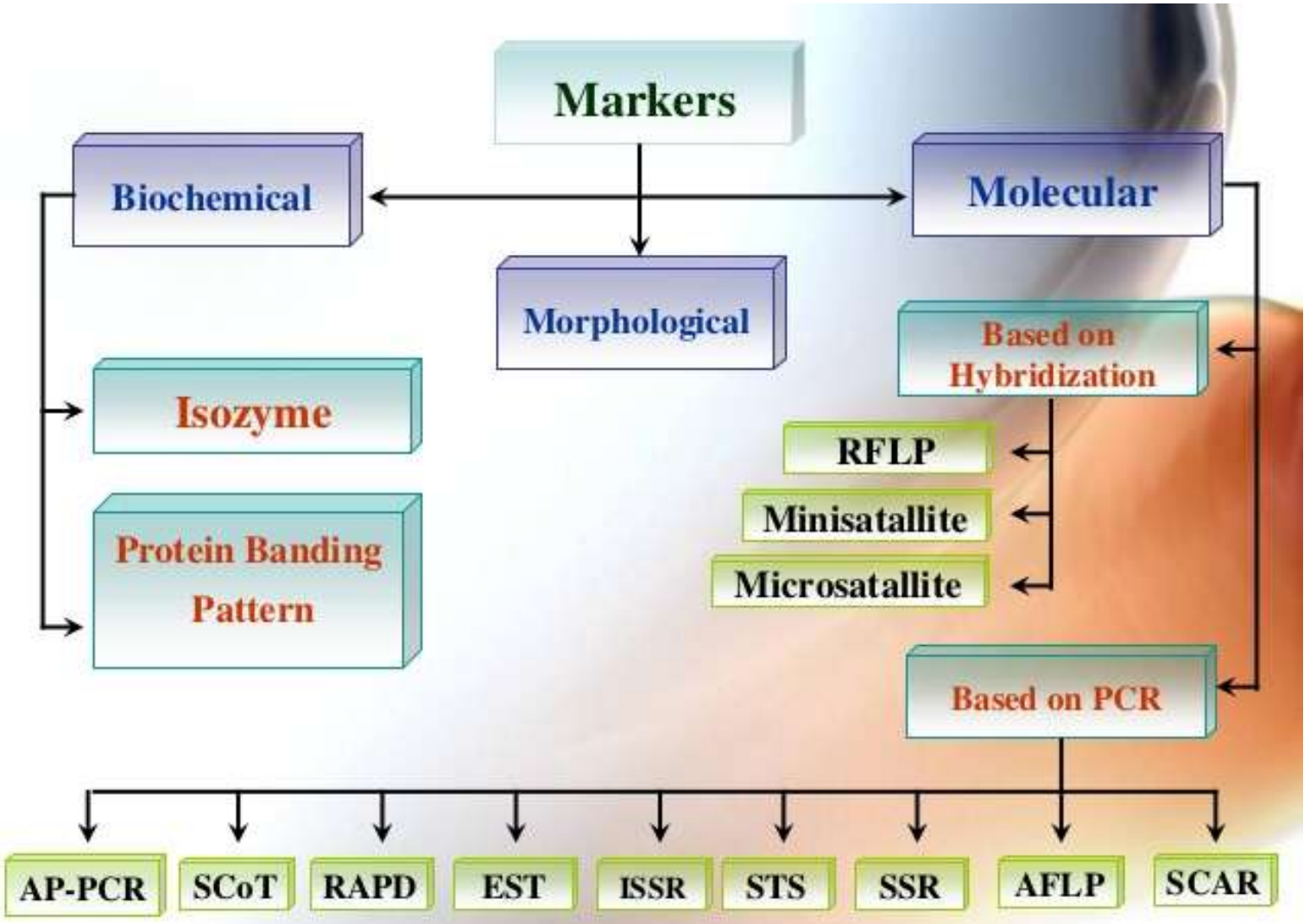
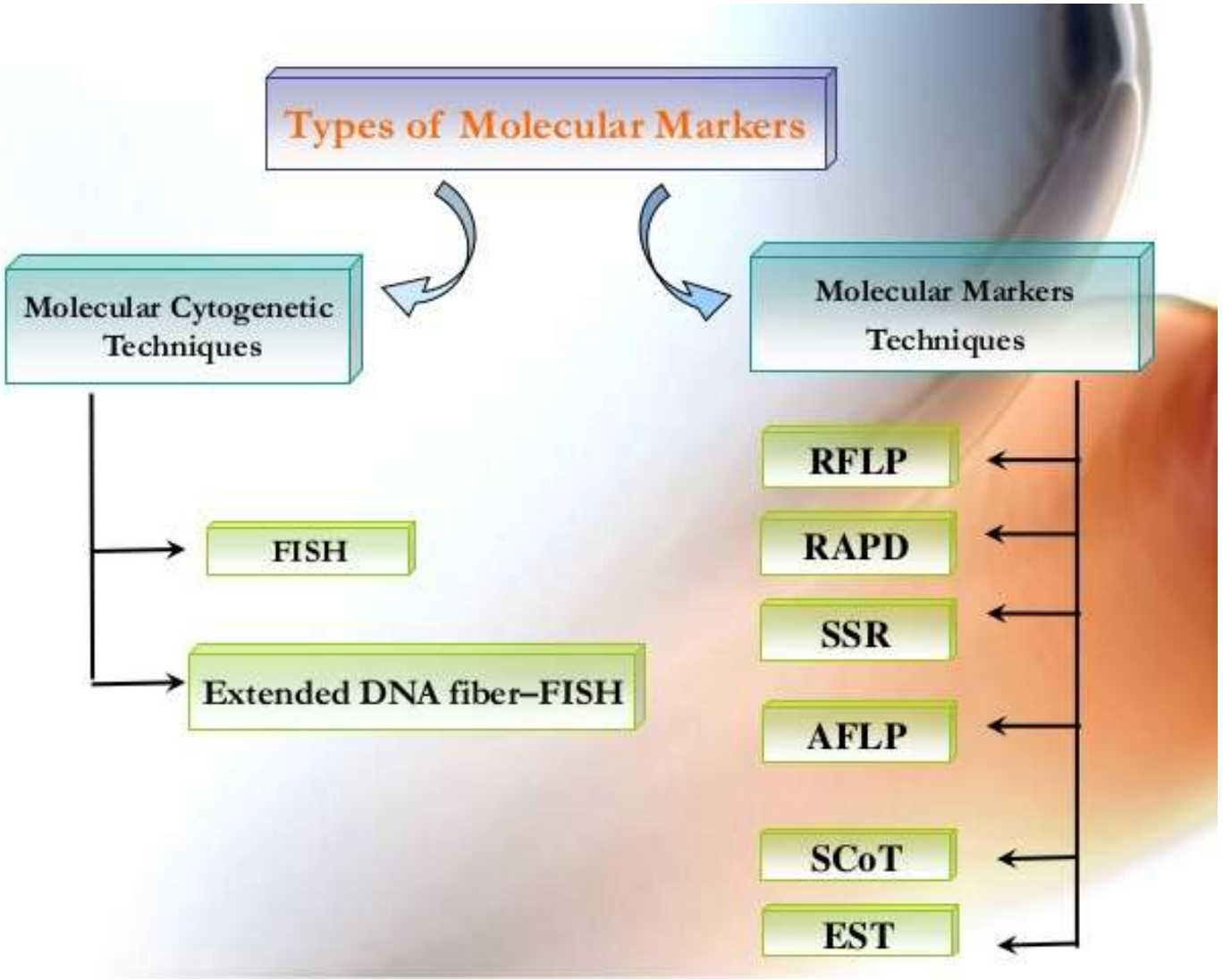
Presented by: Dr.Asma


Markers
Biochemical
Molecular
Morphological
Isozyme
Protein Banding Pattern
Based on Hybridization
RFLP
Minisatallite
Microsatallite
Based on PCR
AP-PCR
SCoT
RAPD
EST
ISSR
STS
SSR
AFLP
SCAR


Types of Molecular Markers
Molecular Cytogenetic Techniques
Molecular Markers Techniques
FISH
Extended DNA fiber–FISH
RFLP
RAPD
SSR
AFLP
SCoT
EST

1. Morphological markers

- These are related to shape, size, colour and surface of various plants parts.
- Such characters used for the varietal identification.

Advantages:

- Readily available.
- Usually require only simple equipment.
- Form the most direct measure of phenotype.

Disadvantages:

- Require expertise on crop or species.
- Subject to environmental influences.
- Limited in number.
- **Limited genomic coverage**

2.Protein (biochemical) markers

- Available since 1950's.
- Such markers are related to the variations in protein and amino acid banding pattern.
- Gel electrophoretic studies used for identification of biochemical markers.

e.g.- Peroxidase, Acid Phosphate, Esterase *etc.*

Advantages:

- ✓ Require simple equipment.
- ✓ A vigorous complement to the morphological assessment of variation.

Disadvantages:

- ✓ Subject to environmental influences.
- ✓ Limited in number.

Molecular Markers

Reflect heritable differences in homologous DNA sequences among individuals.

They may be due to:

- **Base pair changes.**
- **Rearrangements (translocation or inversion).**
- **Insertions or deletions.**
- **Variation in the number of tandem repeats.**

Advantages of Molecular Markers

- ✓ Ubiquitous.
- ✓ Stably inherited.
- ✓ Multiple alleles for each marker.
- ✓ Devoid of pleiotropic effects.
- ✓ Detectable in all tissues, at all ages.
- ✓ Long shelf life of the DNA samples.

Differences b/w conventional and molecular breeding

Particulars	Conventional breeding	Molecular breeding
Type of markers used	Morphological markers	Molecular or DNA markers
Laboratory required	Simple laboratory	Sophisticated laboratory
Effect of environment	Very high effect of environment on conventional markers	No effect of environment on identification of DNA markers
Accuracy of method	Medium to high	Very high
Time required to develop new variety	Very long time (10-15 years)	Very short time (3-5 years)
Cost involved	Low to medium	Very high
Health hazards	Only in mutation breeding	With technique involving radio active labeling
Effect of gene interaction	Very high	No effect
Mapping of QTL	Not possible	Possible
Screening at seedling stage for economic traits	Not possible	Possible

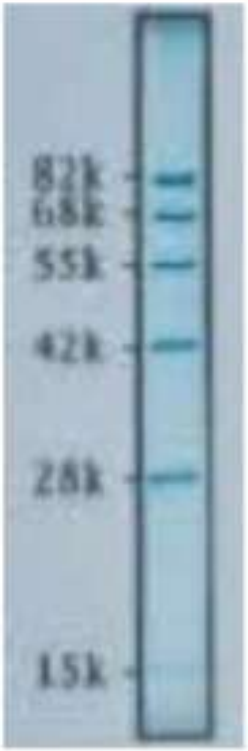
Southern, Northern and Western blotting

What is blotting?

- Blots are techniques for transferring DNA , RNA and proteins onto a carrier so they can be separated, and often follows the use of a gel electrophoresis. The Southern blot is used for transferring DNA, the Northern blot for RNA and the western blot for PROTEIN.

1. Southern Blotting

- This method involves seperation, transfer and hybridization.
- The Southern blotting is used to detect the presence of a particular piece of DNA in a mixture of sample.
- The DNA detected can be a single gene, or it can be part of larger piece of DNA such as viral genome.
- The key to this method is Hybridization.
- **HYBRIDIZATION:** Process of forming a double stranded DNA molecule between a single-stranded DNA probe and a single stranded target patient DNA.

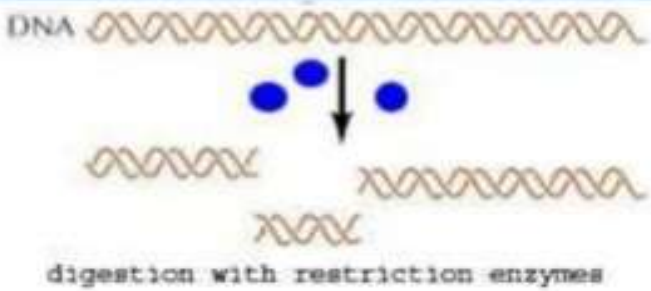


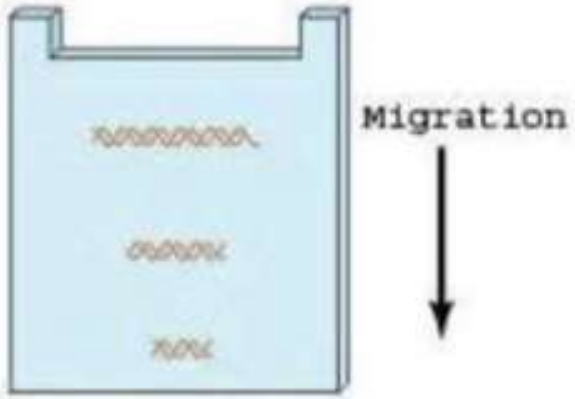
Principle

1. The mixture of molecule is separated.
2. The molecules are immobilized on a matrix transferred to a solid support (blot).
3. The labeled probe is added to the matrix to bind to the molecule.
4. Any unbound probes are then removed.
5. The place where the probe is connected corresponds to the location of the immobilized target molecule.

Steps in Southern blotting

1. The DNA to be analyzed, such as the total DNA of an organism, is digested to completion with a restriction enzyme.
2. The complex mixture of fragments is subjected to gel electrophoresis to separate the fragment according to size.





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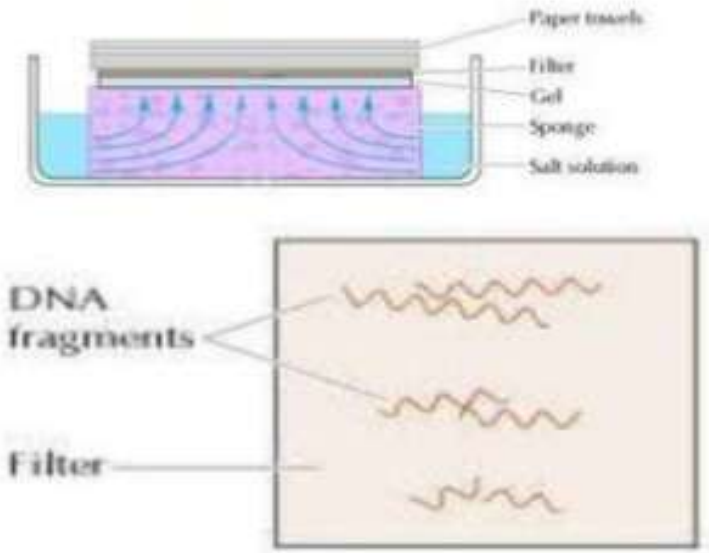
3.The restriction fragments present in the gel are denatured with heat or alkali and transferred onto a nitrocellulose or nylon membrane by blotting.

- This procedure preserves the distribution of the fragments in the gel, creating a replica of the gel on the filter.



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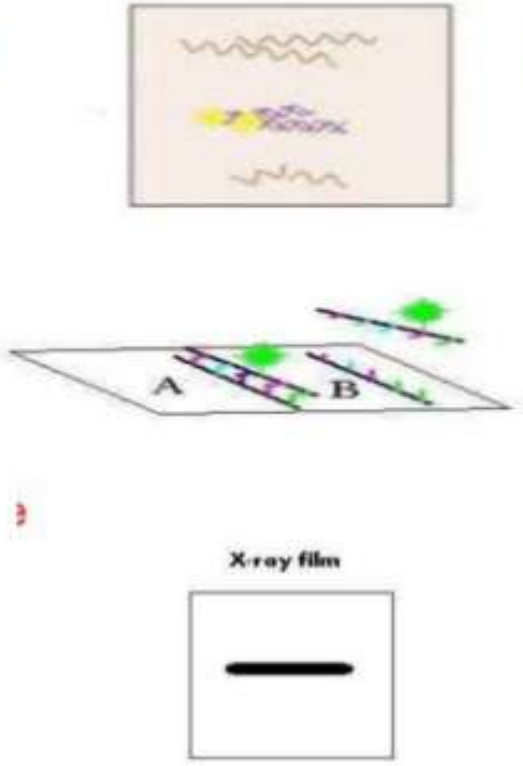
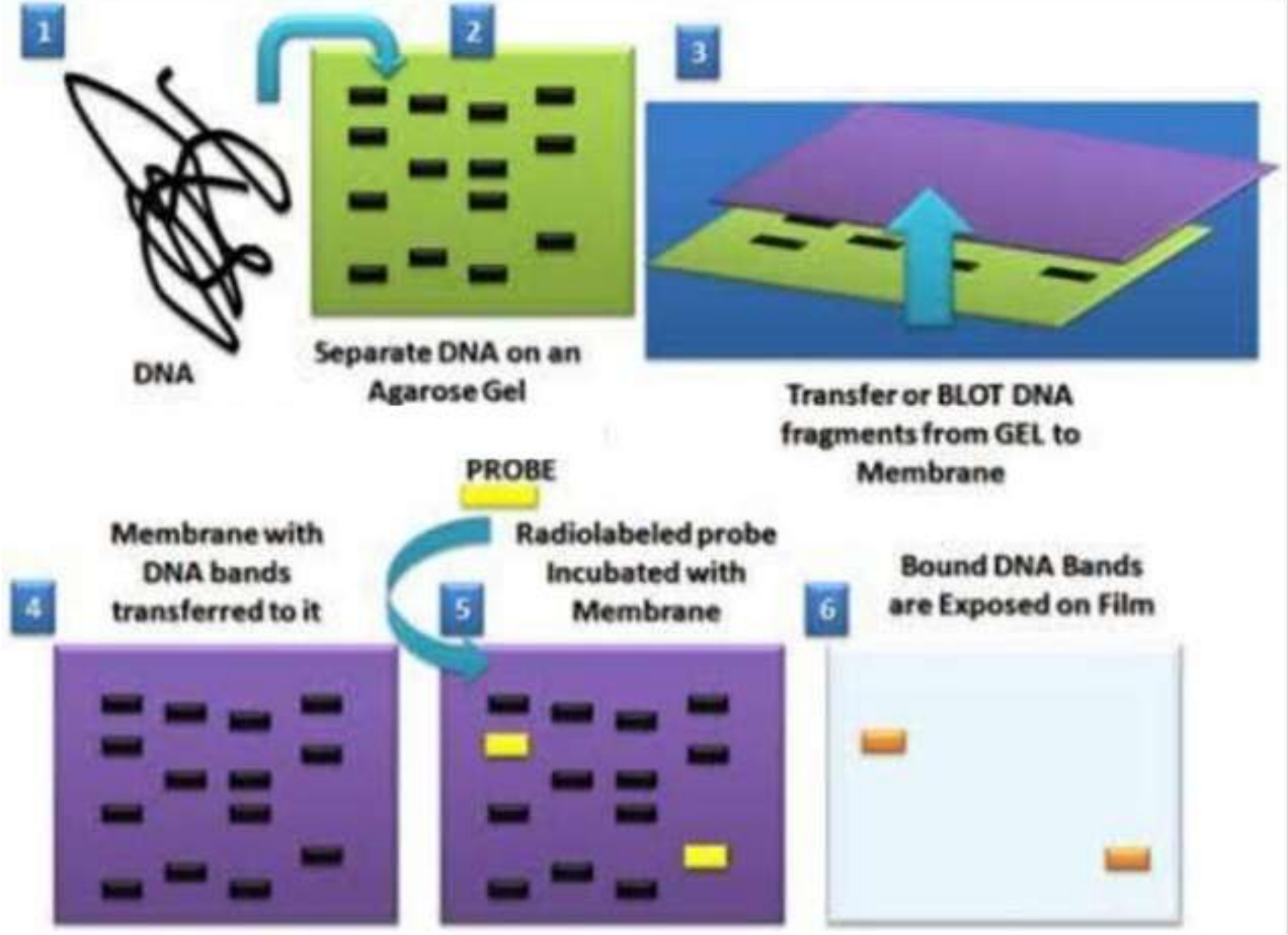
4.The filter is incubated under hybridization conditions with a specific radio labeled DNA probe.

5.The probe hybridizes to the complementary DNA restriction fragment.

6. Excess probe is washed away and the probe bound to the filter is detected by autoradiography, which reveals the DNA fragment to which the probe hybridized.




1
DNA
2
Separate DNA on an Agarose Gel
3
Transfer or BLOT DNA fragments from GEL to Membrane
PROBE
4
Membrane with DNA bands transferred to it
5
Radiolabeled probe Incubated with Membrane
6
Bound DNA Bands are Exposed on Film

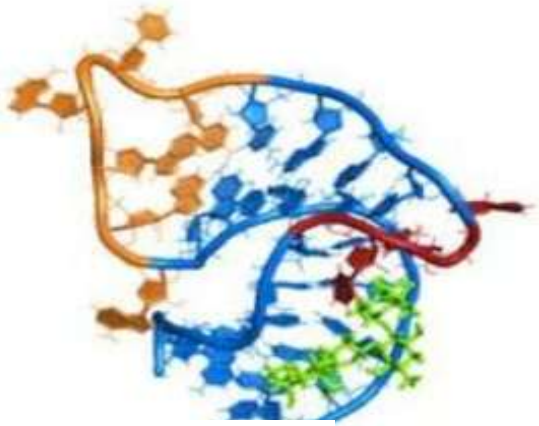
2. Northern Blotting

- Northern blotting is a technique for detection of specific RNA sequences.
- Northern blotting was developed by *Jamse Alwine* and *George Stark* at Stanford University and was named such by analogy to Southern blotting.

Steps involved in Northern blotting

1. RNA is isolated from several biological samples (e.g. various developmental stages of same tissue etc.)

* RNA is more susceptible to degradation than DNA



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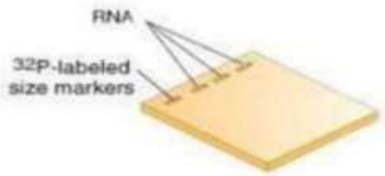
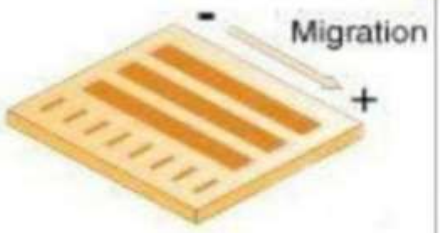
2. Sample's are loaded on gel and the RNA samples are separated according to their size on an agarose gel .

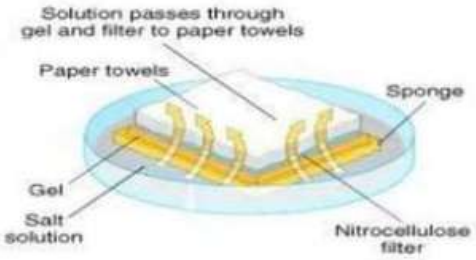


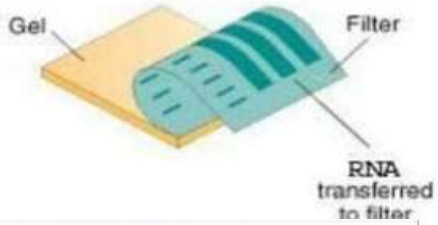
- The resulting gel following after the electrophoresis run.



CONT.....

3. The gel is then blotted on a nylon membrane or a nitrocellulose filter paper by creating the sandwich arrangement.

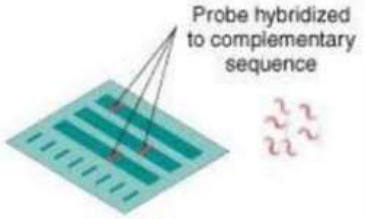




CONT.....

4. The membrane is placed in a dish containing hybridization buffer with a labeled probe.
   - Thus, it will hybridize to the RNA on the blot that corresponds to the sequence of interest.

5. The membrane is washed to remove unbound probe.



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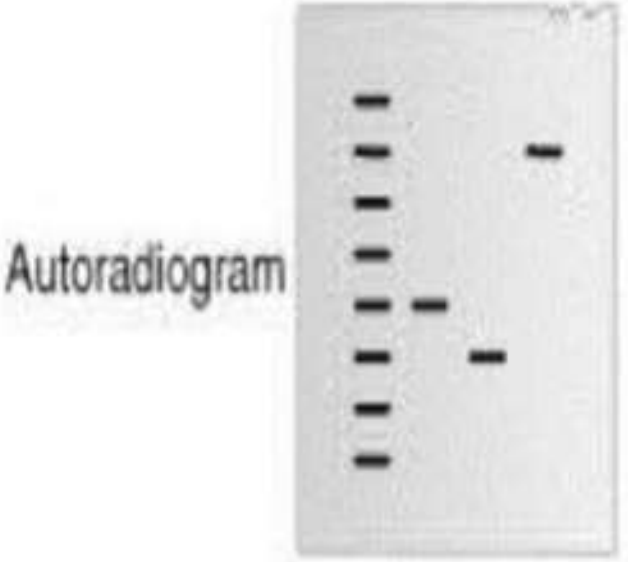
6. The labeled probe is detected via autoradiography or via a chemiluminescence reaction (if a chemically labeled probe is used). In both cases this results in the formation of a dark band on an X-ray film.

- Now the expression patterns of the sequence of interest in the different samples can be compared.

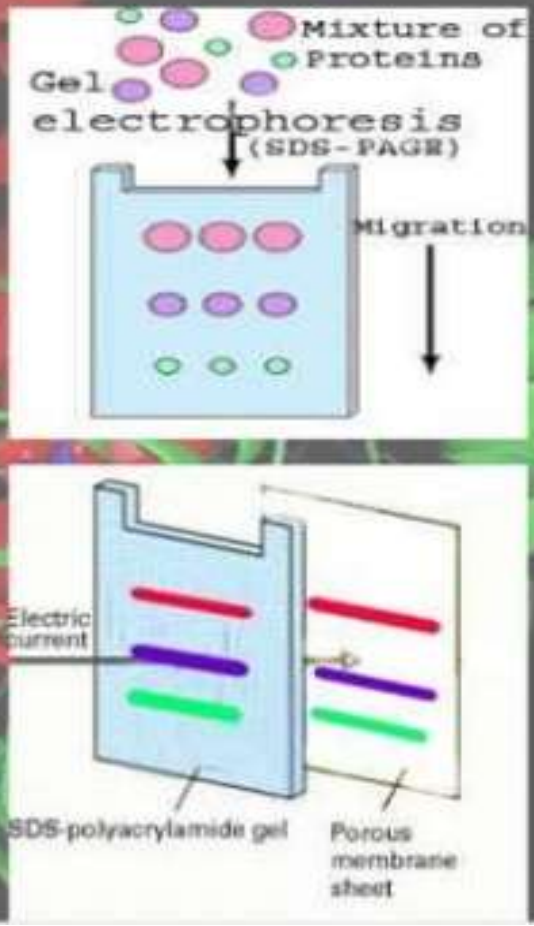


Western blotting

- Western blotting is an Immunoblotting technique which rely on the specificity of binding between a molecule of interest and a probe to allow detection of the molecule of interest in a mixture of many other similar molecules.
- In Western blotting, the molecule of interest is a protein and the probe is typically an antibody raised against that particular protein.
- The SDS PAGE technique is a prerequisite for Western blotting .

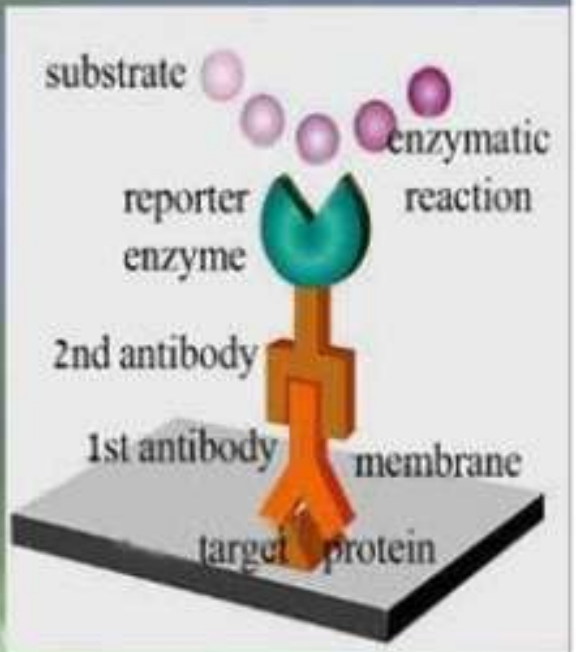
Steps in western blotting

- A protein sample is subjected to electrophoresis on an SDS-polyacrylamide gel
- Electroblotting transfers the separated proteins from the gel to the surface of a nitrocellulose membrane.



3. The blot is incubated with a generic protein (such as milk proteins or BSA) which binds to any remaining sticky places on the nitrocellulose.
4. An antibody that is specific for the protein of interest (the primary antibody = Ab1) is added to the nitrocellulose sheet and reacts with the antigen. Only the band containing the protein of interest binds the antibody, forming a layer of antibody molecules.

5. Following several rinses for removal of non-specifically bound Ab1, the Ab1-antigen complex on the nitrocellulose sheet is incubated with a second antibody (Ab2), which specifically recognizes the Fc domain of the primary antibody and binds it. Ab2 is radioactively labeled, or is covalently linked to a reporter enzyme, which allows to visualize the protein-Ab1-Ab2 complex.



Hybridization based (non-PCR) Technique

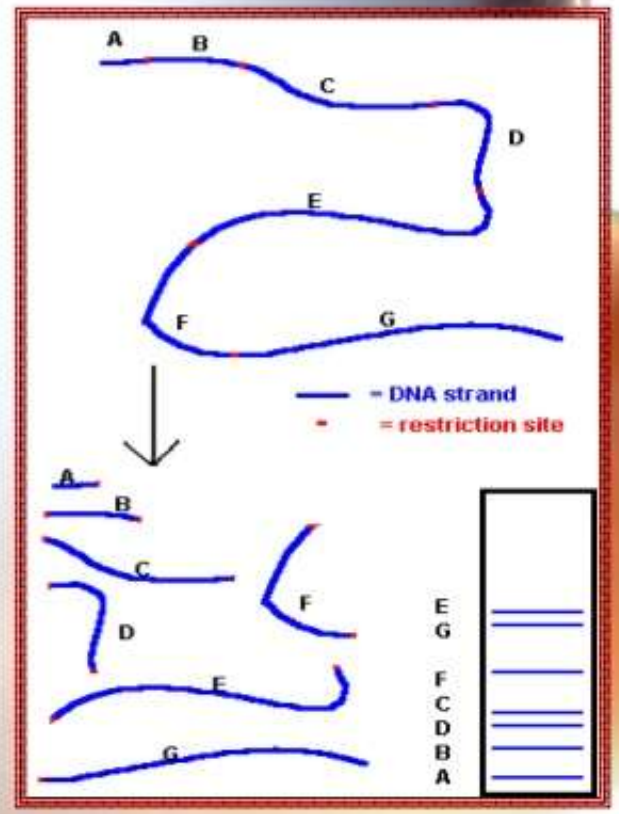
RFLPs

Restriction Fragment Length Polymorphism analysis

Botstein et al. (1980)

RFLPs :

Genetic markers resulting from the variation or change in the length of defined DNA fragments produced by digestion of the DNA sample with restriction endonucleases



RFLPs

(restriction fragment length polymorphisms)

Electrophoretic comparison of the size of defined restriction fragments derived from genomic DNA

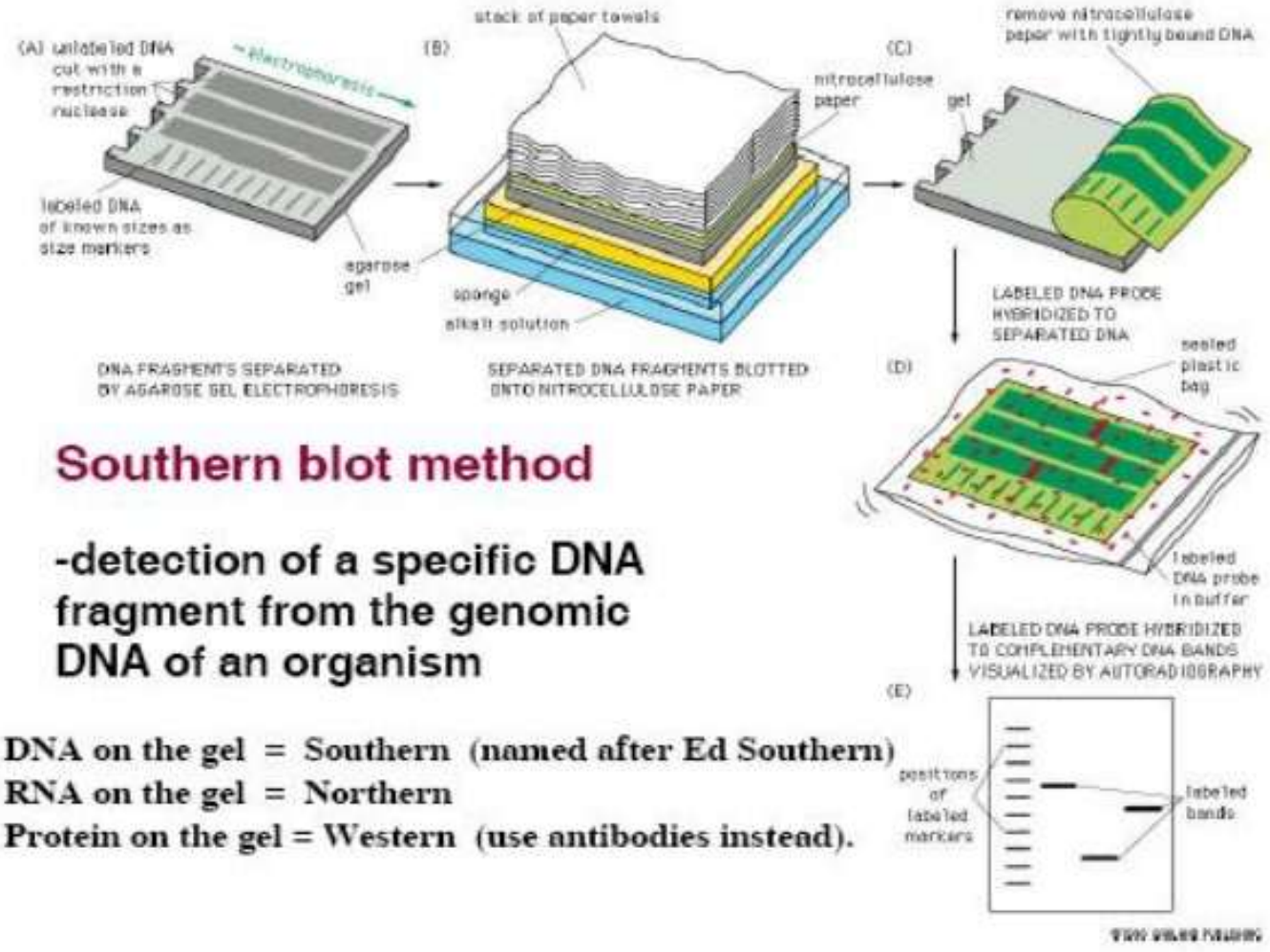
1. Isolate high quality DNA

2. Digest with a combination of restriction enzymes

3. Fractionate digested samples by electrophoresis

4. Transfer fragments to membrane

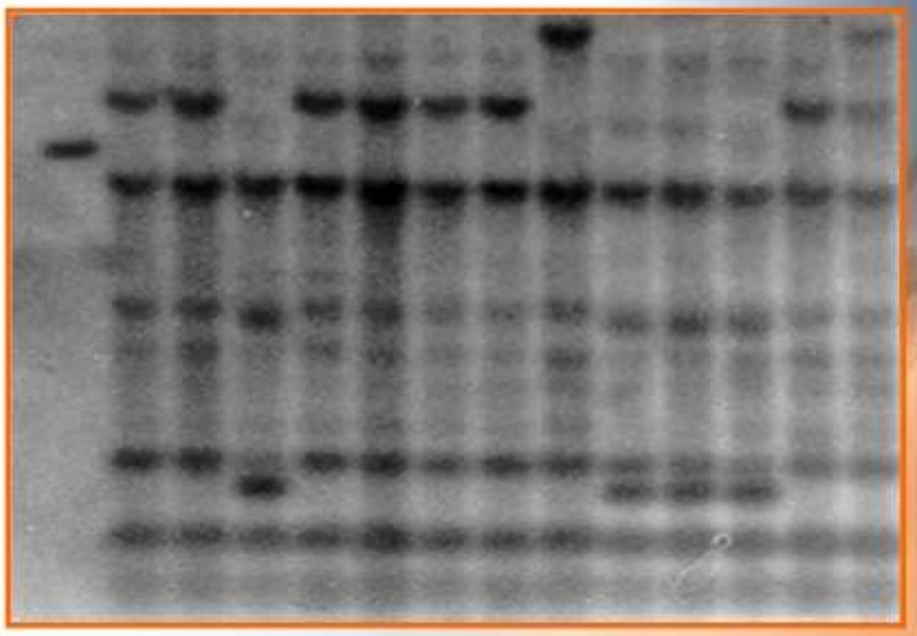
5. Hybridize with radioactively labeled DNA probe(s); detect by autoradiography. Can also use non-radioactive labeling systems



Southern blot method

-detection of a specific DNA fragment from the genomic DNA of an organism

DNA on the gel = Southern (named after Ed Southern)
RNA on the gel = Northern
Protein on the gel = Western (use antibodies instead).



RFLP analysis

Polymorphism revealed by different probe/enzyme combinations among 13 different accessions.

Considerations for use of RFLPs

- Relatively slow process

-Use of radioisotopes has limited RFLP use to certified laboratories (but non-radioactive labeling systems are now in wide use)

- Co-dominant markers; often species-specific
- Need high quality DNA
- Need to develop polymorphic probes
- expensive

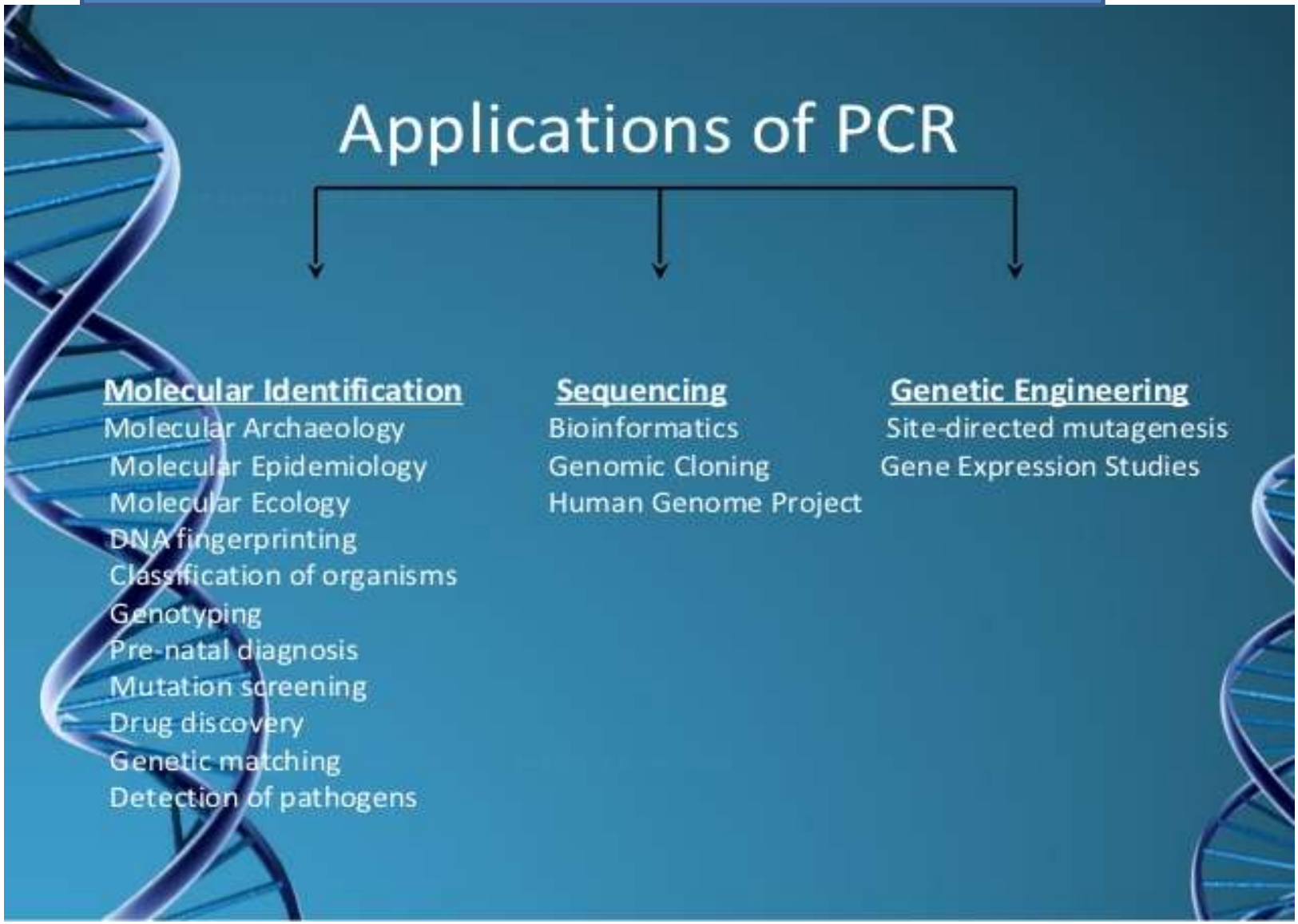
Applications

- Intraspecific level or among closely related taxa
- Presence and absence of fragments resulting from changes in recognition sites are used for identifying species or populations
- Estimating genetic distance and fingerprinting
- Forensic - biological parentage, paternity cases
- Disease status
- Genetic mapping

PCR based techniques

(RAPD, ISSR, SSR, AFLP, EST ,SCoT)

Polymerase Chain Reaction (PCR)



Principle of PCR

- Purpose:
- Condition:
- Components:

Purpose

- To amplify a lot of double-stranded DNA molecules (fragments) with same (identical) size and sequence by enzymatic method and cycling condition.

Condition

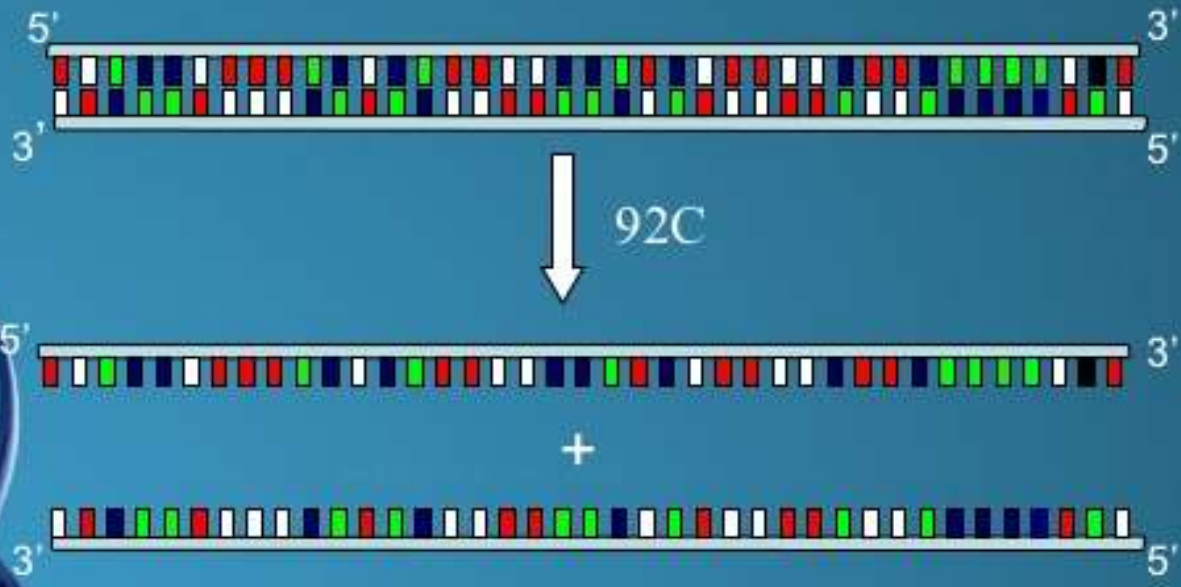
- 1. Denaturation of ds DNA template
- 2. Annealing of primers
- 3. Extension of ds DNA molecules

Three Aspects of PCR

- Specificity
- Efficiency
- Fidelity

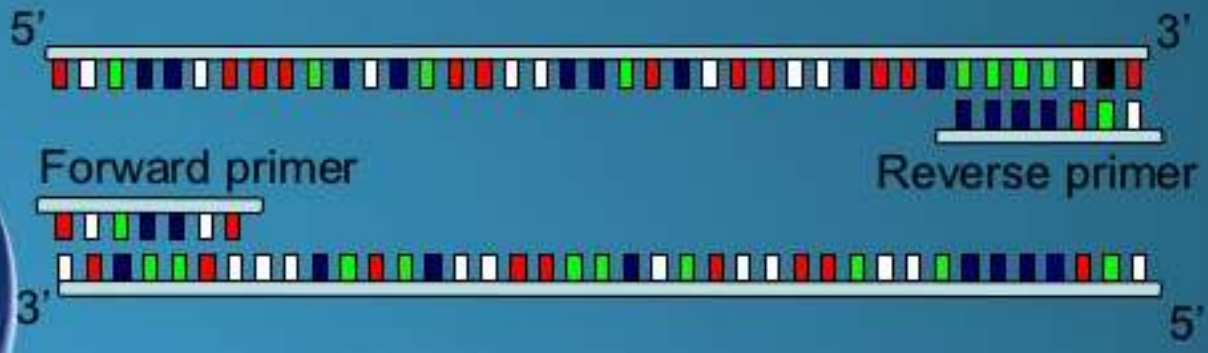
Denaturation

- Temperature: 92-94C
- Double stranded DNA melts ⟶ single stranded DNA



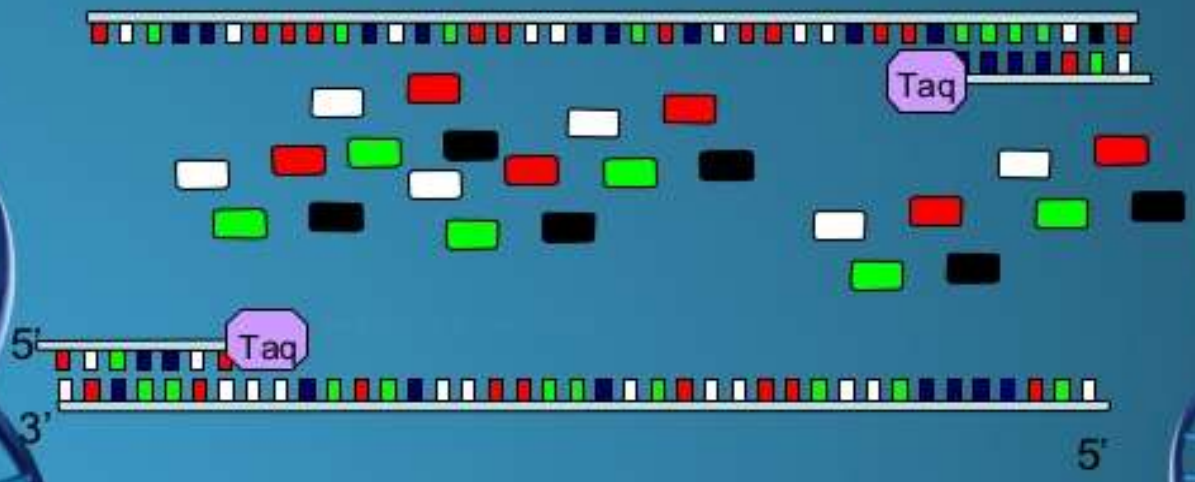
Annealing

- Temperature: ~50-70C (dependant on the melting temperature of the expected duplex)
- Primers bind to their complementary sequences



Extension

- Temperature: ~72C
- Time: 0.5-3min
- DNA polymerase binds to the annealed primers and extends DNA at the 3' end of the chain



Cycling

Chemical Components

- Magnesium chloride: .5-2.5mM
- Buffer: pH 8.3-8.8
- dNTPs: 20-200µM
- Primers: 0.1-0.5µM
- DNA Polymerase: 1-2.5 units
- Target DNA: ≤ 1 µg

Basic requirements for PCR reaction

- 1) DNA sequence of target region must be known.

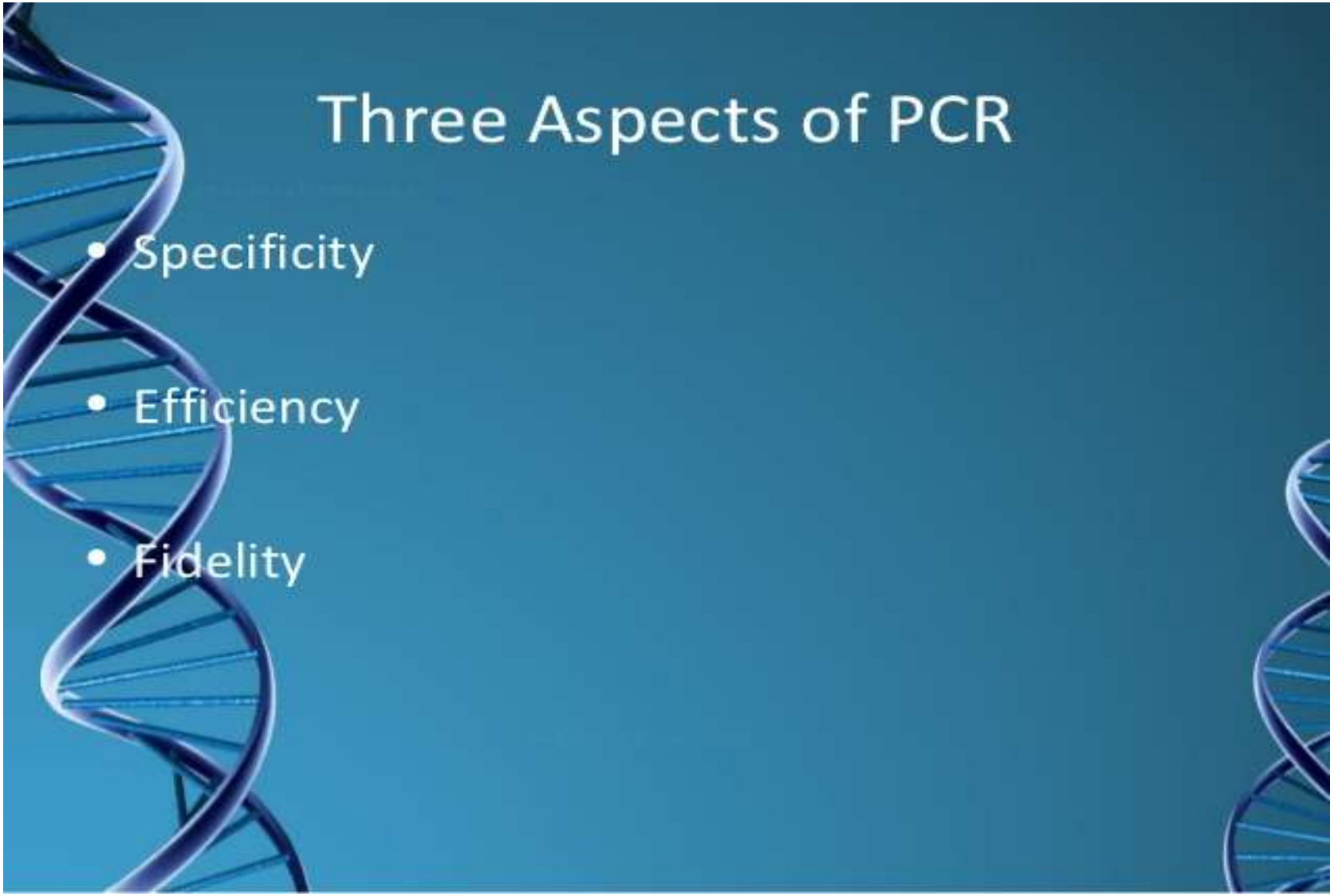
2) Primers - typically 20-30 bases in size. These can be readily produced by commercial companies. Can also be prepared using a DNA synthesizer

Basic requirements for PCR reaction

- 3) Thermo-stable DNA polymerase - eg *Taq* polymerase which is <u>not</u> inactivated by heating to 95C

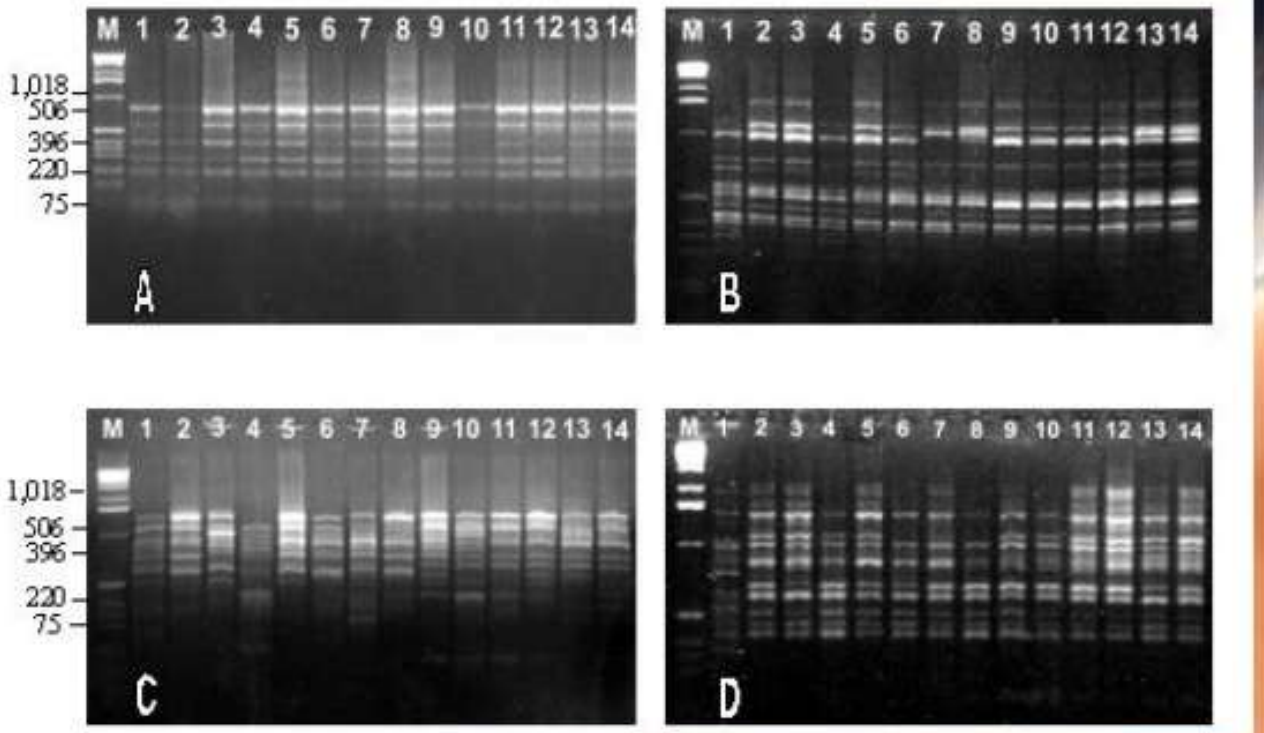
4) DNA thermal cycler - machine which can be programmed to carry out heating and cooling of samples over a number of cycles.

Instrumentation


Three Aspects of PCR
• Specificity
• Efficiency
• Fidelity

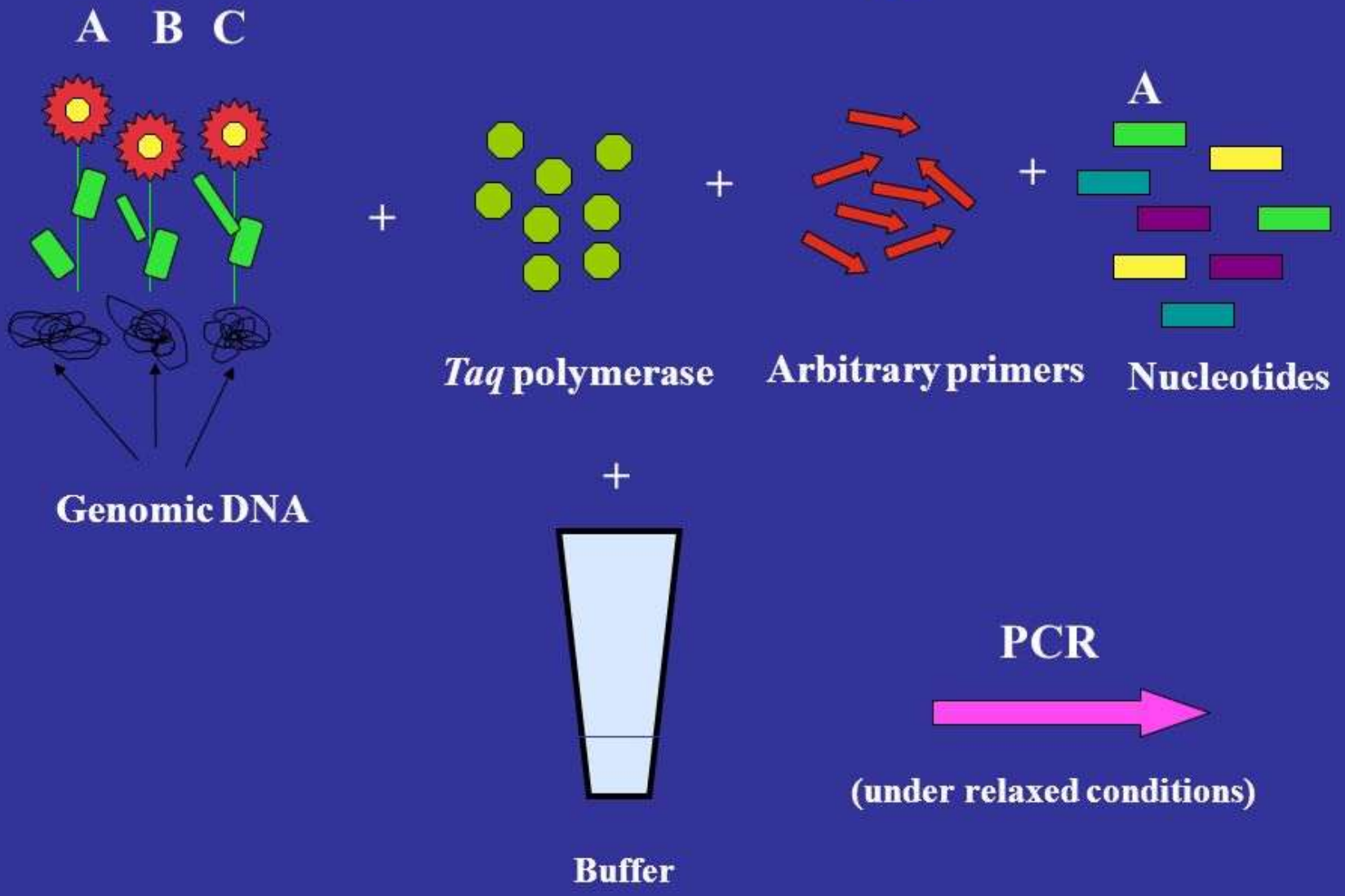
Random Amplified Polymorphic DNA (RAPD)

- Randomly Amplified Polymorphic DNA (RAPDs) are genetic markers resulting from PCR amplification of genomic DNA sequences recognized by ten-mer random primers of arbitrary nucleotide sequence (Williams et al., 1990).
- RAPDs are dominant markers that require no prior knowledge of the DNA sequence, which makes them very suitable for investigation of species that are not well known (Williams et al. 1993).



RAPD profiles for the 14 Date Palm accessions as detected with primers OPB-06 (A), OPB-08 (B), OPB-11 (C), and OPO-07 (D). Lanes 1 to 14 represent: SAK-AK, SAK-AB, BRT-AK, BRT-AB, MLK-AK, MLK-AB, GND-AK, GND-AB, SIW-KH, SIW-DK, SIW-HB, SIW-TZ, FRA-HB and FRA-TZ. M: 1 Kb ladder DNA marker.

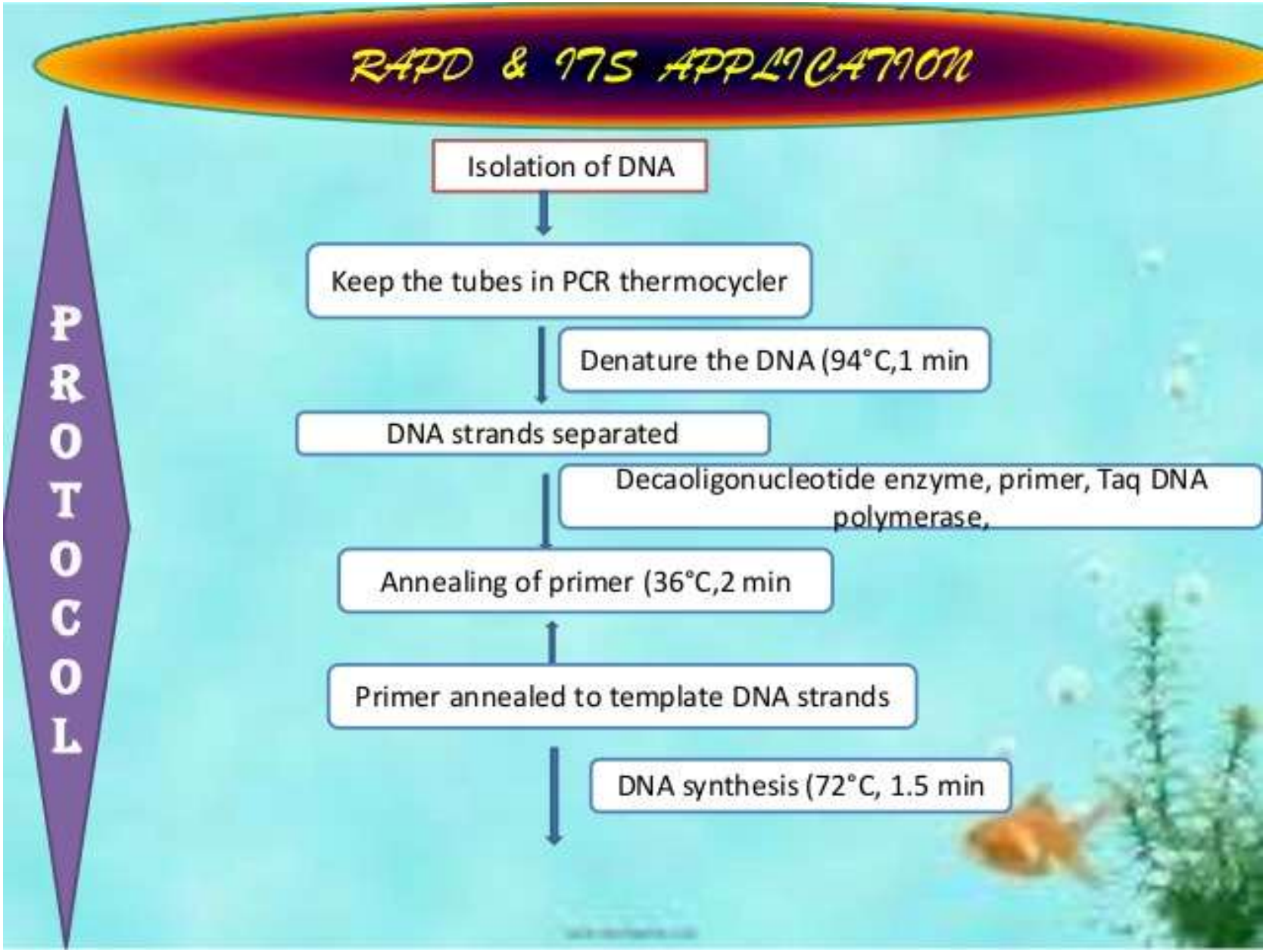
RAPD technology



RAPD & ITS APPLICATION

DEFINITION

- **DEFINITION:-** RAPD that is defined by differences between individuals in terms of DNA regions either being or not being amplified in a polymerase chain reaction primed by random oligonucleotides sequences.
- It is a type of PCR reaction, but the segments of DNA that are amplified are random.
- **RAPD creates several arbitrary, short primers (8–12 nucleotides), then proceeds with the PCR using a large template of genomic DNA**
- **RAPD**- The full form of RAPD is **RANDOM AMPLIFIED POLYMORPHIC DNAs** are obtained by using a PCR equipment or a thermo cycler.
- **RAPD -** is a lab technique used to amplify unknown(random) DNA segments


RAPD & ITS APPLICATION
PROTOCOL
Isolation of DNA
Keep the tubes in PCR thermocycler
Denature the DNA (94°C,1 min
DNA strands separated
Decaoligonucleotide enzyme, primer, Taq DNA polymerase,
Annealing of primer (36°C,2 min
Primer annealed to template DNA strands
DNA synthesis (72°C, 1.5 min

RAPD & ITS APPLICATION

PROTOCOL

Complementary strand synthesis

↓

35 to 45 cycles

Amplified products separated by gel electrophoresis

↓

Bands detected by Ethidium bromide staining

MICROSATELLITE

What is a microsatellite?

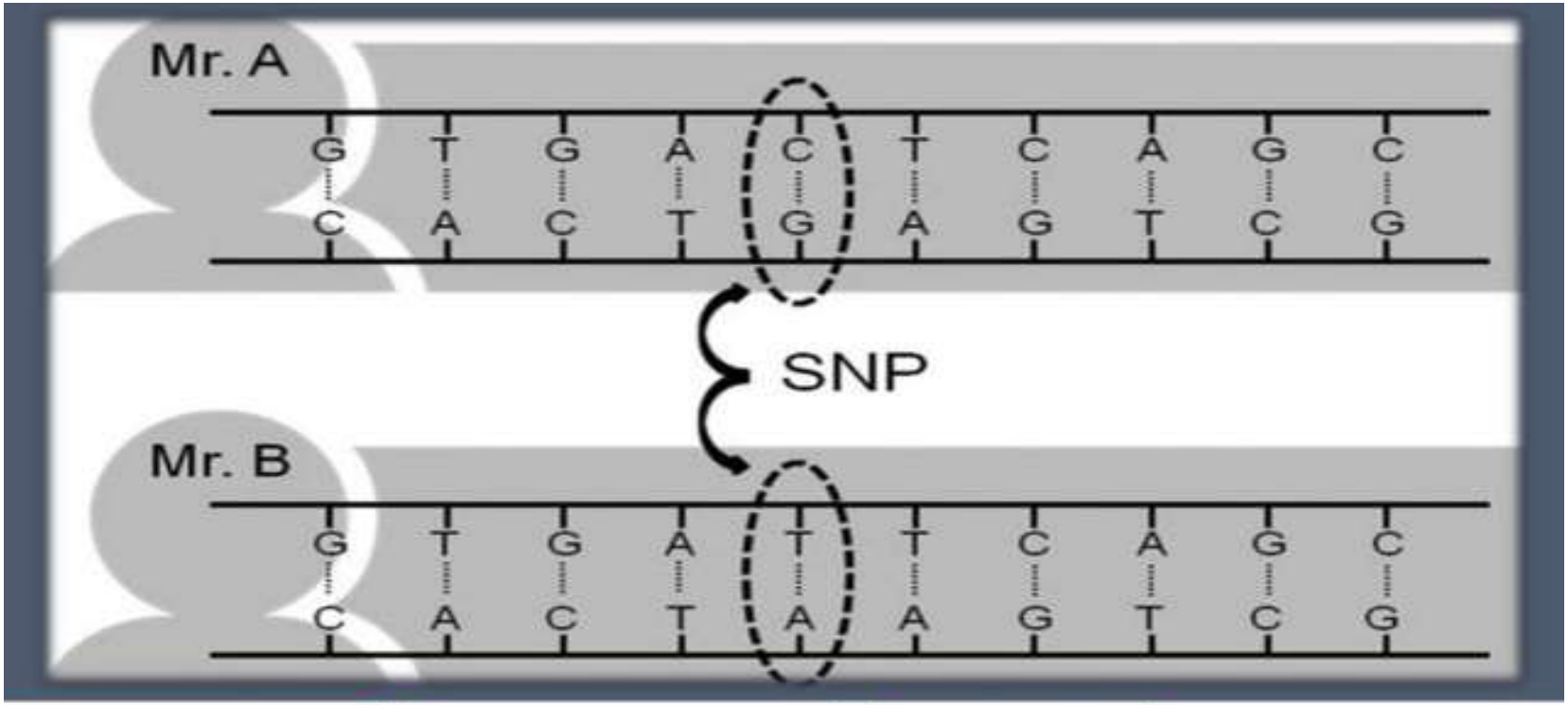
- Tandemly repeated DNA (**may see in the literature as STRs -** Short tandem repeats)
  - Poly A/T most common
  - 1-10 bp tandemly repeated = 'micro' satellite
  - >10 = 'mini' satellite
- Types of microsats
  - Di, tetra and tri nucleotide (**used in that order**)
  - Perfect
  - Imperfect/interrupted
  - Compound
    - Varying levels of variation associated with each type
    - Difficulty in scoring

Single nucleotide polymorphism

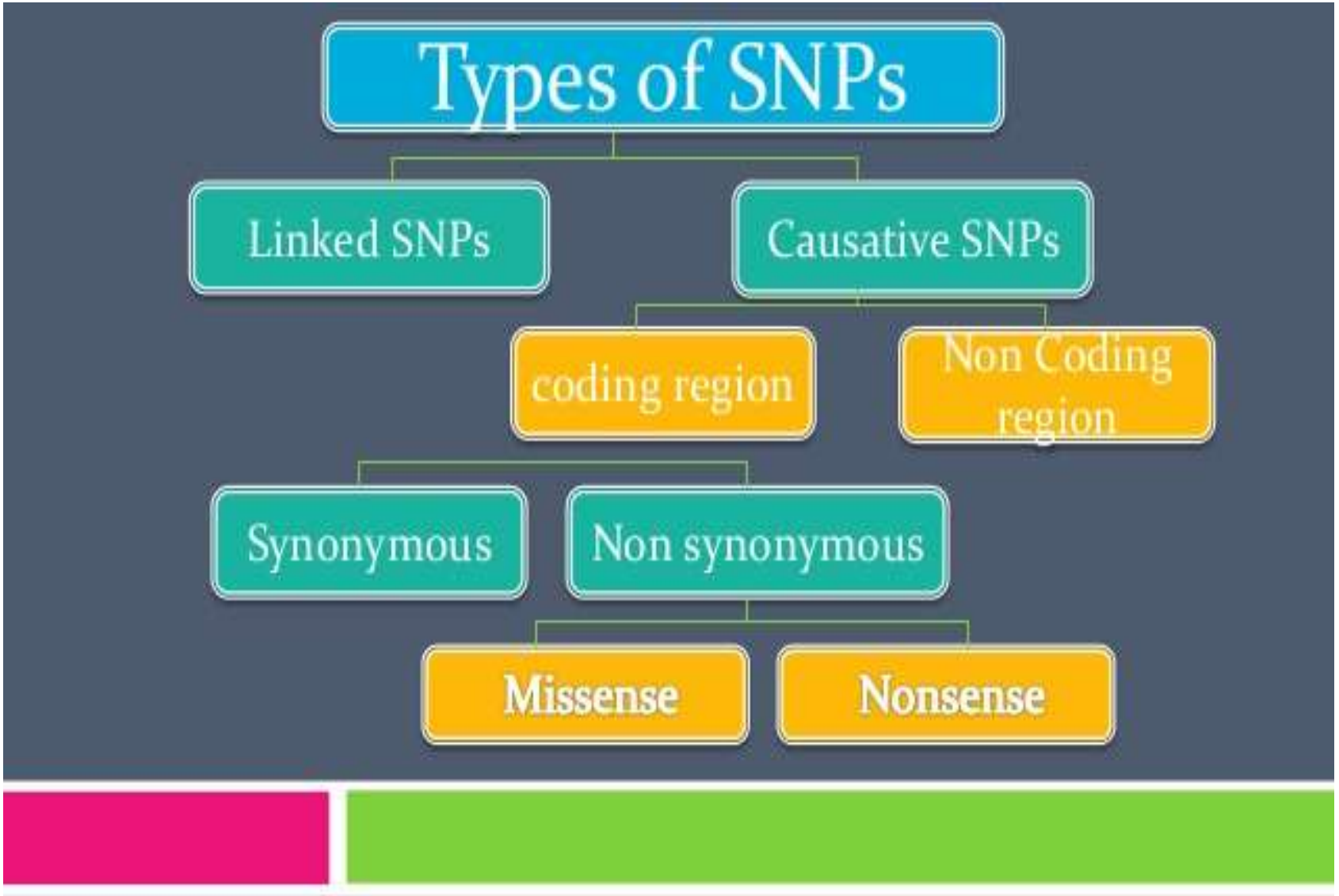
- A Single Nucleotide Polymorphism, also known as Simple Nucleotide Polymorphism, is a DNA sequence variation occurring commonly within a population (e.g. 1%) in which a single nucleotide — A, T, C or G — in the genome differs between members of a biological specie .
- Pronounced snips

Common type of genetic variation among people

- Each SNP represents a difference in a single DNA building block called as nucleotide



Example


Types of SNPs
Linked SNPs
Causative SNPs
coding region
Non Coding region
Synonymous
Non synonymous
Missense
Nonsense

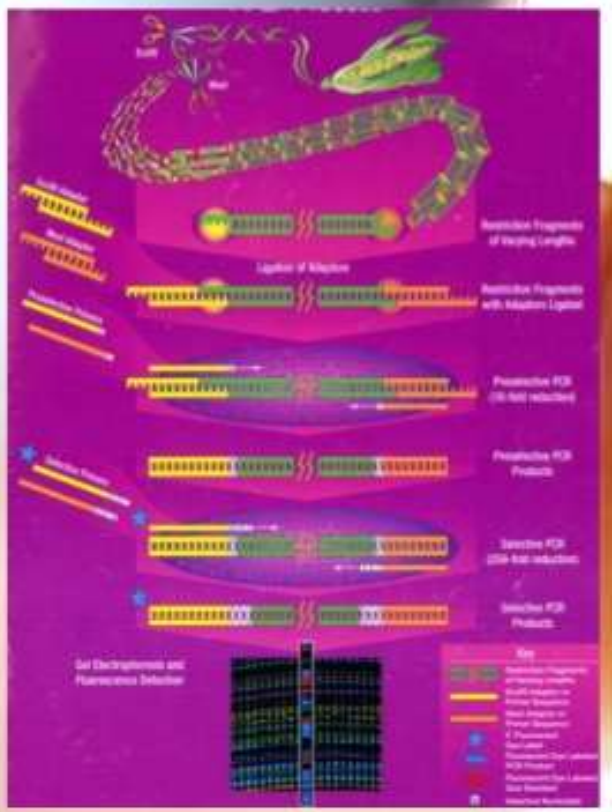
AFLP

(Amplified Fragment Length Polymorphisms)

- A combination of PCR and RFLP
- Informative fingerprints of amplified fragments

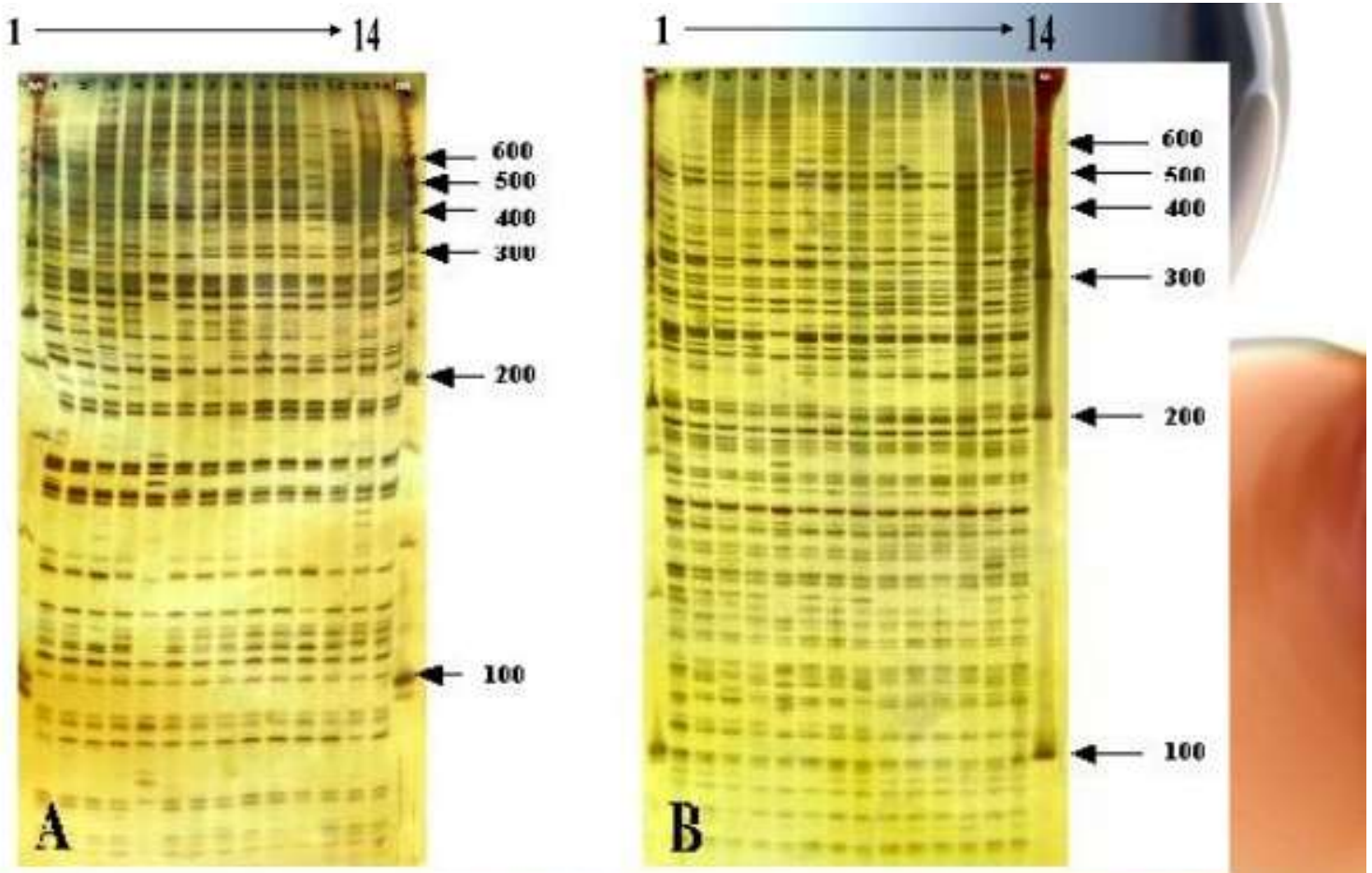
Amplified Fragment Length Polymorphism (AFLP)

AFLP technology is a DNA fingerprinting technique that combines RFLP and PCR. It is based on the selective amplification of a subset of genomic restriction fragments using PCR.



AFLP process

1. Digest genomic DNA with restriction enzymes
2. Ligate commercial adaptors (defined sequences) to both ends of the fragments
3. Carry out PCR on the adaptor-ligated mixture, using primers that target the adaptor, but that vary in the base(s) at the 3′ end of the primer.



AFLP profiles of the 14 Date Palm accessions as revealed by the primer combination Eact X Mcta. (A) and the primer combination Eagc X Mcaa. (B). Lanes 1 to 14 represent: SAK-AK, SAK-AB, BRT-AK, BRT-AB, MLK-AK, MLK-AB , GND-AK, GND-AB, SIW-KH, SIW-DK, SIW-HB, SIW-TZ, FRA-HB and FRA-TZ. M: DNA molecular weight marker (100 bp Ladder).

Advantages of AFLP's

- Very sensitive
- Good reproducibility but technically demanding
- Relatively expensive technology
- Discriminating homozygotes from heterozygotes
- Requires band quantitation (comparison of pixel density in images from a gel scanner)
- Bands are anonymous

Applications

1. Monitoring inheritance of agronomic traits
2. Diagnostic in genetically inherited disease
3. Pedigree analysis,
4. Forensic typing - Parentage analysis
5. Identifying hybrids
6. Species level relationship
7. Also in some case at higher level relationship

Properties of Different MM

Features	RFLP	PCR-RFLP	DFP	RAPD	Microsatellite	SNP
Detection method	Hybridization	PCR	Hybridization	PCR	PCR	PCR
Type of probe/primer used	g DNA/ cDNA sequence of structural genes	Sequence specific primers	Mini satellite synthetic oligos	Arbitrarily design primer	Sequence specific primers	Sequence specific primers
Requirement of radioactivity	Yes	No/Yes	Yes	No/Yes	No/Yes	No/Yes
Extant of genomic coverage	Limited	Limited	Extensive	Extensive	Extensive	Extensive
Degree of polymorphisms	Low	Low	High	Medium to High	High	High
Phenotype expression	Co dominant	Co dominant	Co dominant	Co dominant/Dominant	Dominant	Co dominant
Possibility of automation	No	Yes	No	Yes	Yes	Yes

DNA marker applications

- Fingerprinting .
- Diversity studies .
- Marker-assisted selection .
- Genetic maps .
- Gene tagging .
- Novel allele detection .
- Map-based gene colning .
- F1 identification .
- Comparative maps .
- Bulk segregant analysis .
- Seed testing .


Thank
You