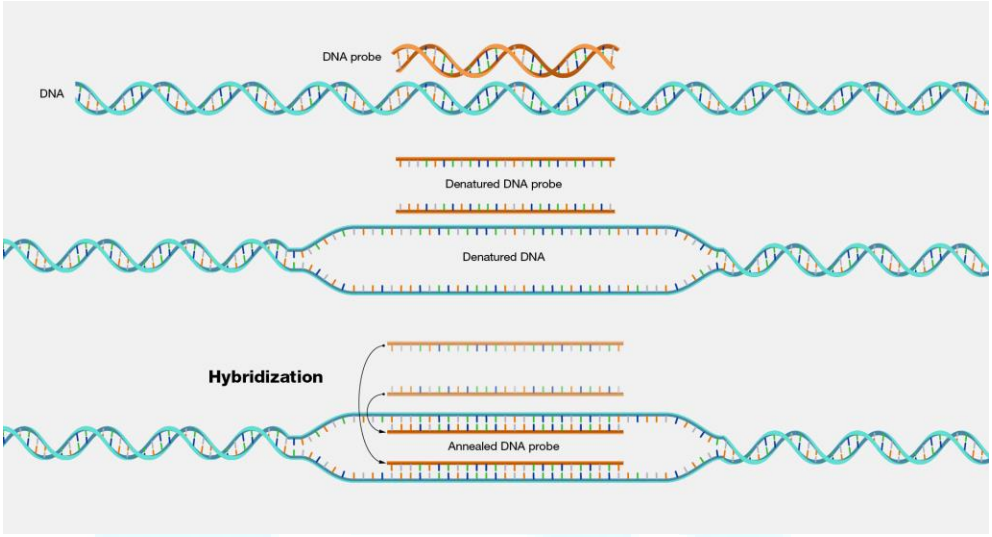
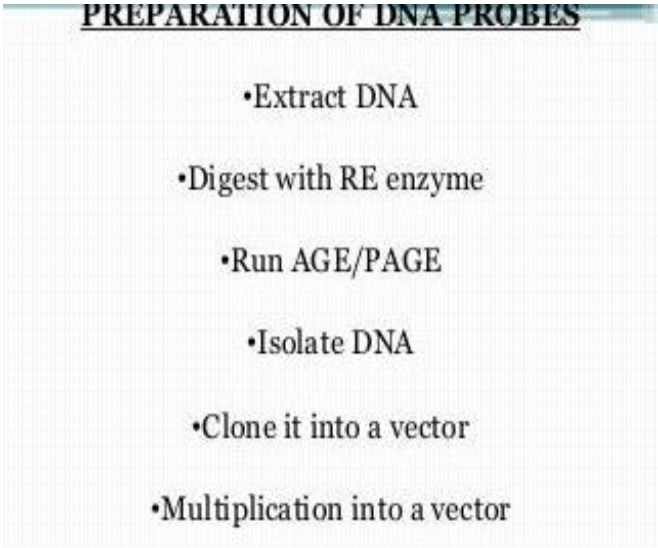
TECHNIQUE OF DNA ANALYSIS

DNA PROBE



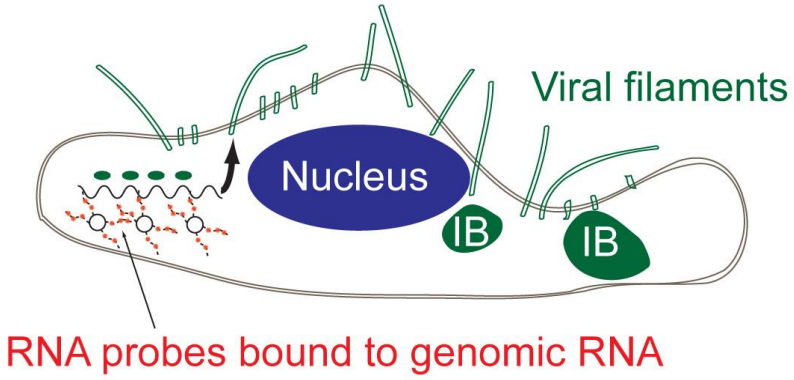
- A DNA probe is a fragment of DNA that contains a nucleotide sequence specific for the gene or chromosomal region of interest.
- DNA probes employ nucleic acid hybridization with specifically labeled sequences to rapidly detect complementary sequences in the test sample.
- A variety of methodologies for labeling DNA have been described.
- In short, these methods are used to generate end-labeled or continuously labeled probes.
- Most enzyme-mediated labeling techniques are very much dependent on polymerase activity, which is responsible for incorporation of the labeled nucleotides.
- Furthermore, the use of Taq or other thermostable DNA polymerases permits labeling reactions to be performed at higher temperatures via PCR, thereby reducing the incidence of enzyme-mediated point mutations during probe synthesis.

- PCR is an excellent method for probe synthesis, requiring very small quantities of template material.
- In the presence of appropriately labeled nucleotide primers, PCR products are labeled as they are being synthesized.
- Alternatively, the primers themselves may be labeled non-isotopically during their own synthesis, negating the requirement for the inclusion of labeled nucleotide precursors as part of the reaction mix.
- Random priming is a type of primer extension in which a mixture of small oligonucleotide sequences, acting as primers, anneal to a heat-denatured double-stranded template.
- The annealed primers ultimately become part of the probe itself, because the Klenow fragment of DNA polymerase I extends the primers in the 3′ direction and, in so doing, incorporates the label.
- Nick translation is one of the oldest probe labeling techniques.
- It involves randomly nicking the backbone of a double-stranded DNA with dilute concentrations of DNase I.
- At extremely low concentrations, this enzyme nicks a template at four or five sites, producing a free 3′-OH group that can act as a primer at each nicking location.
- Next, the enzyme DNA polymerase I removes the native nucleotides from the probe molecules in the 5′→3′ direction (exonuclease activity) while replacing them with labeled dNTP precursors by virtue of its 5′→3′ polymerase activity.
- Nick translation is efficient for both linear and covalently closed DNA molecules, and labeling reaction are completed in less than an hour.
- The ready-to-use NT Enzyme Mix is user friendly and minimizes error from pipetting.



- Probes labeled by nick translation can be used in many different hybridization techniques including: in situ hybridization (ISH), fluorescent in situ hybridization (FISH), screening gene banks by colony or plaque hybridization, DNA or RNA transfer hybridization, and re-association kinetic studies.

RNA probes



- RNA probes are stretches of single-stranded RNA used to detect the presence of complementary nucleic acid sequences (target sequences) by hybridization.
- RNA probes are usually labeled, for example with radioisotopes, epitopes, biotin or fluorophores to enable their detection.

- RNA probes as hybridization tools remain popular because of several key advantages associated with their use.
- These probes are synthesized by in vitro transcription and can be substituted for DNA probes in nearly all applications.
- High specific activity RNA probes or riboprobes may also be synthesized from DNA templates cloned in expression vectors such as SP 6 and T 7 systems.
- RNA probes are single-stranded and offer several advantages over DNA probes including improved signal or hybridization blots.
- Compared to the diverse methods for DNA probe synthesis, there is only one reliable method for labeling RNA probes, namely in vitro transcription.
- Because of the intrinsically labile nature of RNA and the susceptibility to RNase degradation, RNA probes must be treated with the same care as any other RNA preparations.
- in vitro transcription is a reliable and economical method for generating RNA probes.
- Large amounts of efficiently labeled probes of uniform length can be generated by transcription of a DNA sequence ligated next to an RNA promoter.
- One excellent strategy is to clone the DNA to be transcribed between two promoters in opposite orientations.
- This allows either strand of the cloned DNA sequence to be transcribed in order to generate sense and antisense RNA for hybridization studies.
- One alternative method to generating continuously labeled RNA probes by in vitro transcription is to label the 5′ end of the molecule.
- This method of 5′ end-labeling is colloquially known as the kinasing reaction; it specifically involves the transfer of the γ phosphate of ATP to a 5′-OH substrate of RNA or DNA (forward reaction).
- The forward kinasing reaction is far more efficient than the exchange reaction which involves the substitution of 5′ phosphates.
- Probe synthesis by 3′ end-labeling involves the addition of nucleotides to the 3′ end of either DNA.

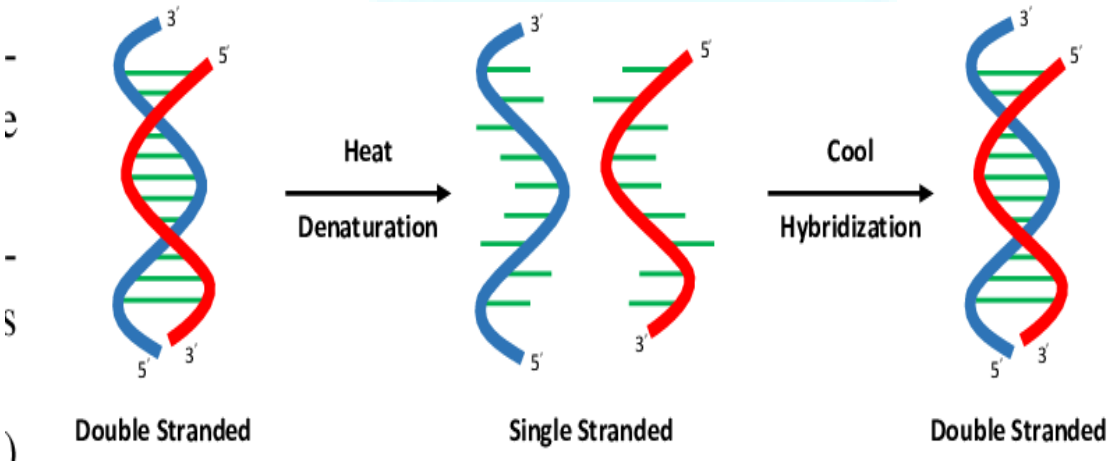
- DNA 3′ end-labeling is most often catalyzed by terminal transferase.
- Single- and double-stranded DNA molecules are labeled by the addition of dNTP to 3′-OH termini.
- RNA can also be 3′ end-labeled using the enzyme poly(A) polymerase.
- This enzyme, which is naturally responsible for nuclear polyadenylation of many heteronuclear RNAs, catalyzes the incorporation of Adenosine Mono Phosphate.
- Isotopic labeling requires α-labeled ATP precursors.
- In addition to its utility in RNA probe synthesis reactions, poly(A) polymerase can be used to polyadenylate naturally poly(A)– mRNA and other RNAs in order to support oligo(dT) primer-mediated synthesis of cDNA.
- Nucleic acid probes are either a single stranded DNA or an RNA with a strong affinity towards a specific DNA or RNA target sequence.
- This affinity and complementary sequence allows binding to specific regions of a target sequence of nucleotides.
- The degree of homology between target and probe results in stable hybridization. In developing a probe, a sequence of nucleotides must be identified, isolated, reproduced in sufficient quantity, and tagged with a label that can be detected.
- In theory, any nucleic acid can be used as a probe provided it can be labeled to permit identification and quantitation of the hybrid molecules formed between the probe and sequence to be identified.

Choice of Label

- Probes can be labeled either by radioactive isotopes or can also be labeled with nonradioactive molecules such as biotin, digoxegenin etc.
- However, the use of radioisotope labeled probes is limited by the short half-life of the isotope, and economic and environmental aspects of radioactive waste disposal.
- Advances in nucleic acid technology offer alternatives to radioactively labeled probes.

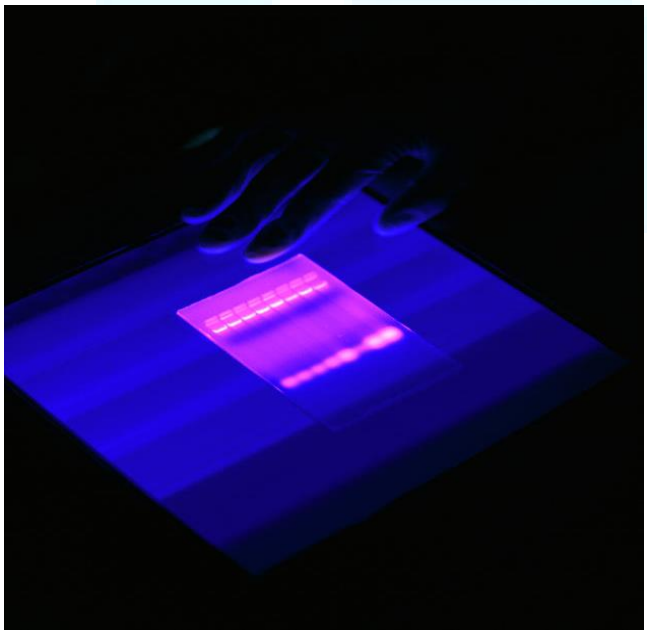
- The use of nonradioactive labels have several advantages such as safety and higher efficiency of the labeling reaction.
- One example is biotin labeling of nucleic acids. This system exploits the affinity that the glycoprotein avidin has for biotin. These probes can be prepared in advance in bulk and stored at -20°C for repeated uses.
- Digoxigenin is another chemical derived from plants and used for non-radioactive labeling of probes.
- An antibody associated with an enzyme (antidigoxigenin - alkaline phosphatase conjugate) is used for the detection of the presence of digoxigenin.
- Progress in sequence-specific DNA imaging by fluorescence microscopy has been achieved by employing the fluorescent hybridization in situ (FISH) method.
- This type of label is especially useful for the direct examination of microbiological or cytological specimens under the microscope.

DNA HYBRIDIZATION



- DNA hybridization was the first DNA-based technique proposed for the molecular discrimination of Eimeria parasites .
- A typical protocol consisted of genomic DNA digestion with different restriction enzymes, separation through agarose gel electrophoresis, blotting and hybridization with DNA probes composed of repetitive regions.
- The final result was a DNA fingerprinting comprising multiple band profiles.
- Similarly to enzyme variation detection, this approach also required large numbers of parasites and was highly time demanding.
- Also, the method was inherently unable to deal with mixed samples, since overlapping band profiles are not informative.

AUTORADIOGRAPHY



- Bands of radioactive DNA separated by acrylamide gel electrophoresis may be detected by autoradiography.

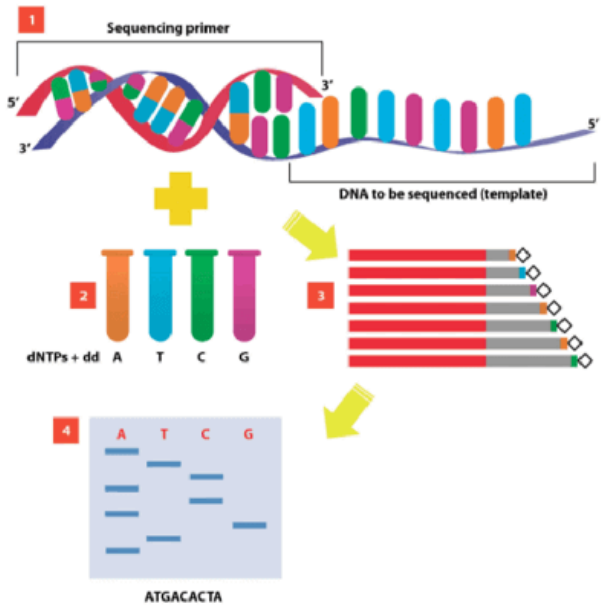
- Analytical polyacrylamide gels containing radioactive DNA are usually fixed and dried before autoradiography.

However, if bands of radioactive DNA are to be recovered from the gel, the gel should generally not be fixed or dried .

DNA SEQUENCING

- DNA sequencing is the process of determining the sequence of nucleotides within a DNA molecule.
- Every organism's DNA consists of a unique sequence of nucleotides.
- Determining the sequence can help scientists compare DNA between organisms, which can help show how the organisms are related.
- This means that by sequencing a stretch of DNA, it will be possible to know the order in which the four nucleotide bases – adenine, guanine, cytosine, and thymine – occur within that nucleic acid molecule.
- The necessity of DNA sequencing was first made obvious by Francis Crick's theory that the sequence of nucleotides within a DNA molecule directly influenced the amino acid sequences of proteins.

- At the time, the belief was that a completely sequenced genome would lead to a
- quantum leap in understanding the biochemistry of cells and organisms.
- Modern DNA sequencing consists of high-throughput methods which allow entire DNA sequences to be discovered in a matter of hours.
- This technology has allowed many companies to start offering at-home DNA testing.
- Many of the "results" found by these tests are simply correlations found between a genetic variant and a certain condition.
- However, technology has also allowed scientists to test the DNA of many organisms to better understand evolutionary relationships.
- Further, the first full sequence of human DNA took around 3 billion dollars. Now, certain companies will sequence your entire genome for less than $1,000.

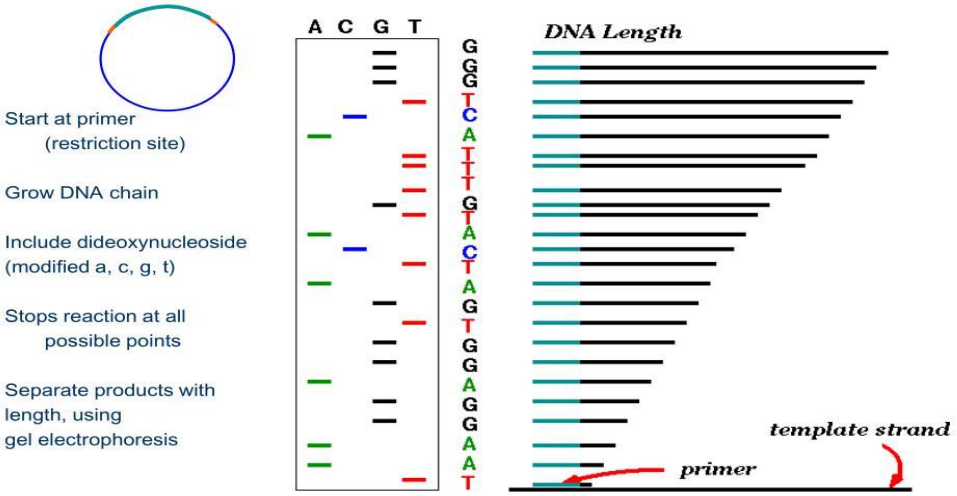


DNA Sequencing Methods

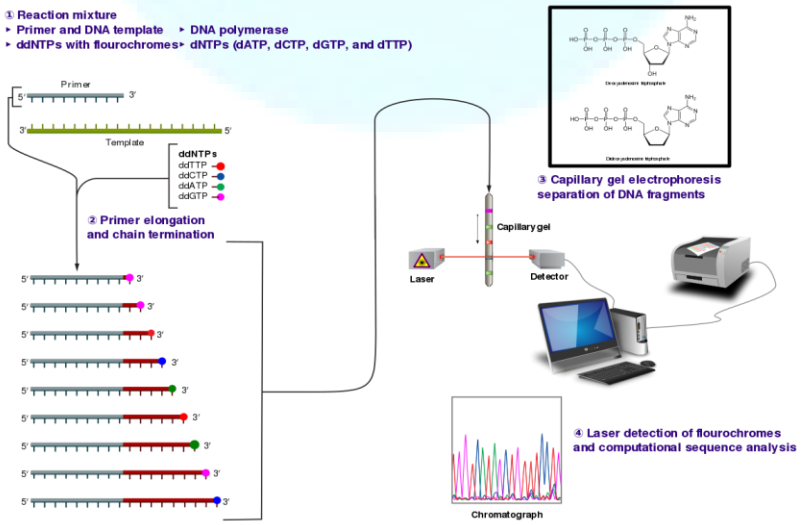
- There are two main types of DNA sequencing.

- The older, classical chain termination method is also called the Sanger method.
- Newer methods that can process a large number of DNA molecules quickly are collectively called High-Throughput Sequencing (HTS) techniques or Next-Generation Sequencing (NGS) methods

DNA sequencing – gel electrophoresis

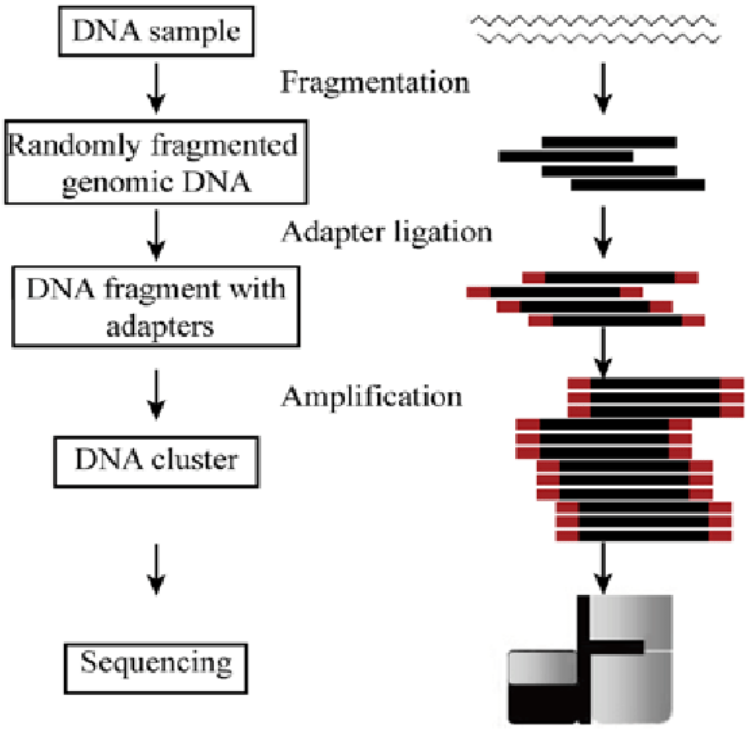


Sanger Sequencing



- The Sanger method relies on a primer that binds to a denatured DNA molecule and initiates the synthesis of a single-stranded polynucleotide in the presence of a DNA polymerase enzyme, using the denatured DNA as a template.
- In most circumstances, the enzyme catalyzes the addition of a nucleotide. A covalent bond, therefore, forms between the 3′ carbon atom of the deoxyribose sugar molecule in one nucleotide and the 5′ carbon atom of the next. This image below shows how this bond is formed.
- A sequencing reaction mixture, however, would have a small proportion of modified nucleotides that cannot form this covalent bond due to the absence of a reactive hydroxyl group, giving rise to the term 'dideoxyribonucleotides', i.e., they do not have a 2′ or 3′ oxygen atom when compared to the corresponding ribonucleotide.
- This would terminate the DNA polymerization reaction prematurely. At the end of multiple rounds of such polymerizations, a mixture of molecules of varying lengths would be created.
- In the earliest attempts at using the Sanger method, the DNA molecule was first amplified using a labeled primer and then split into four test tubes, each having only one type of ddNTP.
- That is, each reaction mixture would have only one type of modified nucleotide that could cause chain termination. After the four reactions were completed, the mixture of DNA molecules created by chain termination would undergo electrophoresis on a polyacrylamide gel, and get separated according to their length.

High Throughput Sequencing



- Sanger sequencing continues to be useful for determining the sequences of relatively long stretches of DNA, especially at low volumes.
- However, it can become expensive and laborious when a large number of molecules need to be sequenced quickly.
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- Ironically, though the traditional dye-terminator method is useful when the DNA molecule is longer, high-throughput methods have become more widely used, especially when entire genomes need to be sequenced.
    - There are three major changes compared to the Sanger method. The first was the development of a cell-free system for cloning DNA fragments.
    - Traditionally, the stretch of DNA that needed to be sequenced was first cloned into a prokaryotic plasmid and amplified within bacteria before being extracted and purified.
    - High throughput sequencing or next-generation sequencing technologies no longer relied on this labor-intensive and time-intensive procedure.

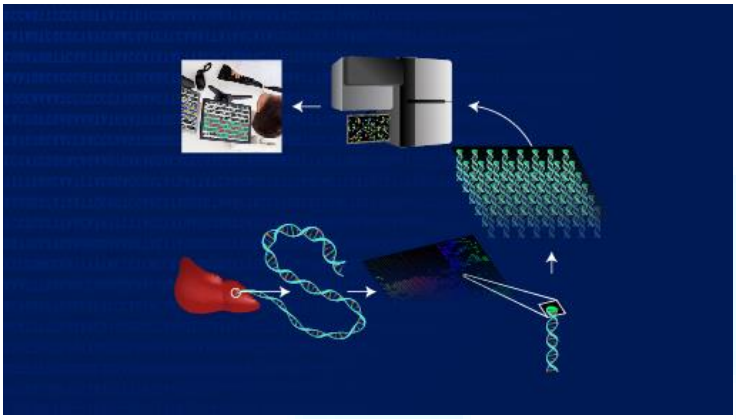
- Secondly, these methods created space to run millions of sequencing reactions in parallel.
- This was a huge step forward from the initial methods where eight different reaction mixtures were needed to produce a single reliable nucleotide sequence.
- Finally, there is no separation between the elongation and detection steps. The bases are identified as the sequencing reaction proceeds.
- While HTS decreased cost and time, their 'reads' were relatively short. That is, in order to assemble an entire genome, intense computation is necessary.
- The advent of HTS has vastly expanded the applications for genomics. DNA sequencing has now become an integral part of basic science, translational research, medical diagnostics, and forensics.

Uses of DNA Sequencing

- Traditional, chain-termination technology and HTS methods are used for different applications today.
- Sanger sequencing is now used mostly for de novo initial sequencing of a DNA molecule to obtain the primary sequence data for an organism or gene.
- The relatively short 'reads' coming off an HTS reaction (30-400 base pairs compared to the nearly a thousand base pair 'reads' from Sanger sequencing methods) make it difficult to create the entire genome of an organism from HTS methods alone.
- Occasionally, Sanger sequencing is also needed to validate the results of HTS.
- On the other hand, HTS allows the use of DNA sequencing to understand single-nucleotide polymorphisms – among the most common types of genetic variation within a population.
- This becomes important in evolutionary biology as well as in the detection of mutated genes that can result in disease.

- For instance, sequence variations in samples from lung adenocarcinoma allowed the detection of rare mutations associated with the disease. The chromatin binding sites for specific nuclear proteins can also be accurately identified using these methods

Overall, DNA sequencing is becoming an integral part of many different applications.



- Diagnostics
- Genome sequencing is particularly useful for identifying the causes of rare genetic disorders.
- While more than 7800 diseases are associated with a Mendelian inheritance pattern, less than 4000 of those diseases have been definitively linked to a specific gene or mutation.
- Early analysis of the exon-genome, or exome, consisting of all the expressed genes of an organism, showed promise in identifying the causal alleles for many inherited illnesses.
- In one particular case, sequencing the genome of a child suffering from a severe form of inflammatory bowel disease connected the illness to a mutation in a gene associated with inflammation – XIAP.
- While the patient initially showed multiple symptoms suggestive of an immune deficiency, a bone marrow transplant was recommended based on the results of DNA sequencing. The child subsequently recovered from the ailment.

- In addition, HTS has been an important player in developing a greater understanding of tumors and cancers.
- Understanding the genetic basis of a tumor or cancer enables doctors to have an extra tool in their kit for making diagnostic decisions.
- The Cancer Genome Atlas and International Cancer Genome Consortium have sequenced a large number of tumors and demonstrated that these growths can vary vastly in terms of their mutational landscape.
- This has also given a better understanding of the kind of treatment options that are ideal for each patient. For instance, the sequencing of the breast cancer genome identified two genes – BRCA1 and BRCA2 – whose pathogenic variants have an enormous impact on the likelihood of developing breast cancer.
- People with some pathogenic alleles even choose to have preventive surgeries such as double mastectomies.

Molecular Biology

- DNA sequencing is now an integral part of most biological laboratories.
- It is used to verify the results of cloning exercises to understand the effect of particular genes.
- HTS technologies are used to study variations in the genetic compositions of plasmids, bacteria, yeast, nematodes or even mammals used in laboratory experiments.
- For instance, a cell line derived from breast cancer tissue, called HeLa, is used in many laboratories around the world and was earlier considered as a reliable cell line representing human breast tissue.
- Recent sequencing results have demonstrated large variations in the genome of HeLa cells from different sources, thereby reducing their utility in cell and molecular biology.
- DNA sequencing gives insight into the regulatory elements within the genome of every cell, and the variations in their activity in different cell types and individuals.

- For instance, a particular gene may be permanently turned off in some tissues, while being constitutively expressed in others.
- Similarly, those with susceptibility for a specific ailment may regulate a gene differently from those who are immune. These differences in the regulatory regions of DNA can be demonstrated through sequencing and can give insight into the basis for a phenotype.
- Recent advances have even allowed individual laboratories to study structural variations in the human genome – an undertaking that needed global collaboration two decades ago.

Forensics

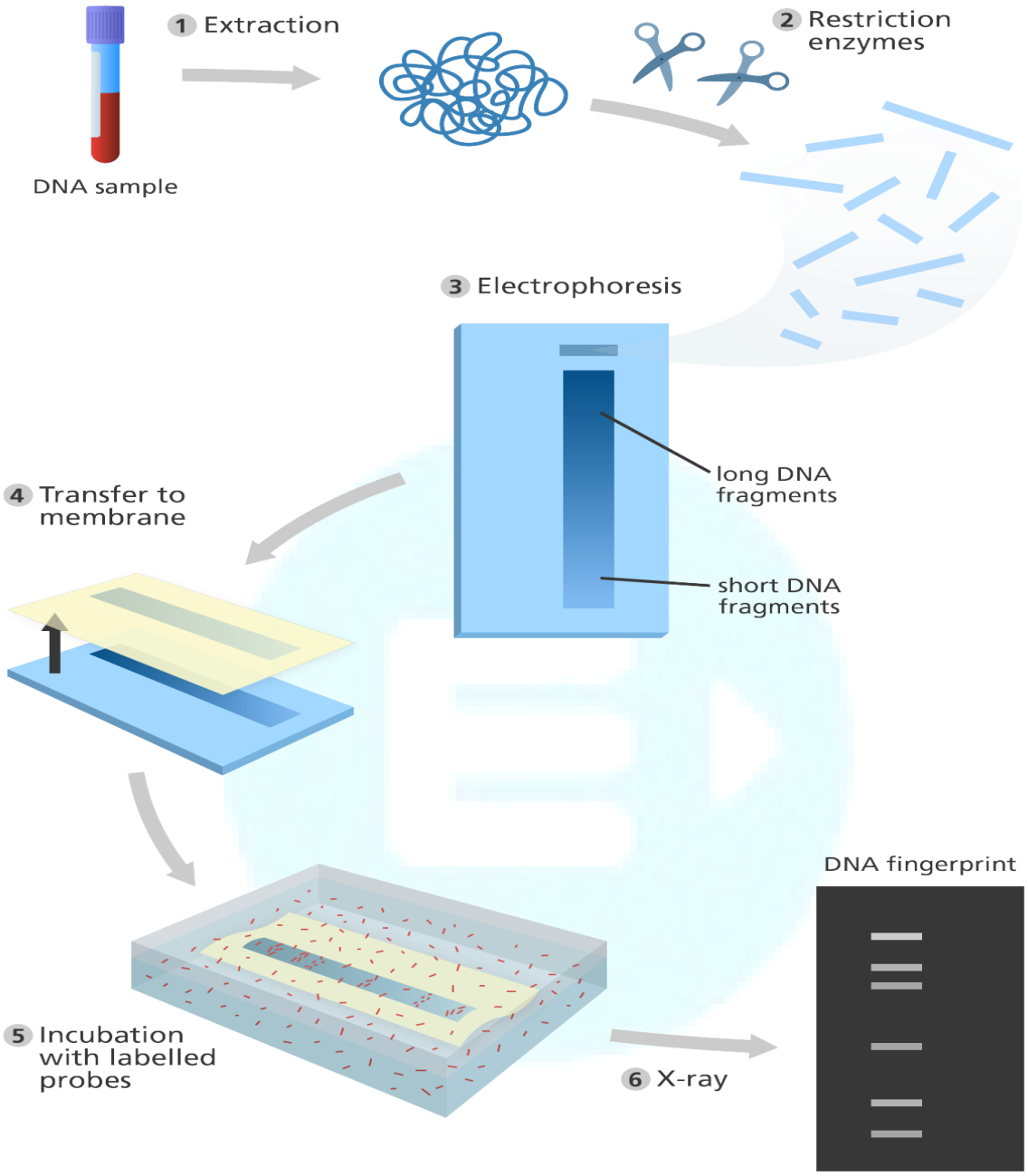
- The ability to use low concentrations of DNA to obtain reliable sequencing reads has been extremely useful to the forensic scientist.
- In particular, the potential to sequence every DNA within a sample is attractive, especially since a crime scene often contains genetic material from multiple people.
- HTS is slowly being adopted in many forensics labs for human identification. In addition, recent advances allow forensic scientists to sequence the exome of a person after death, especially to determine the cause of death.
- For instance, death due to poisoning will show changes to the exome in affected organs.
- On the other hand, DNA sequencing can also determine that the deceased had a preexisting genetic ailment or predisposition.
- The challenges in this field include the development of extremely reliable analysis software, especially since the results of HTS cannot be manually examined.

DNA FINGERPRINTING

- DNA fingerprinting is a chemical test that shows the genetic makeup of a person or other living things.
- It's used as evidence in courts, to identify bodies, track down blood relatives, and to look for cures for disease
- The process of DNA fingerprinting was invented by Alex Jeffreys in 1985.
- Blood ,Hair ,Saliva ,Semen ,Body tissue ,cells ,DNA samples have been obtained from vaginal cells transferred to the outside of a condom during sexual intercourse.
- There are 8 steps for DNA Fingerprinting Step 1: Isolation of DNA :DNA must be recovered from cells or tissue. Only a small amount of blood, hair, or skin is needed to isolate DNA
- Digestion of DNA by restriction endonucleases The DNA is cut into fragments using restriction enzymes. Each restriction enzyme cuts DNA at a specific base sequence.
- The sections of DNA that are cut out are called restriction fragments. This yields thousands of restriction fragments of all different sizes because the base sequences being cut may be far apart (long fragment) or close together (short fragment).
- Commonly used RE are (hae III, Hinf I, Alu I etc.) Reaction mixture is incubated overnight at 37.C
- Electrophoretic separation of different fragments · Fragments are separated on the basis of size using a process called gel electrophoresis. DNA fragments are injected into wells electric current is applied along the gel.

- DNA is negatively charged so it is attracted to the positive end of the gel. The shorter DNA fragments move faster than the longer fragments.
- DNA is separated on basis of size.
- Transfer DNA on Nylon/Nitrocellulose membrane The DNA fragments are transferred to a nylon sheet by placing the sheet on the gel and soaking them overnight by the process southern blot.
- Probing/probe labeling .Adding radioactive or colored probes to the nylon sheet which is complementary to target sequences.
- Each probe only sticks to one or two specific places on the sheet.
- Hybridization labeled probe DNA should be hybridized with the complementary sequences located on nylon membrane for the detection of position of later .
- Membrane is washed to remove non specific binding and clearing of the background
- Autoradiography to detect the sequences in genome bound with the hybridized radioactive probe on membrane,
- Technique involves alignment of hybridized membrane with X-ray film .
- The X-ray film alter its development shows multiple no. of bands that looks like bar codes and known as DNA fingerprints.
- Interpretation of band patterns · Analysis of band patterns of different individuals comparison of position of bans Computer software are also available for for the analysis of DNA fingerprints.

ENTRI


1 Extraction
DNA sample
2 Restriction enzymes
3 Electrophoresis
long DNA fragments
short DNA fragments
4 Transfer to membrane
5 Incubation with labelled probes
6 X-ray
DNA fingerprint