Laboratory for Microbiology
Wageningen UR

The safety guide

Philippe Puylaert

1 Preface

It is a pleasure and a privilege to write a short introduction for the second edition of this book on laboratory practices in the Laboratory of Microbiology. The success of the first edition earlier this year made it necessary to print this next edition. This testified for the foresight of Philippe Puylaert who initiated this activity and deserved all the credit for this !

In this second edition, the opportunity was taken to expand the content with new chapters, including those on transformation, cloning and qPCR. We are all engaged in research and education in our focus area, which includes biotransformations and interactions of microorganisms as well as their control. This requires experimental approaches and here this book will be of great help as it provides important background information. Not by describing cook book recipes but by providing a clear perspective how to implement the experimental approaches and actually do it in a safe and sustainable way.

We expect this practical book to be a living document and more editions are to be expected. This brings me to the role of you as the reader: please check the book,

provide feedback to Philippe and also think of new chapters that you can contribute yourself !

A last word on how to use this book: all of us have their own research projects but we are also collaborating in various ways, and that is the purpose of the Laboratory of Microbiology. One way is to collaborate in the laboratory. It is essential that you comply with the safety rules as well as respect each other – this requires a social attitude, and I would like you as the reader to follow Philippe's advice: leave the laboratory space in a cleaner state than when you started your experiment !

Good luck ! WILLEM

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2 Contributors

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3 First things first

Your **supervisor** is responsible for you. He/she should introduce you to the rules, the building, safety features and colleagues in the lab.

Before you start working in the lab, you have to make sure to complete the following steps, with help from your supervisor.

- Registration at the secretary.
- Participated in a general tour around the labs.
- Receive safety instructions.
- Introduce yourself to colleagues.

Upon arrival, you should receive a 'starter package' consisting of a lab journal, writing pad, memo bloc, pens, etc. You can ask for it at the secretary's office.

4 Ethics on the lab

Ethics is a science in itself. A very big subject on which many books are written. We will not attempt to write another book but try to summarize how ethics are applied within the lab of Microbiology and Systems and Synthetic Biology. So how do we 'apply' ethics on the lab? How to convert such un undefined subject into a daily practice?

The European Science Foundation (ESF) states "*that any university or institute who employ researches, has a duty to ensure a prevailing culture of research integrity*". To achieve this goal the ESF published a document called 'The European Code of Conduct for Research Integrity' (see references) in which they describe how proper and ethical science should be performed. You are expected to read this document as you sign the 'Letter of Integrity'.

Signing this letter means that you agree to work ethically. That you will contribute to our culture, which is 'to maintain a pleasant work environment'. And that you will respect the lab rules. In this chapter we will look at the Code of Conduct and explains the guidelines given to you.

4.1 Principles

The ESF gives guidelines in there Code of Conduct. They divide these guidelines into two parts, Principles and Practices. But what are those? Starting with Principles, according to the dictionary it is '*a fundamental assumption'*, '*a moral rule or aspect*' or '*a source or origin*'. Principles are fundamental assumptions and are based on moral rules generally accepted within the (life science) community. The Principles as formulated by the Code of Conduct:

• *Honesty* in presenting research goals and intentions, in precise and nuanced reporting on research methods and procedures, and in conveying valid interpretations and justifiable claims with respect to possible applications of research results.
• *Reliability* in performing research (meticulous, careful and attentive to detail), and in communication of the results (fair and full and unbiased reporting).
• *Objectivity* interpretations and conclusions must be founded on facts and data capable of proof and secondary review; there should be transparency in the collection, analysis and interpretation of data, and verifiability of the scientific reasoning.
• *Impartiality and independence* from commissioning or interested parties, from ideological or political pressure groups, and from economic or financial interests.

• *Open communication*, in discussing the work with other scientists, in contributing to public knowledge through publication of the findings, in honest communication to the general public. This openness presupposes a proper storage and availability of data, and accessibility for interested colleagues.
• *Duty of care* for participants in and the subjects of research, be they human beings, animals, the environment or cultural objects. Research on human subjects and animals should always rest on the principles of respect and duty of care.
• *Fairness,* in providing proper references and giving due credits to the work of others, in treating colleagues with integrity and honesty.
• *Responsibility for future science generations.* The education of young scientists and scholars requires binding standards for mentorship and supervision.

4.2 Practices

Now that the Code of Conduct has given us the fundamentals on which we as scientist should base our work on, we can now focus on the Practices. The dictionary says that Practices are '*the ongoing pursuit of a craft or profession*', '*actual operation or experiment*' or '*the form, manner, and order of conducting*'. Compared to Principles, Practices are less abstract: They specifically tell you what to do in order to perform

science along the Principles earlier described. Practices are based on Principles and are formulated in the Code of Conduct:

• *Data practices*, including data management and storage, placing data at the disposal of colleagues who want to replicate the findings, adequate preservation of original data.
• *Research procedures*. Deviations from desired practices include insufficient care for research subjects, insufficient respect to human subjects, animals, the environment, or cultural heritage; violation of protocols; failure to obtain informed consent; insufficient privacy protection; improper use of laboratory animals; or breach of trust (*e.g.* confidentiality). Improper research design, carelessness in experimentation and calculations that lead to gross errors, may also be classified under this heading, although the partition-wall between incompetence and dishonesty may be rather thin here.
• *Publication-related* conduct, including authorship practices. It is unacceptable to claim or grant undeserved authorship and to deny deserved authorship, or to inadequately allocate credit. Breaching of publishing rules, such as repeated publication, salami-slicing of publication, no or a too long delay in publication, or insufficient acknowledgement of

contributors or sponsors, fall within this category as well.

• *Reviewing* and *editorial* issues, including independence and conflict of interests, personal bias and rivalry, appropriation of ideas.

4.3 Culture

Culture in the dictionary: "*the arts, customs, and habits that characterize a particular society or nation*." and "*the beliefs, values, behaviour and material objects that constitute a people's way of life.*" So if we distil what culture is for our lab it would be the customs, habits, beliefs, values and behaviour that shape how we work. Our culture is summarised in the Letter of Integrity but what do we actually mean? What is our culture? It is very difficult to specify a concept like culture, but more or less we can describes our culture in the following way:

You are here to learn new skills and knowledge. Asking questions is good and helps you to improve. We help you and you will help others on the lab. Feedback is given in a direct manner, but should never be taken personally. We want you to work in a safe environment for yourself and your colleagues. Your experiment is not more important than that of others. Leave the laboratory space in a cleaner state than when you started your experiment.

You will agree on this by signing the Letter of Integrity which says "*I will contribute to maintain the Laboratory a pleasant work environment*".

4.4 Lab rules

Now you understand what the Principles and Practices are and how they contribute to our culture. But these are still too abstract for everyday use on the lab, so there is a gap. This gap is filled with lab rules.
Some rules are the same in every lab, like wearing your lab coat on the lab. Some are very specific for our lab, like not to touch the autoclaves in the kitchen. However, you will not find a list with all the lab rules in this chapter. The rules and guidelines are spread throughout this book because every specialisation and location has its own lab rules. The rules ensure that many employees and students can perform experiments in a safe environment in a lab with shared facilities.

Violations of the lab rules will have consequences, although each case varies. If you break an Erlenmeyer, a new one will be ordered. This can happen and no further action will be taken. In a serious case like breaking a machine, technicians will find out what happened. If it turns out that this is caused by negligence and/or not following the lab rules action will be taken.

In a serious case a meeting with professor and supervisor(s) will be organized to determine what punishment the perpetrator will get. Ultimately the thesis contract of a BSc- or MSc student will be terminated. PhD-students or other staff will get an official warning. Consequences of violations are quite serious; physically, financially, and your reputation among colleagues will be affected.

The Principles and Practices from our culture on the labs of MIB and SSB. Together with the specific lab rules of our laboratory, they give you guidance, enabling you to perform research in an ethical and safe way on a daily basis.

References:

- The European Code of Conduct for Research Integrity. Search via Google: 'esf code of conduct'
- Ethics and Science. An introduction'. WUR library 224-D-7/2012-006.

5 Lab rules

This is a summary of the lab rules, that you will receive when you arrive at the lab. If you did not receive these, ask your supervisor. If there is something you do not know, ask someone. There is no 'trial and error' concerning safety issues. Use common sense!

5.1 Safety

- Supervisors are responsible for their students!
- Students are also responsible for their own safety!
- Wear a lab coat at all times.
- Wear gloves only **in** the lab.
- No food, drinks, chewing gum, or smoking in the lab.
- No laptops, iPads or any other personal devices in the lab.
- Handle toxic and hazardous compounds in the fume hood.
- Only work with equipment after receiving instructions from your supervisor or technician.
- Some equipment is used by many. Please make a reservation in the agenda of the machine.
- Report defective equipment to the technicians.

- Used needles and scalpels are always disposed of in the special yellow containers.

In case of an emergency call **82345** between 8:00 - 17:00. Outside office hours 112, the general Dutch emergency number.

5.2 Stocks & storage

- Refill / replace what you use.
- Perform your lab-duty.
- Report depleted chemicals.
- Put dirty glassware in the dedicated boxes for washing.
- Chemicals should be returned after use.
- Only store things in places designated to you.

5.3 Building

- Opening hours between 7:30 – 23:00.

- Never work alone in the lab.
- When leaving, clean bench and switch off used equipment.
- Read the green cards spread around the lab, with instructions what to do in case of a fire or accident.
- For further details see ‘Regulations of the Microbiology Lab’, October 2008 on the N:/safety.

6 Safety

Working in a lab means that you are responsible for your own safety and that of your colleagues. That is why it is important to know and to understand the safety rules.

6.1 Behaviour

Nothing is more cumbersome than safety regulations. Slowing you down at daily lab work, increasing the amount of work, most rules seem unnecessary….
However, those rules are there for a reason. When applied, they reduce the risk you face working in the lab. Everyone will know a story about someone who (almost) had an accident in the lab. But of course, accidents will not happen to you! Problem is that all people who had these accidents thought exactly the same…. That is precisely the wrong way of thinking. It is not only about you, it is also about the safety of your colleagues. Your mistakes can have consequences for others; spilled dangerous chemicals, unattended Bunsen burners, pieces of broken glass that are not disposed of. The list is endless.
In a worst-case scenario the consequences can be quite serious; physically, financially, and your reputation can be affected.
If you encounter a dangerous situation, the easiest thing to do is to look away. However, what you should do is

talk about it with your colleague(s). This might be difficult if you, as a BSc-student, have to address this to a Post-Doc, but you should do it anyway.

6.2 Dress up

Wear your lab coat it protects you. Wear safety glasses if needed, you only have two eyes… Use the correct gloves (fig 1).

Latex gloves are for regular use; these are also the least expensive gloves. People with latex allergy can work with colourless vinyl gloves. Blue vinyl for Ethidium bromide / Sybr Safe work, and green is for working with corrosive chemicals.

No gloves in the corridor!

Figure 1 a) Latex, b) Vinyl colourless, c) Vinyl blue and d) Vinyl green

Make sure that gloves fit properly to prevent getting caught between things, or causing contamination in your reactions or cultures. See also 8.4

6.3 Chemicals

Know your chemicals and know their risks. Work with them in a correct way at the correct places. Use the correct gloves. Read MSDS's and know how to dispose them properly See also 6

6.4 Micro-organisms

Know how to work with micro-organisms. If you work with GMO's make sure you are on the list (contact the technicians). Autoclave before disposing waste that was exposed to micro-organisms.
If you work with micro-organisms, or you need to work sterilely, you can use the Flow-cabinet, also known as the Laminar Air Flow (LAF)-cabinet (fig 2). The Flow-cabinet creates a continuous airflow downwards preventing the spread of aerosol / micro-organisms. This also prevents micro-organisms going out, or into the cabinet. Use the burner only for sterilising, not 'to work sterile'. You always work sterile because of the laminar airflow, the burner actually disrupts the airflow and increases the chance of contamination. Please ask for instructions from your supervisors or the technicians when you start to work in the LAF-cabinet.
Using the burner on your lab bench, does create a sterile zone of up to 30cm from the flame.

Working in the ML-II lab. If you work with dangerous pathogens or GMO's you have to work in the ML-II

lab, located on the 1st floor. You have to be registered on a special list. Ask your supervisor for help. See also 5.3 /7

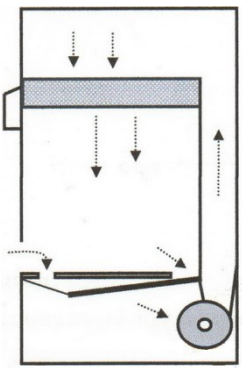

Figure 2 Airflow in a Flow- or LAF-cabinet

6.5 Labelling

If you make buffers, dilutions, media or whatever, label it correctly. Write down the name of the chemical / organism(s), your own name and the date it was made / inoculated.

6.6 Alarm

All over the Microbiology building you will find green plasticised cards hanging. These indicate what you have to do in case of an alarm and what to do if there has been an accident. Read these carefully.
In general, when the alarm sounds, turn off the Bunsen burners and leave the building as soon as possible to the designated areas (= in front of the Computechnion). Do not take (personal) stuff with you!
More info on N:\Safety.

6.7 Spillage

Clean what you spill. This can be some chemical you spilled during weighing, a bottle with hydrochloric acid that falls out of your hands or leaking bacterial cultures. In all cases, you have to take the correct action.
See also 8

7 Waste disposal

7.1 Regular waste

Regular waste is the waste that can be disposed of in the regular black bins found all over the building. This waste does not contain hazardous chemicals, micro-organisms, paper, glass or sharp items. It ends up in the big green waste container in the parking lot.

7.2 Chemicals

Not all chemicals end up in the hazardous waste container (fig 3b). Only toxic or carcinogenic chemicals will end up here. Know what is toxic!
See also 4.3 / 6

7.3 Micro-organisms

All micro-organisms should be autoclaved before being disposed of into the regular waste. Loose plastic containers (tubes, petridishes) are collected in special metal bins with heat resistant bags and can be found in the labs. Glassware with liquid culture is also autoclaved, emptied in the sink and put in the dish washers. See also 7

7.4 Samples

Samples taken from soil, gut or other sources should also be autoclaved and then disposed of. If you store samples, use minimal space and label correctly. This

will prevent that you lose items, moved by someone else who is looking for space.

7.5 Needles & Scalpels

Needles and scalpels are very dangerous and **always** need to be discarded in the sharps container at all times! It does not matter what you use them for or what kind of chemical / micro-organisms it has been in contact with. For these, we have special yellow sharps containers (fig 3a). Know how to disconnect the needle from the syringe using the sharps container.
Only put in the needles, not the whole syringe. Everything else should be deposed of seperately.

Figure 3 a) Sharps container b) hazardous waste container c) paper container

7.6 Disposables

Disposables are all plastics used in lab work, such as pipets, tubes, Eppendorf tubes, qPCR plates etc.
Plastics containing toxic chemicals should be emptied in the proper container (fig 3b).
Plastics which contain micro-organisms should be autoclaved, and then disposed of in the regular waste.

7.7 Glass

All broken glass should be disposed of in glass containers, each lab has its own. Glass contaminated with micro-organisms should be autoclaved, ask a technician for help.
Brown glass, used to store chemicals in, can be thrown into the regular green waste container in the parking lot. Most glass originating from the lab cannot be recycled due to the special mixtures used during production.

7.8 Paper

Paper and cardboard are collected separately and put in the blue paper containers (fig 3c). Fold the boxes to save space.

7.9 Styrofoam boxes

Styrofoam boxes can be thrown into the regular green waste container in the parking lot.

8 Chemicals

Working with chemicals always involves a certain risk. Knowing how to handle a specific chemical reduces this risk significantly.

8.1 MSDS

To know how to handle a substance, just Google / Wikipedia the name of the chemical you work with together with the term MSDS (example: "Sulphuric acid MSDS"). MSDS stands for Material Safety Data Sheet, and it contains all safety info concerning the substance and the precautions you should take. It is very easy, so please use it.

8.2 Hazard signs

In figure 4, you will find the new symbols, now used worldwide, indicating hazardous chemicals. Officially known as the: Globally Harmonised System (GHS) of Classification and Labelling of Chemicals. These symbols can be found on the container of any chemical. You can use these to assess the risks involved in working with these chemicals.
The signs are divided in Physical -, Health – and Environmental hazards. If the chemical has signs from the 'health' and 'environmental' category, you should

Figure 4 GHS symbols

work in a fume hood and dispose of as chemical waste in the hazardous waste container (fig 3b) and **not** in the sink! Liquid chemicals in large quantities should be collected in special containers. Ask the technicians whether you need one.

8.3 Finding Chemicals

If you want to find a chemical in the Microbiology department, open Internet Explorer and type "http://ssb1/Chemicals_Db/" → press enter → click "New! Find GROS chemicals". This opens the GROS chemical database (fig 5). The disadvantage is that all names are in Dutch, due to Dutch legislation. Best thing to do is to look up the chemical on Wikipedia and check the Dutch page, which will give you the correct name for GROS.

Do not enter the full name but only part of the name. This will increase the hits and overcome misspellings in the database. For example sodium chloride. Instead of "natrium chloride" enter "nat" in the first bar and "chlo" in the second bar, see (fig 5)

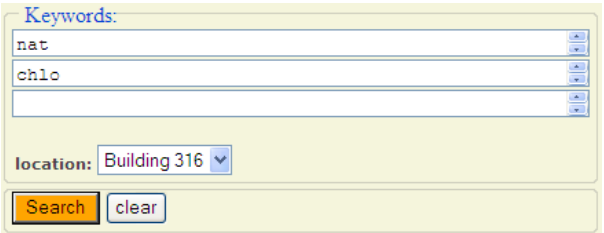

Figure 5 Screenshot GROS database

Do **not** press “Enter” to start the search command but click on the “Search” button. Pressing “Enter” will clear the keyword bars. If you did press “Enter”, refresh the internet page, using the F5 button.

Location	Kost_plaats	Pot_nr	Units		Qual	Gros_art. & code	Description
ATV.DD.316W.MIB.0044.VCC1	61_420	166048	1	kg		WUR 31434-1KG-R	NATRIUMCHLORIDE
ATV.DD.316W.MIB.1005.OS1	61_420	183307	1	kg		WUR 31434-1KG-R	NATRIUMCHLORIDE
ATV.DD.316W.MIB.1009.VCC1	61_420	144301	5000	g	GR	DATV 106404	NATRIUMCHLORIDE
ATV.DD.316W.MIB.1011.OS1	61_420	126015	25	ml		DATV 239305	NATRIUMHYPOCHLORIET
ATV.DD.316W.MIB.1011.OS1	61_420	128244	5	g		DATV D1878	2
ATV.DD.316W.MIB.1014.FVC1	61_420	137325	500	g		106565	NATRIUMPERCHLORAAT
ATV.DD.316W.MIB.1014.FVC1	61_420	126516	1000	g		DATV 22322	NATRIUMCHLORAAT

Figure 6 Screenshot results GROS database, with individual hits

The result page (fig 6) will give you several different hits containing the keywords that you entered. Now you have to know how to read each result. As an example, we take the second hit in figure 6 and analyse the hit. In the hit “ATV.DD.316W.MIB.1044.VCC1” you can analyse the following parts of the code.
ATV.DD.316W.MIB = microbiology building.

1044 = This is the number of the lab: 1 is the first floor, 044 is the room.
VCC1= Ventilated Chemical Cupboard, a special type of storage place for chemical. We have several types listed in the table 1.

Storage place	Codes
Acid / Alkaline Cupboard	AAC
Chemicals Box	CB
Chemicals Cupboard or Cabinet	CC
Desiccator	DES
Fume hood Cupboard	FC
Freezer	FR
Fireproof Ventilated Cupboard	FVC
Gascylinder Clamp	GC
Gas Fireproof Cupboard	GFC
Open Shelves	OS
Poison Cupboard	PC
Refrigerator	REF
Ultra Freezer	UFR
Ventilated Chemicals Cupboard	VCC

Table 1

More info is to be found on the GROS site. Chemicals are stored in alphabetical order.

8.4 Labelling

Label all your dilutions, buffers, etc. with your own name, name of chemical, the concentration and the date of preparation. Correct labelling will keep things orderly and will inform others what is in it. See also 4.5

8.5 Ordering chemicals

If you empty stock chemicals, send the following information (below) in an email to one of the technicians for re-ordering.

1) Chemical name
2) Order number
3) Amount (kg, litre, etc.)

Take the GROS sticker of the (almost) empty container and stick it on the A4 paper for GROS labels at the entrance of the lab.

If you need a chemical that is not in stock or something else, write down the correct full name (use Wikipedia) and contact one of the technicians.

8.6 Calculating with chemicals

In table 2 you will find the basics on calculating with chemicals. Furthermore, you will find some examples of calculating and preparation of some widely used chemicals.

Table 2 Basics of calculating with chemicals

Term	Symbol	Meaning	Example
Mole	mol	An amount containing Avoradro's number of whatever units are being considered. Avogadro's number = 6.023×10^{23}	1 mol H_2O = 6.0^{23} x 1023 H_2O molecules.
Molar or Molarity	mol/litre or M	Concentration of a substance in a liquid. Molarity = moles of substance / litre of solution	A 1 M (mol/litre) solution of Tris in H_2O is prepared by dissolving 1 mol Tris molecules in H_2O to a final volume of 1 litre.
Molar weight	g/mol	Weight of 1 mol (=6.023×10^{23} parts) of a molecule, as defined by the molecular weight of this molecule.	Molecular weight of Tris: 121.1 g/mol. An 1M (mol/litre) solution of Tris in H_2O is prepared by dissolving 121.1 gr Tris in H_2O) to a final volume of 1 litre.

Example: How many grams of NaOH are needed to make up a 100 ml solution of 5 M NaOH
Formula: Needed grams = Concentration x Molecular weight x Final volume
= 5 mol/litre x 40 g/mol x 0.1 litre
= 20 gram

0.5 M Na_2EDTA, 1 litre (pH 8.0)

- Dissolve 186.12 gr disodium ethylenediaminetetraacetate – 2 H_2O in 800ml H_2O; stir vigorously on a magnetic stirrer.
- Adjust to pH 8.0 with NaOH pellets (≈ 20 gr) and adjust volume to 1 litre.
- Note: EDTA will only dissolve when the pH reaches 8.0.

10% SDS, 1 litre (pH 7.2)

- Dissolve 100 gr sodium dodecyl sulphate crystals (SDS) in 900 ml H_2O.
- Heat to 70ºC to dissolve the crystals.
- Adjust pH to 7.2 with HCl (≈50 µl)
- Adjust volume to 1 litre with H_2O.
- When SDS precipitates, heat to 37ºC to dissolve again.

1M Tris. 1 litre (optional pH)

- Dissolve 121.14 gr tris (hydroxymethyl) aminomethane (Tris, MW = 121.14) in 800 ml H_2O.
- Adjust pH by adding concentrated HCl:
  - pH 7.4 ≈ 70 ml
  - pH 7.6 ≈ 60 ml
  - pH 8.0 ≈ 42 ml
- Adjust volume to 1 litre.

8.7 Making chemical solutions

When making solutions take into account the following tips.

Look up all chemicals in the database and check the MSDS's of chemicals that are unknown or new to you. Then collect the chemicals in a plastic box to keep everything together.
Check the content of each jar: Do you have enough?
Are your magnets and spatulas clean?
Only start weighing when you have everything, otherwise you will notice halfway that one of the chemicals is out of stock and you can throw away what you made so far.
Weigh small amounts on special weighing paper, larger amounts in the plastic weighing cups and very large amounts directly in the Erlenmeyer or beaker glass.

Clean balances and spatulas every time you weigh a new chemical. The most accurate balances are sensitive to airflows, so you might want to close doors and prevent people from walking by when you start weighing.
Sometimes, chemicals dissolve better in warm water (**only water! no other liquids**). Take the demi-water (**without any chemicals!**), heat it up in the microwave to about 50°- 60°C, and use this to dissolve the

chemicals. Alternatively, use the heater of the magnetic stirrer.

Adjust the pH with the bottles of 10M NaOH and 37% HCl, to be found near the pH meter in the fumehood. Use glass Pasteur pipets to add the solutions. After setting the pH, adjust solution to the desired volume.

Autoclave:
You might want to autoclave your solutions. To autoclave your stuff, label the continer correctly with the chemical/microbe name, date, and your own name. Bring it to the kitchen and put there on the table. Within 24 hours it is autoclaved. You can find the sterilized materials on the table in the corridor, across the kitchen. REMINDER, the table in the corridor is not a place to store your materials, come and pick it up. Be aware that not all chemicals are stable at high temperatures. These solutions must be filter sterilized.

Store your solution in a bottle, and label. After making and labelling**, clean up** the workspace with balances and stirrer. Put back the chemicals in the right place, in alphabetical order. See also 6

8.8 Chemical spillage

There are different kinds of spillage. This depends on the amount and the concentration of the chemical.

Small spills you can clean up easily yourself, or with the help from colleagues.
Big spills with high concentrations are much more dangerous as they can affect everyone in the lab or even in the building. For example, in the case of dropped bottle of 37% Hydrochloric acid, everyone in the lab should leave immediately, doors to the lab closed and the emergency number **82345** should be called. Also, people working in adjacent rooms or rooms directly under the lab of the accident should be warned. Specially trained employees will then clean the spillage. Go to the (Albron)canteen as data about the substance and other information should be given to these "special cleaners". Furthermore, a headcount will take place to see if all people came out of the lab. See also 4.6

Figure 7 "Chemical spillage"

8.9 Storage

Store your stuff, fully labelled in the designated areas such as your personal box or the shelf above your bench. Label everything you have. See also 3.2 / 4.5

Factor	Prefix	Symbol
10^{18}	exa	E
10^{15}	peta	P
10^{12}	tera	T
10^{9}	giga	G
10^{6}	mega	M
10^{3}	kilo	k
10^{-3}	mili	m
10^{-6}	micro	µ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{-18}	atto	a

Table 3 Metric prefixes

9 Micro-organisms

9.1 Finding micro-organisms

Micro-organisms cannot be found in the GROS database, like chemicals. Usually you will get a strain from your supervisor or colleague. When your micro-organism is not available in the lab you can order one at one of the culture collections. Most micro-organisms at our lab come from the 'German Collection of Micro-organisms and Cell Cultures' also known as DSMZ. Located in Germany and can be contacted via www.DSMZ.com. Besides cells, genomic DNA is available. An alternative is the 'American type Culture Collection', the ATCC. However, these are more expensive than DSMZ. Contact a technician if you want to order. If you are working with micro-organisms you can do this in in ML-I labs. All labs in the Microbiology department are classified as **ML-I labs**.

9.2 Pathogens & GMO's

Working with Genetically Modified Organisms requires (by Dutch law) that you are registered. This should be arranged **before** you start. Ask your supervisor to do this for you. Furthermore, you are only allowed to work with GMO's in the **ML-II lab**, room 1.035. Pathogenic micro-organisms require careful handling, as they can be infectious. The chance of you being actually infected is unlikely. As long as you apply safe microbial

techniques like washing your hands, sterile work and other measures to prevent spreading of micro-organisms. If you don't apply the proper techniques you might spread bacteria outside the lab and infect people at home!

9.3 Finding media

Media stocks are not taken up in the GROS database. They are scattered over the labs. Ask a colleague or supervisor where to find these.

9.4 Choosing media

Before you start preparing, it is wise to collect all ingredients before you start. They can be spread over many locations. Not all media, especially the more exotic ones, are present in our lab. You might need to order it. An alternative is to ask the staff of Microbiology what you can use as an alternative, from the ingredients that are present.

9.5 Preparing media

Media consist of two types of ingredients: chemicals and a group which consists of mixtures of sugars, proteins, etc… Chemicals can be harmful, the other group is usually not that harmful. Check always the label on the jar.

When making media, weigh the ingredients, dissolve, bring to the boil, then autoclave. If you have solid

media, melt it using a hot water bath or using the microwave. When using a microwave, mix / shake with intervals during heating. Be careful with boiling liquids in flasks or bottles. If you don't shake during the heating process the liquid will boil, leaking out of the bottle. You will have to clean the microwave afterwards...

When pouring media, always flame the opening of the bottle. When adding antibiotics, allow the agar to cool down to 50ºC to prevent antibiotic becoming inactive. Put the flasks in a water bath to cool down to the correct temperature.

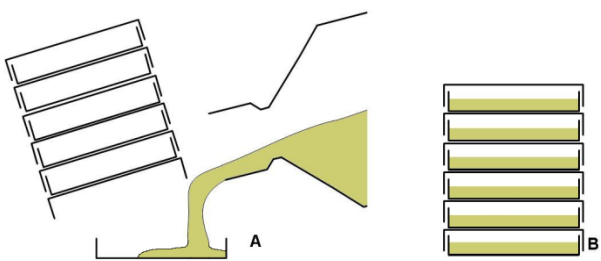

Figure 8 a) pouring plates b) poured plates cooling

If you pour media/agar into plates, you don't have to leave them open to dry. This takes a lot of space in the Flow-cabinet. A good alternative is to stack the empty petri-dishes in stacks of five, to eight (or a number you can handle). Lift the stack of plates of the bottom one

and start pouring the agar (fig 8a), work your way up to the top.
If you incubate or store large stacks of plates, put them back in the empty plastic bag of the petri-dishes. This will (partly) prevent drying of the plates and condensation formation.
Before use, take plates out of the fridge and put them in the stove at 37ºC for 1 hour. This will remove any condensation.

9.6 Anaerobic culturing

Many micro-organisms studied at the department are anaerobic. This requires special methods in preparing media and inoculating. This is not as straightforward as regular culturing and specialized training is required. When you have to work with anaerobic organisms, your supervisor or the technicians of MicFys can instruct you.

10 Cleaning

10.1 Lab-duty

Everyone has to do his or her lab-duty. Your own work is **not** more important than other people's work. So just do it. Plan it in your schedule, take your time, and don't do it hastily.
The schedule can be found on the door of the lab.

10.2 Spillage

In case of a spillage, you have to decide which action you have to undertake. This depends on the chemical or bacteria, the amount and the concentration. A droplet 0.1 M NaOH can be wiped off with a tissue, a droplet of bacterial culture you can wipe off using a tissue with 70% ethanol. Small spills you can be handle yourself.
Big spills can be more serious. A broken bottle of hydrochloric acid means that the lab has to be evacuated. A broken Erlenmeyer with a GMO, inside an incubator, means that the whole incubator has to be cleaned and decontaminated.
Big spills are something you **cannot** do by yourself as you lack the knowledge and equipment to perform these actions in the right way. Notify your colleagues and / or the technicians to clean it up properly. This is one of those things, which you should **not** attempt yourself.
See also 4.7 / 5.2 / 6.8

10.3 Equipment

Equipment (centrifuges, incubators, etc) has to be cleaned with warm water and soap. Ethanol is not a cleaning agent for equipment! Manuals of equipment actually advise to clean their equipment with water and soap, because it is not as aggressive as other chemicals such as bleach, ethanol, acetone or others. These chemicals can affect plastics, rubbers or the metal of the equipment, especially UV cabinets!

To clean the equipment, put some soap (fig 9c) in warm water. Use a yellow cloth and / or sponge to gently clean the equipment. Squeeze out all excess water to prevent water leaking in the machine. Clean inside and outside of the machine. Also clean the surfaces of the bench were the equipment is located. Dry with paper towel, if necessary.

Figure 9 a) ethanol, b) bleach, c) soap

10.4 Lab coat

Do not wear the same lab coat continuously for months. Regularly, you will receive a mail in which is announced that you can hand in your lab coat. In the basement, you can put dirty lab coats in a grey/green container. You can find clean ones in room K.026 in the basement. See also 4.2

10.5 The effect of Ethanol, Bleach & UV-light

Ethanol is used to decontaminate tools (pipettes, etc) and surfaces from micro-organisms. Bacteria have a cell membrane containing a phospholipid bilayer (fig 10). Ethanol is an organic solvent. This means that it can dissolve the phospholipid bilayer. When bacteria come into contact with ethanol, the cell membrane becomes affected, leading to the rupture of the cell and killing the bacteria instantly.

Dilute 96% ethanol to ≈70%. Measure in a measuring cylinder 700 ml 96% ethanol. Add 300 ml demineralized water, mix and divide in ethanol several wash bottles (fig 9a). Water increases the effectiveness of the ethanol. Thus 96% is not better than 70% ethanol.

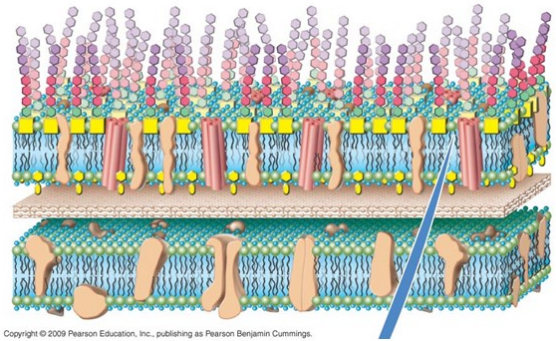

Figure 10 Cell membrane of Gram positive bacteria. Blue arrow points at the phospholipid bilayer.

Bleach is very effective for the decontamination of DNA. The damage caused by bleach is not fully understood but it leads to depurination (A and G break loose) and to general destabilization of the structural backbone of the double helix. Literature describes that use of 10% bleach is enough to damage DNA.
Bleach has to be made freshly each day, it is light sensitive. Make a 10% dilution from the bottle (fig 9b). Wet some paper towel. Clean surfaces and pipets before and after working with DNA.

UV light is very effective for the decontamination of DNA, as it causes significant damage to the DNA. The UV light induces a covalent bond between two pairing thyamines (tt) in a sequence. The DNA polymerase is not able to pass this barrier. Therefore, no unwanted product will be formed (fig 11).
See also 10.1- 10.3

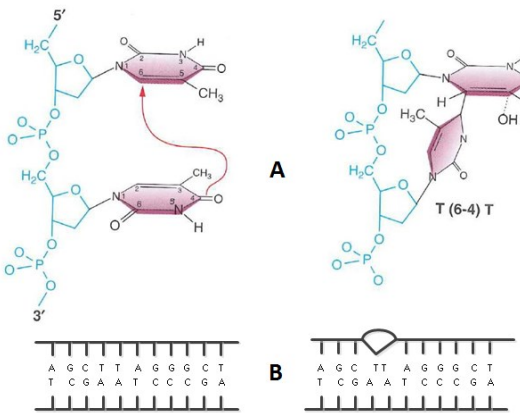

Figure 11 a) Covalent bond formed by UV-light, b) schematic overview.

11 DNA / RNA isolation

Isolating DNA is not that difficult, as long as you know which protocol to choose. Many options are available to you and discussing them all goes beyond the scope of this book. We will therefore focus on some general issues and tips that apply to all DNA isolation protocols.

DNA and RNA are more or less the same. But RNA is difficult to obtain and degrades quickly. This makes RNA isolation quite specialized and the advice is to learn these procedures from experienced people in the lab. However, knowledge of DNA isolation is also applicable in RNA isolation.

11.1 Choosing the protocol

For DNA isolation there are many options available to you. You just have to pick the right one. Start by asking your lab colleagues, or people from other labs, what they use. If your application is quite different, start searching in literature. Choose 2 or 3 protocols, on the basis of what is available in your own lab, experience of colleagues, and if you can perform the techniques described. Try to find out what a protocol can and cannot do. What are the limitations? Then try a couple of samples and determine which protocol gives the highest yield.

11.2 Kits

You might think that everything in the lab can be done with a kit... Well, yes and no. Indeed, there are many kits available on the market, but they are not made especially for you. Developing a kit takes a lot of time and money for the manufactures'. That is why they try to serve as many applications as possible. The result of this is that not every kit is suitable, and a selection has to be made from those available.
Also here, choose on the basis of what is available in your own lab and experience of colleagues. Once in a while, you might want to try a new kit, because it is mentioned more and more in literature.
Sometimes, additional steps are necessary to improve the yield, like lysozyme steps to 'break open' Gram positive bacteria. These can be described in the booklets of the kit, or you can try them yourself.

Whatever you do, read the manuals included in the kit. They provide lots of information, FAQ's and troubleshooting sections. If your question is not answered by the manual, contact the manufacturer of the kit.

Figure 12

11.3 General tips

Mix well to be sure that the fluids of your isolate come into contact with each other. Do not mix for 1 second with the vortex, but mix for 3 second, invert your tube(s) twice and mix for another 3 seconds, should this be described in the protocol. Dissolve pellets properly by pipetting, Vortexing, or leave pellets to soak overnight for a more gentle approach.
When eluting the DNA from a column it can help to run the eluent twice over the column to obtain more DNA (fig 15).

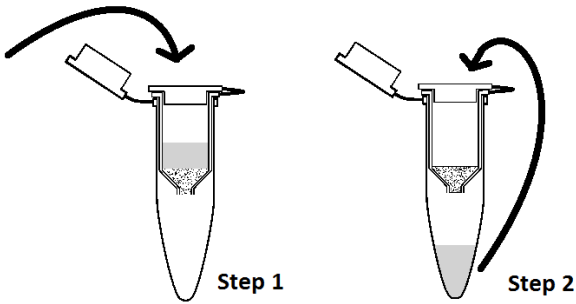

Figure 13 Step 1, eluent from the bottle. Step 2, re-use of the eluent.

11.4 Centrifuges

Centrifuges come in two types. Small, these fit on a lab bench. Big ones, to be found in the basement.

The speed of all centrifuges is expressed in two measures: Rotations Per Minute (RPM) and Relative Centrifugal Force (RCF). You might also encounter G-force, but that depends on the size of the rotor and the speed, which are different for every type of centrifuge. To overcome this, the RCF value was introduced. If you run the same RCF value on two different centrifuges, the sample will experience the same force.

$$RCF = 1.12xR(\frac{rpm}{1000})^2 \quad rpm = 1000\sqrt{\frac{RCF}{1.12xR}}$$

where R = radius of rotor in millimetre.

Big centrifuges. If you want to work with the big centrifuges you have to be instructed by experienced users. Ask technicians for help.
Big centrifuges have to be loaded symmetrically **(always!)** which means you have to divide the load over two bottles, in such a way that the weight of container and sample together are equal. If your rotor of interest is missing, look in the 4ºC room, where people might store them. This keeps the samples cool while running.

Small centrifuges. The small centrifuges on the bench also need to be loaded symmetrically **(always!)** You do not need to be accurate on the gram, but if there is a tube one side, another one should be on the opposite side.

Clean centrifuges with water and soap when they are dirty. Use ethanol only if bacteria are spread in the centrifuges. See also 8.3

11.5 Quality control

You can use several methods to check if your isolate is pure or clean. Each has its own (dis)advantages.

Gel-electrophoresis. Can be used to check if you obtained the right product, and if there are any smears or unwanted products. It's performed after every PCR.
You can retrieve the sample if you want, several kits and protocols can be used. Ask colleagues what their experience is and what they advise you to do.

NanoDrop. The nanodrop is a spectrophotometer in which you can check how much DNA you obtained. It indicates what is in the sample but it is not perfect. The machine gives you an indication, but not more than that!
The Nanodrop needs 1,5 µl of sample to determine the content. The effective range of the Nanodrop is depicted in the table below.

	Min range (ng/µl)	Max range (ng/µl)
ss DNA	2	2400
ds DNA	2	3700
RNA	2	3000

Table 8 Minimum and maximum ranges for the Nanodrop.

If values come below 2 ng/µl, they should be considered as DNA-free samples. When negative values are given, repeat the blanking and measuring procedure for that sample.

A260/A280 ratio. The 260/280 ratio is a measurement to see how clean your sample is. The 260 and 280 indicate the wavelength at which the Nanodrop measures the content of your sample. At 260nm DNA is detected, at 280nm proteins are detected. The more proteins you have, the less clean your sample is. Ratios for DNA of ≈1.8 and of RNA ≈2.0, are considered as clean.

11.6 Storage conditions

DNA. DNA is much easier to work with as it is very stable. DNA / PCR products can be stored overnight at 4ºC, for longer storage the -20ºC is recommended. Storing at -80ºC for DNA samples is unnecessary and takes precious space in the -80ºC freezer, so don't!

RNA. RNA degrades much more rapidly than DNA. RNA should therefore be handled more delicately then DNA.
When working with RNA, always keep it cool, and for the least amount of time possible on your bench. If you are finished with the RNA sample, put it in the -80ºC freezer. RNA should always be stored at -80ºC to

minimize degradation. Before storing in the -80ºC, ask the technicians where you can put your samples. RNA (and to some extent DNA) is sensitive for repeated freezing and thawing cycles, so this should be avoided. RNases are a problem as well. They are present all over the lab and humans are also a source for RNases. Great care should be taken not to contaminate these samples.

11.7 Storage Buffers

After isolation, the DNA / RNA has to be stored in a buffer. Most kits provide a special elution buffer in which your DNA / RNA can be stored. If this is not provided there are several choices.

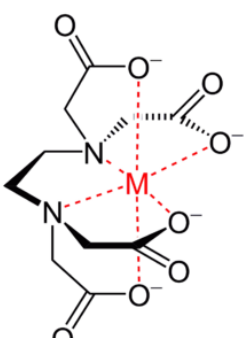

Figure 14 Structure of EDTA. Ethylenediaminetetraacetic acid is able to 'catch' metal ions (shown as the red M) from a solution. Metal ions are crucial for most enzymes to work properly.

DNA. DNA stored in water for longer periods of time, tends to get acidic, which may lead to auto-degradation. TE-buffer prevents the acidification. However, TE-buffer contains EDTA which can 'catch away' the magnesium ions in the PCR, causing inhibition (fig 16). This is not a problem when the DNA is stored in high concentration as these have to be diluted anyway. If concentrations are low, PCR water can be also used to store DNA for shorter periods of time. But in general both will work fine.

RNA. Same issues as with DNA. Literature also advises to use commercial storage buffers that are guaranteed RNase free.

12 Primer design

In the following chapter, we will look at the design of primers. This is an important step for different techniques such as (q)PCR, DGGE and sequencing. Good primer design is necessary to obtain accurate experimental results. Primers can be designed by hand, or professional software. In this chapter we will guide you through the procedure.

12.1 Guidelines for primer design

Make primers according to the following criteria for optimal result. The average primer length is between 18-30 nucleotides. The sequence should not allow for hairpin formation (fig 17).

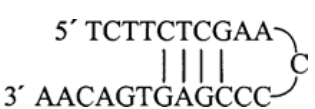

Figure 15 Primer 'hairpin'

Primer-primer hybridisation should also be avoided. These primers easily bind to each other instead of to the target. The GC-content should be around 50%. This allows the annealing temperature to be well below 72ºC, the elongation temperature. The annealing temperature for both primers should also be within 5ºC of one another. Avoid primers ending with a C or G.

Wobble primers are primers that contain variable nucleotides. These can be necessary when targeting a gene in a wide range of bacteria. Instead of the regular A,T,C or, G, other letters are used to specify what you need (table 4). During production of these primers, instead of one nucleotide a couple of nucleotides are added to the primers during fabrication, depending on the chosen wobble. For example: if the wobble is Y, a Cytosine and a Thymidine are introduced. There are now two different primers, one with C and one T on the place of the wobble. You should also this into account when calculating primer concentrations.

IMPORTANT! Ordering of your primers should be done via the technicians.

Code	Represents	Complement
A	Adenosine	T
G	Guanine	C
C	Cytosine	G
T	Thymidine	A
Y	Pyrimidine (C&T)	R
R	Purine (A&G)	Y
W	weak (A&T)	W
S	strong (G&C)	S
K	keto (T&G)	M
M	amino (C&A)	K
D	not C	H
V	not T	B
H	not G	D
B	not A	V
X/N	unknown	X/N

Table 4 Codes for the different nucleotides which can be used in wobble primers.

12.2 Primer design with Clone Manger

Clone Manager is a software program that is used in the lab of Microbiology for many applications such as aligning, cloning, and primer design. It has to be installed on your computer because of licensing issues. Ask Wim Roelofsen to do this for you. When it is installed you can follow the instructions below:

First, import your sequence with Ctrl+C and File → Import from clipboard, Clone manger does not require FASTA format. There are two main options for primer design within the Clone Manager software. The first option is to use the 'Design…' option that is found in the submenu 'Primer'.

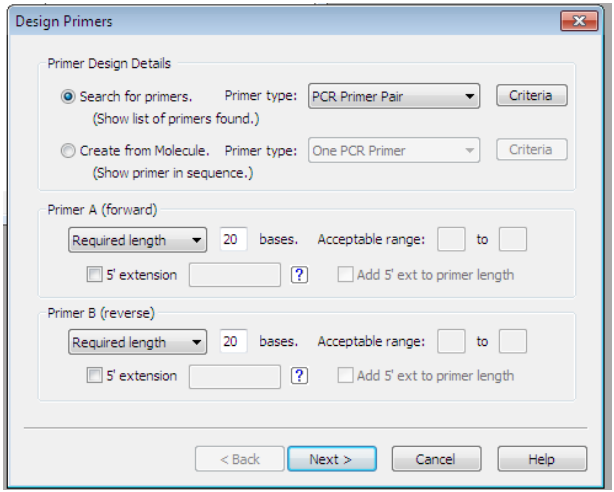

Figure 16

In this window (fig 18), select whether you want to create a primer pair or a single primer or probe. Select the required primer length (important for specificity). On the 5' extension sequence of your primers you can give additional input (for example an additional restriction site). The criteria used for the desirred primer are found under the button 'Criteria'.

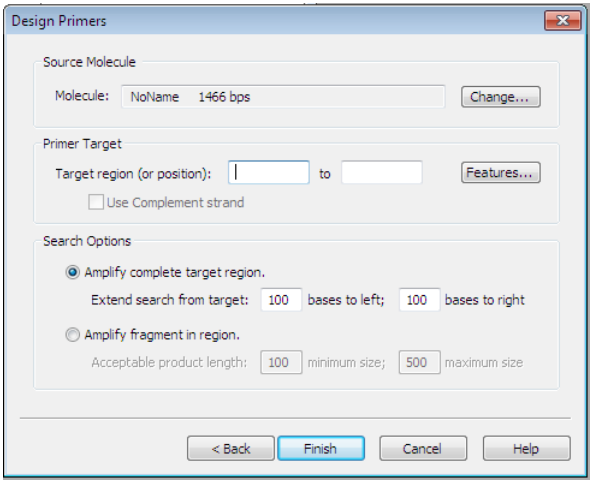

Figure 17

In the next window (fig 19), select your preferences regarding the size and location of your PCR product. When you click 'Finish' the program will produce a list of primers that meet the given criteria, ordered by best match. If you select one of the primer sets (clicking 'Primer report') it produces a screen summarizing the selected primer set (use the tabs in the bottom left). Furthermore, you can click 'Enter to primer list' that allows you to save the primer in your personal database. Remember to check one of the boxes; "add to collection file" or "add to disk file" (fig 20, see arrows).

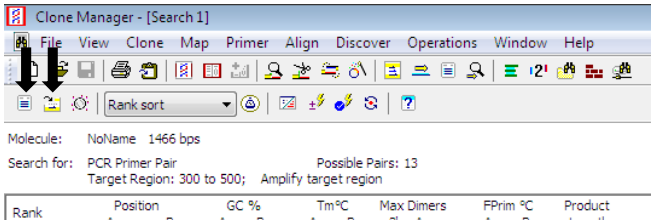

Figure 18

12.3 Manual primer design

Another and maybe better option is to manually design your primers. The advantages are that you have direct influence on the primer design criteria, length and location of the primer. Especially this last feature can be very useful wen designing Reverse Transriptase-qPCR primers or primers for a cloning project.

Afther loading your sequence (molecule file) into the program you can select the sequence location (fig 21, see arrow) were you like to design your primer. Choose one of the options under the lightning button (Primer, Complement Primer).

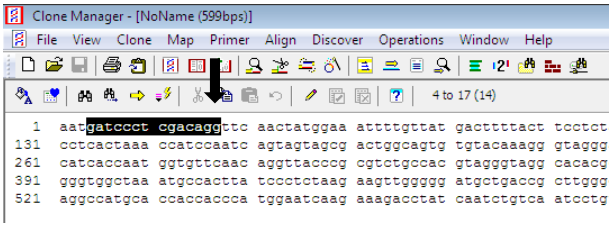

Figure 19

You now arrive at a window that depicts a part of your template sequence with the hypothetical primer attached. In the bottom (fig 22) left you can see if this current primer meets the standard preferences for primer design. Examples are; Tm, GC percentage, GC clamp and more.

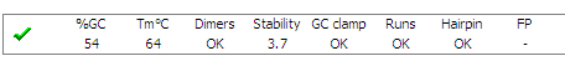

Figure 20

To view or edit these preferences (for example a more narrow Tm range for a qPCR primer pair) click the button with the blue dot with the √ (fig 23, see arrow). Furthermore you can 'walk' your primer over the template sequence with the blue arrows and shorten or extend the primer with + and - buttons. The specificity of your design in progress can be directly validated with the option Primer-Blast primer (NCBI).

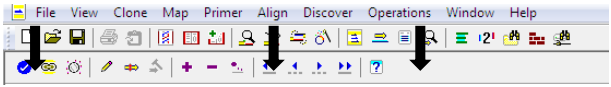

Figure 21

When the primer design is finished you can save it to your primer data base with File → Save. Furthermore you can check the quality of your primer set as a pair, by selecting Primer → Analyze → Primer pair. Here you find out about primer dimers, homology, hairpins and other important primer flaws.
In addition to these two options there are also 'specialized' tools for e.g; RT-primer design, that can be found under the submenu 'Primer'. But these are nothing more than the regular design tool with the additional option to input intron-exon boundaries.

13 PCR

The Polymerase Chain Reaction (PCR) is probably the most commonly applied technique in the lab. Being wide-spread in the lab, most people take PCR for granted. However, it is still important to understand the basics, especially when things go wrong.

13.1 Preventing contamination

The most common and time consuming problem to anyone performing a PCR is having a contamination. The best method is to prevent contamination in the first place! There are several stages in the PCR process in which you can take the correct measures to prevent contamination.

Storage: Keep all your reagents in a separate box and be the sole user of your stuff. Other people should not be allowed to use your reagents. Do not keep target DNA in the same freezer as your PCR reagents. If possible, aliquot your H2O, primer and probes.

Decontaminate: PCR plates or / and tubes can be decontaminated by UV light. This can be done in the UV-hood, or in the Stratalinker, which is much more powerful. Put the Eppendorf tubes straight up to allow maximum light intensity inside the tube. If you use the Stratalinker, use an old plastic box, for example from

the filter tips, for transportation. Use 10% bleach to decontaminate your workspace and pipettes. See also 8.5

Workspace: Separate your work into four workspaces to reduce contamination.
1) where you isolate your DNA = your bench in the lab.
2) where you prepare your master mixes = the UV hood
3) where the PCR machines are = the PCR room
4) where you analyse your DNA= Gel-electrophoreses lab.

Work Method: Keep all tubes with your reagents closed, as they are clean on the inside. Then arrange everything outside the UV cabinet first (paper, notes etc.) Then put on proper fitting, clean gloves and start preparing your master-mix.
Never shake your reagents, as possible contamination from the top of your tube might spread through the whole tube.
Open an Eppendorf tube in controlled way, avoiding aerosols. Don't put screw cap lids on the table, keep them in your hands.
Use filter tips for DNA work. Always insert a new tip into your reagent tubes, never enter twice with a used tip, even for the same reagent.
Always use a negative control in every PCR that you do, to check for contamination. No exceptions!

13.2 Working in the UV–cabinet

There are some rules for working in a UV cabinet:
- **No target DNA** is allowed in the UV cabinet**.**
- Leave dedicated pipettes, racks, etc in the UV-cabinet.
- Clean with 10% bleach.

No DNA is allowed in the UV-cabinet! (except primers). Template DNA is added outside the UV-cabinet, this prevents contamination originating from other experiments.
Leave everything, that is dedicated, in the UV-cabinet. Only your plastics and reagents are allowed in. Other stuff is **not**.
Cleaning with ethanol is **useless**. Decontamination of tools is done with 10% bleach. Decontamination of plastics is done by UV light. General cleaning of the UV-cabinet is done with water and soap. See also 8.5

Follow the rules! Your work is not more important than that of others!

If you not follow these rules, the technicians are authorised to stop you from doing your experiment, and exclude you from using the UV-cabinet.

13.3 Handling contamination

Every PCR goes well until your negative control is positive. Other than a ruined experiment, you have to

spend some time solving the problem. Follow the flow sheet (fig 24) to overcome the contamination.
This flow sheet gives you the quickest way of localising the contamination in your PCR. You can do the tests quite rapidly, usually in 2 days. Of course, you can throw everything away and start all over again, but it is expensive and primers have to be ordered, made and delivered to you. Moreover, maybe the contamination is not in your reagents but in the way you work...

Know how clean your workspace, including your tools (pipettes, tube blocks). Practice ‘clean’ working and apply the UV protocol. Use filter tips, sterilized tubes etc.

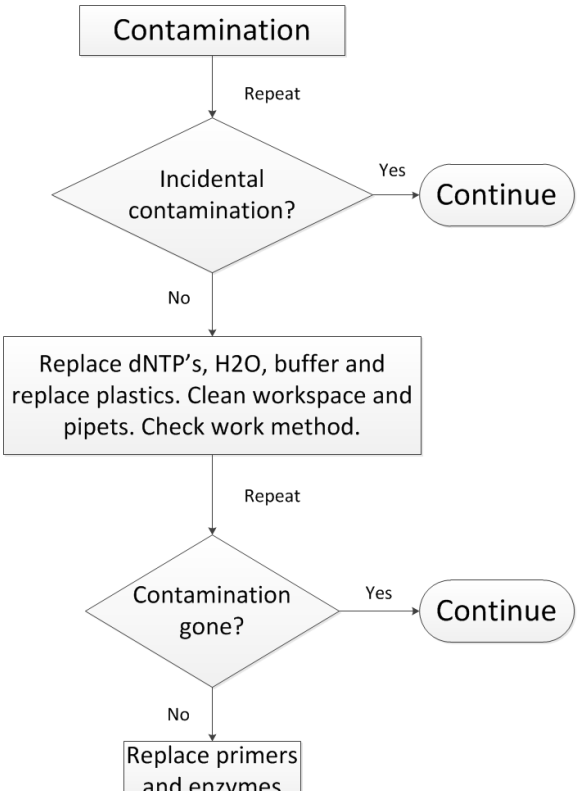

Figure 1 Flowsheet contamination

13.4 Setting up a PCR

Setting up a PCR starts with choosing your target. As this depends on the particular project you work on, you will have to discuss this with your supervisor. Add 1 µl of target DNA to your master-mix with a concentration of 20 ng/µl. This may be changed according to your own experience.

Primer design: Has been discussed. See also 11

Master-mix: A great deal of literature describes how to create the optimal master-mix combinations. But in fact, these days most scientists use off-the-shelf mixes that are optimized already by the manufacturers, including the dNTP's mixes. Most used in this lab are Go-Taq (Hotstart) from Promega, FastTaq from Roche and PhusionII & Phire II from Finnzymes. Only water, dNTP's and primers need to be added to prepare your master-mix.

A general composition of a PCR mixture for 50µl:

	concentration	max. range
Buffer	5x/10x	
dNTP's	10 – 100 µM	
primers	0.1 – 0.5 µM	0.05 – 1.0 µM
enzyme	1 U	0.25 – 2.5 U
H_2O	-	
DNA	20 ng/ul	1 copy to 500 ng/ul

Table 5 Ranges for setting up a PCR

Template. The amount of template strongly influences the PCR. The recommended amount of template for a standard is:

- max 500 ng of human DNA
- 1 – 10 ng of bacterial DNA
- 0.1 – 500 ng of plasmid DNA

Cycler Program. A standard protocol for a normal PCR consists of the following steps:

	Temp	*Time*	*Cycles*
Hot-start	95ºC	2-10 minutes	1
Denaturation	95ºC	30-60 seconds	
Annealing	40-70ºC	30-60 seconds	
Elongation	70-75ºC	60-120 seconds	30-40
Extension	70-75ºC	5-15 minutes	1

Table 6 Ranges for setting up a cycler program

The mentioned protocol needs some fine-tuning, but you can use it as a start. Most of the time you will use or adapt a protocol described in literature.
The melting temperature of DNA is usually 95ºC. The annealing temperature depends on the sequence of the primers. This can be calculated with free software available on the internet. The elongation temperature is usually 72ºC, although this is enzyme dependent. The extension temperature is always the same as the elongation temperature. Every enzyme manufacturer will recommend different protocols, so check their manuals. Programming of the cyclers is simple. Ask someone in the lab to explain to you how to do this.

Next to your samples, you must **always include a negative control** to prevent false-positive results. This will allow you to check if you have contamination in your master-mix. If so, you will have to redo the PCR and check where the contamination is coming from.

A positive control is advised, especially when you start with setting up a PCR, or when you work with 'difficult samples'. Positive controls will prevent you from having false-negative results and making sure that the PCR worked.

13.5 Gel-electrophoresis

Important! *Working at the gel-electrophoreses lab requires knowledge of the safety rules. Learn these before you start!*

Casting the gel. For analysing the PCR you will need to perform a gel-electrophoresis. For this, you need to make a gel slab of agarose. A standard gel is made of 1% agarose. For this, add 1gr agarose in 100ml 0.5x TAE buffer in Erlenmeyer. Boil in the microwave until the agarose is dissolved. Be careful with boiling, otherwise the whole microwave can be cleaned afterwards….
Meanwhile, prepare the casting tray (fig 25) wearing blue gloves. Set the combinations of combs in the way you like, depending on the amount of samples you have. When the agarose has dissolved, take it out of the microwave and cool down, holding the Erlenmeyer under running tap-water, to about 60ºC.
Then add SYBR safe to the agarose solution: 1µl SYBR safe per 10ml agarose solution is sufficient. Mix well, pour the solution into the tray, and leave it to solidify for 10-15 minutes.
After cooling, remove the combs, lift the tray with the gel from the casting and place the gel in the reservoir with 0.5xTAE buffer (fig 25).

Loading the gel. Cut a small piece of parafilm and make little dots of 1 µl of 6x loading buffer. Take 5 µl of PCR product and mix this with the dot of 1 µl loading buffer. After mixing, put 5 µl of the mix in one of the slots. Complete this for all your samples. Leave one slot open, in every row, for the marker. Add the markers at the end, 5 µl per lane.

Running the gel. Close the reservoir with the lid. A standard gel can be run at 100 volt for 10-40 minutes, depending on the size of the expected products. Remember, DNA is negatively charged, so it runs to the positive pole.

Scanning the gel. The following step can only be done if you know the correct protocol (ask a technician if you don't). Use your marker as a reference to estimate the size of your PCR product (fig 26).

Adjusting the protocol. Standard gels are made of 1% agarose, but this can be adjusted. 0,5% can be used for separation of large fragments >5 kb, 2% for very small fragments < 300bp. Gels can also be made smaller, around 50 ml when only few samples are analysed.
Adjusting the run of the gel can be done with the voltage and time. Larger fragments separate better using lower voltages ≈ 50 volt, smaller fragments with higher voltages ≈ 150+ volt.

Figure 2 Gel-electrophoresis equipment

If your PCR products have not separated as much as you wanted, just put the gel back into the reservoir and let it run on the same setting, for a longer period.

On the next page you will find the most commonly use DNA markers from Fermentas.

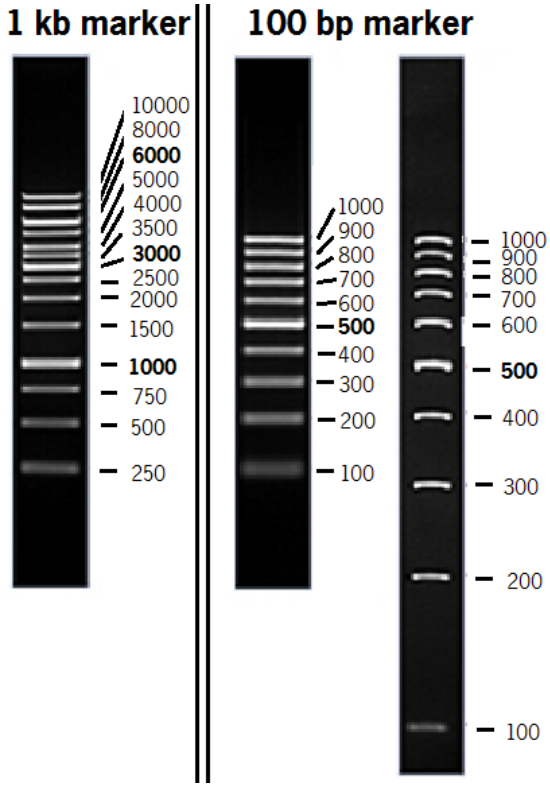

Figure 3 Fermentas 1kb and 100 bp markers

13.6 PCR optimization

A PCR is not always successful. It is possible, you will have a vague product, a smear, or no product at all. Some fine-tuning is then needed to make a PCR work. There are many things that you can adjust in a PCR. But what to do when?

Determining the problem: There are two kinds of problems;

1) the setup of you PCR
2) problems with specific sample(s)

The first problem probably occurs when you design a new primer set, which you are testing for the first time. These problems start at the beginning. The second problem occurs when your PCR is working fine, but with certain samples you have a problem.

Note: there is a lot of literature on PCR optimization in which they mention adjustment of concentrations of: $MgCl_2$, buffers, dNTP's and salts. But usually off-the-shelf mixes are used, which are already optimized by the manufacturer. Therefore, these options will not be discussed here.

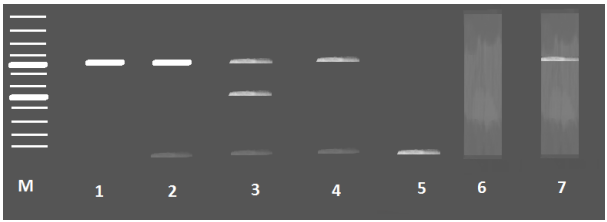

Figure 4 Several possible results of a PCR. M) marker 1) perfect band 2) good band with primer dimers 3) poor band with a-specific product and primer dimers 4) poor band with primer dimers 5) no band 6) smear 7) difficult sample or spiked with another sample.

In the figure above, several outcomes of a PCR can be seen. M) is the marker 1) shows a nice band with no extra by-products. 2) Still a good band, but with primer dimers, simple optimizations needed. 3) Poor band and next to aspecific product, also primer dimers; Touchdown PCR for optimization. 4) A poor band; possible inhibition and more optimization needed. 5) No band; likely to be inhibition if PCR usually works fine. Or, much optimization is needed if it is a newly designed primer set. Possible redesigning of the primers. 6) Smear, same issues as band nr. 5. 7) Result of too much enzyme added to the master-mix. Also a possible result if a difficult sample is spiked with template DNA from the positive control.

Optimizing a new PCR. There are a few things you can adjust when optimizing a new primer-set for a PCR. Firstly, apply a temperature gradient 5ºC under and above the theoretical annealing temperature. This can be easily programmed on most PCR machines. The annealing temperature is not always the correct temperature. Products of the different temperatures have to be analysed via gel-electrophoresis. The best result corresponds with the optimal temperature.
Another option is to increase the primer concentration up to a concentration of 1.0 µM. Increasing the concentration of the enzyme is also a solution, but should be done as a very last resort.
Heaving a smear or by-products in your PCR can be the result of too much template DNA, enzyme and / or primers. Decreasing the concentration is then the solution.
When testing a new PCR it is always a good idea to use template DNA which you know is clean. For example, if you target a piece of 16S rRNA gene (position 391F to 583R) you might want to test it on the whole 16S gene. To do this, you first amplify the 'whole' 16S rRNA gene with primers 27F and 1492R. This product (1500bp) is then purified and used to test the primers 391F and 583R. This practice is also known as 'nested PCR'. This product can also be used as a positive control in future PCR runs.

Optimizing difficult samples. Normally you will not have a problem with your samples, but once in a while you will have 'a difficult' sample, one that does not work as expected, giving a vague or no product at all.
To check if your sample contains high quality DNA, spike your sample with DNA from the positive control or a sample that worked earlier. When you get a PCR product from the spiked sample, you know that there are no inhibitors. If there is no product, you know that there are inhibitors present in your sample.

The problem causing these 'difficult' samples is usually the presence of inhibitors. These interfere with the DNA polymerase, resulting in less or no product. Inhibitors are substances that are present in the sample of origin, for example faeces, soil or sponges. The substances were not excluded during isolation and are present in the PCR reaction. To avoid this, lower the amount of DNA by diluting. This will also dilute the concentration of inhibitors. Sometimes it works to add more sample to the mixture. So with difficult samples, make a dilution series of 5, 10, 20, 40 and 60 ng/µl of template DNA of the same sample.
If lowering the DNA concentration is not an option, add Bovine Serum Albumin (BSA), 1µl from the tube per reaction. The exact mode of action of this protein is not fully understood, but it cancels out the inhibition, enabling the polymerase to work and produce product.

Dimethyl sulfoxide (DMSO) is another chemical used in PCR reactions, 2-10% (vol/vol). DMSO destabilises any secondary structures in the DNA, allowing access for primers and polymerases. It also lowers the melting temperature of your primers.
This Changes a standard protocol into something completely different. So be careful! The above substances are used the most, but other additives can be found in literature:

Substances	Concentration
Betaine	0.5 – 2 M
BSA	100ng/ 50 ul reaction mix
DMSO	2-10% (vol/vol)
Glycerol	1-5% (vol/vol)
Formamide	2-10% (vol/vol)

Table 7 Reaction additives to improve a PCR

To overcome aspecific binding of your primers (resulting in clear band(s) of different sizes) you might try a touch-down PCR. In this case you increase the annealing temperature in the first 5-10 cycles of your PCR run. With higher annealing temperatures the primers will not attach to DNA template, other than your target. After these first cycles, enough PCR product has been formed to outnumber a-specific products, resulting in a clear band in your gel.

How much do you optimize? That is up to you, where you work and what you want to do with the product. If you work in a hospital you have to do the same test every time perfectly, because you diagnose people. You determine if they are healthy or not, and changes in the protocols are not allowed in these kinds of diagnostic testing. In our case at the university, you just want a proof of concept and carry on to the next experiment.
What you at least want is a product that (dis)proves your hypothesis and carry on. If you need the product for further analysis you want to make sure that you amplify your target. Positive and negative controls are your quality check in this case. But be aware that changes to a protocol can result in a different outcome, making a comparison with previous results impossible.

More info can be found in the book: '*Principles and Technical Aspects of PCR amplification*' Pelt-Verkuil et al. 2007. Available as an electronic book on the WUR library site.

14 qPCR

In this chapter a strategy is presented for setting up a qPCR experiment. It will not give ready-to-go protocols!

14.1 Introduction

With quantitative PCR (qPCR) it's possible to quantify the amount of DNA in a sample. This is done by measuring the accumulation of dsDNA in real-time. The accumulation of dsDNA is detected using fluorescent dyes or fluorescent probes which can be detected by the machine.

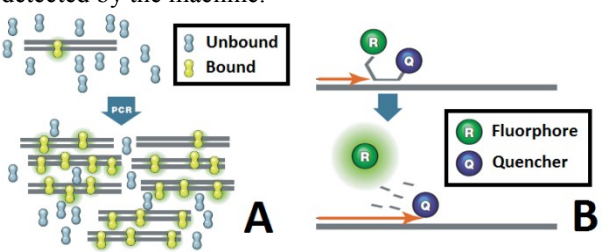

Figure 5 a) SYBR emits fluorescents when bound to dsDNA. b) Fluorphore is initially silenced by the quencher but emits light when released from the probe.

There are two types of detection systems. Fluorescent dyes and fluorescent probes.

Fluorescent dyes: SYBR green which binds unspecifically to any dsDNA. When unbound it doesn't emit any light; only when it attaches to dsDNA does become fluorescent. Assays using only SYBR Green are called 'singleplex assays'.

Fluorescent probes: This is a short piece of DNA, like a primer, with a fluorphore on one end and a quencher on the other. When the fluorphore is near the quencher, it transfers all its energy to the quencher and no light is emitted. When the probe is incorporated in the new DNA strain, the quencher and probe are cleaved of by the DNA polymerase. Now the fluorphore can emit light enabling the detection of new formed DNA. Up to 5 probes can be detected in one sample, which are called 'multiplex assays'. However, they require a lot of time to set up and are expensive, making them unsuitable for WUR microbiology. So we will not further discuss them.

14.2 Deciding on an analysis assay

There are two types of analysis assays that you can choose from: 'absolute quantification' and 'relative quantification'.

Absolute quantification: Determines the amount of DNA (copy nr / µg) per amount of sample (per cell / µg). This method, can be used, for example to

determine the amount of a specific type of bacteria in a sample. With the help of a serial dilution and the standard curve the number can be determined. Most assays done at WUR Microbiology are singleplex assays for absolute quantification.

Relative quantification: Gives a ratio (x-fold difference) between control sample A and sample B. For example: this method is applied when one wants to measure gene expression under different growth conditions in a cell. To measure the fold difference, a standard curve, and one or two housekeeping genes are necessary besides the target gene.

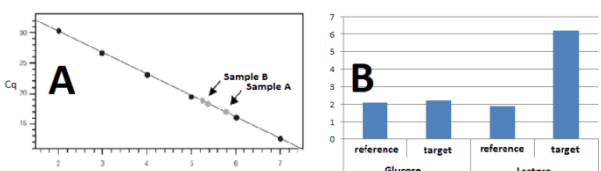

Figure 6 Examples: a) determine number of bacteria in samples. b) Expression of gene X in a bacteria.

Reverse Transcriptase: This step is necessary when performing a relative quantification. To measure the gene expression you look at the produced RNA. However, RNA cannot be used to perform a qPCR. The RNA sequence is converted by the enzyme Reverse

Transcriptase into copyDNA (cDNA). This cDNA is then used in the qPCR reaction.

14.3 MIQE guidelines

Setting up and publishing the result of your qPCR assay should be done by accordingly to the MIQE guidelines. These guidelines were written by a group of experts to help prevent inadequate reporting of experimental details, frequent use of flawed protocols; and attempt to standardize qPCR related publications. Throughout the years there were many terms floating around in literature for the same concept. For example the quantitative cycle (Cq) value. This was also known as: threshold cycle (Ct); crossing point (Cp); take off point (TOP). Furthermore, material & method descriptions were usually too incomplete for another scientist to repeat the experiment.

The MIQE guidelines provide a list of items that should be included in your experiment and publication. However, this list is very elaborate and not everything is necessary for every type of assay. It also depends on what the aim is for your experiment. If you work in a hospital the qPCR assay should be much more elaborate than when you run a simple experiment at WUR Microbiology. The MIQE guidelines have marked each item with an 'E' for essential or 'D' for desired. The decision of which items should be included in your publication, is based on a combination of the MIQE

guidelines, common sense and advise of your supervisor.
We expect everybody to follow the MIQE guidelines in their publications, whether it's a BSc thesis report or an article in Nature!

14.4 Setting up an assay

In the following paragraph, we will look at the steps involved in setting up a qPCR assay and provide tips & tricks for each item. Not all items discussed have to be performed as this depends on your type of assay.
General tips can be found in other chapters.

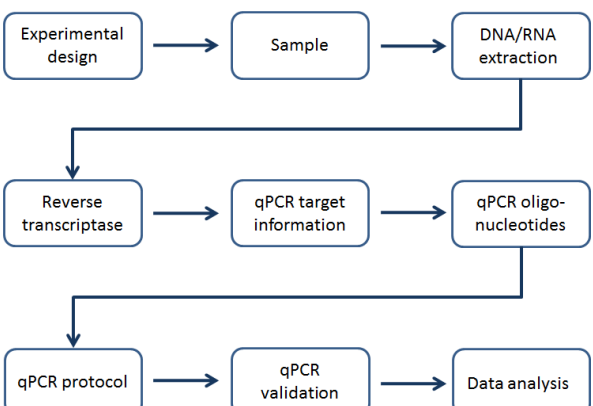

Figure 7 Scheme of steps involved in setting up a qPCR.

Experimental design. Discussed in previous section.

Sample. What is the origin of your sample? If you want to perform a relative quantification, you should choose your reference- or household gene carefully. Also be aware of possible inhibition from the source of you sample. A positive and a negative control are essential to test the assay on. Are you ordering a strain / DNA from DSMZ or are you isolating the organism yourself?

DNA/RNA extraction. Here you have to decide how to isolate your DNA / RNA from the sample. Which kit will you use, or do you make or use a custom protocol? See if you can increase the yield by optimizing the procedure. How do your measure the quality of your DNA / RNA? How will you overcome inhibition? Do not forget to include a negative control. This is to make sure that the isolation procedure does not cause DNA contamination with your samples.

Reverse transcriptase. This is only applicable if you do a relative quantification assay. Will you use a separate RT kit, or a combination kit where the RT and qPCR are combined? What are your references genes? Do not forget to include a no template control (NTC) next to a negative control. A negative control contains water instead of the sample. The NTC is the negative control of the RNA isolation step before. This is necessary to

check if your isolation procedure caused RNA contamination .

qPCR target information. This is the bioinformatics section. Which gene is your target? What is the sequence, or where can people find it online? What is the length and other relevant info. Also search for homologs and secondary structures.

qPCR oligonucleotides. Also known as your primers (and probes). You design them based on the information you obtained from the step above. Check for the correct criteria (length, C-G content, etc...). It is also important to know the annealing temperature, which you need with the next step.

qPCR protocol. Your protocol is not dictated by what you find in literature, but by the enzyme that you use. Here at WUR Microbiology we use BioRad Supermix. BioRad have the following guidelines (table 8)

Cycling Step	Temperature	Hold time (min:sec)	# of Cycles
Initial denaturation and enzyme activity	95°C	2:00 - 3:00	1
Denaturating	95°C	0:10 - 0:15	40
Annealing	55-60°C	0:15 - 0:30	
Elongation	72°C	0:30	
Melt curve (optional)	55-95°C (in 0.5°C increments)	0:10 - 0:30	1

Table 8 Guideline for BioRad Supermix.

The time that you run each step is also dependent on the size of your product, so check the BioRad Supermix manual.

After choosing the protocol, you have to optimize the annealing temperature and the primer concentration by performing a gradient PCR. The BioRad machine can perform a temperature gradient along the rows (A to H) of a qPCR plate. You program the temperature 5°C above and below the theoretical annealing temperature. Along the columns (1 to 12) you make a gradient of the primer concentration. You can look what is used in literature, otherwise the concentration is somewhere between 100-200 µM per reaction. Make the gradient 50 or 100 µM per step. The well with the highest fluorescence (RFU) indicates the optimal temperature and primer concentration. Do not include negative or positive controls, a standard curve is not necessary.

After this optimization step you will perform your first real qPCR including a negative-, positive control, NTC, standard curve and a couple of samples. Here you will look if everything runs well, especially if the sample concentrations are in range of the standard curve. Other issues are discussed in the next paragraph.

qPCR validation. At WUR Microbiology, validation is limited, but some parameters are essential to check. Important are the run values, which depends on the brand of the qPCR machines. For BioRad see table.

	Range	Optimal
Efficiency	90-105%	95-100%
Slope	as close to	-3.3
Y-value	as close to	0.999

Table 9 Run values for BioRad.

Another tool to check how well your qPCR performed is the melt curve that can substitute a gel-electrophorese. Each peak represents a product (fig 31). In the example below, a nice peak can be seen for each well (one line = one well) and no other peaks are visible. If a smaller peak would appear around 70°-80°C, it would indicate the formation of primer dimers. This is corrected by changing the annealing temperature and primer concentration.

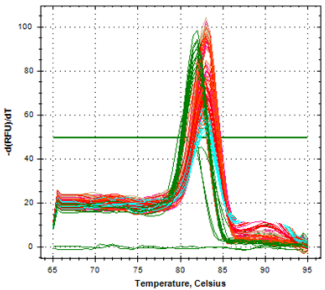

Figure 8 A meltcurve

Data analysis. It is important to realize that once you have the results of your qPCR experiment, you also have to perform some statistical analysis on these results. The statistical analysis of qPCR results is out of the scope of this booklet and will not be discussed. Ask your supervisor for more information.

14.5 Further reading

Dorak M.T. Real-time PCR (book)

Bustin S.A. A-Z of quantitative PCR (book)

MIQE guidelines. (2009)Bustin *et al*. Clinical Chemistry 55:4 611–622

MIQE précis. Practical implementation ... PCR experiments. (2010) Bustin *et al*. BMC Molecular Biology, 11:74

15 Cloning genes

When you want to clone a gene or DNA fragment, check your DNA fragment for sites that are present in your vector (for example pUC19) but absent in your DNA fragment of interest. This can be done using the "Nebcutter" website http://tools.neb.com/NEBcutter2/ or with Clone Manager software. Preferably select two enzymes that produce different 4 base overhangs and that use compatible reaction conditions (buffers and temperature). If no suitable pair of enzymes can be found, check for the presence of isoschizomers (enzymes with the same cleavage site but from a different organism) or enzymes producing compatible cohesive ends (same overhang. note that fragments can be ligated but not re-cleaved).

Order primers that contain your restriction site (for example GAATTC EcoRI)

GCGGCGAATTCTCGATTTCACAGGTTCGAA,
Tm 60º C

Follow the procedure below:

1. PCR amplify your target gene with restriction sites containing primers (usually 3x 50µl per

gene of interest, 30 cycles, genomic template DNA, 100ng/µl primer, negative control should contain no genomic DNA. If PCR doesn't work, change annealing temperature (40-60) or amount of template).

2. To check for product load 5µl onto an agarose gel alongside a suitable marker.

3. Purify the PCR product over a anion exchange column using a PCR purification kit. Elute in 50µl MQ.

4. Check amounts of PCR purified using nanodrop.

5. Obtain microgram amounts of vector/plasmid by Plasmid miniprep (Qiagen). (Usually 3.6 ml culture per miniprep column, elute in 50 µl EB or TE).

6. Set up a 3-4 hour digestion of 1 to 2 µg of your insert and vector at 37 °C in a stove (to prevent condensation in the lid) using the digestion buffers recommended by the supplier.

7. After digestion treat **only the vector** with alkaline phosphatase (CIAP) by adding 1/5

volume of CIAP enzyme for 10 min at room temperature.

8. Incubate for 1 hour at 37°C.

9. Prepare a thick gel using the wide comb, then load both digested vector and insert onto the gel.

10. Run the gel and excise the desired band. Voltage and time depend on the size of the product.

11. Purify the band according to the Fermentas extraction kit protocol. Elute in 30-50 µl MQ.

12. Check yield on Nanodrop and load 10 µl of the insert and vector onto a gel. Estimate the relative amounts of DNA from intensities of the bands, for example vector : insert, 2:1 or 3:1.

13. Calculate the composition of ligation mixtures based on 3 (recommended, 5 or 10 fold molar excess of insert (sometimes 1:1 is required especially when using very small inserts). See below.

14. Setup ligations according to the formula below and always include a negative control containing MQ instead of insert.

15. Incubate overnight at 15-20°C (ML-II lab).

16. Transform the DNA to the competent *E. coli* cells by heat shock or electroporation.
(When transforming to new competent cells for the first time, include a negative control of just cells and no DNA. Use maximally 1 µl ligation mix for electroporation to prevent "explosions". Otherwise microdialyze the desired amount before electroporation.

On the next page you will find a formula to calculate for DNA ligations.

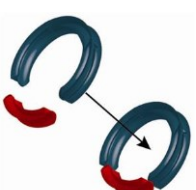

Figure 9 DNA ligation

Table 10 Ligation formula

a = insert/vector	Molar ratio chosen in ligation (1, 3, 5, 10)
b = vector/insert	Size ratio (eg. 5300/1050)
c = vector/insert	Estimated ratio from band intensities on gel
If you use 1 ml vector use $a/b \times c = d$ ml insert	
To get a 10 ml ligation you need $7/(1+d) = e$ ml vector and $7-e = f$ ml insert	
So you get	e ml vector
	f ml insert (or MQ for negative control)
	2 ml T4 ligase buffer (prevent droplets on the tip!)
	1 ml T4 ligase

16 Transformation

Transformation is change in the genetic content of a cell as a result of uptake and expression of exogenous DNA. 'Competence' is the ability of a cell to take up exogenous DNA. Competence varies greatly among bacterial species: some bacteria are capable of natural DNA uptake (natural competence), but most bacterial cells have to be made competent (artificial competence). DNA can be taken up by cells in various ways, but chemical transformation and electro-transformation are the most common procedures in a laboratory to transform a bacterium of choice. During these procedures cells are treated in such a way that their membrane becomes permeable, after which DNA can find its way into the cell.

In this chapter, transformation techniques for *Escherichia coli* are discussed. However, these techniques and the theory behind them might also apply to other bacteria.

16.1 Competent cells

Before transformation can take place, cells have to made competent. Sometimes competent cells are ordered from companies, but it is fairly easy (and a lot cheaper) to make competent cells yourself.

To make competent cells, a growing culture is selected in its early- to mid-log growth phase, when cells are healthy and active. Cells are washed by multiple centrifugation and pellet resuspension steps.

Chemically-competent cells are made by washing cells with a solution containing divalent cat-ions (e.g. Mg^{2+}, Ca^{2+}) which bind membrane-molecules (promoting pore formation), resulting in better DNA uptake.

Electro-competent cells are made by washing the cells in a salt-less or salt-poor solutions: salts may cause 'arcing' of the electroshock, resulting in a failed electro-transformation.

During both procedures, it is important to keep cells at or below 4°C to get high numbers of competent cells. In the final washing step competent cells are usually washed in a glycerol containing solution. Glycerol prevents ice crystal formation, making it possible to store aliquots of competent cells at -80°C.

16.2 Heat-shock transformation

Chemically-competent cells are thawed on ice and mixed with the DNA, usually a plasmid solution. Up to 5µL of DNA solution can be added. A chemical transformation can be performed with high amounts of salts in the DNA sample, which makes it particularly

useful for the transformation of cells with ligation mixtures.
The competent cell-DNA mixture is incubated on ice, the time according to protocol or experience. During this incubation, the divalent cations in the cell sample mask the negative charge of the DNA, improving transformation efficiency. After this incubation a heat-shock is applied to the cells by transferring the mixture to a 42°C water-bath or heat block. During this heat-shock, pores in cell membrane open and DNA flows into the cell. After 90 seconds the cells are returned on ice, which ensures a slow pore closure, resulting into maximum DNA uptake.

16.3 Electro-transformation

During an electro-transformation, electro-competent cells are thawed on ice and mixed with DNA. Both the cells and the added DNA should contain very little to no salt to prevent the sample from 'arcing' during the electroshock. If you want to transform cells with a ligation mixture, never add more than 0.25µL of this mixture.

The competent cells-DNA mixture can directly be transferred to an electroporation cuvette. Once the sample is loaded in the cuvette, sweep the cuvette with a tissue to make sure it is dry. Place it in the in the electroporator and apply an electroshock with the

desired settings. The electroshock causes the membrane to form pores and pushes the charged DNA into the cells.

Figure 10 Electroporator and electroporation cuvettes

Different electroporation cuvettes are used and each cuvette requires different amounts of mixture to be loaded as well as different electroporator settings. Pre-cooling cuvettes in the freezer improves transformation efficiencies because low temperature slow down pore-closure.

16.4 Recovery

Pre-warmed 37°C medium is added to chemically- or electro-transformed cells. Different media which promote cell survival and pore closure can be used. These cultures are incubated at 37°C in a shaker incubator for at least an hour. During this recovery step, cells are able to both close pores formed and possibly

express antibiotic resistance markers located on the inserted DNA. For some antibiotics, recovery time has to be increased to improve transformation efficiency. After recovery, cells are plated in a dilution range on plates with antibiotics corresponding to the antibiotic marker on the inserted DNA. The plates are incubated overnight at 37°C.

16.5 Confirming transformation

Cells, which have successfully taken up DNA containing an antibiotic marker, are able to form colonies on plates with the corresponding antibiotic marker. Each of these cells will form a colony, and is therefore called a Colony Forming Unit (CFU). Efficiency of transformation is usually determined as CFU per ng of DNA.

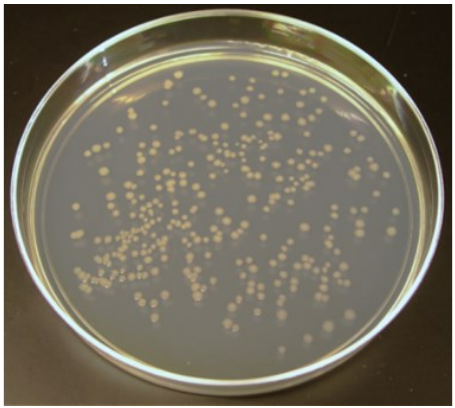

Figure 11 A successful transformation

16.6 Transformation protocols

More detailed protocols for competent cells and transformations, and protocols for the transformation of other organisms can be found at N:\BacGen\Protocols and N:\MolEco\Protocols\transformation.

16.7 Improving transformation efficiency

Transformation efficiency might be improved by applying these rules during your transformation experiments:

Use electroshock instead of chemical transformation
Electro-transformations usually result in more transformants than chemical transformation.

Use more DNA for transformation
Keep in mind that in electro-transformations amounts of salts in sample should be as low as possible.

Use other competent cells
Sometimes competence of cells is low, so always compare the competence of a new batch, with that of an old batch of competent cells.

Keep cells cool
Always keep cell samples on ice (also thaw on ice!) and pre-cool electroporation cuvettes.

Pre-warm medium
Pre-warm recovery medium and plates to 37°C.

Recover in shaker incubator
Recovery can be improved by putting recovery samples horizontally in a shaker incubator rather than a heat-block or water bath.

16.8 Transformation troubleshooting

Problem	Possible explanation	Solution
No colony formation	-Wrong competent cells	*Use electro-competent cells for electro-transformation and chemically-competent cells for heat-shock transformation*
	-Wrong antibiotic in plate	*Marker on DNA should match antibiotic in plate*
	-DNA does not replicate in bacterium	*Use a plasmid with an origin of replication corresponding to bacterium*
	-Toxic gene insert	*Use a plasmid that allows inducible expression of insert*
Smear formation/too many colonies	-No or too little antibiotics in plate, or plates are too old (antibiotics degraded)	*Use fresh plates with corresponding antibiotics*
	-Recovery step too long	*Never recover longer than 2 hours*
	-Cells have a genomic antibiotic marker or contain a plasmid with an antibiotic marker	*Choose cells suitable for your transformation*
	-Too many transformants	*Use less DNA or dilute sample prior to plating*
Electroshock 'arced'	-Too much salt in cells-DNA mixture	*Use less DNA mixture, or dialyse DNA sample. Use good quality electrocompetent cells*

Table 11 Transformation troubleshooting

17 Sequencing

17.1 Introduction

Sequencing is the process of determining the nucleotide order of a given DNA fragment. In the last decade different sequencing methodologies have been developed. Each technique has its own advantages and disadvantages (tab 1), which makes it more or less suitable for a given task. Therefore, it is best to decide on which method is most applicable before starting with the necessary sample preparations.

17.2 Sequencing of genes or plasmid inserts

Sequencing of genes (e.g. from single bacterial isolates) or cloned plasmid inserts is relatively easy. The target DNA fragment is simply amplified by PCR using primers that are specific for the target DNA. PCR products are then sent to a facility for sequencing applying the traditional Chain Termination (Sanger) methodology using either the forward or the reverse primer that was used for the PCR to obtain the DNA fragments of interest is used in the sequencing reaction. This method yields around 700 bp sequences.
Bidirectional sequencing (using a forward and reverse primer) is necessary when the DNA fragment is longer

than 700 bp (e.g. 16S rRNA genes which are 1500 bp). The obtained sequences can be assembled using a program such as Clone manager.

17.3 Sequencing complex samples

When dealing with complex samples containing multiple (similar) genes of interest the traditional Sanger methodology cannot be applied directly. An additional cloning step is necessary to obtain individual clones, each with a single cloned gene of interest (insert). The insert sequence can be determined by amplification by PCR and subsequent sequencing. However, this process is time consuming and costly.

An alternative is pyrosequencing to be determine the sequence of the DNA fragments in complex samples. A full description of the principle of pyrosequencing is beyond the scope of this text. For details about pyrosequencing methodology. See also 17.5 Margulies M, *et al.*

Pyrosequencing yields ~1-1.5 million sequences of ~400 bp per run using the titanium chemistry.

Pyrosequencing starts with a PCR that employs primers (specific for the DNA fragment of interest) attached with sample specific barcodes and adaptors. The former are used for sorting purposes of the pyrosequences per sample, whereas the latter are necessary for pyrosequencing itself. Subsequently, PCR products are

analysed by agarose gel electrophoresis to confirm product size, followed by PCR product purification and DNA yield is measured using the Nanodrop. To obtain an equal amount of sequences per sample, all purified PCR products have to be mixed equimolarly. PCR primer dimers still present in the purified PCR samples may hamper pyrosequencing of the specific PCR products. To remove primer dimers, the equimolarly prepared pool of PCR products should be run on an agarose gel followed by extraction and purification of the specific PCR products. Finally the resulting PCR product can be sent to a sequencing facility.

The main disadvantage of pyrosequencing is the relatively high error rate. See also 17.5 Niu B *et al.* Originally, pyrosequencing was used to obtain a high coverage of sequence data for genome sequencing projects. Nonetheless, assembly of the pyrosequencing reads produces highly accurate concensus sequence. However, the high error of pyrosequencing is detrimental for studies that use the individual pyrosequences to determine the natural variation of particular gene in complex samples. Therefore, it is important to remove erroneous sequences using specialized tools that are implemented in pyrosequencing analysis pipelines such as QIIME or MOTHUR. See also 17.5 Caporaso JG*, et al.*

An alternative to the pyrosequencing methodology is illumina sequencing. See also 17.5 Bennett S. This

technique yields shorter reads, but produces more than 10 times the number of reads per run as pyrosequencing. Although the principles for both techniques are different, preparation of the equimolar pool of PCR products is virtually the same.

17.4 Genome sequencing

Previously, genome sequencing involved cloning of DNA fragments into bacterial vectors, amplification and purification of individual templates, followed by Sanger sequencing. Pyrosequencing and Illumina sequencing are currently frequently used for genome sequencing, since both techniques circumvent the laborious cloning steps. However, obtaining high quality genomes with pyrosequencing is challenging due to the high error rate, whereas assembly of genomes from Illumina reads is challenging due to the relatively short sequence length.

The best option to get high quality genomes is to combine both techniques and first assemble the pyrosequence reads followed by removing the errors and improve the assembly with the illumina reads.

Please note that genome sequencing requires high molecular weight DNA, which sometimes can be difficult to isolate from bacteria with rigid cell walls. See the DNA extraction section for details about

obtaining high molecular weight DNA for genome sequencing.

Sequencing methodology	Sequence length	Number of sequences	Advantage	Disadvantage
Chain termination (Sanger) sequencing	700	1 per reaction	Longer sequence with generally a good quality	
Pyrosequencing using titanium chemistry	~400	1-1.5 million per run on a full picotiterplate		Relatively high error rate
Illumina sequencing	~150 bp paired end reads	10-15 million sequences per run	Yields millions of sequences which makes it applicable for high resolution 16S rRNA gene based community profiling	Yields shorter sequences (~250 bp) compared to pyrosequencing

Table 12 Characteristics of sequencing methodologies

17.5 Further reading

Margulies M*, et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. (Translated from eng) *Nature* 437(7057):376-380

Niu B, Fu L, Sun S, & Li W (2010) Artificial and natural duplicates in pyrosequencing reads of metagenomic data. (Translated from eng) *BMC bioinformatics* 11:187

Caporaso JG*, et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. (Translated from eng) *Nature methods* 7(5):335-336

Bennett S (2004) Solexa Ltd. (Translated from eng) *Pharmacogenomics* 5(4):433-438

18 Presentations

Everybody has to give a presentation at some time. For the average presentation at Microbiology you have approximately 10 minutes. Giving a good presentation is not easy, so here are a couple of guidelines.

18.1 PowerPoint vs. Prezi

Microsoft PowerPoint is the standard for making presentations. A nice alternative is Prezi, which is partially free software for academic use. It is available from prezi.com, were you need to register. Make sure you click on the 'Student / Teacher License' button and choose the 'Edu Enjoy' version of the program. Register and you are able to login and work with the program. It has tutorials but the best way to learn is to work with it!

WARNING! Prezi and Microsoft PowerPoint have many nice options involving movement and animations. Do not use these options too much! The more you put in, the less professional your presentation will be. See also 18.2 → Layout

18.2 Making a presentation

The audience. Starting with a presentation should start with thinking about who your audience is. Is it your group? First year BSc students? Your family? Every audience has a certain level of knowledge and

according to that you make your presentation. Your family will not know what a PCR is, but if you explain at a group meeting what a PCR is, they will get bored....

Length. Try to use max. 1,5 slide per minute of presentation, so 10 minutes = 15 slides. Do not put on to much text. This will prevent you reading the story to the audience. Use the presentation / slides as a support for the story you tell.
As a rule: A maximum of 8 sentences with each 8 words, so 64 words per slide.

Layout. Presentations are made in MS PowerPoint or Prezi, which has many features for movements, actions, strange layouts. These are great, but are terrible to look at and do not appear very professional, so leave them out.
Do not put in as many picture or symbols in as you can find. Keep the slides 'clean', using only the essential information.

Pictures. Pictures say more than a 1000 words, but don't use to many as things can get confusing. Make important pictures as big as possible to clearly show the details.
Pictures containing dark colours should be brightened (fig 35). On your computer screen they look perfect, on a large screen they always look darker and details you

wanted to show, cannot be seen. The same for colours, red and orange will appear brownish on the big screen. Use colours that differ from each other.

Practice. Few people are very good at giving presentations, but usually those people have already given 'tons' of presentations. Practice before you do a presentation, at least two times and preferably in the room you have to give the presentation. Ask someone to come and listen to your presentation to give feedback. If you know your presentation you will be less nervous.

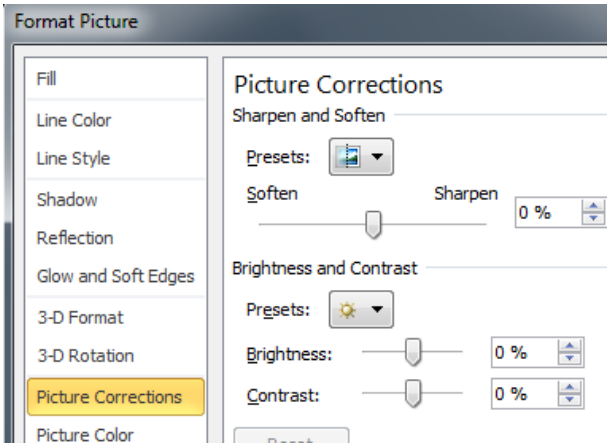

Figure 12 To brighten the picture, right-click mouse button on the specific picture, click format picture and adjust the brightness.

18.3 Giving presentations

Some people like to have some water during the presentation, so bring this with you if you like that. Arrange a laser pointer or a pointer stick.
Make sure your presentation works on the particular computer you have to use and put it on the computer before the meeting starts. Have a back-up on a N-drive / Gmail / 2nd USB stick. If it is really important, make a PDF copy of the presentation to overcome MS PowerPoint problems.

Nerves. To control your nerves, good preparation is essential. Practice the presentation in front of a colleague. Prepare a small paper with some keywords / important points to guide yourself through the presentation. Control your breathing, inhale deeply once in a while to gain control.

Beginning. You are given the floor and can start your presentation.
Start by giving a **short** introduction about yourself, why you are here, and what you are doing. If you don't like being asked questions during the presentation, just say that **after** the presentation there is room for questions and discussion.

Giving. During the presentation you speak loud enough for the people in the back. You face the audience, do not look at the screen continuously. Important! Keep track of the time! Do not exceed the time limit.
Maintain the tempo. Too slow and people will lose interest, too quick, and people will not understand everything.
When you indicate something on a slide, use a stick or laser. If you use the laser **hold it still**, you're not a Jedi warrior fighting the Dark force. It is incredibly irritating for people to look at.
Important! Keep track of the time! Do not exceed the time limit.

Ending. At the end of your presentation the audience is allowed to ask questions. You can point out the people, who have a question, yourself. Listen carefully, ask people to repeat if you don't understand them.
Some people are really annoying, they keep asking multiple questions and you want them to shut up. Do not say "shut up", but you say: "I think this is an interesting discussion, but I suggest we continue the discussion after the presentation/meeting" or "My time is limited and I would like to give other people the opportunity to ask questions". Right after saying this you point out the next person and your problem is solved.

19 Notes:

20 Index

This is the safety guide of Microbiology and Systems and Sythetic Biology ofWageningen UR. It contains all information necessary, for those who work in the lab on a daily basis.
This book is for all (new) employees at all levels, from BSc-student to PostDoc.

Puylaert, et al.

version 2.1

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