Unit-IV

Molecular Diagnosis

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Nucleic Acid Amplification Method

- Nucleic acid amplification is a pivotal process in biotech and molecular biology and has been widely used in research, medicine, agriculture and forensics.

- Nucleic acid amplification procedures can be used to overcome the limitations of direct probe hybridization assays.

- Each method makes use of in vivo mechanism of nucleic acid replication and repair to effect the amplification of nucleic acids in vitro.

- The strength of nucleic acid amplification technology is that it can make as many copies of sequence present initially at a low concentrations.

- Various modern methods have been adopted to reduce contamination risks of amplified assay products allowing the adaptations for routine use in the clinical laboratory.

- PCR (Polymerase Chain Reaction) is the preferred method.

Polymerase Chain Reaction (PCR)

- PCR is a revolutionary method developed by Kary Mullis in year 1980.
- It is based on the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand.
- PCR is a method widely used in molecular biology to rapidly make billions of copies of a specific DNA samples.
- Scientists take a very small sample of DNA and amplify it to a large enough amount to study in details.

Working Principle of PCR

- As the name implies, it is a chain reaction, a small fragment of the DNA section of interest needs to be identified which serves as the template for producing the primers that initiate the reaction. One DNA molecule is used to produce 2 copies, then 4, then 8 and so forth.

Components of PCR

- Target DNA Template
- Pair of Primers
- 4 dNTPs (deoxynucleosides triphosphate)
- Buffer system (containing Magnesium ions)
- DNA polymerase

Steps in PCR in DNA sequence

Step1: Denaturation by heating.

Step2: Annealing Primer to target sequence.

Step3: Extension.

Step4: End of the first PCR cycle, New strand is obtained.

Types of PCR

- Colony PCR
- Nested PCR
- AFLP PCR
- Hot Start PCR
- In Situ PCR
- Inverse PCR
- Asymmetric PCR
- Long PCR
- Long Accurate PCR
- Reverse Transcriptase PCR
- Allele Specific PCR
- Real Time PCR

Some PCR Techniques

- **Long PCR:** Used to amplify DNA over the entire length up to 25kb of genomic DNA segments cloned.

- **Nested PCR:** It involves 2 consecutive PCR reactions of 25 cycles. The first PCR uses primers external to the sequence of interest.

- The second PCR uses the product of the first PCR in conjunction with 1 or more nested primers to amplify the sequence within the region flanked by the initial set of primers.

- **Inverse PCR:** Used to amplify DNA of unknown sequence that is adjacent to known DNA sequence.

- **Quantitative PCR:** Product amplification with respect to time, which is compared with a standard DNA.

- **Hot Start PCR:** Used to optimize the yield of the desired amplified product in PCR and simultaneously to suppress non- specific amplifications.

Applications of PCR Technique

Medical Diagnostics

- Diagnosis and characterization of infectious diseases (pathogens).
- Diagnosis and characterization of genetic diseases.
- Diagnosis and characterization of Neoplasia.

Forensics

- Identify criminal suspects.
- Paternity cases.

Hybridization Technique

- In molecular biology, hybridization is a phenomenon in which single-stranded DNA or RNA molecules anneal to complementary DNA or RNA.
- By decreasing the surrounding temperature allows the single- stranded molecules to anneal or ‘hybridize’ to each other.
- Replication and transcription of DNA into RNA both rely upon nucleotide hybridization.
- The techniques include are –
- PCR
- Southern blots
- Northern blots
- DNA sequencing

DNA Sequencing Methods

- DNA sequencing is a laboratory method used to determine the order of the bases within the DNA. Differences in the sequences of these 3 billion base pairs in the human genome lead to each person's unique genetic makeup.

- In medicine for healthcare practitioners DNA sequencing is used for a range of purposes, including diagnosis and treatment of diseases, to determine gene mutations linked to genetic disorders.

- The technique generally consists of breaking long strands of DNA into many small fragments, using different test to determine the order of nucleotide bases in order of the original DNA strand.

- ‘Sanger Sequencing’ was developed in 1970’s which was used in Human Genome Project to completely sequence the DNA of a human for first time. It relies on chain terminating nucleotide (dideoxynucleotides).

- For many years Sanger sequencing had been gold standard for clinical DNA sequencing but its limitation was that it could only read one short section of DNA from 1 person at a time.

- Then ‘Next- Generation Sequencing’ was introduced which was time and cost effective, also are faster than ‘Sanger sequencing’ as it can sequence millions of small DNA fragments from different parts of genome all at the same time. It is also referred to as massively parallel sequencing.

Blotting

- Blotting is used to detect different types of macromolecules. It is common and widely used technique in field of molecular biology.

- The technique depends upon the size of molecule and their binding ability to the solid support by using probe to detect the molecule of interest.

- In this nucleic acids i.e., RNA and DNA or proteins are transferred onto a specific membrane. It can be done either after gel electrophoresis or done directly and observed by using different stains.

- **Southern Blotting** is used for DNA analysis.

- **Northern Blotting** is used for RNA analysis.

Southern Blotting

- Southern blotting is widely used for detection of specific genes in cellular DNA. The technique was developed by E.M.Southern.
- The DNA to be analyzed is digested with a restriction endonuclease and then the fragments are separated by gel electrophoresis.
- The gel is overlaid with nitrocellulose filter/nylon membrane, to which the DNA fragments are blotted to yield a replica f the gel.
- The filter is then incubated with radiolabeled probe, which hybridizes to DNA fragments containing complementary sequence.
- Then fragments re visualized by exposure of filter to X-ray film.

Northern Blotting

- Northern blotting is a variation of southern blotting, used for analysis and detection of RNA instead of DNA.
- This was given by Alwine. It is frequently used in studies of gene expression.
- In this method, total cellular RNAs are extracted and fractionated according to size by gel electrophoresis.
- Then RNAs are transferred to a filter and detected by hybridization with radioactive probe.

In Situ Hybridization

- In situ hybridization is a technique that is used for localization and detection of specific homologous DNA and RNA sequences in cells, preserved tissue sections, in chromosomes, intact cells or entire tissue by hybridizing the complementary strand of a nucleotide probe to a particular sequence.
- Fluorescence in situ hybridization (FISH) is a molecular cytogenic technique that uses fluorescent probes that bind to only those parts of a nucleic acid sequence with a high degree of sequence complementarity.
- It is also used to detect specific mRNAs in different types of cells within a tissue. In this case, the hybridization of radioactive or fluorescent probes to specific cells or subcellular structures is analyzed by microscopic examination.
- FISH is often used for finding specific feature in DNA for use in genetic counseling, medicine, and species identification.

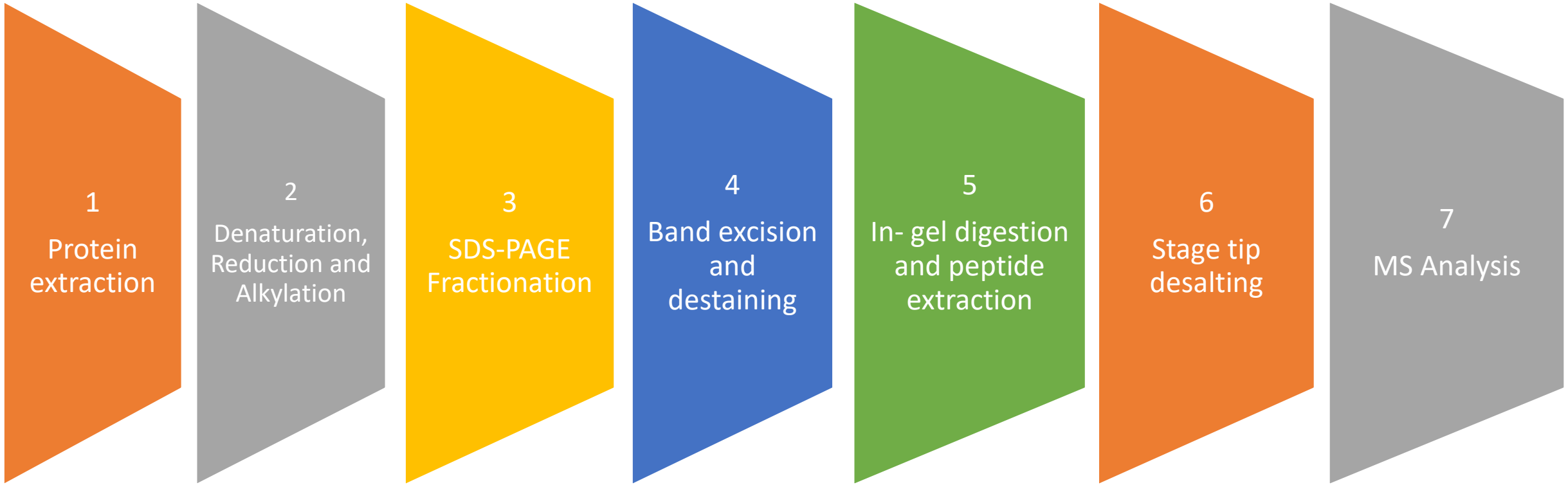
Protein Extraction

- **Proteins** are the biomolecules composed of amino acid, forming building block of the system and performs most of the biological functions of the system.

- **Protein extraction** is the process by which proteins from the cell are recovered for the analysis purpose.

- They are extracted from tissues for wide range of purposes such as comparison, purification, identification, resolve, physiological studies, diagnosis, mechanism of action and so on.

Procedure


1
Protein extraction
2
Denaturation, Reduction and Alkylation
3
SDS-PAGE Fractionation
4
Band excision and destaining
5
In- gel digestion and peptide extraction
6
Stage tip desalting
7
MS Analysis

SDS-PAGE Analysis

- **SDS-PAGE** (*Sodium dodecyl sulphate polyacrylamide gel electrophoresis*)
- It is a common technique used to separate, visualize, and therefore compare the relative polypeptide chains contained in different fractions.
- The combined use of sodium dodecyl sulphate and polyacrylamide gel allows to eliminate the influence of structure and charge, and proteins are separated solely on the basis of different in their molecular weight.
- It is a reliable method for determining the molecular weight of an unknown protein. It yields the mass of each individual subunit derived from the denatured complex.

Thank You…