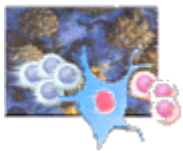
MOLECULAR BIOLOGICAL METHODS



There is a variety of protocols of the molecular biological techniques, which use in both immunogenetics and sister fields. Analogous to antigen-antibody reactions, the molecular biological methods are based on the interactions of complementary nucleic acids (cDNA and cRNA). Target DNA (analyte) is commonly dsDNA, which upon the denaturation become ssDNA. Two formed ssDNA are complementary to each other, but they both contain unknown sequences of nucleotides.

Hybridization, a method of the detection, represents the precise ligation of nucleotides of the target ssDNA to cDNA or cRNA with known nucleotide sequences labeled by a radioactive element, fluorochrome or enzyme for visualization. This label is usually termed as a probe. The target nucleotide sequence may also be read using gel electrophoresis and directly by sequencing.

The ***blotting***, Southern and Northern blotting, is an ancillary technique by which target nucleic acid, analogous to proteins during the Western blotting, may be electrophoretically transferred from the liquid phase to the solid nitrocellulose membrane.

Analyte molecules first are separated by size using polyacrylamide gel electrophoresis; next also transferred electrophoretically directly onto solid nitrocellulose membrane for hybridization to cDNA. After that, the film is washed away, to remove any unbound cDNA.

Since nucleotide sequences in the cDNA are common, the blots may be then detected by a certain method of visualization (see Table).

Table. Blotting Techniques

Method	Analyte	Detection	Possibilities
Southern blot (developed by E.M. Southern)	DNA	DNA hybridization to a radioactive (or other) cDNA probe	Detection of a particular gene of interest in genomic DNA
Northern blot	RNA	RNA hybridization to a radioactive (or other) cDNA probe	RNA isolation from different tissue to see which tissue express a certain gene (e.g., in primary immuno-deficiencies)
Western blot	Proteins (antigens)	Binding to antibodies conjugated to any label	Detection of a particular protein

Restriction Fragment Length Polymorphism (RFLP) is used for detection of molecular anomalies (mutations) in genes, mapping the human chromosomes and identification of persons including forensic use. The technique exploits cleavage of the DNA strand with restriction enzymes, which target a specific DNA recognition sequence.

The classical principle of ***Polymerase Chain Reaction (PCR)*** according to 1993 Nobel Laureate K. Mullis is composed in the multifold copying of target DNA with the use of *primers* through their polymerization by DNA polymerase to amplify billions of copies (*amplicons*) required for the detection of the target gene. Analyte must contain the well-characterized target gene with the target sequence of nucleotides (on dsDNA). Primer is a short piece of DNA (20-30 bps) synthesized in vitro, which is complementary to this target sequence. Pair of the primers includes

a forward primer and different reverse primer, but each primer is complementary to the 3' ends of each of the target sequence on both sense and anti-sense strand of the DNA. Primers will be annealed and extended to form then dsDNA under the influence of thermostable *Thermus aquaticus (Taq)* or *Pyrococcus furiosus (Pfu) DNA polymerase* in an amplifier (or thermocycler). The amplifier is a thermostat in which heat regime can be changed automatically, and copying cycles be repeated. Polymerase starts synthesizing new DNA strand complementary to the target sequence from the end of each primer.

Any PCR cycle includes:
(1) DNA denaturation, or separation of strands (at 94°C) to break all H bonds and get ssDNA;
(2) Annealing of the pair primers (at 40-60°C) to find and bind complementary sequence on each ssDNA;
(3) Elongation of the pair primers (at 70-74°C) by DNA polymerase rextension.
Such cycles repeat 20-30 times. As a result of amplification, after the first few cycles, there are formed excessively long sequences. After that, since the primers bind to their targets on these long sequences, they begin to shorten until most of the amplicons in the mixture reaches the expected length of the target gene. The nucleotide sequence in these copies (amplicons) may be identified by sequencing, gel electrophoresis, hybridization or other methods. Hybridization allows performing the detection by a radioactive element, fluorochrome or enzyme (ELISA). For the intermediate bridge, biotin and avidin (or streptavidin) may be used as they have the strong ability to bind up to each other. Also, internal control (or internal standard) is required in any cases.

As contrasted to conventional PCR, ***real-time PCR*** operates the amplification of a targeted DNA molecule during the PCR, not at its end, that allows this modification to use as a quantitative or semi-quantitative approach. In addition, there is ***quantitative PCR (qPCR).***
PCR uses for: (1) selective DNA isolation and cloning genes; (2) detection of mutations; (3) HLA typing for transplantation; (4) disputed paternity and person's identification (including forensic use); (5) infection diagnostics except for RNA viruses; (6) scientific research of ancient sources, etc.
PCR modification, ***Reverse Transcription PCR (RT-PCR)***, implies extraction of mRNA from cellular suspension, synthesis of cDNA by *reverse transcriptase (RT)* and further common protocol (see Fig).

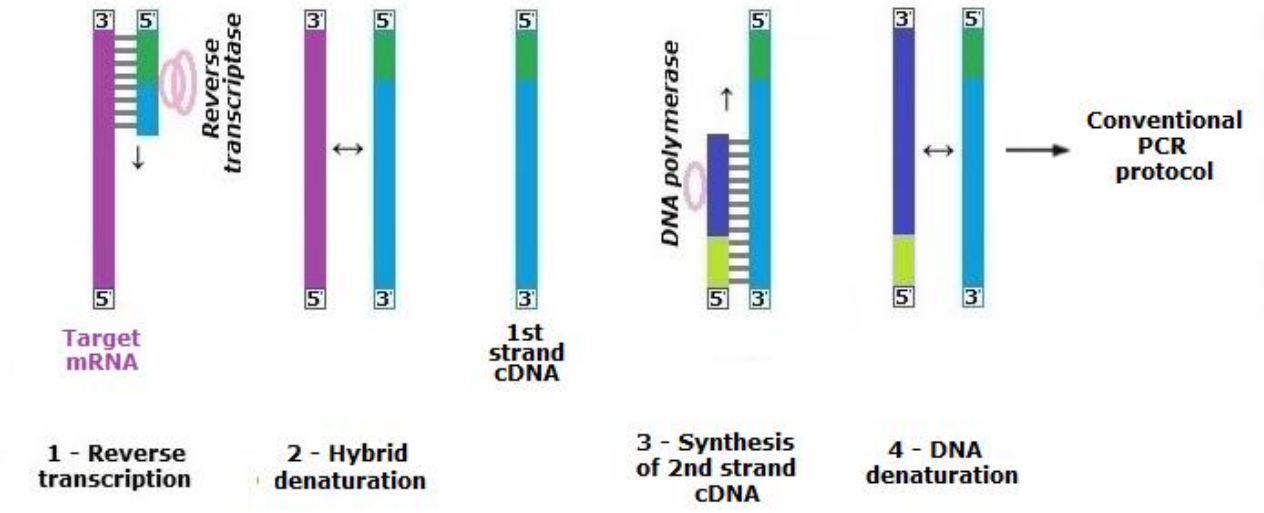


Fig. Reverse Trascription PCR (RT-PCR)

This quantitative and in fact functional technique allows the diagnose of RNA viral infections such as *HIV* and *Hepatitis C Virus*, and analysis of m RNA transcripts such as those produced by synthesis of molecules of the immune system. A well-known limitation to RT-PCR is the RNAase activity. Assess of the gene expression and correspondingly mRNA by Northern blot and RT-PCR differs by details of received information. However, RT-PCR is more sensitive.
Combination of PCR and RFLP (***PCR-RFLP analysis***) uses for the detection of intraspecies and interspecies variations, e.g., when there is a single nucleotide polymorphism.

Gene DNA Chip* (or *DNA Micro-array*) *Technology based on a collection of attached to a solid surface small DNA spots is a tool for effective simultaneous analysis of the enormous number of human genes including those encoded immune system's molecules. Analogous to computer microchips (photolithography), the technique enables to synthesize *in vitro* lots of different small pieces (30 bps) of DNA (or oligonucleotides) on a single chip. The oligonucleotides are designed to detect mRNA from a significant amount of genes using their hybridization with known sequences, biotin and streptavidin-fluorochrome labeling and laser scanner reading the probes.