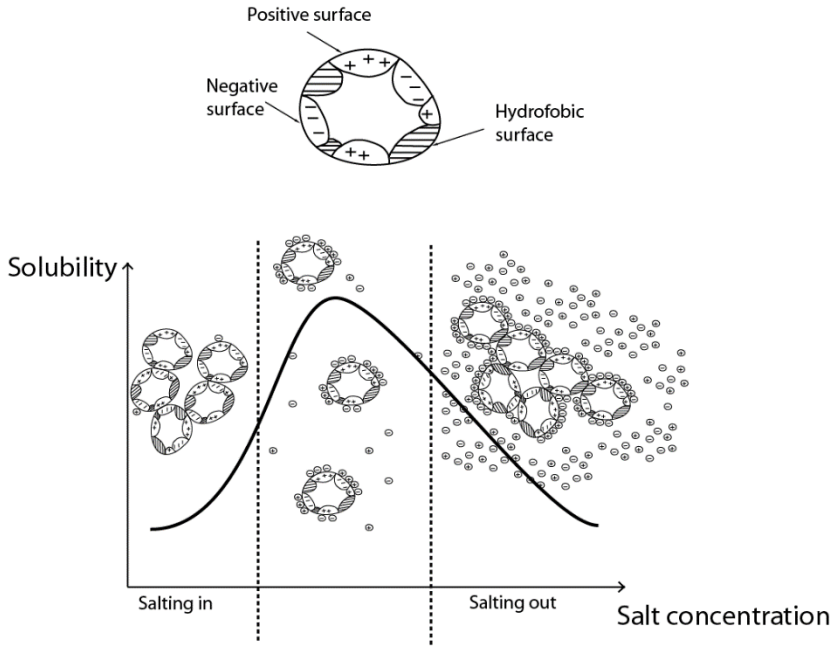


Figure 10.2 Effect of salt concentration on protein solubility. At low salt concentration the proteins interact with each other through their charged surfaces and form precipitates. At high ion concentration the hydrophobic surfaces of the proteins interact with each other leading to their precipitation.

Therefore, salting out could be applied as a protein purification step. Since salt can be added to large volumes of samples this method is

robust, although more proteins have the similar hydrophobicity, hence it has low resolution.

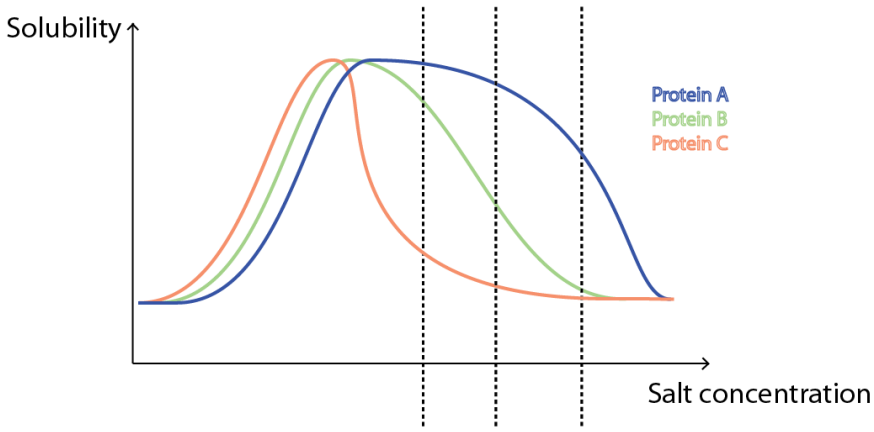


Figure 10.3 Different proteins can be precipitated at different salt concentrations. At the ionic concentration of the first dotted line most of Protein C can be precipitated. At the second line half of the Protein B and all of protein C can be precipitated. At the third line Protein B and C can be precipitated with some Protein A contaminations.

Returning to our case of Pfu polymerase: it can be precipitated, then collected by centrifugation and dissolved in a small amount of buffer. The excess ions could be removed by dialysis.

A few useful practical comments:

Ions can be classified based on their effect on protein solubility. The list was made by Hoffmeister and it is called Hoffmeister series. Ammonium sulphate is ideal for protein precipitation, it's cheap, it does not denature proteins and it's easily removable. Ammonium sulphate

has good solubility, the concentration of 4 M could be achieved at room temperature. The amount of ammonium sulphate that is required to precipitate a specific protein is usually expressed in the percentage of the saturation. A 4 M solution is 100 % saturated. The exact weight of the required amount of ammonium sulphate to reach a given saturation percentage can be determined by using the following table:

	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
Starting percent	Amount of ammonium sulfate to add (grams) per liter of solution at 20°C saturation																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

Figure 10.4 The table shows the weight (g) of ammonium sulphate added to one litre of solution to produce a desired change in the concentration (% saturation) of ammonium sulphate.

(https://www.sigmaaldrich.com/technical-documents/protocols/biology/size-exclusion-chromatography/sample-preparation.html)

Note that the temperature influences the solubility of the salt. Since the protein precipitate is collected by centrifugation, the solution should not be too dense, therefore use of glycerol or saccharose should be avoided if salting out of a protein is attempted.

Practical workflow and protocol

In this practice the class will form three large groups. Each group has a slightly different protocol to follow. Be sure to identify your group and carefully follow the protocol. Group 1 will monitor the sonication efficiency, Group 2 will follow the heat treatment efficiency, while Group 3 will be responsible for the data collection of the salting out procedure.

(For a graphical representation of the protocol see Figure 10.6)

Protocol for Group 1

1. Resuspend the previously collected cells (30 ml induced samples stored at -20 °C) in 5 ml lysis buffer.

2. Collect 15 to 20 ml sample in a 50 ml falcon tube by combining more 5 ml cell suspension. At the end of this process there should be 3 combined samples in the class, one for each workgroup. Transfer 100 ul of sample from the mixed cell suspension to an Eppendorf tube, centrifuge it and add 100 ul of 2x SDS loading buffer to it. Sonicate the samples (see 'Chapter 6').

> **Note:** To sonicate 5 ml of sample by a larger head (probe) of the sonicator is difficult since foam could be easily formed. Sonication of a 15-20 ml volume is more convenient and time is also spared by combining 3-4 samples.

3. During sonication transfer 100-100 ul from the samples to Eppendorf tubes after every 2nd cycle.

100-100 ul samples collected so far:

3.1 Centrifuge the samples at 13,000 rpm for 10 min.

3.2 Pipette the supernatants into new Eppendorf tubes.

3.3 Add 100 ul 2x SDS loading buffer to the supernatants, and resuspend the pellets in 200 ul 2x SDS loading buffer.

Sample left in the falcon tube:

4. Centrifuge at 5,000 rpm for 5 min. Recover the supernatant in a new centrifuge tube.

5. Boil the supernatant at 75 °C for 20 min (proteins are denatured), then cool the sample on ice.

Transfer 100 ul of sample in a new Eppendorf tube

100-ul sample collected:

5.1 Centrifuge the sample at 13,000rpm for 10 min.

5.2 Pipette the supernatant into a new Eppendorf tube.

5.3 Add 100 ul 2x SDS loading buffer to the supernatant, and resuspend the pellet in 200 ul 2x SDS loading buffer.

Sample left in the falcon tube:

6. Centrifuge at 5,000 rpm for 5 min.

7. Place the supernatant into a beaker. Slowly add 4.3 g fine crystals of solid ammonium sulphate per 10 ml supernatant (constant stirring).

Transfer 100 ul of sample in a new Eppendorf tube.

100 ul sample collected:

7.1 Centrifuge the sample at 13,000rpm for 10 min.

7.2 Transfer the supernatant into a new Eppendorf tube.

7.3 Add 100 ul 2x SDS loading buffer to the supernatant, and resuspend the pellet in 200 ul 2x SDS loading buffer.

Sample left in the falcon tube:

8. Centrifuge at 15,000 rpm for 10 min at 4 °C. Remove the supernatant.

Protocol for Group 2

1. Resuspend the previously collected cells (30 ml induced samples stored at -20 °C) in 5 ml lysis buffer.

2. Collect 15 to 20 ml sample in a 50 ml falcon tube by combining more 5 ml cell suspensions. At the end of this process there should be 3 combined samples in the class, one for each workgroup. Transfer 100 ul of sample from the mixed cell suspension to an Eppendorf tube, centrifuge it and add 100 ul of 2x SDS loading buffer to it. Sonicate the sample (see 'Chapter 6').

Note: To sonicate 5 ml of sample by a larger head (probe) of the sonicator is difficult since foam could be easily formed. Sonication of a 15-20 ml volume is more convenient and time is also spared by combining 3-4 samples.

3. Transfer 100 ul of samples after sonication into new Eppendorf tubes.

100-100 ul-samples collected so far:

3.1 Centrifuge the samples at 13,000 rpm for 10 min.

3.2 Pipette the supernatants into new Eppendorf tubes.

3.3 Add 100 ul 2x SDS loading buffer to the supernatants and resuspend the pellets in 200 ul 2x SDS loading buffer.

Sample left in the falcon tube:

4. Centrifuge at 5,000 rpm for 5 min. Recover the supernatant in a new centrifuge tube.

5. Boil the supernatant at 75 °C for 20 min (proteins are denatured), then cool the sample on ice.

Transfer 100 ul of sample in every five minutes during the heat treatment.

100-100 ul-samples collected:

5.1 Centrifuge the samples at 13,000rpm for 10 min.

5.2 Pipette the supernatants into new Eppendorf tubes.

5.3 Add 100 ul 2x SDS loading buffer to the supernatants and resuspend the pellets in 200 ul 2x SDS loading buffer.

Sample left in the falcon tube:

6. Centrifuge at 5,000 rpm for 5 min.

7. Place the supernatant into a beaker. Slowly add 4.3 g fine crystals of solid ammonium sulphate per 10 ml supernatant (constant stirring).

Transfer 100 ul of sample in a new Eppendorf tube.

100 ul sample collected:

7.1 Centrifuge the sample at 13,000rpm for 10 min.

7.2 Transfer the supernatant into a new Eppendorf tube.

7.3 Add 100 ul 2x SDS loading buffer to the supernatant and resuspend the pellet in 200 ul 2x SDS loading buffer.

Sample left in the falcon tube:

8. Centrifuge at 15,000 rpm for 10 min at 4 °C. Remove the supernatant.

Protocol for Group 3

1. Resuspend the previously collected cells (30 ml induced samples stored at -20 °C) in 5 ml lysis buffer.

2. Collect 15 to 20 ml sample in a 50ml falcon tube by combining more 5 ml cell suspensions. At the end of this process there should be 3 combined samples in the class, one for each workgroup. Transfer 100 ul of sample from the mixed cell suspension to an Eppendorf tube, centrifuge it and add 100 ul of 2x SDS loading buffer to it. Sonicate the samples (see 'Chapter 6').

Note: To sonicate 5 ml of sample by a larger head (probe) of the sonicator is difficult since foam could be easily formed. Sonication of a 15-20 ml volume is more convenient, and time is also spared by combining 3-4 samples.

3. Transfer 100 ul of samples after sonication into new Eppendorf tubes.

100-100 ul-samples collected so far:

3.1 Centrifuge the samples at 13,000 rpm for 10 min.

3.2 Pipette the supernatants into new Eppendorf tubes.

3.3 Add 100 ul 2x SDS loading buffer to the supernatants and resuspend the pellets in 200 ul 2x SDS loading buffer.

Sample left in the falcon tube:

4. Centrifuge at 5,000 rpm for 5 min. Recover the supernatant in a new centrifuge tube.

5. Boil the supernatant at 75 °C for 20 min (proteins are denatured), then cool the sample on ice.

Transfer 100 ul of sample in a new Eppendorf tube.

100 ul sample collected:

5.1 Centrifuge the sample at 13,000rpm for 10 min.

5.2 Pipette the supernatant into a new Eppendorf tube.

5.3 Add 100 ul 2x SDS loading buffer to the supernatant and resuspend the pellet in 200 ul 2x SDS loading buffer.

Sample left in the falcon tube:

6. Centrifuge at 5,000 rpm for 5 min.

7. Place the supernatant into a beaker. Slowly add 4.3 g fine crystals of solid ammonium sulphate per 10 ml supernatant (constant stirring).

Transfer 100 ul of sample when ¼, ½, ¾ and all of the ammonium sulphate is added, respectively.

100-100 ul-samples collected:

7.1 Centrifuge the samples at 13,000 rpm for 10 min.

7.2 Transfer the supernatants into new Eppendorf tubes.

7.3 Add 100 ul 2x SDS loading buffer to the supernatants and resuspend the pellets in 200 ul 2x SDS loading buffer.

Sample left in the falcon tube:

8. Centrifuge at 15,000 rpm for 10 min at 4 °C. Remove the supernatant.

The pellet contains the precipitated protein. The pellet could be frozen until the next practice.

Figure 10.5 Precipitated proteins seen as pellet at the bottom of the tubes

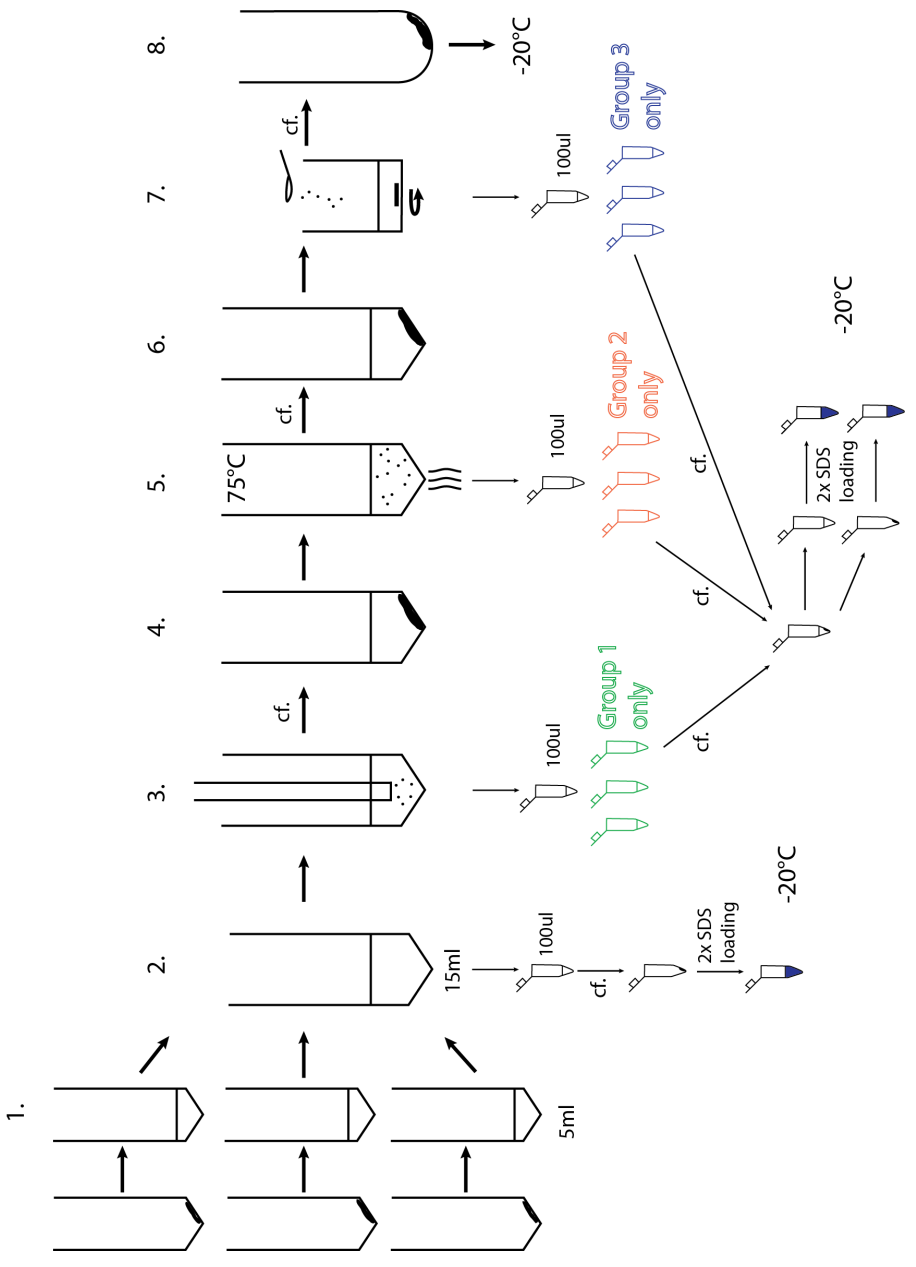


Figure 10.6 Graphical representation of the protocol

Materials

Lysis buffer:

- 25 mM Tris-HCl pH 7.6
- 100 mM NaCl

Lysozyme

2x SDS loading buffer

Ammonium sulphate

IX. Lab report

Date: _____

Name: _____

Title: _____

Aim: _____

_____

Short summary (2-3 sentences about the experimental setup):

_____

_____

_____

_____

Used materials (You should check all the solutions and materials before you start the experiment. You should know for what and why we use them during the experimental process.)**:**

Lyzis buffer:

- Tris:_____
- NaCl:_____
- Lysozyme:_____

2x SDS loading buffer:

- Tris:_____

- DTT (b-Me): _____
- SDS: _____
- Bromophenol blue: _____
- Glycerol: _____

Changes in the protocol: (the experiment could be repeated only, if we write down everything):

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Observations and Conclusion:

_____

_____

_____

Additional information

Denaturation, precipitation

Heat treatment denatures proteins, which results in their precipitation. SDS also denatures proteins, although it does not lead to the precipitation of the proteins. During salting out, proteins are precipitated but are not denatured. It might be confusing, but the behaviour of the molecules perfectly explains these phenomena.

Heat:

Proteins are composed of non-branching chains of amino acids, which are also referred to as polypeptides. However, proteins are rarely present in the cells in linear, fibre-like form. They would be rather folded into complex 3D structures. The folding of the proteins mainly depends on the intramolecular interactions among the amino acids they are composed of. When a protein solution is heated, the interactions between the side chains of the amino acids could be disrupted and the protein could lose its structure, in other word it is partially or completely unfolded. This is the loss of the natural state of the protein, called denaturation. In this state amino acids are still able to form interactions but instead of creating bounds, which stabilize the native state, they form various inter- and intermolecular interactions. Through these not specific interactions a huge amount of proteins can cling to each other, forming an aggregate, which is large enough to lose its solubility and precipitate. Precipitated proteins can be sediment by centrifugation.

SDS:

Similar to heat, SDS can also unfold the proteins but by a different mechanism. SDS interacts with the amino acid side chains while it is unfolding the protein. Due to that, proteins cannot interact with each other, thereby they remain in solution. They are denatured but soluble.

Salt:

When the salt concentration is increased, the hydrate shell of the proteins is disturbed and the hydrophobic surfaces of the proteins are exposed. Through these surfaces, the proteins will interact with each other and will be precipitated. This process is reversible, since if the salt concentration is reduced, the hydrate shell and the structure of the protein are restored and the interactions that have kept the precipitate together are diminished. Therefore, the solubility of the proteins is also restored. This is different than the earlier mentioned heat treatment, by which new interactions are formed upon cooling, therefore the temperature reduction cannot restore the original structure of the proteins. In other words, the process is irreversible.

Questions

What were the principles we used to purify the Pfu DNA polymerase?

_____

_____

Which method was used to concentrate the Pfu DNA polymerase?

_____

Describe the behaviour of proteins during “salting in” and “salting out”!

_____

_____

_____

_____

What are the Hoffmeister series?

_____

How do the selectivity and capacity of the applied methods usually change during protein purification?

_____

_____

Chapter 11
Research project in molecular biology
10th practice

Dialysis, visualizing the efficiency of the sonication and following the purification of the Pfu polymerase by SDS-PAGE

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will understand the principles of dialysis and why desalting is important after salting out.

Skill:

2. They will be able to perform a dialysis experiment.
3. They will be able to prepare and run their samples on SDS-PAGE.
4. They will be able to evaluate their gels and draw conclusions for the improvement of their protein purification protocol.

Attitude:

5. They will try to improve their protein purification experiments.

Responsibility and autonomy:

6. They will be able to handle toxic materials with care.
7. They will learn how to write their lab reports and evaluate polyacrylamide gels on their own.

Dialysis and visualizing the steps of Pfu DNA polymerase purification by SDS-PAGE

In the previous practice, we purified Pfu DNA polymerase from induced *E. coli* cells. We disrupted the cells then heat-treated the cellular extract to precipitate the non-thermostable proteins. To increase the concentration of the heterologous protein, we precipitated it with salt. The protein pellet is the most concentrated form of our protein, but it could not be used in reactions. For that, we should dissolve the pellet in the appropriate buffer to get a concentrated solution, which will still have high salt concentration that inhibits PCR reactions. Therefore, before using the enzyme the salt concentration should be decreased. During this practice our aim is to separate the proteins from the salt to make the protein suitable for further downstream reactions.

Our other goal is to monitor the purification process and to improve our protocol based on the available information. For this, we have to compare the samples collected so far and run them on PAGE as it was introduced in the previous practice. By evaluating the gel, we can get information about the optimal cycle number of sonication, the optimal time-span of heat treatment and the efficiency of salting out at different salt concentrations.

To desalt our samples, we will apply dialysis and to visualize protein from samples collected in the previous practice, we will use SDS-PAGE.

Theoretical background

Dialysis is a method used to change small molecule components of a solution containing macromolecules. You can imagine how it works by thinking about a filter on which dust from peas can be removed. During dialysis, two solutions are separated from each other by a semipermeable membrane, also known as the dialysis membrane. This membrane serves as a sieve, since it lets the small molecules go through its small pores, while it prevents the movement of the large molecules. The size of the pores of the membrane determines the separation range.

The principles of the SDS PAGE can be found in ‘Chapter 7’.

Practical workflow and protocol

1. On the day before the practice, dissolve the frozen protein pellet collected after the salting out procedure in 1.5-2 ml dialysis buffer, place it onto a dialysis membrane bag, close it tightly and place it into a litre of dialysis buffer solution and swirl it overnight.

2. Recover the samples from the membrane bag. Take 30 ul from the sample for SDS-PAGE and supplement it with 30 ul 2x SDS loading buffer.

3. Add glycerol to reach 50 % final concentration to the remaining samples and store them at -20 °C. (The glycerol stock solution is 87 %)

The samples collected last time will be run together with sample prepared today on PAGE and will be visualized by Coomassie Brilliant Blue staining.

1. Sample preparation

- Add the same volume of 2x SDS loading to the sample (if it is necessary)
- Boil it for 10 min at 95 °C

Samples:

	Group 1	Group 2	Group 3
Cells without any treatments	1 sample	1 sample	1 sample
Sonicated cell supernatant	4 samples	1 sample	1 sample
Sonicated cell pellet	4 samples	1 sample	1 sample
Supernatant of boiled samples	1 sample	4 samples	1 sample
Pellet of the boiled supernatant	1 sample	4 samples	1 sample
Supernatant of salted out samples	1 sample	1 sample	4 samples
Pellet of salted out samples	1 sample	1 sample	4 samples
Concentrated sample (after dialysis)	1 sample	1 sample	1 sample

That means that each group should have 14 samples. There are differences between the groups in the distribution of samples, therefore evaluate all three groups' results in your lab report.

The samples obtained from salting out contain high salt concentration that interferes with polyacrylamide gel electrophoresis and results in a smeary gel.

2. Gel casting and running

We will use a 10 % polyacrylamide gel.

Assembly of the gel casting apparatus:

Wash the two glass parts with alcohol to remove the stains!

Assemble the two glasses and put it into the frame!

Stabilize the glasses in the frame by using the white hand-screws. (It will form a tank for the buffer.)

Fix the frame to its silicon base! (It will prevent the gel from leaking until it is polymerised.)

Gel casting:

We mix the gel in 40 ml final volume. The volume of space between the two glasses is around 15 ml. This amount of mixture is enough for two gels (15 ml + 15 ml). (The residual 10 ml of mixed gel solution will be used to check whether the gel has polymerised.)

Before the gel is casted mark the top of the separating gel on the glass. Put the comb between the glasses. Mark the glass 1-2 mm below the bottom of the comb with a marker. Remove the comb.

Prepare the gel matrix.

Recipe for 40 ml of separating gel:

- 13.3 ml acrylamide (30 %)
- 10 ml 4x separating buffer– 1.5 M Tris pH 8.8, 0.4 % SDS
- 16.6 ml H_2O
- 150 ul 10 % APS
- 75 ul TEMED (induces the polymerisation)

Pour the gel between the two glasses until the gel volume reaches the desired level (marked line).

Layer isopropanol to the surface of the polymerizing gel. It flattens the surface of the gel and ensures anaerobe environment.

During polymerisation, the gel loses volume that could deform the glass. To avoid this, put the comb between the glasses. The comb should not reach the isopropanol layer.

While the gel is polymerising, mix the solution for the stacking gel.

Recipe for 10 ml of stacking gel:

- 6 ml H_2O
- 2.5 ml 4x stacking buffer (0.5 M Tris pH 6.8)
- 1.2 ml acrylamide
- 60 ul APS
- 30 ul TEMED (do not add it until the separating gel is polymerized!)

If the separating gel polymerizes in the tube, remove the isopropanol from the top of the gel and wash the surface of the gel with tap water. Remove all the water by blotting it.

Put the comb between the glasses and add TEMED to the stacking gel. Mix and pour the solution into the mould.

While the stacking gel is polymerizing, mix the SDS-PAGE ELFO buffer: (stock 5X SDS-PAGE ELFO buffer)

- 200 ml 5X buffer
- 800 ml H_2O

After the gel is polymerized, remove the frame from the base and put it into the electrophoresis chamber. Fill the upper and the lower tank with 1x SDS-PAGE ELFO buffer.

Gently remove the comb by pulling it vertically with a constant speed. Wash the pockets with 2x SDS loading buffer (it marks each well and the subsequent washing removes the residual gel contamination).

Load 15 ul of each sample onto the gel! Don't forget that the dialysed sample is much more concentrated than the other ones, therefore 15 ul from that may overload the gel.

The order of the samples should be discussed. Plan the sample order and write it down in your lab report. Do not forget to load the protein ladder.

Run the gel at 120 V for 1.5-2 hours.

3. Gel staining with Coomassie Brilliant Blue

- Disassemble the apparatus.
- Put the gel into the fixing solution (50 % methanol, 10 % acetic acid) and put it on a shaker for 15 min.

- Exchange the fixing solution to the Coomassie Brilliant Blue solution. Put it on a shaker for 30-120 min.
- Exchange the dye solution to differentiating solution (10 % acetic acid), add pieces of sponge to promote the more efficient washing and shake the gel overnight. (The sponge will absorb the Coomassie dye.)

Materials

Dialysis buffer:

- 25 mM Tris-HCl pH 7.9
- 50 mM KCl
- 0.2 mM EDTA
- 0,05 % Tween-20
- 0,05 % NP-40
- 1 mM DTT

2X SDS loading buffer:

- 100 mM Tris pH 6.8
- 200 mM DTT
- 4% SDS
- 0.2 % Bromophenol blue
- 20 % Glycerol

Buffer I:

- 100 mM Tris pH 7.5
- 2 mM EDTA

30 % acrylamide-0.8% bisacrylamide

4x separating gel buffer

4x stacking gel buffer

10 % APS

TEMED

5 x SDS ELFO buffer (2 l)

- 20 g SDS
- 30.2 g Tris
- 188 g Glycine
- H_2O
- pH should be 8.3

Fixing solution

- 50 % methanol
- 10 % acetic acid

Coomassie Brilliant Blue

- 50 % methanol
- 10 % acetic acid
- 0.05 % Coomassie Brilliant Blue R-250

Differentiating solution

- 10 % acetic acid
- (5 % methanol)

X. Lab report

Date: _____

Name: _____

Title:_____

Aim: _____

_____

Short summary (2-3 sentences about the experimental setup):

_____

_____

_____

_____

Used materials (You should check all the solutions and materials before you start the experiment. You should know for what and why we use them during the experimental process.)**:**

Dialysis buffer:

- Tris:_____
- KCl:_____
- EDTA:_____
- TWEEN 20: _____
- NP-40:_____
- DTT: _____

2xSDS loading buffer: _____

Coomassie Brilliant Blue:_____

Separation gel buffer: _____

Stacking gel buffer: _____

SDS ELFO buffer: _____

Changes in the protocol: (the experiment could be repeated only, if we write down everything):

_____

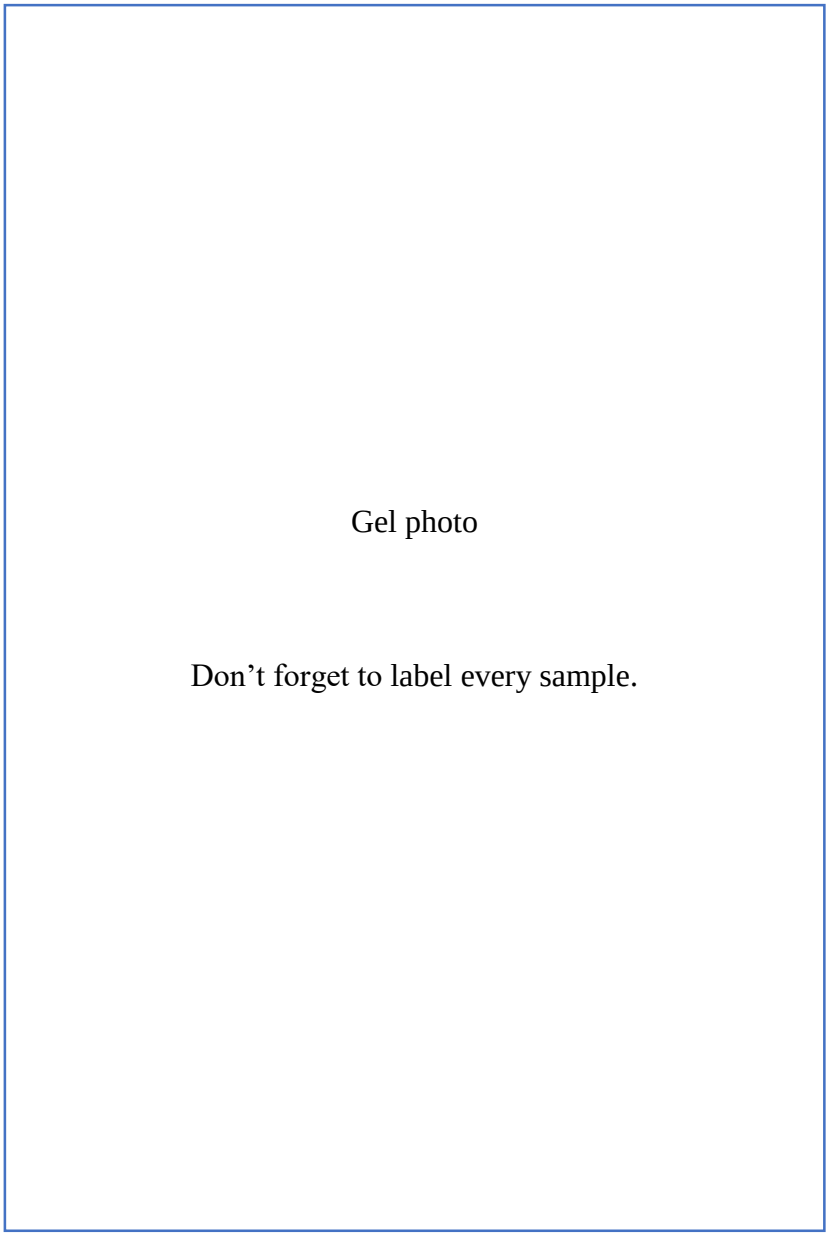
_____

_____

_____

_____

Observations and Conclusion (labels!):



(Write down: which samples are compared, what is expected, are the results in accord with the expectations?)

Cell lysis: _____

_____

_____

Purification: _____

_____

_____

Concentration: _____

_____

_____

_____

Further observations: _____

_____

_____

_____

_____

_____

Questions

Why is dialysis important after salting out?

_____

How does dialysis work?

_____

_____

What kind of information can you get by comparing

- the series of sonicated samples?

_____

- the series of heat-treated samples?

_____

- the series of salt-precipitated samples?

_____

- the sonicated supernatant and the boiled supernatant?

_____

- the boiled supernatant and the dialysed sample?

_____

Chapter 12

Technique for protein detection and identification

Western blot

Learning outcomes

At the end of the practice, students will acquire the following learning outcomes:

Knowledge:

1. They will be familiar with the principles of Western blot.

Skill:

2. They will be able to understand the basics of Western blot.

Attitude:

3. They will inquire about the background of Western blot.

Western blot

Western blot is a frequently used technique for identifying specific protein(s) separated by SDS-PAGE by using specific antibodies.

Occasionally Western blot also refers to immunoblotting. We use the prefix ‘immuno‘, since specific immune reagents (antibodies) are applied in the reaction and the suffix ‘blotting’ is used, since proteins after electrophoresis are transferred to a specific membrane from the gel. In this property, it is similar to Southern and Northern blot, although Southern blot is used for the detection of specific DNA and Northern blot is applied for the detection of RNA molecules. At first Southern blotting had been developed by Edwin Southern, and the other blots were later named in reference to Southern's name. In this chapter, we are going to speak about Western blot, which was developed by Harry Towbin.

Western blotting is a very specific and sensitive method, since specific antibodies are used to identify the protein of interest. To perform a Western blot, two antibodies are required. The first one is the primary antibody, which is specific for the protein that we would like to identify. The other is the secondary antibody, which is raised against the constant chain of the primary antibody. The secondary antibody is also conjugated to an enzyme, which in the most frequent cases is the horse radish peroxidase (HRP), which makes the detection of the antibody possible.

However, antibodies determine the specificity and sensitivity of the Western blot method, the proper preparation of the protein sample is

required to use them, which starts with SDS-PAGE. As it is detailed in Chapter 7, SDS-PAGE separates proteins based on their molecular mass. Then proteins are transferred to a nitrocellulose or a PVDF membrane. The transfer is called electroblotting and performed in an arrangement that preserves the pattern of proteins as separated by the SDS-PAGE, therefore following the blotting step, a replica of the gel is obtained on a filter. Nitrocellulose and PVDF membranes bind proteins with high affinity therefore all the still free protein binding sites of the membrane should be filled, in order to avoid the non-specific binding of the antibodies. It is done by soaking the membrane in a solution containing proteins in high concentration (usually milk proteins are used at this step) and by this the membrane is ready to incubate it with the antibodies. First the primary antibody solution is added to the membrane to let the antibody molecules bind to the specific target proteins. Then the membrane is washed to remove the non-bound primary antibodies and then the membrane is soaked in secondary antibody solution. At this step, secondary antibodies attach to all positions, where primary antibodies have bound to, as well. After washing steps, the proteins can be detected, most often by a chemiluminescent reaction catalysed by the HRP enzyme conjugated with the secondary antibody.

Steps of Western blot:

1. *Sample preparation for SDS-PAGE*

Usually, the firs step of sample preparation is to release proteins from the cells. There are several cell lysis methods available, although the optimal choice depends on the current experiment we are going to perform. During cell lysis we can use either non-denaturing or denaturing lysis buffer. These differ from each other in the detergent components involved in it: non-denaturing lysis buffer contains Triton X-100 or NP-40, which are non-ionic detergents, while in denaturing lysis buffer ionic detergent, such as SDS or Na-DOC can be found. In addition, before loading the samples into the gel, we mix the samples with SDS loading buffer and boil them to completely denature the proteins. SDS denatures the proteins and provides them a uniform negative charge. We usually add some kind of reducing agent, such as 2-mercaptoethanol to the loading buffer to remove disulphide bonds, thereby reducing the proteins.

2. *Determination of protein concentration*

Western blot is often used to compare protein levels in different samples. For instance, if we want to follow the expression of a specific protein over time we should load equal amount of total proteins into the wells of the gel. Otherwise the observed changes in protein level are explained with the difference in the amount of loaded proteins and not with the actual changes in the expression. To normalize our samples to protein levels we should first determine the protein concentration, which could be done by Bradford reaction before adding the SDS loading buffer to

the samples. Bradford reaction is a colorimetric assay, which uses CBB dye. This dye can exist in three different form: cationic (red), neutral (green) and anionic (blue). The Bradford buffer also contains phosphoric acid, which ensures an acidic environment for the reaction. Under acidic conditions, the red CBB converts to its blue form by interacting the proteins in the solution. If there is no protein in it, it remains red-coloured. The intensity of the blue colour will be proportional with the protein conenctration. To reveal the exact concentration of the proteins we should measure the absorbance of the mixed solution by a photometer and compare it to the absorbance of reactions with known concentrations. The anionic form of CBB has an absorption maximum at 595 nm, therefore it is measured at this wavelength. Generally, bovine serum albumin (BSA) is frequently-used to create a protein standard. The protein standard is a series of samples that contains BSA in different but known concentrations, usually in a series of dilution. Based on the absorbance values of the BSA standard a standard curve can be calculated, by wich we can determine the protein concentration of the unknown sample. After normalizing for protein concentrations and loading the gel, some pipetting errors can still occur. To check this possibility we have loading controls, which are used in order to see whether the loading was uniform in case of every sample. For this, we use an antibody against a protein in the sample that is constitutively expressed at a high level. In case of whole cell extracts, actin or GAPDH, in case of nuclear fraction H3 histone or Lamin B1 and in case of serum transferrin are commonly used controls. After the blotting step, the level of these proteins is checked by using the appropriate antibodies.

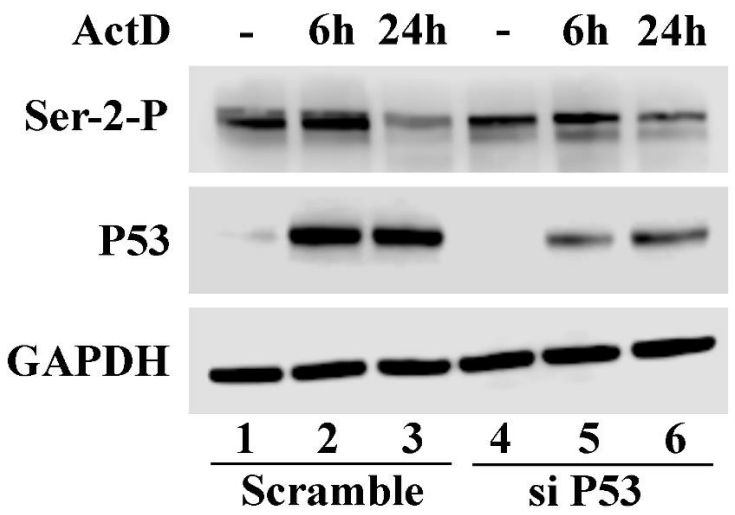


Figure 12.1 Detection of the protein level of P53 and S2P RNAPII in non-treated and Actinomycin D-treated samples (6 and 24 h). As a loading control, antibody against GAPDH was used.

https://www.ncbi.nlm.nih.gov/pubmed/28102346

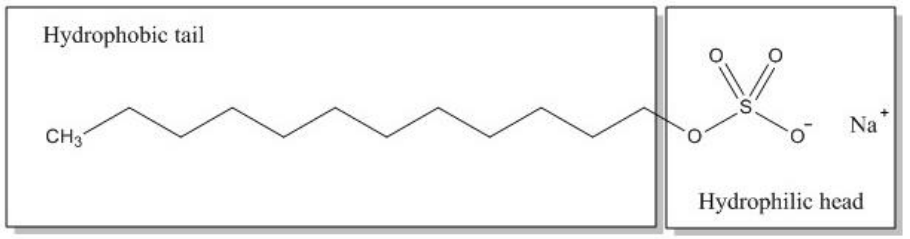


Figure 12.2 Schematic structure of Sodium dodecyl sulphate (SDS)

https://commons.wikimedia.org/wiki/File:SDS_with_structure_description.jpg

3. Polyacrylamide gel electrophoresis

Before blotting the proteins are separated by their mass. It is important for several reasons. First if the western blot signal is at the correct molecular weight the detected protein is most probably the one we want to study. If we want to monitor multiple proteins in the same sample (for instance our protein of interest and a loading control) first we should separate them otherwise the signals of different proteins would be indistinguishable. Additionally, if the protein of interest has multiple forms (post translational modifications, isoforms, or degraded proteins), these could be identified after the separation. However, antibodies are very specific they can bind to proteins, which they are not supposed to. If the proteins are separated by SDS-PAGE we can visualise the non-specific bindings.

As described in Chapter 7, Laemmli gels has two different gel sections: stacking and separating/resolving gel. In stacking gel (pH 6.8) the proteins are concentrated in a thin band while in the separating gel (pH 8.8) the separation of the proteins happens. For details see the description of PAGE in Chapter 7.

4. Electrotransfer/Blotting

In a polyacrylamide gel, proteins are not accessible for the antibodies used in Western blot. In addition, it is almost impossible to get the antibodies into the gel and wash them away from there. To overcome this problem proteins should be transferred onto the surface of a membrane where they can be exposed to the antibodies. The membrane used for this can be either nitrocellulose or PVDF and the technique of the transfer is called electroblotting. Proteins can be moved in the gel

under electric conditions, therefore electricity could be used to remove them as well. During electroblotting instead of a vertical electrophoresis we perform a horizontal one, which transfers the proteins from the gel to the surface of a membrane. For this, both the gel and the membrane are placed between electrodes in the correct arrangement: the negatively charged proteins will run towards the positively charged anode therefore the membrane should be placed between the gel and the anode, so the protein will be trapped on it. It is important to position the membrane facing to the positive electrode (since in the reversal arrangement the proteins will be not trapped in the membrane, they will be lost in the electrophoretic buffer). To do this, the gel and the membrane should be placed in a horizontal electrophoresis chamber (transfer tank or blotting device). However, for the optimal transfer, a so-called "sandwich" should be assembled, which consists of filter papers at the bottom, then the gel and the membrane, which are all covered with some more filter paper on the top. The gel should be placed on the membrane directly in a close contact avoiding any bubble formation. Air bubbles do not conduct electricity therefore they inhibit the protein transfer from the gel to the membrane. The sandwich also contains sponges to protect the gel and the membrane and keep them close together, while permitting movement of the ions.

The transfer is usually performed in a glycine buffer supplemented with methanol. For larger proteins, lower level of methanol (10 %) is suggested, because it promotes the swelling of the gel, therefore allowing easier transfer of these proteins. In addition, larger proteins can form precipitate in the gel, which can be avoided by adding some more

SDS (0.1 %) to the transfer buffer. On the other hand, blotting of smaller proteins requires 15-20 % methanol content of the transfer buffer.

After blotting step, proteins can be visualized by Ponceau staining. This can give us information about the success of the blotting. Since it is a reversible staining, this dye can be easily removed by washing the membrane with apropriate buffer or water.

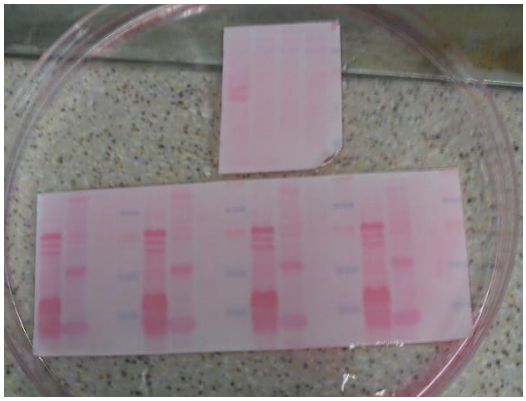


Figure 12.3 Ponceau stained nitrocellulose membranes

	Nitrocellulose	PVDF
Protein size	Mainly mid or low molecular mass proteins	Mainly high molecular mass proteins
Sensitivity	Strong binding capacity	Higher protein binding capacity and sensitivity
Strip/re-probe	Possible, but it can lose sensitivity in the process	Very good
Durability	Less durable	More durable
Background noise	Very low	Higher

Table 12.1 Differences between nitrocellulose and PVDF membranes

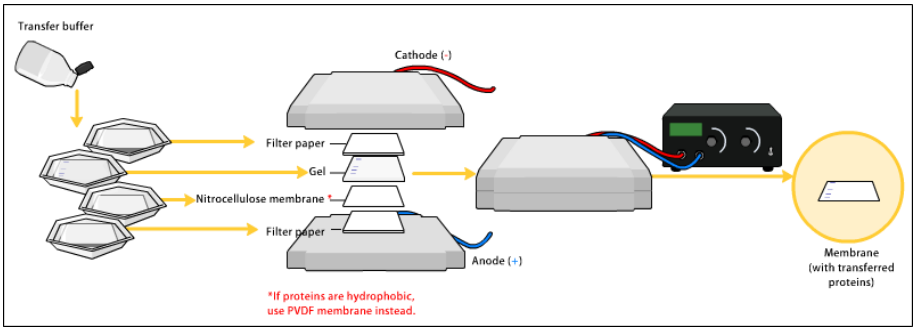


Figure 12.4 Blotting 'sandwich'

https://upload.wikimedia.org/wikipedia/commons/9/93/Western_blot_transfer.png

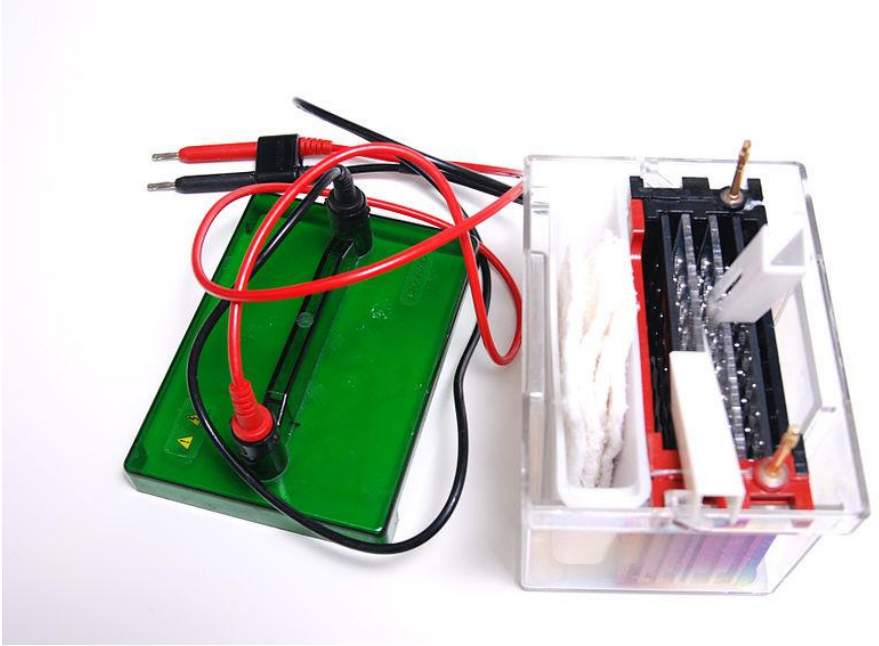


Figure 12.5 Blotting system

https://commons.wikimedia.org/wiki/File:Western_blot_wet_transfer_system_Criterion-06.jpg

5. Membrane blocking

The aim of the blocking step of Western blot is to prevent the non-specific binding of antibodies to the free protein binding sites of membrane. Antibodies are also proteins and they could attach to the membranes as any other proteins, unless we saturate the membrane with other polipeptides. Consequently, after this step the antibodies will be co-localised only with the protein of interest and will not bind non-specifically to the entire membrane. A solution of an abundant amount of cheap protein is used for blocking step. It is most frequently performed by using 5 % BSA or 5 % non-fat dry milk. Milk is a

preferred choice, since it contains several proteins, therefore higher blocking efficiency could be reached with it than with BSA-containing blocking solution. However, since milk contains casein, which is a phosphoprotein, antibodies against phosphorylated proteins could non-specifically bind to it. Therefore, in case of phosphorylated protein detection, blocking with BSA is recommended.

6. *Incubation with primary and secondary antibodies*

After blocking step the membrane is ready for incubation with the primary and secondary antibodies.

Before use the commercially available antibodies should be diluted as recommended by the manufacturer. The dilution buffers are always supplemented with 1 % non-fat dry milk or 1 % BSA, which helps to maintain the solubility of the antibody (highly diluted proteins can attach to the surface of plastic ware and proteins have a stabilizing effect on each other) and it also helps to avoid the detection of non-specific signals.

After the addition of the antibodies, they could specifically recognise and bind to the protein of interest. The binding is affected through a small region, generally no more than 5-6 amino acid length surface, which is called epitope. If an antibody recognises only one epitope of the protein it is a monoclonal antibody. These types of antibodies are generated by a specific technique using clones of antibody-producing B cells. On the other hand, polyclonal antibodies raised in animals contain a mixture of antibody molecules and recognise more than one epitope.

Polyclonal antibodies are produced by several B-cells therefore they are different antibody molecules targeting the same protein through multiple epitopes. Polyclonal antibodies are therefore more likely to form non-specific bindings. However, in such cases when the target protein is not denatured (e.g. immunoprecipitation) targeting different epitopes is a preferred choice. Monoclonal antibodies are produced by only one type of cells called hybridomas. Hybridomas are made of the fusion of a B cell and a myeloma cell, which made the B cells possible to proliferate while producing only one type of antibody. Additionally, monoclonal antibodies could be more specific than polyclonal antibodies. In 1975, Georges Köhler and César Milstein received Nobel Prize for hybridoma technology.

It is very important to use extensive washing steps between blocking and primary or secondary antibody application, respectively.

7. *Protein detection*

The secondary antibody is usually conjugated with an enzyme, which catalyses an easily detectable reaction. By using horse radish peroxidase (HRP), we can detect the protein of interest by chemiluminesce. After the addition of a reaction mix of peroxide and luminol onto the membrane, H_2O_2 will be produced in the positions where the HRP is active. H_2O_2 oxidises luminol and during this reaction, light will be emitted. Finally, the signal can be captured on a film or with instruments specifically developed for this technique (e.g. LiCor Western blot scanner).

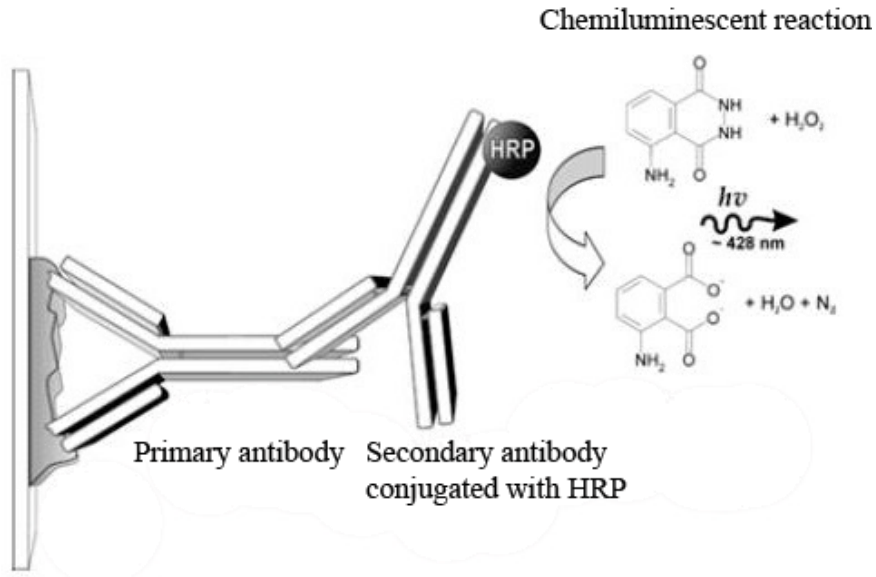


Figure 12.6 Signal detection with chemiluminescent detection

https://de.wikipedia.org/wiki/Western_Blot

Figure 12.7 Western blot scanner

Questions

What is the purpose of Western blot?

_____

_____

Why is blocking with BSA a better choice than blocking with milk in case of phosphorylated proteins?

_____

_____

What is the name of the small surface of the protein which is recognized by the primary antibody?

_____

What is the difference between the monoclonal and polyclonal antibodies?

_____

_____

_____

How could we detect a chemiluminescent signal at the end of Western blot?

_____

_____