Methods of nucleic acid analysis

Nucleic acid analysis generally involves isolation as a first step. DNA can be isolated from any biological material including teeth, hair, saliva, archived tissue in paraffin or tiny blood spot.

Isolation of DNA/RNA

Biological sample is retrieved and protected from further degradation by providing cold environment and/or by addition of a special chemical environment. The main goal is to obtain DNA/RNA molecule purified from co-extracts (proteins, lipids, steroids, etc). A typical sample in medical departments is fresh blood, 3ml of blood provide app. 50ng/µl of DNA. Quantity and quality of isolated nucleic acids has a huge impact on all downstream analyses. Concentration and purity can be measured by spectrophotometry. The length of DNA/RNA molecules could be assessed on electrophoresis.

Electrophoresis

Physical **method of separation** of molecules based on their length. A mixture of DNA/RNA of all different sizes is put in a specific gel providing physical barrier for molecules migrating in electrical field. Based on the fact, that DNA/RNA have negative charge they tend to migrate towards positive pole of an electrophoretic machine. Smaller sizes migrate faster and even one-base difference between two fragments could be visualized. Visualization is achieved by different strategies. Most commonly by intercalating dyes (ethidium bromide, etc.) or fluorophores incorporated in nucleic acid structure (HEX, NER, FAM, etc.). 16 simultaneously amplified fluorescently labeled STR loci are evaluated in forensic laboratories that focus on personal identification.

RNA – cDNA conversion

Several analytical approaches prefer DNA instead of RNA molecules. For that reason, single stranded RNA molecules of interest (total RNA, rRNA, mRNA, etc.) could be converted into double stranded cDNA (complementary DNA) molecule by reverse transcriptase, an enzyme typical in retroviral biology.

PCR

PCR – polymerase chain reaction – This in vitro method mimics processes that are physiological during replication phase of a cell cycle. It allows researchers to copy a specific fragment or several fragments of DNA making them more abundant it the sample and thus capable of analysis. Once the sample is enriched by fragments of specific regions, all downstream methods characterize only the selected amplified piece of DNA. It can focus on presence (qualitative measure), length (specific length can define an allele), nucleotide composition (sequencing, restriction, different behavior in double strand melting), etc…

qPCR

qPCR - quantitative PCR, also called real-time PCR, combines amplification of a particular DNA segment with simultaneous fluorescent detection of the resulting product. The instrument records the growth of the fluorescence signal in each cycle and thus the rate at which the PCR product is generated. By monitoring reaction during the exponential phase, the initial quantity of target DNA segment can be determined.

Sequencing

Nucleotide composition of PCR-amplified fragment is revealed by basically two principles.

Sanger method

Sanger method is a chain-termination method that uses classic PCR components plus chemically modified fluorescently labeled dideoxynucleotides (4 different fluorescent dyes for each base) than once incorporated, polymerase cannot propagate to add another nucleotide. All possible lengths (primer+1 base until last nucleotide of amplified sequence) are made during sequencing PCR and then are separated according to their length by electrophoresis in an instrument, that is capable of reading fluorescent signal of each fragment.

NGS

NGS – next generation sequencing is a high-throughput sequencing currently represented by a variety of usable platforms. This massively parallel sequencing is a complicated procedure capable of sequencing millions of different short DNA fragments in one run. Technical principle is NGS platform-specific, but they all provide huge amounts of data, that must be evaluated by sophisticated bioinformatical softwares or linux pipelines. They provide the most comprehensive picture of species genome.

RFLP

RFLP – restriction fragment length polymorphism. Bacterial endonucleases are capable of cutting DNA strand at specific region defined by short DNA sequence (palindrome structure – sequence that is read the same from both sides, eg.:ACCA). Nucleotide substitution can cause appearance/disappearance of a restriction site. This approach can serve a common mutation detection assay, or as a tool in indirect DNA analysis. Long time ago, after DNA was digested by specific restriction endonuclease, the resulting fragments were then separated by electrophoresis, transferred to the membrane (Southern blot), and visualized with labelled probe.

Southern blot

Southern blot – is the transfer of DNA fragments separated by electrophoresis on the membrane support. Transmission on the membrane enables further manipulation, especially hybridization with labelled probe.

Other methods

A variety of methods (**DGGE**, **TGGE**, **SSCP**, **Heteroduplex Analysis**, **Melting Curve Analysis**, etc.) have been developed to detect unknown variants in selected DNA fragments (PCR products). These methods utilize the difference in chemical or physical properties of double-stranded completely complementary DNA fragments over double-stranded DNA fragments with mismatch, or single-stranded DNA fragments mutated against non-mutated DNA fragments. The difference is transformed into a different mobility of the DNA fragment in the polyacrylamide gel. Techniques for detection of non-balanced deletions/duplication not retrievable by classic karyotyping are based on the fact that DNA hybridizes to its complementary counterpart whenever single stranded and in appropriate environment (temperature, ionic strength). These methods include Comparative Genomic Hybridization (CGH), Multiplex ligation-dependent probe amplification (MLPA), or Real time PCR (qPCR).

Techniques for detection of balanced structural aberrations of smaller extent (hard to reveal by classic karyotyping) are less abundant, since most of the molecular techniques focus on quantitative measurements. Fluorescently labeled in-situ hybridization (FISH) or Multicolor FISH (mFISH) – multiple fluorescently labelled probes are hybridized to condensated chromosomes and the final multicolor chromosome should consist of defined color band picture.

Karyotyping – classic method of cytogenetic departments. Natural process of chromosomal condensation during cell replication is exploited in this method of visualization of chromosomal bodies in light microscope. Recognition of specific banding pattern helps to identify each chromosomal pair and evaluate possible numerical or structural aberrations.

Cloning – Unknown organism´s DNA is fragmented with restriction enzyme and then cloned into a plasmid vector and amplified. New genomes could be read by assemblies of overlapping sequences, so called contigs.

Mitochondrial and **Y chromosome-specific sequences** have only one appearance and form a so called haplotype. These sequences serve as a maternal and paternal lineages codes and are widely used as a tool in evolutionary studies.