Laboratory protocols for the practical part of the course

Methods of molecular biology in plant ecology and systematics

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This set of laboratory protocols has been created for the practical part of the course. It does not contain all protocols and modifications used in our laboratory nor is it the set of general protocols of the methods included. The protocols are written for the specific material (plant species) that will be used during the practical course and some optimization will often be needed when working with other species.

General laboratory and safety rules

- **Common sense is needed in a laboratory. Always take care of and make an effort to (1) not harm yourself nor your colleagues, (2) not damage laboratory equipment, (3) not degrade your as well as colleagues' samples and material.**
- Never eat, drink, or smoke in the laboratory.
- Use laboratory (indoor) shoes.
- Use adequate lab clothing; use protective gloves and other equipment when needed (working with poisonous substances, GMOs, etc.)
- When working with GMOs, always follow the specific working protocols, make a proper record of your experiments and handle the waste according to GMO-specific rules; only authorised persons may work with GMOs.
- Keep your work space clean. At the beginning of work, clean your table with 2% bleach and pipettes with 70% ethanol; after finishing the work, clean your table with 2% bleach and wash all used laboratory glass (tap water + distilled water).
- Be careful when working with electric equipment (e.g. electrophoresis), always switch power supplies off before any manipulation with the material, gels, wires, etc.
- With volatile compounds work only in a fume hood (e.g. chloroform, isoamyl alcohol, isopropanol, 2-mercaptoethanol, TEMED, concentrated acetic and hydrochloric acid).
- DNA stains (SybrGreen, GelRed, loading buffers containing the stains ("GelRed" and "LB+SG"), DNA ladders) are potential mutagens (although claimed harmless). Always use protective gloves, never touch any equipment with contaminated gloves, store the waste (gels) in the special containers; gels stained with GelRed may be recycled. Wash all contaminated equipment immediately (gel beds, combs, etc.).
- Acrylamide is highly poisonous (neurotoxin). Work with extreme care, always use protective gloves and coat. Store any waste material in a special container (must not be disposed among ordinary waste!), wash all equipment with plenty of water. Stock solution is stored in a fridge (2–8°C) in a special bottle.
- When working with DNA, always use sterile (sterilized) plastic (pipette tips, tubes…).
- For material extremely sensitive to contamination (historical DNA samples, non-specific primers, common stock solutions, etc.) always use filter (barrier) tips.
- Never touch sterile plastics with hand (gloves); for PCR strips / tubes always pour the needed amount from the storage bottle on the clear table, store any excess in a separate bottle and never return it back into the sterile storage bottle.
- For PCR, DNA sample dilution, primers, etc., use always sterile water.
- Keep DNA, primers or enzymes on ice; some enzymes and their buffers (esp. ligase, restriction endonucleases, some types of DNA polymerases) are extremely sensitive to heating.
- Always lock the laboratory when you are the last person leaving.

Contents

DNA isolation

A) DNA isolation using the Macherey Nagel NucleoSpin Plant II Kit

The method is based on a patented technology. It allows rather fast and efficient isolation of high quality genomic DNA from fresh, dry or frozen plant tissue. The DNA is bound to the silica membrane, washed and finally eluted with low salt buffer or sterile water. DNA binding / release are driven by altering the salt concentration and pH.

Protocol for dry material (e.g. silica-dried samples)

→ *see page 2 for modifications with other type of material*

General information

- The following version is the most frequently used protocol. Individual steps (amount of starting material, number of washes, etc.) may be optimized according to the material used. Two lysis buffers are provided. The buffer PL1 is preferred as it has been verified to work for wide range of taxa.
- Make sure that ethanol has been added to the wash buffer PW2 before the first use (check the label on the bottle). If not, add the required volume of 96% or 100% ethanol.
- Decant the waste liquids into a beaker.
- Collect used microtube pestles into a beaker with bleach (SAVO). When finished, wash with detergent and rinse with distilled water. Collect used steel beads in a Petri dish, rinse with water and dry with a paper towel. Do not leave the beads in water as they may corrode!
- Transfer the required amount (for all samples) of elution buffer (PE) into a sterile 1.5 ml Eppendorf tube and preheat at 65°C on heat block.

Protocol

1) Transfer dry material (5–15 mg, not more than 20 mg) into the 1.5 ml Eppendorf tube and add 2 steel beads (or use more beads for resilient tissues).

   ! *The optimal amount of the starting material depends on the taxon analysed. Using too much material may decrease the efficiency of DNA purification. If possible, use similar amounts across all samples analysed.*

2) Place the tubes into the white adaptors of the Retsch 400MM mill. Insert the adaptors into the mill (use both adaptors to counterweight the machine). Grind the samples for 1 min. at maximum speed (or increase the time for resistant tissues).
3) Add 400 µl of lysis buffer PL1. Close the tubes, mix thoroughly (!) by flicking or vortexing and incubate for at least 10 min at 65°C (at shaking incubator; if using a heat block instead, mix the tubes occasionally).
5) Place the NucleoSpin Filters (violet ring) into new 2.0 ml collection tubes (both provided in the kit).
6) Load the lysate onto the filter. (If the lysate contains a lot of plant debris, centrifuge the tubes for 1 min at 12 000 rpm and transfer only the supernatant). Transfer the steel beads into a Petri dish, wash with water and dry with a paper towel.
7) Centrifuge for 2 min at 12 000 rpm (or longer if necessary). Transfer the filtrate into new 1.5 ml Eppendorf tubes. If a pellet is visible in the filtrate, transfer the clean supernatant only! Discard the filters, DNA remains in the filtrate!

8) Add 450 µl of buffer PC and vortex thoroughly.
9) Place the NucleoSpin Plant II Column (green ring) into new 2.0 ml collection tubes (both provided in the kit). Load 600 µl of the sample onto the membrane. The DNA binds to the membrane. The remaining sample volume will be processed in subsequent steps.
10) Centrifuge for 1 min at 12 000 rpm, discard the flow-through and place the column back into the 2.0 ml collection tube.
11) Load the remaining sample volume onto the membrane and repeat previous centrifugation step. (The maximum loading capacity is 600 µl. If needed, repeat loading + centrifugation step again).
12) Add 400 µl of wash buffer PW1.
13) Centrifuge for 1 min at 12 000 rpm, discard the flow-through and place the column back into the 2.0 ml collection tube.
14) Add 650 µl of wash buffer PW2.
15) Centrifuge for 1 min at 12 000 rpm, discard the flow-through and place the column back into the 2.0 ml collection tube.
16) Add 200 µl of wash buffer PW2.
17) Centrifuge for 2 min at 12 000, discard the flow-through and place the column into a new 1.5 ml collection tube (not provided in the kit; safe-lock tubes can be used for long term storage).
18) Pipette 50 µl of elution buffer PE (preheated at 65°C) directly onto the membrane. Incubate for 5 min at 65°C.

    ! *The DNA is eluted from the membrane. Using smaller volume of PE (e.g. 30 µl) results in higher DNA concentration but reduces total DNA yield.*

19) Centrifuge for 1 min at 12 000 rpm.
20) Optional: for maximum yield pipette the filtrate onto the membrane again, incubate for 5 min at 65°C, and repeat the centrifugation (step 19).

    Alternatively, pipette another 50 µl of fresh PE (instead of the filtrate) onto the membrane to obtain the second isolate (with lower DNA concentration but maximum total yield).

21) The DNA quality and quantity may be checked by agarose gel electrophoresis.
22) DNA isolates can be stored in a fridge (up to several weeks) or in a freezer (-20°C, long-term storage).

Modifications

- Fresh material can be used, but not more than 100 mg.
- Dry (!) samples can be ground using liquid nitrogen. Immerse the tube with the sample and microtube pestle in liquid nitrogen and grind. Continue with step 3.
- Samples with big amounts of starting material can be ground using a mortar and a pestle. Add lysis buffer to the sample and grind. Transfer the homogenate into 1.5 ml Eppendorf tube and continue with incubation for at least 10 min at 65°C (step 3). If the material absorbs the lysis buffer so that no liquid homogenate can be transferred, add another 1–2 volumes of the lysis buffer. The volume used should be adjusted to recover ca 400 µl of the homogenate. Dry (!) samples can be ground using liquid nitrogen (a glass mortar and pestle must be kept in a freezer before use as it may crack!). Using sterile scalpel, transfer the crushed plant tissue into 1.5 ml Eppendorf tube and continue with the step 3.

- Samples with small amount of starting material can be ground in 1.5 ml Eppendorf tubes using microtube pestles. Add small volume of the lysis buffer (10–20 µl), small amount of sterile sand, and grind with the pestle. Add the remaining volume of the lysis buffer, and continue with incubation for at least 10 min at 65°C (step 3).
- DNA isolates can be further purified by commercial clean-up kits (e.g. MoBio PowerClean DNA Clean-up Kit) or concentrated using sodium acetate precipitation.
- For RNA removal (if needed), add RNase A; recommended final concentration is 10 µg/ml.

B) DNA isolation using CTAB method

The original principle of the method was based on the ability of CTAB (cetyl trimethylammonium bromide) to form a complex with DNA. The CTAB–DNA complex is soluble at high salt concentrations (0.7M NaCl) but precipitates at lower salt concentrations (0.45M NaCl). The following simplified protocol involves isopropanol DNA precipitation, with CTAB acting as a detergent to remove cell membranes and proteins. The method is cheap and provides usually rather high DNA yield. The DNA purity may not be sufficient in difficult taxa (additional purification may be needed).

Protocol for dry material (e.g. silica-dried samples)

→ *for modifications see notes in the previous section (p. 2–3)*

1) Transfer the dry material (5–15 mg) into the 1.5 ml Eppendorf tube and add 2 steel beads (or use more beads for resistant tissues).
2) Place the tubes into the white adaptors of the Retsch 400MM mill. Insert the adaptors into the mill (use both adaptors to counterweight the machine). Grind the samples for 1 min. at maximum speed (or increase the time for resistant tissues).
3) Add 700 µl of the CTAB buffer and 10 µl of 2-mercaptoethanol; use fume hood.
4) Close the tubes, mix by flicking and incubate on shaking incubator for 30 min at 50°C (if using a heat block instead, mix the tubes occasionally).
5) Spin the tubes for 2–3 s and transfer the supernatant into new 1.5 ml Eppendorf tubes (use prolonged centrifugation for samples with higher amounts of starting material – e.g. 1 min at 10 000 rpm). Transfer the steel beads into a Petri dish, wash with water and dry with a paper towel. Do not leave the beads in water as they may corrode!
6) Add 500 µl of chloroform : isoamyl alcohol (24:1) mixture. Use fume hood!
7) Close the tubes, invert several times and incubate for 5 min at room temperature.
8) Centrifuge for 10 min at 10 000 rpm.
10) Transfer the upper aqueous phase (ca 500 µl) into new 1.5 ml Eppendorf tubes. Use fume hood, discard contaminated tips and tubes into the bottle for toxic waste. Decant the lower chloroform phase into the "odpad – chloroform" waste bottle.

! *Only the upper aqueous phase contains DNA. It is necessary to avoid touching and taking the coloured borderline and lower chloroform phase as it contains impurities (cell debris, hydrophobic molecules such as fatty acids, proteins, etc.). In case of taking the impurities, repeat the centrifugation and transfer the upper aqueous phase into a new tube. The steps 6–10 may be repeated several times.*

11) Add 500 µl of ice-cold isopropanol (stored at -20°C).
12) Close the tubes, invert several times and incubate for ca 30 min at -20°C.
13) Centrifuge for 5 min at 13 000 rpm.
14) Discard the supernatant into a beaker. Use the fume hood and 200 µl tips. Be careful not to disturb the DNA pellet! (whitish or slightly coloured, should be visible on the bottom of the tube). Decant the waste supernatant into the "odpad – isopropanol" waste bottle.
15) Add 400 µl of ice-cold 96% ethanol (stored at -20°C). Mix by flicking to detach the DNA pellet from the bottom of the tube.
16) Incubate for 15 min at 37 °C (heat block).
17) Centrifuge for 5 min at 13 000 rpm.
18) Discard the supernatant into a beaker.
19) Add 200 µl of ice-cold 70% ethanol (stored at -20°C). Mix by flicking to detach the DNA pellet from the bottom of the tube. Incubate for ca 5 min at room temperature.
20) Centrifuge for 5 min at 13 000 rpm.
21) Discard the supernatant into a beaker.
22) Leave the tubes opened for ca 15 min at room temperature to dry the DNA pellets. When no drops of ethanol are visible, continue with the following step.
23) Add (10–)30(–200) µl of TE buffer or sterile water.

    The volume can be adjusted based on expected DNA yield. Decrease the volume for samples with small pellets or increase for samples with big pellets.
24) To dissolve the DNA pellets, leave the tubes in fridge overnight, or incubate the tubes at room temperature for ca 30 min and flick occasionally.
25) Always flick and briefly spin the tubes with DNA samples before use. The DNA quality and quantity may be checked by agarose gel electrophoresis.
26) DNA isolates can be stored in a fridge (up to several weeks) or in a freezer (-20°C, long-term storage).

C) DNA isolation DNA using NaOH

Very fast and cheap method, suitable especially for samples with small amounts of starting material. Sodium hydroxide denatures proteins and releases DNA to the solution. The resulting DNA is denatured (ssDNA) and suitable for routine PCR only (including DNA sequencing and PCR-RFLP). The NaOH isolates are not usable for methods more dependent on DNA quality and quantity (e.g. AFLP). The DNA can not be checked by agarose gel electrophoresis or spectrophotometry, and is not suitable for long-term storage (degradation may occur).

1) Add 10–20 µl of 0.5M NaOH to the sample (< ca 0.5 cm^2; the volume of NaOH should be adjusted to amount of material used). Grind the sample using a microtube pestle and small amount of sterile sand (alternatively, liquid nitrogen may be used for dry samples – see the chapter dealing with kit isolation, page 2)
2) Add 20 µl of 0.5M NaOH and continue grinding (1−2 min) if necessary.
3) Centrifuge for 2 min at 13 000 rpm.
4) Aliquot 100mM Tris-HCl, pH 8.3 into the set of new tubes or PCR strips. The volume used depends on the required final volume of the DNA isolates. The suspension from previous steps will be diluted at the ratio 1:10 (example: transfer 2 µl of the supernatant to the 20 µl of 100mM Tris-HCl).
5) Transfer the required volume of the supernatant into the tubes with 100mM Tris-HCl.
5) If necessary, samples with strong coloration can be further diluted with 100mM Tris-HCl or sterile water.
6) Store at freezer (-20°C).

D) DNA check by agarose gel electrophoresis

- For details on ELFO, see p. 7–9.
- Prepare 1.5 % agarose gel (use recycled agarose if possible), see page 6.
- Mix 0.8 µl of loading buffer ("GelRed" or "LD+SG") with 2 µl of DNA sample.
- Load 6 µl of λ DNA-HindIII ladder.
- One distinct band of large fragments (≥ 20 kbp) indicates high molecular weight DNA. Smear indicates fragmented DNA and/or residual RNA.
- Not possible to use for NaOH isolates (DNA is single stranded – cannot be stained).

E) Measurement of DNA concentration and DNA dilution

Measurement of DNA concentration

DNA concentration is usually reported in ng/µl. The most accurate is fluorometric quantification using "Qubit" fluorometer. The spectrophotometric quantification using "NanoDrop" spectrophotometer is less reliable and DNA concentration is usually overestimated. The spectrophotometric quantification involves calculations based on UV absorbance at 260 nm (maximum absorbance of DNA). The following additional values may be informative:

- Absorbance ratios A_{260} / A_{280} a A_{260} / A_{230}

  ! *Both ratios should be within the range 1.8–2.0(–2.2) for pure DNA. Lower values indicate presence of impurities, which may inhibit subsequent enzymatic reactions. Additionally, the impurities may show some absorbance at 260 nm, leading to artificially inflated values of DNA concentration!*

- Absorbance values measured (260 nm, 280 nm, 230 nm)

  ! *Absorbance values <0.01 are too low for reliable calculations of DNA concentration etc. Use less diluted samples for accurate measurement.*

Estimation of DNA concentration by agarose gel electrophoresis

DNA concentration can be **roughly** estimated from band intensities. Load 6 µl of λ DNA-HindIII ladder (see page 8) and 2 µl of the sample. The sample bands with intensity similar to the two lowermost ladder bands (2027-2322 bp) have concentrations approx. 20–150 ng/µl, more distinct bands have concentrations of several hundreds of ng/µl. The estimation is suitable for high molecular weight DNA, but becomes more difficult in samples with fragmented DNA, as the smear cannot be easily compared with ladder bands.

DNA dilution

- Dilution to obatain required concentration:

  $V_{sample} = c_{final} / c_{sample} * V_{final}$ $V_{water} = V_{final} - V_{sample}$

  V_{sample} is the volume of the starting (undiluted) sample, c_{sample} is concentration of the starting sample, c_{final} is concentration needed, and V_{final} is volume needed.

- Example: We need to prepare 50 µl of DNA solution with concentration 50 ng/µl. The starting sample has concentration 215 ng/µl. The volumes of sample and sterile water needed is as follows:

  $V_{sample} = 50 / 215 * 50 = 11.63\ \mu l$ $V_{water} = 50 - 11.63 = 38.37\ \mu l$

DNA electrophoresis

Horizontal agarose gels

Recycled agarose gels:

Gels used for samples stained with the non-toxic stain "GelRed" (not other stains such as SybrGreen!) can be used repeatedly. Collect such used gels in an Erlenmeyer flask. Do not use gels with a lot of dust impurities or gels with strong blue coloration. Recycled gel can not be used for *post-staining* visualization and gel extraction (for that use fresh agarose!).

Gel preparation:

1) Choose the suitable size of tray and comb(s). The volume of the gel depends on tray size.
2) Seal the tray with a tape and place it on the table. Set the table to horizontal position.
3) If using recycled gels, continue with step 5. If you need fresh gel, calculate the amount of agarose needed (or see the table below) and transfer it into a clean Erlenmeyer flask.

   Optimal concentration for PCR products (100–2000 bp) is 1.0–2.0% gel, for genomic DNA 0.7% gel is most suitable. Anyway, both types of DNA can be analyzed using 1.5% gels.

concentration %	**agarose [g] and gel volume [ml]**				
	30	**50**	**60**	**120**	**200**
0.7	0.21	0.35	0.42	0.84	1.40
1.0	0.30	0.50	0.60	1.20	2.00
1.2	0.36	0.60	0.72	1.44	2.40
1.5	**0.45**	**0.75**	**0.90**	**1.80**	**3.00**

4) Add required volume of 1×TBE buffer. Optional: incubate for several min at room temperature (hydration facilitates melting of agarose).

   1×TBE buffer is in a bottle on the shelf above the electrophoretic table. The 10× concentrated stock solution is on the central table.
5) Heat in a microwave to melt the agarose. Check the flask to see if the agarose is completely dissolved.
6) Cool the flask to ca 50°C under running tap water (cool until you can put your hand on the flask without feeling too much pain).
7) Pour the cooled gel into the tray and insert comb(s). Remove air bubbles using a pipette tip. Let it solidify for ca 30 min.
8) Remove the tape and insert the tray into the electrophoresis chamber. If needed, add 1×TBE buffer to immerse the gel completely. Remove the comb(s).

Loading the samples, running the electrophoresis

1) Use a microtiter plate or a piece of parafilm. Using a single filter tip, aliquot 0.8 µl drops of the blue loading dye with GelRed (tube labeled as „GelRed“) according to the number of samples.
2) Add 1.5 µl of the sample DNA. Use filter tips and mix by pipetting up and down three times. (The ratio sample:GelRed should be ca 1.5:1–4:1. Adjust the volume of GelRed if need to analyse different sample volumes).
3) Leave the first well empty for a ladder (for larger gels with big set of samples also leave the last well empty for a ladder).
4) Load the sample mixtures into the wells of the gel. It is not necessary to change the tip between samples. Instead, wash the tip by pipetting the buffer in electrophoresis chamber up and down several times.

   ! *Be careful not to disrupt the gel! The pipette tip may be placed just above the well. The sample mixture will sink into the well because of its higher density. Do not hurry. Although the blue colour may diffuse into the gel, the DNA remains inside the well.*

5) Load 6 µl of the ladder into the first (and last) well. Use λ DNA / Hind III ladder for analysis of large DNA fragments (e.g. genomic DNA), or 100 bp ladder for shorter fragments (e.g. PCR products) – see page 9.
6) Close the lid of the electrophoresis chamber and connect the electrical wires (red to red, black to black). Turn on the power supply, press "SET" to set the voltage – up to 60 V for the smallest chamber, or 140 V for bigger chambers. Press "SET" again and then press "START". Wash the microtiter plate with detergent and rinse with distilled water.
7) Press "STOP" when the blue band reaches about 0.5–1 cm from the end of the gel. Turn off the power supply, remove the lid of electrophoresis chamber and place the gel into the UV transilluminator chamber. Close the lid of the electrophoresis chamber to avoid buffer evaporation.

Gel Imager UV transillumitator (controlled by Scion VisiCapture software)

1) Place the gel into the UV transilluminator chamber (remove the gel tray!). Rotate the gel so that the wells are aligned along the left part of the UV chamber. Gently touch the gel to remove air bubbles.
2) Discard used gloves and start Scion VisiCapture software.

   ! *Do not touch any lab device with gloves contaminated by gels and buffers from the electrophoretic chamber!*

3) Choose "Image" and "Start Live Capturing". If no image is visible on the screen, disconnect and reconnect the cable between camera and computer (thin cable on the top of the camera).
4) If necessary, set zoom (the thin middle ring on the camera) and focus (the lowermost wide ring on the camera).

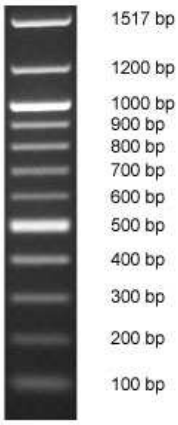
5) Close the chamber and turn on UV light. If necessary, adjust the aperture (the uppermost ring on the camera). Alternatively, set gain and exposure time in "Image" – "Properties" (if possible, do not set more than 1.2 s exposure – otherwise the image transfer will be slow). In case of low image intensity check the UV light button on the UV chamber (should be set on maximum).
6) Choose "Image" and press "Snap" to make an image.
7) Save the image (.tif) by choosing "File" and "Save As" (preferably the folder "gely" on Desktop). Image can be edited using IrfanView or other software.
8) Turn off the UV light, remove the gel and wipe the UV chamber with a paper towel moistened with 70% ethanol.

! *Gels used for samples stained with non-toxic stain "GelRed" (not other stains such as SybrGreen!) can be used repeatedly. Collect such used gels in an Erlenmeyer flask. Use the waste bottle "gels with SybrGreen" for gels stained with "LD+SG". Gels stained by concentrated GelRed solution (post-staining, see page 22) goes into mixed waste.*

DNA ladders

λ DNA / Hind III Digest (NEB)

100 bp DNA ladder (NEB)



PCR (Polymerase Chain Reaction)

PCR allows amplification of a certain part of DNA *in vitro.* The amplified region is defined by *primers* – oligonucleotides (single strand) usually 10-25 bases long complementary to a certain sequence of the template DNA; reaction starts from this sequence (DNA polymerase used cannot synthesise a DNA strand *de novo*, it can only extend the existing strand in the direction 5' → 3'). Usually a pair of two different primers is used; primers in a pair are complementary each to one of the opposite DNA strands, thus the sequence between the primers is amplified.

In its basic version, PCR needs a specific mixture of components (PCR buffer, DNA polymerase, $MgCl_2$, nucleotides, a pair of primers, template DNA, possibly some other additives) and cyclic changes of temperature (three basic steps: denaturation, annealing of primers, elongation; usually longer initial denaturation and longer final elongation are added to the beginning and end of the cycle, respectively). During PCR the DNA region delimited by the primers is exponentially amplified.

For each material (species), region sequenced and primers used it is necessary to find optimal reaction conditions (amount of the template, concentration of mix components, temperature and length of individual steps of the cycle, additional steps and modification of the cycle may be tested) → optimization of PCR.

PCR protocol with the use of Plain PP Master Mix (Top-Bio)

- Plain PP Master Mix contains all components of PCR reaction except for primers and template DNA. Using this mix (or similar products) we save time needed for preparation of the PCR and reduce number of pipetting steps and thus possibility of errors. The stock solution is 2× concentrated, final concentrations of the components in the reaction are: 75 mM Tris-HCl pH 8.8; 20 mM $(NH_4)_2SO_4$; 0.01% Tween20; 2.5 mM $MgCl_2$; 200 μM dNTP of each type; Taq DNA polymerase (0.05 U / μl); stabilizers and additives.
- The same protocol can be used also with the 2× concentrated mix of Fermentas company (it differs especially by lower concentration of DNA polymerase and is thus cheaper).
- For special applications all PCR components are also available separately in the laboratory, which allows to prepare a PRC mix with different concentrations or using a different type of DNA polymerase. Also mixes with Taq polymerase blocked by antibodies to allow "hot-start" reaction are available (e.g. "Combi PP Master Mix").
- PCR may be performed in different volumes, based on our experience (more difficult pipetting of small volumes) and planned use of the PCR product. Try to use as little volume as possible (cheaper); composition of the mix per sample for the most frequently used volumes is presented in a table on the next page.

1) Work on ice. Use only sterile plastics. Let all chemicals defreeze slowly, mix gently with a finger and centrifuge briefly. Mark PCR tubes or strips or plates with sample numbers.

2) Prepare PCR mix into one tube. Calculate amount of components for the planned number of samples + 1 (negative control and a reserve for pipetting errors; use + 2 for larger sets of samples). See the table on the next page.

chemicals	total volume of reaction					final conc.
	20 µl	**15 µl**	**10 µl**	**7.5 µl**	**5 µl**	
PCR (sterile) water	6.8	5.1	3.4	2.55	1.7	
forward primer (5 pmol/µl)*	1.2	0.9	0.6	0.45	0.3	0.3µM*
reverse primer (5 pmol/µl)*	1.2	0.9	0.6	0.45	0.3	0.3µM*
2× Plain PP Master Mix	10.0	7.5	5.0	3.75	2.5	1 ×
total mix	***19.2 µl***	***14.4 µl***	***9.6 µl***	***7.2 µl***	***4.8 µl***	
DNA**	0.8 µl	0.6 µl	0.4 µl	0.3 µl	0.2 µl	

** Valid for the most often used concentration of primer solution 5 pmol/µl (= 5µM) and final concentration 0.3 µM. If other concentrations (primer solution / final concentration) are used, adjust the composition of the mix with changing the volume of water (to compensate for different volume of primers).*

*** **Volume of template DNA may be adjusted**, compensate with volume of water.*

3) Pipette individual chemicals in the order: water, primers, PP Master Mix. Mix gently, centrifuge briefly.

4) Aliquot the mix into 0.2 ml PCR tubes, strips or plates. Use the volume from the "total mix" row of the table or other optimized volume; in general, total reaction volume minus DNA. The excess of the mix is used as a negative control. Add the required amount of template DNA (except for the negative control). Mix gently, centrifuge briefly.

5) Run the required PCR program on a thermocycler (se p. 44–45 for operation instructions). Wait until the lid is heated and the temperature of the block reaches ca 80°C. Put samples into the devise and tighten the lead using the screw.

An example of a PCR program:

1×	94°C	3 min	(initial denaturation)
35×	94°C	0.5 min	(cycle denaturation)
	[Ta]°C	1 min	(annealing; specific for each primer pair)
	72°C	1 min	(cycle elongation)
1×	72°C	10 min	(final elongation)
1×	15°C	hold	(cooling of the mixture after PCR)

6) Check the PCR results using electrophoresis. Mix1.5(–2.0) µl of a sample (including negative control) with 0.5(–0.8) µl of the loading dye "GelRed" and load on 1.5% agarose gel in TBE buffer (the gel may be recycled). Load 6 µl of 100 bp ladder to the first well.

There should be only one band of DNA of ± expected size; a band of primer dimers <100 bp may also occur. All other bands are non-specific products: the same DNA region amplified from contaminating organisms (such as endophytic fungi, epiphytic algae, etc.) or other region amplified with the same primers (e.g. mismatched priming or duplication of the region). There must not be any PCR product in the negative control (presence of any band in the control means contamination of the PCR mixture with unknown DNA; the same contamination is likely to occur in all samples).

Concentration of PCR product may be roughly estimated from the gel. If 6 µl of the 100 bp ladder and 1.5 µl of the PCR product are used, the concentration of PCR product whose intensity is similar to 500 bp and 1000 bp bands of the ladder is about tens of ng/µl.

DNA sequencing

DNA sequencing consists of several steps:

1. PCR & purification. We amplify certain region using PCR. Only specific products must be present. The product must be purified from unincorporated primers and dNTPs (except for very strong products from very efficient PCR where nearly no primers / nucleotides remain).
2. Sequencing reaction (cycle sequencing). Modified PCR in which only one primer is present and dNTPs are mixed with ddNTPs that are fluorescent labelled. Concentrations of all substances (including the template DNA) must be precisely balanced. Only one DNA strand is synthesised. When ddNTP is incorporated, elongation of the strand is terminated. The reaction results in a mixture of DNA strands of different length terminated with a fluorescent label corresponding to the last nucleotide.
3. Purification of sequencing reaction. Before analysis in an automatic sequencer, the reaction mixture must be purified, e.g. using Sephadex.
4. Analysis in an automatic sequencer. Products of the sequencing reaction are separated by their length using capillary electrophoresis. The fragments go through a laser beam and the fluorescent signal of the last base is detected. The sequence of fluorescent signals corresponds to the original DNA sequence. Sequencer allows detecting of about 600–800 bases (depending on the quality of the PCR product and the sequence itself). If longer region is sequenced, it is usually necessary to run two separate sequencing reactions with different primers (forward and reverse). The product is sequenced from both directions; the two sequences are then combined by specialized software based on the overlapping part.

In our laboratory, we perform only PCR, purification and preparation of a mixture (template + primer) for sequencing reaction. Other steps are performed by commercial companies.

A) PCR amplification

See the general protocol, p. 10–11. For sequencing the sample must contain only one specific PCR product (or at least the specific product must be much stronger than non-specific products).

For purification of a PCR product, we use either enzymatic purification ("Exo–AP", protocol B) or commercial purification kits (protocol C).

B) Enzymatic purification of PCR products (Exo–AP)

This purification method combines 2 enzymes: Exonuclease I cleaves single-strand DNA (unincorporated primers), Fast Alkaline Phosphatase cleaves unincorporated dNTP.

- Enzyme mixture is stored at -20°C. Work on ice, take the enzymes from a freezer just before use, mix gently (never vortex!), put back into the freezer immediately after use.

1) Aliquot the Exo-AP enzyme mixture by 0.4 µl to new PCR tubes / strips (other optimized volume may be used; generally, the ratio between the enzymes and the PCR product should be about 1.5 : 5, respectively).
2) Add 1.4 µl of the PCR product (or other volume, e.g. more in case of weak bands). Mix gently, centrifuge briefly.
3) Put the samples into a thermocycler and run the 'Exosap' program (37°C – 15 min, activity of the enzymes; 85°C – 15 min, deactivation of enzymes; 15°C – hold).

C) Purification of PCR products using the QIAquick PCR Purification Kit (Qiagen)

The method is based on patented technology ("spin column technique"). The DNA is bound to the silica membrane, washed and finally eluted with low salt buffer or sterile water.

General information

- Make sure that ethanol has been added to wash buffer PE before the first use (check the label on the bottle). If not, add the required volume of 96 or100% ethanol.
- Decant the waste liquids into a beaker.
- If possible, use recycled spin columns to save money.

Protocol

1) Add 5 volumes of buffer PB1 to 1 volume of PCR product (e.g. 50 µl of PB1 to 10 µl of PCR product). Mix and briefly spin.
2) Place a QIAquick spin column into a provided 2 ml collection tube. Load the sample onto the membrane.
3) Centrifuge for 1 min at 12 000 rpm.
4) Discard the flow-through (the DNA is bound to the membrane).
5) Place the column back in the same tube and add 200 µl of buffer PE.
   Use 500 µl of PE for PCR volumes >20 µl.
6) Centrifuge for 1 min at 12 000 rpm.
7) Discard the flow-through (the DNA is being washed).
8) Place the column back in the same tube and centrifuge for 1 min at maximum speed (13 000 rpm) to remove residual ethanol from buffer PE.
9) Place the column in a clean 1.5 ml Eppendorf tube (not provided by the kit) and pipette 30 µl of elution buffer PE or sterile water directly onto the membrane.
10) Incubate for 1 min at room temperature.
11) Centrifuge for 1 min at 12 000 rpm (DNA is eluted from the membrane).
12) DNA can be stored in fridge (up to several weeks) or in a freezer (-20°C, long-term storage).
13) Place the used columns into the bottle with 1M HCl and shake to mix.

D) Preparation of sequencing reaction

For sequencing reaction, both amount of DNA and relative concentrations of DNA and primer must be within certain optimal range. Especially for the amount of DNA significant deviations from the optimum in both directions (i.e., too little / too much of DNA) may distort the sequencing reaction (low quality or short incomplete sequences).

General remarks:

- Sanger sequencing (i.e. sequencing using fluorescently labelled ddNTPs) requires certain (optimal) ratio of dNTP a ddNTP concentrations (besides DNA and primer concentrations). Usually (at least for ABI sequencers) BigDye Terminator v3.1 Cycle Sequencing Kit is used. With this type of chemistry, about the first 30 bases of the sequence cannot be read. If the very beginning of the sequence is important, different

chemistry might be used, such as BigDye Terminator v1.1 Cycle Sequencing Kit, but in this case only short sequences are produced (unsuitable for most applications in botany, we usually sequence longer DNA regions).

- Sequencing reaction is performed by commercial sequencing companies. In our laboratory we only prepare a mix of the DNA template (purified) and sequencing primer (only one!). Recipes of two sequencing companies we use are as follows (both for 3.1 kit):

SEQme	
total volume: **10 µl**	
type of the template	**amount**
PCR product < 500 bp	50 ng
PCR product 500–1000 bp	100 ng
PCR product > 1000 bp	200 ng
plasmid	500 ng
primer (in total)	**25 pmol** e.g. 5 µl for concentration of 5 pmol/µl

GATC Biotech	
total volume: **10 µl**	
type of the template	**amount**
PCR product	100-400 ng
plasmid	400-500 ng
primer (in total)	**25 pmol** e.g. 5 µl for concentration of 5 pmol/µl

Protocol:

1) Calculate the required amount of sequencing primer (based on concentration of the stock solution); see the table above.
2) Select a proper amount (volume) of a purified sample. It must be optimized for each material and region sequenced (or DNA concentration may be directly measured).
   In general, for samples from enzymatic (Exo-AP) purification, we use 1–2 µl for concentrated products (strong bands on an electrophoretic gel) while we use maximum possible volumes for weak products. For samples from "spin-column" purification kits, we often use larger amounts of DNA, e.g. 3 µl, due to dilution of DNA during purification (for example, when we purify 10 µl of PCR product with a kit and use 30 µl of elution buffer, the final DNA concentration is 3× lower; in fact, it is even lower due to some DNA loss during purification).
3) Calculate the amount of sterile water to complete the total volume of the reaction.
   For example, for 10 µl and 5 pmol/µl primers we use 5 µl of a primer and 5µl remains for DNA (so, this is the maximum volume) + water; for 10 pmol/µl primers the volume of a primer is 2.5 µl and 7.5 µl remains for DNA + water.
4) To 1.5 ml Eppendorf tubes or 96-sample PCR plates (for SEQme company), pipette the sequencing mix in the order: water – primer – template DNA.
5) Mix gently, centrifuge briefly.
6) For SEQme company, fill an ordering form on-line (www.seqme.eu), print it. Put the order + samples into an envelope / bag and put it into the company's mailbox at the entrance to Biology Centre. For GATC Biotech company, mark the tubes with the sticky barcodes (available in the laboratory; put the second part of the barcode with the sequence number in your laboratory notebook or note the numbers), put the samples into an envelope / bag and put it into the company's mailbox at the entrance to Biology Centre.

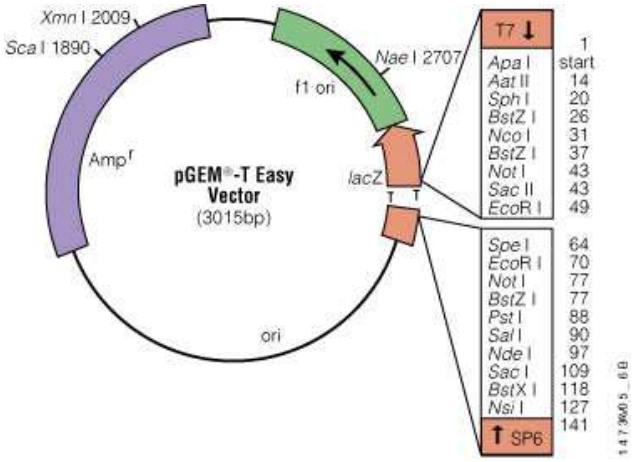
Molecular cloning

Principle

Molecular cloning is a method of genetic engineering that creates a recombinant molecule by introducing a DNA fragment into a vector (plasmid). It can be used to separate individual molecules of heterogeneous PCR product, which is essential for sequence analysis of multi-copy regions, heterozygous individuals, contaminated samples, etc.

Molecular cloning of PCR products can be easily performed using commercial kits such as pGEM-T Vector System I Promega (the kit includes the plasmid pGEM-T - see fig. below, T4 DNA ligase, ligation buffer and a control insert). Vectors are linearized molecules with a single T-overhangs at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products with A-overhangs generated my most Taq polymerases. Selection of transformed bacterial cells is ensured by ampicillin resistance gene (Amp^r) of the pGEM-T vector. Additionally, the LacZ gene is located at the insertion site. Insertional inactivation of the gene allows identification of recombinants by blue/white screening on indicator plates. Only the transformed cells can grow on plates with ampicillin. The transformed cells with empty plasmid (= functional LacZ gene) can utilize the substrate (X-gal) and form blue colonies, whereas those with insert remain white.

Fig. pGEM-T Vector. A DNA fragment is ligated into multiple cloning site (MCS) flanking the insertion site. The MCS sequence involves numerous motifs recognized by restriction enzymes. If the ligated DNA fragment is generated by restriction of given enzyme, it can be subsequently released by the same restriction.



A) PCR amplification

See the general protocol for PCR. To reduce PCR artifacts, minimize number of PCR cycles, use 2× longer extension time and a polymerase with higher fidelity (*proofreading* etc.).

B) Ligation

B1) According to the manufacturer's instructions

This protocol may be inefficient for some types of DNA fragments and requires PCR products purified by spin-column kit (e.g. QIAquick PCR Purification Kit – see page 12), which is not suitable for products with low DNA concentration. The following reactions were rescaled to smaller volume to save money. The optimal amount of input PCR product can be calculated using equation below (rescale the mass to the reaction volume used – for example below, use 10 ng for 1/4 reaction and 20 ng for 1/2 reaction). Anyway, using maximal volume of the product (= no need to add sterile water) usually works well.

Preparation of ligation reaction

Component	**1/4 reaction**	**1/2 reaction**
2× ligation buffer (vortex thoroughly!)	1.25 µl	2.5 µl
pGEM-T Vector	0.25 µl	0.5 µl
T4 ligase (keep on ice!)	0.25 µl	0.5 µl
Insert (PCR product)	0.75 µl (Optional: calculate – see below)	1.5 µl (Optional: calculate – see below)
(Optional: sterile H_2O)	(for the total volume of 2.5 µl)	(for the total volume of 5 µl)
Total	**2.5 µl**	**5 µl**

Calculation of the optimal amount of input DNA for ligation

$$\text{mass of insert needed [ng]} = \frac{\text{vector mass [ng]} \times \text{insert size [kbp]}}{\text{vector size [kbp]}} \times \text{(molar ratio) insert : vector}$$

Example: PCR product - 800 bp long, 1 full-volume reaction contains 50 ng of pGEM-T Easy Vector (Promega) - 3 kbp long, optimal ratio insert:plasmid is 3:1.

$$\text{mass of insert needed [ng]} = \frac{50\,\text{ng} \times 0.8\,\text{kbp}}{3.0\,\text{kbp}} \times \frac{3}{1} = 40\,\text{ng}$$

(For measurement of DNA concentration see page 5.)

Ligation

1) Prepare the reaction mixture (see the table above). Multiply the volumes by number of samples + 1. Ligase buffer is in single-use aliquots, discard the unused volume! (The buffer contains ATP, which may degrade due to repeated thawing/freezing).
2) Mix by gentle pipetting up and down, briefly spin.
3) Add the PCR product (= insert) and water if needed.
4) Incubate overnight in a fridge (Optional: incubate for 1 h at room temperature – the efficiency of ligation may be somewhat lower).

B2) Protocol using band excision from low-melting agarose

The protocol is very efficient even for PCR products with low DNA concentration. Moreover, products with multiple bands can be excised and ligated separately. The only disadvantage is the rather high cost of low-melting agarose.

1) Prepare new (not recycled!) 1.5 % *low-melting* agarose gel in 1× TAE buffer (not TBE; the TAE buffer is stored in the table with electrophoresis). Let it solidify for ca 45 min, then insert the tray into the electrophoresis chamber. Add 1×TAE buffer to immerse the gel completely.
2) Mix 6 µl of the PCR product with 2 µl of loading buffer with SybrGreen ("LD+SG"). Load on the gel and run the electrophoresis.
3) Prepare a set of sterile 0.5 ml tubes.
4) Preheat the heat block to 65°C.
5) Visualize the gel using blue-light transilluminator, use orange glasses. Excise the target bands using a scalpel and transfer into 0.5 ml tubes. Wipe the scalpel between samples (use a paper towel moistened with ethanol)!
6) Incubate the tubes on a heat block for 10 min at 65°C to melt the gel.
7) Prepare a set of new sterile 0.5 ml tubes. Prepare following mixture. Multiply the volumes by number of samples + 1:

   sterile water..... 1.75 µl
   pGEM-T Vector..... 0.25 µl

   Mix and spin briefly. Pipette 2 µl of the mixture into the tubes prepared. Add 1.25 µl of the sample (melted gel + DNA), mix and briefly spin. Incubate the resulting "melted gel mixture" for 10 min at 65°C.
8) Work on ice. Prepare the ligation mixture. Multiply the volumes by number of samples:

   2× ligation buffer (vortex thoroughly!) ..... 2.5 µl
   T4 ligase (keep on ice!) ..... 0.25 µl

   Mix and briefly spin. Pipette the ligation mixture 2.5 µl into new PCR tubes or strips.
9) Add 2.5 µl of the "melted gel mixture" to the tubes with ligation mixture. When pipetting the "melted gel mixture", wait 3 seconds before mixing with ligase mixture (to avoid the damage of ligase by the hot mixture). Mix by pipetting immediately.
10) Start the thermocycler – program "ligace16" (12–14 h at 16°C). When the temperature inside the block drops to 16°C, insert the tubes and fasten the lid. The remaining volumes of melted bands can be stored at -20°C freezer.
11) After ligation, heat the tubes for 15 min at 65°C (a thermocycler or a heat block) and add 20 µl of sterile water to prevent solidification.

C) Transformation

1) Obtain required number of *E. coli* K12 DHα aliquot tubes. One tube contains 100 µl of the cells and is sufficient for 2(–3) cloned samples. Thaw the tubes on ice.
2) Work in laminary box. Prepare a set of sterile 1.5 ml Eppendorf tubes. Add 50 µl of competent cells into each tube. For samples ligated using protocol according to the manufacturer's instructions add 2.5 µl of ligation mixture. For samples ligated using band extraction from low-melting agarose add 12.5 µl of the ligation mixture (the remaining volumes can be stored at -20°C).

   Alternatively, use 30 µl of competent cells and decrease the volume of ligation mixture proportionally (i.e., 1.5 µl or 7.5 µl, respectively).
3) Gently flick the tubes and spin for 1 s. Incubate for 20 min on ice (stabilization of the cells).
4) Preheat the heat block to 42°C.
5) Heat-shock the cells by incubation for 45 s at 42°C (a heat block), immediately return on ice for at least 2 min. Do not shake the tubes!

   Heat-shocked cells must be handled as GMOs (genetically modified organism). Do not open the tubes or plates with GMO cell cultures outside a laminary box. All tips and tubes contaminated with GMOs have to be collected in a beaker labelled "GMO" *and sterilized by autoclaving*.

D) Cultivation

1) Work in laminary box. Add 300 µl of SOC medium (room temperature).
2) Place the tubes in horizontal position on a shaking incubator and incubate for 45 min at 37°C, 150 rpm (cell recovery, activation of gene expression).
3) Prepare LB/Amp/IPTG/X-Gal plates (one for each sample). Heat the bottle with 250 ml of solid LB medium on a heated magnetic stirrer to melt the medium. Once melted cool the medium to ca 65°C and add 250 µl of stock solution Amp (ampicillin, 0,1g/ml) – work in laminary box! Pour the medium into Petri plates.
4) Mix 64 µl of X-Gal (12,5 mg/ml) and 3,5 µl of IPTG (240 mg/ml), vortex and pipette 67,5 µl of the mixture on the plate. Use a sterile hockey stick to spread the mixture on the surface of the plate.

   If processing more samples, multiply the volumes of X-Gal and IPTG by number of samples +1.
5) Once incubation is complete, transfer 100 µl of the SOC cell suspension on the plate and spread using a sterile hockey stick. Sterilize the stick between samples using a flame.

   To obtain higher number of colonies, centrifuge the SOC cell suspensions for 2 min at 3980 rpm (= 1500×g). Discard ca 250 µl of the supernatant, re-suspend the pellet of cells by pipetting, and spread the remaining volume on the plate.
6) Invert the plates so that the agar is in the upper half of the plate and incubate overnight (ca 15 h) at 37°C. Continue with picking the colonies next day in the morning.
7) The LB plates with colonies can be stored for a few weeks at 4°C (fridge). The colonies can be used for direct PCR, plasmid isolation, etc.

E) Picking the colonies and direct PCR

1) Pipette 40 µl of autoclaved distilled water into 0.2 ml tubes of PCR strips or plates.
2) Work in laminary box. Gently touch a white colony with a sterile toothpick and dip the toothpick into the water in the tube. Collect the used toothpicks into a beaker with bleach (SAVO).
3) Denature the tubes at 94°C for 5 min in a thermocycler (program „Denatur“) to release plasmids from cells.
4) Prepare the following mixture according to the general PCR protocol. Multiply the volumes by the number of samples + 1. The primers SP6 and T7 anneal to the flanking region of the insertion site.

   PCR water ..... 1.3 µl
   primer SP6 (5 pmol/µl)..... 0.4 µl
   primer T7 (5 pmol/µl)..... 0.4 µl
   2× Plain PP Master Mix ..... 2.5 µl

5) Mix and spin briefly. Pipette 4.6 µl of the mixture into PCR strips or tubes.
6) Add 0.4 µl of denatured DNA (the remaining volume can be stored at -20 °C freezer). Mix and spin briefly.
7) Start a thermocycler – program „SP6_T7“. When the temperature inside the block reaches ca 80°C, insert the tubes and fasten the lid. The program is as follows:

   94°C – 3 min., 45× (94°C – 30 s, 50°C – 1 min, 72°C – 1 min), 72°C – 10 min

8) Once finished, briefly spin and check the PCR products by agarose gel electrophoresis (see page 6).

F) GMO handling, deactivation and documentation

Heat-shocked cells and plates with colonies must be handled as GMOs (genetically modified organisms). Do not open the tubes or plates with GMO cell cultures outside laminary box. All lab plastics contaminated with GMOs must be sterilized by autoclaving. Collect the used toothpicks into a beaker with bleach (SAVO). Lab protocols dealing with cloning must be archived for 10 years (both printed and electronic version).

ISSR, Inter-simple sequence repeat

The method is based on PCR using primers (16–25 bp long) complementary to microsatellite sequences. Microsatellites (*simple sequence repeat*, SSR) are short segments of DNA with variable number of short (1–6 bp) nucleotide motifs. ISSR amplifies the genomic regions between two microsatellites of the same motive. The resulting product contains pool of genomic fragments of different length. The sequence of ISSR primer may be anchored by motif of 1–3 bases different from the microsatellite sequence to provide more specific primer annealing. Products of PCR amplification are analysed on an agarose gel or using fragment analysis in an automatic sequencer. Variation in fragments is caused by differences in distribution of microsatellites across the genome and indel mutations between adjacent microsatellites. The analysis involves selection and optimization of suitable primers to generate reproducible and scorable patterns. Each sample should be analysed in two independent runs, and only fragments present in both replicates can be scored. For large sets of samples, representative subset of samples (ca 10%) may be used for error rate calculation and loci with poor reproducibility should be omitted.

Protocol

1) Prepare PCR mixture. Multiply the volumes by number of samples + 2. Work on ice. The following mixture is for 1 reaction of the total volume 7.5 µl:

   PCR water ..... 1.55 µl
   primer (2.5 pmol/µl)..... 1.8 µl
   2× Plain PP Master Mix..... 3.75 µl

2) Mix and spin briefly. Pipette 7.1 µl of the mixture into PCR tubes or strips.

3) Add 0.4 µl of sample DNA. Mix and briefly spin.

4) Start the thermocycler – use a program with *touchdown* (see below). When the temperature inside the block reaches ca 80°C, insert the tubes and fasten the lid. The cycling is as follows:

1×	94°C	3 min	
5×	94°C	1 min	
	[T_a +5 to +1]°C	1 min	1°C decrease / cycle
	72°C	2 min	
33×	94°C	1 min	
	[T_a]°C	1 min	
	72°C	2 min	
1×	72°C	10 min	
1×	15°C	hold	

   T_a is the *annealing* temperature specific for particular primer. *Touchdown* protocol starts with the first cycle having T_a + 5°C. The temperature is decreased by 1°C for each of subsequent 4 cycles. The remaining 33 cycles use the temperature of T_a.

5) Once finished, briefly spin and check the PCR products by agarose gel electrophoresis using post-staining (see page 22).

Microsatellite analysis

Microsatellites (**STR =** *short tandem repeats,* **SSR** = *simple sequence repeats*) are short segments of DNA with variable number of short (1–6 bp) nucleotide motifs, e.g. $(A)_n$, $(AT)_n$, $(ATA)_n$. The allelic variation in a particular genomic site (locus) is caused by variable number of repeats. The possible number of alleles in a locus is determined by ploidy level (each chromosome set contains one allele). The analysis is based on PCR amplification using primers annealing to flanking (adjacent) sequences of microsatellite loci. Primers for nuclear microsatellites are usually species-specific, whereas microsatellites in chloroplast DNA occur in more conserved sites and can be amplified by more universal primers. The alleles are detected as PCR fragments of different length, usually using fragment analysis on an automatic sequencer (less frequently using denaturing polyacrylamide gel).

Nuclear microsatellites are considered as one of the most suitable marker due its frequent occurrence across the genome, high level of polymorphism, codominant nature and high reproducibility. The most frequent use of microsatellites is in population biology studies, involving identification of clones, parental analysis, genetic structure of populations etc.

A) PCR amplification

Primers for nuclear microsatellites are usually species-specific and can be used with limited success in closely related taxa. To shorten the duration of PCR, the cycling involves usually short elongation and denaturation steps (sufficient for amplification of rather short microsatellite loci).

The protocol below is a modification using arbitrary M13 primer with fluorescent label (four different colours available). PCR reaction includes 3 primers – labelled forward M13 primer and a pair of locus-specific primers. One of the specific primers (usually forward) is extended by the M13 arbitrary sequence (so-called *tail*) on its 5' end. The first ¾ of the total PCR cycles use the annealing temperature suitable for amplification by specific primers only. The annealing temperature is decreased in the remaining cycles to allow for annealing of M13 forward primer to molecules of the PCR product, which results in most of the molecules being labelled with a fluorescent dye. The advantage of this protocol is in using one set of labelled primers for all species-specific primers having the tail sequence, which greatly reduces the cost paid for fluorescent primers (not necessary to buy specific fluorescent primers for individual taxa analysed in the laboratory).

As the analysis involves amplification of numerous (ca 8–10) microsatellite loci, multiplex PCR may be suitable solution to save money and time. Multiplex PCR amplifies several loci simultaneously in a single reaction. As the process requires multiple primers in a single tube, the primers should have approximately similar annealing temperature and should not form primer dimers. If using labelled M13 primer, the loci multiplexed in a single reaction are stained using the same colour. Therefore, only loci of different length can be multiplexed. (The same principle also applies to single locus amplifications. Loci of overlapping length must be stained with different fluorescent colours).

Protocol with a M13 primer

1) Prepare the following mixture. Multiply the volumes by the number of samples + 2. Work on ice. Keep the tubes with fluorescent primers covered by aluminium to prevent exposure to light. The following mixture is for 1 single locus reaction of the total volume of 5 µl:

PCR water	0.65 µl
M13 forward primer (2.5 pmol/µl; fluorescently labelled)	0.6 µl
'tailed' specific forward primer (2.5 pmol/µl)	0.15 µl
specific reverse primer (2.5 pmol/µl)	0.6 µl
2× Plain PP Master Mix (Top-Bio)	2.5 µl

For multiplex reaction, the total volume (sum) of ´tailed´ specific forward primers is equal to 0.15 µl. All the ´tailed´ specific primers are used in equimolar concentrations – e.g. for multiplex amplification of 3 loci, each primer has 0.05 µl (= 0.15 µl / 3). The same is for specific reverse primer (except that the total volume is 0.6 µl).

2) Mix and spin briefly. Pipette 4.5 µl of the mixture into PCR tubes or strips.

3) Add 0.5 µl of sample DNA. Mix and spin briefly.

4) Start a thermocycler. When the temperature inside the block reaches ca 80°C, insert the tubes and fasten the lid. The cycling is as follows:

1×	94°C	2 min	
33×	94°C	30 s	
	[T_a]°C	30 s	(temperature allowing annealing of specific primers only)
	72°C	60 s	
11×	94°C	30 s	
	46°C	30 s	(temperature allowing annealing of M13 forward primer)
	72°C	60 s	
1×	72°C	10 min	
1×	15°C	hold	

5) Once finished, briefly spin and check the PCR products by agarose gel electrophoresis (see page 6).

B) Sample preparation for fragment analysis (SEQme company)

- If possible, keep the number of samples in multiples of 16. The price for samples in tubes is always rounded up to the nearest 16th sample. Similarly, the price for samples shipped in PCR plates is fixed regardless the number of sample positions used.

1) Mix 0.5 µl of PCR products of all loci of the sample to be analysed (use PCR strips, tubes or PCR plates).

   If the loci differ in band intensity, increase the volume of weak bands (use e.g. 1–3 µl).

2) Fill the on-line form (www.seqme.eu), specify the volume of the mixture to be used for fragment analysis (e.g. 1 µl), select the dye set and plasticware (five-dye set DS-33, 600LIZ, Tubes or Plates) and sample shipment (Ceske Budejovice – Biology Centre).

3) Print the order. Put the samples and the order into a zip-lock bag or paper envelope, and put into sample collection box located at the entrance to Biology Centre.

PCR-RFLP (Restriction Fragment Length Polymorphism)

The method is based on restriction enzymes (endonucleases) that cut DNA at specific nucleotide sequences (restriction sites). The specific genomic region is amplified using PCR and subjected to restriction with selected enzyme(s). The resulting DNA fragments are visualized using agarose gel electrophoresis. The variation in fragments reflects the number of restriction sites. If the target PCR product involves a single restriction site, two fragments are visible on the gel (and accordingly more fragments for higher number of restriction sites). Shared polymorphism of restriction sites (or fragments) reflects genetic similarity among the studied samples. The method can be used for large-scale screening of PCR products at much lower cost than traditional sequencing. Anyway, the method searches for polymorphisms in specific restriction sites only, and cannot detect any other mutations located outside such sites. The best use is for detection of variants (haplotypes, alleles) which can be unambiguously assigned based on given restriction site(s).

A) PCR amplification

- See general protocol for PCR.
- Check the PCR product using 1–1.5% agarose gel. Only one specific band should be visible (non-specific products may complicate interpretation of PCR-RFLP patterns).

B) Restriction

General information

- Follow the manufacturer´s instructions concerning recommended reaction buffer, enzyme concentration and optimum working temperature of the given enzyme.
- Work on ice (or keep enzymes on cooled rack).

Protocol

An example of the method (ferns of the genus Dryopteris*, trnL-trnF region of chloroplast DNA, restriction with* TaiI *enzyme – provided by ThermoScientific)*.

1) Prepare the following restriction mixture. Multiply the volumes by the number of samples + 1.

   Sterile water ..... 2.9 µl
   10× Buffer R (10× concentrated reaction buffer) ..... 0.3 µl
   TaiI (10 U/µl) ..... 0.3 µl

2) Mix and spin briefly.
3) Aliquot 3.5 µl of the mixture into PCR tubes or strips.
4) Add 1.5 µl of the PCR product, mix and spin briefly. Incubate in a thermocycler for 3 h at 65°C.

C) Electrophoresis with *post-staining*

Recycled agarose gels and sample staining using loading buffer (GelRed or LD+SG) result in rather poor band resolution and shifts in DNA migration. For superior resolution, use new agarose gels, loading buffer without DNA dye, and electrophoretic accessories which have not been contaminated with DNA dyes. The DNA fragments are stained by incubation in solution of DNA dye after the electrophoretic separation. The protocol allows for detection of length polymorphisms of several tens of bp and detects fragments ≥100 bp.

Protocol

1) Prepare 1.5 % agarose gel (see page 6). Use new agarose!
2) Add 2,5 µl of the blue loading buffer „LD“ to the DNA sample, mix and load on the gel.
4) Add 6 µl of „JK ladder“ 100 bp (ladder without DNA stain).
5) Set the voltage to 180 V and run for ca 1-3 h.
6) Transfer the gel into the plastic container labelled "GelRed post-staining". Add staining solution (1× GelRed in 1M NaCl – be careful, potentially mutagenic!) and incubate for 15–45 min.
7) Visualise the gel using a UV transilluminator. Used gel goes into mixed waste.

AFLP (Amplified Fragment Length Polymorphism)

AFLP is a restriction method that searches for polymorphism in the whole genome; no prior information on the target genome is needed. Total genomic DNA is digested with two restriction enzymes (restriction step; combination of *Mse*I a *EcoR*I restrictases is most often used). Double-stranded adaptors of known sequence are ligated to the ends of fragments (ligation step). Part of fragments is amplified using two successive PCRs (pre-selective and selective amplification); primers complementary to adaptors extended by 1 to 3 'selective' bases are used and one of the primers is fluorescently labelled. Typically, tens to hundreds of fragments are amplified in one reaction. Fragments are separated according to their length using a capillary sequencer (formerly also on polyacrylamide gels). Presence/absence of a fragment of certain length is scored (length is a "locus", presence/absence are "alleles").

The method is highly reproducible and sensitive for detecting polymorphism at low taxonomic levels (related species, populations within a species). It is often used in phylogeography, population and conservation biology (amount of variation within and between populations) and taxonomy (separation of closely related species). Its major disadvantages are relatively high price, complicated protocol, sensitivity to the quantity and quality of the input DNA and dominant type of the data.

A) Protocol using AFLP kits (Invitrogen)

General remarks:

- Fragment analysis by SEQme company is payed for every 16 samples; thus, the number of samples should be in multiplies of 16.
- Up to four fluorescent dyes can be mixed in one sample for fragment analysis (dyes 6-FAM, VIC, NED a PET). A set of labelled primers is available in the laboratory.
- Work on ice all the time. Enzymes (esp. restrictases and ligase) are kept in a freezer, take them out of it just before use and return back immediately.
- Ligation solution contains ATP that degrades during repeated thawing and refreezing. Only use aliquots for the planned number of samples, do not use the stock solution.
- Fluorescent primers must be protected against light (tubes covered with aluminium foil, put them back into the freezer after use), use aliquots.

Protocol

1) Use AFLP® Core Reagent Kit I for restriction. Prepare restriction mix for the planned number of samples +1 (since the kit is relatively expensive, for low number of samples +0.5 or no excess is recommended). The total volume is 2.5 µl per sample.

   sterile H_2O ..... 1.1 µl
   5× Reaction buffer ..... 1.0 µl
   EcoRI/MseI enzyme mixture ..... 0.4 µl

2) Mix gently, centrifuge briefly, aliquot by 2.5 µl into PCR strips or 0.2 ml PCR tubes.
3) Add 2.5 µl of genomic DNA (~100 ng/µl); the final volume is 5 µl. Mix gently, centrifuge briefly.

   The mentioned concentration of DNA is valid only for the material used in practical course. Optimization is needed; usually 50–250 ng is used. The volume may be increased by avoiding water and corresponding adjustment of the volume of the mix above.

4) Incubate 3 hours at 37°C (e.g. in a thermocycler).
5) Use AFLP® Core Reagent Kit I for ligation. Prepare ligation mix for the planned number of samples +1 (since the kit is relatively expensive, for low number of samples +0.5 or no excess is recommended). The total volume is 5 µl per sample.

   Adaptor/Ligation Solution ..... 4.8 µl
   T4 DNA ligase ..... 0.2 µl

6) Mix gently, centrifuge briefly, aliquot by 5 µl into samples (take a new tip for each sample); the total volume is 10 µl.
7) Incubate 12 hours at 16°C (e.g. in a thermocycler).

   To save time, restriction should be performed afternoon and follow-up ligation overnight. Duration of restriction and ligation (and possibly also temperature of ligation) may be optimized for each material (species, DNA concentration).

8) Check the success of restriction + ligation step using electrophoresis. Run 2 µl of a sample + 0.8 µl of loading buffer ("GelRed") on a 1.5 % agarose gel (recycled gels can be used). Load 6 µl of the 100 bp ladder into first well. A smear of restriction fragments (ca 50–1000 bp) should be visible while non-digested high-molecular DNA and strong signal of non-ligated adaptors should be absent.

   With routine use of the method this step can be omitted (or performed as a backward control only if following pre-selective amplification fails).

9) Use AFLP® Pre-amp Primer Mix I for pre-selective amplification. The mix contains primers and dNTPs. Prepare the reaction mix for the planned number of samples + 1 (+0.5 for small number of samples). The total volume is 4.6 µl per sample.

   PA mix ..... 4 µl
   PCR buffer (10×)* ..... 0.5 µl
   DNA polymerase (1 U/µl)* ..... 0.1 µl
   ** If different concentration is used, adjust composition of the mix (e.g. by sterile water).*

10) Mix gently, centrifuge briefly, aliquot by 4.6 µl into PCR strips or 0.2 ml PCR tubes. Use remaining mix as a negative control (i.e., run PCR without adding DNA).
11) Add 0.4 µl of a sample after ligation; the total volume is 5 µl. Mix gently, centrifuge briefly.
12) Freeze (-20°C) the remaining ligation samples (for repetitions if something fails).
13) Put samples into a thermocycler and run the following program for pre-amplification:

1×	72°C	2 min	
20×	94°C	1 s	
	56°C	30 s	
	72°C	2 min	ramping 2°C/s
1×	60°C	30 min	
1×	10°C	hold	

14) Perform 1:10 dilution of the reaction: transfer 3 µl into a new PCR strip/tube and add 27 µl of sterile H_2O.
15) Check the pre-selective amplification using electrophoresis. Run remaining 2 µl of a sample (after ligation) + 0.8 µl of loading buffer ("GelRed") on a 1.5 % agarose gel (recycled gels can be used). Load 6 µl of the 100 bp ladder into the first well. An intense smear of pre-amplification fragments should be visible; the intensity is variable (some blurred "bands" usually appear). The negative control must not contain any PCR products.

16) Prepare the reaction mix for selective amplification for the planned number of samples + 1 (+0.5 for small number of samples). We usually prepare 4 different mixes that contain different primer combinations and each *EcoR*I primer labelled with different fluorescent dye. The total volume is 3.75 µl per sample (one mix).

Sterile H_2O	2.55 µl
PCR buffer (10x)*	0.5 µl
dNTP (10 mM)	0.1 µl
EcoRI primer (1 pmol/µl)	0.25 µl
MseI primer (5 pmol/µl)	0.25 µl
DNA polymerase (1 U/µl)*	0.1 µl

* *If different concentration is used, the volume of water must be adjusted.*

Primers available in our lab (only last three "selective" bases shown):

EcoRI primers (labelled)		MseI primers
6-FAM (blue)	AAC*, ACG*, AGT	CAC, CAG, CAT, CCA, CCG, CGA, CGG, CTA, CTC, CTG
VIC (green)	AAC*, AAT*, ACG*, ATG	
NED(yellow)	AAT*, ACA*, AGG	
PET (red)	ACA*, AGC, ATC	

primers marked with * are available in two different colours

17) Mix gently, centrifuge briefly, aliquot by 3.75 µl into PCR strips or 0.2 ml PCR tubes. Use remaining mix as a negative control (i.e., run PCR without adding DNA).

18) Add 1.25 µl of diluted pre-amplification, the total volume is 5 µl. Mix gently, centrifuge briefly.

19) Put samples into a thermocycler and run the program for selective amplification:

1×	94°C	2 min	
	65°C	30 s	
	72°C	2 min	ramping 2°C/s
8×	94°C	1 s	
	64–56°C	30 s	touch-down by 1°C
	72°C	2 min	ramping 2°C/s
23×	94°C	1 s	
	56°C	30 s	
	72°C	2 min	ramping 2°C/s
1×	60°C	30 min	
1×	10°C	hold	

20) Check the selective amplification using electrophoresis. Run remaining 2 µl of a sample + 0.8 µl of loading buffer ("GelRed") on a 1.5 % agarose gel (recycled gels can be used). Load 6 µl of the 100 bp ladder into first well. A smear of fragments (~50–600 bp) should be visible; some parts are more intense and the pattern is specific for each primer combination. The negative control must not contain any PCR products.

21) It is recommended to continue immediately with following steps (purification, fragment analysis). If it is not possible, store the selective amplification in a freezer (-20°C).

22) Mix products of selective amplification labelled with different dyes for fragment analysis. Use 1 µl of each product. Any type of plastic may be used (1.5 ml tubes work well). Store the remaining selective amplification in a freezer (-20°C).

Fluorescent dyes / selective amplification products may differ in the fluorescence intensity. The volume of each part in a mixture may be optimized (e.g. 2 µl of more fluorescent or 0.5 µl of less fluorescent dye).

23) Continue with sample purification (protocol B) or send the samples for fragment analysis to SEQme Company. Fill the on-line ordering form (www.seqme.eu), specify the fluorescent labelling and size standard to be used (5-colours set DS-33 + 600LIZ size standard). Print the order, put into the envelope / plastic bag with samples. Put the samples into the company's sample collection box at the entrance to Biology Centre.

B) Sodium acetate purification

General remarks:

- Purification step may be omitted for fragment analysis (compare quality of the result with and without purification). Based on our experience, AFLP profiles are often better (more clear peaks, higher intensities) when purification is used.
- Work on ice. All centrifugation steps are performed in a centrifuge cooled to 4°C (start the cooling at the beginning of work).

Protocol

1) Pipette 1 µl of 3M sodium acetate on a wall of a 1.5 ml tube.
2) Add 3 µl of selective amplification mixture directly to the drop of sodium acetate.
3) Add 25 µl of cold (from a freezer) 96% ethanol.
4) Vortex, centrifuge briefly.
5) Incubate for at least 20 min (up to overnight) at -20°C.
6) Centrifuge 30 min at 13 000 rpm. Orient all the tubes to the same position (e.g. hinge outside) to have the DNA pellets at the same place.
7) Slowly decant (or carefully pipette) ethanol from the tube, DNA remains on the tube wall.

   ! *Work slowly and pour out the liquid through the opposite wall then where DNA is (otherwise it may peel off and be lost). The same in point 10 of this protocol.*

8) Add 100 µl of cold (from a freezer) 70% ethanol.
9) Centrifuge 5 min at 13 000 rpm.
10) Slowly pour out (or carefully pipette) ethanol from the tube, DNA remains on the tube wall.
11) Open the tubes and let them dry for at least 5 min at room temperature (optional: continue drying for ca 10 min in 37°C). There must not be any remaining ethanol.
12) Dried samples may be stored for a few days in a fridge (~4°C). Usually we send the samples for fragment analysis immediately.
13) Fill the on-line ordering form of the SEQme Company (www.seqme.eu), specify that the samples are dried, the fluorescent labelling and size standard to be used (5-colours set DS-33 + 600LIZ size standard). Print the order, put into the envelope / plastic bag with samples. Put the samples into the company's sample collection box at the entrance to Biology Centre.

Isozymes (allozymes)

Isozyme analysis screens for variation of certain group of proteins: of enzymes that can be detected by specific staining. Due to degeneration of genetic code, not all mutations in coding DNA sequence change the structure (amino-acid sequence) of a protein. However, it is still possible to find enzymes (a) that have within a studied organism (population, taxon …) different forms (alleles) that can be detected and (b) these differences are heritable. Detection of isozymes is performed as a "histochemical staining" – not the enzyme molecules themselves but products of their catalytic activity are stained. Most of the enzymes used belong to the base metabolic pathways, which makes the method quite universal (the same enzymes are found through all plants and some even in animals or fungi). Moreover, the basic metabolism must function and is therefore under strong selective pressure – therefore, it may be expected that different alleles (if persist in a population long enough) are not strongly selected for or against, i.e. are more or less selectively neutral. These features together with codominant nature of the data and low price makes isozymes suitable tool for population genetic analyses, which is one of the most common applications of the method in botany (together with identification of clones).

A) Extraction

Collecting samples in the field

- Isozymes are isolated from living tissues, typically from leaves (several cm^2). The tissue must be healthy, free of parasites and in good physiological state (not withered, frozen…).
- Immediately after collection, put the samples to plastic bags (zip-lock), moist slightly (but not too much), store in cold conditions (portable fridge, vacuum bottle with ice, etc.). Isozymes must be extracted within a few days after collection.

General remarks to extraction

- There is variety of extraction buffers (stabilize pH, usually contain antioxidants and other protein-stabilizing chemicals). Several buffers should be tested for the studied species and the most suitable selected. See p. 50 for recipes of buffers often used in our laboratory.
- The buffers may be supplemented by other chemicals, such as small amount of Dowex-Cl exchange resin (lower background on gels, but sometimes lower activity of enzymes).
- All steps are performed on ice. All equipment (e.g. a mortar and a pestle) and chemicals are cooled in a fridge. All centrifugation steps are performed in a cooled centrifuge at 4°C.
- Mark one set of 1.5 ml tubes and 3 sets of 0.5 ml tubes with sample numbers.

Extraction protocol

1) Put ca 50 mg (usually a few cm^2 of a leaf) into a pre-cooled mortar, work on ice.
2) Add 500 µl of ice-cold extraction buffer and a small amount of Dowex-Cl.
3) Grind the tissue in a mortar with a pestle. Tough leaves may be cut with scissors to small pieces. After grinding, no visible pieces of a tissue should remain.
4) Transfer the homogenate (pipetting using tips with lower end cut) to a 1.5 ml tube.
5) Centrifuge 10 min at 13 000 rpm at 4°C.
6) Aliquot the supernatant to 0.5 ml tubes by 90 µl. Usually there will be two series with exact volume while remaining supernatant (more or less) is used as a third series.
7) Store the extracts at -80°C (long-term; for short-term storage -20°C may also be used).

B) Casting of gels and preparation of electrophoresis

General remarks

! ***Acrylamide solution is <u>highly poisonous</u>*** *(neurotoxin).* Work with extreme care, always use protective coat and gloves. All contaminated objects (combs, spacers, a casting stand, etc.) must be washed with plenty of water. Excess of acrylamide solution (including non-polymerized remains on a gel surface, etc.) must be stored in a special bottle for toxic waste, it must not be poured into common sink. Polyacrylamide is not toxic but since the polymerization efficiency is never 100%, all gels and apparatus parts may contain some non-polymerized acrylamide and must be handled in gloves; the waste must be stored in a separate container.

- The apparatus available in the laboratory (Hoeffer 600SE) allows electrophoresis on 1, 2 or 4 gels simultaneously, usually 28 wells (samples) per one gel.
- As a standard, we use discontinuous gels composed from an 8.16% resolving (separation) gel (lower) and 4% stacking gel (upper).
- Gels cannot be stored for long before electrophoresis. We usually prepare the gels in the afternoon before analysis and store (and cool) in a fridge overnight.

Assembling the casting unit (glass "sandwich")

1) Put the gasket into the casting stand ("rubber" side up). Place the stand into a tray (that would collect acrylamide in case of leaking).
2) Carefully check the glass plates, spacers and combs. The surface must be very clean and smooth. If necessary, rinse them with distilled water and ethanol and clean with a soft tissue.
3) Place a pair of spacers on shorter sides of an outer glass plate (thicker, flat upper side). Put an inner glass plate (thinner, concave upper side) on spacers. Place another pair of spacers, put the second outer glass plate on them.

   We usually prepare two "sandwiches", each consisting of two gels (four gels together). For analysis of 2 gels only, prepare 2 "sandwiches" each with one gel (do not use the inner plate and the second pair of spacers) instead of 1 "sandwich" of 2 gels. For analysis of 1 gel, replace the second "sandwich" with a thick glass plate.
4) Put the "sandwich" into clamps and slightly fasten the uppermost and lowermost screws.
5) Put up the "sandwich" on a lower edge, loose the screws, assemble glass plates and spacers so that their edges are flush with each other. Fasten the screws, place the "sandwich" on the upper edge and repeat the assembly. Repeat the procedure until all edges are perfectly flush with each other.

   ! *Check carefully, else the gasket in the casting stand will not provide effective seal, which can lead to leaking of an acrylamide solution when poured between the plates.*
6) Fasten the screws firmly (but not too much, not to break the glass plates), put the "sandwiches" into the casting stand.
7) Place cams into holes on the sides of the casting stand, longer end down. Smoothly turn both cams by 180° (longer end up). The sandwiches will slightly sink into the gasket.

Gel preparation

8) Mark the position of lower gel edge 2.2 cm from the upper margin of glass plates.

9) Prepare fresh 10% solution of APS (ammonium persulfate; cannot be stored). Put the required amount of APS into 1.5 ml tube, use analytical weights. Add distilled water.

	1 gel	2 gels	4 gels
APS	0.022 g	0.033 g	0.067 g
distilled water	200 µl	300 µl	600 µl

10) Prepare solution for the resolving gel, use a 250 ml glass beaker:

resolving gel buffer	6.75 ml	13.5 ml	27 ml
distilled water	11.5 ml	23 ml	46 ml
acrylamide + BIS stock solution	4.75 ml	9.5 ml	19 ml

Acrylamide stock solution may be added using 5 ml pipette (e.g. 4 × 4.75 ml = 19 ml).

11) Mix (whirl) gently, avoid air bubbles (oxygen inhibits acrylamide polymerization). Continue until the solution is homogenous (no "filaments" of acrylamide visible).

12) Add following two chemicals that initiate polymerization:

10% APS	108 µl	215 µl	430 µl
10% TEMED	108 µl	215 µl	430 µl

13) Mix gently. Fill a syringe with the gel solution. Place the syringe between glass plates near one side and slowly push one larger drop of solution out along a spacer. Let it reach the bottom and the lowermost corner (to avoid bubbles in the corner). Continue with slow flow until the surface reaches the marked level. All the time check for air bubbles; if they occur, stop pouring a solution and wait until they burst.

14) When all gels are filled, pour distilled water on an upper surface (to make it flat). Use 5 ml pipette. Push the water along one of the spacer (it will not mix with acrylamide), continue with slow flow until the water level reaches near the opposite spacer.

15) Let the gels and also an excess of acrylamide solution in a beaker polymerize for ca 1 hour. Continue preparing the apparatus and buffers (see below) in that time.

16) After polymerization suck out water using a syringe with a needle. As it contains remains of non-polymerized acrylamide, pour the water into the special bottle for toxic waste. The same applies for the remains in the beaker.

17) Prepare solution for the stacking gel, use a 100 ml glass beaker:

	1 gel	2 gels	4 gels
stacking gel buffer	4,5 ml	9 ml	18 ml
acrylamide + BIS stock solution	0,5 ml	1 ml	2 ml

18) Mix (whirl) gently, avoid air bubbles. Add following two chemicals that initiate polymerization:

10% APS	30 µl	60 µl	120 µl
10% TEMED	30 µl	60 µl	120 µl

19) Mix gently. Fill a syringe with the gel solution. Pour the solution between glass plates until the surface reaches about 5 mm bellow the outer plate margin (= inner plate margin).

20) Place combs between the glass plates. Align the two combs in a "sandwich" exactly (makes loading of samples easier). Check for air bubbles under comb teeth.

21) Let the gels and also an excess of acrylamide solution in a beaker polymerize for ca 1 hour. Continue preparing the apparatus and buffers (see below) in that time.
22) After polymerization, carefully remove the combs. If the walls of wells bend, carefully reposition them with a needle. If there is larges excess of non-polymerized acrylamide solution, suck it out using a syringe with a needle and pour it into the special bottle for toxic waste. The same applies for the remains in the beaker.
23) Fill the wells with electrode buffer.
24) Suck the buffer from wells using a syringe with a needle. Pour it to the beaker; it can be then poured into normal sink (non-toxic). Fill the wells again with an electrode buffer.
25) If you do not continue with sample loading immediately, cover the gels with aluminium foil to avoid drying out and store in the fridge. Gels may be stored overnight.

Preparing the apparatus

26) Prepare the electrode buffer (see Solution & buffers, p. 51)
27) Fill the lower electrophoresis chamber:

electrode buffer .....2.4 l
distilled H_2O .....1.8 l

28) Place the electrophoresis into larger plastic basin and place both into an "electrophoretic" fridge. Place the heat exchanger and magnetic stir bar into the electrophoresis chamber. Let the apparatus cool overnight, do not switch the additional cooling and the water pump on (unless you continue with sample loading immediately).

C) Sample loading & electrophoresis

1) Switch the additional cooling and the water pump on (a switch on the side of the cooling apparatus + plugging the pump wire in the wall socket). Switch the magnetic stir on.
2) Fill the outer basin with ice.
3) Slowly defreeze the samples (takes ca 15 min). Work on ice, mix the samples occasionally with a finger.
4) Place the gels on a table, fill the wells with electrode buffer, place cooling plates around and between the gels.
5) Load 10 µl of each sample (or other optimized volume) using a Hamilton syringe. Wash the syringe between samples with distilled water (sucking in and pushing out).

! *Work as quickly as possible to avoid heating of gels and samples. Usually you will load the same samples in the same order onto all gels. In that case suck the total volume (e.g. 40 µl) and load by 10 µl on all gels.*

6) Place the gaskets in grooves on the upper electrophoresis chamber. It may help to rinse the grooves with a small amount of distilled water, spread the water with fingers into a thin layer and "glue" the gaskets on the chamber.
7) Place the upper chamber on the upper side of glass plates. Loose the cams on the casting stand (slowly turn by 180°, the longer end to point down). Remove cams from the casting stand. Place the cams in the holes on upper electrophoresis chamber, longer ends up. Smoothly turn both cams by 180° (longer ends down).
8) Remove glass plates from the casting stands, remove the gaskets (if attached to the lower edge of the plates). Place the plates + upper chamber into lower electrophoresis chamber, the heat exchanger being between the glass plates.

9) Fill the upper chamber with electrode buffer. Start from the middle and slowly flood the grooves connecting the chamber and the gels (to avoid washing out the samples). The level of the buffer is about 1 cm above the electrode (a thin wire in the middle of the chamber).
10) Cover the chamber with the lid, check it the electrodes fit the lid properly.
11) Plug the wires into the power supply, check the polarity.
12) Run the electrophoresis at the constant current of 80 mA. After stabilizing (ca 1 min), record the values of voltage and resistance.
13) Regularly check the apparatus (position of the front, amount of the buffer in the upper chamber – interrupt the electrophoresis and refill if necessary).
14) Prepare buffers and staining solutions (see below) during the electrophoresis.
15) When the electrophoresis front reaches ca 1 cm from the lower edge of glass plats (ca 4.5–5 hours), stop the electrophoresis. Switch off the power supply, disconnect the wires. Record the time and final values of voltage and resistance.

D) Detection of isozymes (staining)

General remarks

- Some of the chemicals used are poisonous. Work with care, always use gloves and protective coat.
- The staining is relatively sensitive to reaction conditions. Use fresh buffers, check pH value. Weight all the chemicals using analytical (precise) weights.
- Some chemicals are stored at 4°C or -20°C. Remove these chemicals from a fridge/freezer long enough before use and let them warm up to the room temperature (to avoid condensation of water in the bottles).
- Some chemicals and nearly all staining solutions (i.e. when dissolved) are light sensitive. Use beakers covered with aluminium foil for preparing the solutions. Put all chemicals back into the storage places (box, fridge, freezer) immediately after use. Dissolve all the chemicals and work with the solutions in a dark chamber only!

Staining solutions

1) Prepare staining solutions during the electrophoresis.
2) Weight the required amounts of chemicals using analytical weights, following the recipes on the pp. 36–41. Store the weighted chemicals (mixtures for one staining) in 100 ml glass beakers covered with aluminium foil in a dark chamber. If an enzyme (glucose-6-phosphate dehydrogenase) is part of the mixture, add it just before application of a solution on a gel, not during preparation of other components.
3) Prepare required volumes of buffers (p. 52). Use stock solution of the given concentration, adjust pH if necessary.
4) About 10 minutes before the end of electrophoresis pour buffers into weighted chemicals mixtures to dissolve them (stir with a glass rod; it may take up to a few minutes, e.g. with Fast Black K salt). Work in dark chamber. Cover the beakers with aluminium foil and store them in the closed locker. Open the chamber for as short time as possible.
5) Just before stopping the electrophoresis prepare the staining trays. Fill them with cooled distilled water or cooled buffers from a fridge (depending on the enzyme stained).

Removing gels

6) Remove glass plates with the upper chamber from the apparatus, pour the electrode buffer into the sink, put gels into the casting stand, remove cams and upper chamber.
7) Loose the clamp screws, remove the clamps.
8) Remove the spacers. Use the thinner end of a plastic wedge or a knife and push the spacers from the bottom (be careful not to chip the gel).
9) Place the thicker end of the wedge between the glass plates of the upper gel in a "sandwich", about at the middle of their length, fix the opposite site with fingers. Pry the upper glass plate slowly. If the gel is attached to the upper plate, turn it quickly. Be careful not to tear the gel.
10) Cut off the stacking gel. Cut certain corner (e.g. the lower right) to mark the orientation of the gel. Be aware of the opposite orientation if the gel is stuck to the upper plate. If you know the position of the isozymes from previous work, it is possible to cut off the "empty" upper or lower part of the gel.
11) Wash your gloves (fingers) in water. Using a wedge or a knife slightly lift one corner of a gel, take it with fingers, slowly lift the whole gel from the glass plate and put it into the staining tray with water / buffer. Be careful not to tear the gel.

    ! *It is crucial to work with wet gloves; gels stick firmly on dry gloves and tear easily. Work slowly. If a gel is accidentally thorn, transfer all parts into the staining tray and spread them not to overlap each other.*

12) Continue with other gels (first remove all gels and then start staining all of them together).

Staining

13) Wash the gel in water / buffer for a few minutes. Pour out the water (hold the gel in the tray with fingers), transfer the tray to the dark chamber. Pour staining solution on the gel (in the dark!), mix gently. If addition of an enzyme is part of the recipe, add it now.
14) Place the tray into the thermostat at 35°C or 37°C.
15) Mix the solution by shaking the tray occasionally (in the dark!), add substrates (if required). Check the staining occasionally in the minimal illumination.

    ! *Length of the staining is specific for every enzyme system and species. The shortest times are about 20 minutes, the longest about a few hours (rarely overnight). It is crucial to stop the staining in the proper time – otherwise the bands might be too intense (wide, blurred) or strong background may develop, both hampering scoring of bands.*

16) After staining pour the staining solution into the special bottle (some components may be toxic). Rinse the gel with distilled water or in fixation solution. If fixation solution is used, pour it to the special bottle after fixation (toxic waste, contains methanol).
17) Wash the gel in distilled water (usually at least for 1 hour) to remove all remains of staining / fixation solution, exchange the water if necessary. Gels can be stored in water at room temperature overnight before drying and final fixation.

E) Drying and fixation of the gels

1) Pour about 1-2 cm of tap water into large plastic dish.
2) Put 2 cellophane leaves per one gel into dish with tap water. Wash the cellophane for at least 5 minutes.
3) Put one plastic frame into the dish with water.
4) Spread a cellophane leaf over the frame. Remove all bubbles on the upper surface of the cellophane.
5) Spread the gel over the cellophane leaf, remove all air bubbles.
6) Spread the second cellophane leaf over the lower cellophane and the gel. Remove all air bubbles (this is crucial, as bubbles may tear the gel during drying).
7) Put the second plastic frame over the cellophanes and the gel, align it with the lower frame.
8) Align the cellophane leaves with the frames and move the gel to the centre of the frames / cellophanes.
9) Fold one side of both cellophane leaves over the upper frame, remove all air bubbles, fix the cellophane and both frames with clothes pegs. Tighten the cellophanes and continue with two other sides. Be careful of air bubbles.
10) Align the gel to the centre of the frames. Fix the gel with fingers and lift the frames with cellophanes and the gels out of water, the last opened side down. Slightly open the space between the frames to release the excess of water between the cellophanes.
11) Fold and fix the last side of the cellophanes. Add a label with the date, samples identity and enzyme system.
12) Let the gel dry. There are two types of frames used in the laboratory. If you are using the thinner frames, it is necessary to fix them after ca 30 min on a firm wooden plate to prevent bending of the gel.
13) After about two days, the gels might be removed from the frames. Cut the cellophane around the gel (leave about 1 cm wide strip around for writing sample numbers etc.).
14) Press the gels for a few days (e.g. in a thick book). Store the gels in a dry place at room temperature in the dark for long term storage (but gels can be exposed to light, even to strong one, during scoring).

F) Enzyme systems

In this section, all enzyme systems used in our laboratory are summarised. For each enzyme, following information is presented:

- abbreviation + full name
- (separated by hyphens) E.C. number, quaternary structure, localisation within a cell (c – cytoplasmic, m – mitochondrial, p – plastid) and typical number of loci
- practical remarks
- recipe for staining solution; for common buffers see p. 52
- specific information about staining (non-standard procedures, etc.)

ACP – acid phosphatase

E.C. 3.1.3.2 – monomeric, dimeric – c, m, p – 1 to 4

0.05M sodium acetate buffer, pH = 5.0	30 ml (+ ca 50 ml to rinse the gel)
1-naphthylphosphate	50 mg
Fast Black K salt	5 mg

Due to acidic pH rinse the gel with pre-cooled buffer instead of water. You need to prepare ~100 ml (min. 80 ml) of the buffer.

ADH – alcohol dehydrogenase

E.C. 1.1.1.1 – dimeric – c – 1 to 3

often highly variable; sensitive to temperature during sample extraction and ELFO; sometimes simultaneous negative staining of SOD

0.1M Tris–HCl buffer, pH = 7.5	40 ml	
NAD^+	15 mg	
MTT	10 mg	
PMS	1 mg	
ethanol (pure)	10 ml	added later (not in the staining solution!)

Pour the staining solution over the gel, incubate for 2–3 minutes; this time may be prolonged up to 10 min in some species. Add ethanol (still in the dark!). In case of low enzyme activity, it is possible to add another 10 ml of ethanol (substrate) each one hour.

AAT – aspartate aminotransferase (= GOT – glutamate oxalacetate transaminase)

E.C. 2.6.1.1 – dimeric – c, m, p – 1 to 4

often variable; frequent occurrence of secondary bands that may complicate the allelic scoring; relatively sensitive to temperature

Solution A (prepare in advance!)

0.1M Tris–HCl buffer, pH = 8.4	20 ml
aspartic acid	240 mg
α-ketoglutaric acid	40 mg

Solution B:

0.1M Tris–HCl buffer, pH = 8.4	20 ml
pyrodoxal-5-phosphate	25 mg
Fast Blue BB salt	50 mg
Fast Violet B salt	50 mg

Prepare the solution A in advance (in light). It is necessary to heat the solution to ca 50°C to dissolve the substrates and let it cool to the room temperature. Prepare the solution B in the dark. Mix the two solutions (in the dark) just before application on the gel.

DIA – diaphorase

E.C. 1.6.-.- – monomeric, dimeric, tetrameric – c, m – 1 to 4

usually fast staining (check to avoid over-staining); the fastest (lower) locus usually monomeric and easy to score, other loci often weak and hard to read; sometimes secondary bands

0.1M Tris–HCl buffer, pH = 8.0	50 ml	
NADH (reduced)	13 mg	
MTT	5 mg	
2,6 dichlorindophenol	2 mg	(sodium salt hydrate)

ENP – endopeptidase

E.C. 3.4.23.6 – monomeric – ? – 1

usually weak bands

0.2M Tris–maleic acid buffer, pH = 5.5	50 ml (+ ca 50 ml to rinse the gel)	
BANA (β-N-benzoyl-DL-arginine-α-naphthylamide.HCl)	25 mg	dissolve in 2 ml of N,N'-dimethylformamide
Fast Black K salt	20 mg	
$MgCl_2.6H_2O$	50 mg	

Due to acidic pH rinse the gel with pre-cooled buffer instead of water. You need to prepare ~100 ml of the buffer. Dissolve all components except for BANA in the buffer (in the dark). Dissolve BANA in N,N'-dimethylformamide, mix with the staining solution, pour on the gel.

EST – esterases

E.C. 3.1.1.- – monomeric, dimeric – c – 2 to several

relatively non-specific staining (more enzymes / loci); usually variable, suitable for clone identification but allelic scoring often difficult (overlaps between loci, etc.); relatively sensitive to temperature

Esterase buffer, pH = 6.45	60 ml (+ ca 50 ml to rinse the gel)	
1-naphthyl acetate	25 mg	dissolve in 2.5 ml of 50% acetone
2-naphthyl phosphate	25 mg	dissolve in 2.5 ml of 50% acetone
Fast Blue BB Salt	50 mg	

Due to acidic pH rinse the gel with pre-cooled buffer instead of water. You need to prepare ~110 ml of the buffer. Dissolve both substrate in acetone (in the dark), dissolve Fast Blue salt in the buffer (in the dark), mix, pour on the gel.

G6PDH (= G6PD) – glucose-6-phosphate dehydrogenase

E.C. 1.1.1.49 – dimeric – c, p – 2

often fast staining (check to avoid over-staining)

0.05M Tris–HCl buffer, pH = 8.0	50 ml	
glucose-6-phosphate	50 mg	(disodium salt)
$MgCl_2.6H_2O$	50 mg	
MTT	10 mg	(may be substituted with NBT)
$NADP^+$	6 mg	
PMS	2 mg	

GLU – β-D-glucosidase (= β-D-glucoside glucohydrolase)

E.C. 3.2.1.21 – dimeric – c – 1

0.05M potassium phosphate buff.,pH=6.5	50 ml	(+ ca 50 ml to rinse the gel)
6-bromo-2-naphthyl-β-D-glucoside	50 mg	dissolve in 5 ml of N,N'-dimethylformamide
PVP-40	1 g	
Fast Blue BB salt	50 mg	

Due to acidic pH rinse the gel with pre-cooled buffer instead of water. You need to prepare ~100 ml of the buffer. Dissolve all components except for the substrate in the buffer (in the dark). Dissolve the substrate in N,N'-dimethylformamide, mix with the staining solution, apply on the gel. Stain for 1 hour at 35°C and then at room temperature (in the dark!)

GDH – glutamate dehydrogenase

E.C. 1.4.1.2 – hexameric, monomeric (?) – c – 1

0.05M Tris–HCl buffer, pH=8,0	50 ml
Na-glutamate	200 mg
$CaCl_2$ (anhydrous)	50 mg
NAD^+	10 mg
MTT	10 mg
PMS	2 mg

HEX – hexokinase (= glucokinase)

E.C. 2.7.1.1 – monomeric – c, m, p – 2 or 3

0.1M Tris–HCl buffer, pH=7,1	50 ml	
glucose	45 mg	
$MgCl_2.6H_2O$	50 mg	
ATP (disodium salt)	12,5 mg	
$NADP^+$	12,5 mg	
NBT	10 mg	
PMS	1.5 mg	
glucose-6-phosphate dehydrogenase	40 µl	(= 40 U, stock solution of 1 U/µl)

Add the dehydrogenase to the solution just before application on the gel (in the dark). Many different staining recipes exists (higher pH, substitution of some chemicals)

IDH – isocitrate dehydrogenase

E.C. 1.1.1.42 – dimeric – c – 1

0.1M Tris–HCl buffer, pH=8,0	50 ml	
isocitric acid	50 mg	(trisodium salt)
$MgCl_2.6H_2O$	80 mg	
$NADP^+$	10 mg	
MTT	8 mg	
NBT	8 mg	
PMS	2 mg	

LAP – leucine aminopeptidase (= AMP – aminopeptidase)

E.C. 3.4.11.- – monomeric – c – 2 or 3

usually variable; sensitive to temperature during sample extraction and ELFO and to sample quality; usually quite weak bands (weak staining)

Solution A:

0.2M Tris–maleate buffer, pH = 6.0	30 ml	(+ ca 50 ml to rinse the gel)
$MgCl_2.6H_2O$	60 mg	dissolve in the buffer (in light)
L-leucyl-β-naphthylamide hydrochloride	35 mg	dissolve in 2.5 ml of 50% acetone (in the dark)

Solution B:

0.2M Tris–maleate buffer, pH = 6.0	30 ml	
Fast Black K salt	25 mg	

Due to acidic pH rinse the gel with pre-cooled buffer instead of water. You need to prepare ~120 ml of the buffer. Dissolve $MgCl_2$ in the buffer. Dissolve the substrate in acetone (in the dark), mix with the buffer (solution A) and pour on the gel. Dissolve Fast Black K salt in the other part of the buffer (solution B). Add the solution B to the gel after 10 min.

MDH – malate dehydrogenase

E.C. 1.1.1.37 – dimeric – c, m – 1 to 3

Roztok A:

0.1M Tris–HCl buffer, pH=7.5	20 ml	
malic acid	150 mg	prepare in advance, adjust pH with Na_2CO_3 to 7.5

Roztok B:

0.1M Tris–HCl buffer, pH=7.5	30 ml	
NAD^+	15 mg	
NBT	15 mg	(may be supplemented with 10 mg MTT)
PMS	2 mg	

Prepare the solution A in advance (in light), adjust pH to 7.5 using Na_2CO_3. Prepare the solution B in dark, mix the two solutions, pour on the gel.

ME – malic enzyme

E.C. 1.1.1.40 – tetrameric – c – 1

0.05M Tris–HCl buffer, pH=8.0	50 ml
malic acid	150 mg
$MgCl_2.6H_2O$	50 mg
$NADP^+$	5 mg
MTT	10 mg
PMS	2 mg

NADHDH (= NDH) – NADH dehydrogenase

E.C. 1.6.99.- – monomeric, dimeric, tetrameric (?) – c, m, p – 1 to 4 (?)

sometimes not distinguished from DIA (catalyse similar reaction, sometimes but not always the same enzymes are stained with both procedures)

0.1M Tris–HCl buffer, pH=8.4	30 ml	
menadion	15 mg	dissolve in 2.5 ml of 50% acetone (in the dark)
NADH (reduced)	17,5 mg	
MTT	20 mg	

PRX – peroxidase

E.C. 1.11.1.7 – monomeric – c – 2 to several

0.05M sodium acetate buffer, pH=5.0	50 ml (+ ca 50 ml to rinse the gel)	
$CaCl_2$ (anhydrous)	11 mg	dissolve in the buffer (in light)
3% H_2O_2	250 µl	
3-amino-9-ethyl carbazole	25 mg	dissolve in 5 ml of N,N'-dimethylformamide

Due to acidic pH rinse the gel with pre-cooled buffer instead of water. You need to prepare ~100 ml of the buffer. Dissolve the substrate in N,N'-dimethylformamide (in the dark). Mix with the buffer, add H_2O_2. Incubate at 4°C (in the fridge).

PGI – phosphoglucoisomerase (= GPI – glucosephosphate isomerase, phosphohexose isomerase)

E.C. 5.1.3.9 – dimer – c, p – 1 or 2

sometimes simultaneously stained other enzymes following after PGI in glycolysis (6-PGDH or G6PDH) but interpretation possible if the loci do not overlap

0.05M Tris–HCl buffer, pH=8.0	50 ml	
fructose-6-phosphate	20 mg	
$MgCl_2.6H_2O$	24 mg	
$NADP^+$	10 mg	
MTT	10 mg	
PMS	2 mg	
glucose-6-phosphate dehydrogenase	7 µl	(= 7 U, stock solution of 1 U/µl)

Add the dehydrogenase to the solution just before application on the gel (in the dark).

PGM – phosphoglucomutase

E.C. 2.7.5.1 – monomeric – c, p – 1 or 2

sometimes simultaneously stained other enzymes following after PGI in glycolysis (6-PGDH or G6PDH) but interpretation possible if the loci do not overlap

0.05M Tris–HCl buffer, pH=8.5	50 ml	
glucose-1-phosphate	50 mg	
$MgCl_2.6H_2O$	24 mg	
$NADP^+$	10 mg	
MTT	10 mg	
PMS	2 mg	
glucose-6-phosphate dehydrogenase	7 µl	(= 7 U, stock solution of 1 U/µl)

Add the dehydrogenase to the solution just before application on the gel (in the dark).

6-PGDH (= 6-PGD, PGD) – 6-phosphogluconate dehydrogenase

E.C. 1.1.1.44 – dimeric – c, p – 2
often fast staining (check to avoid over-staining); sometimes simultaneous negative staining of SOD

0.1M Tris–HCl buffer, pH=8.4	30 ml
6-phosphogluconic acid	10 mg
$MgCl_2.6H_2O$	30 mg
$NADP^+$	5 mg
MTT	5 mg
PMS	1 mg

SKDH (= SKD) – shikimate dehydrogenase

E.C. 1.1.1.25 – monomeric – c, p – 1 or 2
often fast staining (check to avoid over-staining); sometimes simultaneous negative staining of SOD

0.1M Tris–HCl buffer, pH=8.4	30 ml
shikimic acid	30 mg
$NADP^+$	5 mg
MTT	6 mg
PMS	1 mg

SOD – superoxide dismutase (= TO – tetrazolium oxidase)

E.C. 1.15.1.1 – dimeric, tetrameric – c, p – 2 or 3
negative staining

0.05M Tris–HCl buffer, pH=8,2	50 ml
NBT	5 mg
EDTA	4.5 mg
riboflavin	1.5 mg

Incubation at 35°C in the dark for 20 min. Transfer to intense light at room temperature (e.g. under the table lamp). Stop staining when dark background develops, bands of the enzyme remain colourless.

Operation manuals

Measurement of DNA concentration using „Qubit 2.0“ fluorimeter

The following protocol use the Qubit dsDNA BR Assay Kit, which enables **very accurate quantification of double-stranded DNA with concentration ranging from 0.1 to 1000 ng/µl**. The samples are measured in tubes placed in sample chamber (covered by black tipping lid). The instrument allows to use as little as 1 µl of the sample, which is mixed with 199 µl of the working solution of DNA dye. For measuring bigger volumes of the sample decrease the volume of the working solution to keep the total volume of 200 µl (e.g. 5 µl of the sample + 195 µl of working solution). All the solutions prepared (working solution, solutions of standards, solutions of samples) are stable for 3 h.

1) Prepare the working solution. Multiply the volumes by number of samples + 4:
   Qubit Buffer ..... 199 µl
   Qubit Reagent ..... 1 µl
   Shake to mix. Cover the tube with aluminium to prevent exposure to light!
2) Prepare two 0,5 ml Axygen (!) PCR tubes and label the tube caps as S1 and S2. Prepare the standards as follows:
   S1: 190 µl of working solution + 10 µl of Standard 1 (stored in a fridge)
   S2: 190 µl of working solution + 10 µl of Standard 2 (stored in a fridge)
3) Vortex for 3 s and spin briefly. Incubate for cca 5 min at room temperature in the dark (cover the tubes with a piece of paper).
4) Prepare one set of 0,5 ml Axygen (!) PCR tubes for samples. Pipette 199 µl of the working solution and label the tube caps.
5) Add 1 µl of the samples, vortex for 3 s and centrifuge briefly. Incubate for cca 5 min at room temperature in the dark (cover the tubes with a piece of paper).
6) Turn the instrument on and select "*DNA*" – "*dsDNA Broad Range*" – *(Read New Standards?)* "*Yes*". Calibrate the instrument with standards. Insert the tube S1, close the lid and press "*Read*". Insert the tube S2, close the lid and press "*Read*". The standards ("○" symbol) must appear within the range of the calibration curve and the option "*Insert Assay Tube*" must be visible. (If the message "*Standard Incorrect*" appears, prepare new standards).
7) Continue with sample measurements. Insert the first sample tube and press "*Read*". Choose the option "*Calculate Stock Conc.*". Using the volume roller wheel in "*Volume of Sample Used*", select the volume of the sample used (e.g. 1 µl). If necessary, convert the sample concentration into units required by opening the pop-up window for units.
8) Insert the next sample, close the lid and press "*Read Next Sample*" (it is not necessary to set the volume of the sample again).
9) When finished, turn off the instrument (button placed on the left upper corner of the display).

Measurement of DNA concentration using „NanoDrop“ spectrophotometer Avans Biotech UVS-99

1) Turn on the computer (login and password written on the side of the computer).
2) Turn on the instrument (power switch is on the right bottom rear of the machine).
3) Start the NANODROP-UVS-99.exe software (If an error message appears, disconnect and reconnect the USB cable between the instrument and computer).
4) Choose "*Nucleic Acid*".
5) Lift the silver sampling arm. Wipe both pedestals of the arm using a paper towel moistened with distilled water (the pedestals are located in the direction of the black fibre).
6) Pipette (1–)1.5(–2.5) µl of the blank sample (the liquid used to dissolve your samples – elution buffer, TE buffer, sterile water, etc.) onto the lower pedestal. Lay down the sampling arm and make sure the solution bridge the gap between both pedestals. Press "*Blank*".
7) When the measurement is complete, open the sampling arm and wipe the blank sample from both pedestals using a paper towel moistened with distilled water. Pipette the sample onto the lower pedestal – use the same volume as for blank sample. Lay down the sampling arm and make sure the solution bridge the gap between both pedestals. Press "*Measure*".
8) Record the values of sample concentration. All the other parameters such as absorbance values, ratios, absorbance curve etc. can be easily saved using "*Save Graph*" (saved as a printscreen).
9) Wipe both pedestals between the samples using a paper towel moistened with distilled water.
10) The absorbance curve may appear somewhat ´toothed´ after a few measurements. This can be improved by repeating the blank measurement (step 6). Anyway, the toothed shape of the curve has usually no impact on the measured concentrations.
11) When finished, wipe both pedestals using a paper towel moistened with distilled water. Choose "*Exit*" and turn off the instrument. Save your data to a USB flash drive and turn off the computer.

TC-XP Bioerg thermocycler (gradient block; two blocks)

How to operate the instrument

- Power switch is on the right bottom rear of the instrument.
- Use F1–F5 (below the display) and arrow keys to select options, and Enter key to confirm.
- Three main options are available on the display:
  - *File* – to open the list of *Users* and programs (*File name* and *Save date* is shown).
  - *System* – to show system settings, e.g. lid temperature, sound alarm at the end of program etc.
  - *Run* – to run the program.
- For two blocks version, press "Shift" to select the block (otherwise the interface is the same).

How to set a program

- Choose *Edit* to change parameters of existing program.
- Press F2 (New File) to set a new program.
- Press F1 (+Seg) to add new cycle steps (segments); for each step, set temperature (Temp), duration (Time), ramping (Ramp = speed of temperature change between segments; default value „#.#“ indicates maximum speed and can be used for most applications).
- Press F2 (+Cycle) to set the number of cycles, and set the beginning and end point of the cycling (e.g. from step 02 to step 04).
- Set +Temp and +Time for *touchdown* PCR or gradual change in step duration, respectively.
- Gradient block only: the value specified in the Grad column indicates the temperature gradient ranging from first to last vertical row of wells. The gradient steps across the rows are set automatically.
- Press F5 (Save), set the user name and program name. Confirm by pressing F3 (Save).
- If the new program can not be saved, there is not enough memory available. Delete some of the existing programs. The two block version allows to edit and run an existing program without saving it.

How to start the program

- Insert the PCR tubes, strips or plates.
- Close the lid and revolve the knob clockwise to fasten tightly (do not use excessive power!); for the two block version revolve the knob clockwise till it sounds "dang, dang.....".
- Press F1 (*File*), choose the program and press F5 (*Run*).
- Details of the running program will appear on the display (user name, name of the program, total time, remaining time, graphical representation of the run, number of cycles, lid temperature etc.)
- When the program has finished, press F1 (*Stop*) and confirm by pressing F1 (*Stop*).

Biometra T3000 thermocycler (three blocks)

How to operate the instrument

- Power switch is on the left.
- Use four white keys (below the display) and arrow keys to select options, *Enter* to confirm, and *Cancel* to return back
- Four main options are available:
  - *Info* – to display information about blocks; *active* (the block is in use) or *inactive*
  - *System* – system settings, sound alarm at the end of program, language settings, display contrast etc.
  - *Start*/*stop* – start/stop the program
  - *Edit* – make a new program or change parameters of existing ones

How to set a program and edit existing ones

- Press *Edit,* choose the folder and press *Enter*.
- Select the subfolder and press *Edit* (for new program) or select an existing program and press *Edit*
- Set the name of the program and press *Name OK*.
- Set *Lid Temperature* to 99°C and *Preheating ON*, confirm by *Enter*.
- Set the temperatures (*Temp*), duration of the steps (*Time*; example: „90“ for 90 s, „3.30“ for 3 min 30 s, „3.0.0“ for 3 h); for hold (infinite duration, "*Pause*") set "0".
- Set the cycling using a back-loop. In the row labelled with „←“ enter the target step for a back-loop for cycling and press Enter. In the row labelled with # enter the number of back-loops. Note: total cycler number = (n back-loops) + 1, e.g. enter 29 for a total number of 30 cycles.
- If necessary, set other parameters in "Options -->". Use arrow keys to set temperature increment (dT[C; for *touch-down* use negative value!), duration increment (dt[S]) or ramping ([°C/s], speed of temperature changes between cycles).
- Press „*Save pgm*“ to save.

How to start the program

- Insert the PCR tubes or strips
- Close the lid and revolve the knob clockwise till it sounds "dang, dang.....".
- Press *Start/Stop*, select the block to be used; open the folder and select the program, press *Start*.
- While lid preheating, the block is held constantly at 25°C. The program starts as soon as the lid has reached the set temperature (usually 99 °C). During the preheating phase the display of the lid temperature alternates between the current lid temperature and "preheating".
- When the lid has reached the set temperature and the program starts to run, stay close to the instrument for 2–3 minutes. The tube may open during this phase, especially the block 1! If you hear sound like cracking, revolve the knob counter-clockwise, open the lid and carefully close the tubes! Then close the lid and revolve the knob again.
- To see the remaining time, press "*Info*".
- When the program has finished (= the program enters a pause), an audible signal starts. Press any white key to stop the signal. Press *Start/Stop*, select the block and confirm by pressing *Stop*.

pH-Meter Hanna pH 211

Calibration

- Recommended at least once a week. Calibrate before each use if high accuracy is required.
- Use the buffer pH = 7.01 toghether with a second buffer. If measuring in the acid range, use pH = 4.01 buffer as the second buffer. If measuring in the alkaline range use pH = 10.01 as the second buffer. (All the solutions are stored in the table below the instrument).
- Remove the protective cap from the electrode. Rinse the electrode and thermometer with distilled water (keep both instruments on the red holder; use a beaker to collect the rinse water). Gently dry with a paper towel.
- Move the red arm to insert electrode and thermometer into the bottle with pH = 7.01 buffer. The hole in the lower part of the electrode (above the glass bulb) must be immersed in the liquid.
- Turn the instrument on and wait until the pH and temperature values 0stabilise.
- Press "CAL" to start the calibration.
- The "Not ready" indication will blink on LCD until the reading has stabilised. When the reading is stable, "Ready CFM" will blink. Press "CFM" to confirm the calibration.
- Use ▲°C / ▼°C to set the pH of the second buffer (shown in right part of LCD).
- Remove the electrode and thermometer from the solution, rinse with distilled water and gently dry with a paper towel. Insert electrode and thermometer into the second buffer.
- The "Not ready" indication will blink on LCD until the reading has stabilised. When the reading is stable, "Ready CFM" will blink. Press "CFM" to confirm the calibration.
- If not continuing with the measurement, turn off the instrument. Remove the electrode and thermometer from the solution, rinse with distilled water and gently dry with a paper towel. Cover the electrode with the protective cap filled with storage solution (if necessary, add the solution to completely immerse the glass bulb).

pH measurement

- Remove the protective cap from the electrode. Rinse the electrode and thermometer with distilled water (keep both instruments on the red holder; use a beaker to collect the rinse water). Gently dry with a paper towel.
- Move the red arm to insert electrode and thermometer into the sample. The hole in the lower part of the electrode (above the glass bulb) must be immersed in the liquid.
- Turn on the instrument.
- Wait until the pH and temperature values are no longer changing.
- When finished, turn off the instrument.
- Remove the electrode and thermometer from the solution, rinse with distilled water and gently dry with a paper towel. Cover the electrode with the protective cap filled with storage solution (if necessary, add the solution to completely immerse the glass bulb).

Common solutions & buffers

1) General solutions

1M HCl (hydrochloric acid)

	100 ml	250 ml	500 ml
distilled H_2O	~ 70 ml	~ 200 ml	~ 400 ml
concentrated HCl (35%)	8.83 ml	22.07 ml	44.14 ml
add H_2O to get the final volume	*100 ml*	*250 ml*	*500 ml*

Always add acid into larger amount of water, never do it opposite way (water into the acid – the solution heats quickly and may splash from the bottle).

1M H_3PO_4 (phosphoric acid)

	100 ml	250 ml	500 ml
distilled H_2O	~ 70 ml	~ 200 ml	~ 400 ml
concentrated H_3PO_4 (85%)	6.74 ml	16.86 ml	33.71 ml
add H_2O to get the final volume	*100 ml*	*250 ml*	*500 ml*

Always add acid into larger amount of water, never do it opposite way (water into the acid – the solution heats quickly and may splash from the bottle).

1M acetic acid

	100 ml	250 ml	500 ml
distilled H_2O	~ 70 ml	~ 200 ml	~ 400 ml
acetic acid (pure)	5.72 ml	14.30 ml	28.60 ml
add H_2O to get the final volume	*100 ml*	*250 ml*	*500 ml*

Always add acid into larger amount of water, never do it opposite way (water into the acid – the solution heats quickly and may splash from the bottle).

NaOH (sodium hydroxide)

	0.5M NaOH		1M NaOH		10M NaOH	
	100 ml	250 ml	100 ml	250 ml	100 ml	250 ml
distilled H_2O	~ 70 ml	~ 220 ml	~ 70 ml	~ 220 ml	~ 70 ml	~ 200 ml
NaOH (anhydrous)	2 g	5 g	4 g	10 g	40 g	100 g
add H_2O to get the volume	*100 ml*	*250 ml*	*100 ml*	*250 ml*	*100 ml*	*250 ml*

For 0.5M NaOH for DNA isolation, use sterile water or use Milli-Q water and sterilize by autoclaving (special program for liquids).

2) DNA isolation and purification

2% CTAB

	100 ml	250 ml	500 ml	1000 ml	(conc.)
CTAB (cetyl trimethyl ammonium bromide)	2.0 g	5.0 g	10.0 g	20 g	(2% w/v)
Tris-base	1.21 g	3.03 g	6.06 g	12.12 g	(0.1M)
Na_2-EDTA	0.74 g	1.86 g	3.72 g	7.44 g	(0.02M)
NaCl	8.18 g	20.45	40.91 g	81.82 g	(1.4M)
PVP	2 g	5 g	10 g	20 g	(2% w/v)
distilled H_2O	~ 80 ml	~ 220 ml	~ 450 ml	~ 900 ml	
add H_2O to get the final volume	*100 ml*	*250 ml*	*500 ml*	*1000 ml*	

100mM Tris-HCl buffer, pH = 8.3

	50 ml	100 ml	250 ml
Tris-base	0.61 g	1.21 g	3.03 g
distilled H_2O	~ 35 ml	~ 80 ml	~ 220 ml
adjust pH using 1M HCl		*pH = 8.3*	
add H_2O to get the final volume	*50 ml*	*100 ml*	*250 ml*

TE buffer (10× concentrated stock solution)

	50 ml	100 ml	250 ml	1000 ml	(conc.)
Tris-base	0.61 g	1.21 g	3.03 g	12.11 g	(0.1M)
Na_2-EDTA	0.19 g	0.37 g	0.93 g	3.72 g	(0.01M)
distilled H_2O	~ 35 ml	~ 80 ml	~ 220 ml	~ 900 ml	
adjust pH using HCl or NaOH			*pH = 8.0*		
add H_2O to get the final volume	*50 ml*	*100 ml*	*250 ml*	*1000 ml*	

Dilute 10× (1 part of the buffer + 9 parts of distilled water) before use to get the final contrantrations of 10 mM Tris and 1 mM EDTA

Sodium acetate

	3M		0.05M	
	100 ml	100 ml	200 ml	500 ml
Sodium acetate (anhydrous)	24.61 g	0.41 g	0.82 g	2.05 g
distilled H_2O	~ 70 ml	~ 80 ml	~ 180 ml	~ 450 ml
adjust pH using 1M acetic acid	*pH = 5.0*		*pH = 5.0*	
add H_2O to get the final volume	*100 ml*	*100 ml*	*200 ml*	*500 ml*

0.5M NaOH

See part 1) Common solutions & buffers

3) DNA electrophoresis

TBE buffer (10× concentrated stock solution)

	10× conc.
Tris-base	108 g
Boric acid	55 g
Na_2-EDTA	9.3 g
distilled H_2O	~800 ml
add H_2O to get the final volume	*1000 ml*

TAE buffer (10× concentrated stock solution)

	10× konc.
Tris-base	48.46 g
Na_2-EDTA	3.72 g
acetic acid (pure)	11.42 ml
distilled H_2O	~800 ml
add H_2O to get the final volume	*1000 ml*

Dilution of 10× TBE / TAE to the working concentration (1×)

	1 l	5l
10× TBE / TAE	100 ml	500 ml
distilled H_2O	900 ml	4500 ml

Blue loading buffer without DNA stain – „LD“

Bromphenol Blue	0.025 g
Glycerol	3 ml
add sterile H_2O to get the final volume	*10 ml*

Aliquot by 1 ml, store at -20°C.

Blue loading buffer with SybrGreen („LD+SG“) or GelRed („GelRed“)

Bromphenol Blue	0.025 g
Glycerol	3 ml
SYBR Green I / GelRed	20 µl
add sterile H_2O to get the final volume	*10 ml*

Aliquot by 1 ml, store at -20°C.

DNA ladders (100 bp ladder or **λ DNA Hind III digest)**

ladder stock solution (500 µg / ml)	20 µl
sterile distilled H_2O	180 µl
LD+SG	40 µl

Store in fridge (short-term and working aliquots) or at -20°C (long-term).

4a) Isozymes – extraction buffers

Extraction buffer "Viola" (isozyme lab of the Institute of Botany of CAS, Průhonice)

	30 ml	50 ml	100 ml	(konc.)
Tris-base	0.36 g	0.61 g	1.21 g	(0.1M)
ascorbic acid	58 mg	97 mg	194 mg	(11mM)
sodium metabisulfite	148 mg	247 mg	494 mg	(26 mM)
adjust pH using HCl		*pH = 8.0 (before PVP is added!)*		
2-mercaptoethanol	148 µl	246 µl	492 µl	(70 mM)
PVP40	1.2 g	2 g	4 g	(4% w/v)

Short-term storage in 4°C, long-term storage impossible (must be fresh).

Extraction buffer "Gentiana" (isozyme lab of the Institute of Botany of CAS, Průhonice)

	30 ml	50 ml	100 ml	(konc.)
Tris-base	0.36 g	0.61 g	1.21 g	(0.1M)
L-glutathione reduced	0.3 g	0.5 g	1 g	(1% w/v)
$MgCl_2.6H_2O$	60 mg	100 mg	200 mg	(10 mM)
adjust pH using HCl		*pH = 8.0 (before sucrose is added)*		
2-mercaptoethanol	30 µl	50 µl	100 µl	(0.1% v/v)
sucrose	1.5 g	2.5 g	5 g	(5% w/v)

Short-term storage in 4°C, long-term storage impossible (must be fresh).

Extraction buffer "Luzula" (isozyme lab of the Institute of Botany of CAS, Průhonice)

	30 ml	50 ml	100 ml	(konc.)
Tris base	0.27 g	0.45 g	0.91 g	(75mM)
ascorbic acid	15 mg	25 mg	50 mg	(3 mM)
1,4-Dithioerythritol (DTE)	36 mg	60 mg	120 mg	(7.8 mM)
adjust pH using H_3PO_4		*pH = 7.5 (before PVP is added!)*		
2-mercaptoethanol	30 µl	50 µl	100 µl	(0.1% v/v)
PVP	1.2 g	2 g	4 g	(4% w/v)

Short-term storage in 4°C, long-term storage impossible (must be fresh).

Extraction buffer "Soltis 1983, variant c"

	30 ml	50 ml	100 ml	(konc.)
Tris-base	0.36 g	0.61 g	1.21 g	(0.1M)
$MgCl_2.6H_2O$	61mg	101.7 mg	203.3 mg	(10 mM)
KCl	22.4 mg	37.3 mg	74.6 mg	(10 mM)
Na_2-EDTA	11.2 mg	18.6 mg	37.2 mg	(1 mM)
adjust pH using HCl		*pH = 7.5 (before PVP is added!)*		
2-mercaptoethanol	30 µl	50 µl	100 µl	(0.1% v/v)
PVP40	1.2 g	2 g	4 g	(4% w/v)

Short-term storage in 4°C, long-term storage impossible (must be fresh).

4b) Isozymes – electrophoresis

Tris-Glycine (electrode buffer)

	1 l	3 l
Tris-base	2.5 g	7.5 g
Glycine	18 g	54 g
distilled H_2O	~ 900 ml	~ 2700 ml
adjust pH using HCl	*pH = 8.3*	
add H_2O to get the final volume	*1 l*	*3 l*

Dilution for the lower electrophoresis chamber: Tris-Glycine buffer 2400 ml + dH_2O 1800 ml

Resolving gel buffer (1.82M Tris-HCl, pH = 8.9)

	30 ml	50 ml	100 ml	200 ml
Tris-base	6.61 g	11.05 g	22.1 g	44.2 g
distilled H_2O	~ 23 ml	~ 35 ml	~ 80 ml	~ 170 ml
adjust pH using HCl		*pH = 8.9*		
add H_2O to get the final volume	*30 ml*	*50 ml*	*100 ml*	*200 ml*

Stacking gel buffer (69mM Tris-H_3PO_4, pH = 6.9)

	30 ml	50 ml	100 ml	200 ml
Tris-base	0.25 g	0.42 g	0.83 g	1.66 g
distilled H_2O	~ 23 ml	~ 35 ml	~ 80 ml	~ 170 ml
adjust pH using H_3PO_4		*pH = 6.9*		
add H_2O to get the final volume	*30 ml*	*50 ml*	*100 ml*	*200 ml*

Acrylamide stock solution (40:1)

	100 ml	200 ml
Acrylamide	40 g	80 g
N,N′-methylenebisacrylamide (BIS)	1 g	2 g
distilled H_2O	~70 ml	~150 ml
add H_2O to get the final volume	*100 ml*	*200 ml*

Both chemicals are very toxic! Always work in gloves and protective coat, surgical mask is recommended when weighting the powders.

4c) Isozymes – staining

Tris-HCl buffers

	0.1M Tris-HCl			0.05M Tris-HCl		
	100 ml	250 ml	500 ml	100 ml	250 ml	500 ml
Tris-base	1.21 g	3.03 g	6.06 g	0.61 g	1.51 g	3.03 g
distilled H_2O	~ 80 ml	~ 220 ml	~ 450 ml	~ 80 ml	~ 220 ml	~450 ml
adjust pH using HCl		*pH = 8.4*			*pH = 8.5*	
add H_2O to get the volume	*100 ml*	*250 ml*	*500 ml*	*100 ml*	*250 ml*	*500 ml*

The recipe is for buffers with the highest pH used. For buffers of the same concentration but lower pH use the high-pH stock solution and only adjust pH using HCl.

Tris-Maleate (Tris-Maleic acid)

	0.1M Tris-Maleate. pH = 5.5			0.2M Tris-Maleate. pH = 6.0		
	100 ml	200 ml	500 ml	100 ml	200 ml	500 ml
Tris-base	1.21 g	2.42 g	6.06 g	2.42 g	4.84 g	12.12 g
maleic acid	1.16 g	2.32 g	5.80 g	2.32 g	4.64 g	11.61 g
distilled H_2O	~ 80 ml	~ 180 ml	~ 450 ml	~ 80 ml	~ 180 ml	~450 ml
adjust pH using NaOH		*pH = 5.5*			*pH = 6.0*	
add H_2O to get the volume	*100 ml*	*200 ml*	*500 ml*	*100 ml*	*200 ml*	*500 ml*

Esterases buffer, pH = 6.45

	100 ml	200 ml	500 ml	(konc.)
NaH_2PO_4 . 2 H_2O	1.56 g	3.12 g	7.80 g	(0.1M)
Na_2HPO_4 . 12 H_2O	0.72 g	1.43 g	3.59 g	(0.02M)
distilled H_2O	~ 80 ml	~ 180 ml	~ 450 ml	
adjust pH using NaOH		*pH = 6.45*		
add H_2O to get the volume	*100 ml*	*200 ml*	*500 ml*	

Potassium phosphate buffer (prepare the solutions A and B, mix to get the required pH)

(A) 0.1M K_2HPO_4

	100 ml	250 ml	500 ml
K_2HPO_4 (anhydrous)	1.74 g	4.35 g	8.71 g
distilled H_2O	~ 80 ml	~ 220 ml	~ 450 ml
add H_2O to get the volume	*100 ml*	*250 ml*	*500 ml*

(B) 0.1M KH_2PO_4

	100 ml	250 ml	500 ml
KH_2PO_4 (anhydrous)	1.36 g	3.40 g	6.80 g
distilled H_2O	~ 80 ml	~ 220 ml	~ 450 ml
add H_2O to get the volume	*100 ml*	*250 ml*	*500 ml*

solution of the final concentration of 0.05M prepare by dilution of 0.1M solution(s) and adjusting pH using either K2HPO4 (increase) or KH2PO4 (decrease)

	mixing the solutions	
pH	K_2HPO_4	KH_2PO_4
5.8	8.5 ml	91.5 ml
6.0	13.2 ml	86.8 ml
6.2	19.2 ml	80.8 ml
6.4	27.8 ml	72.2 ml
6.5	32.95 ml	67.05 ml
6.6	38.1 ml	61.9 ml
6.8	49.7 ml	50.3 ml
7.0	61.5 ml	38.5 ml
7.2	71.7 ml	28.3 ml
7.4	80.2 ml	19.8 ml
7,6	86.6 ml	13.4 ml
7,8	90.8 ml	9.2 ml
8,0	94 ml	6 ml