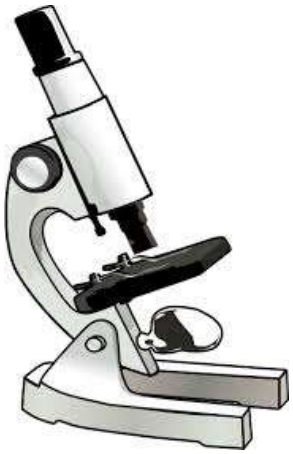
Lab Skills

Genetics

SKI2088

UCM 2017/2018
Period 1

Contents

1. General information

In the course “Lab Skills Genetics” the student is offered a hands-on training that aims at developing practical competences in widely-used, basic genetic techniques and theoretical understanding of these techniques. Students who wish to take this course should enrol in “*SCI2022: Genetics and Evolution*” or should have taken “*SCI2022: Genetics and Evolution*” before. No general prior knowledge of laboratory skills is needed, although basic knowledge of general biology, chemistry and genetics is recommendable.
The practicals take place at the laboratory facilities at the UM Randwijck campus (**UNS50**, Universiteitssingel 50, 6229 ER Maastricht).
The course will consist of 7 practical trainings that will take place over 6 sessions.

1.1 Course coordinator and contact information

Dr. Rita Brandão,
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1.2 Aims of the Skills Training Genetics

This Skills Training is a practical introduction in basic and widely used genetic techniques. It aims to develop laboratory skills in the field of molecular genetics, as well as competences in the planning and understanding theoretic principles of these techniques. The course starts with an introductory lecture providing information on the assignments as well as an introduction into Good Laboratory Practice (GLP) and Safe Laboratory Practice (SLP).

1.3 Learning outcomes

After completing the Skills training the student should:
- understand elementary laboratory rules and correctly apply laboratory safety regulations when handling clinical specimens or hazardous chemicals;
- be competent in performing basic laboratory handlings such as buffer preparation, pH measurement, making dilutions, pipetting of small volumes etc;
- understand the principles DNA isolation, DNA amplification by PCR, DNA gel electrophoresis, DNA spectrophotometry, RNA isolation and RT-PCR;
- be able to perform these techniques independently and be able to modify existing protocols for specific experiments;
- search for the sequence of a specific gene, transcript or protein for different species;
- perform genetic alignments
- use terminology related to these genetic techniques appropriately and have background knowledge of the applications of these techniques in diagnostic testing and scientific research. A non-exhaustive list of genetic and laboratory terms in included as Appendix 2. Consider this list as a guideline only. Not all terms may come along in the Skills training and you may encounter other terms that are also relevant.
- be able to report and discuss experimental methods and results in a scientific correct

manner.

1.4 Assessment

The assessment for the Skills Training will consist of

- written assignment (45% of the final mark)
- a written test at the end of the course (45% of the final mark)
- general behaviour and attitude during lab work (10%)

If a resit is necessary, a new written report and/or written assignment will be necessary. You should have a positive in all assessment forms.

The written test will contain questions about both practical aspects as well as theoretical background of the techniques applied in the course and will be similar to the "Questions to answer before or during the Skills training" given for each session.

Guidelines for written practical reports:

Aim at a maximum of 8 pages in total for your final report describing the practical work you have been doing using scientific writing. Share your experimental results with other students to increase the amount of data that you can interpret in your written report. Compare results, calculate means and standard deviations (if applicable), explain differences, etc.
The questions given with each protocol will help you to determine the scientific level that is expected for the practical report.

The report should be uploaded in EleUM.

Use the following lay-out:

• Front page: *name, ID#, experiment, experimental date, report date*
• Introduction: Aim of the experiments and theory behind them, Hypothesis (if applicable)
• Materials and Methods: *the protocol in (academic) scientific language. Describe the protocol rather than copy-paste the protocol provided. For an example of how to scientifically describe methods, see a scientific publication on a genetic subject. Ask the coordinator or a supervisor if you are unsure!*
  o Apparatus (*incl. manufacturer and the registration number of the apparatus)*
  o Reagents used (*incl lot no., date of preparation etc*.)
• Results: *including observations, data, calculations, etc. Describe the results in a scientific manner.*
• Discussion & Conclusion: *Do the results meet the expectations of the hypothesis? How can the difference between hypothesis and actual findings be explained? Explain the observations and data.*
• Literature: *When you use information from a scientific article, textbook or internet source, make sure you include the reference! Not doing so, is a form of scientific plagiary.*

Use numbers throughout the text in order of appearance. A good program to help you with this is EndNote.

1.5 Attendance requirement

There is a 100% attendance requirement for practical meetings unless a valid reason is given for absence. In case you are unable to attend please notify the course coordinator in due time.
Please attend the introductory lecture as a definitive schedule of the experiments will be provided there and an explanation of the aim of the course will be given.

1.6 Before you attend the practical trainings:

1) Please **prepare** for the Practical Trainings. **Read the descriptions** before you are going to do the actual experiments in the laboratory.
Each description has some questions about the technique in general and specific questions on the protocol ("***Questions to answer before or during the Skills Training***"). It is helpful for the understanding of the techniques to prepare these questions at home. Doing so will help you to better understand why you are doing certain steps in the protocol and why you are adding certain reagents. Aim to have a basic understanding of the experiments you are going to perform upfront. For answering these questions, you can use standard genetic or biochemical text books, scientific publications, wikipedia or other internet sources and the literature sources given in 1.8 in this course manual. Remember to always mention the source of the information that you found. We estimate you need approx. 0,5-2 hours for answering these questions, depending on the practical training.

2) Make sure you understand **Elementary Laboratory Rules** and **Laboratory Safety Regulations**. These are described in the Appendix of this Course Description and we urge you to read these carefully at the beginning of the course. You may be handling infectious agents, toxic substances, carcinogenic chemicals or aggressive reagents during the course and knowledge of how to handle such substances is of utmost importance. To prepare for the practical training, search the S and R sentences of reagents that will be used.

3) Make sure that you **wear a white lab coat** that you can wear during the practical work. These are available at the university labs.

4) Take a note book to **make notes** during the practical course. It is important to directly write down if there is a deviation from the protocol, an error in the protocol or if you make a mistake. Write down what you do during the experiments. Furthermore, take notes of machines (manufacturer, UM number, model) and reagents (concentration, manufacturer etc.). You will need to include this information in your final written report.

1.7 Preliminary schedule

*__Week 1__: Introduction + Practising basic lab skills + DNA isolation

__*Week 2__: Bioinformatics I

*__Week 3__: Spectrophotometry + Polymerase Chain Reaction (PCR)

*__Week 4__: DNA gel electrophoresis + RNA isolation

*__Week 5__: RNA isolation (cont.) + Measure RNA quantity and quality

*__Week 6__: Bioinformatics II

*__Week 7__: Exam

1.8 Some recommended literature and internet sources

1. **Alberts et al.** Molecular Biology of the Cell, 5th edition, 2008; Garland Science, NY. Freely available at: www.pubmed.com (choose “Books” from scroll list instead of the “Pubmed” default).
2. **International Chemical Safety Cards** available at: www.ilo.org/dyn/icsc/showcard.home
3. **Molecular Biology Protocols** available at: www.molecularstation.com
4. Online Mendelian Inheritance in Men (**OMIM**): http://www.ncbi.nlm.nih.gov/omim/
5. **Gene**: http://www.ncbi.nlm.nih.gov/gene/
6. **Pubmed**: http://www.ncbi.nlm.nih.gov/pubmed/

Practical training 1: Basic laboratory techniques

The first session of the Skills Lab training in Genetics will be focused on explanation of basic laboratory techniques

Questions to answer before or during the Skills training:

1) For some experiments (e.g. making a reference curve) it is necessary to precisely pipet small volumes. A pipet needs therefore to be precise. How would you check if a pipet is calibrated?

2) In the upcoming experiments you will be working with human biological specimens. What biological risks are associated with this? What personal protection would you choose/need to minimize these risks?

*** Practicing with pipetting of small volumes (if applicable).**

1) Use an analytical balance to determine the mass of an empty Eppendorf tube.
2) Pipet 10 µl of sterile water into that same tube and repeat a certain amount of times (e.g.) 50 times. Different volumes are also allowed, as long as you keep a record of them. Pipet precisely! Ask a supervisor how to pipet if in doubt.
3) close the tube and determine the mass of the Eppendorf tube
4) Collect data from all participants of the practical training and determine who did the most precise pipetting.

Practical training 2: DNA isolation

DNA isolation methods are either based on extraction of the DNA from cells (e.g. using silica or magnet particles that bind the DNA, but not other cell components) or on DNA precipitation after removal of proteins and cell debris.
In this training you will be isolating DNA by a widely used commercial kit (Promega), based on the DNA precipitation principle.

Questions to answer before or during the Skills training:

1) What are the main steps needed for DNA extraction using the DNA precipitation principle?

2) What reagents could be in the cell lysis buffer you will be using in the DNA isolation protocol described below (it is not necessary to know the exact chemicals and concentration, but rather to understand what *could* be a chemical that is in the solution and why it would be used).

3) In the DNA precipitation principle, how to we get rid of the protein?

4) After the blood is taken from the arm of a patient by venepuncture it should be prevented from clotting, otherwise it is difficult to isolate DNA from it. How is clotting prevented (i.e, what reagents can be present in the collection tube and what are their modes of action)?

4) What biological risks are involved when working with blood? How would you protect yourself from these risks? What would you do if you accidently injured yourself with a used needle that was used for drawing a patient's blood?

5) How should you store the genomic DNA that you isolated? For how long can genomic DNA be stored?

6) You will have to centrifuge at 2000xg during DNA isolation. With most centrifuges it is not possible to program the g-force of centrifugation. Instead you will have to set the RCF (relative centrifuge force) or RPM (revolutions per minute). How do you calculate the RPM needed if a g-force of 2000xg is needed (what information do you need to calculate the RPM?).

7) what is the colour of pure DNA?

Materials and equipment needed:

Centrifuge
1,5 ml eppendorf tubes
Tube racks
Nalgene tubes
vortex
Water bath

thermoshaker
PBS
cell lysis solution (Promega)
nuclei lysis solution (Promega)
protein precipitation solution (Promega)
proteinase K (optional)
isopropanol
70% ethanol
gloves

Procedure for DNA isolation from cell cultures:

wear gloves!

1. Collect cells from the cell culture flask and transfer them to an 1,5 mL Eppendorf tube.

2. Centrifuge at 16,000 xg for 10 seconds at room temperature (RT) to pellet the cells.

3. Remove the supernatant, leaving behind the cell pellet and a very small amount of residual liquid.

4. Add 200μL of PBS and vortex shortly to wash the cells

5. Centrifuge at 16,000 xg for 10 seconds at RT. Remove the PBS leaving a very small amount behind.

6. Vortex vigorously to resuspend the cells.

7. Add 600 μl of cell lysis solution and pipet to lyse the cells. Pipet until no cell clumps are visible. If necessary heat the sample to 65°C (no longer than 10min!)

8. Add 3μl of RNase Solution to the nuclear lysate and mix the sample by inverting the tube 2–5 times.

9. Incubate the mixture for 15–30 minutes at 37°C.

10. Allow the sample to cool to room temperature for 5 minutes before proceeding.

11. To the room temperature sample, add 200μl of Protein Precipitation Solution and vortex vigorously at high speed for 20 seconds.

12. Chill sample on ice for 5 minutes.

13. Centrifuge for 4 minutes at 13,000–16,000 × g. The precipitated protein will form a tight white pellet.

14. Carefully remove the supernatant containing the DNA (leaving the protein pellet behind) and transfer it to a clean 1.5ml microcentrifuge tube containing 600μl of room temperature isopropanol. Note: Some supernatant may remain in the original tube

containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

15. Gently mix the solution by inversion until the white thread-like strands of DNA form a visible mass.

16. Centrifuge for 1 minute at 13,000–16,000 × g at room temperature. The DNA will be visible as a small white pellet. Carefully transfer the supernatant to a clean1,5 mL tube (just to be sure you don't lose the DNA!).

17. Add 600µl of room temperature 70% ethanol, and gently invert the tube several times to wash the DNA. Centrifuge for 1 minute at 13,000–16,000 × g at RT

18. Carefully aspirate the ethanol, transferring it to a clean tube. The DNA pellet is very loose at this point, and care must be used to avoid aspirating the pellet into the pipette.

19. Invert the tube on clean absorbent paper, and air-dry the pellet for 10–15 minutes.

20. Add 100µl of DNA Rehydration Solution, and rehydrate the DNA by incubating overnight at room temperature or at 4°C.

21. Store the DNA.

Practical training 3: Bioinformatics I

In this practical, we will explore OMIM, how to search for the sequence of a gene, how to find where are the exons and introns, how many transcripts do certain genes have, how to find the RNA sequence and the protein it codes for. We will also explore how to design primers for amplification by PCR (a technique that we will also use).

Additionally, we will also explore the SNP database and how to design simple experiments to determine the genotype.

The assignments will be given during the practical work. We will not be programming and no special computer skills are required. We will use user-friendly online-available databases and tools.

In practical training 5, several dilutions and solutions have to be prepared. If there is need and if time allows it, we will discuss some basic formulas needed for the calculations and we can practice with some examples.

Questions to answer before or during the Skills training:

1) Where can we search for a gene sequence?

2) What are exons and introns?

3) Why would we want to know where variants are located?

4) What are primers? Which are their characteristics?

5) How can we know if a primer sequence is specific?

6) What is a genetic alignment and why would we want to do it?

Practical training 4: DNA spectrophotometry

For use in downstream reactions, e.g. DNA restriction or DNA amplification by PCR (see Practical Training 5), it is necessary to measure DNA concentration and DNA purity. A cheap and fast method to do so, is spectrophotometry.
Spectrophotometry is based on absorbance of light of a certain wavelength by chemical compounds. Purine and pyrimidine bases in nucleic acids absorb UV light at 260 nm (designated as “A260”). This feature which can be used to determine concentration of DNA (or RNA): the higher the absorbance, the higher the DNA concentration in a sample. Similarly, the peptide bond between adjacent amino acids in proteins absorbs light at 280 (“A280”), which can be used to assess protein contamination of purified DNA samples.

The relation between concentration and light absorbance is described by the Lambert-Beer law:

$$\mathbf{A = \varepsilon \cdot c \cdot l}$$

A = absorbance value (“light in (I_0) – light out (I_i)”)
ε = extinction coefficient for a given substance,
c = concentration of substance
l = light path length

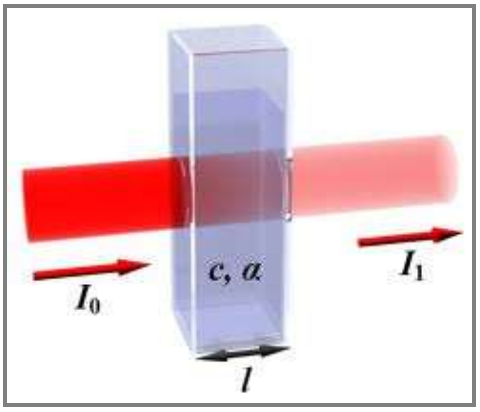

Figure 1 – An image demonstrating the Lambert-Beer law.

DNA concentration:
For DNA, the extinction coefficient ε is 0.020 per µg of double stranded DNA (dsDNA) per ml of solution per cm of light path (or 0.020 per µg/ml/cm). In other words: a reading value of 1.0 at 260nm corresponds to 50 µg/ml of double stranded DNA (at light-path of 1 cm).

DNA purity:
An aqueous solution of pure DNA has and A260/A280 ratio of approx. 1.8-2.0. In case of contamination with protein, this ratio will be significantly less than the values given above and accurate quantitation of the amount of nucleic acid will not be possible.

Questions to answer before or during the Skills training:

1) Why do you think it is important to know DNA concentration? Why DNA purity? What impurities could be present in DNA?

2) Suppose you have two DNA solutions, both have the same concentration of 200 ng/µl and both contain 100 µl of the DNA solution. The first tube contains human genomic DNA (gDNA), the second tube contains *Escherichia coli* gDNA. Which tube contains the most genome equivalents? What is the molarity (mole/µl) of the human gDNA solution?

3) Can you think of other methods to quantify DNA?

4) Describe how you can quantify RNA by spectrophotometry (i.e. determine the amount of RNA in a solution in ng/ul)

Materials and equipment needed:

DNase- and RNase-free water (biological grade)
Methylacrylate cuvette (disposable) or a quartz cuvette (non-disposable)
Spectrophotometer
Tube racks
DNA isolate

Procedure:

1. The concentration and purity of the DNA isolated in practical training 1 will be determined by a technician of the dept. of Clinical Genetics using a Nanodrop spectrophotometer (see http://www.nanodrop.com/Overview.aspx). The Nanodrop is quicker and more precise than spectrophotometers available at the UM student laboratories.

2. A260 and A280 values of your DNA sample will be provided and you will have to calculate concentration and purity of the DNA, to prepare DNA dilutions of different concentrations (PCR).

3. A demonstration of an “old-style” spectrophotometer available at the UM laboratory (Genequant, GE Health Care) will be given during the session. Students will have the opportunity to practise spectrophotometry themselves using the Genequant.

Practical training 5: Polymerase chain reaction (PCR)

In the early 1980s Kary Mullis invented a cyclic reaction that enables amplification of specific parts of DNA. This reaction has revolutionised biological research and diagnostic testing (e.g. in microbiology, genetics, pathology, forensics and plant science). PCR and molecular techniques derived thereof are still widely in use today. PCR is an extremely sensitive technique, which can detect as little as 1 or 2 molecules of target DNA. On the other hand, it can yield enormous amounts of product after the amplification is finished (up to 1000 billion copies).
In this training, you will amplify DNA that was isolated and quantified in previous experiments, using different DNA concentrations as input to test PCR sensitivity. Each group will use a different amount of $MgCl_2$ and/or annealing temperature to test their effect on the PCR. The target you will be amplifying is an exon from *BRCA1* gene. Germline mutations in this gene are associated with hereditary early-onset of breast and/or ovarian cancer syndrome. The amplified DNA will be visualised in Practical Training 6: DNA gel electrophoresis. Combining the results of all the groups will allow to determine the best PCR conditions and the sensitivity of the technique.

Questions to answer before or during the Skills training:

1) Describe the principle of the Polymerase Chain Reaction (PCR). Use at least the following keywords: *primers, Taq polymerase, dNTP (deoxyribonucleotide triphosphate), $MgCl_2$, denaturation, annealing, elongation, 5'- end, 3'-end, reaction cycle.* Why are 2 primers needed in the PCR reaction? Explain via a schematic drawing.

2) Imagine you start with 10 copies of a certain piece of DNA, that you are going to amplify by PCR. With how many copies will you have generated after 5 reaction cycles? And after 10 reaction cycles? Make a graph of the cycle number (X-axis) vs. the number of DNA copies present after the cycles (Y-axis). Describe the mathematical relationship between X and Y variables. What is the end product of PCR?

3) Is PCR suited for detection or amplification of RNA? Why?

4) What control reactions (which samples) would you include in the PCR set-up in order to monitor if everything has gone well? Why?

5) Why do you think that we need to do PCR?

6) As explained above, PCR is extremely sensitive and can be used to amplify very low quantities of target DNA. This also implies however, that PCR is viable to contamination, e.g. with DNA from the laboratory worker himself, PCR products from previous reactions or omnipresent DNA from non-human sources. Such contamination can lead to false positive results. How would you avoid that your PCR becomes contaminated with this foreign DNA? Try to find as many solutions as possible and explain the rationale behind them.
7) Give an example of a specific application of PCR in microbiology, genetics, pathology/oncology and forensic science.

8) Why is MgCl2 required in the PCR? What do you expect to happen if you add more? and what if you add less? What do you expect to happen if you decrease the annealing temperature in the PCR described below (e.g. around 50°C), what if you increase this temperature (e.g. around 72°C)?

9) Do you still remember how we can test if the primers used are specific for the region to be amplified?

Reagents, materials and equipment needed:

- Purified DNA obtained in Practical Training 2 and quantified in Practical Training 4
- 75% Ethanol
- Taq polymerase (5 U/µl)
- dNTPs mix (each at a final concentration of 10 mM)
- 10 x PCR buffer
- $MgCL_2$ 25 mM
- Primer solution (10 pmol/µl)
- sterile water
- Ice and ice bucket
- PCR machine (Thermocycler)
- PCR tubes and racks
- 1,5 ml reaction Eppendorf tubes (plastic) and racks
- micropipette sets
- gloves
- calculator

Procedure:

Wear gloves! Before you start, program the PCR program on the Thermocycler as explained below under item 7).

1) Clean your lab bench with ethanol and dry with a paper towel.

2) Dilute the DNA to 50 ng/µl in H_2O (use a 1,5 ml tube to make a total volume of 100 µl). *Hint:* Calculate the volume of water and the volume of DNA you want to add together first. If you are unsure you made the correct calculation ask a supervisor to check this before you proceed!

3) From the 50 ng/µl DNA solution, make further dilutions of 5 ng/µl, 0,5 ng/µl, 0,05 ng/µl and 0,005 ng/µl and 0,0005 ng/µl (all in water, all in new tubes). Each time you have made a new dilution, mix well by vortexing for approx. 5 seconds.

4) Add 3 µl of water and 2 µl of the different DNA dilutions (ranging from 50 ng/ µl to 0,5 pg/µl) to different PCR tubes (open only one tube at a time in order to minimize risk of contamination). Add first the water to all tubes. Then add the DNAs. Don't forget to change

the pipet tip. Be sure to label the tubes or to write down the order you pipetted. Also pipet a positive control at the end and a negative control (=2 µl of water, instead of DNA). *Hint*: make a scheme of the tube sequence and samples ("sample sheet") before you start pipetting. Close the PCR tubes with DNA and store them on ice.

6) Working on ice, prepare the PCR mix (all reagents, except the DNA and some water) for a total of 9 reactions in a 1,5 ml tube. You will receive the concentrations required in a reaction. Using that information, you will need to calculate the volume needed for each reaction of 25 µl (final volume) and how much you need to prepare the PCR mix.

7) Carefully add 20 µl of PCR mix to each of the 8 PCR tubes, changing the pipet tip, in order not to contaminate the mix, since the DNA is already in the tubes. Pipet very carefully so all of the solution is in the bottom of the tube, without air bubbles.

7) Program the Thermocycler as follows:

	95°C for 5 min
35 cycles	95°C for 30 s $T_{annealing}$ for 45 s* 72 °C for 45 s
	72 °C for 10 min
Hold at	10°C

*different groups will be using different temperatures of annealing.

8) Place the PCR tubes with PCR mix and DNA in the Thermocycler and start the program

9) Store the amplified samples for subsequent use in Practical Training 6: DNA gel electrophoresis

10) Report the results in your written practical report and show a photo of the gel. Interpret the results of all groups together!!

Practical training 6: Agarose gel electrophoresis of DNA

The PCR reaction creates many copies of the amplified fragment. To check the PCR results, we need to separate the DNA according to length and visualize it.
(Eventually one can also isolate PCR product with specific lengths from the gel when required.)

A good and relatively simple method to check if our PCR worked and if it was specific is to pull the PCR product through an agarose gel with the aid of an electric current. The electric charge makes the DNA travel through the agarose gel and the DNA fragments will become separated according to their size. The larger fragments will travel slower through the gel than the shorter fragments. A sample with many DNA fragments with different lengths (DNA ladder or molecular weight) is used next to the samples to be analysed. The two main components that determine speed and quality of the DNA separation are the amount of electric charge that we apply and the pore size of the gel matrix that we use.

Questions to answer before or during the Skills training:

1) If you know that DNA is an acid, which direction will the DNA molecules travel? To the anode (+) or the cathode (-)? Why to this direction?

2) For our DNA samples we will use an agarose gel. What other type of gel is also commonly used to separate DNA fragments? Why do you think we will use agarose?

3) What are the main advantages and disadvantages of the two mainly used gel types?

Besides gels, one can also use narrow silica capillaries with a polymer matrix inside to separate DNA fragments according to their size (fragment size analysis). This is often used in automated systems in modern labs.

To make an agarose gel, agarose is dissolved in a buffer. The percentage of the gel determines characteristics and the ability to separate DNA fragments. For small DNA fragments in the range of 150 to 2000bp a 2% agarose gel is normally used, for longer (2 to 10kb) a lower percentage is used (1%).

For our experiment we will use a 1% agarose gel in TAE buffer. Agarose dissolves and ‘melts’ in the TBE buffer when heated. After cooling it becomes a solid gel.

4) You need to prepare a 2% agarose gel. How much agarose (in grams) do you need to make a 100 ml gel? What do you think would happen with DNA separation if you would make a 4% gel? What if you make a 1% agarose gel ?

The TAE buffer is a mixture of Tris base (tris(hydroxymethyl)aminomethane), acetic acid and EDTA (Ethylenediaminetetraacetic acid).

The function of the Tris in the buffer is to keep the DNA deprotonated and soluble in water. The EDTA is a chelating agent that removes certain metal ion from enzymes that are thus deactivated and inhibited from destroying the DNA.

Of course we need a way to make the DNA visible once it's traveling through the agarose gel. To do that, we will use ethidium bromide. This compound works as a fluorescent tag for DNA. Ethidium bromide 'lights up' (fluorescence) with an orange colour when exposed to ultraviolet light (UV). When bound to DNA the intensity of the fluorescence increases a 20-fold, thus making DNA visible with UV light.

Warning: ethidium bromide is toxic and may be a mutagen, a carcinogen, and a teratogen. Make sure that it never touches your skin. Dispose/change your gloves after working with it, without touching anything else in between!

Warning: ultraviolet light is dangerous and minimize exposure to it. In particular, avoid looking at it as much as possible. Use eye protection!

Reagents, materials and equipment needed:

- 10x TBE buffer
- Agarose
- DNA ladder
- loading dye
- ethidium bromide
- 1,5 mL tube racks
- Gel mold + comb
- Horizontal electrophoresis tank
- Electric power source
- micropipettes
- deionized water

Procedure:

Making the agarose gel

1) Dilute 10x TBE buffer to 1x with deionized water. Make enough to fill the electrophoresis tank and to make the agarose gel.

How much?

2) Take a gel mold and calculate how much you need for a 1cm thick agarose gel.
Use the balance to take the right amount of agarose to make a 2% gel and mix this with the amount of TBE buffer needed in an Erlenmeyer.

Heat the TBE buffer in a microwave oven to dissolve the agarose. Be careful not to have it boil over the rim! **HOT SOLUTION** ! Wear glasses and gloves for the heat. You might have to cool down the solution and warm it again a few times.

4) A small amount of ethidium bromide is added to the fluid agarose gel, but after it cooled a bit (until you can hold the flask without the heat glove). Mix well and pour immediately into the mold.

3) To 'load' our DNA samples on the gel, we make a series of holes or 'wells' at one side by inserting a 'comb' in the mold.
Select a comb and insert it in the gel mold. Remove gel bubbles with a pipette tip. In particular check for air pockets around the comb.

5) Once the gel has solidified, the comb is removed. The gel tray is then placed in the electrophoresis tray with 1x TAE buffer. The gel needs to be slightly submerged with 2-3mm buffer on top. The gel is ready to be used.

DNA loading on the agarose gel:

6) Before a sample can be loaded, we need to add a dyed loading solution. This serves two purposes: to make your sample visible with a dye so that you can see your sample going into the well and to make the sample 'heavier' than the TAE buffer to have it sink to the bottom of the well.

How much PCR product and how much loading dye?

Mix 10 µl PCR product with 5 µl dyed loading solution.

Carefully bring the mix into a well of the gel. Keep the first and the last well of each row free for the DNA ladder (see next point)!
Be careful not to damage the well and keep track of what sample goes into which well!

Because we want to determine the length of our DNA PCR products, we need a 'reference' For that we will use a DNA ladder mixture.

A DNA ladder contains DNA fragments of known lengths. For example, DNA fragments with 100 bp, 200 bp, 300 bp, 400 bp. etc.

Question: What DNA ladder would you prefer to use for our experiment?

At least one well in each agarose gel line is to be filled with the DNA ladder. Even better is to use two: one on each side. Be careful when loading the samples with loading dye on the gel! Do not spill into adjacent wells and do not damage the well itself.

7) load 3 µl of the DNA ladder in each well reserved for the ladder.

8) Once all samples are loaded, the electrophoresis tank is closed and electric current is applied.

Make sure the correct polarity is respected!

Set the transformer to 120Volt.

9) With the loading dye it is possible to check how far the samples have run. Note that the dye runs faster than the sample.

10) After the fragments are well separated, a photograph of the gel under UV light is made.

Remove the gel from the tray (avoid spilling and wear gloves!).

Place the gel on the UV plate.

Make a photograph.

Question: What do you see?

Question: What is the undefined ‘blob’ at the bottom (that has travelled the farthest)? Explain…

Determine the fragment length.
It’s possible to make a reasonable ‘guess’ of the DNA fragment size on the gel by comparing the position with the DNA ladder fragments.

Practical training 7: RNA extraction

Total RNA consists of different classes of RNA: ribosomal RNA, transporter RNA, messenger RNA and other non-coding RNAs.

Messenger RNA is often used in genetics to perform gene expression analysis and to evaluate RNA splicing, i.e. removal of introns from the pre-mRNA molecule leaving exons adjacent to each other.

In this practical training, we will isolate total RNA from cultured cells, check its degradation by electrophoresis.

Questions to answer before or during the Skills training:

- What are the differences between DNA and RNA?
- How can you test if there is DNA contamination in the RNA sample?
- What can be done to remove any DNA contamination from the RNA sample?
- What is the best storage condition for RNA?
- What do we have to do before we can do gene expression or RNA splicing analysis? Do we need enzyme(s)? if so, name them.
- There is a G>A variant in the last nucleotide of exon 4 of the *ABC* gene. How can we test its effect on RNA splicing?
- Interpret figure 2.
- There is a variant in the promoter region of the *ABC* gene. How can we test if it affects the gene expression of ABC?

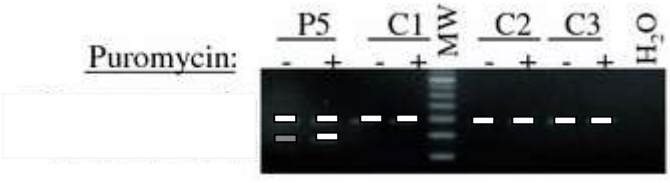

Figure 2- Electrophoresis results of amplification of cDNA of a sample with a germline heterozygous variant (P5) and control samples (C1, C2 and C3, who do not carry the variant). Primers were localized in exons 3 and 7. Puromycin is a drug that is added to avoid degradation of RNAs containing premature stop codons.

Materials and equipment needed:

Centrifuge
1,5 ml eppendorf tubes and racks
vortex
thermoshaker
Bleach solution to clean
Trizol
chloroform
isopropanol
75% ethanol (cold)
Baxter water at 40°C
ice and ice buckets
micropipettes and filter tips
agarose, TBE and gel electrophoresis system
Erlenmeyer flask
cuvette and spectrophotometer

Procedure for RNA isolation from cell cultures:

1. Start by cleaning the lab benchtop and the fume-hoods with bleach.
2. Collect cells from the cell culture flask and transfer them to an 1,5 mL Eppendorf tube.
3. Centrifuge at 16,000 xg for 10 seconds at room temperature (RT) to pellet the cells.
4. Remove the supernatant, leaving behind the cell pellet and a very small amount of residual liquid.
5. Add 200μL of PBS and vortex shortly to wash the cells
6. Centrifuge at 16,000 xg for 10 seconds at RT. Remove as much PBS as possible.
7. Working in the fume-hood, add 1mL of TRIzol reagent and vortex to dissolve the pellet. Let it stand for 5 min at room temperature (RT) to lyse cells.
8. Add 0.2 ml chloroform.
9. Vortex vigorously (2seconds) and incubate them for 5 min at RT.
10. Spin eppendorfs at 12 000xg for 15 min at 4 ^{o}C.

11. Working in the fume-hood, transfer the top aqueous phase to a clean 1,5ml eppendorf tube (should be approximately 0,6ml). Be careful not to take the interphase, as it contains DNA and will contaminate the RNA sample.
12. Add 0.5 ml 2-propanol and mix by gently inverting the tube 25 times.
13. Allow the RNA to precipitate at -20°C until the following week*.

RNA isolation (cont.)

14. Precipitate RNA by spinning for 20 min at 16 100xg and 4 ^{O}C,
15. Remove the supernatant.
16. On ice, wash the RNA pellet with 500µL 75% EtOH (cold) and mix by pipetting. Add more 500µL 75% EtOH.
17. Spin for 7 min at 16 100 xg, 4 ^{O}C
18. Aspire supernatant and wash pellet with 100 µl 75 % EtOH
19. Spin 7 min 16 100 xg, 4 ^{O}C.
20. Aspire supernatant and air dry the RNA pellet (maximum for 10 min, preferably, but always check it!)
21. Dissolve RNA pellet in 25 µl Baxter H_2O, previously warmed at 40 ^{o}C.
22. Check the quality of the sample by gel electrophoresis in 2% agarose gel.
23. Check the quantity of the sample using spectrophotometry as in practical training 4. There is one difference. Which is it?

* Note that the precipitation is time is longer than usually.

Practical training 8: Bioinformatics II

In this practical training we will analyse sequences obtained from Sanger sequencing (see en.wikipedia.org/wiki/Sanger_sequencing).

We will start by confirming, using online-available tools, that the sequence is from the expected fragment. Then we will:

- analyse the sequence to detect genetic variants (each group will have a different sequence) and determine if they are homozygous or heterozygous variants.
- determine their impact on protein.

The results should be reported using currently accepted nomenclature (see www.hgvs.org/varnomen).

We will also interpret results from a gene expression experiment.

Questions to answer before or during the Skills training:

1) What type of mutations exist? Discuss at DNA-level.
2) Which main processes are needed in the cell to produce protein?
3) How can the variants affect the protein?
4) How to determine if a genetic variant is pathogenic?
5) A gene expression experiment was performed and the results are shown in figure 3. Interpret the figure.

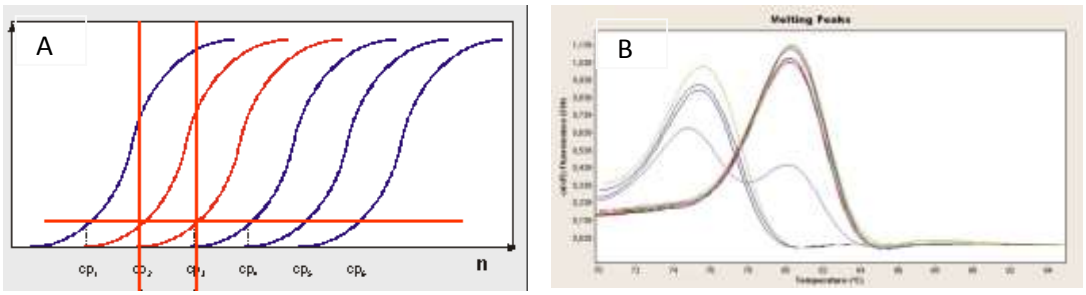

Figure 3 – Panel A shows the amplification plots. X-axis shows the PCR cycle and the y-axis shows the fluorescence intensity. Panel B shows the melting curves of the fragments.

6) The average Ct values per sample group are shown in a table. Calculate the fold-changes using the ΔΔCt method

Table 1- Averaged Ct values and standard errors obtained in a RT-qPCR experiment

Sample condition	TP53 average Ct	GAPDH average Ct
HeLa: no treatment	30,5 ± 0,15	23,4 ± 0,09
HeLa: drug A	26,7 ± 0,2	22,6 ± 0,03

Appendix 1

Elementary laboratory rules

- In principle, the practical work takes place at UNS 50 (Randwijck) in a lab room, computer room or other locations at the Randwijck campus (for details see the schedule on MyUM)
- Lab coats are available and should be worn **at all time** while present in the laboratory.
- It is **not** allowed to eat or drink in the laboratory.
- Safety glasses should be worn when working with chemicals and biological material.
- Gloves should be worn when working with chemicals and biological material. It is **not allowed** to wear gloves or lab coats outside of the lab.
- Volatile liquid chemicals should only be handled in a fume-hood.
- Be present on time.
- Coats and bags should be stored in the lockers.
- Only the participating students are allowed access to the laboratory.
- Coffee or tea breaks are only allowed in consultation with the laboratory staff.
- At the end of each practical, laboratory tables need to be cleaned

Safe Laboratory Practice (SLP)

In laboratories you always find a high concentration of hazardous substances. Materials that are poisonous (**toxic**) for humans, animals and plants, **aggressive** (caustic/corrosive) – like strong acids and bases – **infectious** (pathogenic/contaminated) – meaning that they contain micro-organisms that could cause disease in humans, animals or plants – **inflammable** and/or **explosive**, emitting **radiation** (UV, IR, electromagnetic, radioactive), very hot or very cold, or **carcinogenic**, **teratogenic** (causing damage to posterity) or **mutagenic** can all be present in a laboratory. Therefore, it is essential to work safely. In order to be able to work safe, you need to be familiar with safety rules and regulations. Safe Laboratory Practice does not concern the experiment itself, but the person who is conducting it and everybody around. When you work in a safe way, you will be able to prevent many dangerous situations. A few guidelines can help to achieve SLP.

Personal conduct

- Eating, drinking, smoking, mobile chatting or using make up in the laboratory is **strictly forbidden**.
- Materials which are not required for the experiment should be kept in lockers. This includes mp3-players and mobile phones.
- Wearing a white laboratory coat is **obligatory**.
- Always keep your coat closed.
- Make sure the sleeves of your lab coat cover your own clothes.
- **Never** wear your lab coat outside the laboratory.
- Remove rings, watches, bracelets, etc.
- Long hair should be put together!
- **Always wear gloves** when working with chemicals or biological materials – **remember**: gloves do not offer complete protection, so keep working as careful as if you were not wearing them.
- Instructions from the laboratory staff should be followed immediately at all times.
- In case of accidents, immediately notify the laboratory staff; this includes accidents that seem harmless.
- In case of accidents involving eyes (e.g. chemicals in the eye), first flush with water, then warm the laboratory staff: "**first water, then bother**".
- **Always wash your hands** before leaving the laboratory; use disinfecting soap and disposable drying paper.

Work area

- **Clean and disinfect the working area** before each experiment.

- Put **only the necessary equipment** in the working area.
- **Always clear away unused materials**, not only at the end of the practical but also during the practical.
- **Immediately remove and clean spilled solutions or chemicals**.
- Mark bottles with solutions: name, description of contents, date of preparations, hazard symbols (if applicable).
- **Always label or mark glassware** - write down the content (even if it is merely water) and the concentration, if applicable.
- **Always close** bottles or jars immediately after using it.
- **Never** lift a bottle or a jar by its stopper, lid or neck.
- When using aggressive, volatile or inflammable substances, **always work inside a fume hood**.
- **Always make sure** that people around you know what you are working with.
- **Always** work in a logical, tidy manner, **minimizing risks**.
- **Always remain focused** on your work. Carelessness produces accidents!

Waste handling

Proper segregation of laboratory waste is essential to good chemical hygiene and a safe workplace environment. You should aim to minimize the quantity of waste products and correctly dispose of all waste products. Many researchers often tend to put all of their wastes into the same cabinet or fume hood. Doing so can have disastrous results!

The guidelines for temporary storage of chemical wastes in the laboratory are really no different than those that you use for the storage of your usual lab chemicals. There are 6 main categories in chemical waste:

CHEMICAL WASTE CATEGORIES:

Category 1

Acidic and neutral inorganic waste in solution

Category 2

Alkaline inorganic waste in solution

Category 3

Halogen-deficient organic waste

Category 4

Halogen-rich organic waste

Category 5

Special waste

Category 6

Waste with exceptional risks

NEVER store the following types of wastes near each other:

- Acids and bases.
- Organics and acids.
- Cyanide, sulfide or arsenic compounds and acids.
- Alkali or alkali earth metals, alkyllithiums etc. and aqueous waste.
- Powdered or reactive metals and combustible materials.
- Mercury or silver and ammonium containing compounds.

If a bottle broke in a waste storage area where incompatibles were present, the results could be disastrous. Remember: incompatible bottles of wastes should be stored in separate cabinets, preferably as far apart as possible.

The rules and regulations regarding various categories of waste products are presented below.

- All glassware must be rinsed with tab water and labels and marks must be removed before placing it into the white container.
- All reaction tubes must be rinsed with tab water. All contents must be removed – if necessary by using a tube brush – and put upside-down in the baskets.
- All stoppers must be rinsed and deposited into the containers marked with “VUIL”.
- All glass pipettes must be rinsed with tab water and put upside-down into the pipette-holders.
- Chemical waste products must be stored in the appropriate containers (ask the laboratory staff).
- Risk waste products and all materials contaminated with those substances are disposed in special yellow bags (ask the laboratory staff).
- “Biohazard” waste products, i.e. biological substances, and all materials contaminated with those substances disposed in stainless steel sterilization jars, equipped with transparent plastic bags.
- All needles and scalpels are discarded in the small yellow/red containers present on the laboratory tables.
- Lab tables must be thoroughly cleaned and dried – when hazardous materials or bio-materials have been used, tables should be disinfected with 70% ethanol.
- All lab tables must be left neat and tidy, turn-off electrical instruments (electrical instruments can remain plugged-in).

Chemical safety

You need to know what you are doing when working with chemicals. Therefore, it is essential to know where to find chemical information, risk indicators and safety advice. The category or risks of substances can be judged from the pictogram of logo displayed on bottles or jars. A few of the most frequently used logos are shown below:

In addition to theses logos, bottles and jars usually contain safety risk codes, referring to Risk and Safety Statements. Theses statements contain information on risks (R-phrases) and safety (S-phrases).

R-phrases

R1 Explosive when dry
R2 Risk of explosion by shock, friction, fire or other sources of ignition
R3 Extreme risk of explosion by shock, friction, fire or other sources of ignition
R4 Forms very sensitive explosive metallic compounds
R5 Heating may cause an explosion
R6 Explosive with or without contact with air
R7 May cause fire
R8 Contact with combustible material may cause fire

R9 Explosive when mixed with combustible material
R10 Flammable
R11 Highly flammable
R12 Extremely flammable
R14 Reacts violently with water
R15 Contact with water liberates extremely flammable gases
R16 Explosive when mixed with oxidizing substances
R17 Spontaneously flammable in air
R18 In use, may form flammable/explosive vapor-air mixture
R19 May form explosive peroxides
R20 Harmful by inhalation
R21 Harmful in contact with skin
R22 Harmful if swallowed
R23 Toxic by inhalation
R24 Toxic in contact with skin
R25 Toxic if swallowed
R26 Very toxic by inhalation
R27 Very toxic in contact with skin
R28 Very toxic if swallowed
R29 Contact with water liberates toxic gas.
R30 Can become highly flammable in use
R31 Contact with acids liberates toxic gas
R32 Contact with acids liberates very toxic gas
R33 Danger of cumulative effects
R34 Causes burns
R35 Causes severe burns
R36 Irritating to eyes
R37 Irritating to respiratory system
R38 Irritating to skin
R39 Danger of very serious irreversible effects
R40 Limited evidence of a carcinogenic effect
R41 Risk of serious damage to eyes
R42 May cause sensitization by inhalation
R43 May cause sensitization by skin contact
R44 Risk of explosion if heated under confinement
R45 May cause cancer
R46 May cause heritable genetic damage
R48 Danger of serious damage to health by prolonged exposure
R49 May cause cancer by inhalation
R50 Very toxic to aquatic organisms
R51 Toxic to aquatic organisms
R52 Harmful to aquatic organisms
R53 May cause long-term adverse effects in the aquatic environment
R54 Toxic to flora
R55 Toxic to fauna
R56 Toxic to soil organisms
R57 Toxic to bees
R58 May cause long-term adverse effects in the environment
R59 Dangerous for the ozone layer
R60 May impair fertility
R61 May cause harm to the unborn child
R62 Possible risk of impaired fertility
R63 Possible risk of harm to the unborn child
R64 May cause harm to breast-fed babies
R65 Harmful; may cause lung damage if swallowed
R66 Repeated exposure may cause skin dryness or cracking
R67 Vapors may cause drowsiness and dizziness
R68 Possible risk of irreversible effects

S-phrases

(S1) Keep locked up
(S2) Keep out of the reach of children
S3 Keep in a cool place
S4 Keep away from living quarters
S5 Keep contents under ... (*appropriate liquid to be specified by the manufacturer*)
S6 Keep under ... (*inert gas to be specified by the manufacturer*)
S7 Keep container tightly closed
S8 Keep container dry
S9 Keep container in a well-ventilated place
S12 Do not keep the container sealed
S13 Keep away from food, drink and animal feeding stuffs
S14 Keep away from ... (*incompatible materials to be indicated by the manufacturer*)
S15 Keep away from heat
S16 Keep away from sources of ignition - No smoking
S17 Keep away from combustible material
S18 Handle and open container with care
S20 When using do not eat or drink
S21 When using do not smoke
S22 Do not breathe dust
S23 Do not breathe gas/fumes/vapor/spray (*appropriate wording to be specified by the manufacturer*)
S24 Avoid contact with skin
S25 Avoid contact with eyes
S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S27 Take off immediately all contaminated clothing
S28 After contact with skin, wash immediately with plenty of ... (*to be specified by the manufacturer*)
S29 Do not empty into drains
S30 Never add water to this product
S33 Take precautionary measures against static discharges
S35 This material and its container must be disposed of in a safe way
S36 Wear suitable protective clothing
S37 Wear suitable gloves
S38 In case of insufficient ventilation wear suitable respiratory equipment
S39 Wear eye/face protection
S40 To clean the floor and all objects contaminated by this material use ... (*to be specified by the manufacturer*)
S41 In case of fire and/or explosion do not breathe fumes
S42 During fumigation/spraying wear suitable respiratory equipment (*appropriate wording to be specified by the manufacturer*)
S43 In case of fire use ... (*indicate in the space the precise type of fire-fighting equipment. If water increases the risk add - **Never use water***)
S45 In case of accident or if you feel unwell seek medical advice immediately (show the label where possible)
S46 If swallowed, seek medical advice immediately and show this container or label
S47 Keep at temperature not exceeding ... °C (*to be specified by the manufacturer*)
S48 Keep wet with ... (*appropriate material to be specified by the manufacturer*)
S49 Keep only in the original container
S50 Do not mix with ... (*to be specified by the manufacturer*)
S51 Use only in well-ventilated areas
S52 Not recommended for interior use on large surface areas
S53 Avoid exposure - obtain special instructions before use
S56 Dispose of this material and its container at hazardous or special waste collection point
S57 Use appropriate containment to avoid environmental contamination
S59 Refer to manufacturer/supplier for information on recovery/recycling
S60 This material and its container must be disposed of as hazardous waste
S61 Avoid release to the environment. Refer to special instructions/safety data sheet
S62 If swallowed, do not induce vomiting; seek medical advice immediately and show this container or label
S63 In case of accident by inhalation; remove casualty to fresh air and keep at rest
S64 If swallowed, rinse mouth with water (only if the person is conscious)

Appendix: Non-exhaustive list of relevant laboraty terms and genetic terms

Annealing
BLAST
Cell cycle
Cell cultures
Cell lysis
CNV: Copy Number Variant
Chromosome
Denaturation
DNA: gDNA, dsDNA, ssDNA, cDNA
dNTPs
Electrophoresis
Elongation
Enzyme
Exon
Gene
Hybridisation
Intron
Lambert-Beer law
Leucocytes
Molarity
Mole
Mutation
Monosomy
Mutation
OMIM
PCR
Polymerase
Pubmed
pH
phytoheamagglutinin (PHA)
Precipitation
Primer
Promoter
Proteinase
RNA
RNA-splicing
Reverse transcriptase
RT-PCR
SNP: Single Nucleotide Polymorphism
SNV: Single Nucleotide Variant
Transcription
Translation
UCSC Genome browser
3'-end
5'-end