BASICS OF DNA DIAGNOSTICS

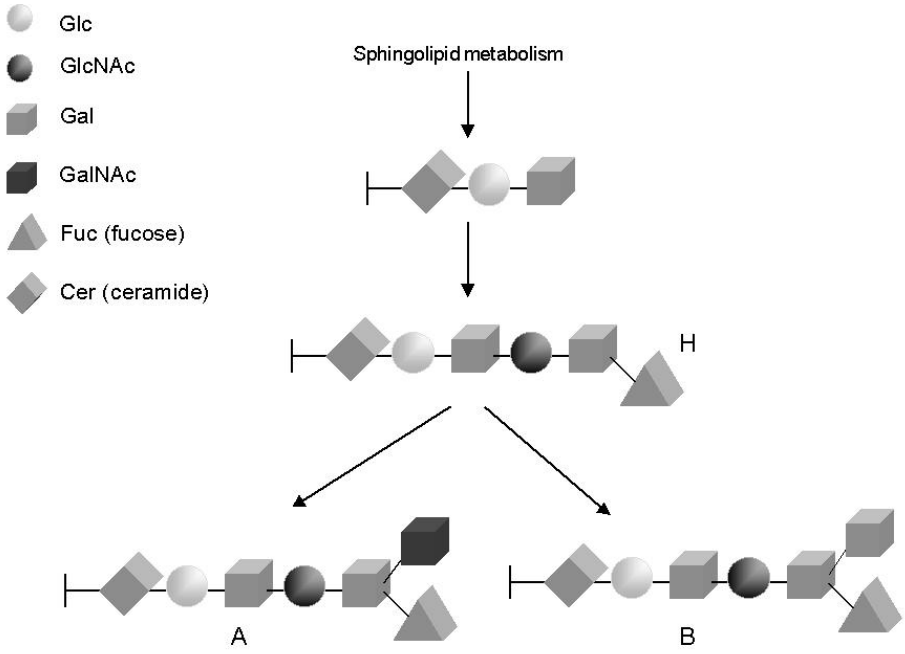
(by MUDr. Martin Vejražka)

THEORY:

Concise genetics of blood group system AB0

The AB0 system of blood groups was discovered by Austrian physician Karl Landsteiner in 1900 (Nobel Prize in 1930). Independently, it was described also by Czech physician Jan Janský in 1907. Felix Bernstein examined heredity of blood groups in families in the middle twenties of last century and discovered that blood groups are determined by a single locus that may carry alleles A, B or 0.

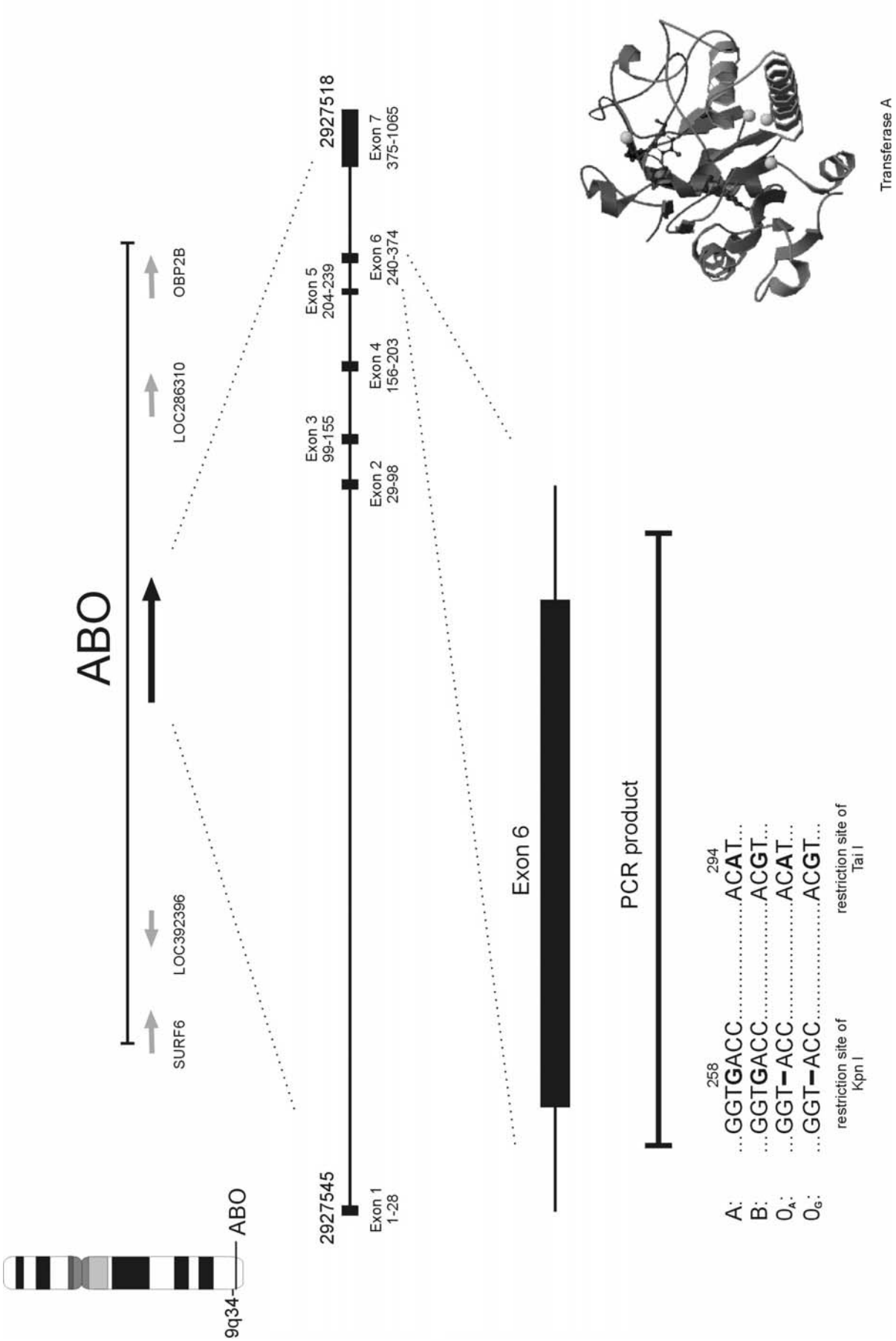
The biochemical background of blood groups was elicited in the fifties of last century. Alleles A and B code for specific glycosyl transferases. These enzymes attach either N-acetyl-D-galactoaminosyl (to yield antigen A) or D-galactosyl (antigen B) residue to an oligosaccharide chain (so called antigen H) attached to the cell membrane.



The allele corresponding to blood group 0 does not code for any functional enzyme so that cells carry only the "basal" antigen H on their membranes.

The locus of AB0 system was identified in the seventies of last century. It was found near the telomere of the long arm of the 9th chromosome, in the band 9q31.3. Finally, the AB0 gene was sequenced and its main polymorphisms were described in the nineties.

The human gene AB0 consists of seven exons stretched over more than 19 kbp. The first six exons are rather short, from 28 to 135 bp. The last, seventh exon is 688 bp long and codes for the catalytic portion of the enzyme. The total length of translated DNA is 1065 bp which corresponds to 354 amino acids.


ABO
9q34
ABO
SURF6
LOC392396
LOC286310
OBP2B
2927545
2927518
Exon 1 1-28
Exon 2 29-98
Exon 3 99-155
Exon 4 156-203
Exon 5 204-239
Exon 6 240-374
Exon 7 375-1065
Exon 6
PCR product
258
294
A: ...GGTGACC.....ACAT...
B: ...GGTGACC.....ACGT...
O$_A$: ...GGT–ACC.....ACAT...
O$_G$: ...GGT–ACC.....ACGT...
restriction site of Kpn I
restriction site of Tai I
Transferase A

More than eighty alleles of the gene have been described up today. We will deal with the most important four of them: alleles coding for transferase A (α1-3-N-acetylgalactosaminyl transferase, E.C. 2.4.1.40), for transferase B (α1-3-galactosyl transferase, E.C. 2.4.1.37), and two alleles corresponding to the group 0.

Alleles A and B differ in 7 base pairs. One difference is located in the sixth exon (allele A carries adenine while allele B carries guanine in position 294), however the same amino acid is joined to the translated protein. Other six differences are located in the seventh exon and two of them (266 and 288) are responsible for the change in specificity of the enzyme.

Allele 0 differs from alleles A and B first of all by a single base deletion in the sixth exon. This deletion is in the position 258, i.e. relatively near to the N-terminal amino acid of the synthesised protein. A frame shift during translation results and after incorporating 22 "erroneous" amino acids, the translation is prematurely terminated. The product possesses no transferase activity. The remaining sequence may correspond either to allele A or B. According to nucleotide A or G in the position 294 (see above) alleles 0_A ("relative" to allele A) or 0_G ("relative" to B) may be distinguished.

There are many rare suballeles. Let us mention the blood group A_2. It is a variation of allele A with a single nucleotide deletion near the C-terminal residue. A frame shift leads to change in amino acid sequence and prolongation of the translated chain. Consequently, activity of transferase is significantly decreased. The phenotype might be described as "incompletely expressed group A" or "a phenotype between 0 and A".

Another rare allele is denoted as cis-AB. Two bases in the seventh exon are changed. The transferase is capable to transfer both galactose and N-acetylgalactosamine so that both antigens A and B are synthesised. In the sixties this allele was found in families in which a descendant had the blood group AB even though one of the parents had the group 0.

Isolation of DNA and determination of its purity

If molecular-genetic examination starts with polymerase chain reaction a very small amount of DNA is sufficient as a sample. In some cases directly a portion of tissue or several cells lysed using tensides or repetitive freezing and thawing may be used. However, quality of template DNA influences the amplification in polymerase chain reaction. Impurities from the sample may slow down the reaction significantly as they act as polymerase inhibitors or bind the template DNA making it inaccessible for the reaction. It is therefore advantageous to isolate DNA from the sample prior to further examination.

Many procedures were established for DNA isolation. A piece of tissue (or for example leukocytes of peripheral blood) is usually lysed using tensides as the first step. Then, DNA is purified from proteins and other contaminating stuffs. Chemical methods (e.g. protein hydrolysis by proteolytic enzymes, denaturation of proteins etc.) as well as physical procedures (different affinity of DNA and contaminants to various substrates) are employed. Two techniques are used most frequently today:

- **Binding DNA to silica column.** DNA has high affinity to silica gel packing of a chromatography column. DNA binds to silica in an environment with high concentration of salts while contaminating substances are washed out. Then, DNA may be recovered e.g. with distilled water or a diluted buffer.
- **Isolation of DNA using phenol-chloroform.** This is the "gold standard" that is still in use despite the fact that it is relatively laborious and inconvenient (work with toxic and bad-smelling substances). This method is, however, reliable, cheap and yields very pure DNA.

Phenol-chloroform method of DNA isolation

The first step is **homogenisation** of tissue in a tenside (e.g. sodium dodecyl sulphate, SDS) containing buffer. The tenside dissolves cell membranes. **Proteinase K** is added to enhance cell lysis and to partially hydrolyse contaminating proteins. It is a potent bacterial enzyme with temperature optimum about 60 °C. Proteinase K does not require calcium or magnesium ions and is not inhibited by concentrated tensides.

Denaturation and precipitation of proteins with phenol-chloroform follows. The mixture of phenol and chloroform is immiscible with water. DNA is dissociated at pH 7.4 and stays dissolved in the aqueous phase while proteins are denatured and precipitate to the hydrophobic phase. Small amount of isoamyl alcohol is added in order to facilitate separation of both phases and to prevent foaming of samples rich in proteins. Traces of phenol would strongly inhibit polymerase reaction; therefore shaking with chloroform only at the end purifies the samples.

Then, DNA is **precipitated** from the aqueous solution. A salt (for example, sodium chloride or sodium acetate) is added in a high concentration. Ions of the salt form a hydration coat and remove solvent from DNA in this way (so called salting out). A low-polar compound (e.g. ethanol or isopropanol) is then added. Decreasing polarity of solvent leads to precipitation of DNA.

Precipitated DNA is **washed** with 70% ethanol. This concentration of alcohol removes remnants of salt and protein while DNA remains undissolved.

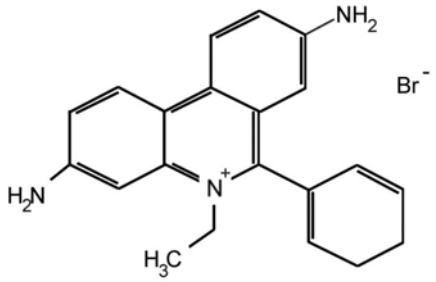
Isolated and purified DNA is usually stocked in an alkaline buffer containing ethylenediaminetetraacetic acid (EDTA). This complex-forming agent facilitates dissolution of DNA. Moreover, it binds Ca^{2+} ions and thus inhibits DNases.

Estimation of concentration and purity of DNA

If the concentration of DNA in a solution is high enough it can be measured by means of direct photometry in UV range. Pure DNA shows maximum absorbance at 260 nm thanks to abundance of purine residues. It can be considered that solution of 50 $\mu g.ml^{-1}$ DNA yields approximately absorbance of 1.

Absorbance spectrum of proteins has, however, a broad peak with a maximum at 280 nm (effect of tyrosine). At 260 nm, absorbance of proteins is quite high. Purity of DNA is often assessed as ratio of absorbances at 260 and 280 nm. A_{260}/A_{280} of pure DNA is approximately 1.8.

Direct photometry is not sufficiently sensitive for diluted samples of DNA. **Intercalation fluorescent dyes** are most frequently employed for determination of DNA concentration in that case. These compounds contain several condensed aromatic rings and so they have a planar structure allowing them to "wedge", intercalate between strands of double helix DNA. Fluorescence of the dye increases after intercalation. Ethidium bromide (3,5-diamino-5-ethyl-6-phenylphenanthridium bromide) may be considered as a prototype of these compounds.



Ethidium bromide

Polymerase chain reaction

Quite a large extent of a particular sequence of a nucleic acid is frequently required for molecular diagnostics. As it was not possible to isolate enough DNA directly from the sample, there was only one method for DNA amplification till 1983: the sequence had to be transferred into a bacterial plasmid and cloned. Today, DNA is amplified in a still growing scale completely *in vitro* by means of polymerase chain reaction (**PCR**).

Kary Mullis discovered PCR when thinking about modifications of sequencing techniques with dideoxynucleotides, driving his car in Californian Mountains. Ten years later, he was awarded with Nobel Prize for this invention. Discovery of PCR was really revolutionary for molecular biology. PCR is, thanks to sensitivity, specificity and quickness, probably the most frequently used technique in this field today.

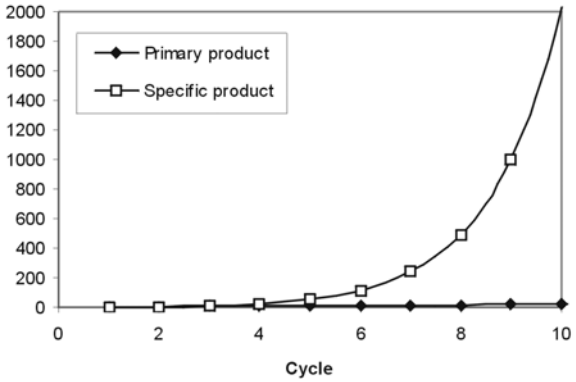
The fundamental principle of PCR is a repetitive controlled denaturation of double stranded DNA followed by renaturing isolated strands with specific oligonucleotides that are in excess in the reaction mixture. Generally, amplification of DNA runs in reiterating cycles consisting of three steps:

1. **Denaturation.** DNA is warmed up to about 95 °C. Hydrogen bonds between strands are cancelled so that double-stranded DNA is split into single-stranded DNA.
2. **Annealing.** DNA is cooled down to 50 – 60 °C. Molecules of DNA renature again. If specific oligonucleotides are added to the mixture in excess they will hybridise with the complementary sequence faster than long single strand molecules. Temperature of hybridisation is critical for result of PCR and must be set suitably for used pair of primers. If the temperature is too low primers hybridise even with not fully complementary sequences and non-specific product is synthesised. If the temperature is too high the primers hybridise reluctantly and only small amount of product is formed.
3. **Elongation**, extension, synthesis. Oligonucleotides that hybridised with single strand DNA (template) in the previous step serve as primers for DNA polymerase in this step. Synthesis of a new strand complementary with template starts from the 3'-terminal base of the primer.

Number of DNA strands in the mixture doubles in the first cycle. Arising strands may play the role of templates in the next cycle so that the amount of product doubles. Quantity of synthesised strands grows exponentially in repetitive cycles.

Synthesis of new DNA starts from the primer. In the first cycle, long molecule of original DNA serves as template. The arising strands (primary product) are shorter than the template but exceed the region limited by both primers. When primary product plays the role of template in further cycles a strand limited on both sides by primers is formed (one primer starts synthesis and in the position of the second primer elongation stops because it is the end of the primary product). The specific product whose length is exactly limited by position of primers increases exponentially, longer strands only in a linear manner. $10^9\times$ more of specific product compared to other strands will be theoretically formed after 30 cycles of PCR reaction. Proportion of longer strands is therefore negligible.

The real gain of PCR reaction is much lower than the mentioned 10^9 copies of every DNA molecule after 30 cycles. Components of the reaction mixture become depleted in the course of amplification. Concentration of the product reaches a plateau after certain number of cycles and then practically stops growing.



Before start of 1st cycle:

template

After 1st cycle:

primer

primary product

After 2nd cycle:

specific product

After 3rd cycle:

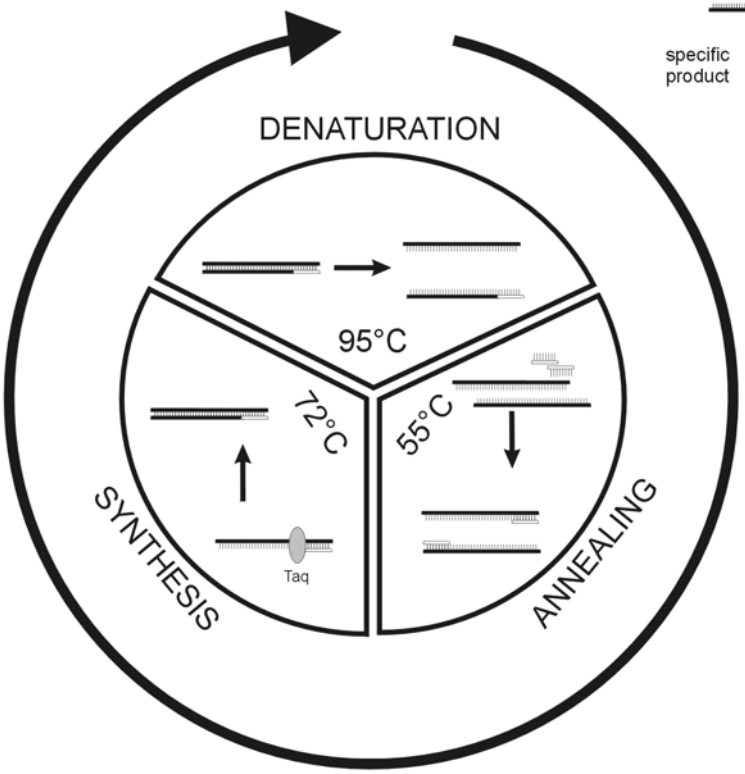
After 4th cycle:

After 5th cycle:

After 6th cycle:
2× ssDNA of template
12× ssDNA of primary product
114× ssDNA of specific product

After 7th cycle:
2× ssDNA of template
14× ssDNA of primary product
240× ssDNA of specific product

After 8th cycle:
2× ssDNA of template
16× ssDNA of primary product
494× ssDNA of specific product



Components of reaction mixture

Template
PCR is not very demanding on template DNA. Just a very small amount of nucleic acid is often sufficient. It is however essential that the sample is not contaminated with improper DNA born from inadequately washed instruments, hands of the worker etc. Even an exiguous amount of contaminating DNA would be amplified in such extent that detectable quantity of product would arise and result of examination could be distorted. Moreover, the sample of template DNA must not contain any compound inhibiting DNA polymerase. Common reagents used for isolation, purification and processing DNA belong to these compounds: phenol, proteinase K, higher concentration of salts, heparin, borate, ethanol, EDTA etc.

Primers
Oligonucleotides 17 to 28 bases long are usually used as primers. They should meet several requirements in order to guarantee good yield of reaction:

- Primers must be specific for the amplified sequence and should be completely complementary to the strand, which they should hybridise with.
- Primers must not be complementary each with other, otherwise they take the role of templates and dimers of primers are formed in PCR. This requirement is twice important for 3'-terminal sequences.
- Primer should not contain inner-complementary sequences; otherwise "loops" are formed.
- Distribution of AT and CG pairs should be even. Especially 3'-terminal part should not be too rich on CG.
- Annealing temperatures of both primers should not differ considerably.

It is obvious that two primes are necessary for PCR reaction. The primer that begins the 5'-terminal strand of the gene and that is elongated in the direction of transcription is denoted as forward, upstream or coding primer. The other is described as reverse, downstream or anticoding.

Polymerase
Any DNA polymerase could be theoretically used for purposes of PCR. However, with regard to high temperature of denaturing step which would destroy the enzyme, it would be necessary to add fresh polymerase in every cycle. Therefore thermostabile DNA polymerases are employed in PCR today. They were originally isolated from bacteria living in hot sea springs. The most important representative is so-called Taq polymerase of *Thermus aquaticus*. The optimal temperature for its work is about 75 °C when it can add about 150 bases per second. At 90 °C it is inactive however resists denaturing.

Polymerases for PCR are manufactured using recombinant technologies today. Besides the mentioned Taq polymerase, several other enzymes are available. They provide higher activity or better thermostability. Some enzymes are able to synthesise longer strands (Taq polymerase "falls away" from the strand after joining several hundreds of bases). Other polymerases posses 3'-exonuclease activity allowing proofreading of inserted nucleotide (Taq polymerase makes one mistake per 10 to 20 thousands inserted bases).

Reaction mixture
The reaction mixture must contain other constituents as well. Besides all deoxynucleotide triphosphates (dATP, dTTP, dCTP, dGTP) a buffering component and salts adjusting the ionic strength are included. Mg^{2+} ions are another important ingredient as magnesium serves as polymerase cofactor. Inadequately low concentration of Mg^{2+} leads to low yield of reaction; on the other hand, too high concentration can cause synthesis of non-specific products. Other components of reaction mixture (albumin, ammonium sulphate, betain etc.) help to stabilise polymerase in solution or improve specificity of the product.

Selected modifications of PCR

Hot start
DNA polymerase is active even at temperatures lower than its optimum even if the reaction rate is lower. At low temperature, primers may hybridise non-specifically with template after setting up the reaction mixture and the polymerase may synthesise non-specific products till the first denaturing step of PCR. To prevent formation of non-specific products, a component of the reaction mixture may be omitted until reaching the denaturing temperature. To avoid opening of test tubes, this separated component may be inserted in a wax capsule that melts at higher temperature. Use of DNA polymerase inactivated with antibody is another possibility. Increased temperature denatures the antibody and active enzyme is released.

Touch down PCR
Excessive formation of non-specific products may be reduced by so-called touch down PCR. Annealing temperature higher than adequate for used primers is used in the first cycles. Primers hybridise less effectively, i.e. yield of reaction is lower, however hybridisation is very accurate and only specific product is synthesised. Annealing temperature decreases step by step in following cycles. Abundance of specific product over original template ensures specificity of reaction and easier hybridisation at lower temperature guaranties that sufficient amount of product is formed.

Amplification of long strands
The most frequently used polymerase for PCR, Taq polymerase, cannot synthesise strands longer than approximately 2000 base pairs. It releases from the template DNA after some time and synthesis is interrupted. Moreover, it cannot amend an erroneously inserted nucleotide and synthesis of long strands would therefore lead to a considerable number of mistakes. Mixtures of several thermostabile DNA polymerases are usually used for amplification of long strands (up to 30 – 40 thousands pairs). Enzymes with and without proof-reading activity are usually combined. The technique is denoted as *long distance PCR* (LD-PCR).

Reamplification
If only a very small amount of template DNA is available for PCR, the yield of an usual reaction may be insufficient. A portion of PCR product may be used as template for further PCR reaction in that case – so-called reamplification. The cost of DNA amplification in the first reaction sometimes is that non-specific products are formed. Another pair of primers is therefore used for the second reaction in order to amplify only the specific product of demand. These primers hybridise between primers of the first reaction (i.e. in the second reaction, shorter strand is amplified). Described technique is called **nested** PCR.

Allele-specific PCR
Sole PCR may be employed for detection of certain allele or mutation. A primer that is complementary with the DNA section differing among alleles or containing the mutation must be available. If examined DNA contains the corresponding sequence amplification runs normally and a detectable amount of PCR product is synthesised. In the opposite case the primer cannot hybridise and the strand is not amplified. Indeed, the test must be accompanied with suitable positive and negative controls.

Preparation of single-stranded DNA
For some techniques, especially for sequencing, it is necessary to prepare single-stranded DNA. **Asymmetric PCR** may be used for this purpose. Primers are added in different amounts (usually in the ratio 50:1 to 100:1). In the course of PCR, double-stranded product will be formed in the regular way at the beginning and it increases exponentially. After approximately 20 cycles the less concentrated, limiting primer is depleted. Then only one strand (starting with "excessive" primer) is synthesised and the amount of this product grows linearly.

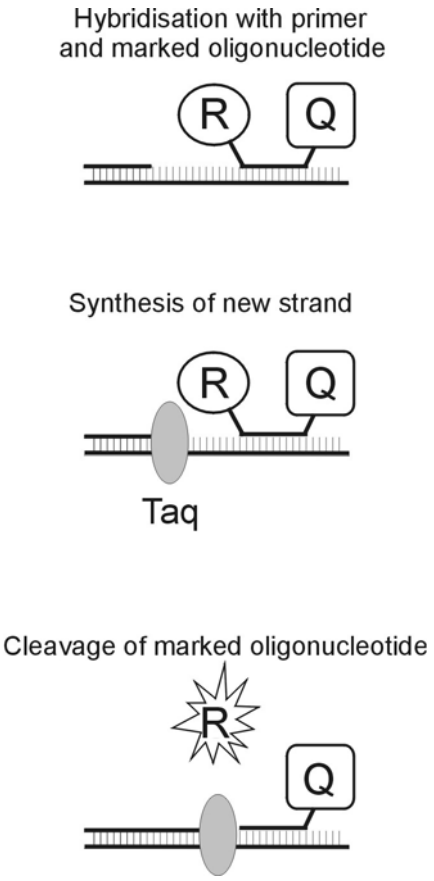
PCR using one of primers marked with biotin is another possibility. The product is then denatured and purified on **solid phase** (e.g. column packed with agarose) with covalently bound streptavidin. Biotin has high affinity to streptavidin and hence the marked strand is trapped on the solid phase while the other strand is eluted together with other components of the reaction mixture.

Reverse transcription PCR (RT-PCR)
If mRNA is to be amplified instead of DNA, RT-PCR may be employed. The first step is isolation of total RNA or mRNA from a tissue sample. Then, mRNA is copied to cDNA using reverse transcriptase. A common PCR follows. This method is much more demanding than DNA amplification alone, even though transcription of RNA to cDNA itself is not very difficult: RNA is quickly destroyed by ribonucleases that are ubiquitous contaminants of samples, consumables and chemicals. RNases are, moreover, thermostable and resist even sterilisation in the autoclave. Activity of RNases withstands cleaning of material with some denaturing agents. Solutions and materials must be treated with inhibitors of RNases (e.g. diethylpyrocarbonate, DEPC) to prevent degradation of RNA.

Quantitative PCR
Polymerase chain reaction in its common layout is used first and foremost for qualitative tests. The use for quantitative estimation of a sequence in the sample (e.g. to assess expression of a gene) is limited by the fact that after certain number of cycles the rate of amplification slows down (see above). In spite of it there are procedures allowing quantification of template. Measuring concentration of PCR product in course of

amplification is the general principle employed (so called **real-time PCR**[1]). Because opening the test tube and taking samples of the reaction mixture would easily lead to contamination of samples, procedures allowing measuring synthesis of product directly in closed tubes are used.

So-called TaqMan PCR may serve as an example. Apart from primers, another oligonucleotide that hybridises with amplified strand is inserted into reaction. This oligonucleotide is marked with a fluorescence dye at one end (reporter dye, in the figure "R"). A quencher ("Q") is bound to the other end. Fluorescence of the reporter dye is suppressed if it is close to the quencher. PCR reaction runs in a regular way until DNA polymerase reaches the marked oligonucleotide. At this moment, the oligonucleotide is replaced with created strand and cleaved down (Taq polymerase lacks proof-reading 3'-exonuclease activity but possesses 5'-exonuclease activity allowing degradation of "interfering" strand). Reporter dye is released into solution and starts to fluoresce. Fluorescence may be measured directly in the test tube during the reaction using a special PCR cycler. Intensity of fluorescence is proportional to the amount of synthesised PCR product.



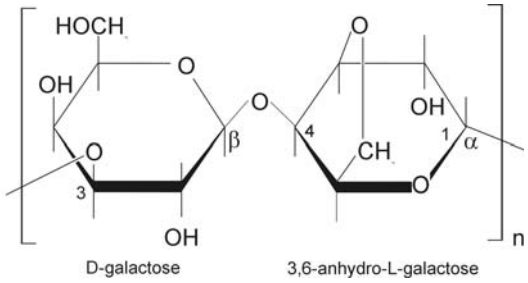
[1] Real-time PCR is sometimes abbreviated as RT-PCR, equally to the reverse transcriptase PCR.

Electrophoresis of nucleic acids

In general, sections of DNA, PCR products or products of nucleic acid restriction can be separated by means of chromatography or electrophoresis. Electrophoresis is much more widely used. It is usually performed in a gel made of either agarose or polyacrylamide. In both cases, molecules of DNA carrying negative charge in an alkaline environment move in electric field from cathode to anode. Polyacrylamide and to a lesser extent agarose gels form a rather dense network. Larger molecules move slower than smaller ones in this network; hence the method is sometimes called molecular sieving.

Electrophoresis in agarose gel

Agarose is a polysaccharide made of D-galactose and anhydro-L-galactose. Some sea algae produce this polysaccharide that is used under name "agar" for preparation of gels in food-processing industry, microbiology, immunology and biochemistry. Gels containing 0.5 to 4% of agarose are used for electrophoresis of nucleic acids. The higher the content of polysaccharide is the better is resolution of gel but the slower is run of electrophoresis and the more difficult is preparation of gel.

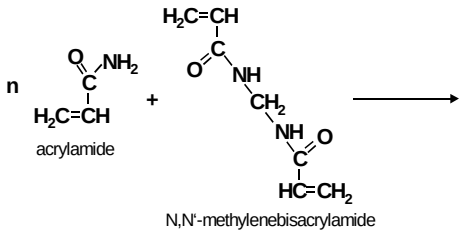


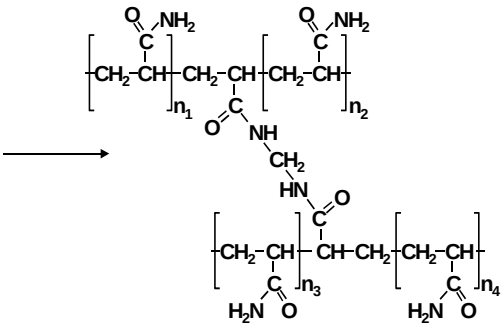
Agarose content	Lenght of DNA
0.5 %	1 – 30 kbp
0.7 %	0.8 – 12 kbp
1.0 %	0.5 – 10 kbp
1.2 %	0.4 – 7 kbp
1.5 %	0.2 – 3 kbp
2.0 %	50 bp – 2 kbp
3 – 4 %	10 bp – 1 kbp

Agarose gel is usually supplemented with ethidium bromide. Individual fractions of DNA may be therefore visualised by UV light. Another possibility is blotting of fractions onto a membrane followed by nucleic acid staining or hybridisation with marked probes.

Electrophoresis in polyacrylamide gel

Polyacrylamide gel is another carrier used for electrophoresis of nucleic acids. Linear molecules of polyacrylamide are produced by polymerisation of acrylamide. N,N'-methylenebisacrylamide is added as copolymer to form cross-links. Both acrylamide and methylenebisacrylamide are relatively stable compounds. Polymerisation runs in absence of oxygen (it is removed by deaeration of solutions using exhauster) and is started by addition of catalyst, ammonium peroxydisulphate (commonly called ammonium persulphate, APS) and N,N,N',N'-tetramethylene diamine (TEMED). In aqueous solution, APS releases free oxygen radicals in presence of basic TEMED. The free radicals attack molecules of acrylamide and bisacrylamide and thus trigger their polymerisation.

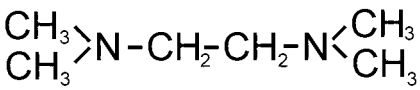




Molecular sieve of polyacrylamide gel is very dense and therefore suitable for separation of shorter fragments.

Polyacrylamide	Length of DNA
3.5 %	1 – 2 kbp
5 %	75 – 500 bp
8 %	50 – 400 bp
12 %	35 – 250 bp
15 %	20 – 150 bp
20 %	5 – 100 bp

Polyacrylamide is less reactive than agarose. Fragments of DNA can be therefore stained, apart from the method mentioned above, with other techniques as well. Among classical ones, staining with silver belongs to the most sensitive. Compared to ethidium bromide, it allows detecting of several orders smaller amounts of DNA.



TEMED (N,N,N‘,N‘-tetramethylenediamine)

Restriction fragment length polymorphism

Analysis of restriction fragment length polymorphism (**RFLP**) is indisputably one of the oldest and till now the most widely used technique in DNA diagnostics. It uses bacterial endonucleases ("restrictases") capable of cleaving DNA if the nucleic acid contains certain exactly defined sequence of nucleotides. Bacteria use these enzymes for protection against viruses: viral DNA can be easily cleaved, in contrast to own nucleic acid that is protected by methylation.

The DNA sequence that is recognised and cleaved by endonuclease is usually only several base pairs long. Frequently, it is a palindromic sequence so it can form a loop. The enzyme can easily find and cut such a loop. If one allele of a gene contains recognised sequence and the other one not, DNA of the former allele is split while the latter stays unchanged. After electrophoresis of fragments we can see two bands of shorter strands originating from the restricted sequence. The sequence that was not split shows one band of the original length.

Two endonucleases are to be employed in our practical exercise. The first enzyme is named KpnI. The germ *Klebsiella pneumoniae OK8* is the source. The recognised sequence for this enzyme is GGTACC (note that it is a palindrome). Both strands are split between cytosine residues (i.e. the resulting DNA double helices are of "uneven ends"):

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5´- G G T A C C  -3´
3´- C C A T G G  -5´
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In the lists of restriction enzymes it is usually displayed as GGTAC^C.

TaiI is another endonuclease used in our course. It has been isolated from germ *Thermus aquaticus Cc1-331*. The recognised sequence is ACGT^ (again, it is a palindrome). Compared with the previous enzyme, TaiI requires a higher temperature (about 65 °C) and a buffer of higher ionic strength.

Detection of mutations

SSCP

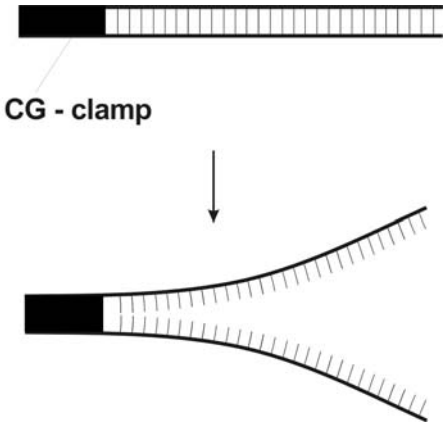
Several methods are employed for detection of mutations. Technique called SSCP (single strand conformation polymorphism) belongs to easy-to-do procedures. The principle of this method is that electrophoresis of single-strand DNA is performed in non-denaturing polyacrylamide gel at low temperature. The strand is folded according to inner complementarities and forms a three-dimensional structure (similar to e.g. tRNA). Mobility of the single-strand DNA in course of electrophoresis depends then on particular conformation. Even a scant change in sequence of nucleotides may lead to a completely different spatial structure of the folded single-strand DNA. SSCP is able to distinguish even a single nucleotide mutation in many cases. Using SSCP, it is possible to detect more than 99% of single nucleotide mutations in DNA molecules 100 – 300 bp long and about 80% in 400 bp long strands. Efficiency of SSCP is the worse the longer is the examined strand; this technique is not suitable for molecules longer than 750 bp.

DNA of most PCR products is separated into two groups of fractions in course of SSCP. The first group is slower and consists of several sharp bands. The second fraction is faster and a single band usually forms it. There are several reasons why one PCR product forms several bands:

- every strand of denatured DNA forms a different conformation
- one strand may form several stable conformations
- a portion of molecules does not fold into a spatial structure
- a portion of molecules renatures into the original double helix so that homoduplexes are formed again
- a portion of molecules may renature forming heteroduplexes in heterozygote

DGGE

Denaturing gradient gel electrophoresis (DGGE) is another very sensitive technique for mutation detection. It is based on the fact that the less hydrogen bridges DNA contains the more susceptible is it to denaturation. Both strands segregate each from other much easier in regions rich in AT pairs while CG-rich regions are more stable. Polyacrylamide gel with continuously growing concentration of denaturing agents (formamide and urea) is used for this method. The velocity of movement of the examined DNA in the electric field corresponds to its molecular weight until its strands start to separate. Denatured strands are much slower. It means that the more susceptible the examined DNA is to denaturing the closer stops it to start. Because completely separated strands of ssDNA would yield blurred bands, primers with so-called CG-clamp are used for amplification of DNA. Double helices of the resulting PCR product contain then a region with CG pairs only at its end. Denaturing is difficult at that spot. Sensitivity of DGGE is nearly 100%.



Temperature gradient gel electrophoresis (**TGGE**) is a similar technique. Gradually increasing temperature of gel is used instead of increased concentration of denaturing agents.

Heteroduplex analysis

Heteroduplex analysis is a method relative to DGGE. A sample is mixed with reference DNA, denatured and then let to re-hybridise. If sequence of the reference DNA differs from the sample both original homoduplexes and heteroduplexes consisting of one strand of reference and one strand of sample DNA are formed. These heteroduplexes contain a segment lacking hydrogen bonds and therefore decompose earlier.

Cleavage of heteroduplexes in non-complementary region is another technique. Chemical or, more reliably, enzymatic procedures are employed (e.g. using T4 endonuclease VII or T7 endonuclease I). This approach is designed as enzyme mismatch cleavage (**EMC**). Sensitivity of this method reaches 90%.