Department of BSBE
Indian Institute Of Technology Guwahati

Techniques in Genetic Engineering

RAPD/RFLP

Dr. Sanjukta Patra

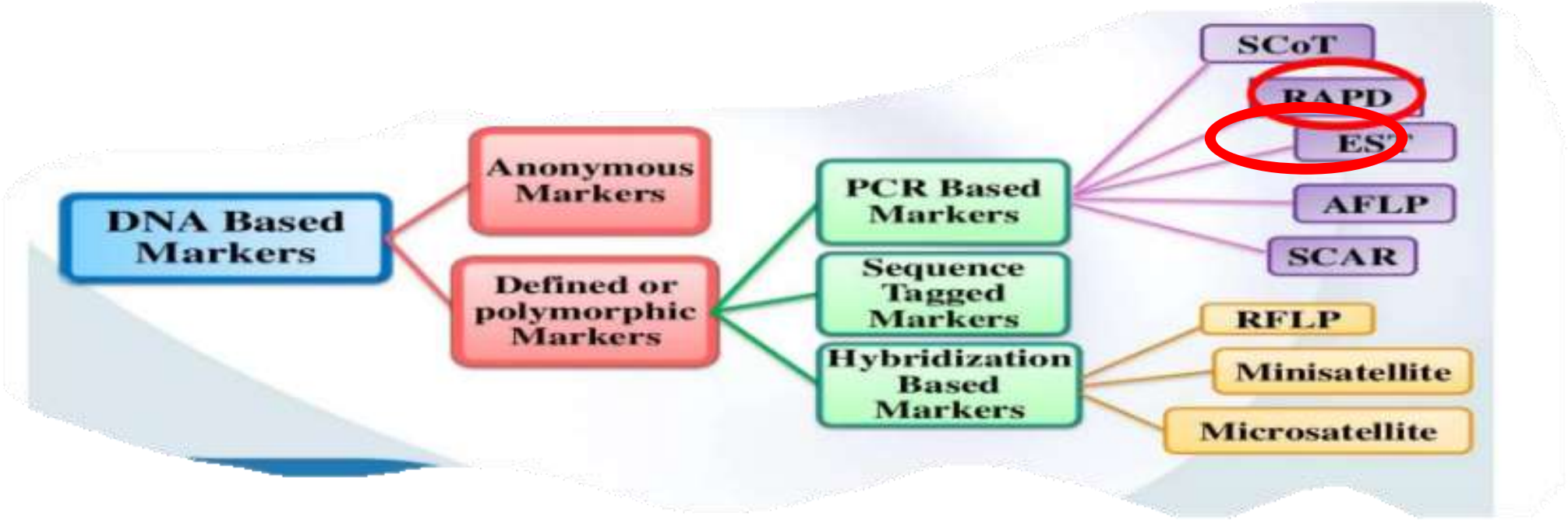
BT 207

Jan - May 2023

What are Markers?

Any genetic trait that can be identified with confidence & relative case and can be followed in a mapping population is called as marker.

Genetic/ Molecular marker is a specific location on a chromosome that is defined by a naked eye polymorphism as differences in electrophoretic mobility of specific proteins or as differences in specific DNA sequence.



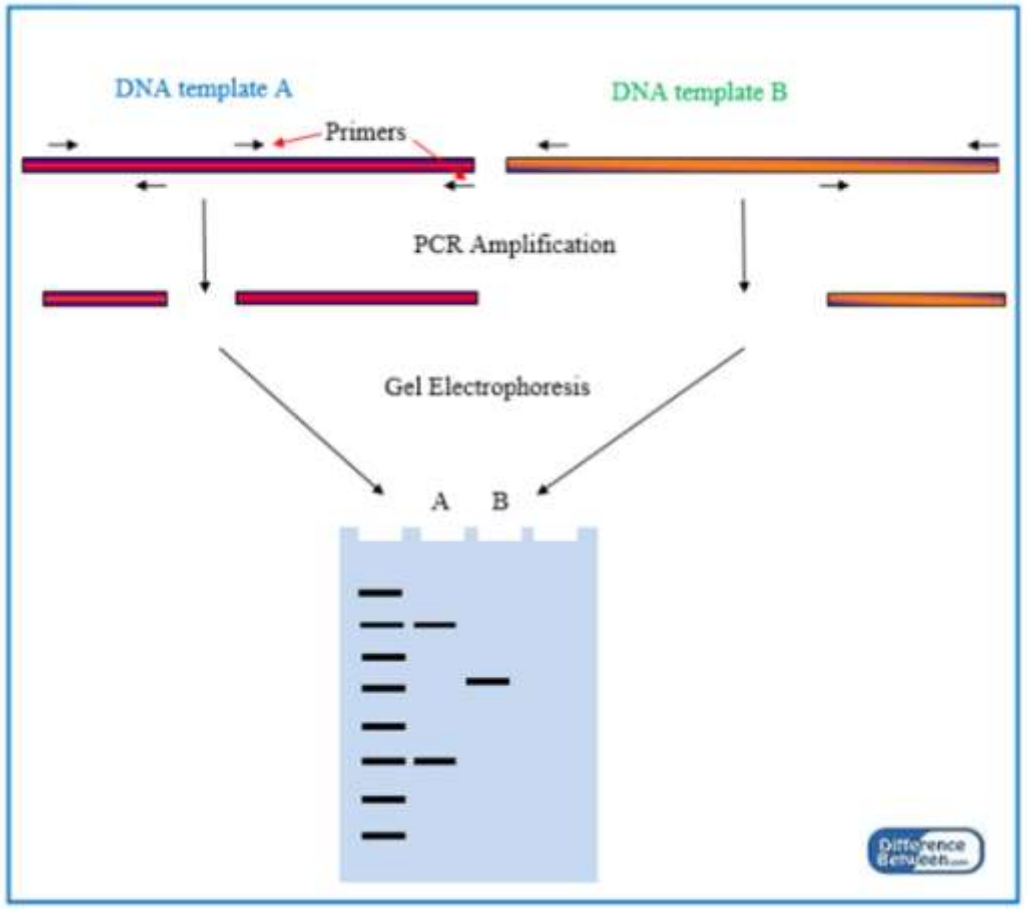
What is RAPD?

RAPD or random amplified polymorphic DNA is a fast, PCR-based method for the detection of DNA variation.

Two laboratories (Williams et. al., 1990; Welsh and McClelland, 1990) independently developed the technique.

Moreover, RAPD uses a single, arbitrary primer for the amplification of many discrete DNA products. The steps involved in RAPD are as follows:

1. DNA extraction
2. Amplification by random primers
3. Gel electrophoresis and visualization of markers



How RAPD works?

- The principle is that, a single, short (10 nucleotide) oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template.

- This means that the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome.

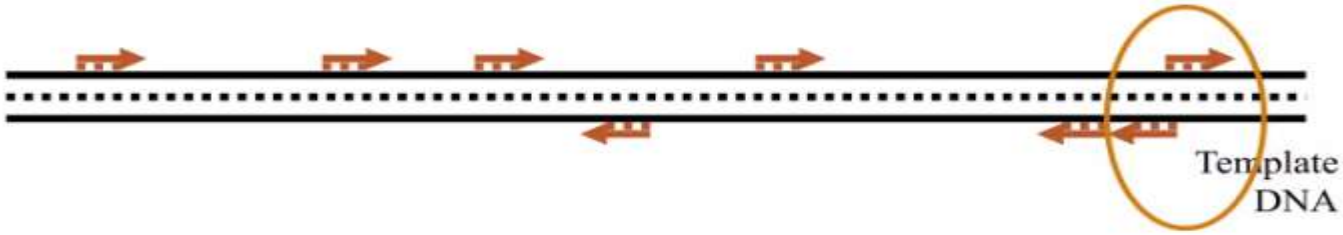
- These amplified products (of up to 3.0 kb) are usually separated on agarose gels (1.5-2.0%) and visualised by ethidium bromide staining.

- Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites.

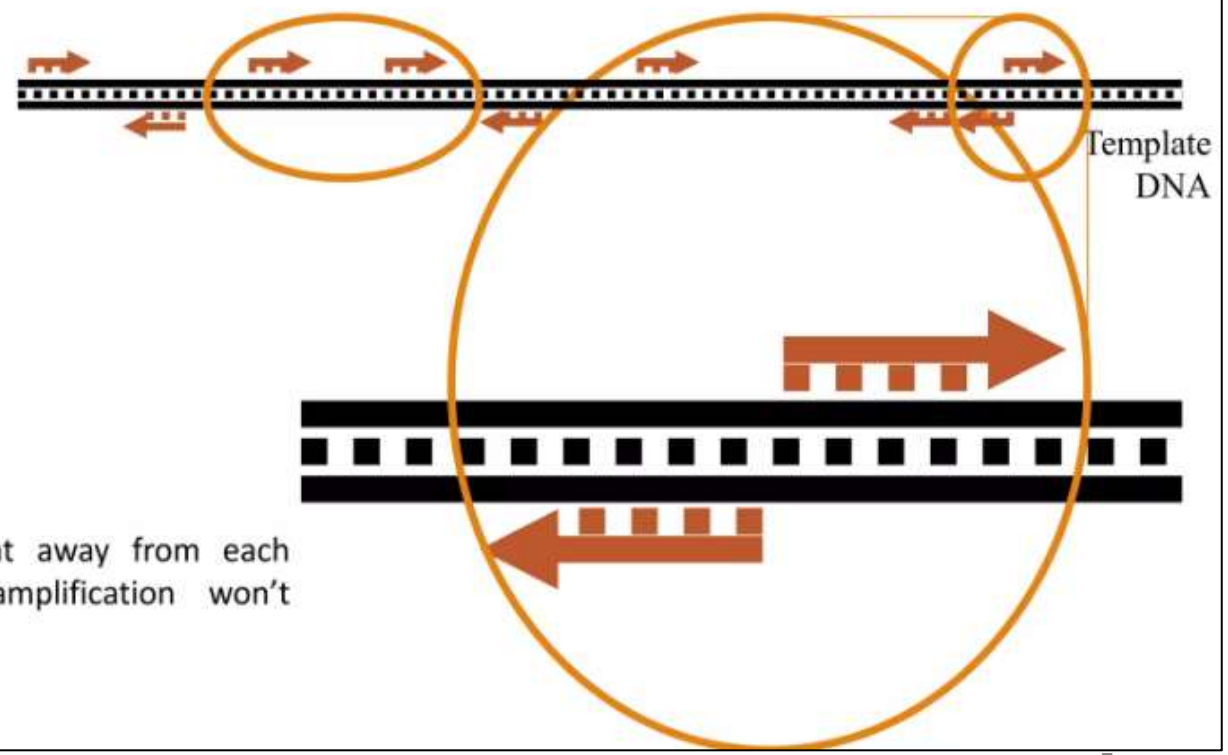
- Recently, sequence characterized amplified regions (SCARs) analysis of RAPD polymorphisms showed that one cause of RAPD polymorphisms is chromosomal rearrangements such as insertions/deletions.

- In order for PCR to occur:

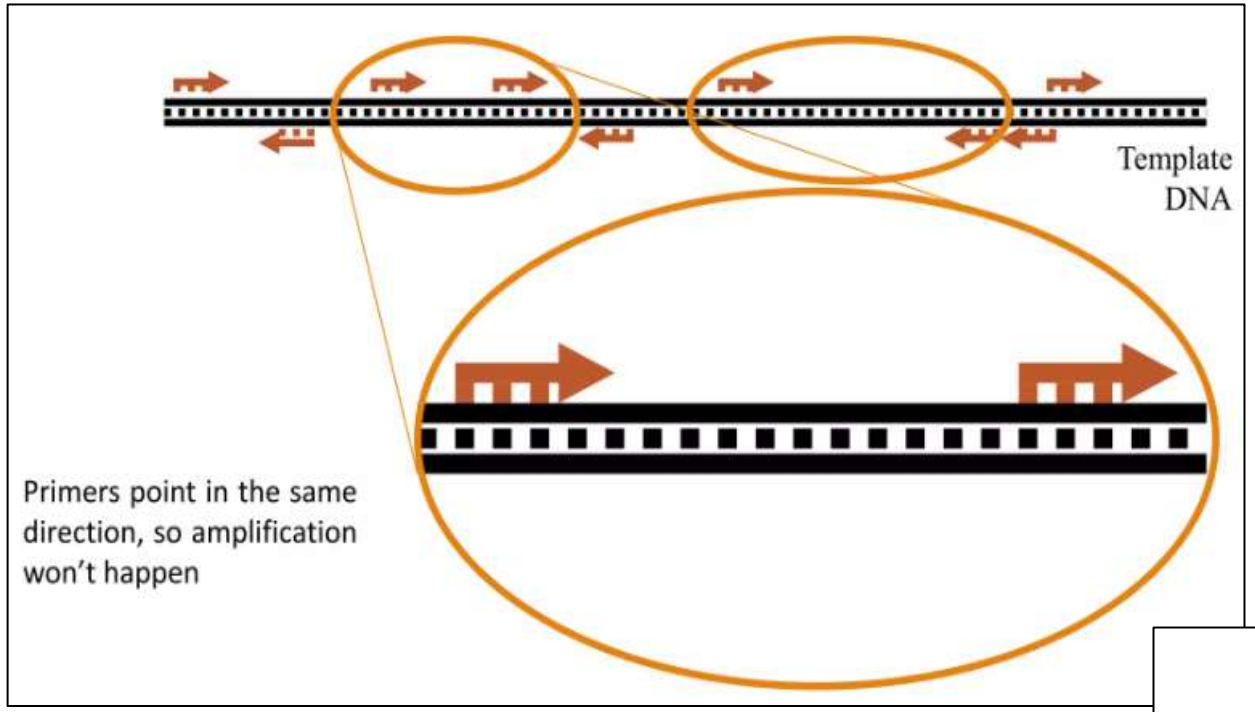
1)the primers must anneal in a particular orientation (such that they point towards each other) and,
2)they must anneal within a reasonable distance of one another.

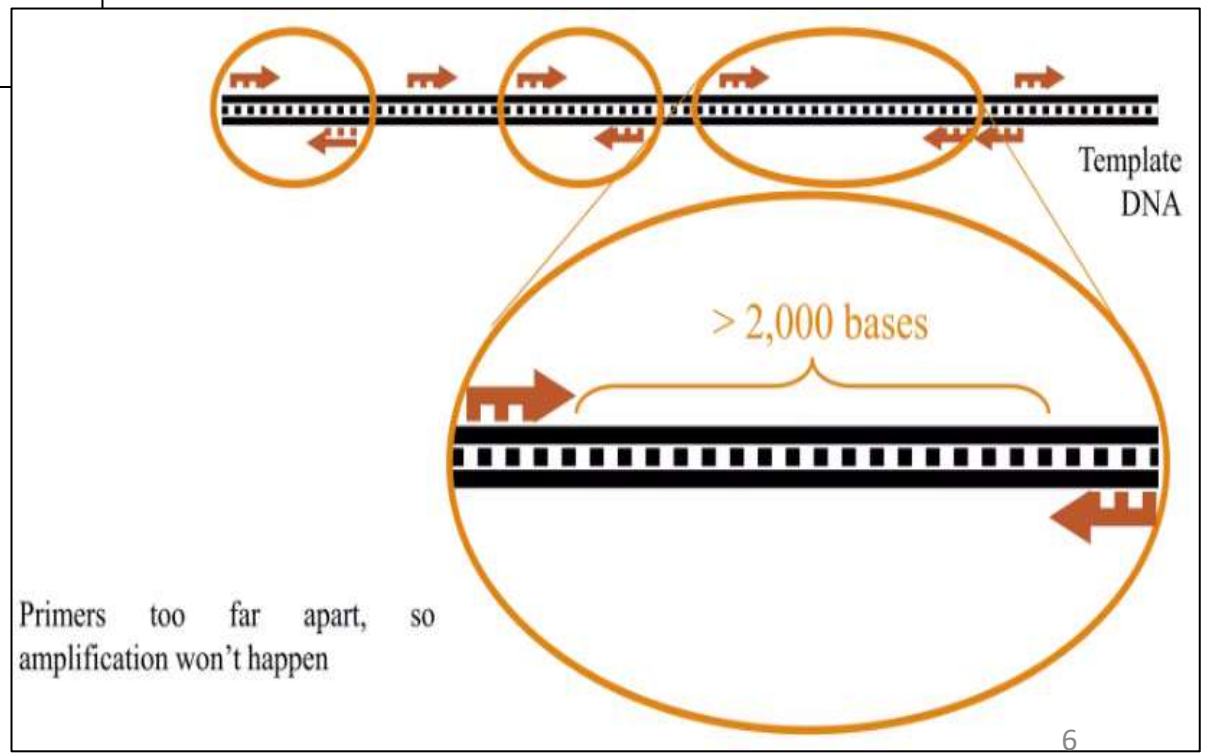


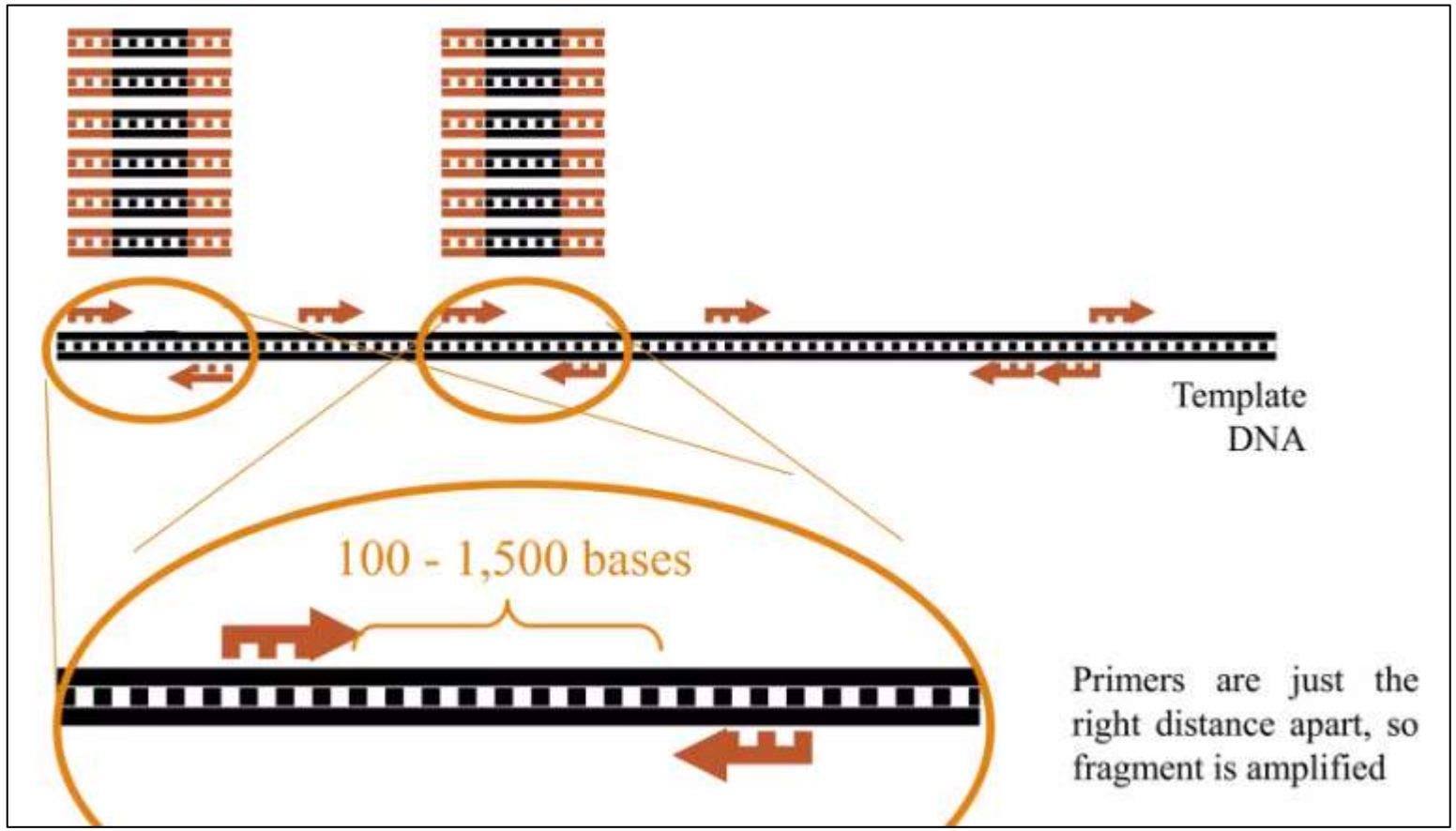
- Primer binds to many locations on the template DNA
- Only when primer binding sites are close and oriented in opposite direction so the primers point toward each other will amplification take place



Primers point away from each other, so amplification won't happen


Template
DNA
Primers point in the same direction, so amplification won't happen


Template
DNA
> 2,000 bases
Primers too far apart, so amplification won't happen


Template
DNA
100 - 1,500 bases
Primers are just the right distance apart, so fragment is amplified

Components of a PCR and RAPD Reaction

PCR

1. Buffer (containing Mg^{++})
2. Template DNA
3. 2 Primers that flank the fragment of DNA to be amplified
4. dNTPs
5. *Taq* DNA Polymerase (or another thermally stable DNA polymerase)

RAPD

1. Buffer (containing Mg^{++}) - usually high Mg^{++} concentrations are used lowering annealing stringency
2. Template DNA
3. 1 short primer (10 bases) not known to anneal to any specific part of the template DNA
4. dNTPs
5. *Taq* DNA Polymerase (or another thermally stable DNA polymerase)

Modifying Thermal Cycler

Two modifications made to typical thermal cycling when RAPD is being done:

1. Annealing temperatures are generally very low, around **36 °C** - This allows very short primers to anneal to template DNA
2. More thermal cycles are used, typically **45** - This compensates for the inefficiency which results from using such short primers.

RAPD

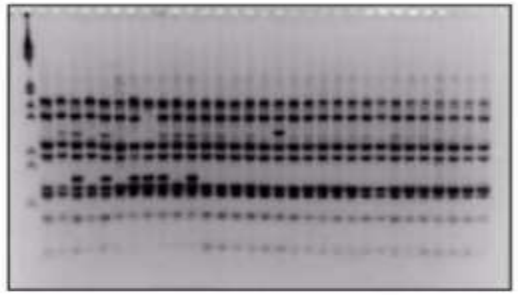
Procedure

1. The DNA of a selected species is isolated.
2. An excess of selected decaoligonucleotide added.
3. This mixture is kept in a PCR equipment and is subjected to repeated cycles of DNA denaturation-renaturation-DNA replication.
4. During this process, the decaoligonucleotide will pair with the homologous sequence present at different locations in the DNA.
5. DNA replication extend the decaoligonucleotide and copy the sequence continuous with the sequence with which the selected oligonucleotide has paired.

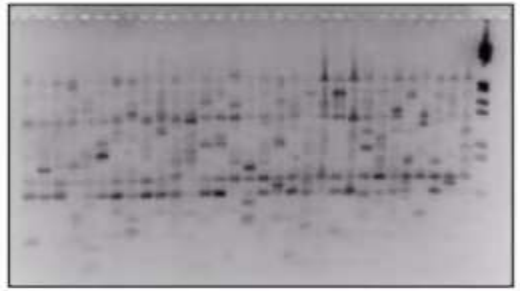
6. The repeated cycles of denaturation - renaturation-DNA replication will amplify this sequence of DNA.
7. Amplification will takes place only of those regions of the genome that has the sequence complementary to the decaoligonucleotide at their both ends.
8. After several cycles of amplification the DNA is subjected to gel electrophoresis.
9. The amplified DNA will form a distinct band. it is detected by ethidium bromide staining and visible fluorescence's under U.V. light

Interpreting RAPD Banding Pattern

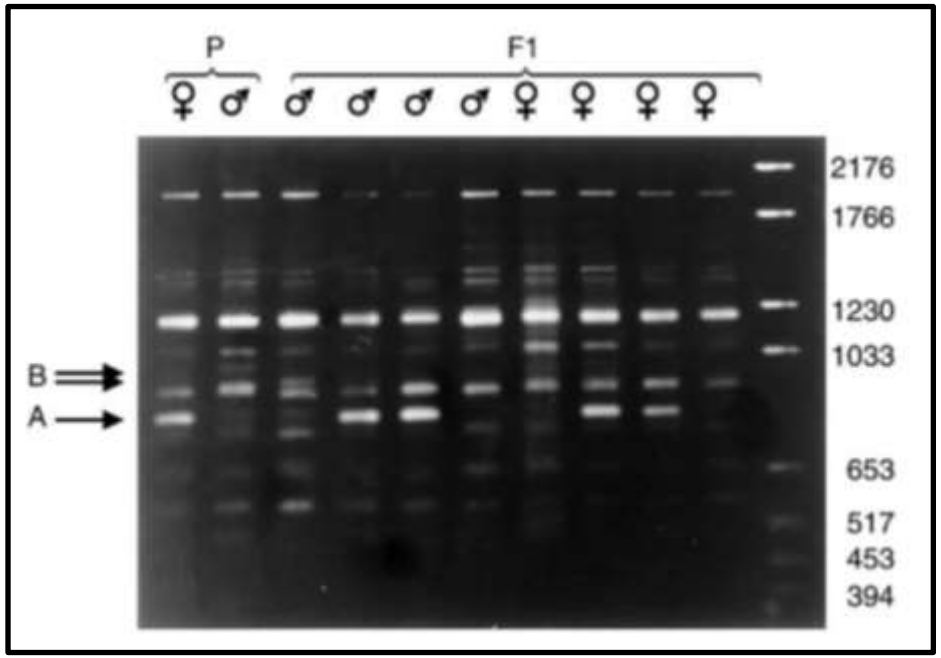
- Each gel is analysed by scoring the present (1) or absent (0) polymorphic bands in individual lanes. The scoring can be done based on the banding profiles which is clear and transparent (Fig.A) otherwise the scoring is very difficult (Fig.B).



A



B



RAPD-PCR banding patterns obtained from individual *Trioxys pallidus* male wasps (lanes 2–5) and individual *Diglyphus begini* (lanes 6–8) provide clear species differences.
Size standards are in lane 1 for reference

The principle of RAPDs is statistically based!

Given any particular five-base sequence, such as ACCGA, how often will this exact sequence appear in any random length of DNA?
Since there are four different bases to choose from, one in every 4^5 (or 4×4×4×4×4=1,024) stretches of five bases will—on average—be the chosen sequence.
Any arbitrarily chosen 11-base sequence will be found once in approximately every 4 million bases.
This is approximately the amount of DNA in a bacterial cell.

In other words, any chosen 11-base sequence is expected to occur by chance once only in the entire bacterial genome.

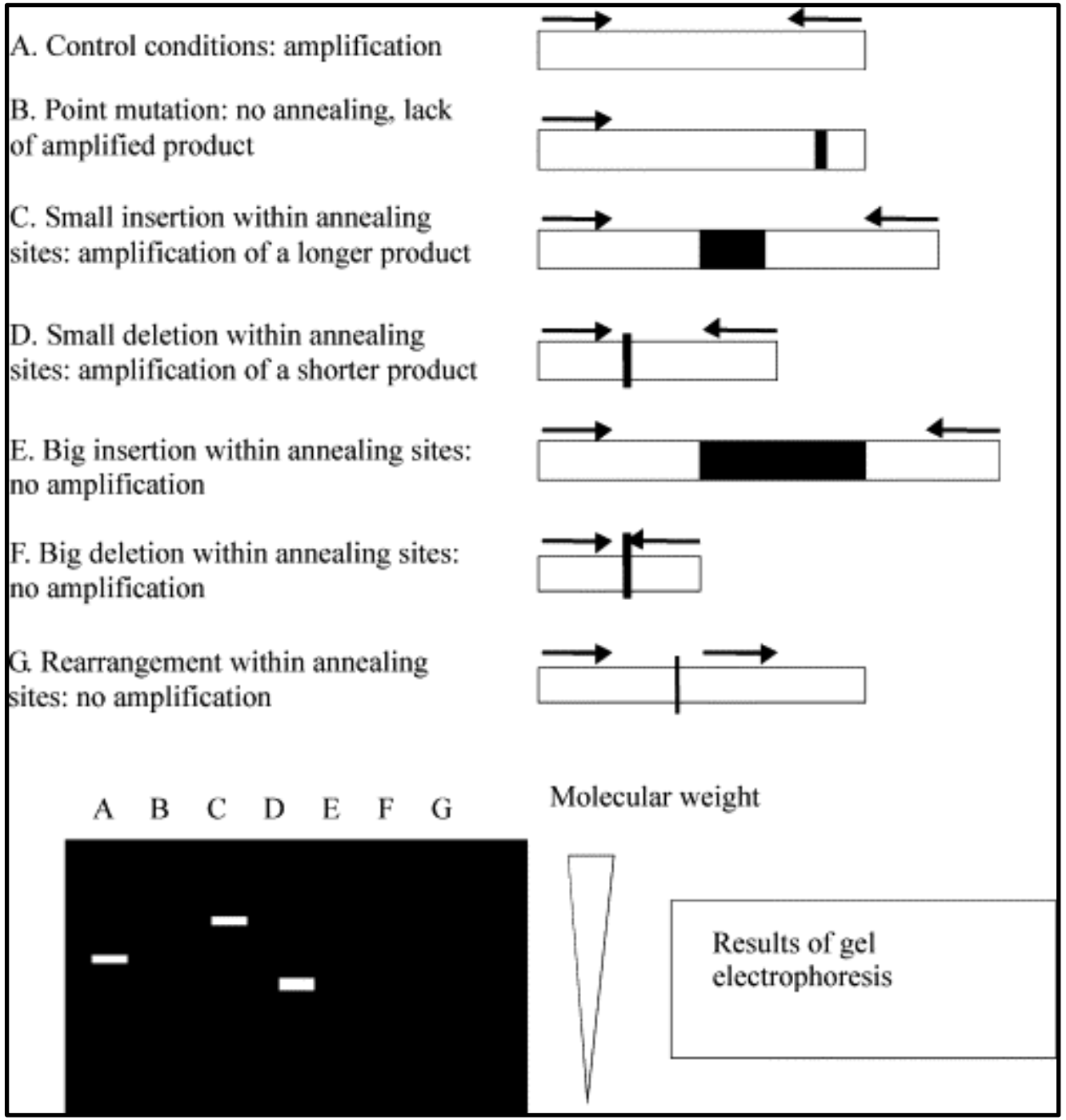
For higher organisms, with much more DNA per cell, a longer sequence would be needed for uniqueness.

Disadvantages

1) Lack of reproducibility

2) RAPD markers are dominant markers.

3)PCR results are very sensitive to amplification conditions and consequently variable between laboratories and even between assays



Possible outcomes of PCR amplification from a single randomly chosen oligonucleotide in **RAPD** technique.

Results of gel electrophoresis evidence appearance or disappearance of bands in the profile according to different types of mutation in the template DNA.

Limitations of RAPD

- RAPDs have low reproducibility; therefore, highly standardized laboratory protocols are required because of their sensitivity to reaction conditions.
- The quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions greatly influence the outcome. So, purified and high molecular weight DNA is required for RAPD analyses.
- Polymorphism by RAPD technique is detected as the presence or absence of a band of a certain molecular weight, with no information on heterozygosity besides being dominantly inherited (Brumlop and Finckh, 2011).
- Nearly all RAPD markers are dominant; hence, it is not possible to differentiate whether a DNA segment is amplified from a locus that is heterozygous or homozygous.

- Codominant RAPD markers are observed as different-sized DNA segments amplified from the same locus and are rarely detected.

- Low reproducibility makes RAPDs unsuitable markers for transference or comparison of results in similar species and subjects.

- RAPD markers are not locus-specific, band profiles cannot be interpreted in terms of loci and alleles (dominance of markers), and similar sized fragments may not be homologous.

- RAPD results may be difficult to interpret, as mismatches between the primer and the template can lead to total absence or merely decreased amount of the PCR product.

Limitation

- Nearly all RAPD markers are dominant, i.e. it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies).
- PCR is an enzymatic reaction, therefore the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome.
- Mismatches between the primer and the template may result in the total absence of PCR product as well as in a merely decreased amount of the product. Thus, the RAPD results can be difficult to interpret.

Application

- Evaluate genetic relationships analysis among native breeds as well between native and exotic breeds of livestock.
- Species differentiation - RAPD markers could differentiate chicken, quail, turkey and other avian species/strains.
- Association with production performance in livestock breeds.
- genetic diversity/polymorphism
- germplasm characterization
- genetic structure of populations & genome mapping
- population and evolutionary genetics
- animal-plant-microbe interactions

What is RFLP?

RFLP or restriction fragment length polymorphism is a method which exploits variation in homologous DNA.

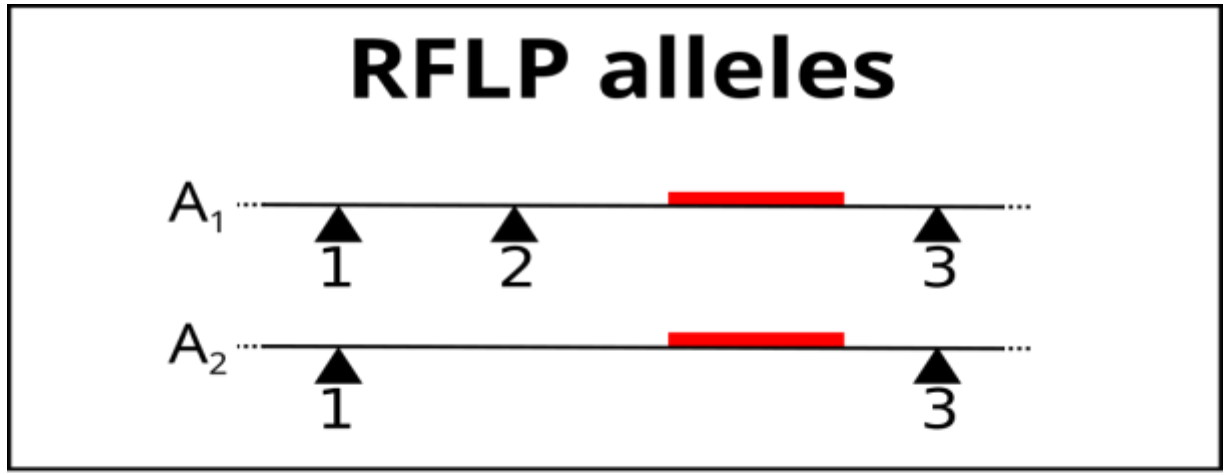
It is also a well-known method for its discrimination power.

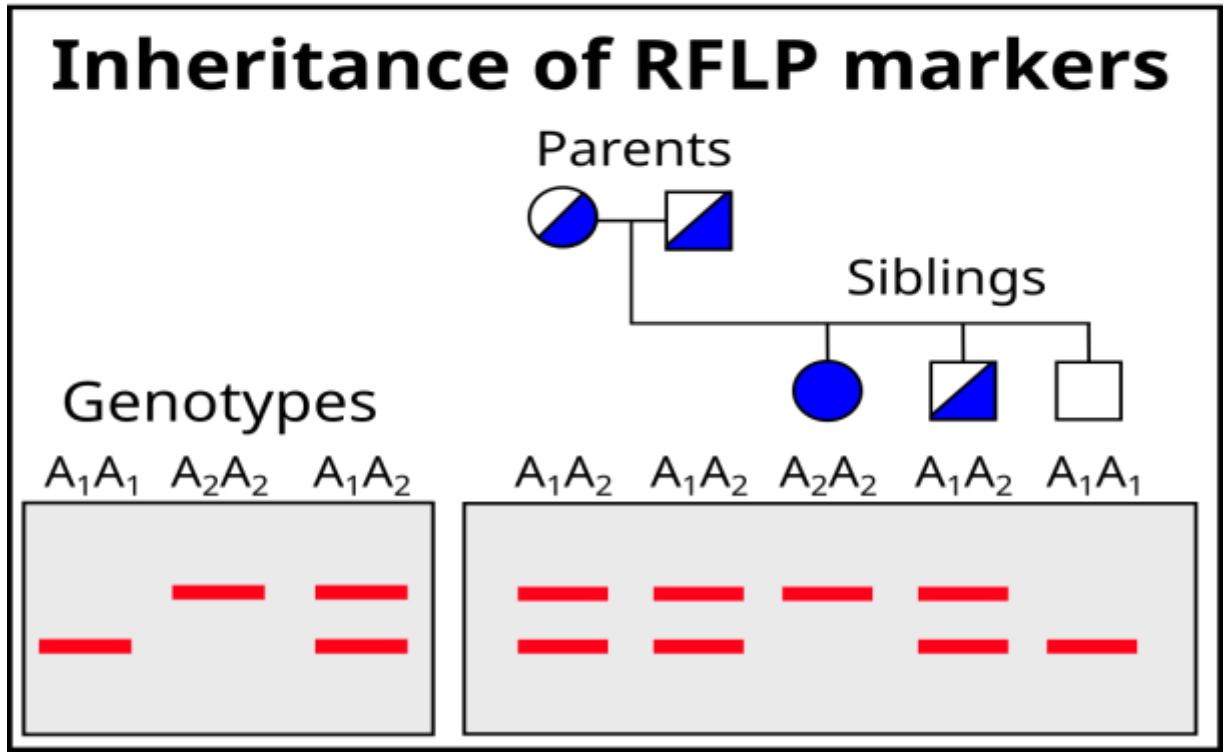
RFLP is a non-PCR-based method, it uses restriction enzymes for the generation of fragments of the genome.

RFLP detects the variations of lengths of restriction fragments among individuals.

The three steps involved in RFLP are as follows:

1. Restriction digestion of DNA
2. Gel electrophoresis
3. Southern blotting by specific probes and detection


RFLP alleles
A1
1
2
3
A2
1
3


Inheritance of RFLP markers
Parents
Siblings
Genotypes
A1A1 A2A2 A1A2
A1A2 A1A2 A2A2 A1A2 A1A1

RFLP

RFLP

What is polymorphism?

• RFLPs have polymorphisms (length differences in homologous fragments between different DNA) which is caused by changes in the primary sequence of DNA.

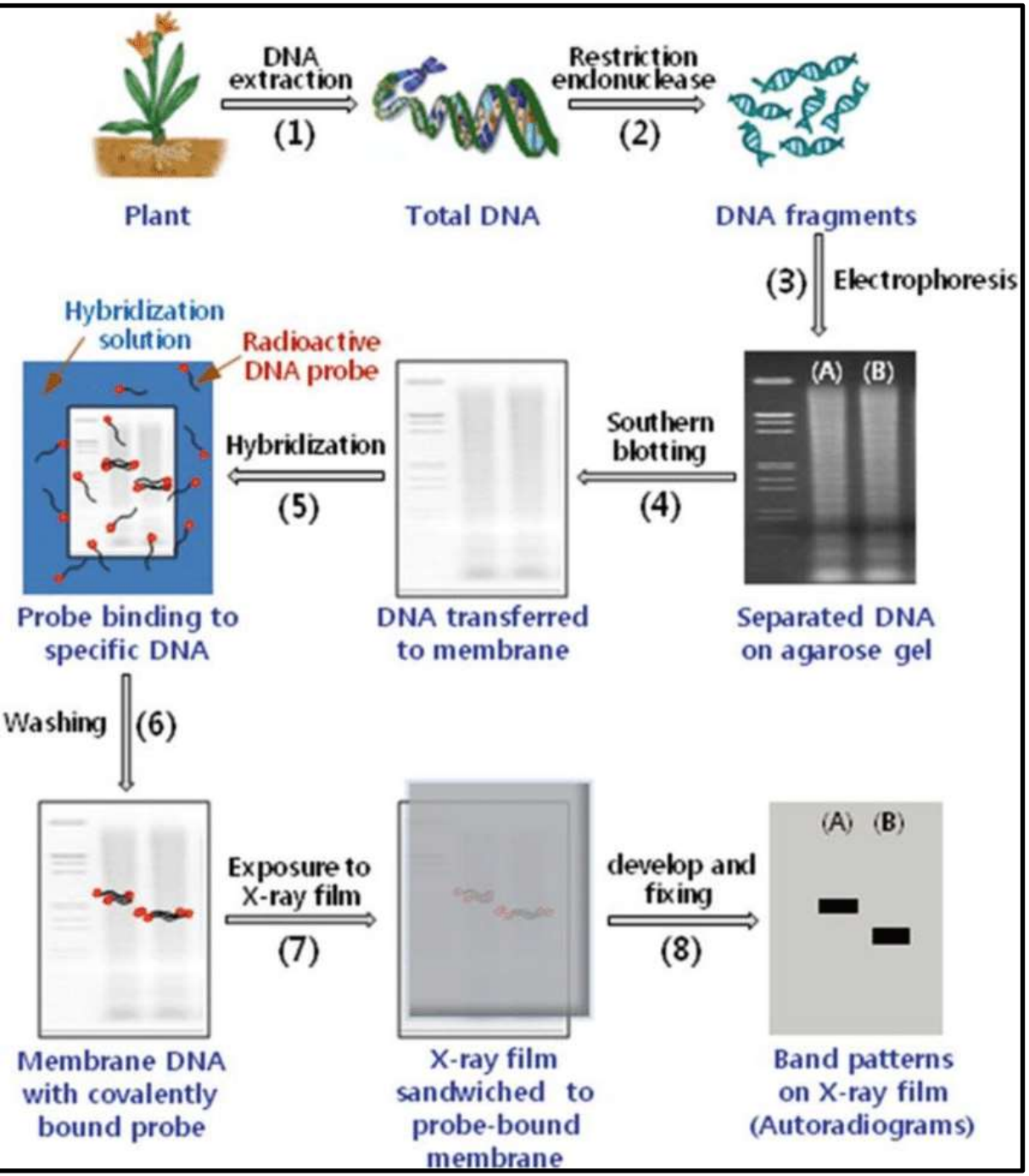
• Polymorphisms can result from –

1. Point mutation resulting in the loss or gain of a restriction enzyme cut site.
2. An insertion or deletion of DNA between two restriction enzyme cut sites
3. A deletion which overlaps a restriction enzyme site
4. A DNA rearrangement where one end of the rearranged segment resides between two restriction enzymes site.

The presence of a particular cut, or uncut, fragment is detected by using a **DNA-probe**, which is a sequence of DNA that is complementary and therefore binds to the respective cut, or uncut, sequence type.

Addition of a marker to the probe, such as a chemiluminescent tag, will mean that the relative position of the fragments on a gel can be detected when the gel is transferred to a membrane and then exposing the membrane to an X-ray film (Southern blotting).

These polymorphisms are called restriction fragment length polymorphisms (RFLPs) and have provided many thousands of new polymorphic markers, enabling major advances in gene mapping in recent years.

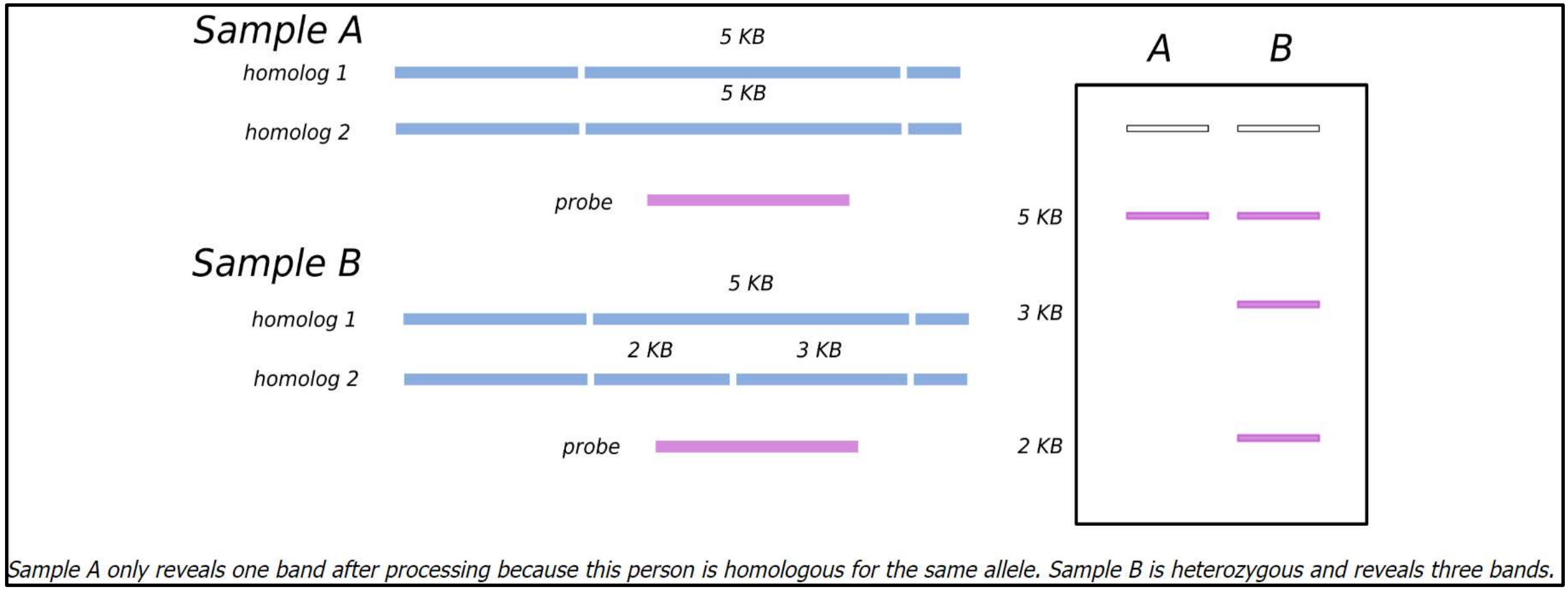


How RFLP works?

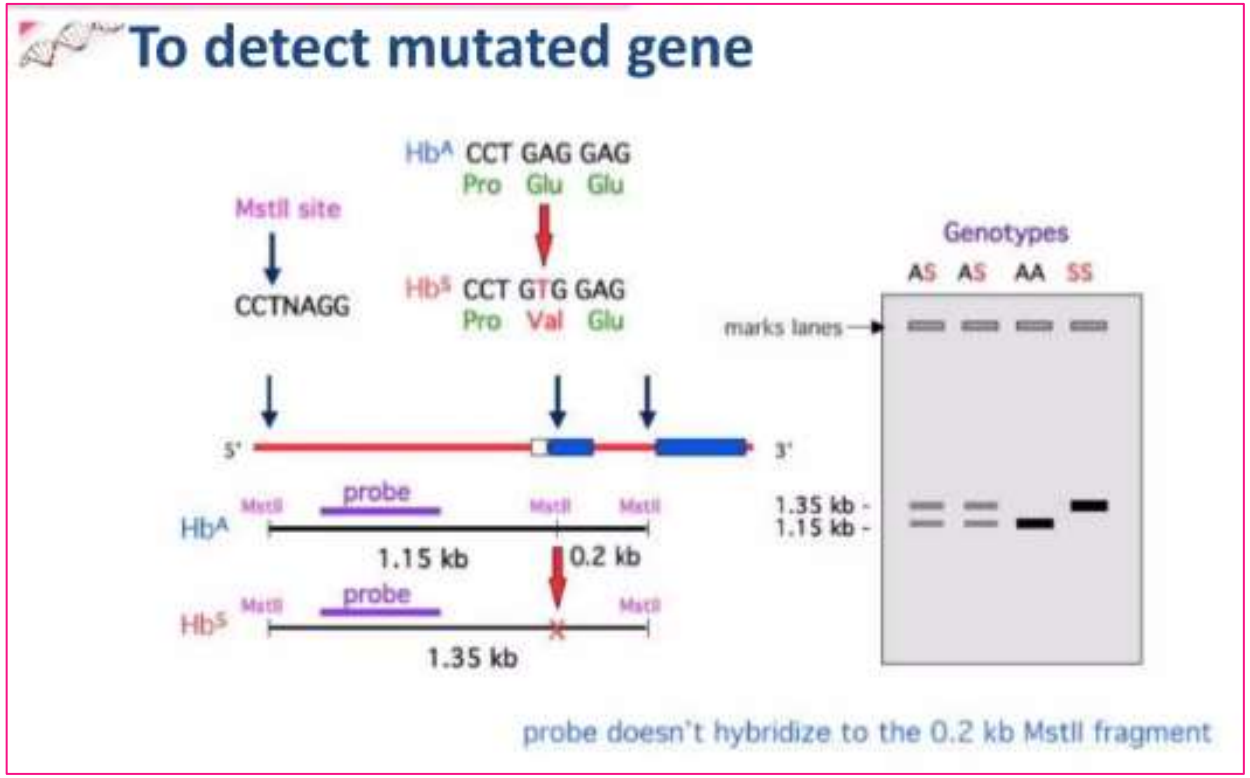
A technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA.

- If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme.

- The similarity of the patterns generated can be used to differentiate species (and even strains) from one another.

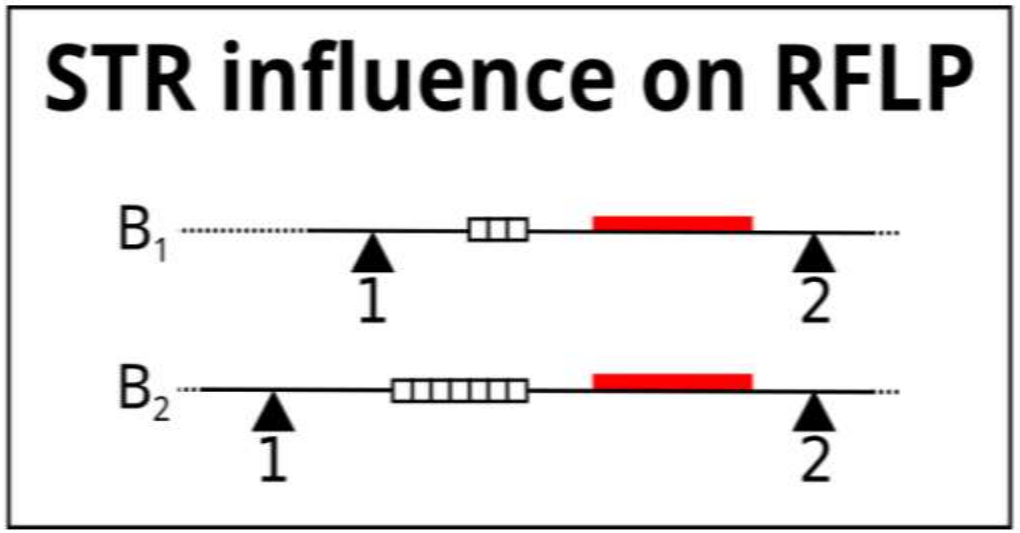


Sample A only reveals one band after processing because this person is homologous for the same allele. Sample B is heterozygous and reveals three bands.



https://www.bio.davidson.edu/genomics/method/RFLP.html

RFLPs represent inheritable markers and can reveal relationships between different individuals. A pedigree can illustrate the relationship of the inherited alleles. The technique can be more informative if using multiple probes simultaneously for different loci or to use **multi-locus probes** that hybridize to multiple locations.



RFLPs may arise from differences in the STR/VNTR repeats between restriction sites. Credit: Jeremy Seto (CC0)

While RFLPs can arise from SNPs, they may also be caused by the expansion or contraction of repeated elements between restriction sites. These repeated elements of DNA are referred to as Variable Number Tandem Repeats (VNTR) and illustrate polymorphisms that normally occur in non-coding regions of the genome.

Advantages

- They are codominant (both alleles in heterozygous sample will be detected) and unaffected by the environment
- Any source DNA can be used for the analysis; no prior informations are needed
- Many markers can be mapped in a population that is not stressed by the effects of phenotypic mutations.
- They don't require high level of skill.
- They have high reliability.
- They have medium polymorphism, allelic information, and robustness

Special advantage (in plant molecular breeding)

- The integration of RFLP techniques into plant breeding promises to: (1) Expedite the movement of desirable genes among varieties,
- (2) Allow the transfer of novel genes from related wild species,
- (3) Make possible the analysis of complex polygenic characters as ensembles of single Mendelian factors,
- (4) Establish genetic relationships between sexually incompatible crop plants.
- (5) In the future, high density RFLP maps may also make it possible to clone genes whose products are unknown, such as genes for disease resistance or stress tolerance.

Applications of RFLP

 can be used in many different settings to accomplish different objectives:

1- In paternity cases or criminal cases to determine the source of a DNA sample. (i.e. it has forensic applications).
2- Determining the disease status of an individual. (e.g. it can be used in the detection of mutations particularly known muations)
3- To measure recombination rates which can lead to a genetic map with the distance between RFLP loci measured in centiMorgans.

Disadvantages
- Only relatively low input can be achieved (Samples per day in the research lab is low (20 sample)).
- Number of samples is moderately low (<300-400)
- Labor intensive.
- Developmental costs ($/sample) are medium (100)
- Running costs ($/sample) are high
- Quantities of high quality DNA are required
- Gel based and thus not easily automable
- Requires relatively large amounts of high quality DNA

Similarities Between RAPD and RFLP

•RAPD and RFLP are two techniques in molecular biology to detect genetic markers, which exploit variation in homologous DNA.

•They detect DNA polymorphism required for genetic mapping, genome fingerprinting, and for the investigation of genetic relatedness.

•Therefore, they help to distinguish individuals, species or populations.

•Moreover, both methods use the total amount of DNA in the genome for the analysis.

Differences

RAPD	RFLP
A PCR-based technique for identifying genetic variation	A molecular method of genetic analysis, which allows individuals to be identified based on unique patterns of restriction enzyme cutting in specific regions of DNA
A PCR-based method	A non-PCR-based method
Involves in the amplification of genetic markers in a genome by using random primers	Involves in the restriction digestion of the genomic DNA
Requires a small quantity of DNA (10-50 ng) for the analysis	Requires comparatively a large quantity of DNA (2-10 µg)
Uses random primers universal for any species	Uses different species-specific probes
Has fewer steps and hence it is a rapid process	Has more steps and therefore, it is a slow process
Less reliable	More reliable
Can detect 1-10 loci	Can detect 1-3 loci
Unable to detect allelic variants	Can detect allelic variants
Important for gene mapping, population genetics, molecular evolutionary genetics, animal and plant breeding, etc.	Important in genotyping, forensics, paternity testing, the detection of patterns in hereditary diseases, and in the detection of disease carriers

Department of BSBE
Indian Institute Of Technology Guwahati

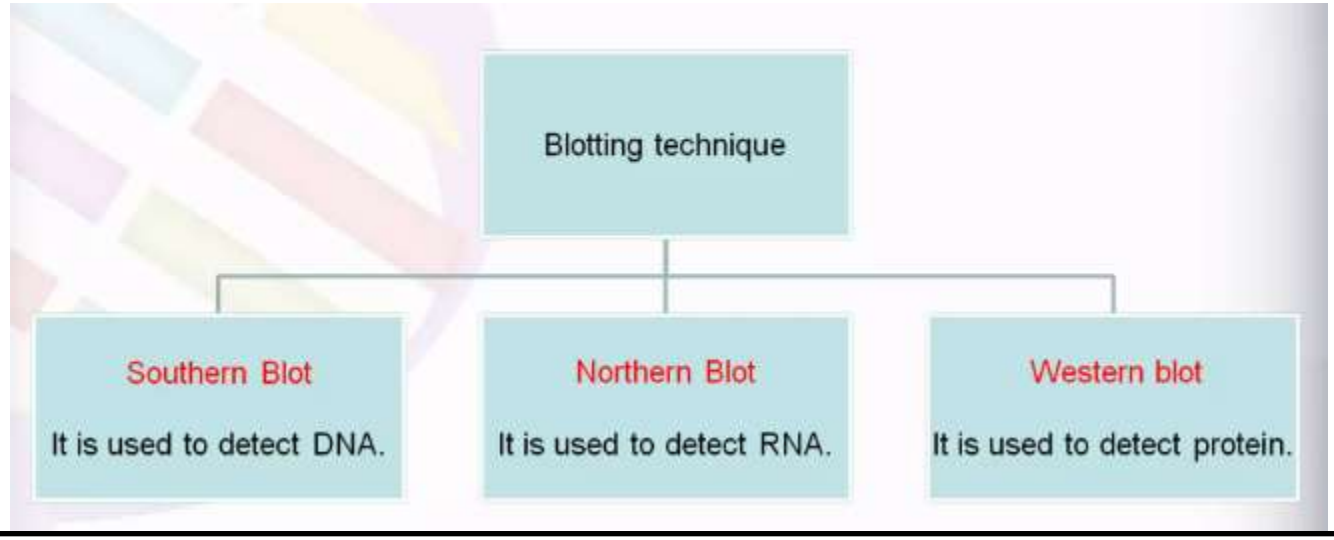
Techniques in Genetic Engineering

Blotting- DNA, RNA, protein

Dr. Sanjukta Patra

BT 207

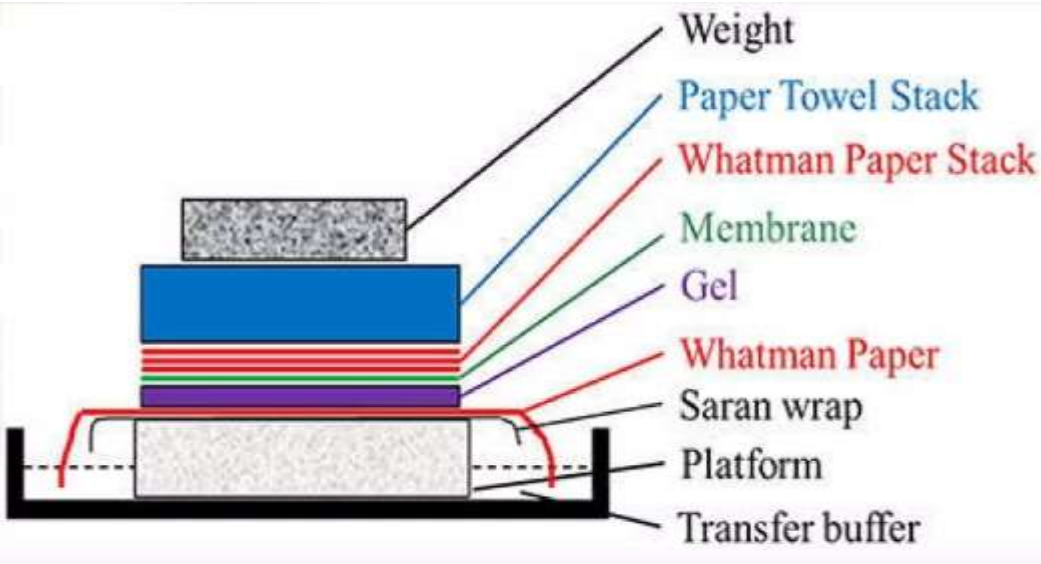
Jan - May 2023

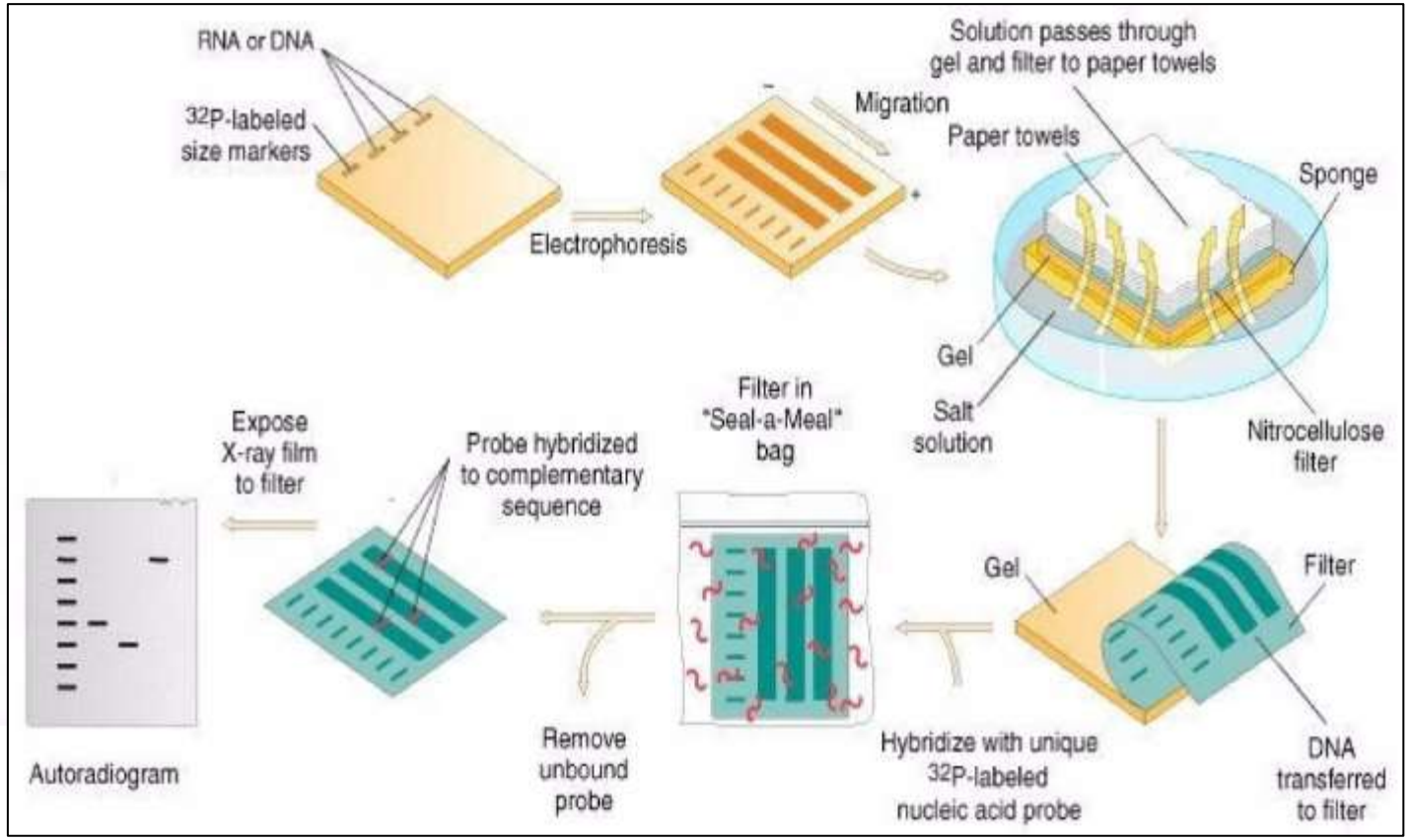


A **blot**, in molecular biology and genetics, is a method of transferring proteins, DNA or RNA, onto a carrier. The term **"blotting"** refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Technique for transferring DNA, RNA and Proteins onto a carrier so they can be separated, and often follows the use of a gel electrophoresis.

	Southern Blot	Northern Blot	Western Blot
Target molecule	DNA	RNA	Protein
Sample preparation	DNA extraction enzymatic digestion	RNA isolation	Protein extraction
Separation	Electrophoresis	Electrophoresis	Electrophoresis
Membrane material	Nylon	Nylon	Nitrocellulose or PVDF
Probe	Nucleic acid probe with sequence homologous to target	RNA, DNA, or oligodeoxynucleotide	Primary antibody
Probe label	Radiolabel, enzyme	Radiolabel, enzyme	Enzyme
Detection methods	X-ray film, chemiluminescence	X-ray film, chemiluminescence	Film, cooled CCD, camera, LED, or infrared imaging system

Southern blotting

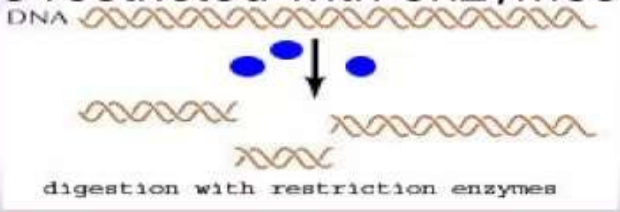




1.Extract and purify DNA from cells

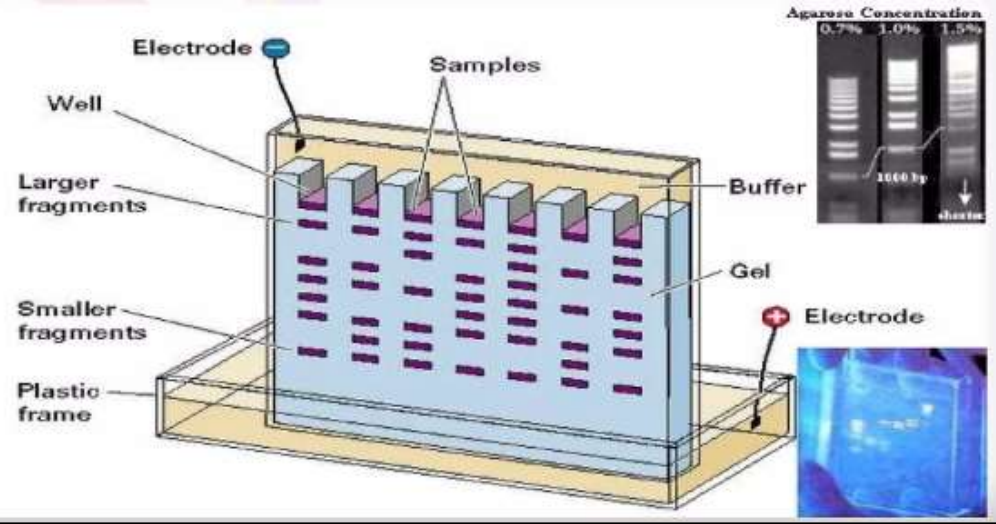
- Isolate the DNA in question from the rest of the cellular material in the nucleus.
- Incubate specimen with detergent to promote cell lysis.
- Lysis frees cellular proteins and DNA.
- Proteins are enzymatically degraded by incubation with proteinase.
- Organic or non-inorganic extraction removes proteins.
- DNA is purified from solution by alcohol precipitation.
- Visible DNA fibers are removed and suspended in buffer.

2.DNA is restricted with enzymes.



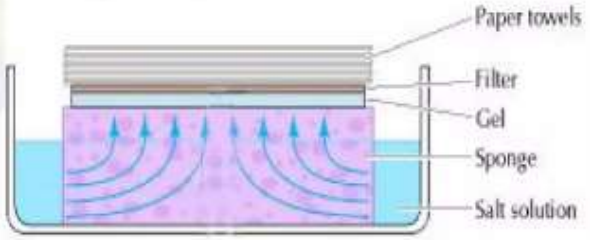
3.Separated by electrophorosis.

- The complex mixture of fragments is subjected to gel electrophoresis to separate the fragments according to size.



4. Denature DNA.

- The restriction fragments present in the gel are denatured with alkali.
- This causes the double stranded to become single-stranded.
- DNA is then neutralized with NaCl to prevent re-hybridization before adding the probe.

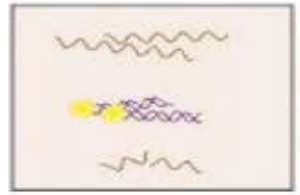


5.Transfer to nitrocellulose paper.

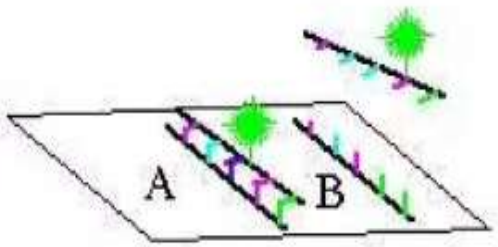
- Transfer the DNA from the gel to a solid support, ie, blotting.
- The blot is made permanent by:
  - Drying at ~80°C
  - Exposing to UV irradiation

6. Add labeled probe for hybridization.

- The filter is incubated under hybridization conditions with a specific radiolabeled DNA probe



- The probe hybridizes to the complementary DNA restriction fragment.

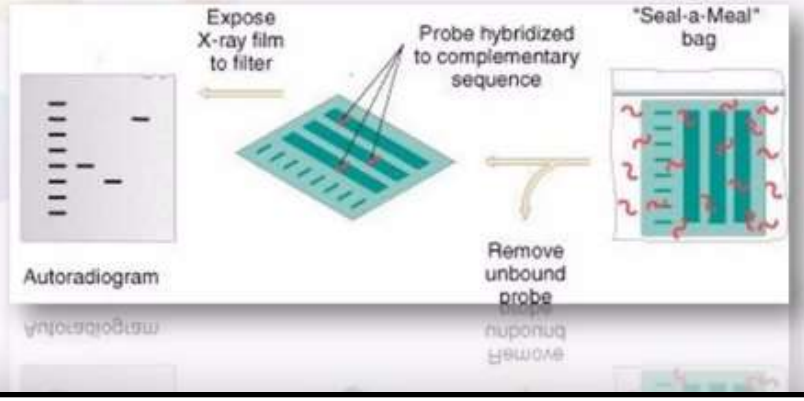


7. Wash off unbound probe.

- Blot is incubated with wash buffers containing NaCl and detergent to wash away excess probe and reduce background.

8. Autoradiograph.

- If the probe is radioactive, the particles emits when expose to X-ray film.
- There will be dark spots on the film wherever the probe bound.



Applications

- Southern blotting transfer may be used for homology-based cloning on the basis of amino acid sequence of the protein product of the target gene.

- Oligonucleotides are designed so that they are complementary to the target sequence. The oligonucleotides are chemically synthesized, radiolabeled, and used to screen a DNA library, or other collections of cloned DNA fragments.

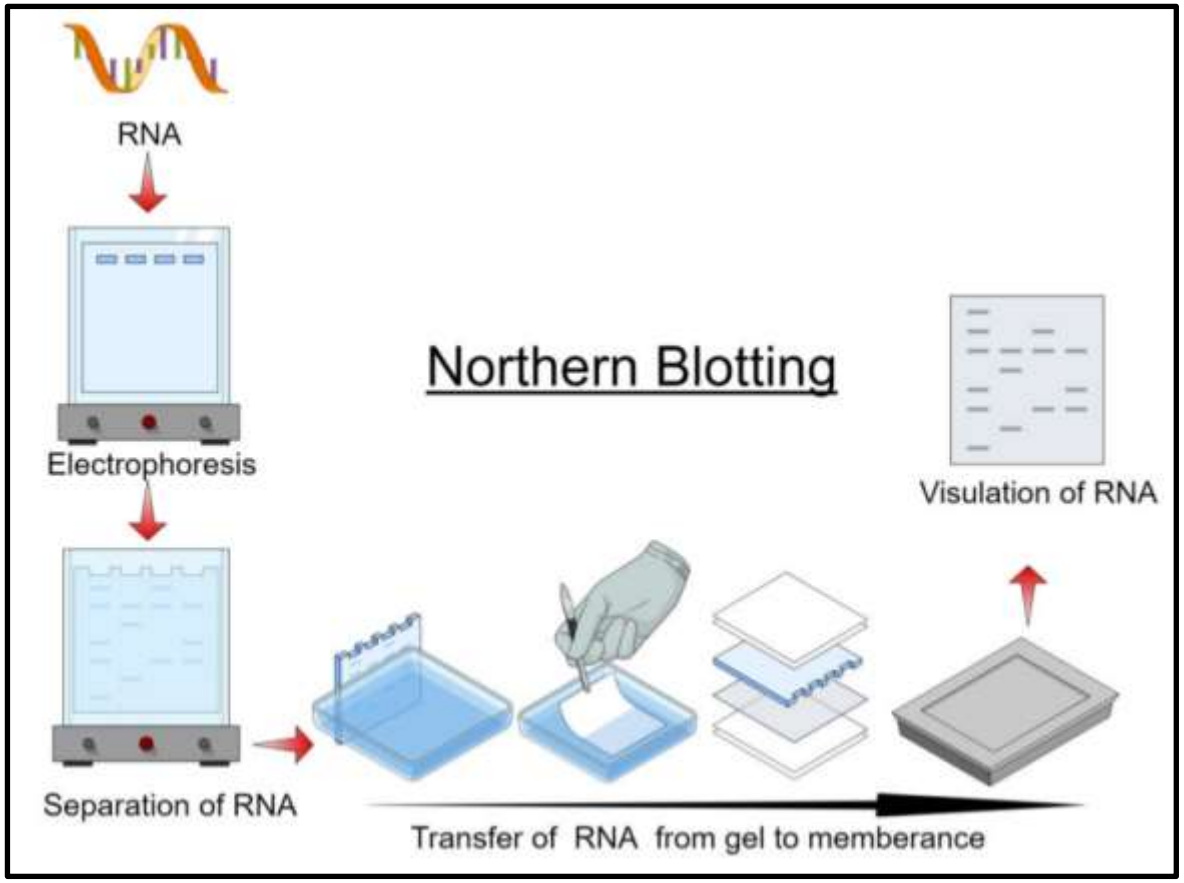
- Sequences that hybridize with the hybridization probe are further analyzed, for example, to obtain the full length sequence of the targeted gene.

- Southern blotting can also be used to identify methylated sites in particular genes. Particularly useful are the restriction nucleases *MspI* and *HpaII*, both of which recognize and cleave within the same sequence. However, *HpaII* requires that a C within that site be methylated, whereas *MspI* cleaves only DNA unmethylated at that site. Therefore, any methylated sites within a sequence analyzed with a particular probe will be cleaved by the former, but not the latter, enzyme.

Northern blotting

The northern blot, or RNA blot, is a technique used in molecular biology research to study gene expression by detection of RNA (or isolated mRNA) in a sample. The northern blot technique was developed in 1977 by **James Alwine, David Kemp, and George Stark** at Stanford University. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot, named for biologist Edwin Southern.

- The RNA samples are most commonly separated on agarose gels containing formaldehyde as a denaturing agent for the RNA to limit secondary structure.
- Probes for northern blotting are composed of nucleic acids with a complementary sequence to all or part of the RNA of interest, they can be DNA, RNA.
- The probes must be labelled either with radioactive isotopes (^{32}P) or with chemiluminescence in which alkaline phosphatase or horseradish peroxidase (HRP) break down chemiluminescent substrates producing a detectable emission of light.



Application of Northern Blotting

- Observe a particular gene's expression, pattern between tissues, organs, development stages, environment stress levels etc.
- Used to show overexpression of oncogenes and down regulation of tumour suppressor gene's in cancerous cells.
- Detecting a specific mRNA in sample used for screening recombinant which are successfully transformed with transfer.
- Also used for studying mRNA Splicing.

Advantages

1. Northern blots are particularly useful to determine conditions under which specific genes are expressed.

2. Only mRNA from cell types that are synthesizing protein will hybridize to the probe.

3. It is useful in detection of mRNA transcript size. Specifity is relatively high.

4. RNA splicing is visible because alternatively spliced transcripts can be detected.

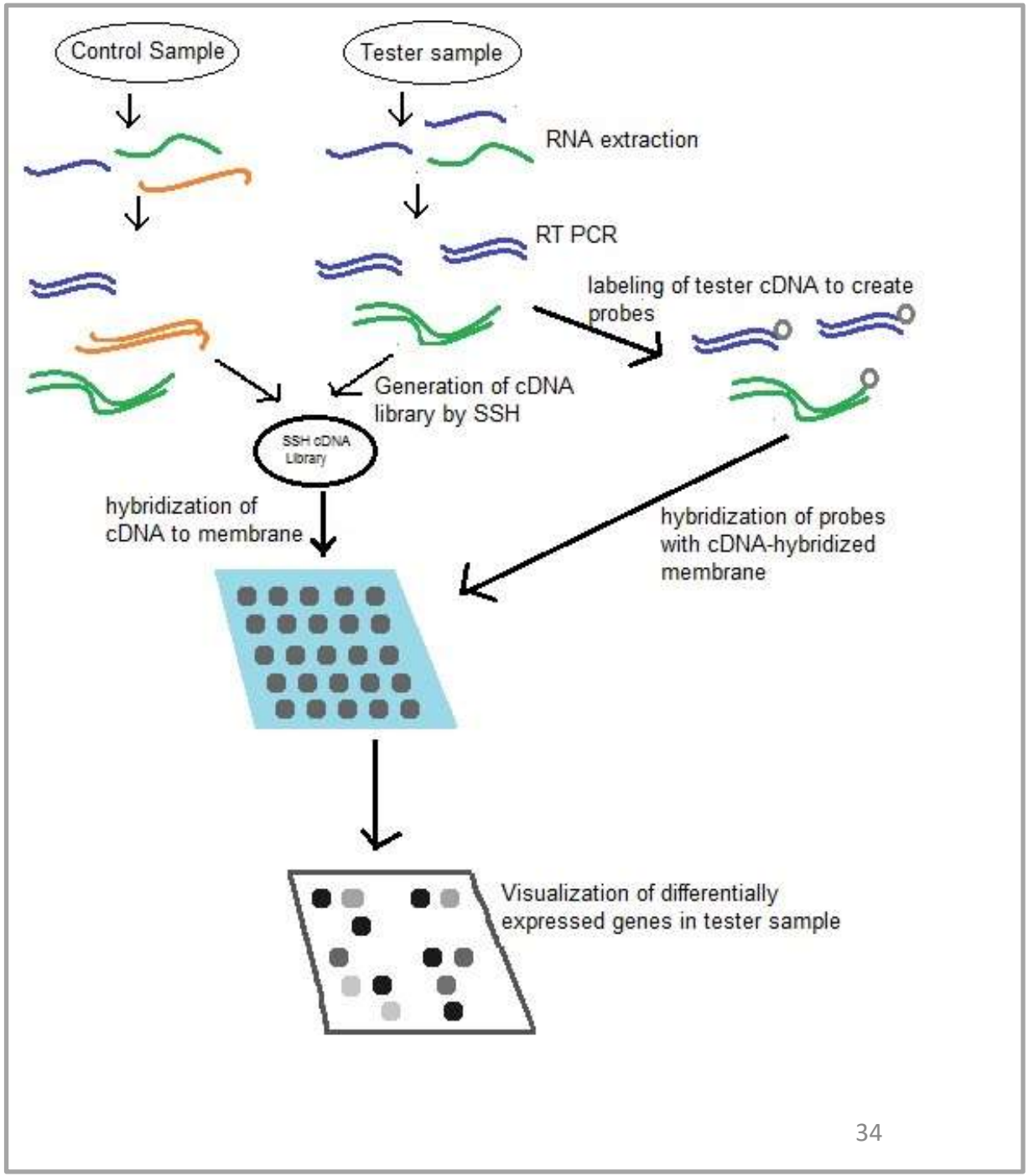
5. Blots can be stored for several years and reprobed if necessary.

Disadvantages

1. Risk of mRNA degradation during electrophoresis: quality and quantification of expression are negatively affected.
2. High doses of radioactivity and formaldehyde are a risk for workers and the environment
3. The sensitivity of northern blotting is relatively low in comparison with that of RT-PCR.
4. Detection with multiple probes is difficult and also time consuming procedure
5. Use of ethidium bromide, DEPC and UV light needs special training and attention.

Reverse Northern Blotting

- The reverse northern blot is a method by which gene expression patterns may be analyzed by comparing isolated RNA molecules from a tester sample to samples in a control cDNA library.
- It is a variant of the northern blot in which the nucleic acid immobilized on a membrane is a collection of isolated DNA fragments rather than RNA, and the probe is RNA extracted from a tissue and radioactively labelled.

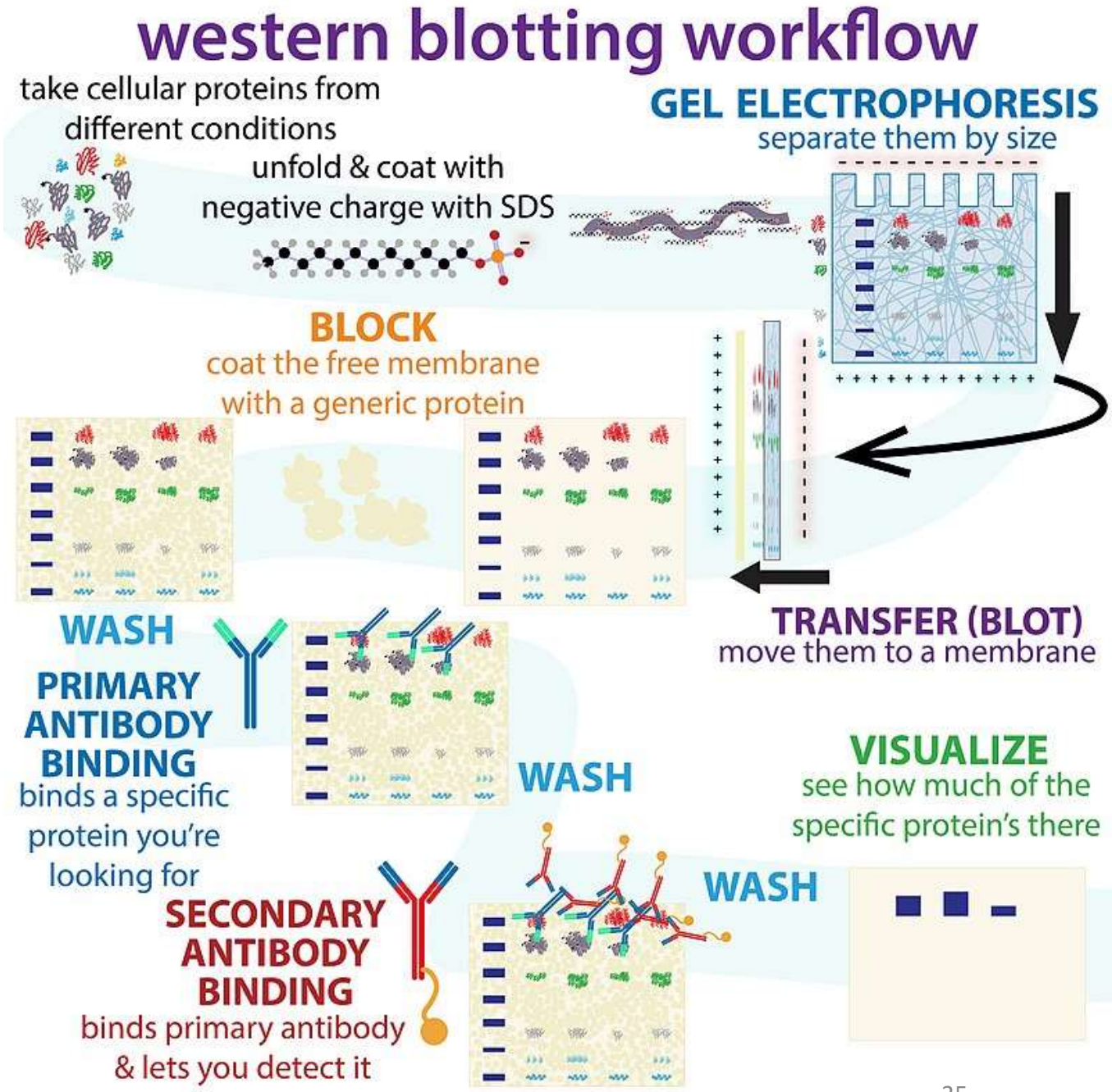


Western blotting

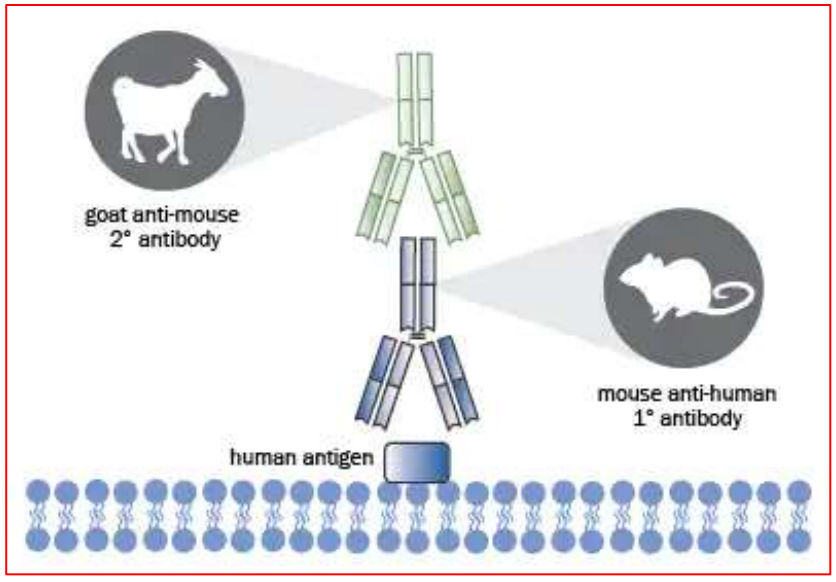
Protein blotting is an analytical method that involves the immobilization of proteins on membranes before detection using monoclonal or polyclonal antibodies. There are different blotting protocols (dot blot, 2D blot); one of the most powerful is western blotting.

While ELISA being a non specific test, Western blotting is a more specific test for detection of HIV.
It can detect one protein in a mixture of proteins while giving information about the size of the protein and so is more specific.

Western blot test is referred to as the 'Gold Standard'



Choice of antibodies for immunoblots



Choose a Commercial Antibody

- Source
- Purity
- Immunogen
- Experimental conditions tested
- Supporting data

Specificity

- Controls
- Genetic verification
- Multiple-epitope approaches
- IP / MS

Selectivity

- Endogenous expression levels
- Antibody dilution and titration
- Blocking buffers

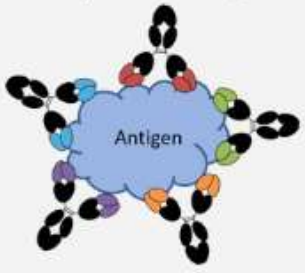
Reproducibility

- Experimental replication
- Biological reproducibility
- Reporting for publication

Polyclonal Antibody

- Cheap to produce
- Mixed population of antibodies
- May bind to different areas of the target molecule
- Tolerant of small changes in protein structure

Polyclonal antibody

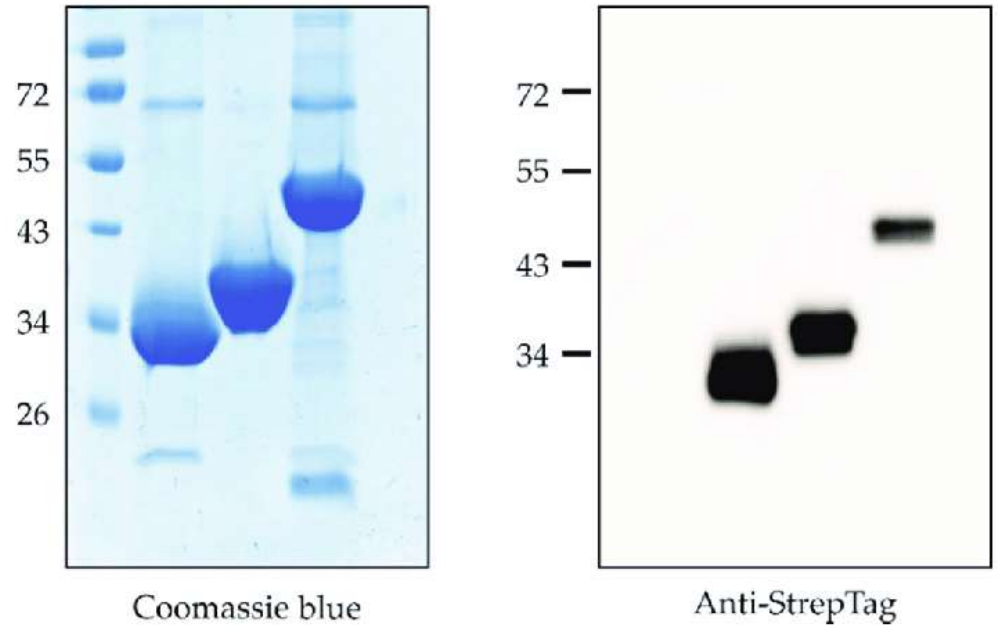


Monoclonal Antibody

- Expensive to produce
- Single antibody species
- Will only bind single specific site
- May recognise a particular protein form

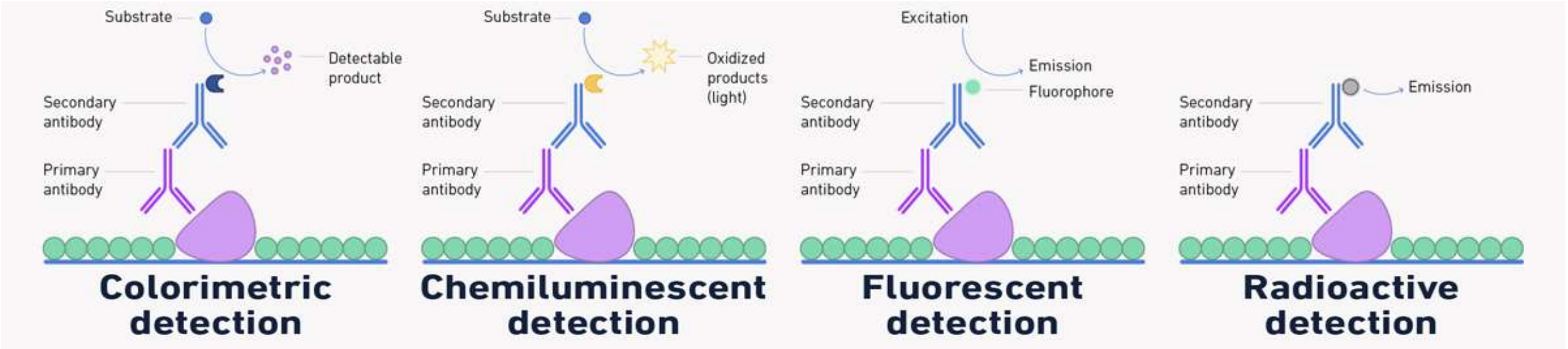
Monoclonal antibody

labclinics



SDS-PAGE and Western blot analyses of recombinant proteins. The recombinant proteins were separated by reducing SDS-PAGE and analyzed by Coomassie staining (left panel) or Western blot (right panel) using an anti-StrepTag specific monoclonal antibody.

Methods for detection of Western blots



Protein analysis platform	Advantages	Disadvantages
Western Blot	Separation of proteins according to molecular weight	Work-intensive, high amounts of protein lysate required, low- or medium-throughput
ELISA	Quantitative, very sensitive	High amounts of protein lysate required
IHC	Cellular localization of protein of interest	Semi-quantitative, sensitivity often not sufficient to detect phosphorylated proteins
Mass spectrometry-based technologies	*De novo* discovery platform, highly multiplex, protein isoforms can be distinguished, analysis of thousands of proteins, no protein binding reagent required	Complex sample preparation, poor analytical sensitivity compared to immunoassays, low-throughput
Forward Phase Protein Arrays	Many analytes can be measured in parallel in a single sample, quantitative	Two highly specific antibodies are needed for every assay, high amounts of protein lysate required
Reverse Phase Protein Arrays	Robust quantification, low amount consumption, high-throughput, highly sensitive, detection of phosphoproteins possible	One highly specific antibody is needed for every assay, special devices needed

Abbreviations: IHC: Immunohistochemistry, ELISA: Enzyme Linked Immunosorbent Assay.