Using molecular marker technology in studies on plant genetic diversity

DNA-based technologies

PCR-based technologies

PCR basics

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The polymerase chain reaction

PCR is a rapid, inexpensive and simple way of copying specific DNA fragments from minute quantities of source DNA material

- It does not necessarily require the use of radioisotopes or toxic chemicals
- It involves preparing the sample DNA and a master mix with primers, followed by detecting reaction products

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The polymerase chain reaction (PCR; Erlich, 1989) is a powerful technique that has rapidly become one of the most widely used techniques in molecular biology because it is quick, inexpensive and simple. The technique amplifies specific DNA fragments from minute quantities of source DNA material, even when that source DNA is of relatively poor quality.

Reference

Erlich, H.A. 1989. PCR technology: principles and applications for DNA amplifications. Stockton Press, NY.

PCR procedures: steps

- *Denaturation*: DNA fragments are heated at high temperatures, which reduce the DNA double helix to single strands. These strands become accessible to primers
- *Annealing*: The reaction mixture is cooled down. Primers anneal to the complementary regions in the DNA template strands, and double strands are formed again between primers and complementary sequences
- *Extension:* The DNA polymerase synthesises a complementary strand. The enzyme reads the opposing strand sequence and extends the primers by adding nucleotides in the order in which they can pair. The whole process is repeated over and over

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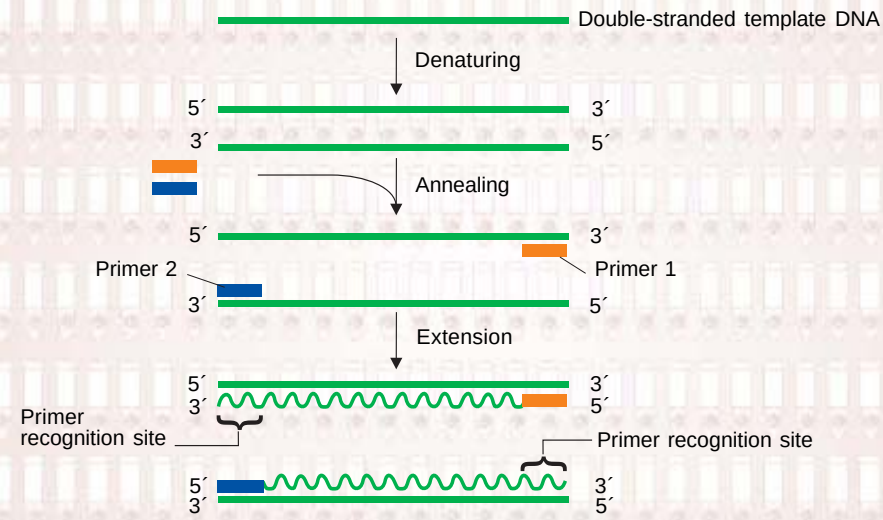
The DNA polymerase, known as 'Taq polymerase', is named after the hot-spring bacterium *Thermus aquaticus* from which it was originally isolated. The enzyme can withstand the high temperatures needed for DNA-strand separation, and can be left in the reaction tube.

The cycle of heating and cooling is repeated over and over, stimulating the primers to bind to the original sequences and to newly synthesised sequences. The enzyme will again extend primer sequences. This cycling of temperatures results in copying and then copying of copies, and so on, leading to an exponential increase in the number of copies of specific sequences. Because the amount of DNA placed in the tube at the beginning is very small, almost all the DNA at the end of the reaction cycles is copied sequences.

The reaction products are separated by gel electrophoresis. Depending on the quantity produced and the size of the amplified fragment, the reaction products can be visualised directly by staining with ethidium bromide or a silver-staining protocol, or by means of radioisotopes and autoradiography.

PCR procedures: cycle 1

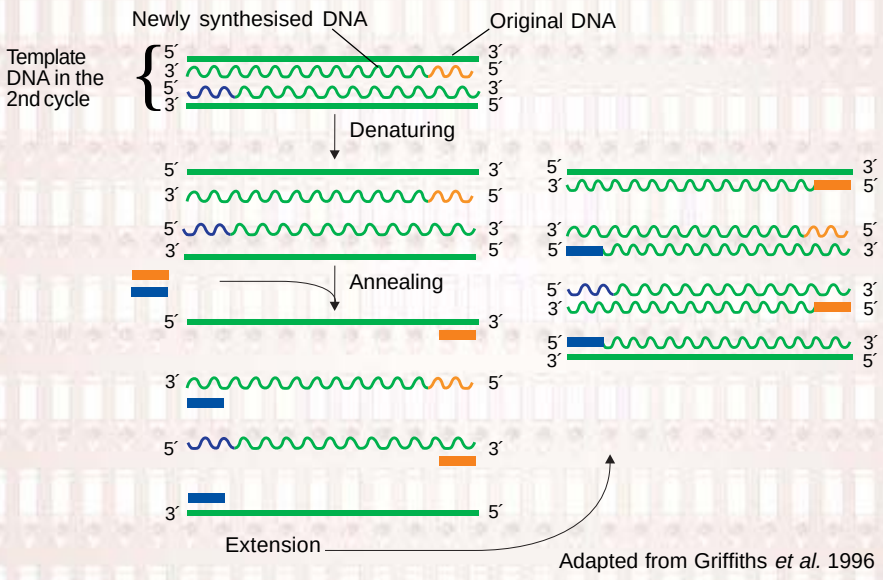


Adapted from Griffiths *et al.* 1996

The PCR steps are all carried out, one after the other, in bouts of cycling. Cycle 1 is as follows:

- During denaturation (about 1 min at 95°C), the DNA strands separate to form single strands.
- During annealing (about 1 min at temperatures ranging between 45°C and 60°C), one primer binds to one DNA strand and another binds to the complementary strand. The annealing sites of the primers are chosen so that they will prime DNA synthesis in the region of interest during extension.
- During extension (about 1 min at 72°C), the DNA synthesis proceeds through the target region and for variable distances into the flanking region, giving rise to 'long fragments' of variable lengths

PCR procedures: cycle 2



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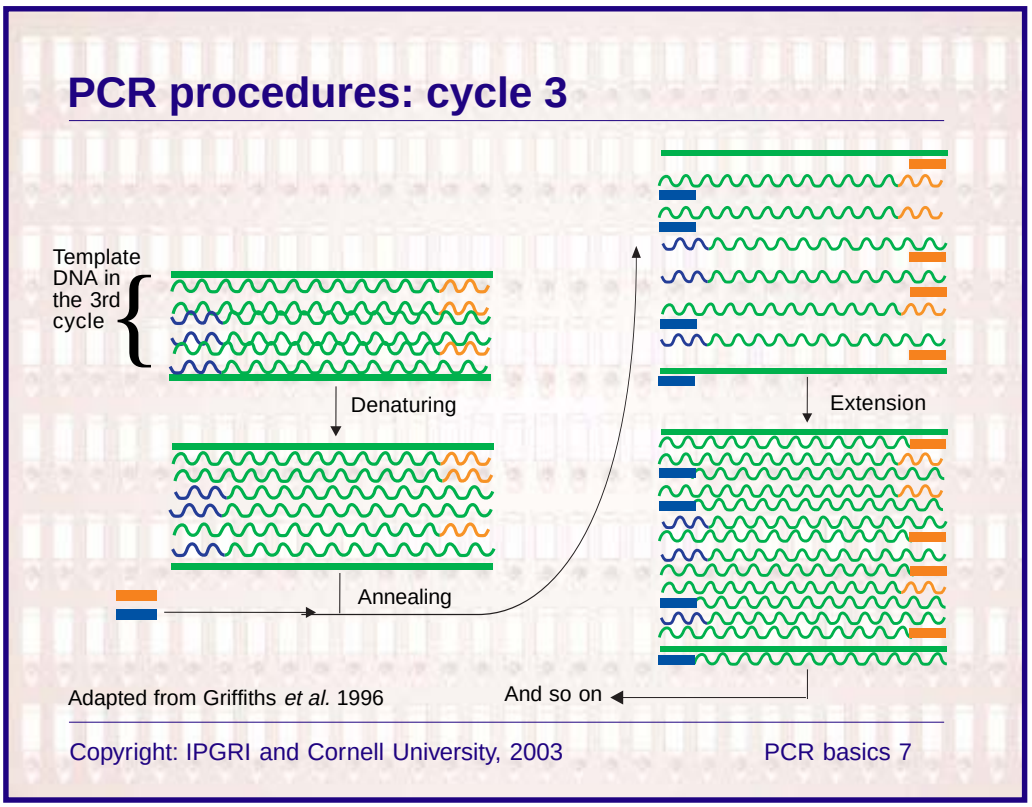
When the second cycle starts, there are effectively two types of template: (1) the original DNA strands; and (2) the newly synthesised DNA strands, consisting of the target region and variable lengths of the flanking region at the 3' end. When the latter template is used in this cycle, only the target region is replicated.



In the third cycle, the newly synthesised target region DNA (i.e. without flanking regions) acts as template. The original DNA molecule is still present, and will be until the end of the reaction. However, after a few cycles, the newly synthesised DNA fragment quickly establishes itself as the predominant template.

Cycles are typically repeated 25 to 45 times. Standardisation of the thermocycler's running conditions is essential for the reproducibility of results.

PCR procedures: conditions for cycling

- Complete denaturation of the DNA template
- Optimal annealing temperature
- Optimal extension temperature
- Number of PCR cycles
- Final extension step

In the initial denaturation step, complete denaturation of the DNA template at the start of the PCR reaction is essential. Incomplete denaturation of DNA will result in the inefficient use of the template in the first amplification cycle and, consequently, poor yield of PCR product.

The annealing temperature may be estimated as 5°C lower than the melting temperature of the primer-template DNA duplex. If non-specific PCR products are obtained in addition to the expected product, the annealing temperature can be optimised by increasing it stepwise by 1-2°C.

Usually, the extension step is performed at 72°C and a 1-min extension is sufficient to synthesise PCR fragments as long as 2 kb (kb = kilobase = 1000 bp). When larger DNA fragments are amplified, time is usually extended by 1 min per 1000 bp.

The number of PCR cycles will basically depend on the expected yield of the PCR product.

After the last cycle, samples are usually incubated at 72°C for 5 min to fill in the protruding ends of newly synthesised PCR products.

PCR procedures: conditions for the reaction mixture

Contamination of the DNA must be prevented by:

- Separating the areas for DNA extraction and PCR
- Using sole-purpose laboratory equipment
- Autoclaving and aliquoting
- Adding a control reaction

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Some useful tips:

- DNA extraction and PCR reaction mixing and processing should be performed in separate areas.
- Use of sole-purpose vessels and positive displacement pipettes or tips for DNA sample and reaction mixture preparation is strongly recommended.
- All solutions, except dNTPs, primers and Taq DNA polymerase, should be autoclaved. Where possible, solutions should be aliquoted in small quantities and stored in designated PCR areas.
- A good practice, to confirm absence of contamination, is to add a control reaction without template DNA.

PCR procedures: components

Components:

- Sterile deionised water
- 10X PCR buffer
- dNTP mix
- Primer
- Taq DNA polymerase
- $MgCl_2$
- Template DNA

Considerations:

- Template DNA
- Primers
- $MgCl_2$ concentration
- Taq polymerase
- dNTPs

Many PCR machines are now available in 48-, 96- or 384-well formats. This, combined with the use of multichannel pipettors, can greatly increase the number of reactions that can be done simultaneously. If several reactions need to be simultaneously prepared, a master mix should be used as follows: water, buffer, dNTPs, primers, $MgCl_2$ and Taq DNA polymerase in a single tube. This will then be aliquoted into individual tubes.

Considerations:

Template DNA. Nearly any standard method is suitable for template DNA purification. An adequate amount of template DNA is between 0.1 and 1 µg for genomic DNA for a total reaction mixture of 100 µl. Larger template DNA amounts usually increase the yield of non-specific PCR products.

Primers. (1) PCR primers should be 10-24 nucleotides in length. (2) The GC content should be 40%-60%. (3) The primer should not be self-complementary or complementary to any other primer in the reaction mixture, to prevent primer-dimer and hairpin formation. (4) Melting temperatures of primer pairs should not differ by more than 5°C, so that the GC content and length must be chosen accordingly. (5) The melting and annealing temperatures of a primer are estimated as follows: if the primer is shorter than 25 nucleotides, the approximate melting temperature is calculated with the formula: $Tm = 4 (G + C) + 2 (A + T)$. (6) The annealing temperature should be about 5°C lower than the melting temperature.

$MgCl_2$ concentration. Because Mg_2^+ ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of $MgCl_2$ has to be selected for each experiment. Too few Mg_2^+ ions result in a low yield of PCR product, and too many will increase the yield of non-specific products. The recommended range of $MgCl_2$ concentration is 1 to 3 mM, under the standard reaction conditions specified.

Taq DNA polymerase. Higher Taq DNA polymerase concentrations than needed may cause synthesis of non-specific products.

dNTPs. The concentration of each dNTP (dATP, dCTP, dGTP, dTTP) in the reaction mixture is usually 200 µM. These concentrations must be checked as being equal, because inaccuracies will increase the degree of misincorporation.

PCR procedures: equipment

- Micropipettes
- Thermocycler
- Electrophoresis units
- Power supply units
- Photographic equipment

PCR technology in pictures

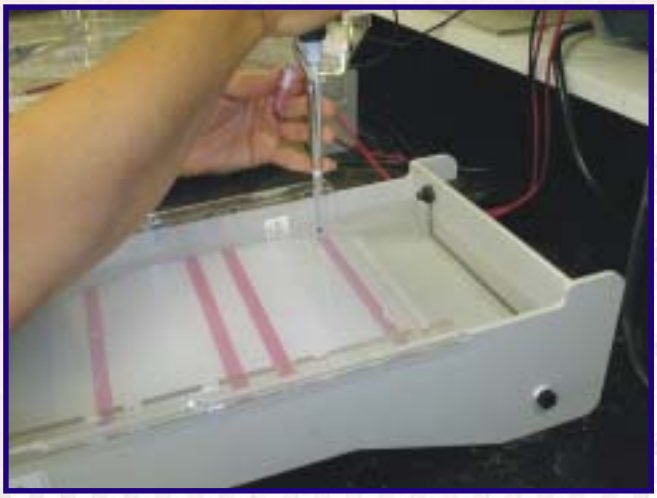
The following photographs demonstrate how PCR technology is carried out

The PCR mixture is prepared on ice. Safety clothing is not required, but may benefit the reaction unless good sterile techniques are practised.

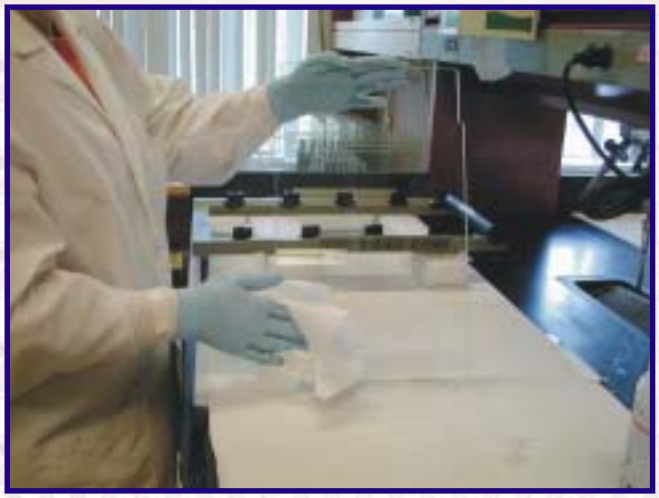
The PCR reactions are loaded into the thermocycler.

The thermocycler is locked shut and programmed.

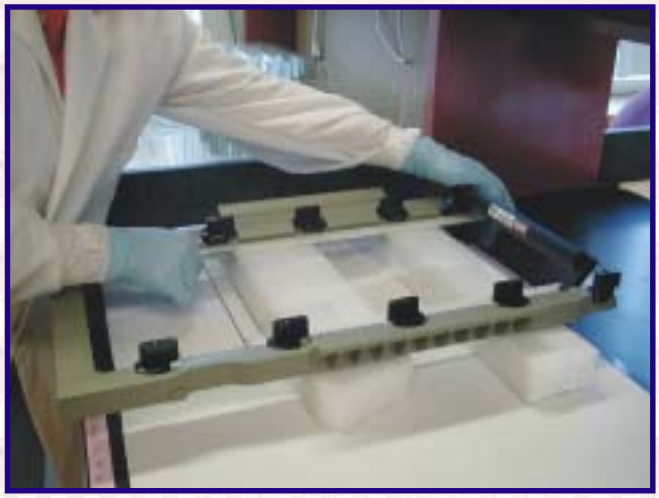
Different types of thermocyclers exist: the black one is a tetrad, in which 4 sets of 96 samples can be run simultaneously. The four smaller white ones each have a 96-well capacity.



Depending on the size of the PCR bands produced and the discrimination needed, band visualisation can be accomplished through either a regular, horizontal, agarose gel or a vertical acrylamide sequencing gel (see next slide). Here, the products are being run on an agarose gel.

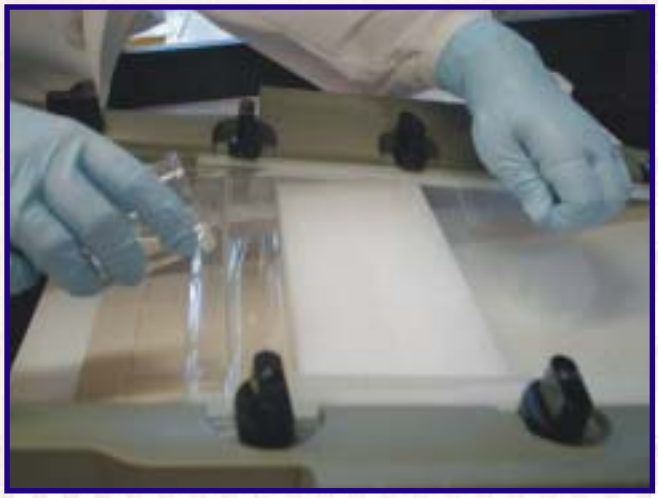


The acrylamide gel may be run in either an independent unit or an automatic sequencer. The preparation of the sequencing gel, although somewhat complicated to set up, is similar for both cases. Here, the glass is being cleaned and wiped before preparing the gel for an automatic sequencer.

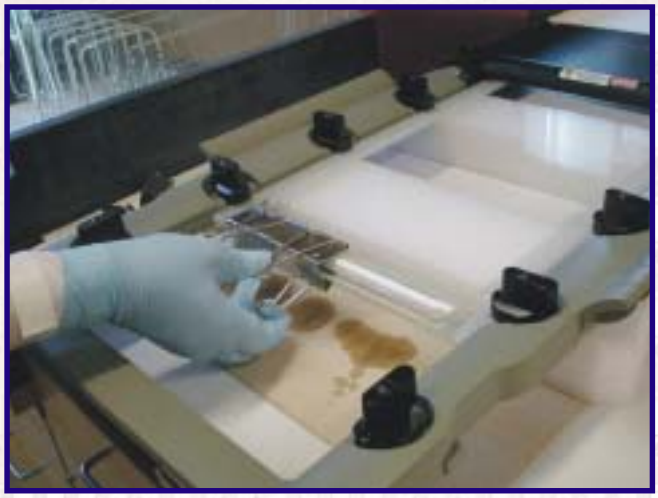


Glass units are being inserted into the frame, which will be fitted into the sequencer.

The glass units are clamped into place, and the entire unit readied for gel to be poured in.



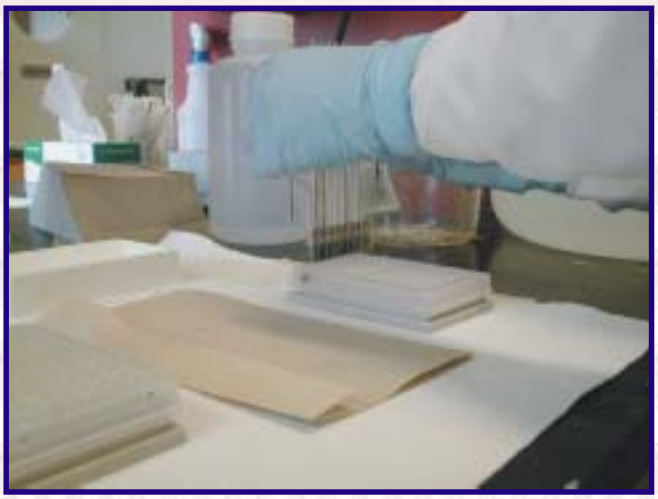
Liquid acrylamide gel is poured into the mould. Because acrylamide is a carcinogen, safety clothing must be worn.



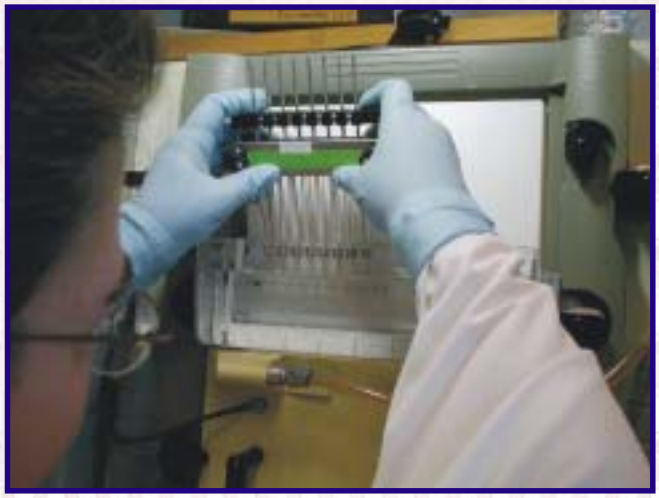
Clamps grip the bottom ends of the glass units to prevent the gel leaking.

A comb is inserted into the top of the gel to create the wells into which the samples will be loaded.

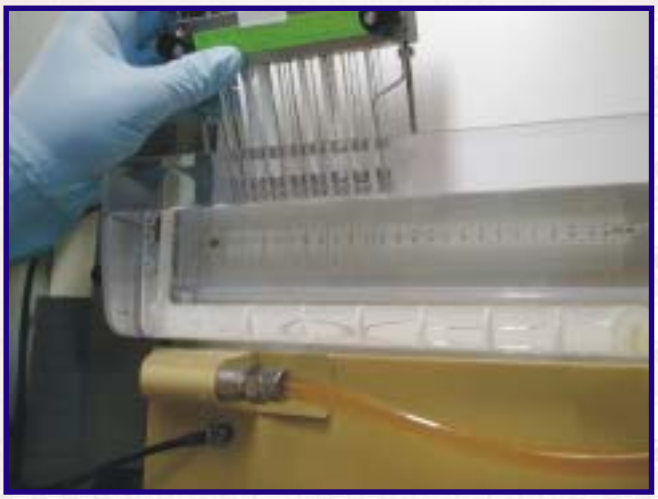
Once the gel is set, the unit is placed into the sequencing machine.



Samples are taken up with a multi-pipettor …



…and loaded into the wells of the gel.



This is a close-up from the previous photograph. Note the extremely small size of the wells and the pipettor, making it possible to load large numbers of samples into each gel.

A heating plate is placed against the gel to ensure a constant performing temperature.

Gel buffer is loaded into the top tank…

…and into the bottom tank.

A final look, then the door is shut and the program begun.

In summary

- PCR is a simple technique to obtain many copies of specific DNA fragments
- Three steps are involved in PCR: denaturation, annealing and extension
- To ensure success, care should be taken both in preparing the reaction mixture and setting up the cycling conditions

By now you should know

- What the basis of PCR is
- Those items that can affect the performance of the PCR, step by step, and what their effects on PCR results would be

Basic references

Erlich, H.A. 1989. PCR technology: principles and applications for DNA amplifications. Stockton Press, NY.

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Newton, C.R. and A. Graham. 1994. PCR, part 1: Basic principles and methods. EngBios Scientific Publishers, Oxford.

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Next

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PCR-based technologies
PCR with arbitrary primers

- DNA-based technologies
  - PCR-based technologies
    - Amplified fragment length polymorphisms (AFLPs)
    - Sequences-tagged sites (STS)
    - Latest strategies
- Complementary technologies
- Final considerations
- Glossary