

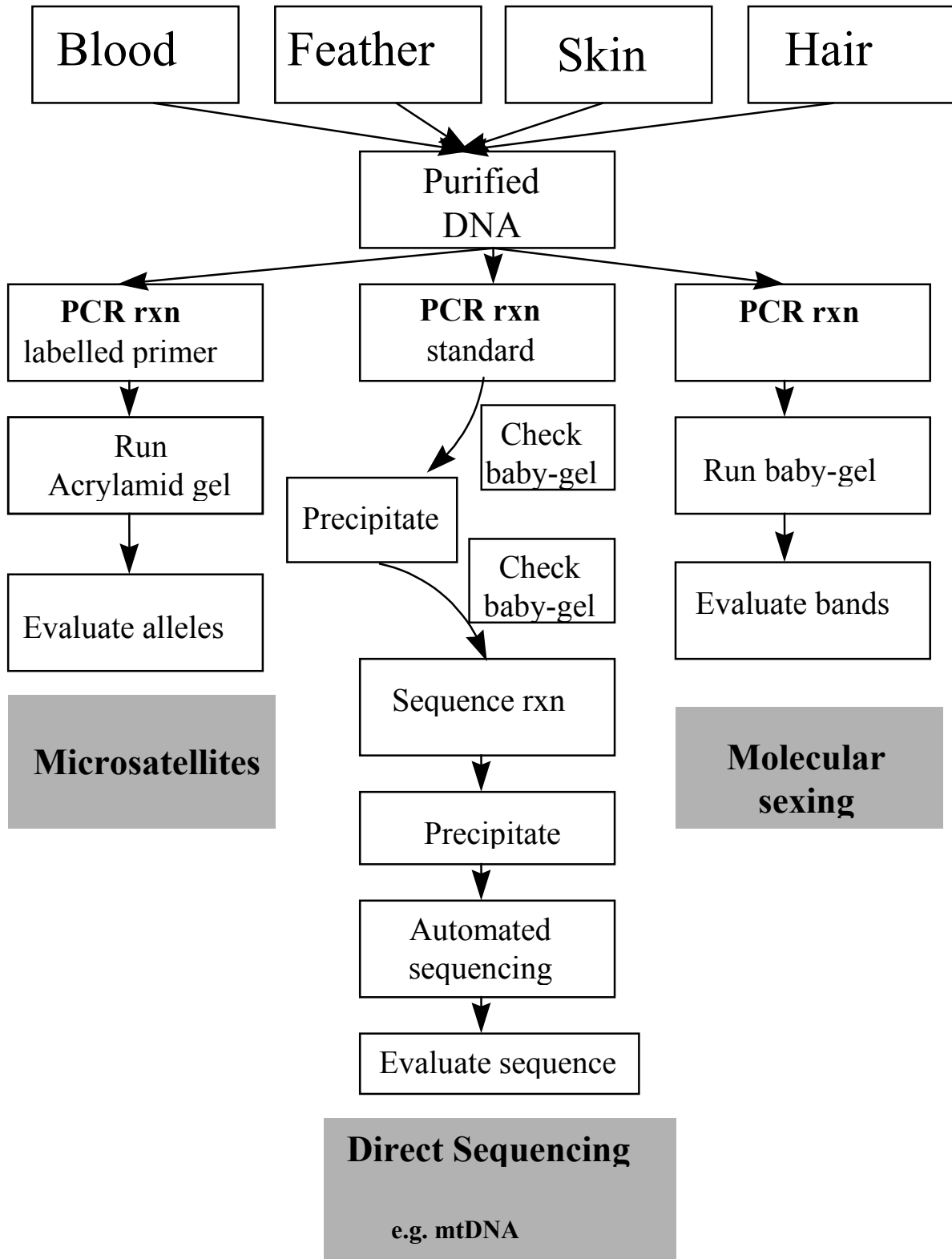
PCR-based Lab Protocols

The invention of the PCR (Polymerase Chain Reaction) technique turned out to be a revolution for evolutionary biologists interested in genetic questions. Suddenly there was a fast, robust and relatively inexpensive technique to get hold of genetic information from small samples of e.g. skin, blood or faeces. An advantage with PCR based molecular studies of DNA is that, once DNA is extracted and purified, the techniques are very similar regardless of the taxonomy of the study organisms. For example, microsatellite typing of great reed warblers is no different than performing it on samples taken from other organisms like apple trees, wasps or humans, given that you have a protocol available for these species. Here, the protocols are exemplified by three projects using bird DNA, but these could easily be adjusted to any other organism.

The aim has been to fill this manual with all the necessary information needed for independent work in our laboratory. However, we realise that this is hard to accomplish, or that it sometimes is difficult to find the necessary information. If you are just a little bit uncertain about how to proceed, do not hesitate to ask any of the researchers that happen to be in the Molecular Ecology and Evolution lab (MEEL). Remember that discussing the lab protocol is indeed an essential part of learning molecular biology!

Contents	Page
Work map	2
General Instructions	3
Examples of Projects	4
1.Molecular sexing	4
2.Direct Sequencing (e.g. mtDNA, parasites etc)	4
3.Microsatellites	5
4.A fluorescein based AFLP protocol	6
5.Cloning of PCR products	10
General Protocols	
1. Extraction of DNA	13
2. Quantification of DNA	17
3. PCR	18
4. Agarose (baby) gels	22
5. Precipitation of PCR products	23
6. Sequencing with Big Dye terminators, incl. precipitation of sequencing PCR	24
7. Polyacrylamide gels	26
8. Taking pictures/scanning gels	28
DNA size ladder	31

Work map



General Instructions for working in the lab

- Always use gloves when working in the lab.
- pre-PCR is carried out in room B221 and B222. All post PCR activity takes place in room C243. Discard gloves used in room C243 or the PCR room before going back to room B221 and B222.
- **BEFORE** stock solutions, kits, gloves, Kleenex etc runs out, it is **your responsibility** to make sure these are replaced (ask someone in the lab if you don't know how to proceed with making solutions or placing orders).
- At the end of the day, remove all your stuff from the lab bench, put these at their appropriate places and clean up your work space.
- **Be extra careful when handling reactions or waste containing hazardous chemicals:**
 1. Waste and contaminated material (including gels) must be placed in separate and labelled waste containers
 2. Always work in a fume hood when handling organic solvents, like phenol and chloroform, or other toxic and/or hazardous chemicals and place waste in appropriately labelled containers.
 3. Make sure that you are handling the chemicals in the appropriate way; read the MSDS and, in case of CMR chemicals, the handling documents.

Dish washing machine

Rinse glass ware with de-ionised water (green tap) after usage and place in the dishwasher/black container. Run the dishwasher preferably during lunch/overnight using program P6, wipe off remaining water on top of lids and measuring glasses and dry by running program P4. Empty the dishwasher and put up the label "dirty".

Five different examples of molecular projects

1. Molecular sexing

To sex birds, one can make use of the fact that females are carrying a unique chromosome (W) in combination with the Z-chromosome, of which males have two copies. There are two useful primer pairs available that work in most species of birds (P2 & P8, Griffiths et al. 1998, *Mol. Ecol.* 7: 1071-1075 and 2550 F & 2718 R, Fridolfsson & Ellegren 1999, *JAB* 30:116-121). These two protocols amplify introns of the CHD gene which in most species has different length in the Z and W copy and thus can be easily evaluated on agarose gels.

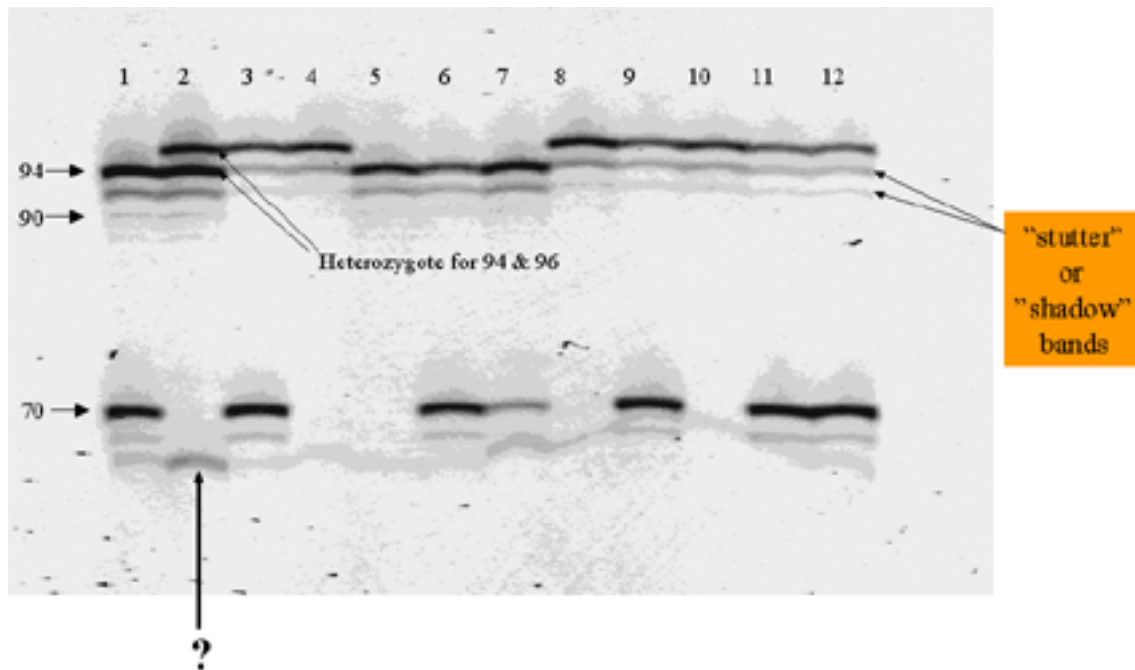
1. Book/confirm that there is a PCR machine available for the next three hours. Turn it on, allowing the block to reach the appropriate temperature. Adjust the program.
2. Set up a standard PCR (10 μ l rxn, p.20). Remember to adjust the $MgCl_2$ if required (default is 1.5 μ l).
3. Place the tray on the pre-booked PCR machine with appropriate markings (date+name).
4. When the PCR is completed, bring the tray to the bench for running baby gels.
5. Prepare and load samples on a 2% agarose baby gel (p. 22) and run for 20-30 minutes.
6. Make a picture of the gel, scanner/camera instructions on p. 28.

2. Direct sequencing (e.g. mtDNA, parasites, etc)

Universal primers for amplifying 306 bp of the cytochrome *b* gene are L14841 and H15149 from Kocher et al. (1989, *Proc. Nat. Acad. Sci. USA* 86: 6196-6200).

2. Book/confirm that there is a PCR machine available for the next three hours. Turn it on, allowing the block to reach the appropriate temperature. Adjust the program.
3. Set up a standard PCR (25 μ l rxn, p. 20). Remember to adjust the $MgCl_2$ if required (default is 1.5 mM).
4. Place the tray on the pre-booked PCR machine with the appropriate markings (name+date).
5. Check the results on a 2% agarose baby gel (p. 22) by taking out 2.5 μ l of the reaction and mix this with 1 μ l of stop mix on a microtiter plate. The product should be approximately as strong as the 500 band of the DNA ladder (p. 30) and no other bands visible.
6. Precipitate the positive reactions with ammonium-acetate/ethanol protocol (p. 24).
7. Dissolve the "invisible" pellet in 20 μ l of ddH₂O.
8. Store sequencing template frozen (avoid contact with the pre-PCR reagents) until ready for setting up sequencing reaction. ☺
9. Use 2 μ l in a cyclic sequencing reaction (p. 24) with one of the primers used in the original PCR (or an internal primer).
10. Precipitate all 10 μ l following the EDTA/ethanol precipitation protocol (p. 26).
11. Put lid/sealing foil on and put it in the freezer in room B240 for sequencing.

3. Microsatellite typing



Microsatellites are short repeated regions that are dispersed at several thousand sites (loci) inside the genome. We use 5'-fluorescein labelled primers (the forward primer as default) to separate the fragments on polyacrylamide gels with 36 wells, and visualise the fragments on a scanner. The polyacrylamide gel should be cast at least 2 hours before use to allow for polymerisation. The gel may be cast one day ahead of usage and stored in the cold room. Safety instructions on p. 26.

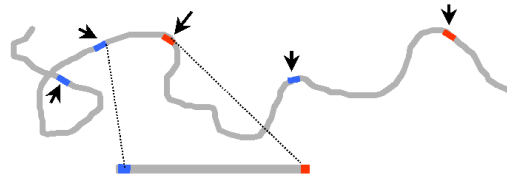
1. Book/confirm that there is a PCR machine available for the next three hours. Turn it on, allowing the block to reach the appropriate temperature Adjust the program. Arrange appropriate number of empty PCR tubes (strips) on a sample tray (do not forget one tube for the blank, containing no DNA).
2. Set up a PCR following the instructions for standard PCR (10 μ l rxn, p. 20) with a pair of microsatellite primers. Adjust $MgCl_2$ if necessary.
3. After thermal cycling, remove tubes and add 5 μ l of form amide-dye. Note that form amide is a toxin, see safety instructions (p. 27). If the samples cannot be analysed immediately, they may be stored at +4°C. ☺
4. Follow the instructions under the heading "7.2. Running polyacrylamide gels" (p. 27).

4. A fluorescein based AFLP protocol

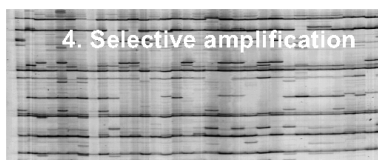
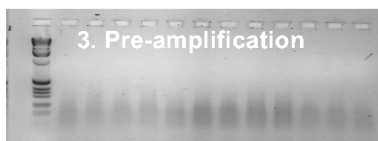
modified from Vos et al. 1995 (Nucl Acids Res 23:4407-4414)



1. Digestion of genomic DNA with two different restriction enzymes



2. Ligation of adaptors



AFLP is a clever combination of two older methods, RFLP and RAPD.

1. As with RFLP, genomic DNA is digested with restriction enzymes, in this case *EcoRI* (sites coloured blue) and *MseI* (red).
2. Synthetically made short fragments of DNA (adaptors; pink for *EcoRI* and black for *MseI*) that have “sticky ends” to the cut sites opened by the enzymes, are ligated (glued) to the thousands of anonymous DNA-fragments.

Arbitrarily selected primers (similar to RAPD) used in the following PCRs will reduce the complexity in two steps:

3. In the pre-amplification step, only fragments exhibiting the chosen bases inside the fragments will be amplified resulting in a reduction of fragment numbers by 1/16 ($\frac{1}{4} \times \frac{1}{4}$) and these can be visualized as a smear when run on an ordinary, 2%, agarose gel.
4. In the selective amplification, a small aliquot of the pre-amplified fragments is used in a second PCR with two primers that extends additionally two bases inwards. This further reduces the number of fragments by 1/ 256 ($\frac{1}{4} \times \frac{1}{4} \times \frac{1}{4} \times \frac{1}{4}$) with typical experiment showing about 100 different fragments.
5. The fragments are then size separated and normally visualised on a polyacrylamid gel by labelling the E-primer with a fluorescent dye.

4.1. Cutting genomic DNA

1. Thaw all reagents (except enzymes) and keep on ice.
2. Make the cocktail (30 rxn + 10%) in a 1.5 ml eppendorf tube (snap-cap). Starting with the ddH₂O and end with the enzymes. Keep the cocktail on ice. Mix by using a pipette set at 150 µl and pipette up and down a few times.
3. Dispense 5 µl of the cocktail in each of the tubes in a PCR strip, or single tubes, arranged in rows of 8.
4. Add DNA and cap tubes.
5. Incubate in for 1h at 37°C in the climate cupboard (underneath the water bath, opposite the telephone).

Reagents	1 rxn (µl)	30 rxn + 10% (µl)	16 rxn + controls (x20)	Recognition sequence for enzymes
ddH ₂ O	3.45	114	69	
TA-buffer* (10X)	1	33	20	
BSA (1µg/µl)	0.5	16.5	10	
EcoRI (50u/µl)	0.025	0.85	0.5	5'-G↓AATTC-3'
TruI (50u/µl)	0.025	0.85	0.5	5'-T↓TAA-3'
DNA (25-50ng/µl)	5			
Total rxn volume	10			

*This buffer provides near-optimal condition for both TruI and EcoRI
(100 mM Tris-Ac pH7.9, 100 mM MgAc, 500mM Kac, 10 mM DTT)

4.2. Ligation of adaptors

6. Thaw ligation buffer and adaptors, keep on ice.
7. Make a cocktail in a 1.5 ml eppendorf tube (keep on ice).
8. Add 2.5 µl of ligase/adaptor cocktail to each of the samples.
9. Continue incubation in the climate cupboard for 3 h at 37°C.
10. Dilute the samples 10 times (i.e. to each rxn of 12.5 µl add 112.5 µl ddH₂O), in PCR tubes in rows by 8. Store at -20°C.

	1 rxn (µl)	30 rxn + 10% (µl)	16 rxn + cont (x20)	Sequence of adaptor:
ddH ₂ O	2.06	68	42	
Ligation buffer (10x)	0.25	16.5	5	
E-adaptor (100 µM)	0.0125	0.41	0.25	5' -CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5'
M-adaptor (100 µM)	0.125	4.1	2.5	5' -GACGATGAGTCCTGAG TACTCAGGACTCAT-5'
T4 ligase (5u/µl)	0.05	1.65	1.0	
Total volume / sample	2.5			

4.3. Pre-amplification

1. Thaw reagents (except the Taq DNA polymerase) and keep them on ice.
2. Make a cocktail in a 1.5 ml eppendorf tube (keep on ice).
3. Dispense 5 μ l of the cocktail in each of the tubes in a PCR strip, or single tubes, arranged in rows of 8.
4. Add the template (water to the blank).
5. Incubate on the PCR machine with the following temperature profile :

[94°C-2 min]+ [94°C - 30 s, 56°C - 30 s, 72°C - 60 s] x 20 cycles + [72°C - 10 min]

Place a tray on the PCR machine with the appropriate markings (name+date).

6. Move 4 μ l to new tubes (to check pre-amp on agarose baby gels).
7. Dilute the samples 10 times (i.e. add 54 μ l of ddH₂O), store at -20°C.

Reagents	1 rxn (μ l)	31 rxn+10% (μ l)	20 rxn+contr (x20)	Primer sequence*
ddH ₂ O	0.9	31	18	
MgCl ₂ (25 mM)	1	34	20	
PCR buffer (10x)	1	34	20	
dNTP (1 mM)	2	68	40	
E-primer (100 μ M)	0.03	1.0	0.6	5' -GACTGCGTACCAATTCN-3'
M-primer (100 μ M)	0.03	1.0	0.6	5' -GATGAGTCCTGAGTAAN-3'
Taq (5 u/ μ l)	0.04	1.4	0.8	
DNA-template (diluted pre- amplification)	5			
Total volume	10			

* The base at the 3' end of the primer "N" is arbitrarily selected. For example, primer E_T is the EcoRI primer with a T at the 3' position

4.4. Selective amplification

1. Thaw reagents (except the Taq DNA polymerase) and keep them on ice.
2. Make a cocktail in a 1.5 ml eppendorf tube (keep on ice).
3. Dispense 7.5 μ l of the cocktail in each of the tubes in a PCR strip, or single tubes, a total of 31 tubes (30 samples and 1 for the blank), arranged in rows of 8.
4. Add the template (pre-amplified blank to the blank).
5. Incubate on the PCR machine with the following temperature profile ("touch down PCR"):

[94°C-2 min]+ [94°C - 30 s, 65°C-0.7°C/cycle - 30 s, 72°C - 60 s] x 12 cycles +
 [94°C - 30 s, 56°C - 30 s, 72°C - 60 s] x 23 cycles + [72°C - 10 min]

Place a tray on the PCR machine with the appropriate markings (name+date).

6. Add one volume (10 μ l) of **formamide dye, safety instructions p.27** (100% formamide, 10 mM EDTA, 0.1% xylene cyanol ff, 0.1% bromphenol blue), to the samples and store at +4°C over night before running on the gel. For some unknown reasons, this makes bands appear sharper than if run immediately after **formamide** has been added.

Reagents	1 rxn (μ l)	31 rxn+10% (μ l)	20 rxn+contr (x20)	Primer sequence**
ddH ₂ O	3.3	113	66	
MgCl ₂ (25 mM)	1	34	20	
PCR buffer (10x)	1	34	20	
dNTP (1 mM)	2	68	40	
E-primer (100 μ M)*	0.06	2.05	1.2	5' -GACTGCGTACCAATTCNNN-3'
M-primer (100 μ M)	0.06	2.05	1.2	5' -GATGAGTCCTGAGTAANNN-3'
Taq (5 u/ μ l)	0.08	2.75	1.6	
DNA-template (diluted pre- amplification)	2.5			
Total volume	10			

* E-primer 5' labelled with fluorescein

** The three bases at the 3' end of the primer "NNN" are arbitrarily selected. For example, primer _{ETGA} is the EcoRI primer with TGA at the 3' position

5. Cloning of PCR products

You will find the equipment, pipettes etc, for cloning in a cupboard by the assign cloning bench. Before starting with the protocol, make plates (p. 11) or borrow from someone else. Heat the plates in the incubator (37°C). Clean the bench with alcohol before working with bacteria. When finished, sterilise all glass (e.g., Pasteur pipettes) with fire before throwing it in the special container for contaminated material. In the small freezer (labelled “A”) in B 221, find the SOC to allow it to rise to room temperature, and the bacteria aliquots (50 µl in purple-cap tubes stored in the – 80 C freezer in B 213, one tube is for two samples) to let them thaw on ice.

5.1. Ligation reaction

Ligation reagents are stored in the DNA lab, freezer A. Use pipettes labelled “cloning” to add the PCR products.

PCR product	1-3 µl
Salt	1 µl
TOPO-Vector	0.7 µl
Add H ₂ O to a total volume of	5 µl

(use 2 ml tubes)
Put in tube, vector last.
Mix gently, let rest for 30 min at room temperature, and then put tubes on ice.

Meanwhile,

1. **Work sterile**, label a set of new sterile tubes (2 ml, use also the purple-cap tubes with bacteria), fetch the bacteria from the -80 °C on ice, let the bacteria thaw on ice and split each 50 µl bacteria into two 25 µl tubes. Keep tubes with bacteria on ice.
2. Thaw SOC medium.
3. Set the water bath at 42°C.

5.2. Transformation and incubation

1. Mix 1 µl ligation reaction in 25 µl of bacteria (mix gently with the pipette tip). Discard the remaining ligation reaction. Use **filtered tips**, you are working with bacteria & PCR products, discard all waste in “bacteria-waste” that is later autoclaved.
2. Let the tubes rest on ice for 30 min (meanwhile, check that the shaking incubator in room B 236 is set at 37°C).
3. Apply heat shock: 45 sec in the water bath at 42°C, keep tubes still.
4. Put tubes on ice for 2 minutes.
5. Add the bacteria to 125 µl SOC at room temperature in large 15 ml sterile tubes, and place the tubes in the shaking incubator for 1 to 1.5 hours at 195 rpm at 37°C.
6. Immediately after placing the tubes in the shaking incubator, add 25 µl X-GAL (stored in freezer A, wrapped with aluminium foil) to the pre-warmed agar plates, spreading it with a bent Pasteur-pipette. Take the plates back to the incubator. **Note:** make sure to check the solvent in the X-GAL – dimethylformamide or DMSO?
7. After shaking incubation, take 75 µl of the mix to the plates, spread it and incubate overnight (< 18 h) at 37°C. Save the remaining bacteria in the cold room, just in case some plates grow too dense.

5.3. DNA extraction (second day)

1. Place the plates in the cold room for 1 h, to allow them to grow thicker.
2. Label a set of tubes and add 150 μl ddH₂O.
3. Pick the colonies at the assign bench in the post PCR lab. Take white colonies to the tubes using an autoclaved toothpick, shaking it to increase yield of DNA (discard toothpicks in the special container for contaminated material). It may be enough with 5-6 clones, but remember that some colonies can contain plasmids that did not incorporate our fragment.
4. Boil the tubes for 3 min or heat in PCR machine for 5 min and place them on ice.
5. Store the tubes (ready for PCR) frozen in the post PCR lab.

5.4. PCR

Template	1 μl
Enzyme	0.1 μl
M13F	1 μl
M13R	1 μl
Buffer	2.5 μl
dNTP	2.5 μl
MgCl ₂	1.1 μl
H ₂ O	15.8 μl
Tot. volume	25 μl

Annealing temperature: 50°C
Extension time depending on fragment length
35 cycles

Use M13F primer for sequencing reaction.

M13F (-20): 5'-GTAAAACGACGGCCAG-3'

M13R: 5'-CAGGAAACAGCTATGAC-3'

5.5. Preparation of plates with LB Medium with AMP (400 ml, for ca. 12 plates)

1. Mix the recipe below in a 500 ml flask, using the magnetic mixer (find the reagents on the top shelf of the chemical cupboard in the PCR room):

NaCl	3.0 g
Tryptone	4.0 g
Yeast	2.0 g
Bacto Agar	6.0 g
Fill up with 400 ml ddH ₂ O	

2. Cap the flask loosely with aluminium foil fixed with autoclave tape, and autoclave it. Meanwhile, find the AMP in the freezer A, and allow it to thaw on ice (you will need 4 ml, about half a tube, for each 400 ml of medium).
3. Let the mix drop to about 50-60°C (when it can be held in the naked hand for a while) on the bench or, if required, in the incubator set at 60°C.
4. Clean the bench with ethanol, light the Bunsen burner and work close to it (always remove the plate lids below the flame level, to avoid contamination).
5. Add 4 ml AMP to the mix using a sterile pipette. Work close to fire.

6. Take sterile plates (seal any plastic bag quickly if you need to open a new one) avoiding exposing them to contamination. Always open them as little as possible, and always close to fire.
7. Pour the medium on the plate (close to fire). Fill up to the level marked by the lid edge. Try to avoid getting bubbles.
8. Let the plates rest overnight on the bench. The day after take them to the cold room to allow them grow thick (place them upside down).

GENERAL PROTOCOLS

1. DNA extraction

1.1 Extracting DNA from avian blood samples

1. Thaw the blood sample (1-40 μ l blood in 500 μ l SET buffer) and spin down. If the blood sample has been stored in EtOH, pick out a piece of dried blood, dry it quickly on a soft paper tissue, add it to a tube and fill up with 500 μ l of SET buffer.
2. Add 13 μ l of SDS (20% in a bottle on the shelf with buffer stock solutions) and 7.5 μ l proteinase K (~20mg/ml, found in a blue box on the top shelf of freezer A). Mix well and spin down.
3. Put samples on water bath (55°C) over night. (Mix samples after one hour may increase yield of DNA, especially if the blood forms clots). ☺
4. Spin down. Add 50 μ l NaCl (5M). Mix well and spin down.

SAFETY

Phenol is classified as acute toxic and will cause skin corrosion. Always work with phenol in the designated hood. Use gloves, and change gloves after working with phenol. Immediately take care of any spill on the bench or elsewhere so your lab-mates are not put at risk.

First aid: Rinse thoroughly with plenty of water. Wipe with polyethylenglycol 400 (big brown bottle in the hood). Moisturize skin with lotion.

5. Take out the required amount of phenol for your samples by transferring it with a plastic measuring pipette to a 13 ml tube, e.g. 10 ml (+ 0.5 ml extra) if you are extracting 20 samples. Pay some attention to the colour; if it is reddish you should consider opening a new bottle, and putting buffer into it. The phenol is stored in fridge B. **Note: The top layer is a buffer. Make sure that you take from the lower phase!!!**
6. Add 500 μ l phenol. Mix well by shaking so that blood/buffer and phenol makes a homogenous mix. Let the samples rest for 40-60 minutes on the bench (in the hood). Shake regularly. If you are looking to extract DNA > 30 kb, consider to use a nutating mixer instead of shaking.
7. Mix again and spin at 11,000 rpm for 15 minutes. Use the assign centrifuge in the hood.

SAFETY

Chloroform is a volatile organic solvent which is classified as cancerogenic, acute toxic and would be hazardous to your health. Work in a hood and use appropriate safety measures like gloves and coat. If spill occurs use Vermiculite, which is then disposed of as toxic waste. Left overs in glass beakers can be left in the hood to evaporate. Be aware of that organic solvents dissolve plastics and is irritating for mucous membrane, respiratory passages, skin and eyes.

First aid: in contact with skin clean with soap and water and moisturize with lotion. In contact with eyes clean with only water. If you inhale, get as quickly as you can into fresh air. When consumed, do not ever evoke vomiting!

8. Label a set of new tubes (2 ml). Pour a small volume of chloroform/isamylalcohol in a beaker and add 500 µl to each tube. Use glass ware with chloroform since it dissolves plastic. Transfer left overs to a tube, put a lid on and put it in the bucket for Chloroform waste. Leave the beaker in the hood to let minor left overs evaporate, rinse it and put it in the dish washer.
9. Use the 1000 µl pipette and pre-cut tips and move the supernatant to the new tubes. **This is where the DNA is. Discard the top layer of proteins that could form in between the phases.** Throw the old tubes, with lids on, containing the phenol phase in a container labelled "Phenol waste".
10. Mix and spin at 11,000 rpm for 15 minutes.
11. Label a set of new tubes (1.5 ml) and add 50 µl of NaAc (3M).
12. Use the 1000 µl pipette and pre-cut tips and move the supernatant to the new tubes.
13. Add 1000 µl of ice cold 95% ethanol.
14. Mix well until you can see a fluff of precipitated DNA.
15. Put in -20°C for at least 1hr or overnight. ☺ **If you plan to keep on working after 1 hr and using the vacuum centrifuge, you should turn on the cooling trap. It takes about 1 hr to get cold.**
16. Spin at 11,000 rpm for 10 minutes. Place the tubes with the labelling facing out from the centre.
17. Locate the DNA pellet and remove the liquid-phase. Work in the hood and put the liquid waste in the assigned waste container.
18. Wash the pellet with 0.5 ml ice cold 70 % ethanol. Extract as much as possible of the ethanol and put it in the same waste container as used in step 17.
19. Dry the DNA pellet in the vacuum centrifuge for 8-10 minutes, or overnight in a cupboard.
20. Dissolve the pellet in approximately 50 µl ddH₂O, or in 50 µl 1xTE buffer. Choose solvent depending on estimated storage time (10-200 µl depending on expected amount of DNA).
21. Put samples in the fridge until ready for DNA-quantification.

SET-buffer: 0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0

1.2 Extracting DNA from skin, hair or feathers

1. Take a small piece of skin/muscle (c 25ng) or feather/hair (c 5 mm from the base). Cut the feather shaft/tissue into smaller strips. Place samples into 1.5 ml tubes with 100 µl of lysis buffer and 1.5 µl proteinase K (~20mg/ml, found in a blue box on the top shelf of freezer A). Mix well.
2. Incubate 3 hours at 56°C, mix again after 1 hour. **If you plan to use the vacuum centrifuge, you should turn on the cooling trap. It takes about 1 hr to get cold.**
3. Spin 10 minutes at 10,000 rpm. Place the tubes with the labelling facing out from the centre.
4. Move the supernatant into new tubes with 10 µl of NaAc (3M) and add 220 µl (two volumes) of ice cold 95% ethanol. Mix well.
5. Spin 10 minutes at 10,000 rpm. Place the tubes with the labelling facing out from the centre.
6. Locate the DNA pellet and remove the ethanol. Remember the orientation of the tubes, the pellet may not be visible.

7. Add 100 µl ice cold 70% ethanol.
8. Spin 5 minutes at 10,000 rpm.
9. Remove the ethanol and let the pellet dry in open tubes in a cupboard over night or in the vacuum centrifuge for 3-5 minutes. Put the ethanol in the assign waste container.
10. Dissolve the pellet in 50 µl ddH₂O, or in 50 µl 1xTE buffer. Choose solvent depending on estimated storage time. Mix thoroughly.
11. Put samples in the fridge until ready for DNA-quantification.

Lysis buffer: 0.1 M Tris, 0.005M EDTA, 0.2% SDS, 0.2 M NaCl, pH 8.5

1.3. DNA extraction on 96-plate with NH₄Ac (ammonium acetate)

Extraction of blood samples, c 20 µl blood kept in 500 µl SET.

1. Thaw the blood samples. If the blood sample has been stored in EtOH, pick out a small piece of dried blood, dry it quickly on a piece of soft paper tissue, add it to a tube and fill up with 15 µl of SET buffer.
2. Mix the thawed samples and spin down.
3. To each tube in the 96 plate add:

	<u>1X</u>	<u>100X (Full plate)</u>
SET buffer	50 µl	5000 µl
Proteinase K(~20 mg/ml)	0.5 µl	50 µl
SDS (20%)	1.5 µl	150 µl
(Add 52 µl of the mix to each tube)		

4. Add 15 µl of the blood+SET buffer mix (the sample) using pre-cut yellow tips. Use plastic caps (not tinfoil!).
5. Vortex heavily.
6. Digest at 55°C with agitation for at least 4 hour (e.g. use the hybridisation oven or a nutating mixer in a heating cupboard).
7. Add 50 µl 4M NH₄Ac to each sample, vortex and leave at room temperature for 15-30 min.
8. Spin at 3300 rpm for 45 min at 10°C to pellet any precipitate.
9. Transfer the supernatant to a new plate.
10. Add 160-200 µl ice cold 95% EtOH to each sample.
11. Mix thoroughly by shaking with a vortex; DNA may be visible at this stage.
12. Spin at 3300 rpm for 45 min at 10° C.
13. Remove the supernatant. BE CAREFUL not to lose the pellet (check the pipette tip before thrown away). Put the ethanol in the assign waste container.
14. Add 160-200 µl ice cold 70% EtOH carefully and remove straight away. Put the ethanol in the assign waste container.
15. Air dry overnight.
16. Dissolve pellet in 50 µl ddH₂O, or in 50 µl 1xTE buffer. Choose solvent depending on estimated storage time. Mix thoroughly.
17. Put samples in the fridge until ready for DNA-quantification.

1.4. DNA Extraction with NH₄Ac (ammonium acetate)

Extraction tested for of avian blood stored in 500 µl SET-buffer.

1. Take 250 µl of the blood+SET buffer mix (include as much of the slime and clots as possible in the sample). If the blood sample has been stored in EtOH, pick out a piece of dried blood, dry it quickly on a soft paper tissue, add it to a tube and fill up with 250 ul of SET buffer.

7 µl 20% SDS.

5 µl 20 mg/ml Proteinase K.

Shake the sample with the two-stand-method (vortex works, but shaking is better) and spin down.

2. Digest in a water bath at 56°C overnight.
3. Add 250 µl of 4M NH₄Ac solution to each sample. **If you plan to use the vacuum centrifuge, you should turn on the cooling trap. It takes about 1 hr to get cold.**
4. Shake WELL and leave at room temperature for at least 40 minutes (60 min better), shaking regularly.
5. Spin sample at 13 000 rpm for 15 minutes.
6. Pour the supernatant into a new labelled 1.5 ml tube (watch out not to get the slime), do not use a pipette.
7. Add 1000 µl of ice cold 95% EtOH using a pipette (tube will be full here).
8. Mix well by shaking. The DNA may be seen at this point. If you need to take a break, this would be a suitable step in the protocol. Put the samples in -20°C until you will be able to continue.
9. Spin the tubes at 13 000 rpm for 15 minutes. Organize the tubes with the labelling facing out from the centre.
10. Pour off the supernatant, being careful not to lose the DNA-pellet. Add 0.5 ml ice cold 70% EtOH to rinse the pellet and remove it straight away (careful with the pellet). Put the ethanol in the assign waste container.
11. Air dry the samples in a cupboard overnight, or in the the vacuum centrifuge for 3-5 min.
12. Add 100 µl ddH₂O and vortex. Let the sample dissolve in the fridge overnight.
13. Quantify the samples.

4M NH₄Ac 100 ml pH 7.5

30.83g NH₄Ac

100 ml dH₂O

Autoclave

pH, if necessary, with glacial HAc (Acetic acid).

2. Quantification of DNA

2.1 The Nano-drop

1. Start by cleaning the reader: Open the arm of the Nanodrop and apply 5 μl ddH₂O to the scanning plateau (bottom). Close the arm and leave for 2 min. Wipe carefully with a soft paper tissue.
2. Open the Nanodrop program on the computer. Select “Nucleic acid”.
3. To blank, apply 1 μl blank solution, either ddH₂O or 1xTE, depending on what your samples were dissolved in, and close the arm. Hit “Blank” (or the F3 key). Wipe carefully with a soft paper tissue after reading. This can be repeated at intervals if you are quantifying a large amount of samples. (Use “Blank”, not “Reblank”.)
4. Apply 1 μl of your sample and close the arm. Type your sample name and hit “Measure” (or the F1 key). Wipe carefully with a soft paper tissue, both top and bottom. Repeat for all of your samples. If you have many samples we recommend that you perform a cleaning every 20 samples.
5. Click “Reports”. Select all your measurements by highlighting them in the list, or Ctrl+A. Choose Export as xml file. Export and name your file, which can be read into Excel.
6. End by doing another cleaning procedure, same as in the beginning.

As a benchmark, DNA of good quality should have a 260:280 ratio between 1.80 and 2.0. Note, however, that the Nanodrop cannot readily indicate if the DNA is degraded (fragmented). If you have reason to suspect this, or if you need to check quality carefully, please run c 100–150 ng DNA with 1 μl stopmix for c. 50 minutes in a 0.8–1.0 % agarose babygel with maximum two lanes.

2.2 The Qubit

Assay kits can be found in the sample fridge in the DNA lab and in the drawer underneath the telephone. In this drawer you will also find the special 0.5 ml tubes for the Qubit.

On the computer in the DNA lab you can find the protocols to make the Qubit working solutions and the standard dilutions.

Use a **clean plastic tube** each time you make Qubit working solution. **Do not mix the working solution in a glass container!**

You **cannot** measure **DNA** and **RNA** from the **same tube** and working solution. If you want to measure both, **two working solutions** need to be made, and a minimum of 2 μl of your sample is needed.

First, guesstimate the amount of DNA or RNA you are expecting:

- If your DNA double stranded and between 10 pg/ μl to 100 ng/ μl then use the dsDNA HS assay kit; otherwise the dsDNA BR assay kit.
- If your DNA is single stranded use the ssDNA assay kit.
- If RNA is between 250 pg/ μl to 100 ng/ μl use the RNA HS assay kit. The RNA BR assay kit works between 1 ng/ μl to 1 $\mu\text{g}/\mu\text{l}$.

Make the working solution according to the specific protocol for your assay kit. The Qubit does not need more than 1 µl of the original sample. Anything between 1 and 20 µl of your sample you can add to the 0.5 ml tubes.

After you made your samples according to the protocols, you can start the measurement. Start the Qubit by touching the screen. Choose either DNA or RNA, and then choose either HS, BR or ssDNA depending on the assay kit used. The standard screen is shown, press “Yes” to run a new calibration or press “No” to use the last calibration. Insert the tube containing Standard #1 in the machine, close the lid, and press **Read**. After the measurement remove the sample and put Standard #2 in the machine, close the lid and press read.

After the standards are read the samples can be measured. Put in sample and press read. The concentration of the assay tube is given. Upon completion of the sample measurement, press **Calculate Stock Conc.** to calculate the concentration of your original sample.

Using the volume roller wheel, select the volume of your original sample that you have added to the assay tube. It will immediately calculate the concentration of your sample.

The units in which the original sample concentration is displayed can be adjusted by, pressing **ng/ml**. A pop-up window showing the current unit selection (as indicated by an adjacent red dash) opens. Select the unit needed by touching the desired unit in the unit selection pop-up window. To close the unit selection pop-up window, touch anywhere on the screen outside the pop-up.

To save the data from your calculation put a USB stick in the Qubit, press **Save** on the dilution calculator screen. The last calculated value of your measurement will be saved as a .CSV file and tagged with a time and date stamp.

3. Polymerase Chain Reaction

3.1. Reagents in PCR

3.1.1 Reagents in regular PCR

A PCR reaction contains the following necessary reagents:

PCR-buffer. Salt and pH-stabiliser. User stock (10X) is distributed along with the Taq DNA.

MgCl₂ Salt which is required for the Taq polymerase to work. The standard rxn concentration is 1.5 mM (range 1-4). Higher concentration makes the Taq polymerase less specific and favours amplifications of short fragments. Too much of MgCl₂ often results in multiple bands. User stock (25 mM) is distributed along with the Taq DNA polymerase.

dNTP's. Free nucleotides (G's, A's, T's and C's) of which the artificial DNA copies are made. User stock (10X, 1.25 mM) is made from the four nucleotides (12.5 µl of each) in 950 µl of ddH₂O. The nucleotide stocks are stored in the same compartment as the Taq and AFLP reagents in the freezer in room B222.

Primers. Single stranded DNA (oligonucleotides), usually the length of 18-30 nt. Primers used for RAPD are normally shorter, 10-15 nt. Stock solutions at 100 μ M are normally stored in the -80° freezers, user stocks at 10 μ M with your PCR reagents at -20°C.

Taq DNA-polymerase. The enzyme that puts the free nucleotides together. It starts at the 3' end of the primer, and uses the complementary DNA strand as a template. The user stock is stored in the freezer in room B222.

Template DNA. The source of DNA for the PCR amplification. This could be DNA extracted from blood, skin, feathers, or PCR products. We use a standard concentration at 25ng/ μ l but depending on organism and protocol might need further adjustments (5-100 ng/ μ l).

Table 3a. Suggested concentration and volumes for PCR, using AmpliTaq DNA Polymerase

Reagents	25 μ l rxn (standard)	10 μ l rxn (standard)
ddH ₂ O	15.4	5.52
MgCl ₂ (25 mM)	1.5	0.6
PCR 10X buffer	2.5	1
dNTP's	2.5	1
Primer Forward (10 μ M)	1	0.4
Primer Reverse (10 μ M)	1	0.4
Taq DNA-polymerase (25 u/ μ l)	0.1	0.08
Template DNA (25 ng/ μ l)	1	1

In addition to the reagents listed in Table 3a, you may find that researchers are adding other reagents to their reactions in hope to get better results:

BSA - bovine serum albumin. Prevent binding of DNA to the test tube. Recommended concentration is 10-100 μ g/ml.

DMSO - dimethylsulfoxide. Increase linearity of DNA (to prevent formation of secondary DNA structure). Recommended concentration is 1-10% DMSO.

3.1.2 Reagents in Qiagen hotstart PCR

Using Qiagen Multiplex Mastermix, all PCR reagents except for the primers are included in the provided solution ("2x Qiagen Mastermix"). The Taq polymerase is enclosed in microscopic lipid droplets, and are released after an initial heating phase. Therefore, the PCR mix is inert, and does not require PCR setup on ice. In fact, you may prepare PCR plates in room temperature and store them in a freezer until you want to run the PCR's.

Recommended starting amount DNA is 10 ng. This can be adjusted, but performance is hampered if too much DNA is included.

Table 3b. Suggested concentrations for PCR reactions with hotstart Taq (Qiagen Multiplex Mastermix)

Reagents	10 μ l rxn (standard)
ddH ₂ O	2.6
2x Qiagen Mastermix	5.0 (vortex before use)
Primer Forward (10 μ M)	0.2
Primer Reverse (10 μ M)	0.2
Template DNA (5 ng/ μ l)	2

In addition to the reagents listed in Table 3b you could also add Q-solution, which is included in the kit from Qiagen. It facilitates amplification of difficult templates by modifying the melting behavior of DNA.

3.2. Set up a PCR

PCRs should only be set up in room B222, the set-up PCR room. Always use the set of pipettes which are exclusively assigned for PCR set ups, and always work on ice.

1. Fill in a PCR protocol and calculate how much you need of your master mix/cocktail (number of rxn + 10%). Don't forget the rxn for the blank (negative control). The master mix should contain everything except the template.
2. Clean the PCR bench.
3. Thaw template DNA and all reaction reagents on the PCR bench, except the Taq polymerase, which should be in the freezer until needed (it contains glycerol and need no thawing).
4. Program the PCR machine while the reagents are thawing.
5. Mix and spin all reagents. Keep on ice, if not using a hotstart Taq.
6. Place an appropriate number of 8 strip microtubes in a PCR tray or use a 96 plate for larger number of samples. If 6 rows or more, use a plate.
7. Make the cocktail in a 1.5 ml eppendorf tube (snap-cap). Observe the end volume, would it fit or do you need a bigger one? Start with adding the ddH₂O, and save the adding of the Taq DNA polymerase as the last step. Mix by using the up to 200 μ l pipette set at 150 μ l and pipette up and down a few times. Do not centrifuge the mix at any time, the mix will separate and the enzyme will not get in contact with the template. **Note:** hotstart Taq PCR mixes, unlike regular ones, should be mixed with vortex.
8. Dispense an appropriate volume of master mix into each of the rxn tubes (total rxn minus amount of template DNA, e.g. 24 μ l). Here you can use the same tip for all tubes.
9. Add the template (change tip between samples!!!). Mix by pipetting up and down a few times. **Do not vortex if regular PCR.**
10. Place tray on ice (regular PCR) and start the PCR machine. Load the tubes, or plate, when heating block is approximately 80°C. Close the lid. Place the tray on the PCR machine with appropriate markings (date+name).

3.3. Programming the PCR machine - choosing a temperature profile

We have one Bioer LifePro, four GeneAmp 9700, one Stratagene Mx 3005P (qPCR) and two BIORAD CFX96 RealTime (qPCR) in room B215, the PCR room. Next to each machine is a booking list with slots for each hour during a working day. Book your planned PCR time (maximum two active bookings!). Don't forget to remove your name from the list if you realise that your plan has to be changed!!!

3.3.1 PCR profiles for regular PCR

The standard PCR starts with a heating phase of 2 minutes at 94°C, to make the template DNA single stranded (denaturated). Then follows the cyclic phase which characteristically consists of three different steps:

- a) 94°C. This is again the *denaturing* step which initiate all cycles and is normally set between 30-60 sec.
- b) 37-70°C. The *annealing temperature* when the primer is allowed to settle on the template DNA. This step is usually set between 30-120 sec. The chosen temperature depends on the melting temperature, T_m , of the primer (length and GC-content).
- c) 72°C. The *elongation temperature* which is the optimal working temperature of the Taq DNA polymerase. This step is set between 5-500 sec depending on the length of the desired fragment. A rule of thumb is that the Taq polymerase builds about 1,000 nucleotides per minute.

The number of necessary cycles usually varies between 20-40 depending on template DNA concentration, quality, length of product and above all, empirical experience with the focal reaction.

The reaction is normally ended with a 10 min phase at 72°C. This will allow the Taq polymerase to add a protruding A at the 3' end of the fragments. This step is very important when cloning the PCR fragments by means of TA-cloning.

3.3.2 PCR profiles for hotstart Taq PCR's

The standard PCR starts with a heating phase of 15 minutes at 94°C, to activate the Taq Polymerase and to make the template DNA single stranded (denaturated). Then follows the cyclic phase which characteristically consists of three different steps:

- a) 94°C. This is again the *denaturing* step which initiate all cycles and is normally set between 30-60 sec.
- b) 37-70°C. The *annealing temperature* when the primer is allowed to settle on the template DNA. This step is usually set between 30-120 sec. The chosen temperature depends on the melting temperature, T_m , of the primer (length and GC-content).
- c) 72°C. The *elongation temperature* which is the optimal working temperature of the Taq DNA polymerase. This step is set between 5-500 sec depending on

the length of the desired fragment. A rule of thumb is that the Taq polymerase builds about 1,000 nucleotides per minute.

The number of necessary cycles usually varies between 20-40 depending on template DNA concentration, quality, length of product and above all, empirical experience with the focal reaction. The reaction is normally ended with a 30 min phase at 60°C.

4. Baby gel electrophoresis

4.1. Running baby gels

We use 2% agarose gels to check the results of the PCR. This is performed in the post PCR room, C243. The agarose solution is stored in the heat cupboard, by the fridge. As a rule, make more than one bottle of 2% agarose solution at a time. There are 2 ways of staining the gel; putting it straight into the hot agarose or staining it in a bath before running. The following instruction includes both.

1. Put together a mould. Pour in agarose solution, alternatively add 5 µl GelRed (15 µl for a 96 gel), spread it evenly, remove any bubbles and put in combs. Cast as many gels as you will need. Let it sit for approx. 20 min in the mould.
2. When ready, and if not already stained, soak the gel in a GelRed bath. Keep the bath in the bottom drawer just below the electrophoresis cells. Make a bath by mixing:

50 ml dH₂O
15 µl GelRed

This should be enough to stain 3-4 gels, one at a time. Write the starting date and the owner of the bath on the lid, and make a mark for each gel. Let it soak for a minimum of 1 h.

3. When ready, move it to one of the mini cells (with the wells towards the windows/ the cathode/ black cord). If there is any piece, i.e. row, that you will not use, and if it is enough for another round, it is ok to put it back in the bath.
4. Take a microtiter plate and add 1 µl of stopmix/loading dye in the number of wells corresponding to the number of your samples. If you find the stop mix to sticky, dilute it. Increase the volume of stop mix with the same number as you diluted the mix, i.e. dilute it 2 times, take 2 µl of stop mix.
5. Add 2.5 µl (if not otherwise stated) of the final PCR reaction to each well (change tips between samples).
6. Add 6 µl of a DNA ladder (1kb Plus DNA ladder, stored in the door-shelf in the fridge) to each of the "ladder wells".
7. Load the first and the last well of each row with ladder, and the in between wells with your samples (PCR rxn + stopmix).
8. Put the lid on the electrophoresis chamber.
9. Turn on the power supply.
10. Adjust the voltage (80 V) and let the gel run for 20-30 min.
11. When finished, examine the gel with the camera(p. 28), the scanner (p. 29) or on a UV light board (in the lion/wolf room).

12. Clean the glass plate with water and then 70% Ethanol, using a Kimwipe. Close the lid/hatch to the scanner/imager.
13. Discard the gel in the waste container for agarose gels (below the sink).

4.1.1. Recipe for 2% agarose gels

Make 300 ml by mixing in a 500 ml Pyrex-bottle

1. Place a beaker on a balance, tare, and add 6.0 g agarose (sample fridge, room B221). Add this to a bottle.
2. Add 15 ml 10X TAE-buffer and 285 ml dH₂O.
3. Cap the bottle with the lid loosely and boil in the microwave oven, in post PCR room, for repeated 30 s period until the agarose is melted and dissolved. Use the blue heat-protection glove when handling the hot bottle.
4. Place the bottle/bottles in the heat cupboard by the fridge in the post PCR room, C243, (65°C) for 1 h before usage.

4.1.2. Electrophoresis buffer 0.5X TAE

The buffer can be reused several times, but should be replaced every second week. Mix 25 ml of 10X TAE with 475 ml dH₂O. Put the old buffer in the assign waste container.

4.1.3. Stopmix / loading dye

0.01 M EDTA

15 % Ficoll (polysaccharide that makes DNA sink in the well)

0.25% brome phenol (blue colour, migrates fast)

0.25% xylene cyanol (blue colour, migrates slow)

To make 50 ml:

0.125 g brome phenol

0.125 g xylene

7.5 g Ficoll

1.0 ml EDTA (0.5 M)

Distribute in 2 ml tubes and store at +4°C. If you suspect that the colour will shade your product it is possible to modify the recipe.

5. Precipitate PCR products

We do this to get rid of excess of salt, free nucleotides and primers. Ammonium acetate has the advantage over sodium acetate that any residuals that may remain after precipitation and washing, has less effect on the activity of the Taq DNA polymerase. By keeping ethanol concentration relatively low and temperature high (room temperature), we can prevent short

DNA fragments from precipitating, a so called differential precipitation. By this mean, primers and primer-dimers can easily be removed with relatively little loss of the target fragment. However, there are other applications when we want to retrieve even the shortest fragments, e.g. when precipitating a sequencing reaction. Then, EDTA and ice-cold ethanol is the desired protocol.

5.1. Differential precipitation with NH₄Ac (ammonium-acetate) (96 plate)

This involves work with PCR products and should therefore be carried out in the post PCR room (C243). Trash gloves when done, so to avoid getting PCR products on places were these can be potential contamination risks.

1. 25 µl rxn:

add 11 µl 8M NH₄Ac, and 37.5 µl 95% ethanol (room temperature). If you use 99% ethanol, instead add 33 µl. Can be pre-mixed.

10 µl rxn:

add 4.4 µl 8M NH₄Ac, and 15 µl 95% ethanol (room temperature). If you use 99% ethanol, instead add 13.2 ul. Can be pre-mixed.

Full plate: 1.2 ml 8 M NH₄Ac and 3.895 ml 95% ethanol (can be mixed).

Full plate: 460 µl 8 M NH₄Ac and 1.56 ml 95% ethanol (can be mixed)

2. Put lids/sealing foil on and mix by a gentle shake with the vortex.
3. Let rest at room temperature for 15 minutes.
4. Spin at 3300 rpm for 30 minutes (remember balance) at 4° C.
5. Remove caps and put the plate upside down on a paper towel and knock it gently against the towel to remove the ethanol.
6. Add 50 µl 70% ice-cold ethanol.
7. Put the plate upside down on a paper towel and knock it gently against the towel to remove the ethanol
8. Put the tray upside down (on a paper towel) and spin 1 min. at 3000 rpm and 4°C.
9. Add ddH₂O (5–25 µl depending on the PCR success, i.e. how strong the bands were on the gel).
10. Vortex well, rest for 5 min, vortex again, and spin down. Samples are now ready for a sequencing reaction (as standard take 2 µl PCR product as template).

6. Sequencing with BigDye™ terminator cycle sequencing kit

The trick is that the terminators are labelled with different colours for each of the four nucleotides (G, A, T & C). This means that we can do the reaction in one tube and yet resolve the sequence.

6.1. Procedure

This involves work with PCR products and should therefore be carried out in room C243, post PCR lab. The exception is the master mix, which should be set up in room B 222, set up PCR. Work on ice!!

Reagents	Quantity
ddH ₂ O	5.0 µl
5xBuffer	1.5 µl
Primer (10 µM)	0.5 µl
Template (40-100 ng)	2 µl
BigDye* Terminator Ready Reaction Mix	1 µl
Total	10 µl

1. Thaw the primer, template and Ready Reaction Mix and place on ice.
2. Arrange an appropriate number of PCR tubes on a PCR tray, and use a PCR Protocol to record the ID of the different samples, and to calculate how much you need of each reagent.
3. Make a master mix of all reagents except the template.
4. Transfer the master mix to the post PCR room.
5. Distribute 8 µl of the mix in each of the tubes.
6. Add the template to each tube.
7. Put caps on and, if need be, fix the tubes on the tray with the special device and cap.
8. The default program (#20 on the 9600) on the PCR machine has the following temperature profile:

[96°C - 10s, 50°C - 5s, 60°C - 4 min] x 25 cycles

The annealing temperature can be adjusted depending on template and primer (between 50 and 55°C). Place a tray on the PCR machine with the appropriate markings (name+date).

- The BigDye mix is sensitive to light - avoid light exposure by keeping tinfoil around the tube.
- 5xBuffer is made from 10xPCR buffer (100µl), 25 mM MgCl₂ (60µl) and H₂O (40µl). You will find ready-to-use ones in the fridge in room B221.

6.2. Precipitate sequencing reaction (96 plate)

In this protocol we want to retrieve even the smallest fragments and uses EDTA instead of NH₄Ac and a longer centrifugation time. This involves work with PCR products and should therefore be carried out in the post PCR room.

1. For 10 µl rxn, add 2.5 µl EDTA (125 mM) and 35 µl 95% ethanol. If you use 99% ethanol, instead add 30 µl.
 - For a full plate: 275 µl EDTA and 3.85 ml 95% ethanol (can be mixed).
2. Put lids/sealing foil on and mix by a gentle shake with the vortex.
3. Let rest at room temperature for 15 minutes.
4. Spin at 3300 rpm for 45 minutes at 4° C (remember balance).

5. Remove caps and put the plate upside down on a paper towel and knock it gently against the towel to remove the ethanol
6. Add 50 µl 70% ice-cold ethanol.
7. Put the plate upside down on a paper towel and knock it gently against the towel to remove the ethanol
8. Put the tray upside down (on a paper towel) and spin 1 min. at 3000 rpm and 4°C.
9. The dried tubes are now ready for sequencing.

6.3. Sequencing on the ABI 3100

This 8 capillary sequencing robot (Microbial Ecology, room B240) has a capacity of approximately of 96 sequences per 24 hours, depending on the length of the fragments. Anna Sterngren operates this facility. Samples in PCR-strips (precipitated and dry pellets) should be placed in the freezer in B240 and the required information emailed to Anna.Sterngren.lu.se.

7. Polyacrylamide Gels

7.1. Casting polyacrylamide gels

SAFETY

The mixture used for making the gels contains acryl amide. This substance is toxic, cancerogenic and can cause hereditary genetic damage. Hence you should not work with this chemical if you are trying to get, or are, pregnant. Work in hood and use thick lab gloves, coat and, if needed, glasses. If spill occurs use Vermiculite and put that in a container labelled “toxic waste, acryl amide”.

First aid: contact with skin or eyes, rinse well with water. Dispose of any contaminated clothes. If consumed, drink plenty of water/milk and induce vomiting. If inhaled, move the person to fresh air in a warm place and let her/him rest.

1. Clean glass plates with 70% ethanol. Make sure that no spots are left; they will cause problems when casting the gel. Make also sure that the spacers and comb are clean. Air-dry for a couple of minutes.
2. Place spacers (0.4 mm) along the sides of the eared plate, put the squared plate on top. Fix position with two clips on one side. Attach tape around sides and bottom, try to make it airtight.
3. Do all the work involving liquid polyacryl amide in the hood in the eastern compartment of room C243. Measure 24 ml UreaGel-6 in a 50 ml measuring glass and 6 ml UreaGel-buffer in a 10 ml measuring glass (or twice as much for two gels). Dispense 120 µl APS (ammonium persulfate) in a 250 ml beaker. Pour the two liquids in the beaker and swirl around to mix. APS triggers the polymerization and the gel-solution must be poured within 10 minutes.
4. Pour gel-solution between the glass plates, put shark teeth comb in place (upside down) and fix with 6 clips along the 2 long edges. Let sit for at least 2 hours.
5. Rinse glass ware in the hood, pour the waste water in the appropriate waste buckets, and bring them back to the dish washer.

6. If you need to keep the gel to the next day, wrap it in saran wrap and put it in the cold room. It could be stored for a maximum of 48 hours.

7.2. Running polyacryl amide gels

SAFETY

The samples contain form amide (CH_3NO), a medium-strength toxin for mammals (LD_{50} on rabbits, external exposure, is 17 g / kg). According to the product information sheet from the manufacturer, exposure of form amide during pregnancy might cause **malfunctional foetus development**. The boiling temperature of form amide is high (210°C) which means that evaporation is a minor problem. After heating the samples, however, do not open the tubes until they have rested on ice for a few minutes. The quantity used per sample in this protocol is very small ($<0.00001\text{g}$) so standard lab safety routines (use of gloves and lab-coat) should sufficient to prevent hazardous exposure.

First aid: On skin: wash with soap and water.

In eyes: wash with water for 10-15 min.

In stomach: consult a doctor immediately – don't provoke vomiting.

1. Remove tape and comb from the glass plates and wash plates gently with cold water to get rid of polyacryl amide spill. Dry with paper towel.
2. Mount the glass plates on the rig (2 clips and 1 holder for the top). Fill the top and bottom buffer tanks with 1xTBE buffer. Start power supply and run the gel for 20 minutes (30 Watt).
3. Heat samples in a PCR machine (program 95 on the 9600) at 95°C for 3 min. Put samples immediately on ice when finished.
4. Turn off power and rinse the well with a syringe. Put the comb in place.
5. Load samples ($3.5\ \mu\text{l}$) and turn on power (30 Watt).
6. Run samples for 1-2 hours (first blue corresponds to 11 bp, second to 98 bp).
7. Start the Typhoon Scanner (p. 28).
8. Turn off power supply.
9. Empty the upper buffer tank using the syringe.
10. Clean the glass plates with 70% ethanol. Place the gel/glass plates on the glass platen. Scan the gel on the Typhoon Scanner (p. 28).
11. Put the gel back on the rig if you are going to use it a second time. If not, separate the glassplates **CAREFULLY**. Throw the used polyacryl amid gel in the labelled container under the sink and wash the plates **WITHOUT** using detergents. Inspect that no acrylamid is left on the plates. Place plates above the sink for drying and when dry, move them to the labelled bench drawer for storage. Empty the buffer tanks, put the used buffer in the assigned waste container.

7.3. Recipe for form amide dye

Mix in a small beaker in a hood (**form amide is toxic**)

10 ml form amide

10 mg xylene cyanol ff

10 mg bromphenol blue
200 µl 0.5 M EDTA pH 8.0

8. Taking pictures/scanning gels

8.1 BioRad camera

The BioRad Molecular Imager is located in the post PCR room (C243). It is a UV-based imager, so take precautions when working with the lightboard (further instructions below). The imager can be turned off/on with a switch on the backside left, but the camera is powered separately and should generally be left plugged in. After use, clean the glass with extra-soft paper tissue and water and ethanol (water is not enough).

1. Turn on the imager (if not already on).
2. Pull out the lower compartment, which holds the glass. Place your gel on the center of the glass.
3. Open the software ImageLab on the computer. On the Start Page, select a protocol from Recent Protocols (or Open...):
 - a. For baby gels: Babygel_GelRed_intense
 - b. For 96-well gels: Large96gel_GelRed_intense
 - c. If you know beforehand or realize after a first scan that your bands are very faint, select the `_faint` versions of the protocols.
4. When the protocol dialogue window opens, click on the yellow button Position Gel. Adjust the position of the gel manually by opening the main front hatch, and viewing the position on the screen.
5. Click on the green button Run Protocol. This will run auto-exposure photography and present a gel picture. (If you are prompted to move the filter to position Filter 1, double-check that the filter (top of the machine, right side) is set in that position.)
6. The default settings give an image in which the background is black and the bands are light. If you want to invert that to look like the Typhoon scans, click the Image Transform icon in the image window, tick Invert image display, and adjust High/Low/Gamma if necessary.
7. Save your gel image by selecting File > Save As. Save in a folder with your name, placed under Documents/GelScanning/Scans/.

For simple printing and/or exporting of your image:

1. Click the Print icon and tear off the printed heat paper for your lab book.
2. For saving/using a digital copy, click the Screenshot icon, select Current Image View, and To File, and save in your personal folder (see above) in TIFF/JPG/PNG/BMP format.
3. Transfer it to a USB drive and have fun!

The software can do a lot more than just producing an image:

- Adjustments – rotate/flip (Image Tools)
- 3D, colour schemes
- Lanes and bands
- Show/hide all

8.2 Typhoon Scanner Instrument

The Typhoon is placed in the western compartment in room C243. The Typhoon instrument is a variable-mode imager that produces digital images of radioactive, fluorescent, or chemiluminescent samples.

1. Turn on the Typhoon instrument and allow 5-10 minutes for the instrument to heat up the lasers and the system components. The on/off switch is on the right side of the instrument.
2. Log in on the computer: Enter username: zoo_dna, password: 85174scan
3. Double-click on the Typhoon Scanner Control shortcut icon on the desktop.
(Alternatively you can start the Scanner Control software using the Start menu)
4. In SCANNER CONTROL window, choose **Template, Load** and choose tray template; MicrosatFAM / Baby-Gel / etc. to get the right grid area.
5. Place the gel on the glass in the Typhoon instrument.
Open the lid on the Typhoon and place the agarose gel directly on the glass in the lower left corner, A1. Check the grid coordinates on the glass platen and make sure you have the same grid area in Scanner Control window. Close the lid. If you scan a microsatellite gel, dry the gel plates with a Kimwipe, place the glass plates on the glass platen in the lower left corner, A1. Check the sample orientation in the Scanner Control window!!
6. Choose scanner control parameters in the Scanner Control window;

	Baby-Gel	Microsat
Acquisition Mode	Fluorescence	Fluorescence
Set up:		
Emission Filter	610 BP, Deep Purple, SYPRO Ruby, EtBr	Depending on dye For FAM :526 SP Fluorescein, Cy2, AlexaFluor 488
PMT voltage	450	450
Laser	Green (532)	Green (532)
Sensitivity	Normal	Normal
Orientation	R	Я
Pixel Size	200 (<i>50 data points/cm and 100 data lines/grid square</i>)	100 (<i>100 data points/cm and 200 data lines/ grid square</i>)
Focal Plane	+3 mm (or Platen)	+3 mm

7. In the Scanner Control window, type comments you want saved with the image.
8. Click **SCAN**, type a file name and click **Save**.

9. The Image Quant Preview window appears. While the instrument scans the sample, a preview image of the sample appears in the window.
10. Scan Completed Successfully. Please wait while.....
11. Image Quant window opens automatically.
12. **View** actual size. Choose **Gray/Color Adjust**, second from the right on the list.
13. In the **Gray/Color Adjust** window place the cursor on the high scale and drag to the left to increase blackness of the bands. Click Apply. To increase lightness put the cursor on the low scale and drag to the right. Apply. Click OK to close the window.
14. Close the Image Quant window.
15. Remove the gel from the instrument and clean the glass platen with a KimWipe. Use only dH₂O. Finish up by wiping the glass platen with some EtOH. Close the lid.
16. Close the Scanner Control window. Turn off the instrument by pressing the on/off switch on the right side of the instrument.
17. LOGOFF / OK

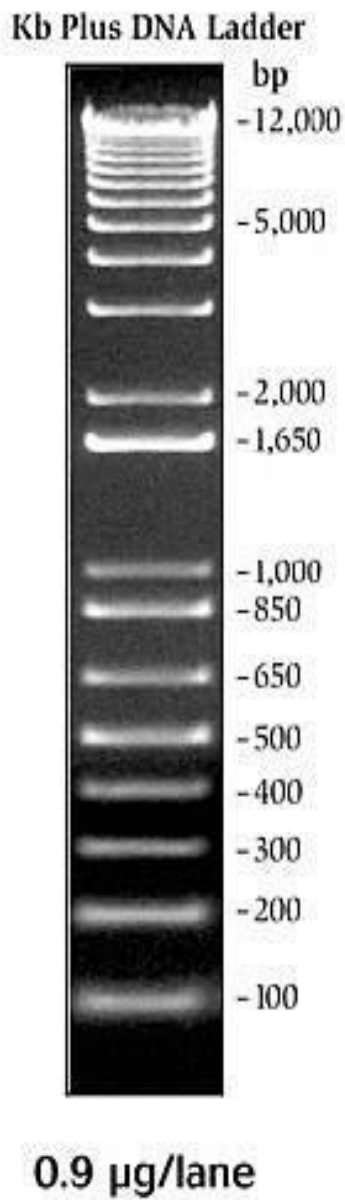


Figure 2. Length of fragments in 1 Kb Plus DNA ladder