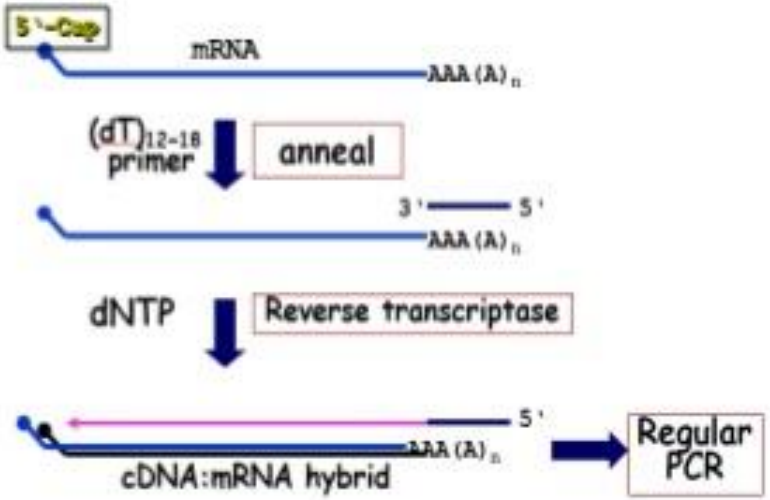
Reverse Transcriptase -PCR

- Detect gene expression through the synthesis of complementary DNA (cDNA) transcripts from RNA.
- RNA template converted into a complementary DNA (cDNA) using a reverse transcriptase.
- Primers – Oligo dt, random primers & gene specific primers.
- One step & Two step RT-PCR.
- Amplified DNA fragments that are produced can by analyzed by agarose gel electrophoresis.
- Amount of amplified fragment produced proportional to the amount of target mRNA in the original RNA sample.

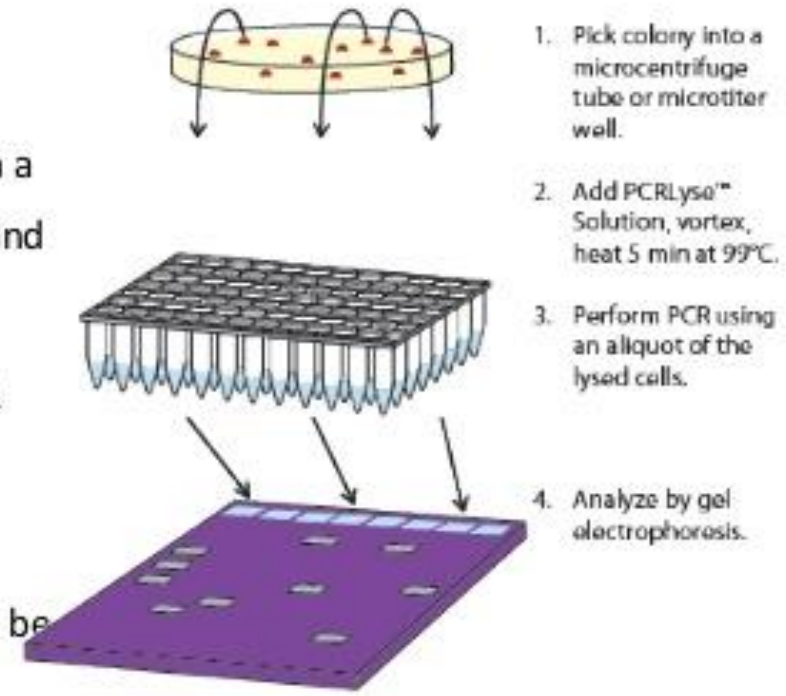


Real Time PCR or (q-PCR)

- In RT-PCR, process of amplification of DNA is monitored in real time.
- PCR with an added probe or dye to generate a fluorescent signal from the product.
- Detection of signal in real time allows quantification of starting material.
- Performed in specialized thermal cyclers with fluorescent detection systems.
- PCR signal is observed as an exponential curve with a lag phase, a log phase, a linear phase, and a stationary phase.

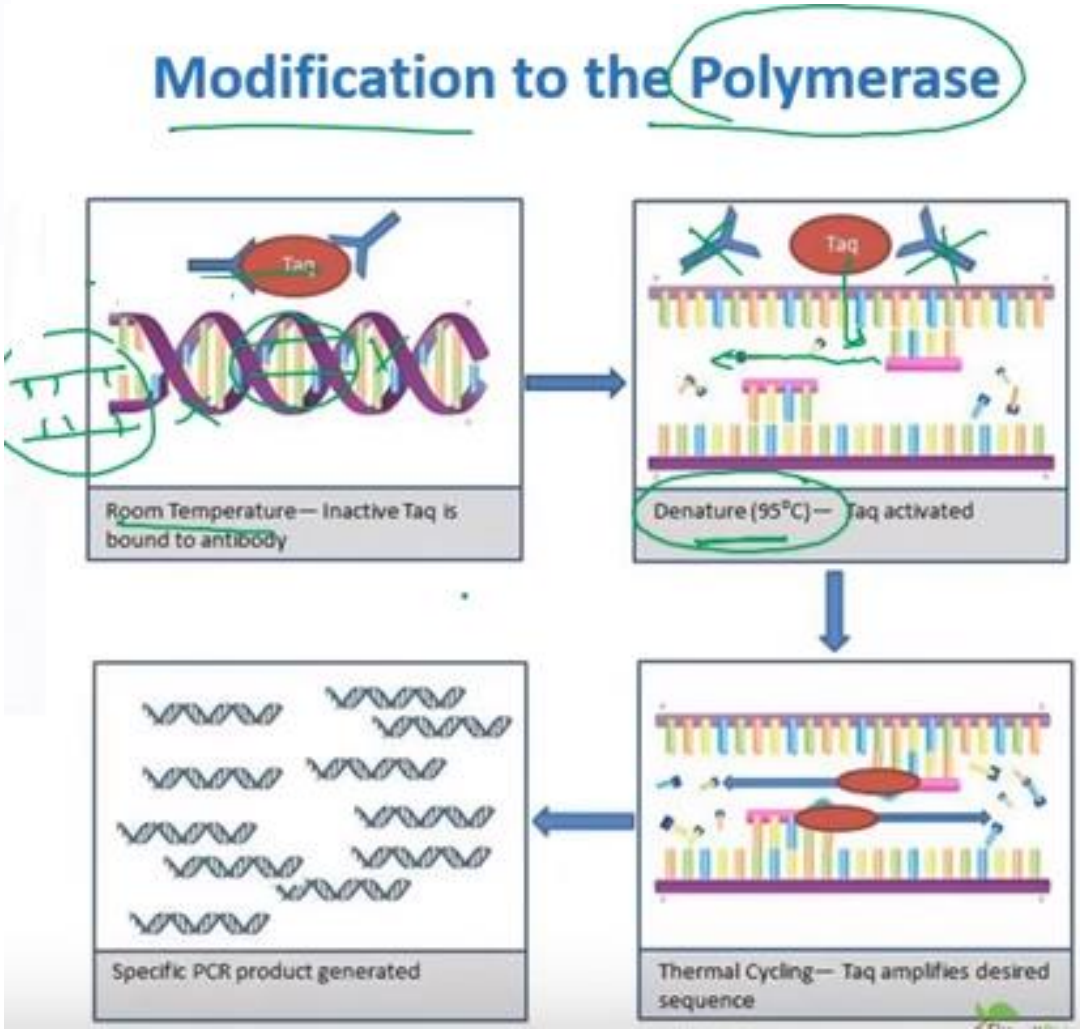
Colony PCR

- Screening of bacteria (E.coli) or yeast clones for correct ligation or Plasmid products.
- Individual transformants can either be lysed in water with a short heating step or added directly to the PCR reaction and lysed during the initial heating step.
- Initial heating step causes the release of the plasmid DNA from the cell, so it can serve as template for the amplification reaction.
- Primers designed to specifically target the insert DNA can be used to determine if the construct contains the DNA fragment of interest and also insert orientation.



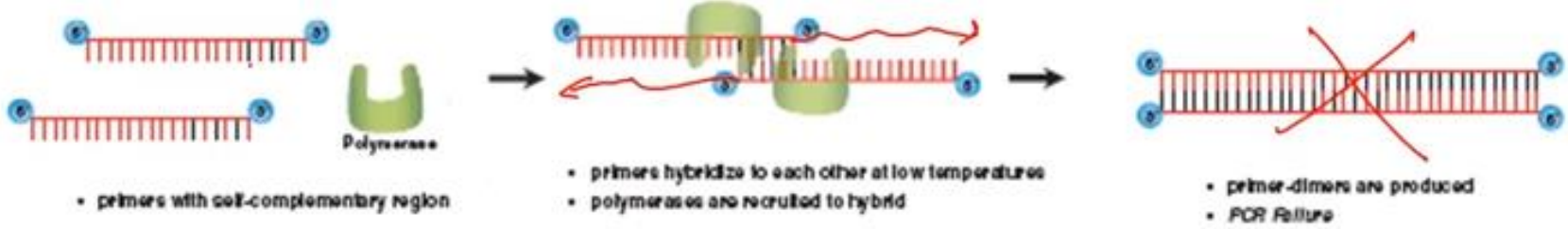
Hot-start PCR

- It is a modified form of PCR which avoids non-specific amplification of DNA by inactivating Taq polymerase at lower temperature
- In the second step in addition to primer and Taq polymerase we add specific antibodies to block Taq polymerase from annealing.
- When temperature raises for amplification at 720 C, the specific antibodies detaches from Taq polymerase & amplification begins with greater specificity.

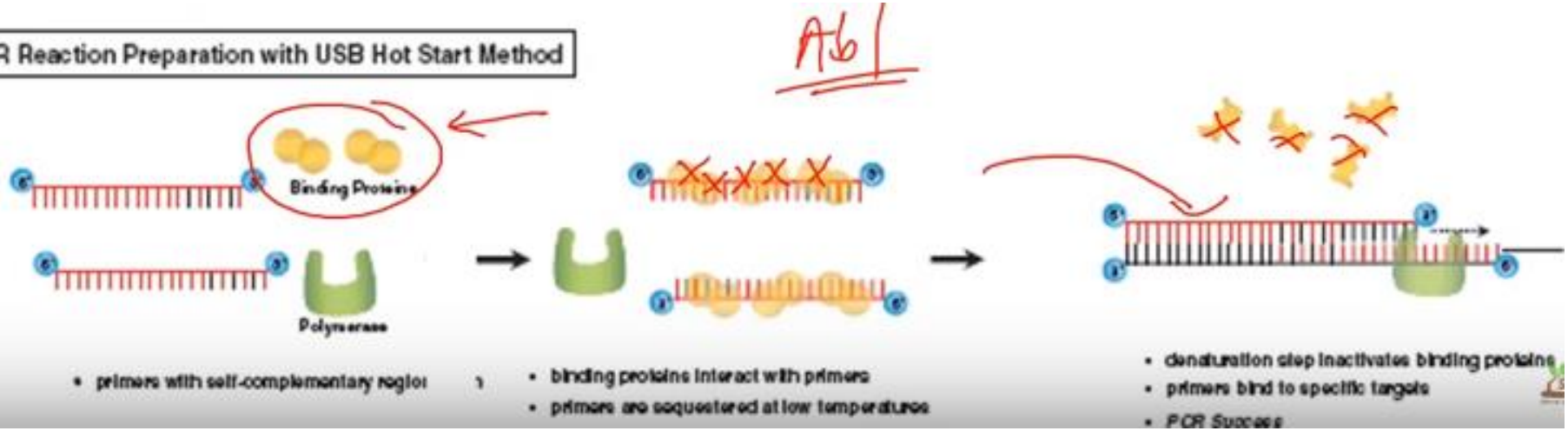


Modification to primers

PCR Reaction Preparation without Hot Start

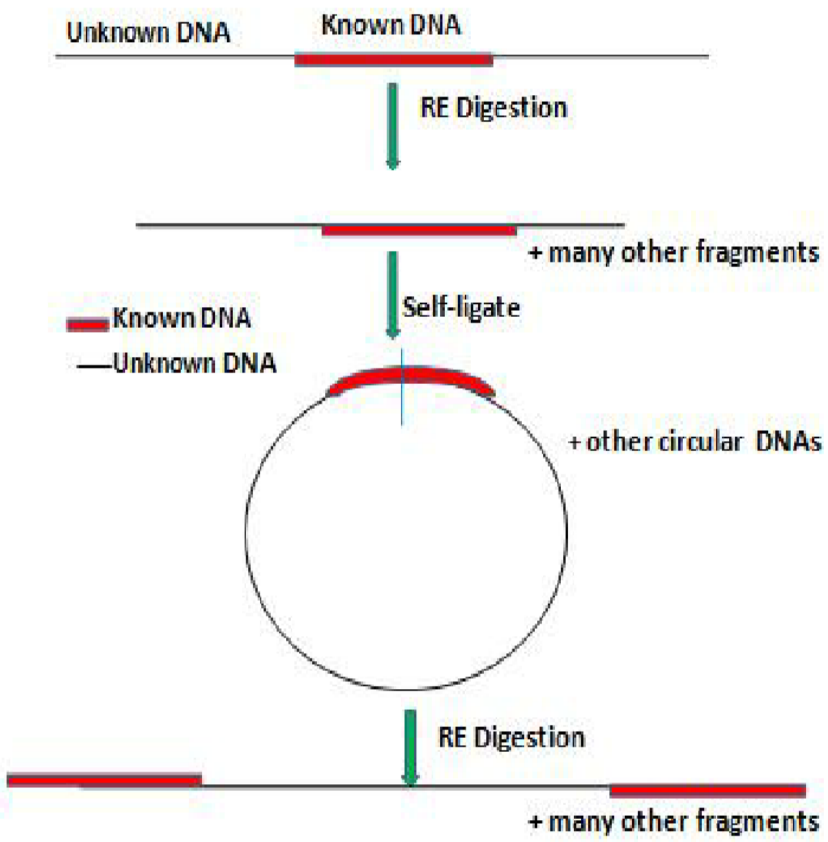


PCR Reaction Preparation with USB Hot Start Method



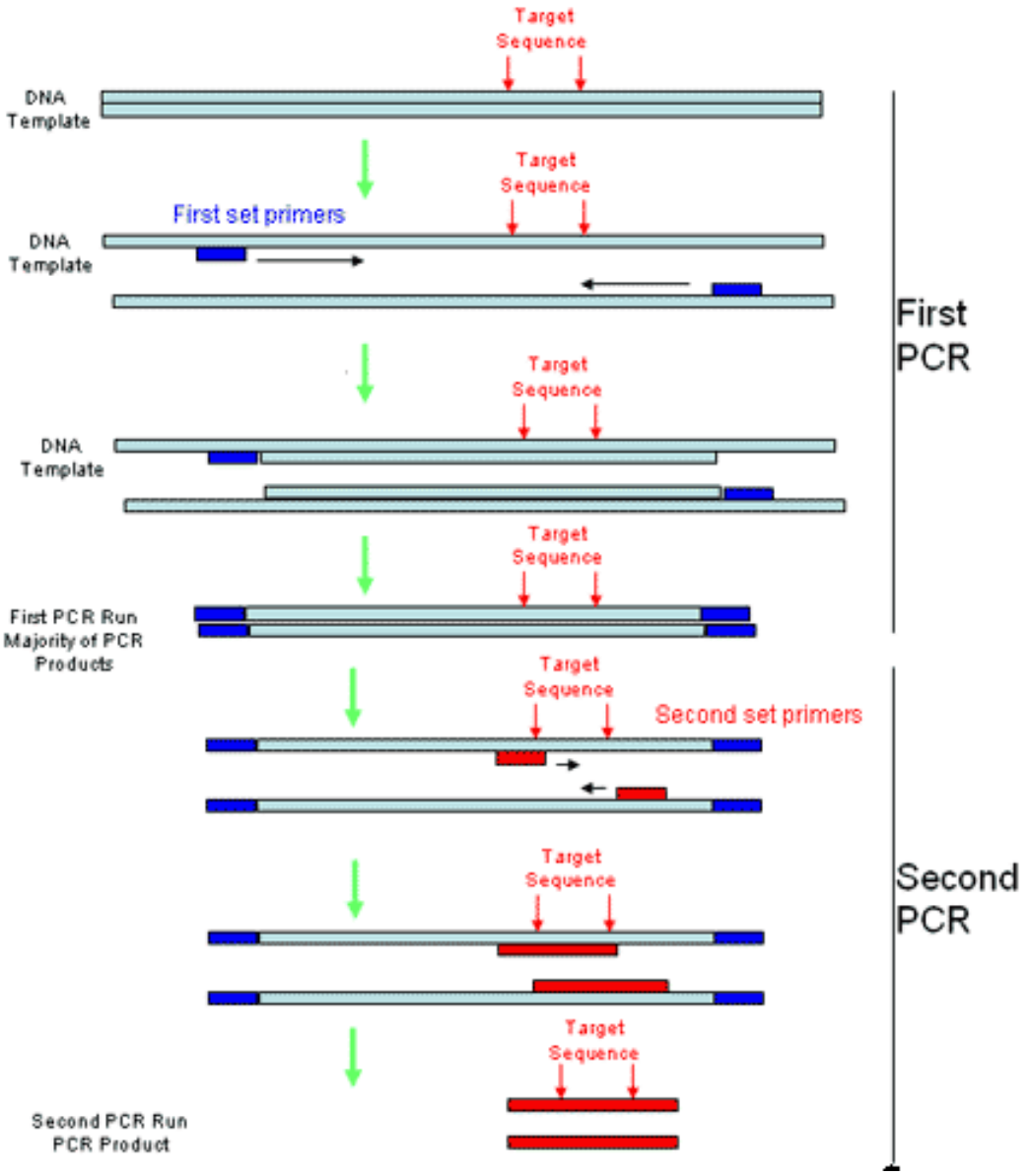
Inverse PCR

- Method used to allow PCR when only one internal sequence is known.
- This is especially useful in identifying flanking sequences to various genomic inserts.
- This involves a series of DNA digestions and self ligation, resulting in known sequences at either end of the unknown sequence.



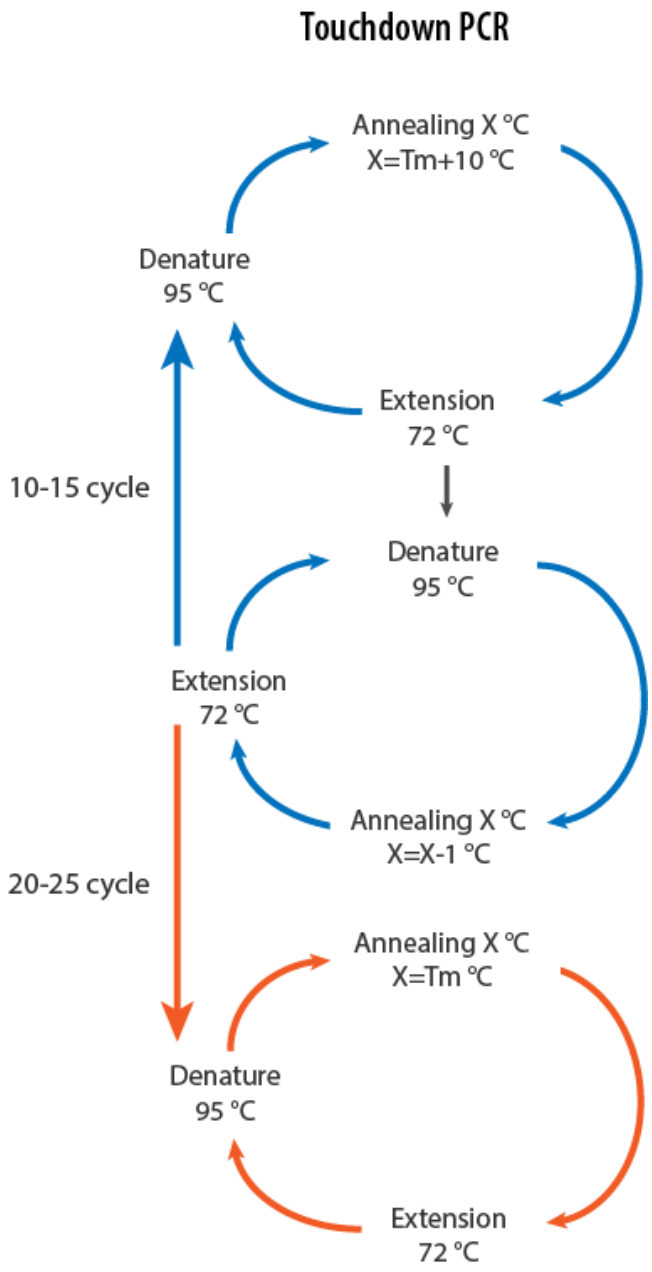
Nested PCR

- Prevents non-specific binding of primer and its amplification.
- Two sets of primers, used in two successive runs of polymerase chain reaction.
- First primer binds to the region far away from Target sequence and product is formed.
- Products are then used in a second PCR reaction with second set of primers whose 3' end complementary to Target sequence.
- second PCR has little contamination from unwanted products of primer dimers, hairpins, and alternative primer target sequences.



Touch down PCR

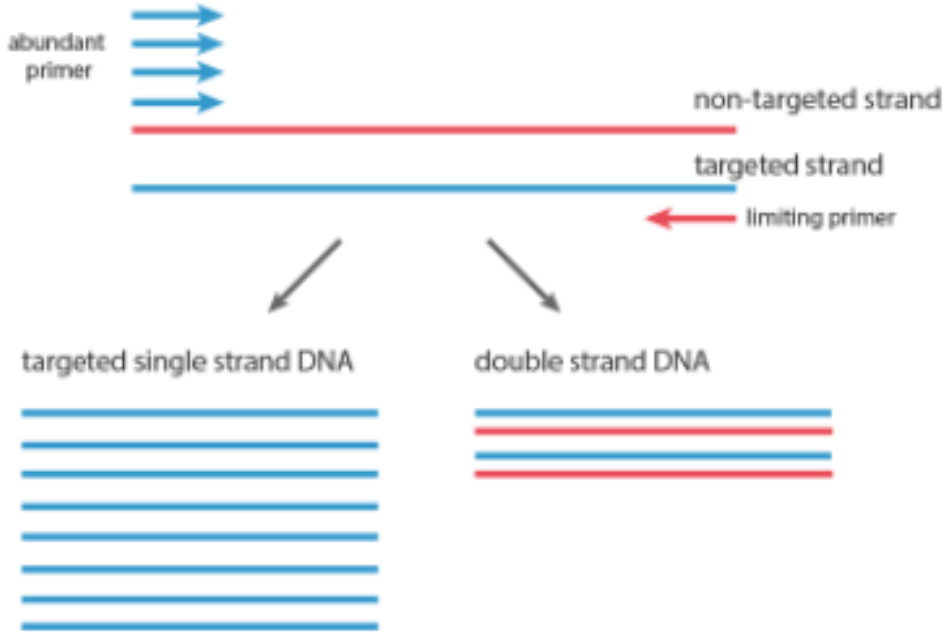
- Reduce nonspecific background amplification -lowering the annealing temperature as PCR cycling progresses.
- Annealing temperature at the initial cycles is usually a few degrees (3-5˚C) above the Tm of the primers used, later cycles, it is a few degrees (3-5˚C) below the primer Tm.
- Higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles.



Asymmetric PCR

In an asymmetric PCR, the reaction preferentially amplifies one DNA strand in a double-stranded DNA template. Thus it is useful when amplification of only one of the two complementary strands is needed such as in sequencing and hybridization probing. The whole PCR process is similar to regular PCR, except that the amount of primer for the targeted strand is much more than that of the non-targeted strand. As the asymmetric PCR progresses, the lower concentration limiting primer is quantitatively incorporated into newly synthesized double stranded DNA and used up. Consequently, linear synthesis of the targeted single DNA strand from the excess primer are formed after depletion of the limiting primer.

Asymmetric PCR is not widely used because it has low reaction efficiency and it is hard to optimize the proper primer ratios, the amounts of starting material, and the number of amplification cycles. Limiting the concentration of one primer lowers its melting temperature below the reaction annealing temperature [8]. Recently, this process has been changed to be known as Linear-After-The –Exponential-PCR (LATE-PCR) where the limiting lower concentration primer has a higher melting temperature than the higher concentration primer to maintain reaction efficiency [9].

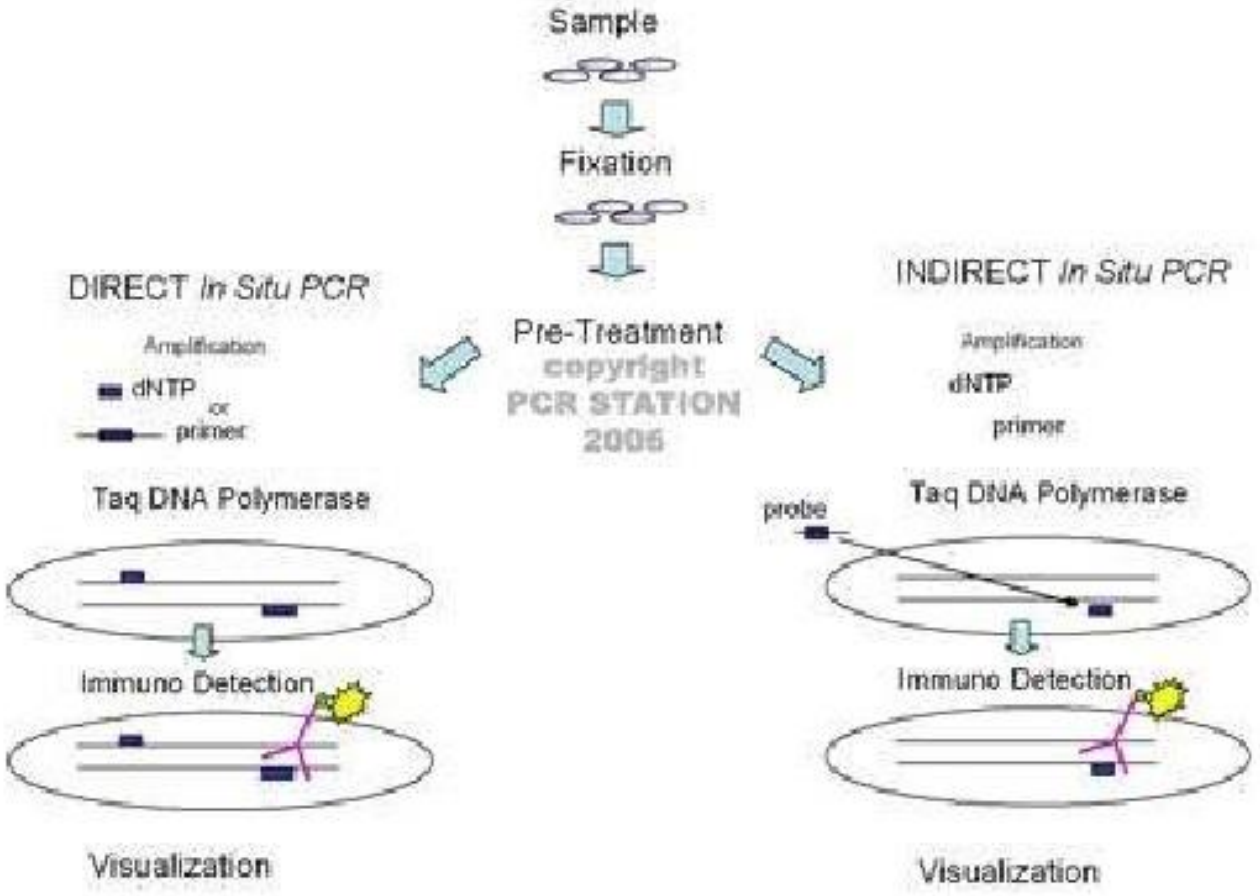


Allele-specific PCR

- Used for identify of SNPs.
- It requires prior knowledge of a DNA sequence, including differences between alleles.
- Uses primers whose 3' ends encompass the SNP
- PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer
- Successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence

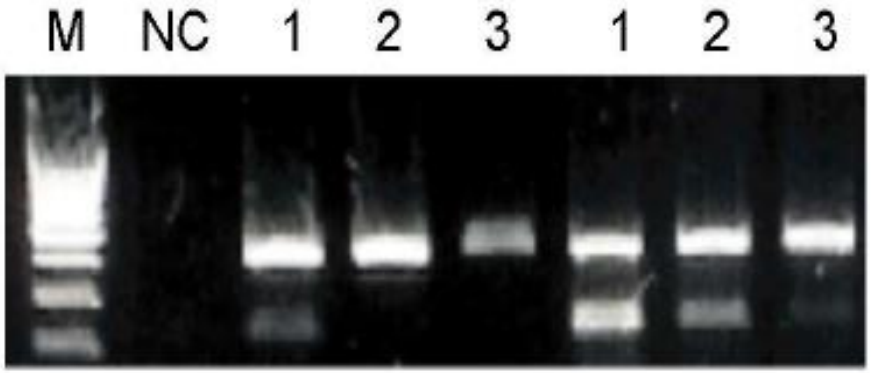
In Situ PCR

- **In Situ PCR (ISH)** is a polymerase chain reaction that actually takes place inside the cell on a slide. In situ PCR amplification can be performed on fixed tissue or cells.
- Applies the methodology of hybridization of the nucleic acids.
- Allows identification of cellular markers
- Limited to detection of non-genomic material such as RNA, genes or genomes

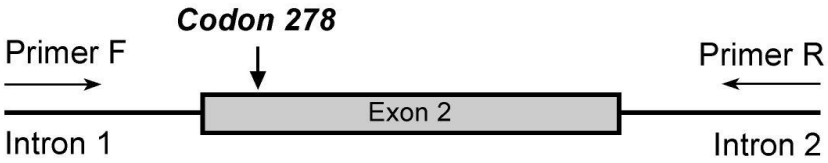


Multiplex PCR

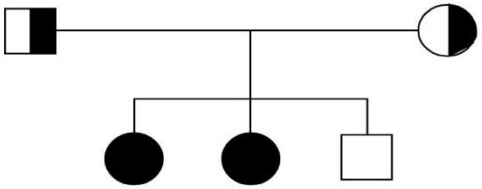
- Multiplex PCR is a variant of PCR which enabling simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers.

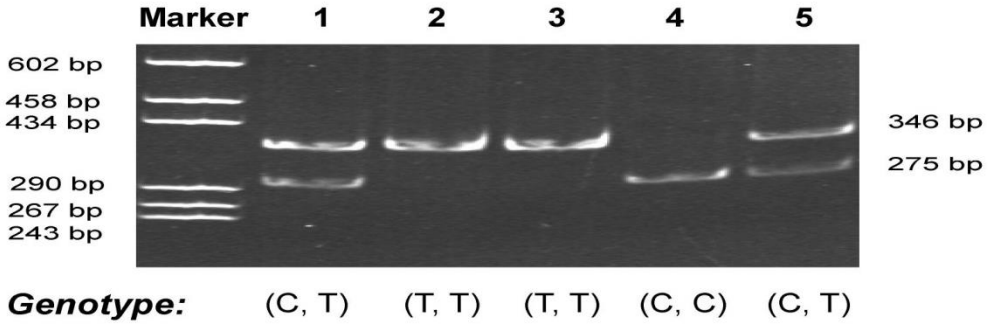


RFLP

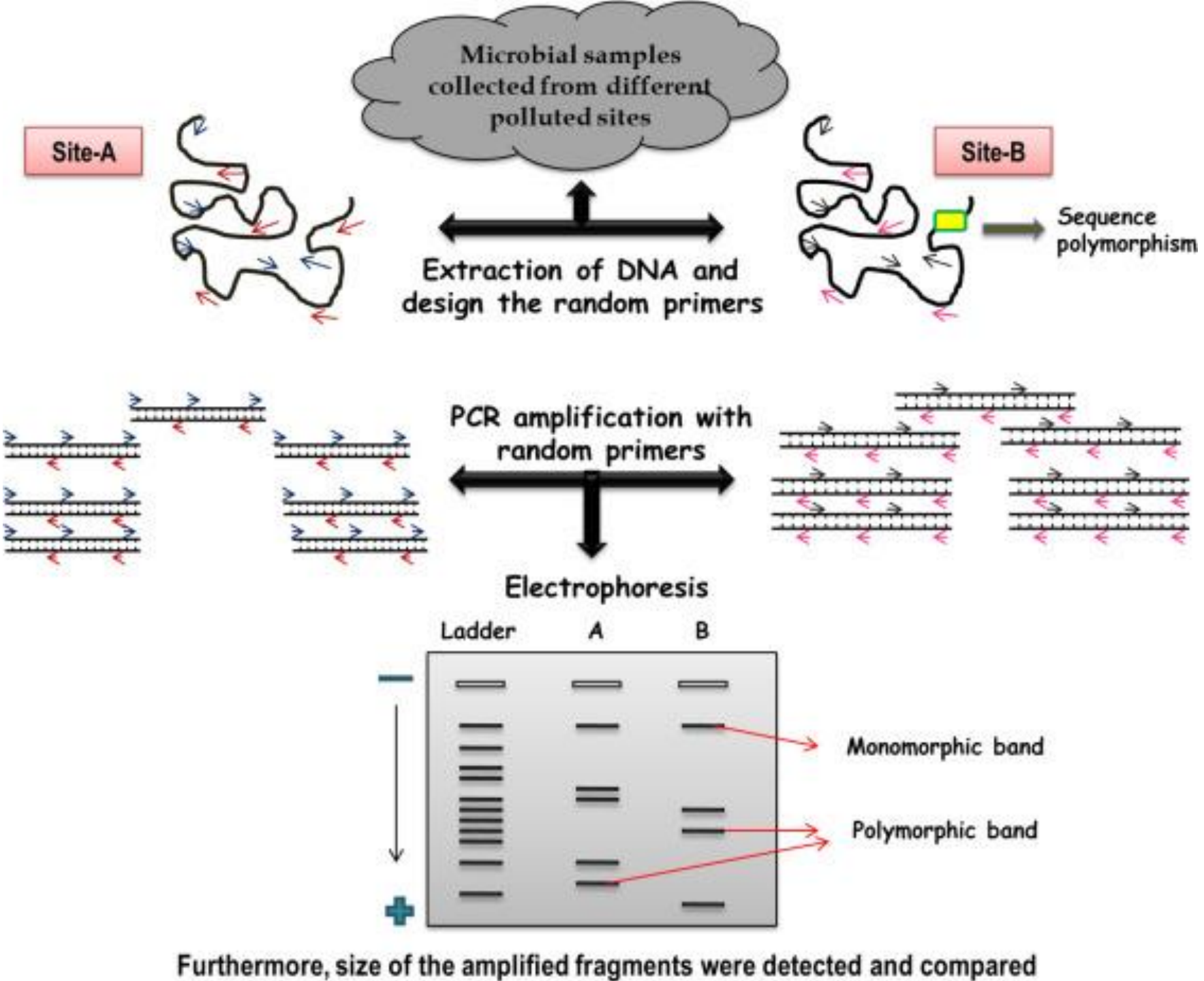


Allele	Codon 278	BsI I site	Digest
C (normal)	CGA (Arg)	+	(275+71) bp
T (mutant)	TGA (stop)	-	346 bp



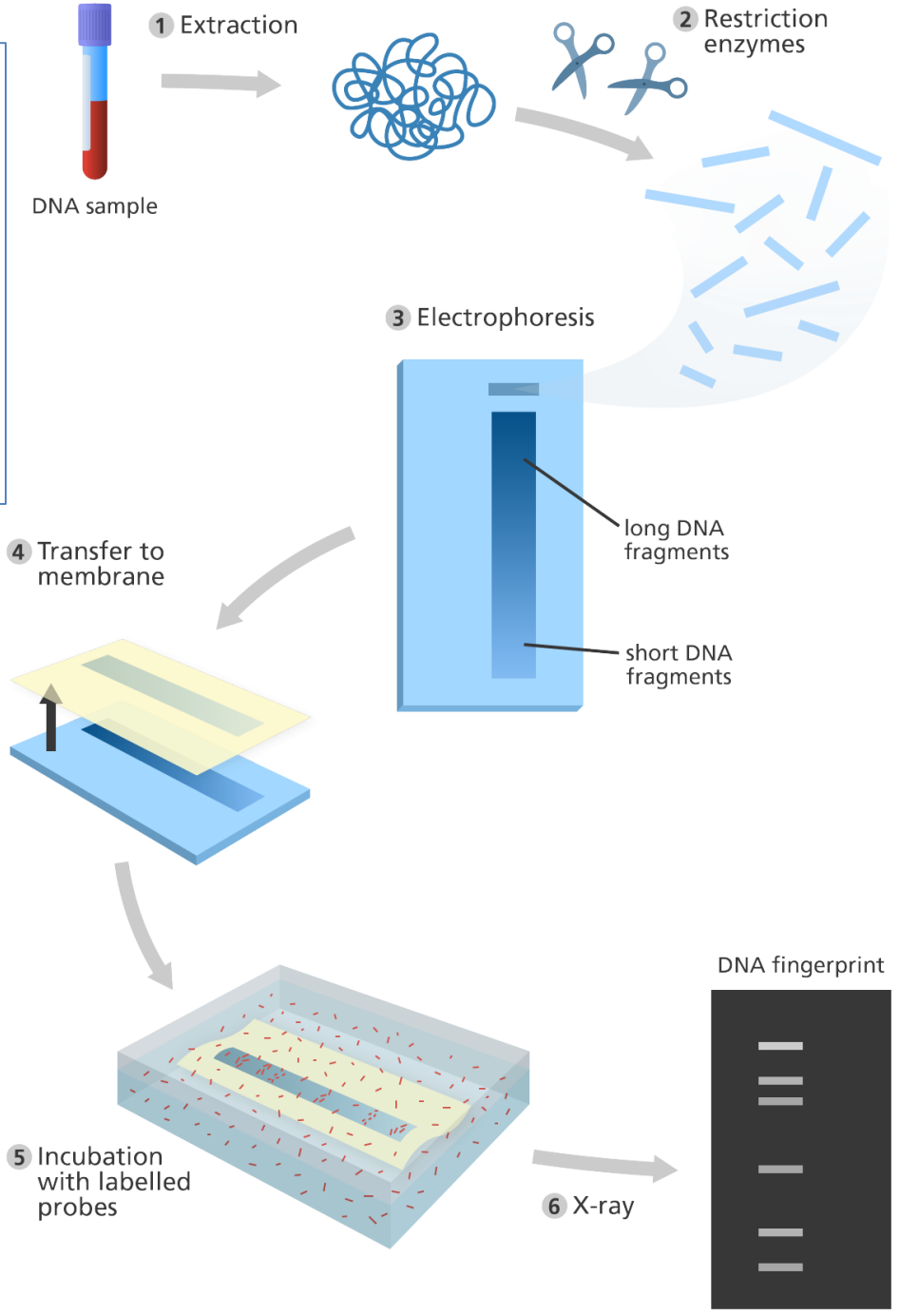


RAPD
(Random Amplified Polymorphic DNA)

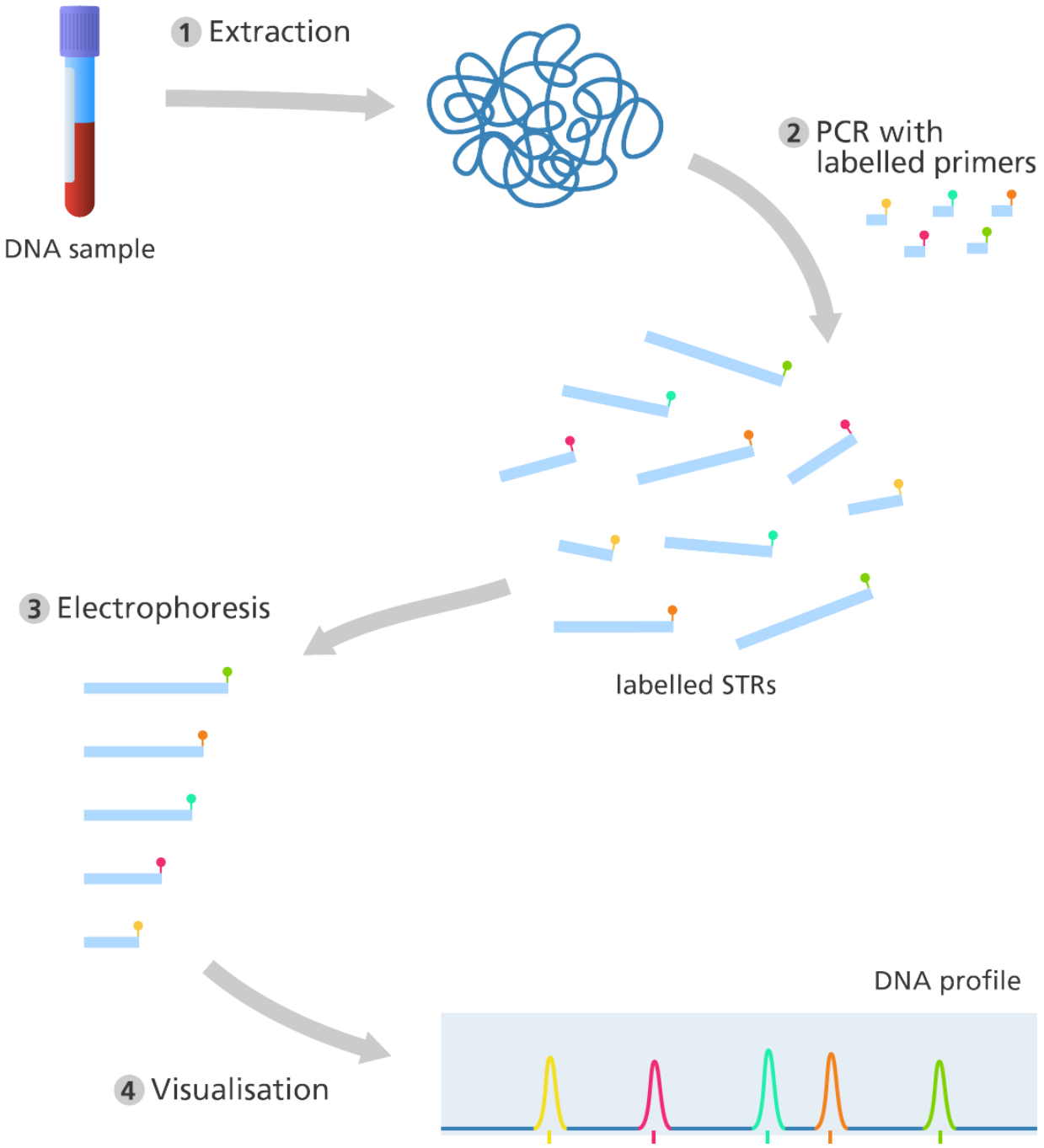

Microbial samples collected from different polluted sites
Site-A
Site-B
Sequence polymorphism
Extraction of DNA and design the random primers
PCR amplification with random primers
Electrophoresis
Ladder
A
B
Monomorphic band
Polymorphic band
Furthermore, size of the amplified fragments were detected and compared

DNA Fingerprinting/ DNA Profiling

The technique was developed in 1984 by British geneticist Alec Jeffreys, after he noticed that certain sequences of highly variable DNA (known as minisatellites), which do not contribute to the functions of genes, are repeated within genes. Jeffreys recognized that each individual has a unique pattern of minisatellites (the only exceptions being multiple individuals from a single zygote, such as identical twins).



https://www.encyclopedia.com/science-and-technology/biology-and-genetics/genetics-and-genetic-engineering/dna-fingerprinting


1 Extraction
DNA sample
2 PCR with labelled primers
labelled STRs
3 Electrophoresis
4 Visualisation
DNA profile

BGM3009 Presentation

On

Electrophoretic Mobility Shift Assay (EMSA):

A Method for Analysing Protein-DNA Interactions

Presenters:

Kate Wisdom, Songo Lolomari, Nicholas Leach & Abhay Jethwani

Aims and Objectives:

- To describe the basic principles behind EMSA
- To highlight methods used in EMSA
- To discuss the applications of EMSA including
  - Kd evaluation &
  - Conformation changes in DNA upon protein binding.

School of Biomedical Sciences

Definition and Basic Principle

- Technique used to study interactions between proteins and DNA.
- Simple, efficient and sensitive technique

- DNA moves through the gel faster when not bound to protein
- A reduction in electrophoretic mobility shows that a complex is formed between DNA and protein

- Can be used to identify DNA-binding proteins present in a nuclear cell extract. For example, transcription factors.

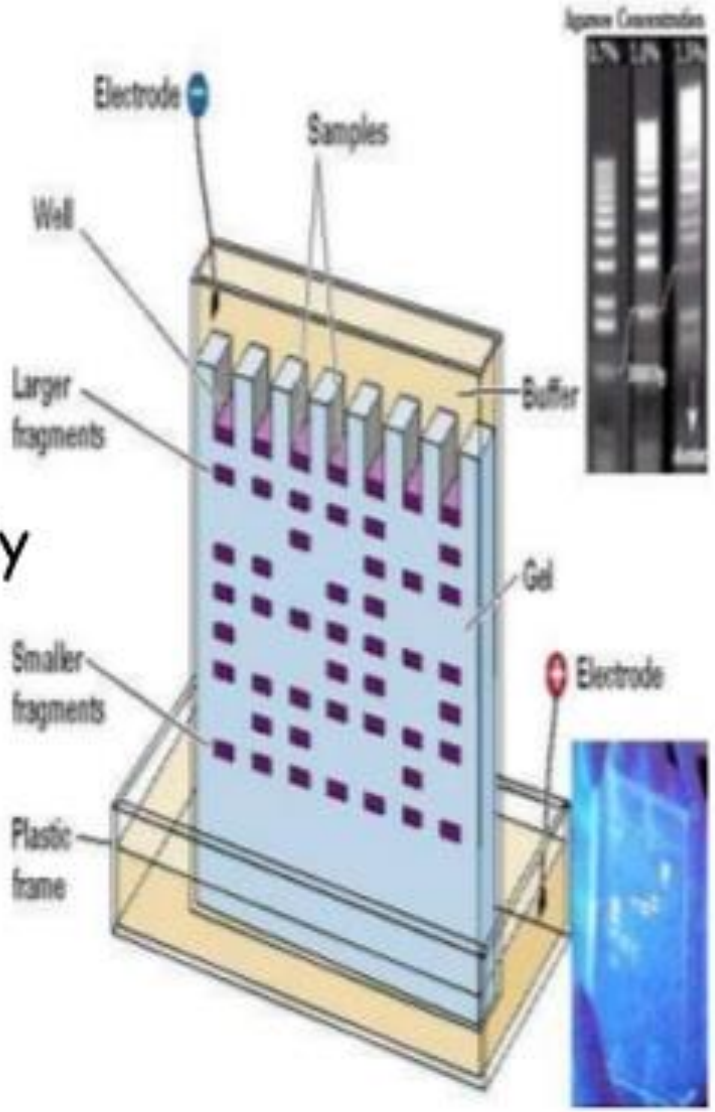


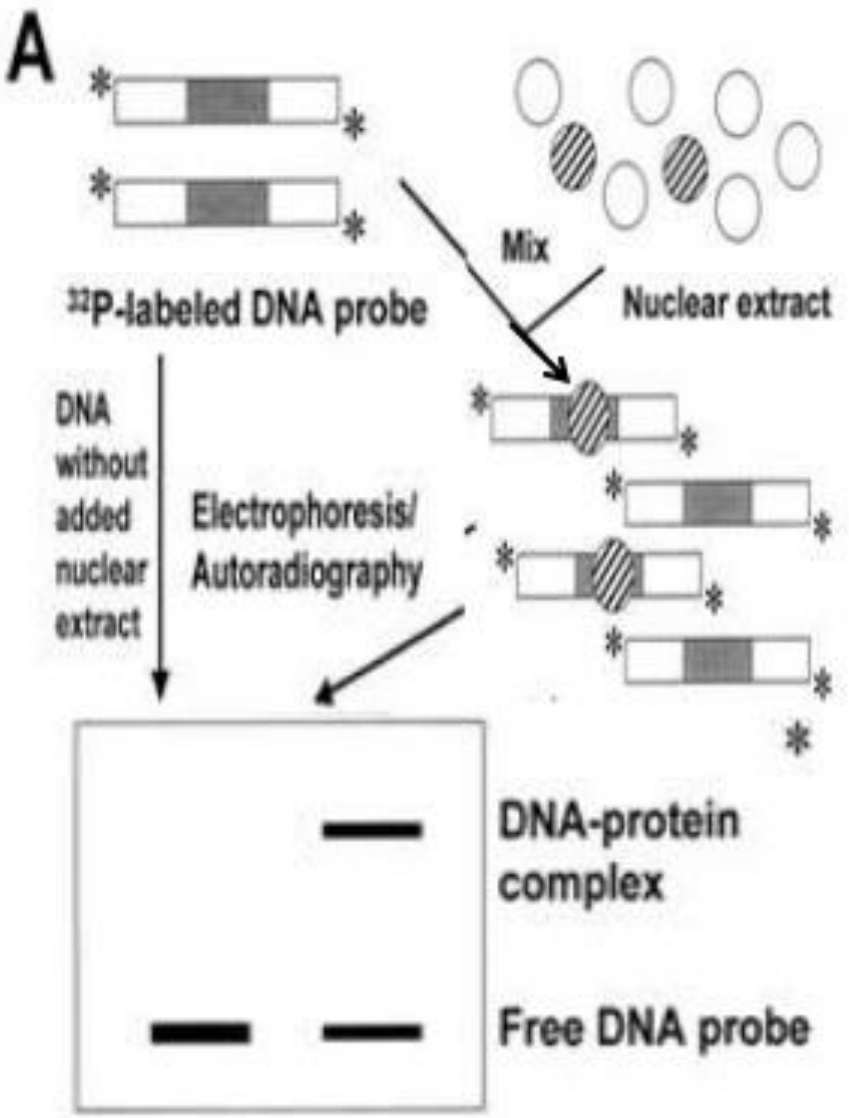
Fig. Source: http://universe-review.ca/R11-16-DNAsequencing.htm

Methods

5 basic steps are in conventional EMSA protocol

- –Preparation of purified or crude protein sample
- –Preparation of nucleic acid
- –Binding reactions
- –Non-denaturing gel electrophoresis
- –Detection of the outcome

EMSA Variant: Supershift assay



Yang, V.W. (1998), *JN*, 128(11), pp. 2045-2051

Advantages and Limitations

Advantages

- It is a simple method to perform but yet is robust enough to include a wide range of conditions.
- Highly sensitive method . Assays could be performed with small nucleic acid concentrations and small sample volumes.
- EMSA could also be used with a wide range of nucleic acid sizes and structures as well as wide range of proteins.
- Finally, it is possible to use both crude protein extracts and purified recombinant proteins.

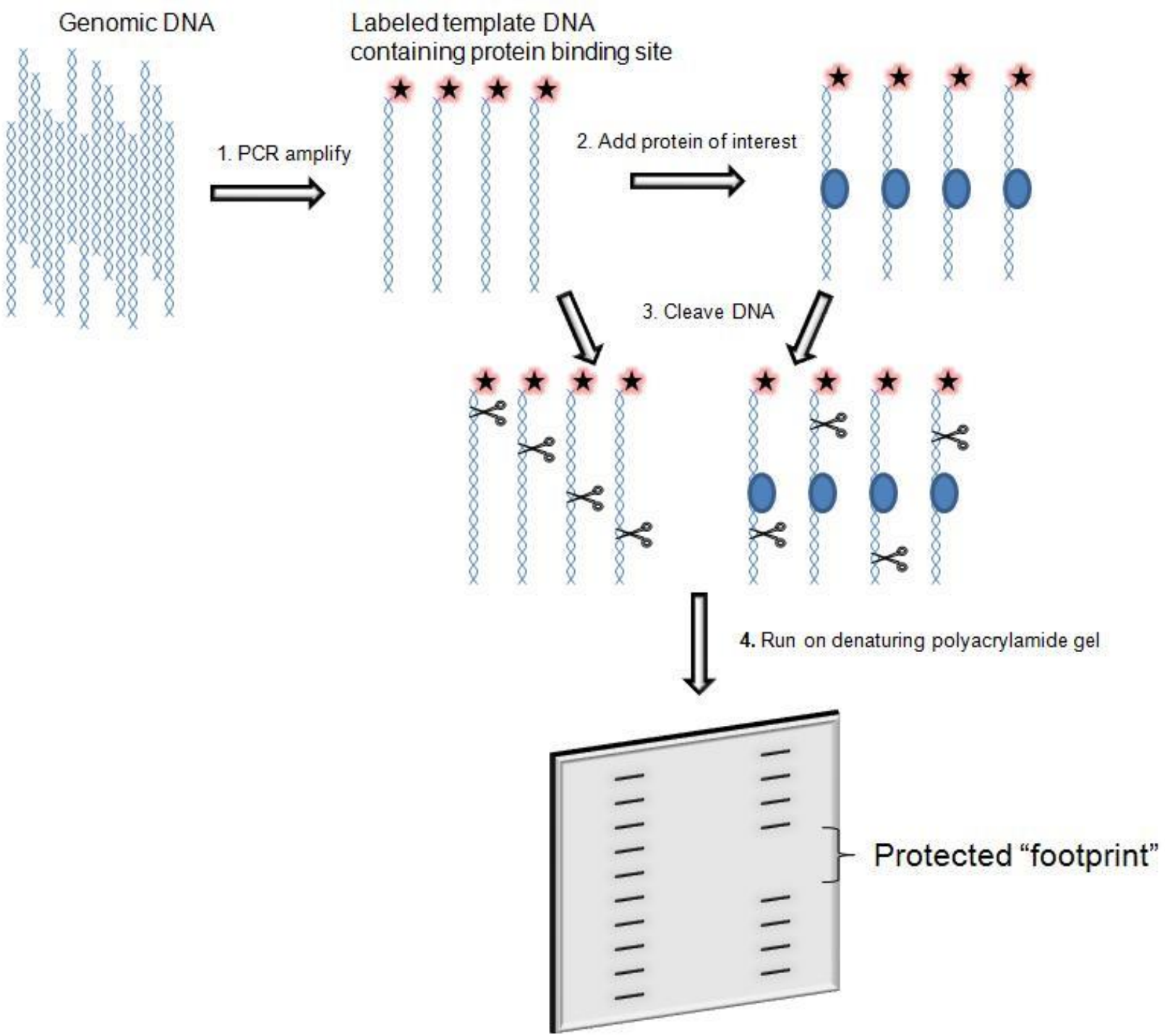
Limitations

- Dissociation is one of the drawbacks of EMSA. It occurs during electrophoresis thus prevents detection.
- EMSA doesn't provide information on the nucleic acid sequence the proteins are bound to.
- Not an appropriate method for Kinetic studies.
- Does not provide a straightforward measure of the weights of the proteins as mobility is influenced by several other factors.

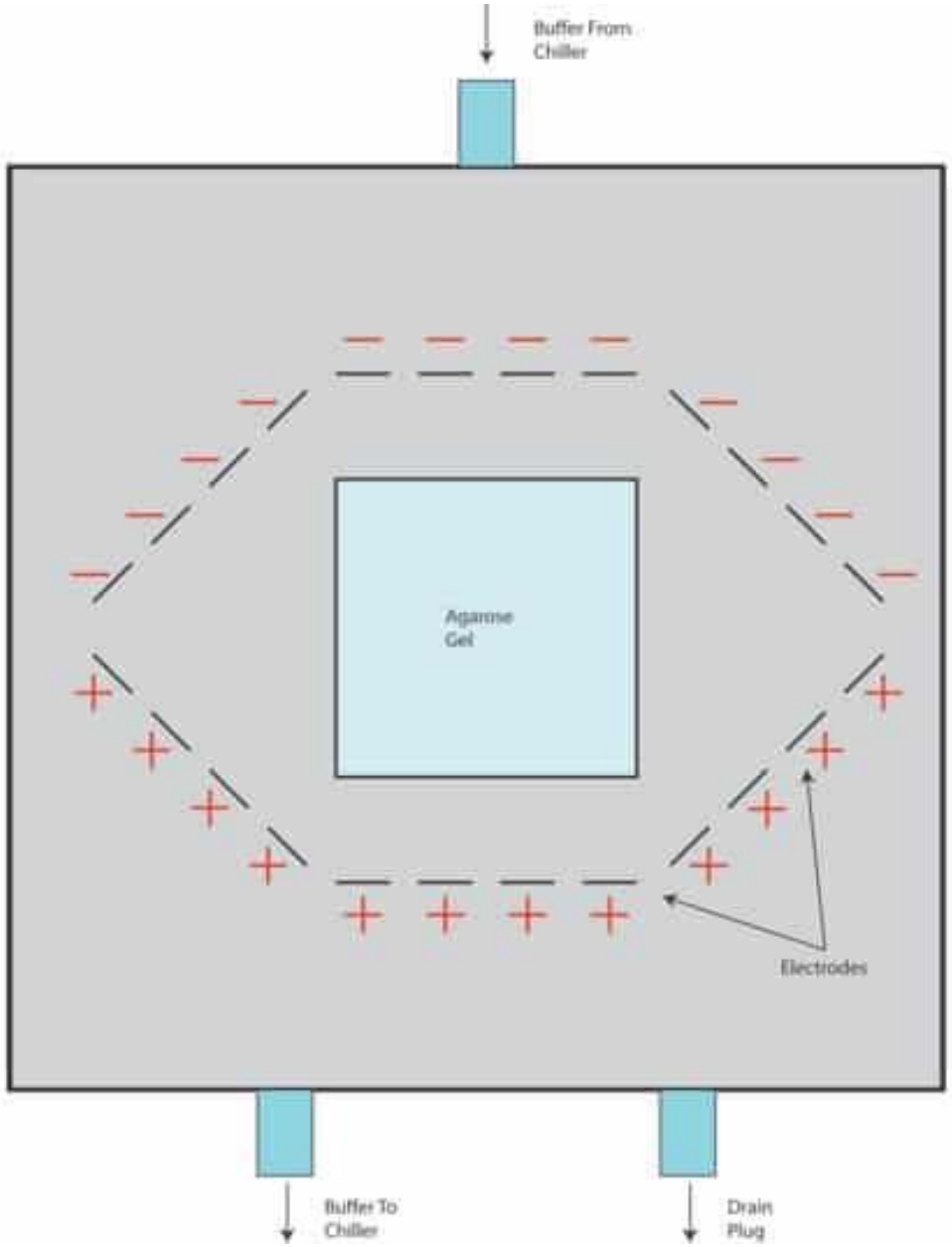
Conclusion

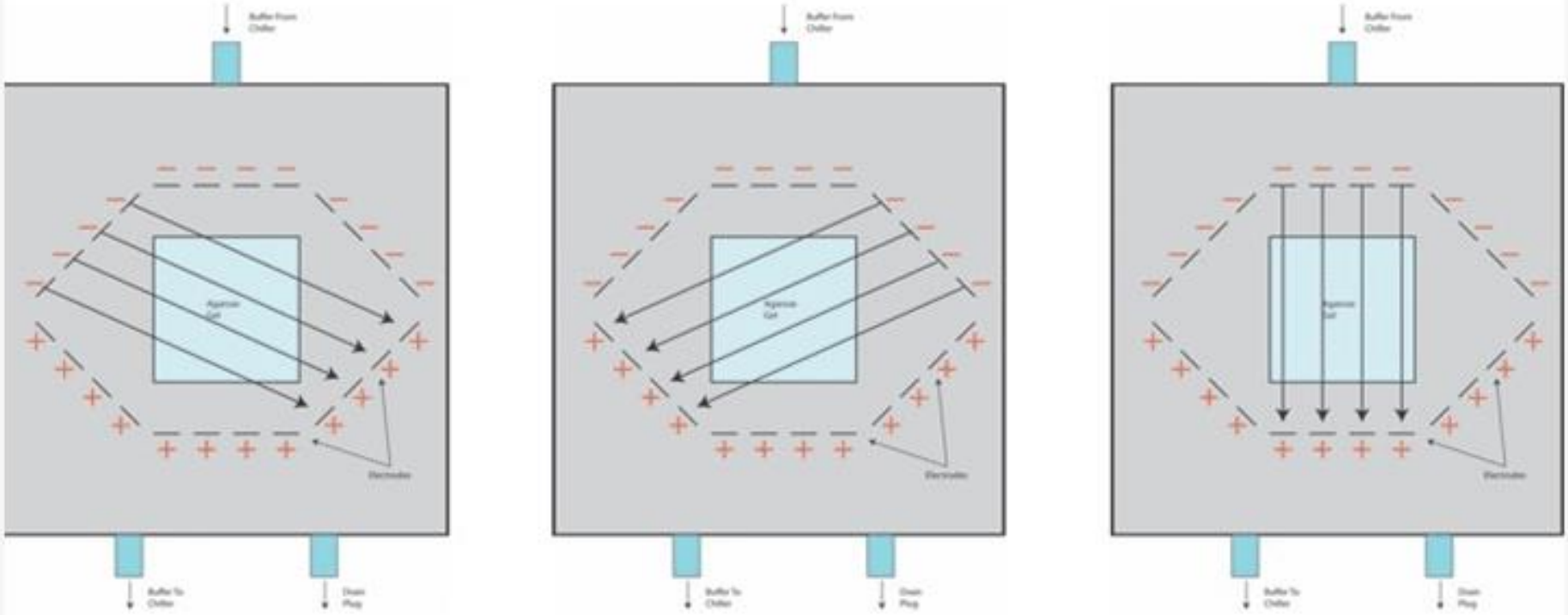
- Electrophoretic Mobility Shift Assay (EMSA) is the most widely used method for the detection of protein-DNA interactions.
- Works on the observation that protein-bound DNA migrate slowly as compared to free DNA when subjected through electrophoresis through a non-denaturing gel.
- Used for various purposes such as quantifying interactions between proteins and DNA, determination of binding affinities but most importantly in the characterization of Transcription Factors.
- There are several alternatives to EMSA which include Foot printing, Yeast hybrid systems, etc.

DNA Footprinting


Genomic DNA
Labeled template DNA
containing protein binding site
1. PCR amplify
2. Add protein of interest
3. Cleave DNA
4. Run on denaturing polyacrylamide gel
Protected "footprint"

Pulsed Field Gel Electrophoresis


Buffer From
Chiller
Agarose
Gel
Electrodes
Buffer To
Chiller
Drain
Plug



1st Pulse

2nd Pulse

3rd Pulse

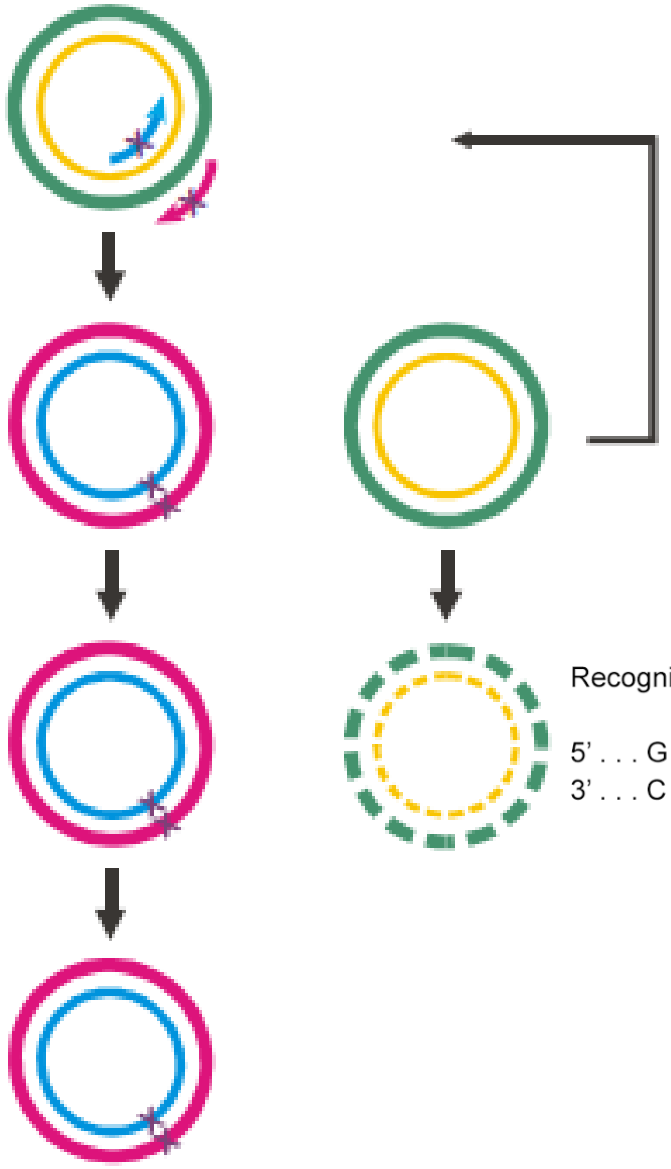
An example of a single PFGE cycle, the arrows indicate which electrodes are active during a certain pulse cycle. The DNA is pulled at different angles throughout the program, with the net result being the DNA moving slowly towards the bottom of the gel.

SDM

Site Directed Mutagenesis

- Site-directed mutagenesis is a molecular biology method that is used to make **specific and intentional changes** to the DNA sequence of a gene and any gene products.
- Also called as site-specific mutagenesis or oligonucleotide-directed mutagenesis.
- This can be done by using oligonucleotides in a primer extension method with DNA polymerase (in PCR), developed by **Michael Smith** who was awarded a Nobel Prize in 1993 for this contribution.

One-Day Method



Mutant Strand Synthesis
Perform thermal cycling to:
1) Denature DNA template
2) Anneal mutagenic primers containing desired mutation
3) Extend primers with *PfuUltra* DNA polymerase

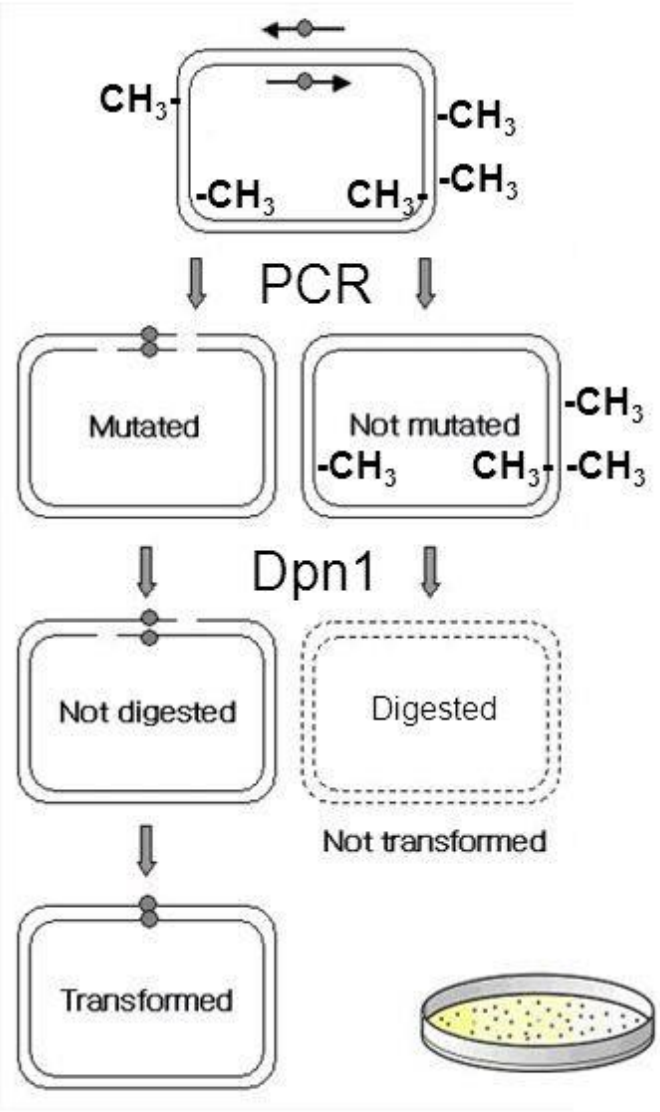
Recognition Site:

5' . . . G A T C . . . 3'
3' . . . C T A G . . . 5'

***Dpn* I Digestion of Template**
Digest parental methylated and hemimethylated DNA with *Dpn* I

Transformation
Transform mutated molecule into competent cells for nick repair

Site-directed mutagenesis



1. Denature methylated template and anneal divergent mutagenic primers.
2. PCR amplify the entire plasmid with a DNA pol lacking 5'-->3' exonuclease.
3. Select against parental strands with Dpn1 restriction enzyme, which cuts methylated DNA.
4. Transform