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BIO203 PPT Slide

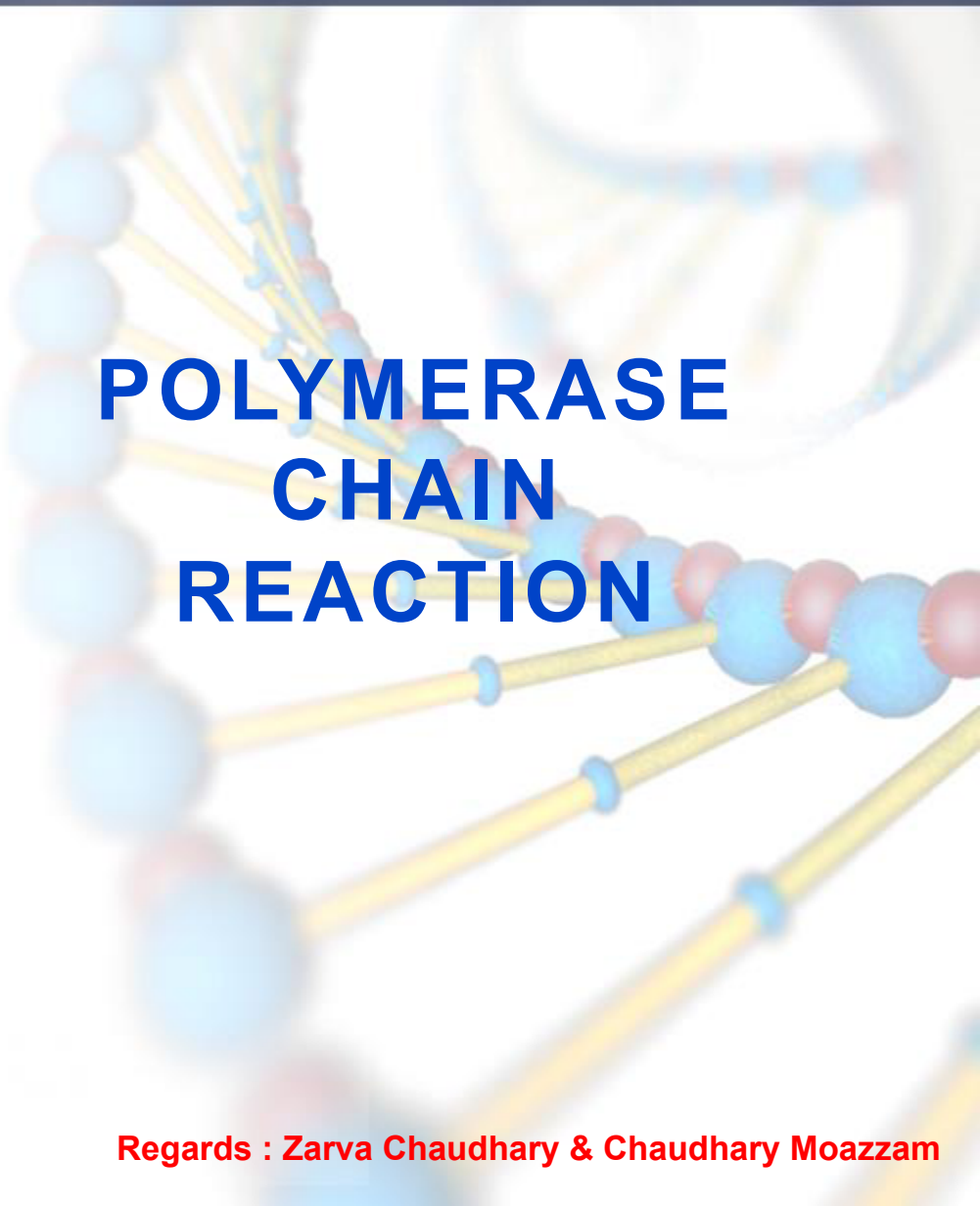
1 To 82

Merged File date 2020

Regards : Zarva Chaudhary

"Chaudhary Moazzam"

Essentials of Genetics



POLYMERASE CHAIN REACTION

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Polymerase Chain Reaction

PCR

- A technique widely used in Molecular Biology and Biotechnology.
- Its name is from one of its key component - DNA polymerase.

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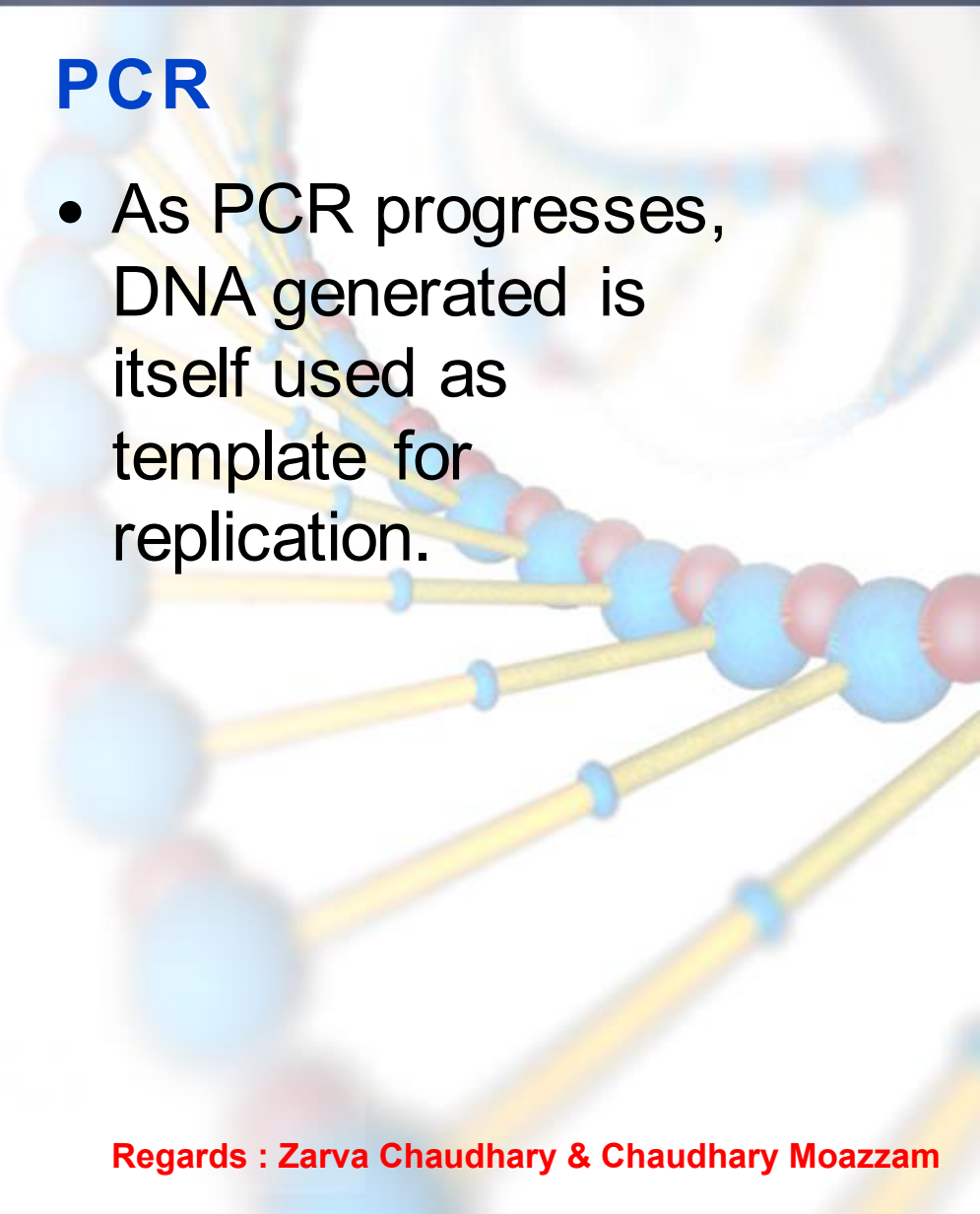
Polymerase Chain Reaction

PCR

- As PCR progresses, DNA generated is itself used as template for replication.



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Polymerase Chain Reaction

PCR- A CHAIN REACTION

- This sets in motion of a chain reaction in which the DNA template is exponentially amplified.

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Polymerase Chain Reaction

HEAT STABLE DNA POLYMERASE

During PCR, a heat-stable DNA polymerase, such as Taq polymerase - an enzyme derived from the bacterium Thermus aquaticus.

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Polymerase Chain Reaction

CONCLUSION

- PCR can be performed to amplify DNA.
- It can be extensively modified to perform a wide array of genetic manipulations.

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STEPS OF PCR

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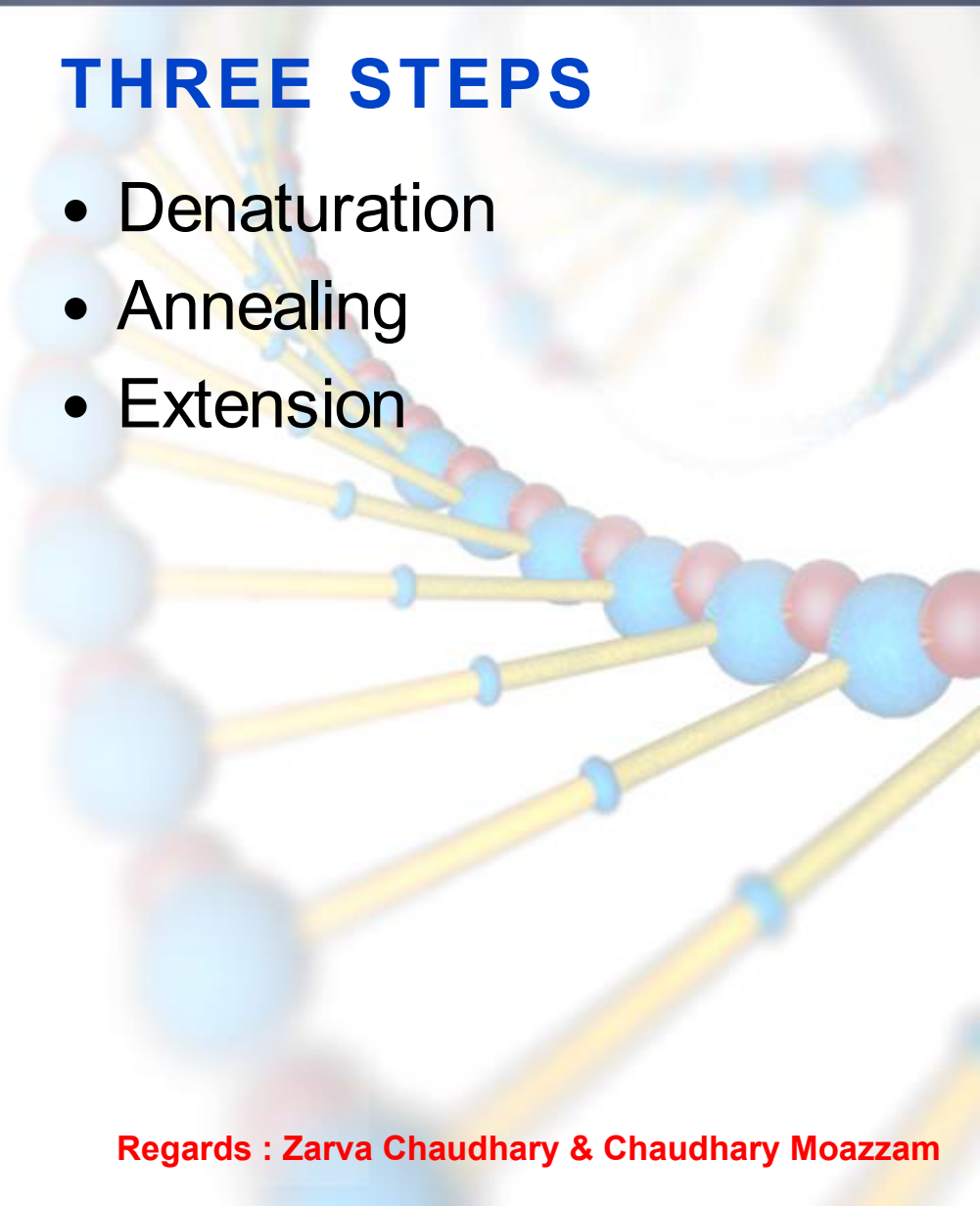
Steps of PCR

THREE STEPS

- Denaturation
- Annealing
- Extension



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Steps of PCR

INITIALIZATION STEP

- This step consists of heating the reaction to a temperature of 93°C - 96°C, which is held for 1-10 minutes.

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Steps of PCR

DENATURATION

- First regular cycling event and consists of heating the reaction to 93°C - 98°C for 20-45 seconds.
- It causes melting of DNA template yielding single strands of DNA.

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Steps of PCR

ANNEALING

- The reaction temperature is lowered to 40-65°C for 20-40 seconds.
- Annealing of the primers to the single-stranded DNA template.

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Steps of PCR

EXTENSION

- The temperature at this step depends on the type of DNA polymerase used.
- Taq polymerase has its optimum activity temperature at 70-80°C.
- Commonly 72°C is used.

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Steps of PCR

EXTENSION

- At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTP's.

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Steps of PCR

FINAL ELONGATION

- Single step is performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

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Steps of PCR

FINAL HOLD TEMPERATURE

- This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

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INGREDIENTS OF PCR

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Ingredients of PCR

MAJOR INGREDIENTS

- Microfuge tube
- Thermal cycler
- DNA template
- Primers
- Buffer
- MgCl2

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Ingredients of PCR

MAJOR INGREDIENTS

- Distilled water
- Deoxynucleotide triphosphates
- DNA polymerase

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Ingredients of PCR

MICROFUGE TUBES

- These are small cylindrical plastic tubes with conical bottoms.

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Ingredients of PCR

THERMOCYCLER

- The thermocycler works on the principle of Peltier effect, which raises and lowers the temperature of the block in a pre-programmed manner.

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Ingredients of PCR

DNA POLYMERASES

- Polymerases
  - Taq polymerase
  - Pfu polymerase
  - Vent polymerase

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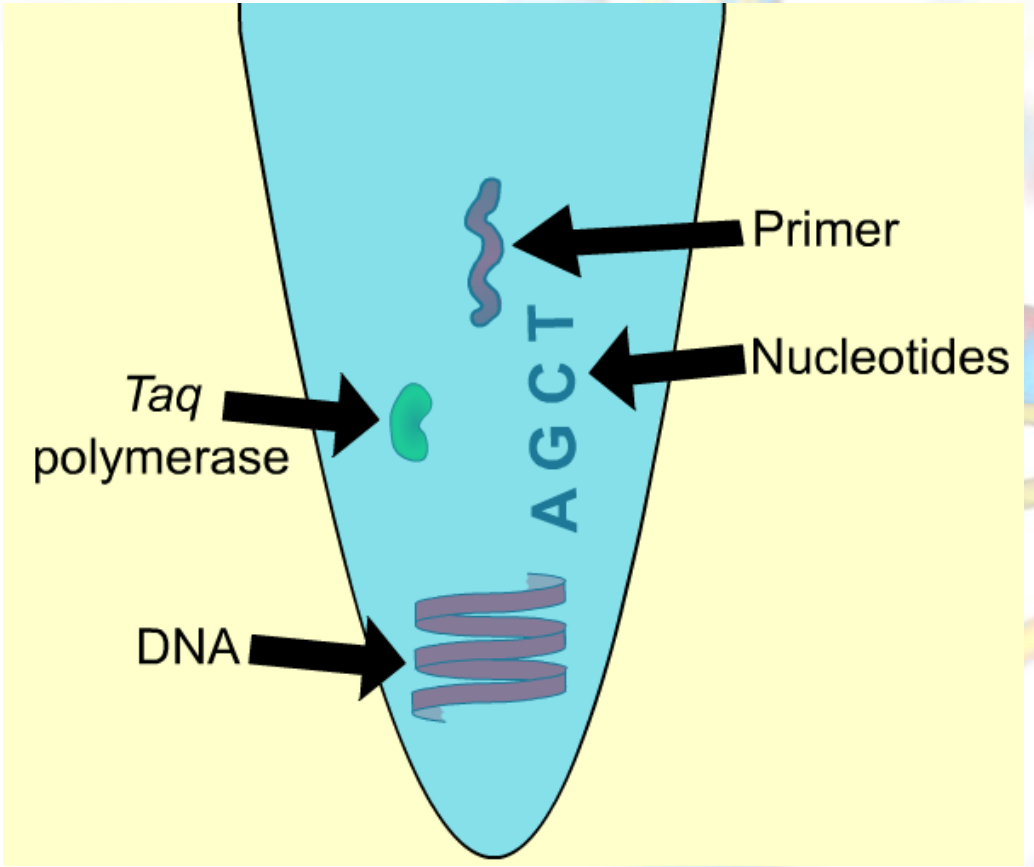
Ingredients of PCR

INGREDIENTS IN PCR TUBE



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Ingredients of PCR

TEMPLATE CAN BE DNA OR RNA

- Template DNA
- RNA in case of reverse transcriptase

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PRIMERS FOR PCR

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PRIMERS FOR PCR

PRIMERS

- Primers are single-stranded 18–30 bp long DNA fragments
- Complementary to sequences flanking the region to be amplified.

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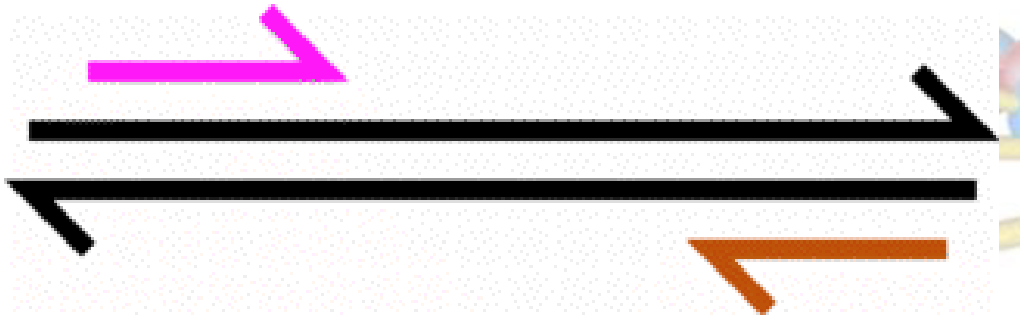
PRIMERS FOR PCR

PRIMERS



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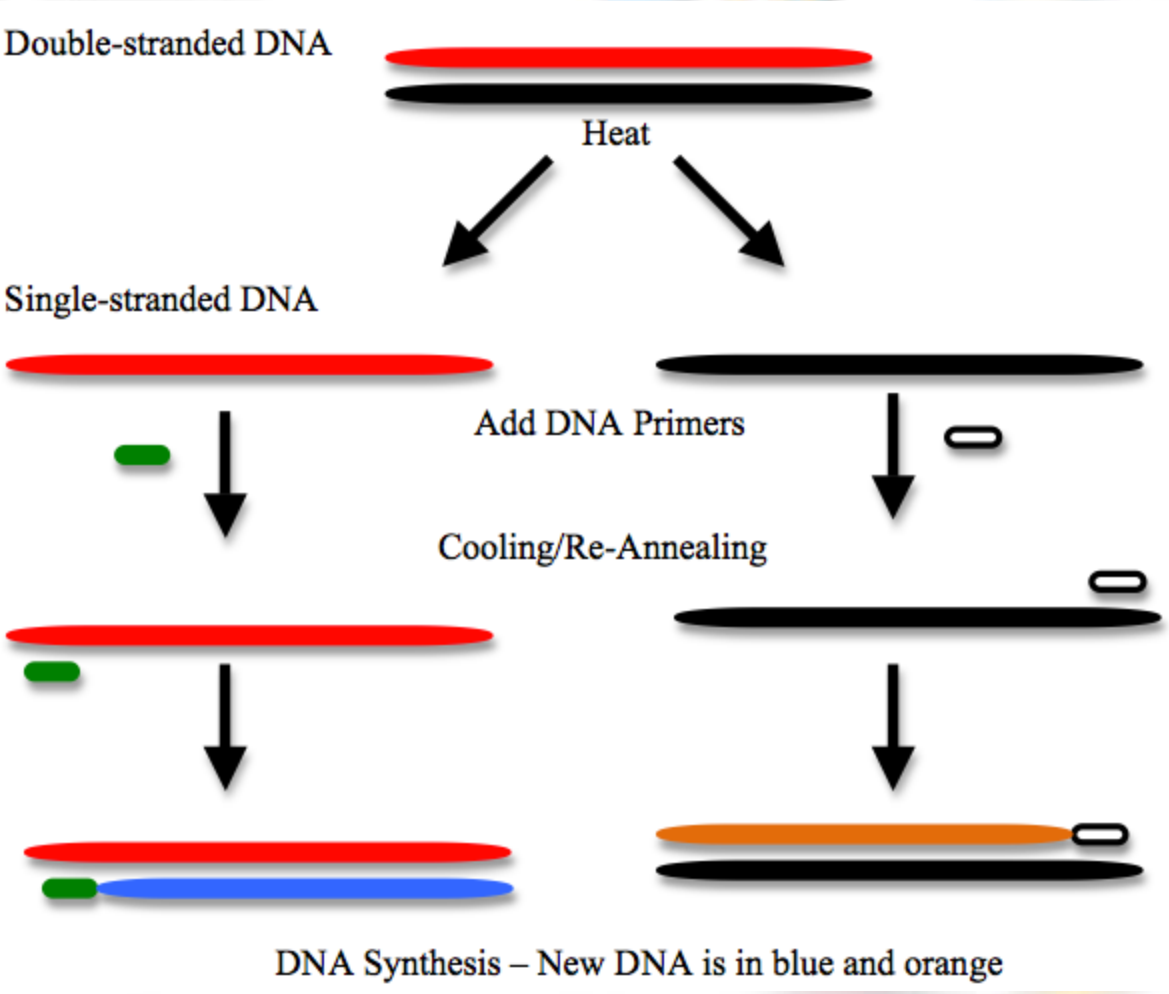
PRIMERS FOR PCR

PRIMERS



DNA Synthesis – New DNA is in blue and orange

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PRIMERS FOR PCR

PRIMERS CAN BE SPECIFIC OR RANDOM

- Primers determine the specificity of the PCR reaction.
- Distance between the primers binding sites will determine the size of PCR product.

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PRIMERS FOR PCR

FEATURES OF PRIMERS

- Types of primers- random or specific
- Primer length
- Annealing temperature
- Specificity
- Nucleotide composition

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PRIMERS FOR PCR

PRIMERS

- Avoid inter-strand homologies
- Avoid intra-strand homologies
- T_m of forward primer = T_m of reverse primer
- G/C content of 20–80%; avoid longer than GGGG
- Product size (100–700 bp)
- Target specificity

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PRIMERS FOR PCR

FORMULA FOR CALCULATING MELTING TEMPERATURE OF PRIMERS

- Tm = 4(G+C) + 2(A+T)

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DNA POLYMERASES

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DNA Polymerases

POLYMERASES

Polymerase	Extension Rate (nt/sec)	Source
Taq pol	75	*T. aquaticus*
Amplitaq (Stoffel fragment)	>50	*T. aquaticus*
Vent	>80	*Thermococcus litoralis*
Pfu	60	*Pyrococcus furiosus*
Tth (RT activity)	>33	*T. thermophilus*

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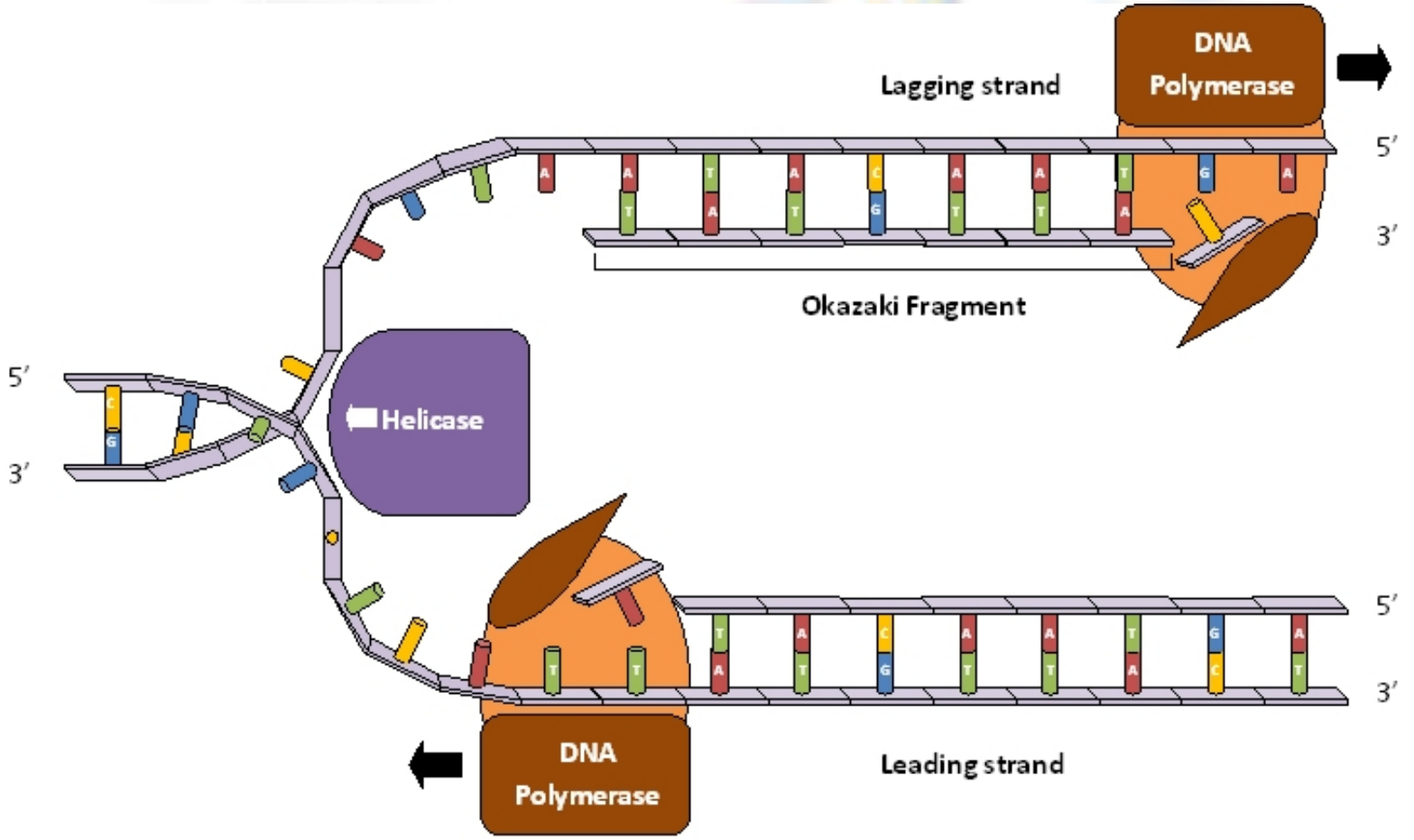
DNA Polymerases

POLYMERASES



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DNA Polymerases

POLYMERASES

- *Taq*: *Thermus aquaticus* (most commonly used)
- *Tfl*: *T. flavus*
- *Tth*: *T. thermophilus*
- *Tli*: *Thermococcus litoralis*
- *Pfu*: *Pyrococcus furiosus* (fidelity)

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DNA Polymerases

DNA POLYMERASES

- Polymerases
  - Taq polymerase
  - Pfu polymerase
  - Vent polymerase

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STANDARD PCR REACTION

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Standard PCR Reaction

STANDARD REACTION

0.25 mM each primer

0.2 mM each dATP, dCTP, dGTP, dTTP

50 mM KCl

10 mM Tris

1.5 mM $MgCl_2$

2.5 units polymerase

10^2 - 10^5 copies of template

50 ul reaction volume

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Standard PCR Reaction

PCR TEMPERATURES

- Denaturation temperature
  - Reduces double stranded molecules to single stranded
  - 90–96°C, 20-45 seconds
- Annealing temperature
  - Controls specificity of hybridization
  - 40–68°C, 20-30 seconds
- Extension temperature
  - Optimized for individual polymerases
  - 70–75°C, 30-45 seconds

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Standard PCR Reaction

TEMPERATURES

Temp	For	Comments
94-60-72	Perfect, long primers	Higher temp can be used; maximum annealling temp
94-55-72	Good or perfectly matched primers between 19-24 nt	Standard conditions
94-50-72	Adequate primers	Allows 1-3 mismatches/20 nt
94-48-68	Poorly matched primers	Allows 4-5 mismatches/20 nt
94-45-65	Unknown match, likely poor	Primers of questionable quality

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Standard PCR Reaction

TEMPERATURES

- Amplification takes place as the reaction mix is subject to an amplification program and temperatures.
- The amplification program consists of a series of 20–50 PCR cycles.

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INTERPRETATION OF RESULTS

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Interpretation of Results

INTERPRETATION

- The PCR product should be of the expected size.
- Misprimes may occur due to non-specific hybridization of primers.

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Interpretation of Results

PRIMERS DIMERS MAY REDUCE AMPLIFICATION

- Primer dimers may occur due to hybridization of primers to each other.

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Interpretation of Results

BLANK REACTION

- Controls for contamination

  contains all reagents except DNA template.

Interpretation of Results

NEGATIVE CONTROL

- Controls for specificity of the amplification reaction

  contains all reagents and a DNA template lacking the target sequence.

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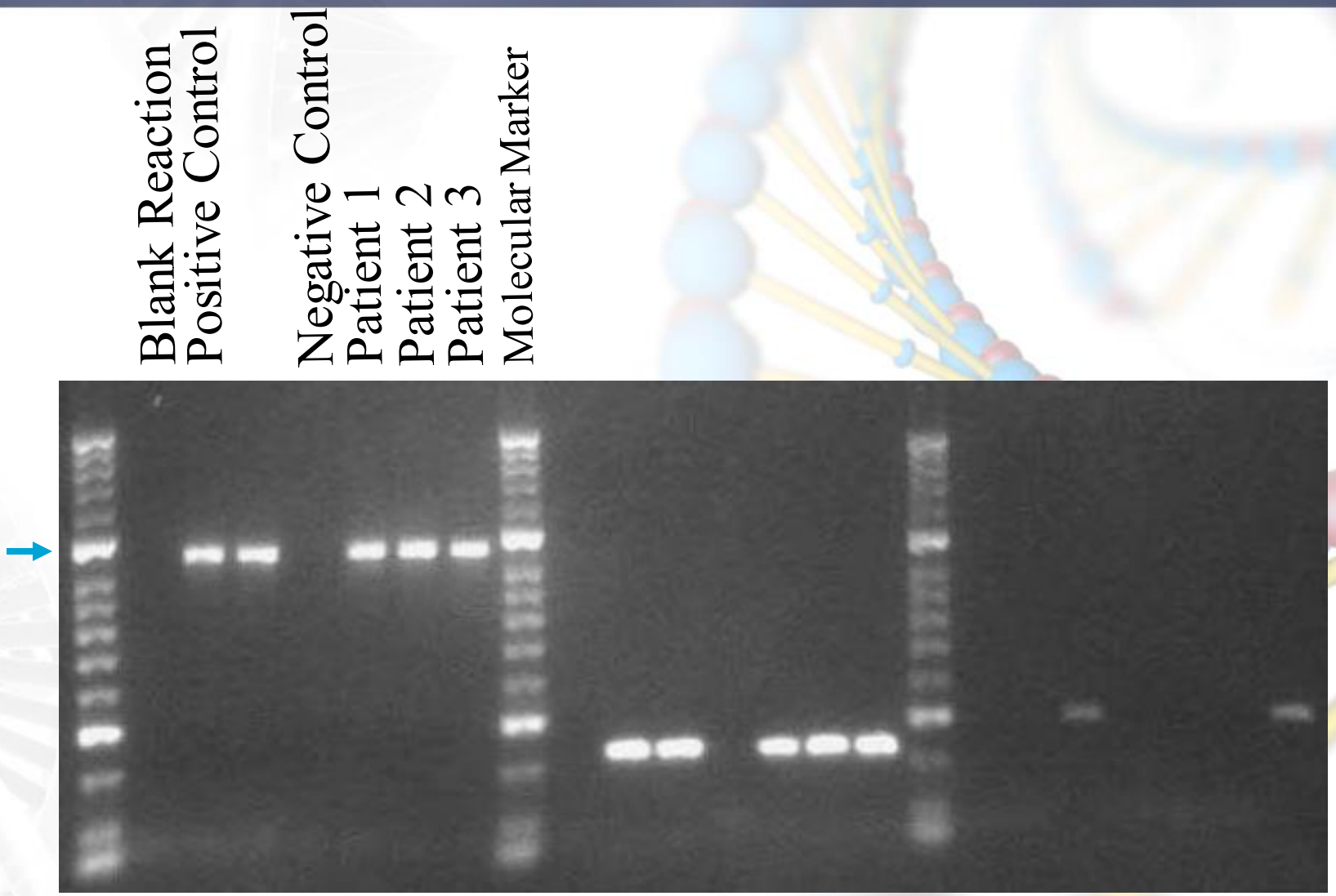
Interpretation of Results



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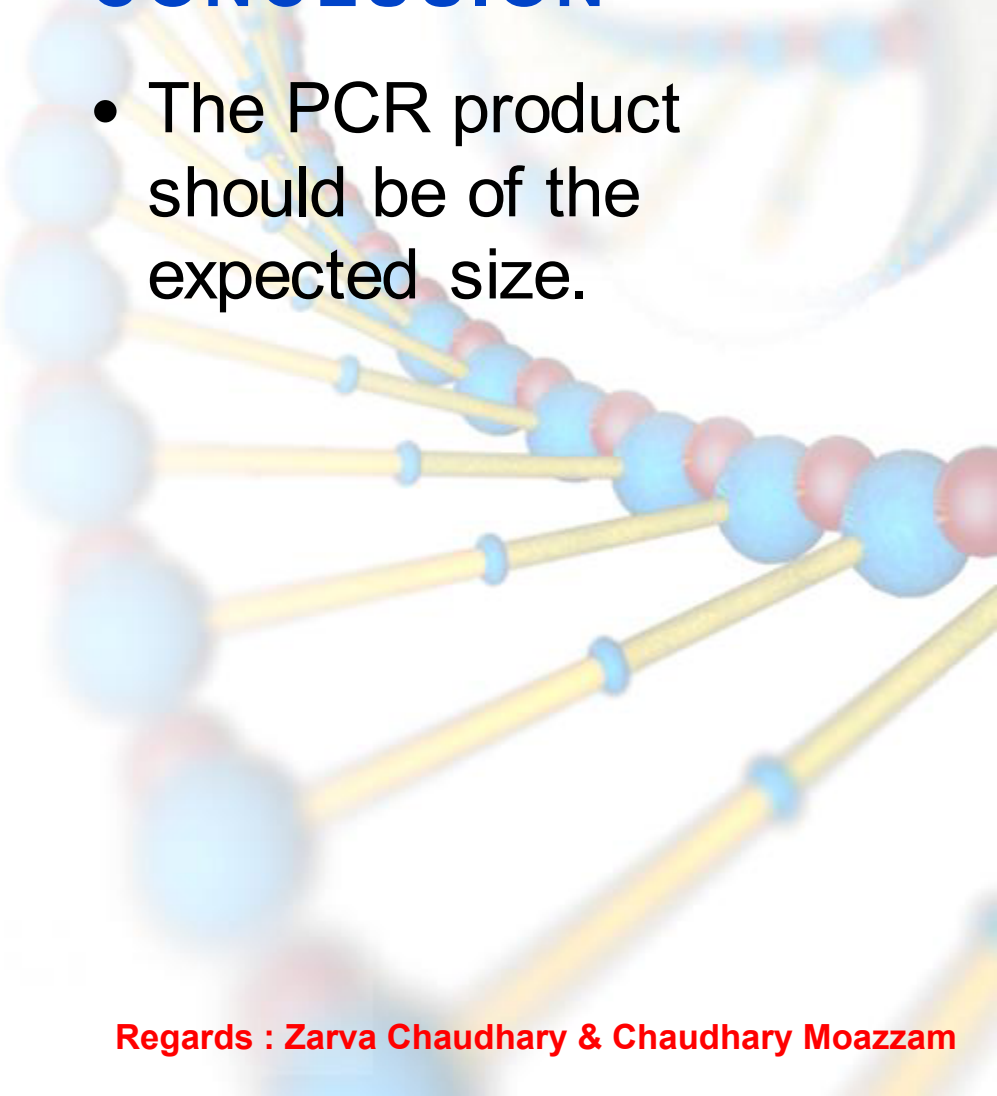
Interpretation of Results

CONCLUSION

- The PCR product should be of the expected size.



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TYPES OF PCR

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Types of PCR

TYPES

- Nested PCR
- Multiplex PCR
- Touchdown PCR
- Sequence-specific PCR
- Reverse-transcriptase PCR

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Types of PCR

TYPES

- Long-range PCR
- Whole-genome amplification
- RAPD PCR (AP-PCR)
- Quantitative real-time PCR

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Types of PCR

TYPES

- Long-range PCR
- Whole-genome amplification
- RAPD PCR (AP-PCR)
- Quantitative real-time PCR

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NESTED PCR

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Nested PCR

NESTED PCR

- Two pairs (instead of one pair) of PCR primers are used to amplify a fragment.
- First pair amplify a fragment similar to a standard PCR.

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Nested PCR

NESTED PCR – SECOND PAIR OF PRIMERS

- Second pair of primers - nested primers (as they lie / are nested within the first fragment).

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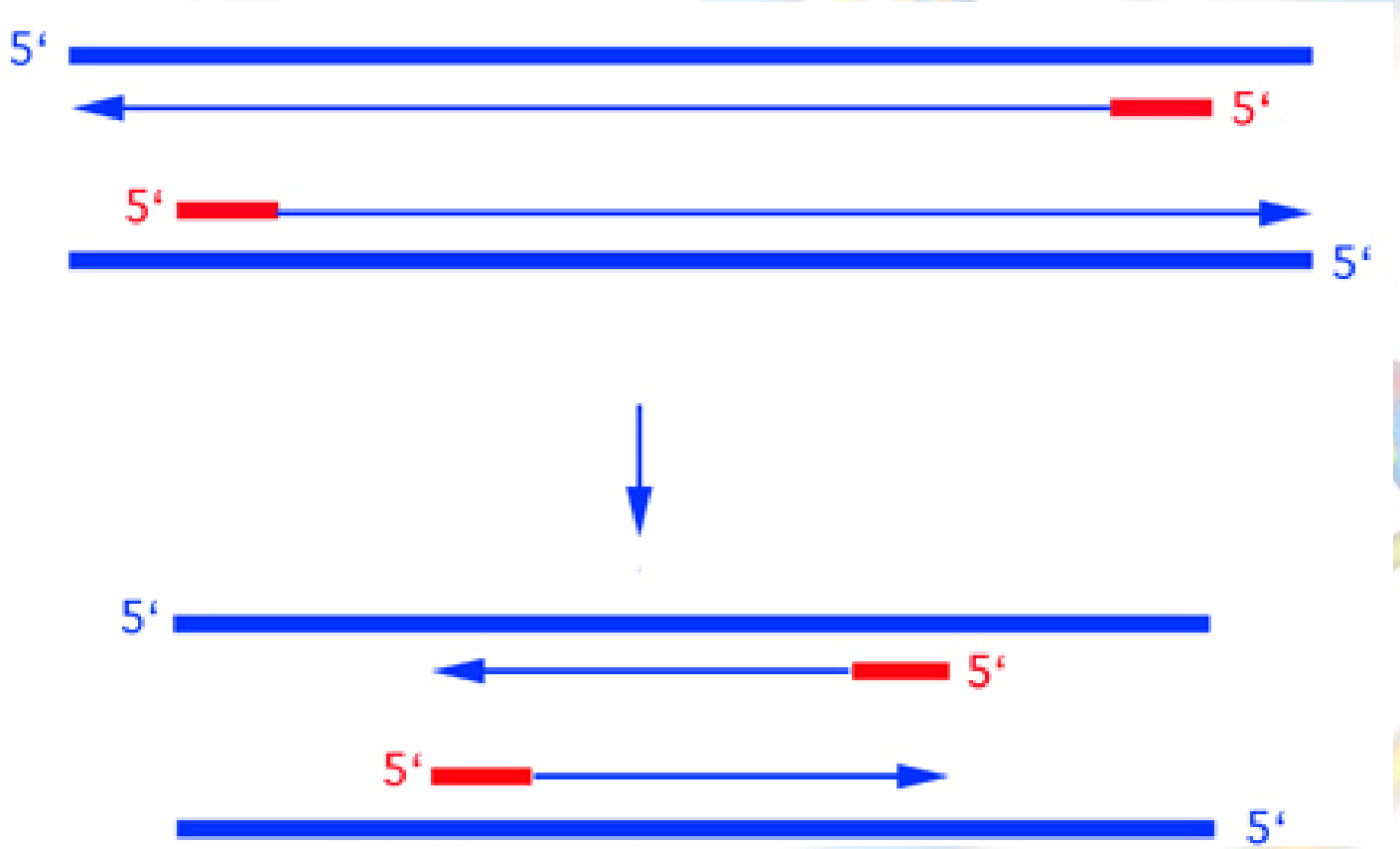
Nested PCR

NESTED PCR



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Nested PCR

SECOND PCR PRODUCT IS SHORTER THAN FIRST ONE

- Second pair of primers bind inside the first PCR product fragment to allow amplification of a second PCR product.

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Nested PCR

SECOND PCR PRODUCT IS SPECIFIC

- increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA.

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Nested PCR

ADVANTAGE OF NESTED PCR

- Very low probability of nonspecific amplification.

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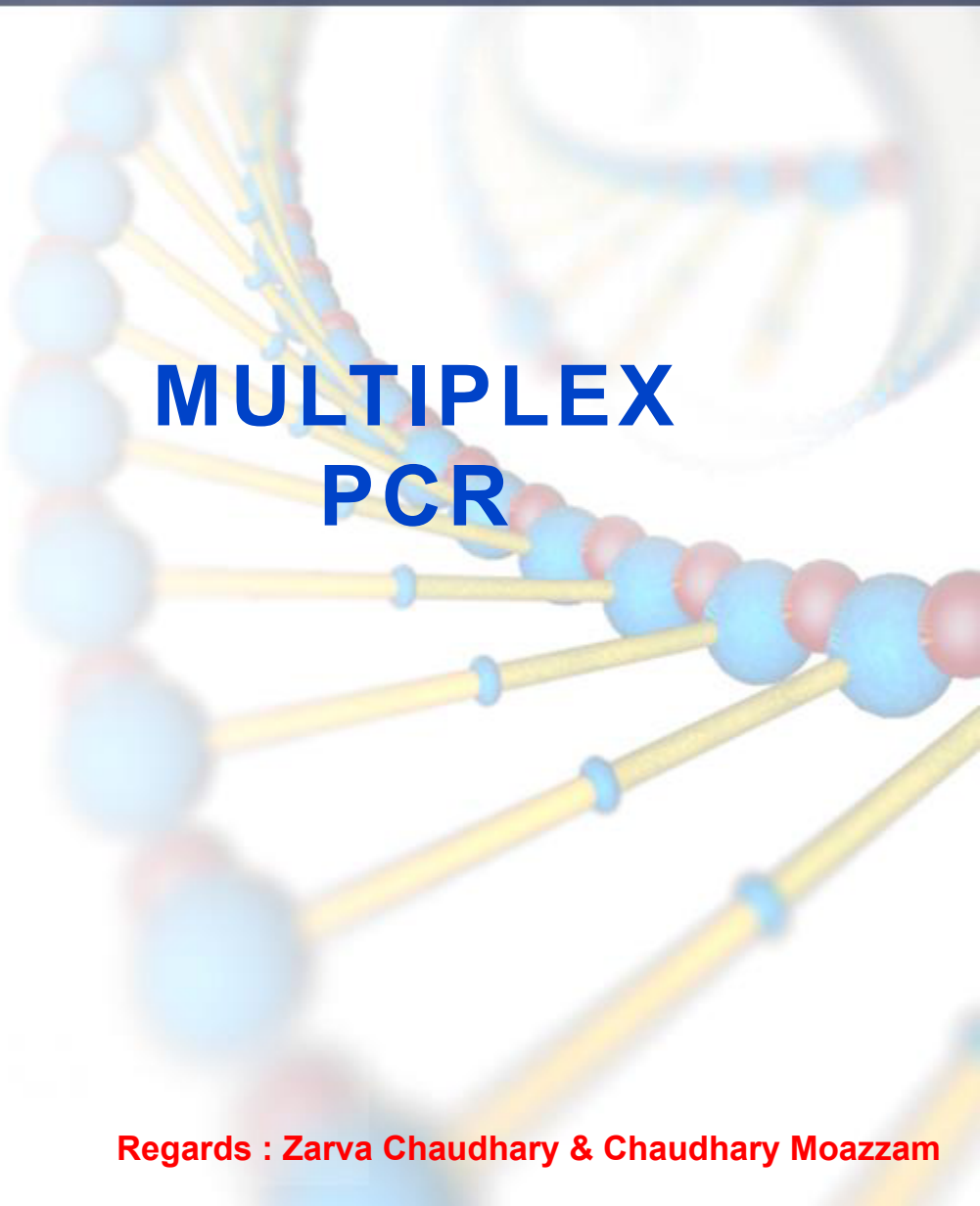
MULTIPLEX PCR



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Multiplex PCR

MULTIPLEX PCR

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

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Multiplex PCR

AMPLICONS OF DIFFERENT SIZES ARE PRODUCED

- Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences.

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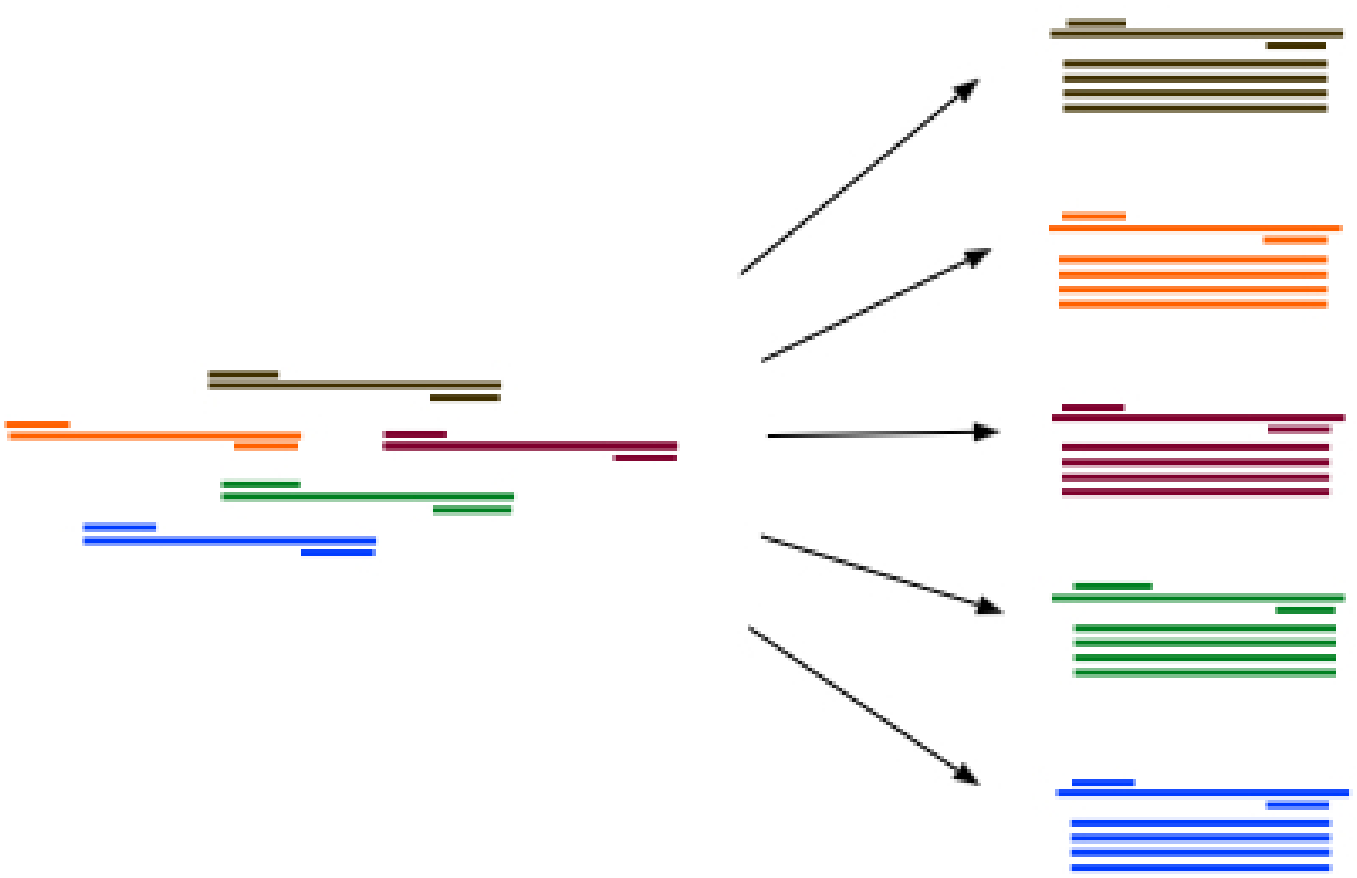
Multiplex PCR

MULTIPLEX PCR



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Multiplex PCR

SINGLE REACTION, MANY AMPLICONS

- By targeting multiple sequences at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform.

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Multiplex PCR

ANNEALING TEMPERATURES BE OPTIMIZED

- Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction.

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Multiplex PCR

AMPLICON SIZE SHOULD BE DIFFERENT OR BE LABELED

- Amplicon sizes should be different enough to form distinct bands when visualized by gel electrophoresis.

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Multiplex PCR

LABELED PRIMERS

- If amplicon sizes overlap, the different amplicons may be differentiated and visualised using primers that have been dyed with different color fluorescent dyes.

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Multiplex PCR

PRIMERS PAIRS BE OPTIMIZED

- The primer design for all primers pairs has to be optimized so that all primer pairs can work at the same annealing temperature during PCR.

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Multiplex PCR

MULTIPLEX PCR

- The use of multiple, unique primer sets within a single PCR reaction to produce amplicons of varying sizes specific to different DNA sequences.

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REVERSE TRANSCRIPTASE PCR

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Reverse Transcriptase PCR

REVERSE TRANSCRIPTASE PCR

- Based on the process of reverse transcription, which reverse transcribes RNA into DNA.

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Reverse Transcriptase PCR

REVERSE TRANSCRIPTASE PCR

- First step of RT-PCR - first strand reaction
- Synthesis of cDNA using oligo dT primers (37°C) one hour.
- Second strand reaction - digestion of cDNA:RNA hybrid (RNaseH)-
- Standard PCR with DNA oligo primers .

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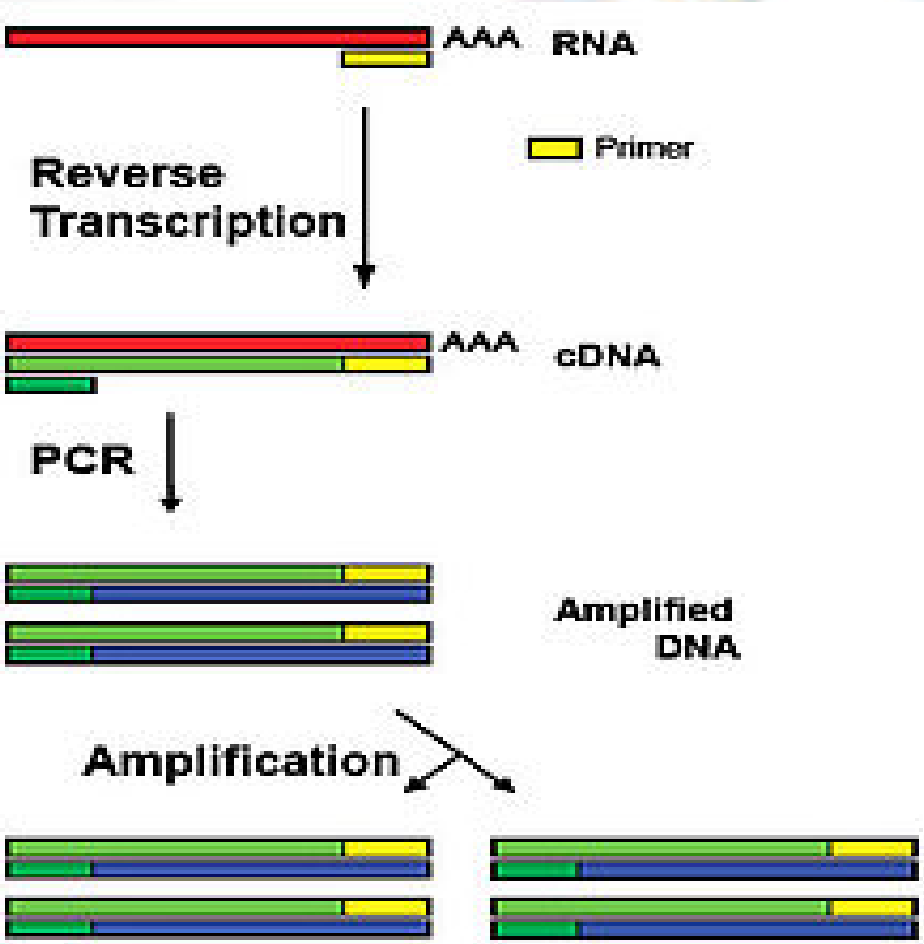
Reverse Transcriptase PCR

REVERSE TRANSCRIPTASE PCR



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Reverse Transcriptase PCR

ENZYME REVERSE TRANSCRIPTASE

- The PCR is preceded by a reaction using reverse transcriptase to convert RNA to cDNA.

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Reverse Transcriptase PCR

REVERSE TRANSCRIPTASE PCR

- RT-PCR is widely used in expression profiling, to determine the expression of a gene
- To identify the sequence of an RNA transcript.

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REAL TIME PCR

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Real Time PCR

REAL TIME PCR

- Real Time PCR is a technique in which fluoroprobes bind to specific target regions of amplicons to produce fluorescence during PCR.

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Real Time PCR

REAL TIME PCR

- Real Time PCR is used to measure the quantity of a PCR product.
- The fluorescence, measured in Real Time, is detected in a PCR cycler with an inbuilt filter flurometer.
- It is the method of choice to quantitatively measure starting amounts of DNA, cDNA or RNA.

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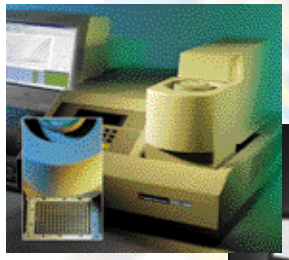
Real Time PCR

REAL TIME PCR

iCycler
BioRad



5700
Applied Biosystems

7700
Applied Biosystems

LightCycler
Roche

FluorTracker
Stratagene

FluorImager
Molecular Dynamics

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Real Time PCR

REAL TIME PCR

- Quantitative real-time PCR is often confusingly known as RT-PCR (Real Time PCR) or RQ-PCR.
- QRT-PCR or RTQ-PCR are more appropriate contractions.
- QRT-PCR methods use fluorescent dyes, such as Sybr Green, or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time.

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Real Time PCR

REAL TIME PCR

- Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample.

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HOT START PCR

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Hot Start PCR

HOT START PCR

- This is a technique that reduces non-specific amplification during the initial set up stages of the PCR.

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Hot Start PCR

HOT START PCR

- The technique may be performed manually by heating the reaction components upto the melting temperature (e.g, 95°C).
- Before adding the polymerase, specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature.
- This is done either by the binding of an antibody or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step.

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Hot Start PCR

HOT START PCR

- DNA Polymerase- Eubacterial type I DNA polymerase, Pfu.
- These thermophilic DNA polymerases show a very small polymerase activity at room temperature .

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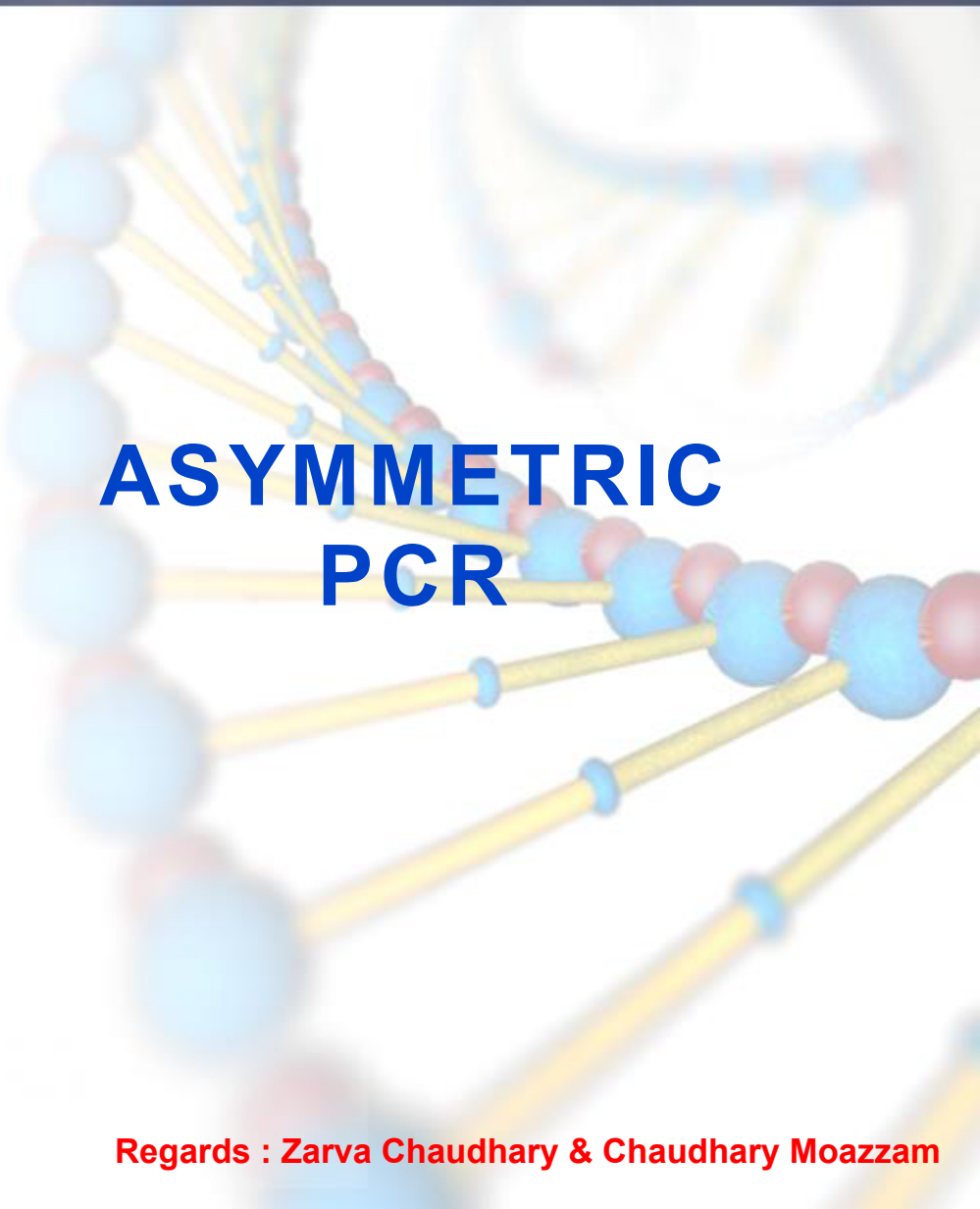

ASYMMETRIC PCR

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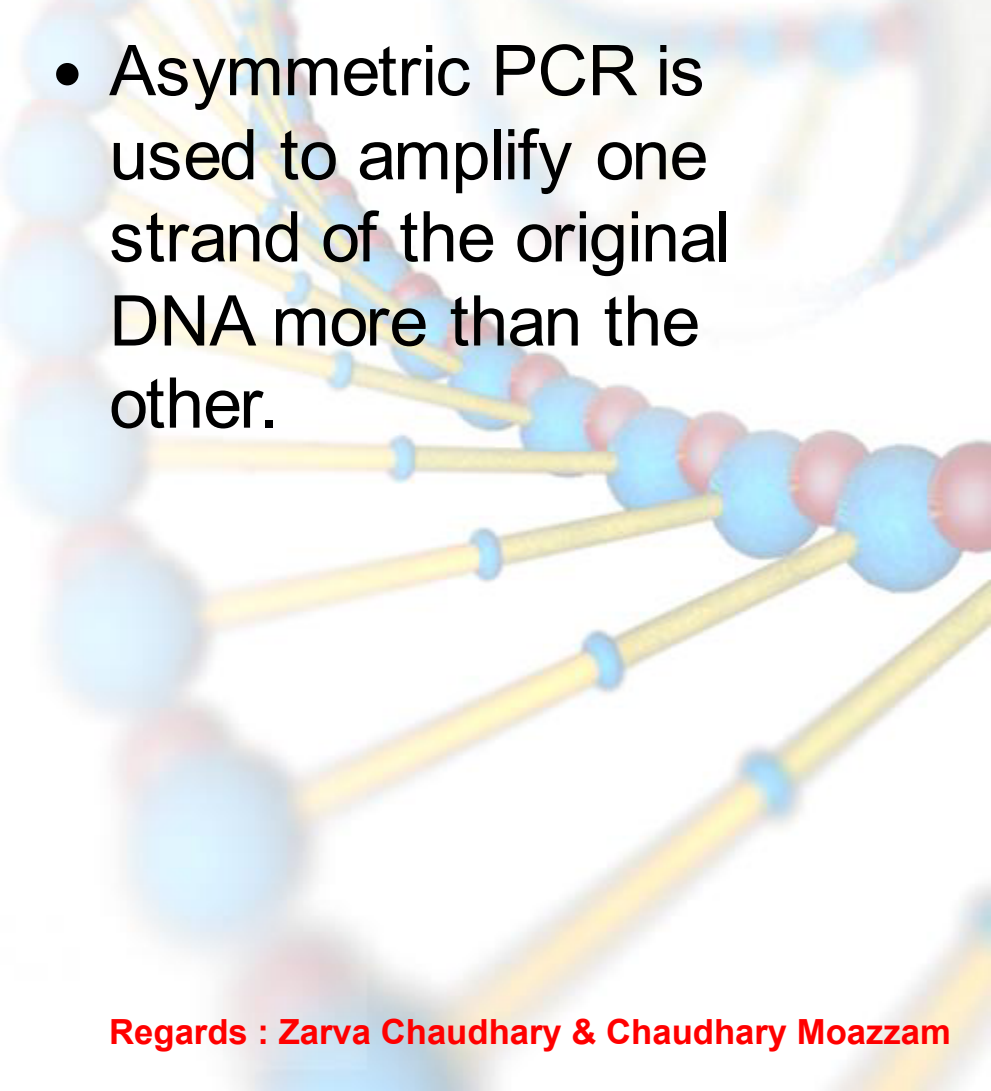
Asymmetric PCR

ASYMMETRIC PCR

- Asymmetric PCR is used to amplify one strand of the original DNA more than the other.



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Asymmetric PCR

ASYMMETRIC PCR

- It is used in some types of sequencing and hybridization probing where having only one of the two complementary strands is ideal.

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Asymmetric PCR

ASYMMETRIC PCR

- PCR is carried out as usual, but with a great excess of one primers for the chosen strand.

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Asymmetric PCR

ASYMMETRIC PCR

- Due to the slow amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required.

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Asymmetric PCR

ASYMMETRIC PCR

- It finds use in some types of sequencing and hybridization probing where having only one of the two complementary strands is required.

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LONG PCR

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LONG PCR

LONG PCR

- Extended or longer than standard PCR, meaning over 5 kilobases (frequently over 10 kb).
- Long PCR is useful only if it is accurate.

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LONG PCR

MIXTURES OF POLYMERASES

- Special mixtures of proficient polymerases along with accurate polymerases such as Pfu are often mixed together.

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LONG PCR

LONG PCR

- Application - to clone large genes not possible with conventional PCR.

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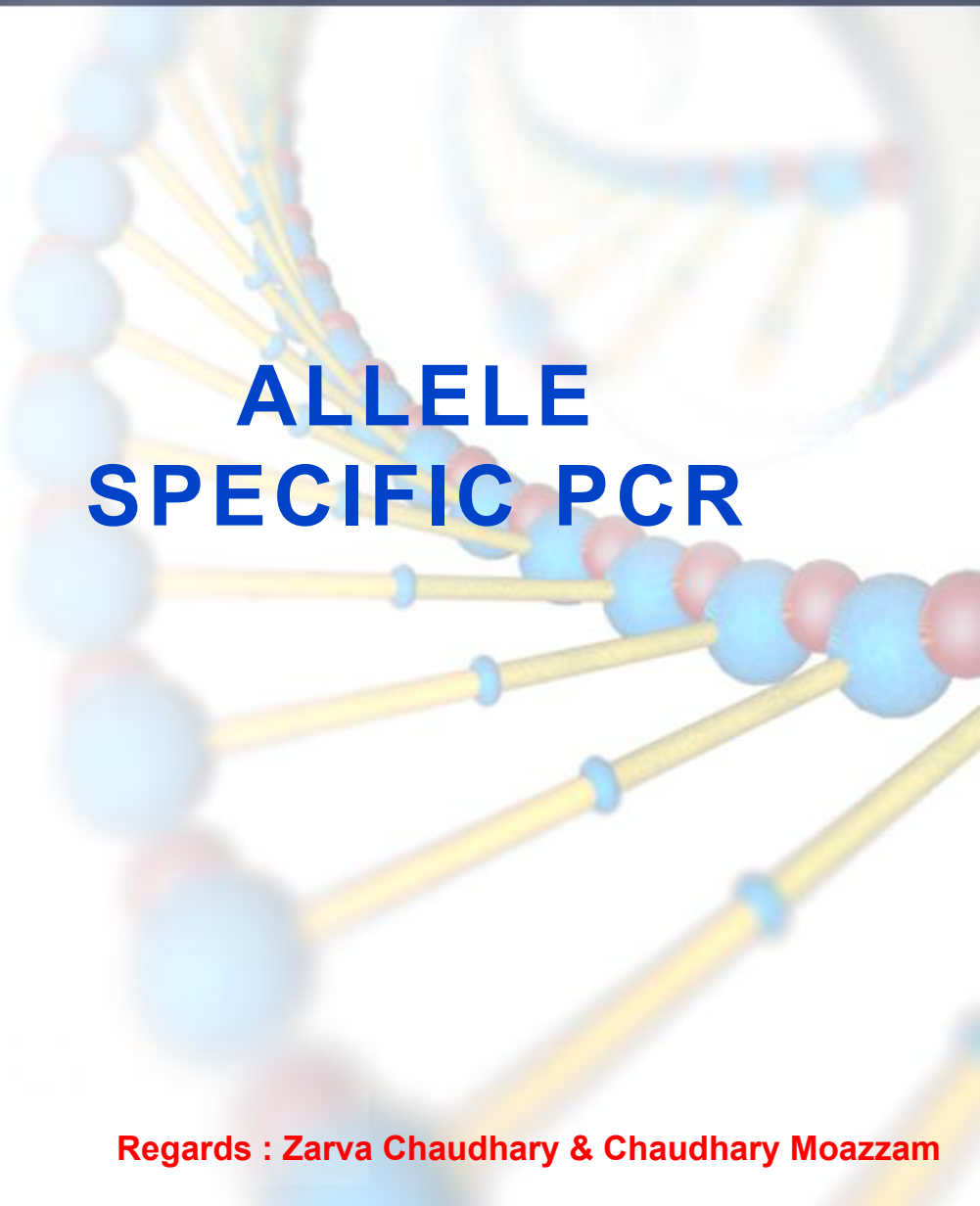
Essentials of Genetics



ALLELE SPECIFIC PCR

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Allele Specific PCR

ALLELE SPECIFIC PCR

- Allele-specific PCR used for identifying of SNPs.
- It requires prior knowledge of a DNA sequence, including differences between alleles.

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Allele Specific PCR

ALLELE SPECIFIC PCR

- 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.

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Allele Specific PCR

AMPLIFICATION WITH SNP SPECIFIC PRIMER

- Successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

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Allele Specific PCR

ALLELE SPECIFIC PCR

- This diagnostic or cloning technique is used to identify or utilize single-nucleotide polymorphisms (SNPs).

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COLONY PCR

Regards : Zarva Chaudhary & Chaudhary Moazzam

Colony PCR

COLONY PCR

- The screening of bacterial or yeast clones for correct ligation or plasmid products.

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Colony PCR

COLONY PCR METHODOLOGY

- The screening of bacterial or yeast clones for correct ligation or plasmid products.
- Pick a bacterial colony with an autoclaved toothpick, swirl it into 25 µl of TE autoclaved dH2O in an microfuge tube.
- Heat the mix in a boiling water bath (90-100^{0}C) for 2 minutes
- Spin sample for 2 minutes high speed in centrifuge.

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Colony PCR

COLONY PCR

- Transfer 20 µl of the supernatant into a new microfuge tube.
- Take 1-2 µl of the supernatant as template in a 25 µl PCR standard PCR reaction.

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Colony PCR

COLONY PCR

- The screening of bacterial or yeast clones for correct ligation or plasmid products.

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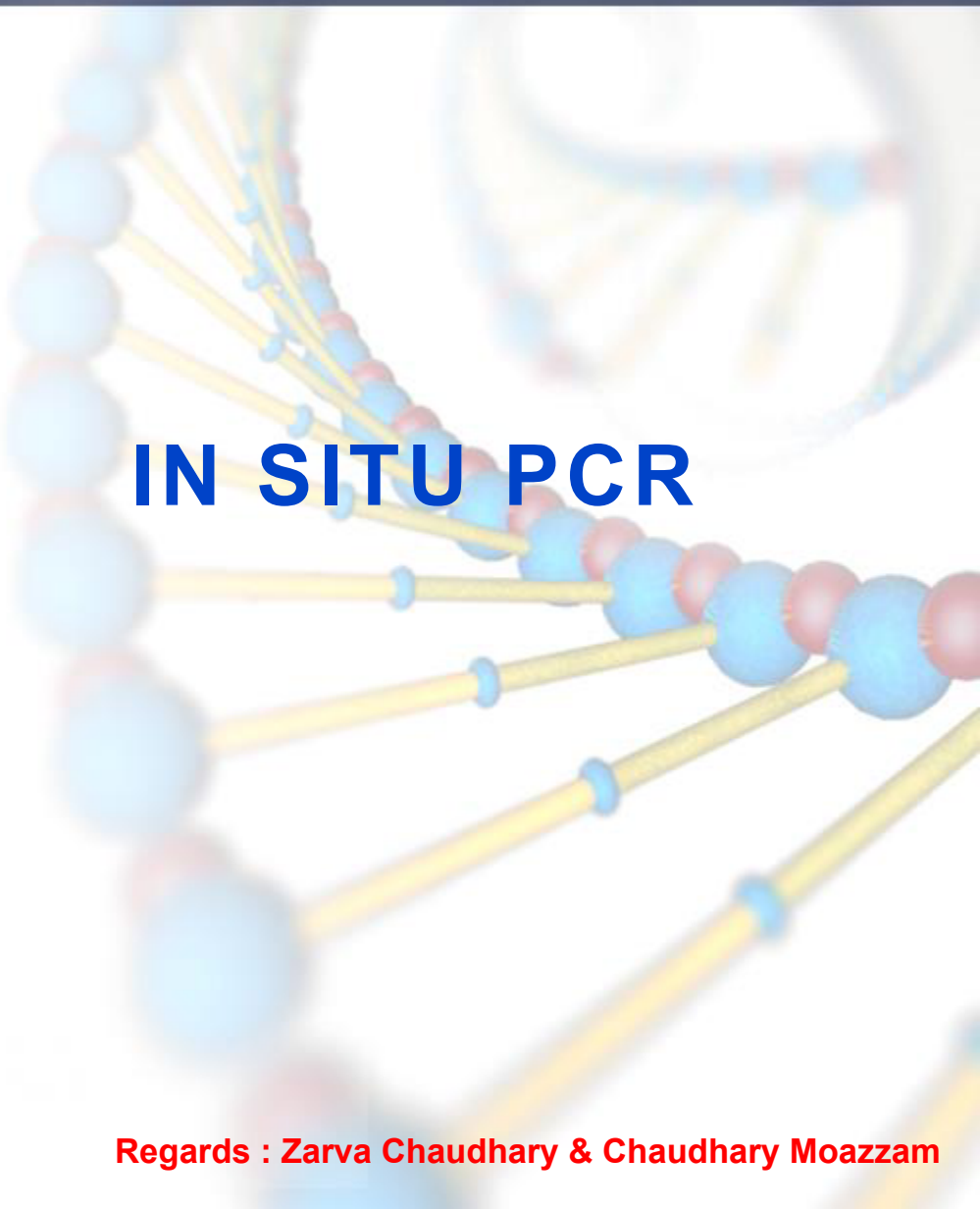

IN SITU PCR

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In Situ PCR

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 .

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In Situ PCR

IN SITU PCR

- Applies the methodology of hybridization of the nucleic acids.
- Allows identification of cellular markers
- Limited to detection of non-genomic material such as RNA.

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INVERSE PCR

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Inverse PCR

INVERSE PCR

- Inverse PCR uses standard PCR primers oriented in the reverse direction of the usual orientation.

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Inverse PCR

INVERSE PCR

- The template for the reverse primers is a restriction fragment that has been self-ligated
- Inverse PCR functions to clone sequences flanking a known sequence.
- Flanking DNA sequences are digested and then ligated to generate circular DNA.

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Inverse PCR

IDENTIFICATION OF SEQUENCES FLANKING TRANSPOSABLE ELEMENTS

- Amplification and identification of sequences flanking transposable elements, and the identification of genomic inserts.

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Inverse PCR

INVERSE PCR

- A method used to allow PCR when only one internal sequence is known.
- Especially useful in identifying flanking sequences to various genomic inserts.

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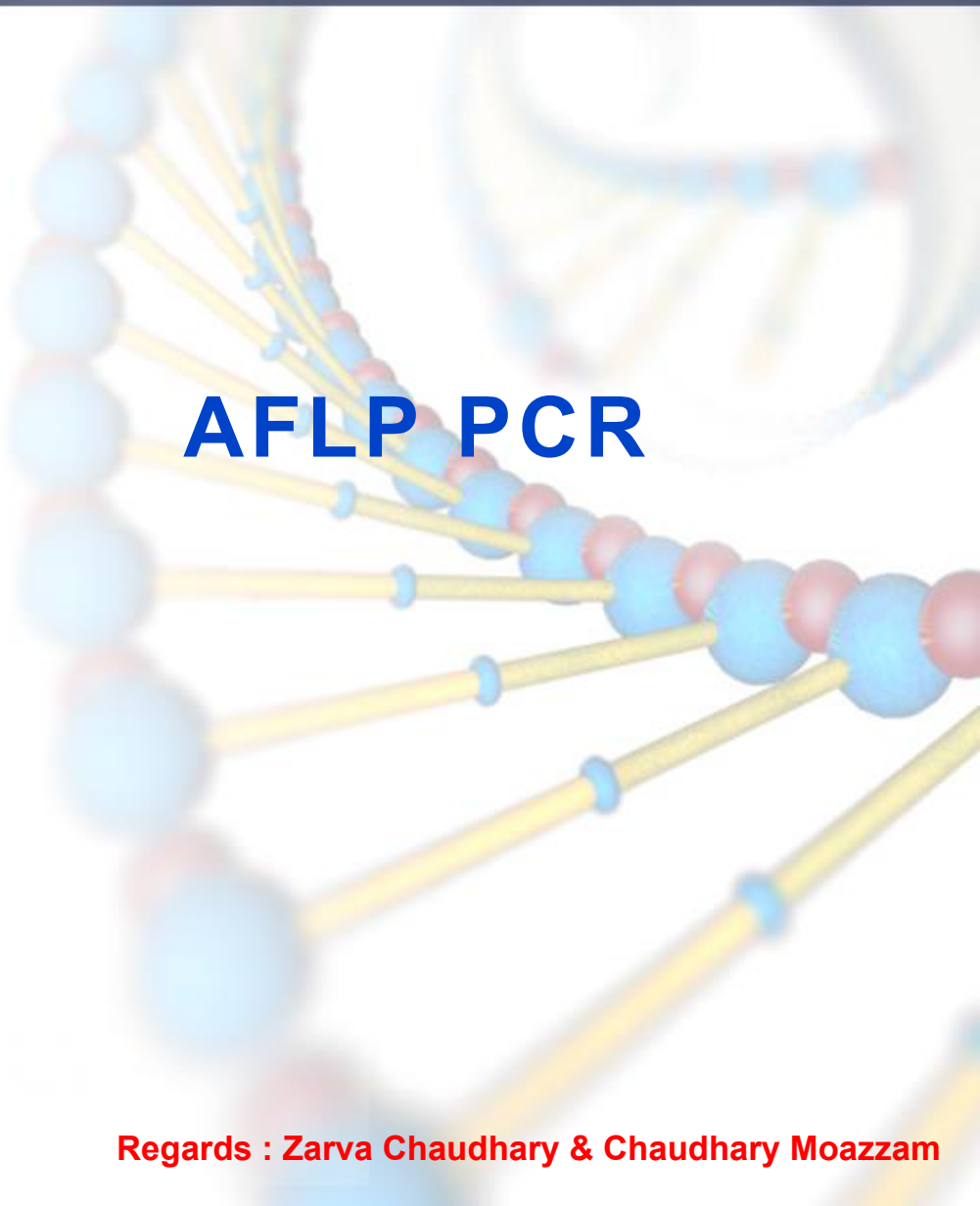
AFLP PCR



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AFLP PCR

AFLP PCR - METHODOLOGY

- Genomic DNA is digested with one or more restriction enzymes. tetracutter (MseI) and a hexacutter (EcoRI).
- Ligation of linkers to all restriction fragments. Pre-selective PCR is performed using primers which match the linkers and restriction site specific sequences.
- Electrophoretic separation and amplicons on a gel matrix, followed by visualization of the band pattern.

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AFLP PCR

AFLP PCR

- AFLP is a highly sensitive PCR-based method for detecting polymorphisms in DNA.
- AFLP can be also used for genotyping individuals for a large number of loci.

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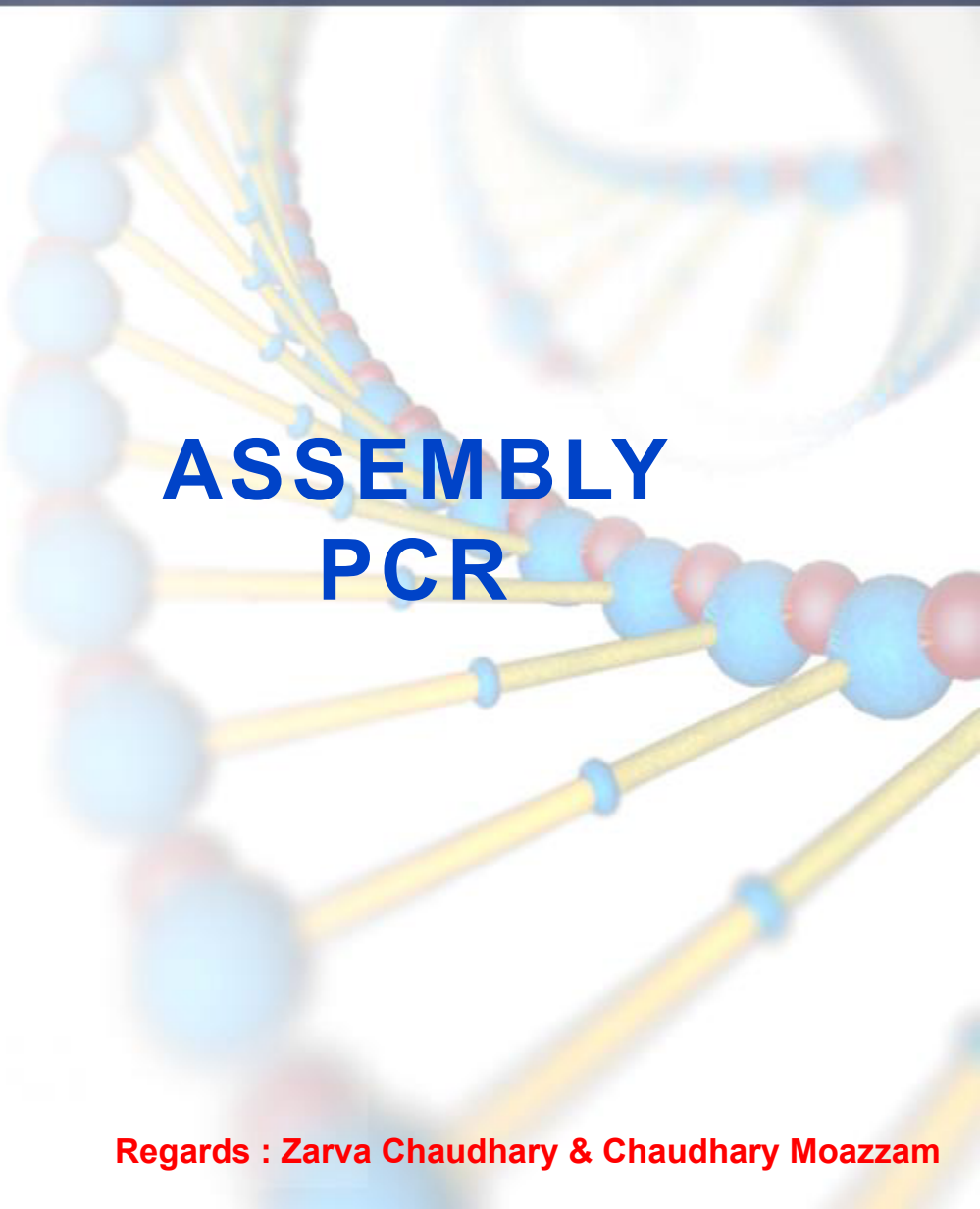

ASSEMBLY PCR

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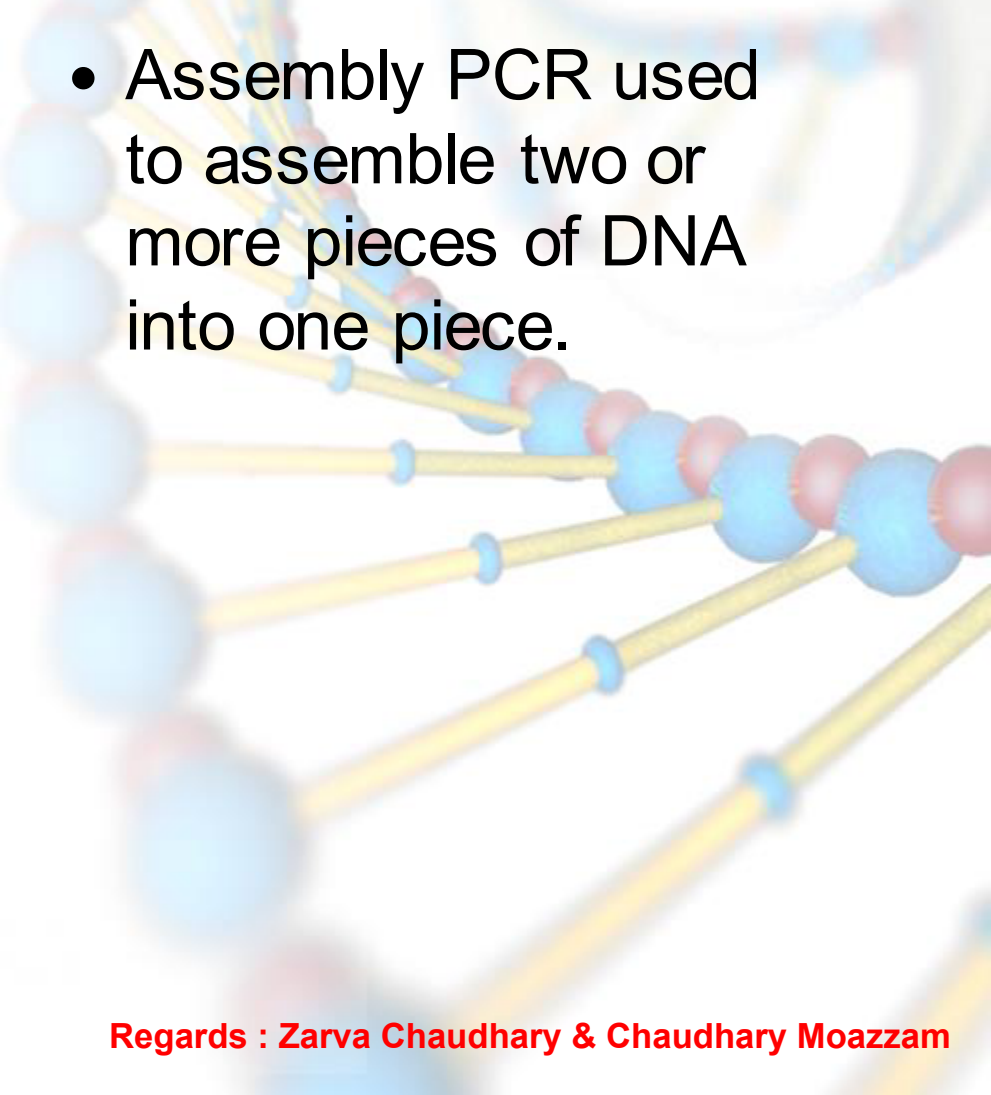
Assembly PCR

ASSEMBLY PCR

- Assembly PCR used to assemble two or more pieces of DNA into one piece.



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Assembly PCR

ASSEMBLY PCR - PRINCIPLE

- Assembly PCR is the synthesis of long DNA structures by performing PCR on a pool of long oligonucleotides with short overlapping segments, to assemble two or more pieces of DNA into one piece.

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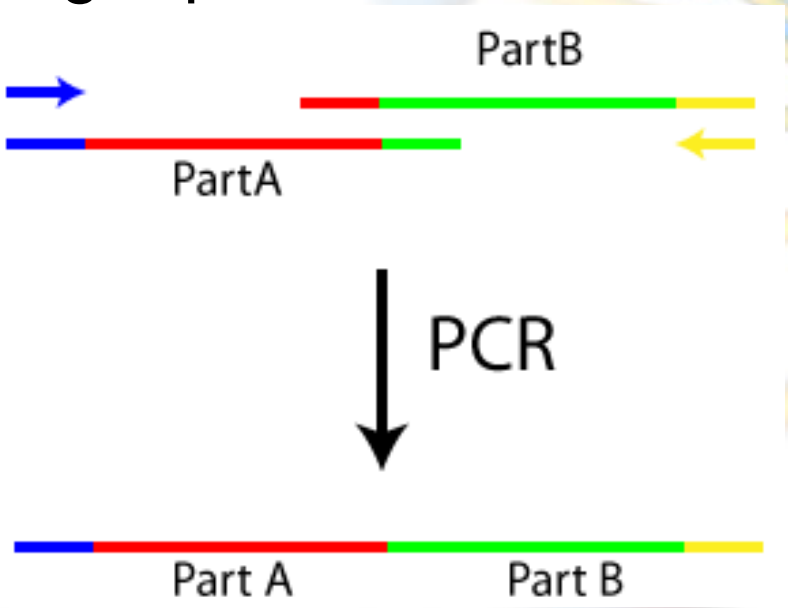
Assembly PCR

ASSEMBLY PCR - METHODOLOGY

- It involves an initial PCR with primers that have an overlap and a second PCR using the products as the template that generates the final full-length product.



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Assembly PCR

ASSEMBLY PCR

- Assembly PCR used to assemble two or more pieces of DNA into one piece.

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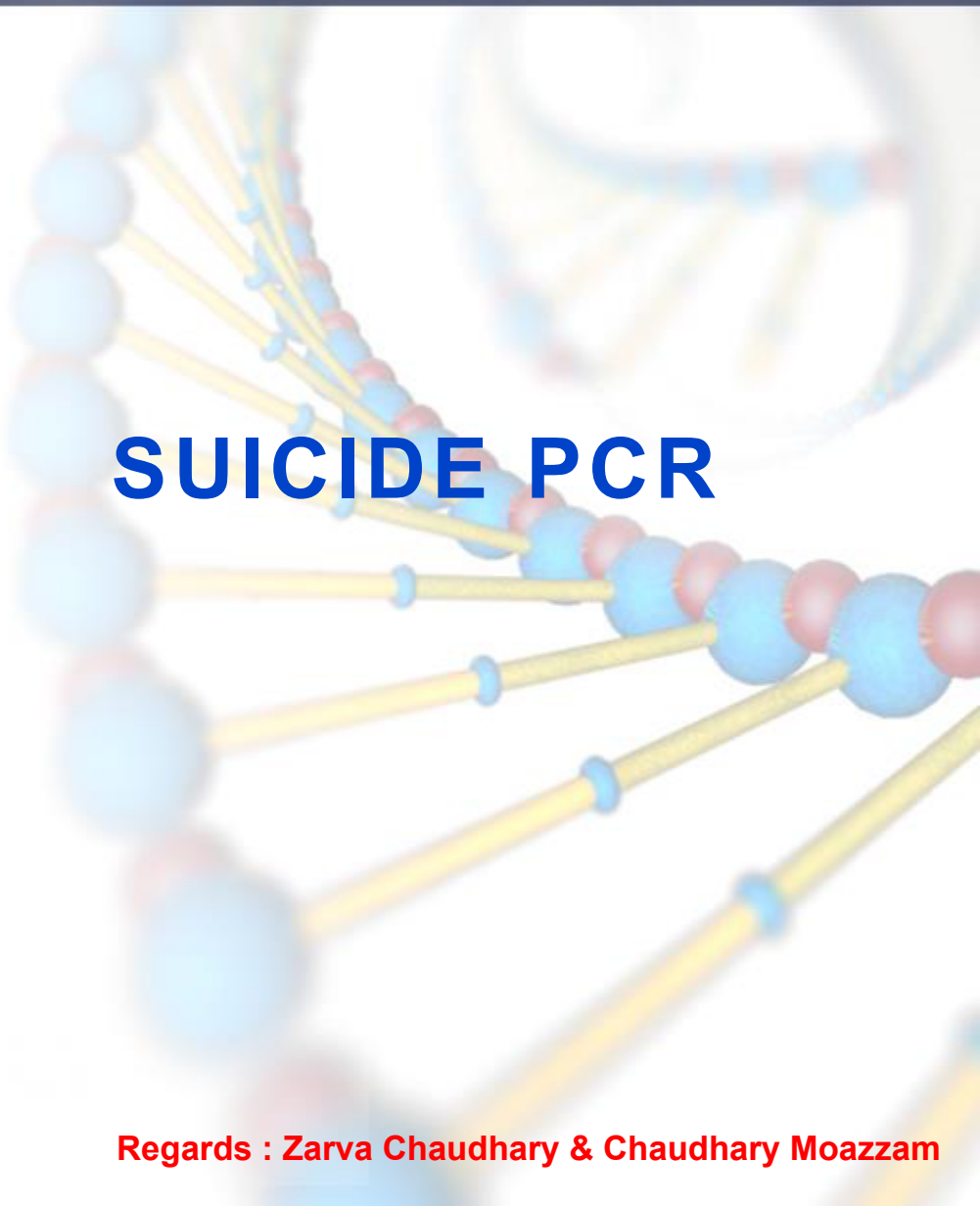

SUICIDE PCR

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Suicide PCR

SUICIDE PCR - PRINCIPLE

- Suicide PCR is typically used in paleogenetics or other studies where avoiding false positives and ensuring the specificity of the amplified fragment is the highest priority.
- The method prescribes the use of any primer combination only once in a PCR, which should never have been used in any positive control PCR reaction.

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Suicide PCR

SUICIDE PCR

- Primers should always target a genomic region never amplified before in the lab using this or any other set of primers.
- This ensures that no contaminating DNA from previous PCR reactions is present in the lab, which could otherwise generate false positives.

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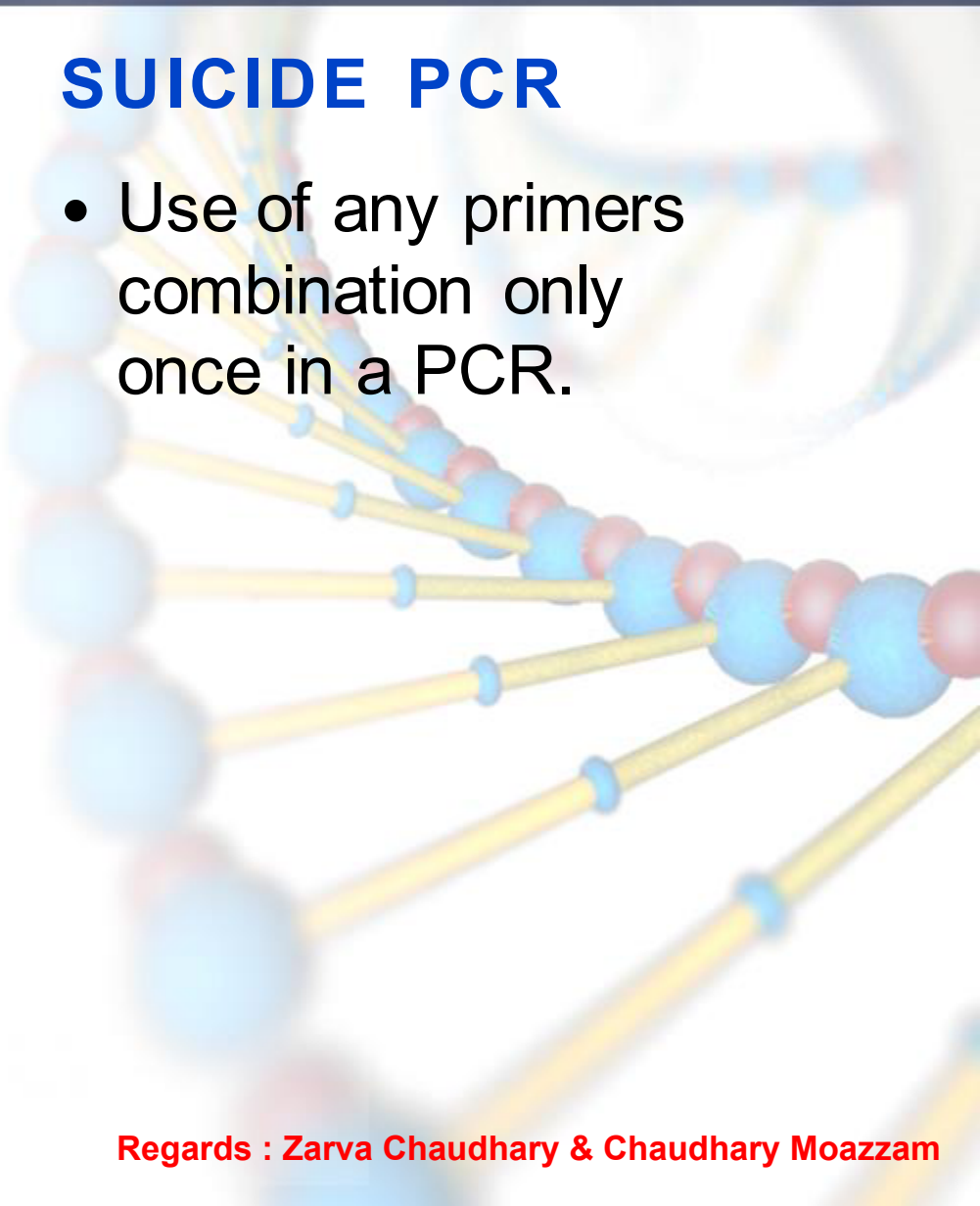
Suicide PCR

SUICIDE PCR

- Use of any primers combination only once in a PCR.



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METHYLATION SPECIFIC PCR

Regards : Zarva Chaudhary & Chaudhary Moazzam

Methylation Specific PCR

METHYLATION SPECIFIC PCR

- Methylation-specific PCR is used to identify patterns of DNA methylation at CpG islands in genomic DNA.

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Methylation Specific PCR

METHYLATION SPECIFIC PCR

- Target DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is complementary to adenosine in PCR primers.
- Two amplifications are then carried out on the bisulfite-treated DNA: One primer set anneals to DNA with cytosines (corresponding to methylated cytosine), and the other set anneals to DNA with uracil (corresponding to unmethylated cytosine).

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Methylation Specific PCR

MSP

- MSP used in quantitative PCR provides information about methylation state of a given CpG island.

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INTER SEQUENCE SPECIFIC PCR

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InterSequence Specific PCR

INTER SEQUENCE SPECIFIC PCR

- A method for DNA fingerprinting that uses primers selected from segments repeated throughout a genome to produce a unique fingerprint of amplified product lengths.
- The use of primers from a commonly repeated segment is called Alu-PCR, and can help to amplify sequences adjacent (or between) these repeats.

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InterSequence Specific PCR

PCR

- PCR used to produce a unique fingerprints of amplified product lengths.

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LIGATION MEDIATED PCR

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Ligation-mediated PCR

METHODOLOGY

- Uses small DNA oligonucleotide 'linkers' (or adaptors) that are first ligated to fragments of the target DNA.
- PCR primers that anneal to the linker sequences are then used to amplify the target fragments.

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Ligation-mediated PCR

PCR

- DNA sequencing
- Genome walking
- DNA footprinting

Regards : Zarva Chaudhary & Chaudhary Moazzam

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WHOLE GENOME AMPLIFICATION PCR

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Whole Genome Amplification PCR

WHOLE GENOME AMPLIFICATION PCR

- Primers can be designed to be 'degenerate'

- able to initiate replication from a large number of target locations.

- Whole genome amplification (WGA) is a group of procedures that allow amplification to occur at many locations in a genome.

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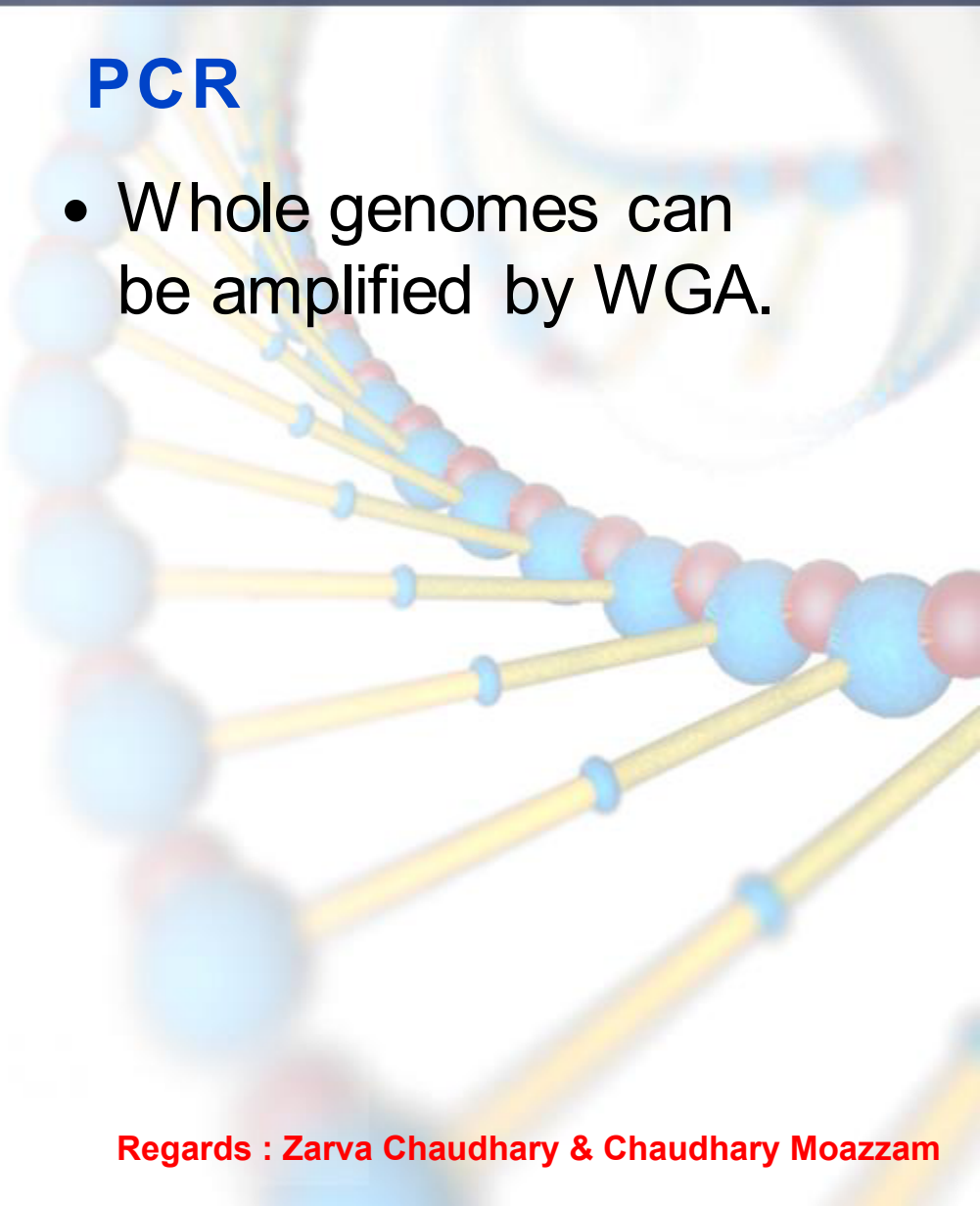
Whole Genome Amplification PCR

PCR

- Whole genomes can be amplified by WGA.



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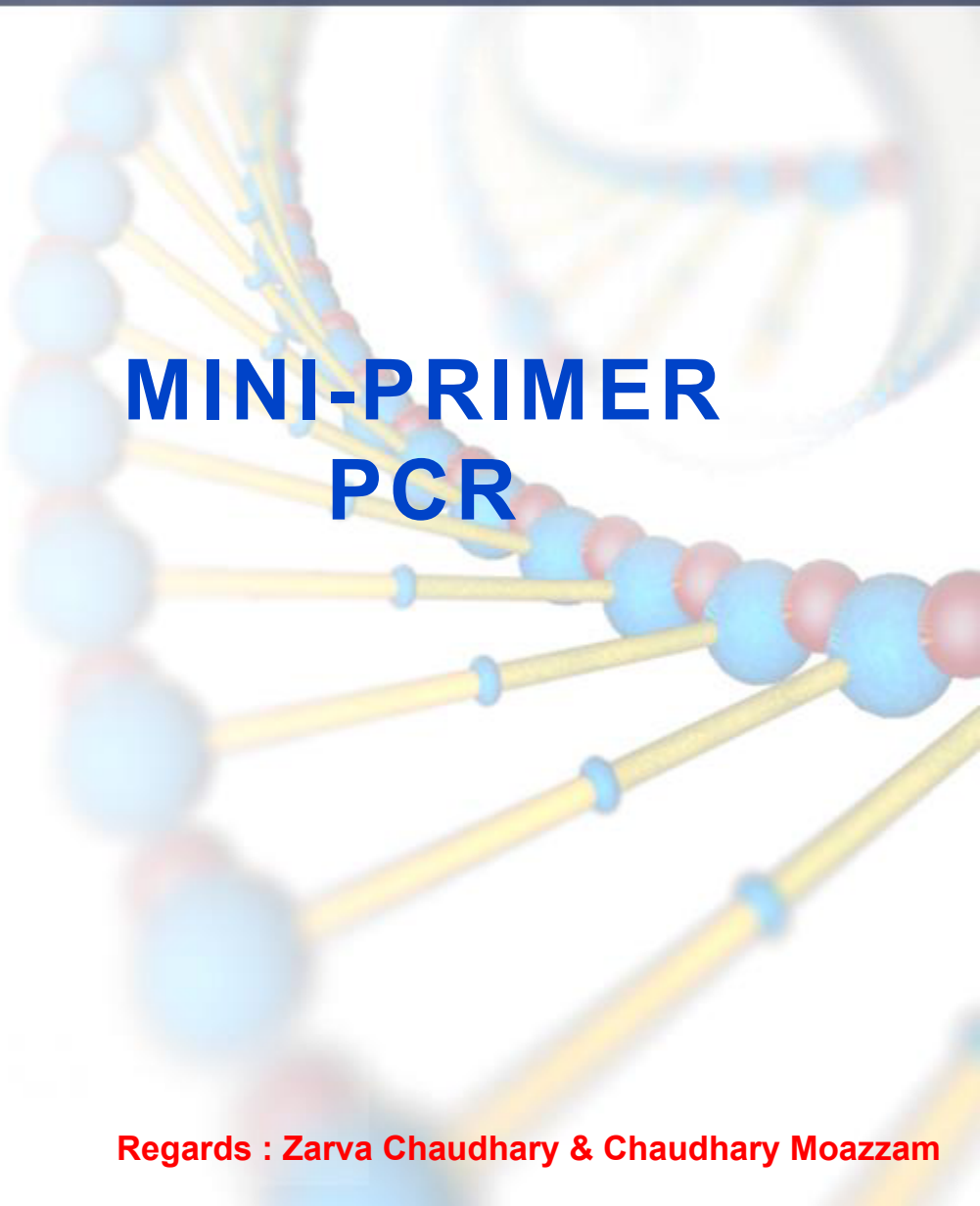

MINI-PRIMER PCR

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Mini Primer PCR

MINI PRIMER PCR

- Mini Primer PCR uses a thermostable polymerase (S-Tbr) that can extend from short primers as short as 9 or 10 nucleotides.
- This method permits PCR targeting to smaller primer binding regions, and is used to amplify conserved DNA sequences, such as the 16S (or eukaryotic 18S) rRNA gene.

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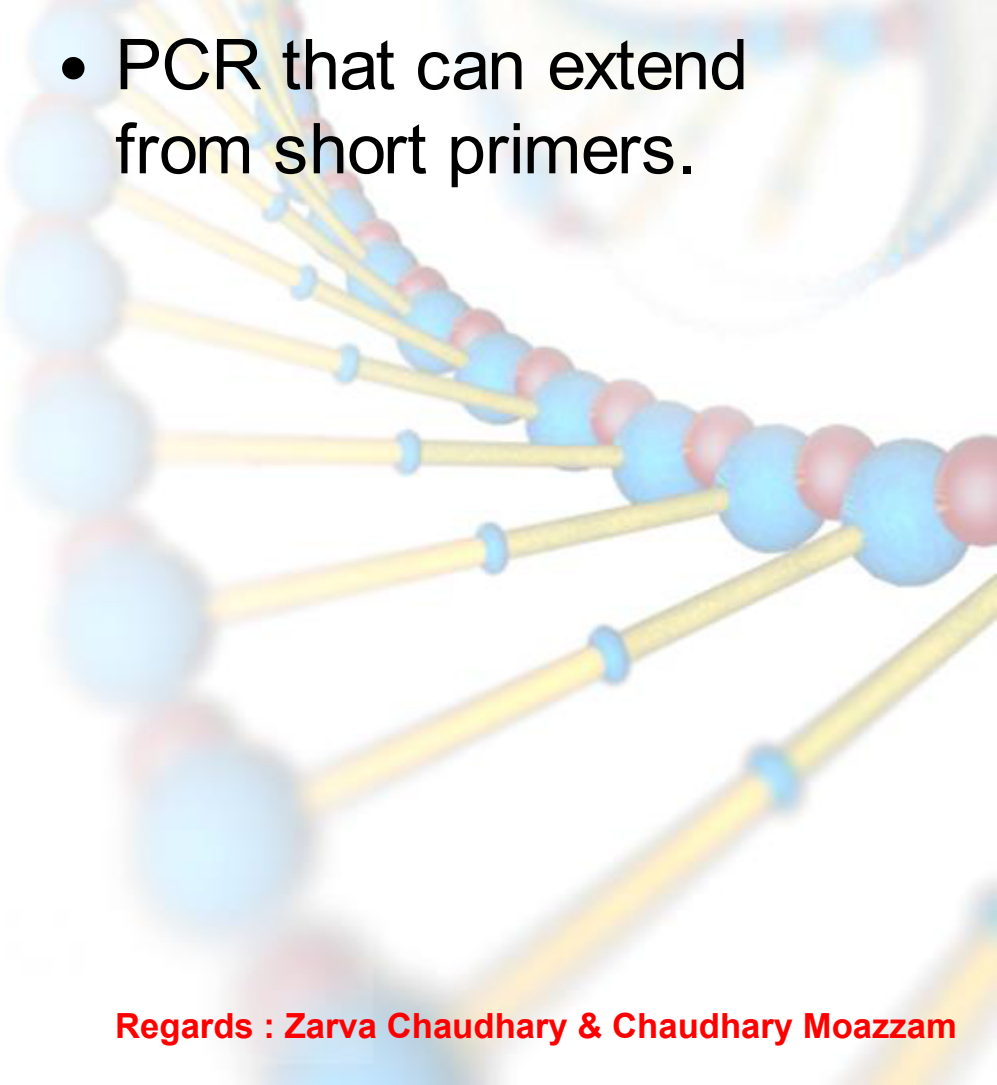
Mini Primer PCR

MINI PRIMER PCR

- PCR that can extend from short primers.



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ADVANTAGES AND LIMITATIONS OF PCR

Regards : Zarva Chaudhary & Chaudhary Moazzam

Advantages & Limitations

ADVANTAGES

- Specific
- Simple, rapid, relatively inexpensive
- Amplifies from low quantities
- Works on damaged DNA
- Sensitive
- Flexible

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Advantages & Limitations

LIMITATIONS

- Contamination risk
- Primer complexities
- Primer-binding site complexities
- Amplifies rare species
- Detection methods

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APPLICATIONS OF PCR

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Applications of PCR

APPLICATIONS

- Detection of Infectious diseases: AIDS, TB, CMV, H1N1, etc Viral, Bacterial and fungal infections,
- Diagnosis of latent viruses.
- Forensic applications: DNA finger printing

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Applications of PCR

APPLICATIONS

- Detection of Mutations: Inherited disorders & carriers Track DNA abnormalities
- Prenatal diagnosis of genetic disorders.

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Applications of PCR

APPLICATIONS

- Detection of pathogens Pre-natal diagnosis DNA fingerprinting Gene therapy Mutation screening
- Drug discovery
- Classification of organisms Genotyping

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Applications of PCR

APPLICATIONS

- Molecular Archaeology
- Molecular Epidemiology
- Molecular Ecology
- Bioinformatics
- Genomic cloning
- Site-directed mutagenesis
- Gene expression studies

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Applications of PCR

CLINICAL MICROBIOLY

- Identification of bacteria:
- Slow growing bacteria
- Bacterial antibiotic resistance genes.
- VIRUS: almost all viruses identification and viral load.

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Applications of PCR

CLINICAL MICROBIOLY

- FUNGUS:
- Aspergillus spp
- Candida spp
- Plasmodium spp
- Trypanosoma spp
- Leishmania spp
- Babesia spp

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Polymerase chain reaction (PCR)

Applications of PCR

- PCR has widespread applications in various fields of life sciences including genetic engineering, medical, forensic, agriculture, environment etc.

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Polymerase chain reaction (PCR)

PCR-Gene cloning and expression

- PCR has been used in gene cloning and screening of genomic libraries

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Polymerase chain reaction (PCR)

PCR-Medicine

- PCR has major impact on medicine especially in the field of clinical microbiology or diagnosis
- Molecular tools have also allowed to perform prenatal genetic diagnosis

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Polymerase chain reaction (PCR)

PCR-Forensic sciences

- Forensic science is the application of scientific procedures to solve criminal and legal matters
- Molecular methods are used to established the filiations of a person or to obtain evidence from minimal samples of saliva, semen or other tissues

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Polymerase chain reaction (PCR)

PCR-DNA profiling

- DNA profiling or DNA fingerprinting is a forensic technique used to identify individuals by characteristics of their DNA

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Polymerase chain reaction (PCR)

PCR-Agricultural sciences and environment

- PCR has also facilitated research in detection of pathogens in plants, animals and environment

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Polymerase chain reaction (PCR)

PCR-Molecular paleontology

- Molecular paleontology refers to the recovery and analysis of DNA and protein from ancient human, animal and plant remains

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Cutting DNA molecules

Types of restriction and modification (R-M) system

- At least four R-M systems are known
- Type I
- Type II
- Type III
- Type IIs

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Cutting DNA molecules

Type I

- Type I systems were the first to be characterized from *E. coli* K12
- The active enzyme consists of two restriction subunit, two modification subunit and one recognition subunit
- Type I systems are of little value for gene manipulation

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Cutting DNA molecules

Type II

- Most of the useful R-M system is Type II
- Type II enzymes recognize a defined sequence and cut within it

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Cutting DNA molecules

Type III

- Type III enzymes have symmetrical recognition sequences but otherwise resemble type I systems and are of little value

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Cutting DNA molecules

Type IIs

- Type IIs systems have similar cofactors and structure to type II but restriction occurs at a distance from recognition site that limits their usefulness

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Cutting DNA molecules

Nomenclature

- A suitable system was proposed by Smith and Nathans (1973)
- The species name of host organisms is identified by the first letter of genus and first two letters of specific epithet
- *E. coli* = Eco
- *H. influenzae* = Hin

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Cutting DNA molecules

Nomenclature

- Strain identification is written as *Eco*K
- In case, host strain has several restriction and modification systems, these are identified by roman numerals, for example, in case of *H. influenzae*
- *HindI, HindII, HindIII* etc

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Cutting DNA molecules

Nomenclature

- All restriction enzymes have general name endonuclease R and modification-methylase M followed by the system name, for example, in case of *H. influenzae*
- R. *HindIII* or M. *HindIII*

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Examples of restriction endonuclease nomenclature

Enzyme	Enzyme source	Recognition sequence
SmaI	*Serratia marcescens*, 1st enzyme	CCCGGG
HaeIII	*Haemophilus aegyptius*, 3rd enzyme	GGCC
HindII	*H. influenzae*, strain d, 2nd enzyme	GTPyPuAC
HindIII	*H. influenzae*, strain d, 3rd enzyme	AAGCTT
HamHI	*Bacillus amyloliquefaciens*, strain H, 1st enzyme	GGATCC

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Cutting DNA molecules

Target sites

- Type II endonucleases recognize and cleave DNA within particular sequences of four to eight nucleotides which have a twofold axis of rotational symmetry i.e. referred as *palindromes*

5′-GAATTC-3′

5′-CTTAAG-3′

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5′-G A A T T C-3′

3′-C T T A A G-5′

5′-G/ A A* T T C-3′

3′-C T T A* A/ G-5′

5′-G 5′- A A T T C-3′

3′-C T T A A-5′ G-5′

Single stranded breaks by *Eco*R1

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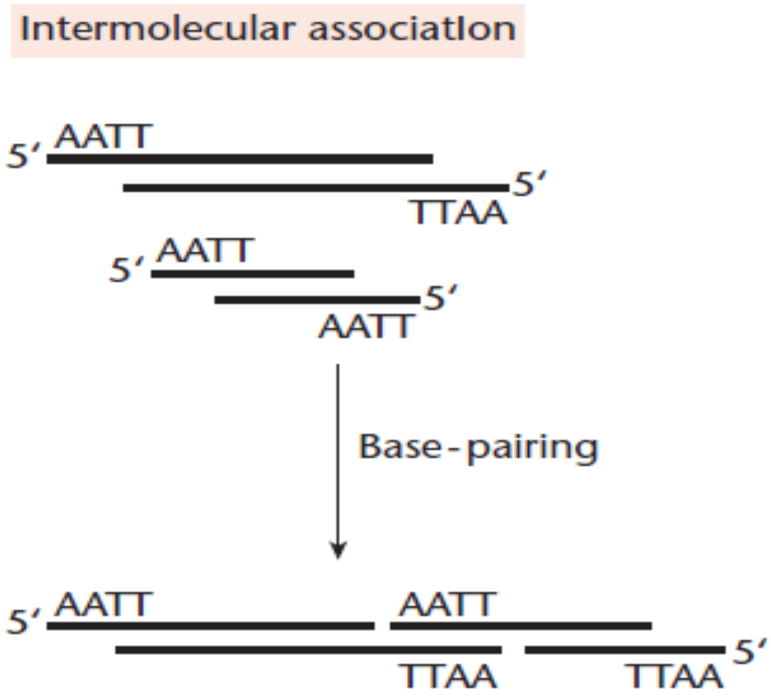

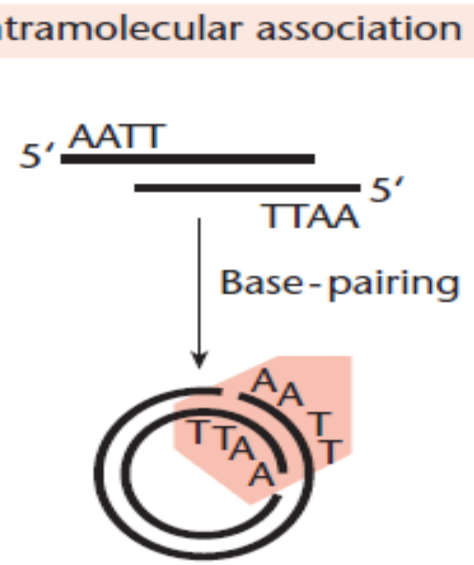

Cohesive fragments of DNA produced by digestion with EcoR1

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Cutting DNA molecules

Number and size of restriction fragments

- The number and size of fragments generated by restriction enzyme depend on the frequency of occurrence of the target site in the DNA to be cut

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Cutting DNA molecules

Number and size of restriction fragments

- Four base recognition site occurs every 4^4 (256) bp
- Six base recognition site occurs every 4^6 (4096) bp
- Eight base recognition site occurs 4^8 (65,536) bp

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Average fragment size (bp) produced by different enzymes

Enzyme	Target	Arabidopsis	E. Coli	Human
*Apa*I	GGGCCC	25000	15000	2000
*Bam*HI	GGATCC	6000	5000	5000
*Spe*I	ACTAGT	8000	60000	10000

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Cutting DNA molecules

Summary of restriction endonucleases

- Type II restriction enzymes are heavily responsible for the current explosion in the field of gene manipulation in that they are essential in forming recombinant DNA molecules

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Joining DNA molecules

DNA modifying enzymes

- Nucleases
- DNA Polymerase
- Reverse transcriptases
- DNA ligases

Regards : Zarva Chaudhary & Chaudhary Moazzam

Joining DNA molecules

Nucleases

- Nucleases or DNases are the enzymes that degrade DNA
- Two broad classes of nucleases
  - i). Exonucleases
  - ii). Endonucleases

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Joining DNA molecules

DNA Polymerase

- An enzyme that catalyzes template-dependent synthesis of DNA

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Joining DNA molecules

Reverse transcriptases

- An RNA-directed DNA polymerase in retroviruses; capable of making DNA complementary to an RNA

Joining DNA molecules

DNA ligase

- An enzyme that creates a phosphodiester bond between the 3′ end of one DNA segment and the 5′ of another

Joining DNA molecules

Methods of joining DNA fragments

- Mainly three methods are used for joining DNA in vitro

i) . Joining covalently annealed cohesive ends by DNA ligase

ii) . Joining blunt-ended fragments by DNA ligase from phage T4 infected *E. coli*

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Joining DNA molecules

Methods of joining DNA fragments

iii). The third utilizes the enzyme terminal deoxynucleotidyltransferase to synthesize homopolymeric 3′ single stranded tails at the ends of fragments

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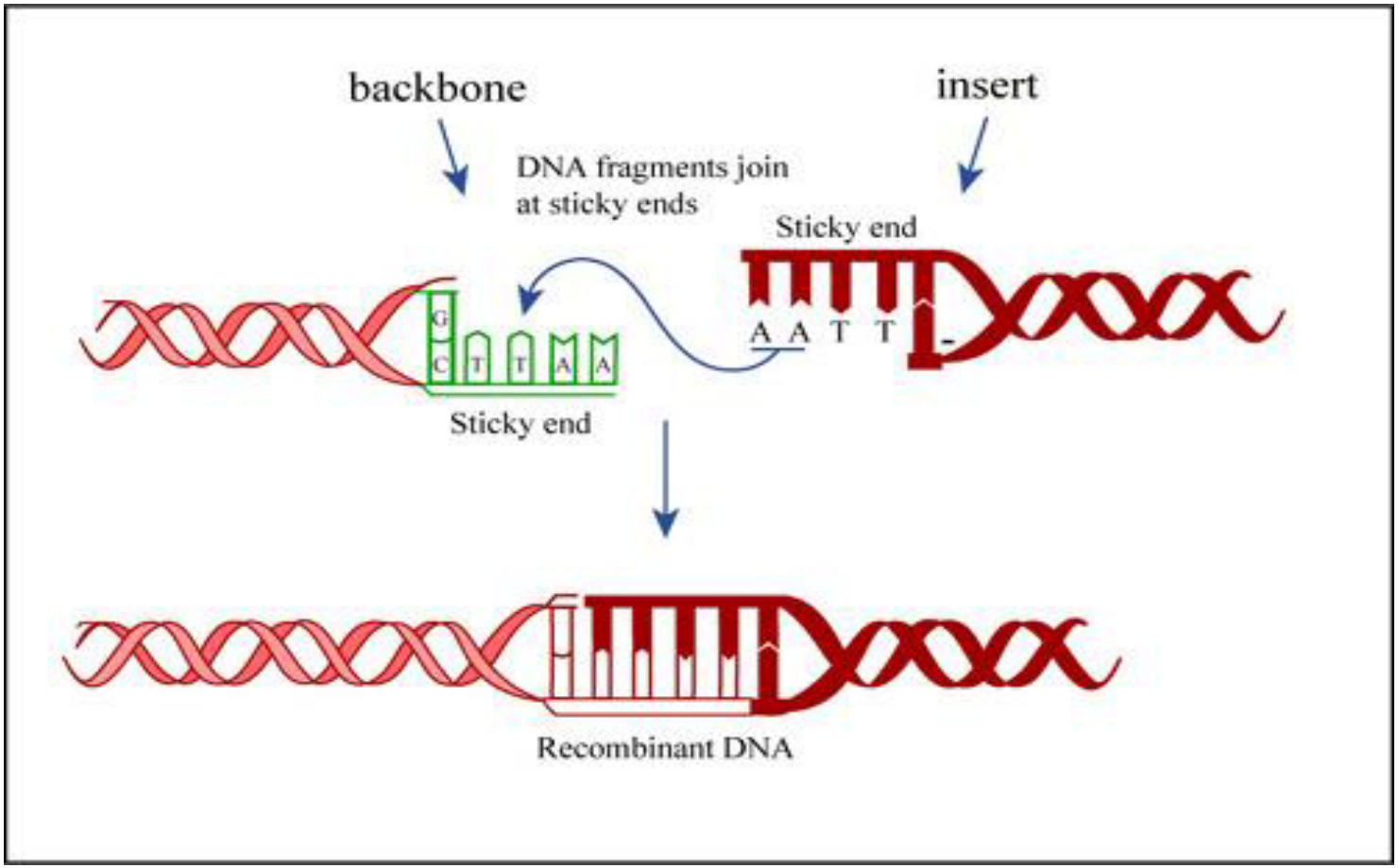
Joining DNA molecules

DNA ligase

- *E. coli* and phage T4 encode enzyme, DNA ligase, which seals single stranded nicks between adjacent nucleotides in a duplex DNA chain



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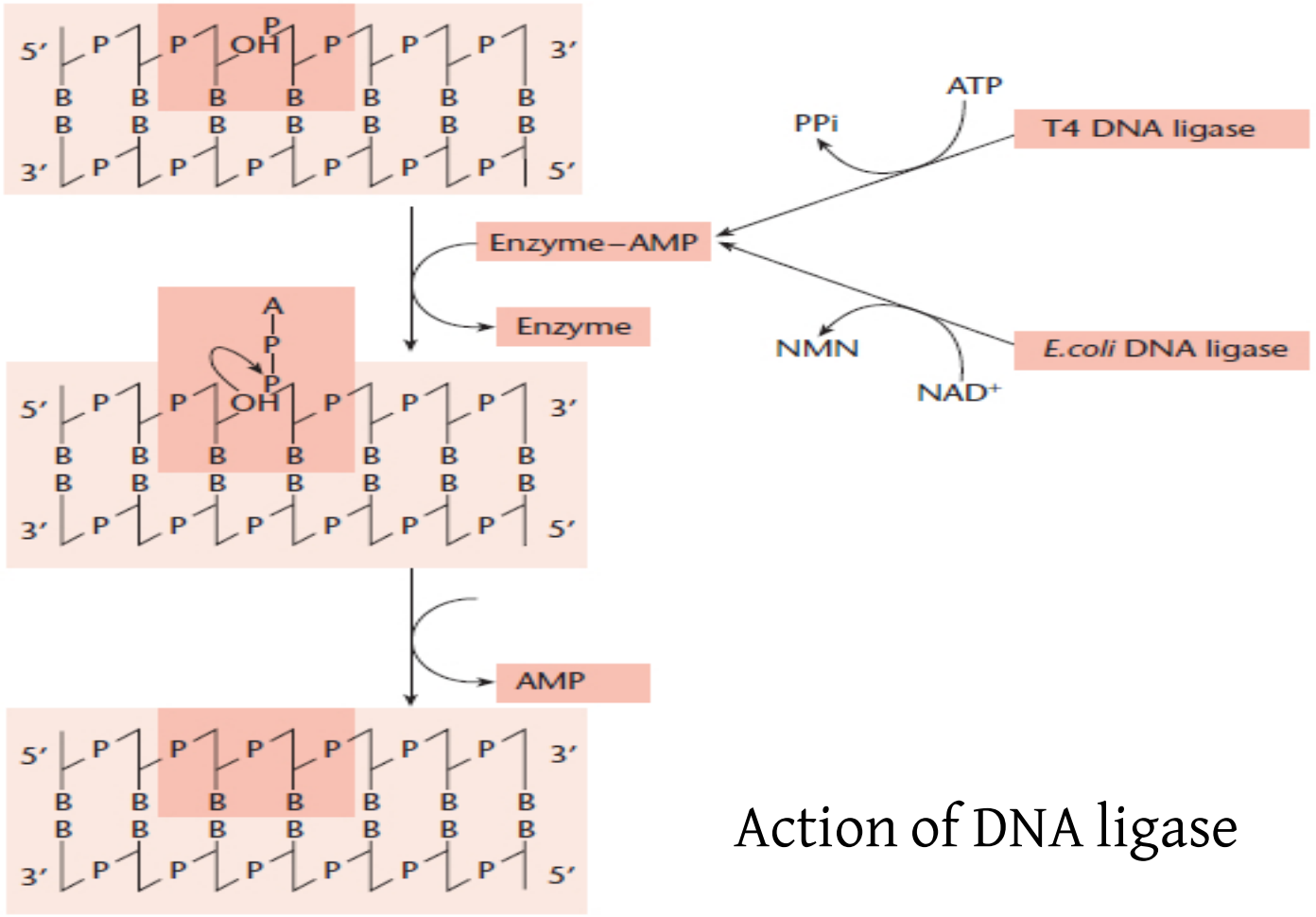

Action of DNA ligase

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Joining DNA molecules

DNA ligase to create covalent recombinant DNA

- DNA ligase can repair the nicks produced after the association of cohesive ends of DNA strands

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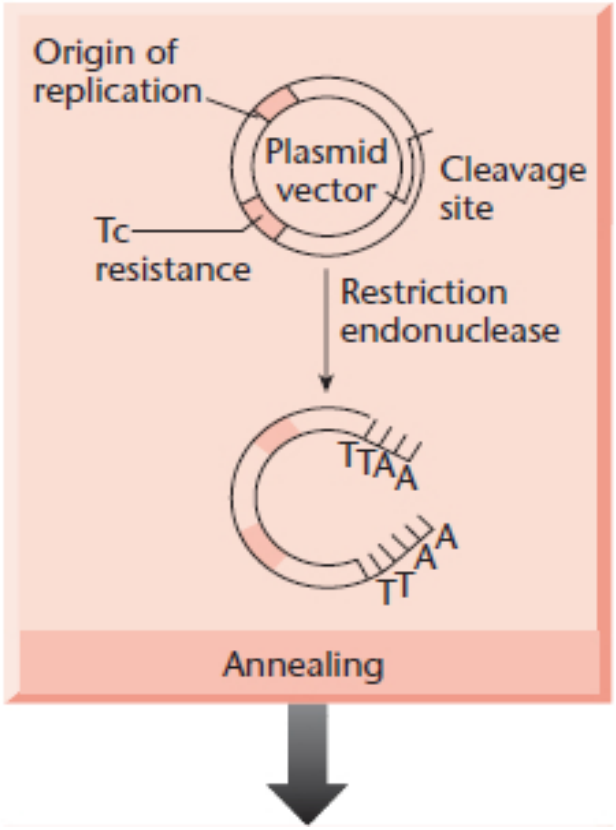
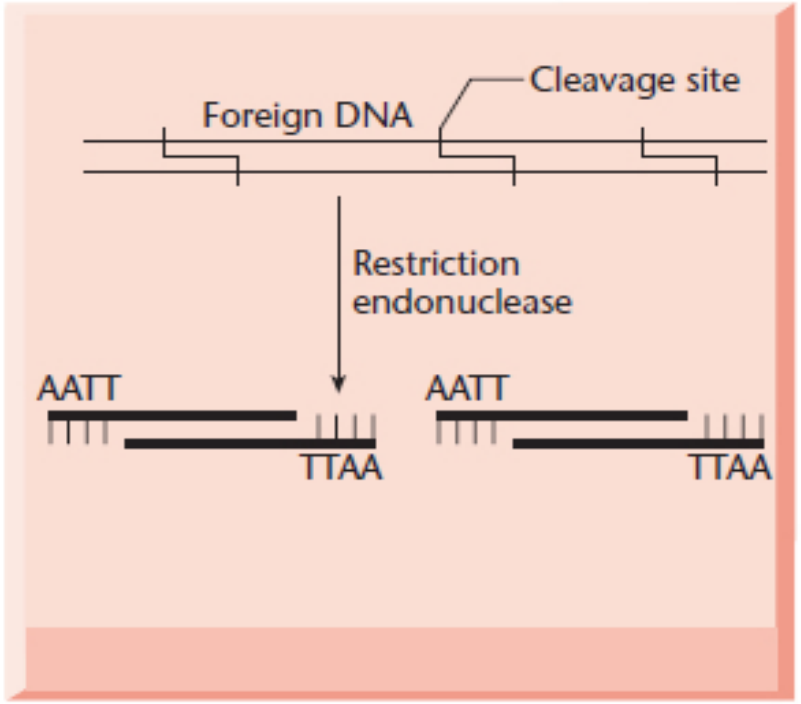
Use of DNA ligase to create a covalent DNA recombinant





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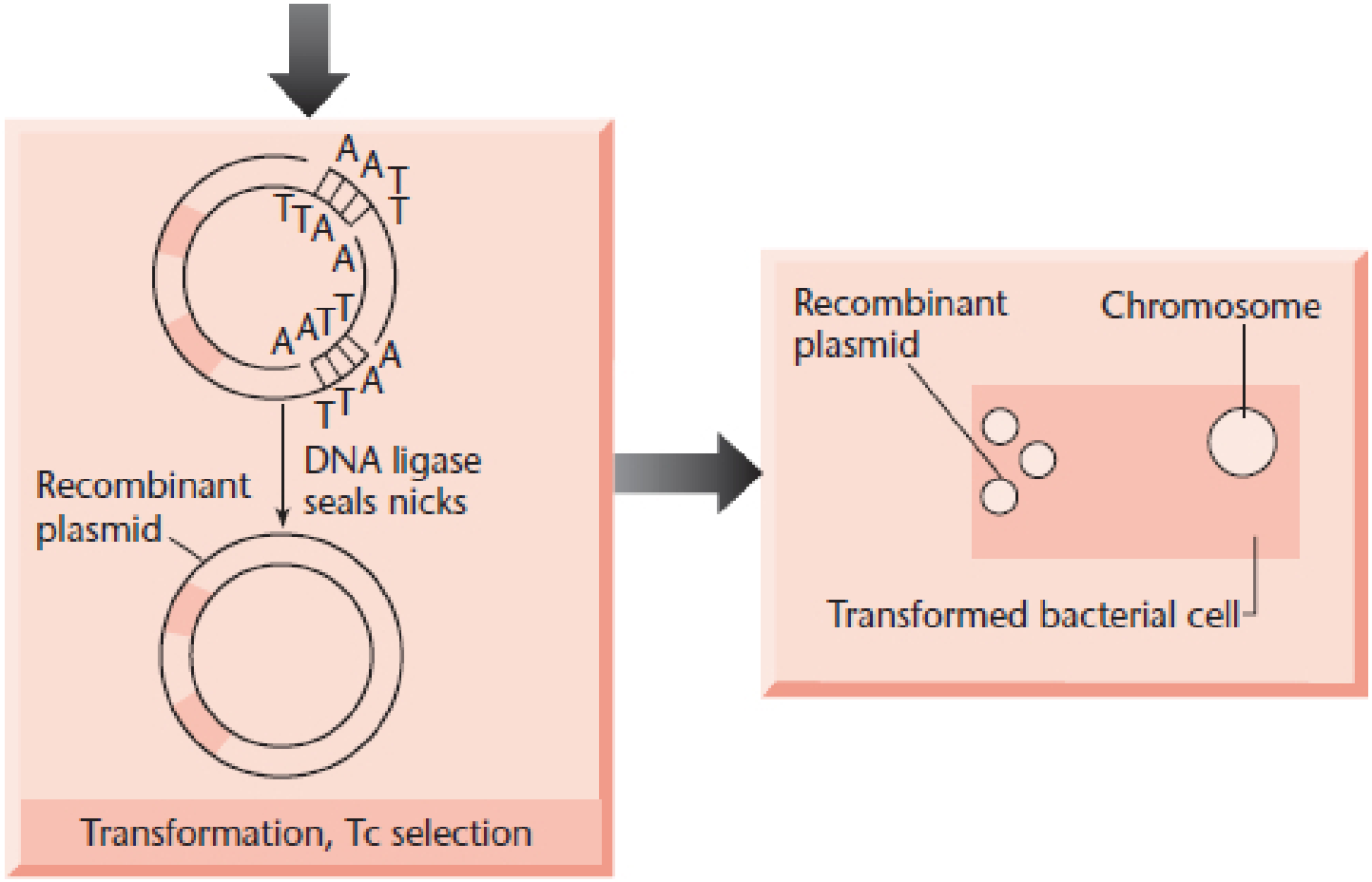

Joining DNA molecules

Alkaline Phosphatase

- An enzyme responsible for removing phosphate groups from many types of molecules including DNA
- Treatment of linearized plasmid vector DNA with alkaline phosphatase to remove 5′-terminal phosphate groups will prevent recircularization and plasmid dimer formation

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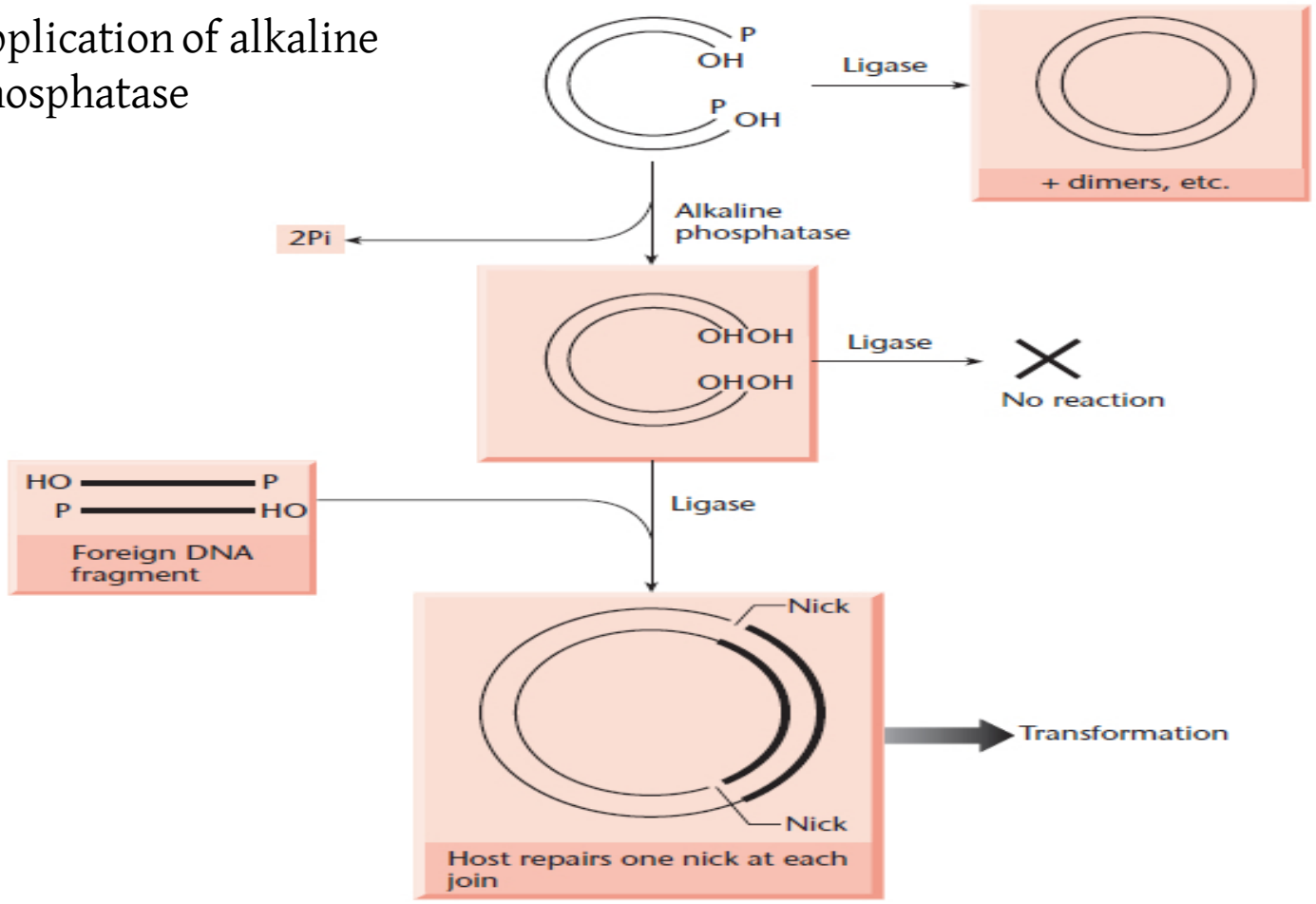
Application of alkaline phosphatase



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Joining DNA molecules

Blunt end ligation via linker molecules

- Joining DNA fragments with cohesive ends by DNA ligase is a relatively efficient process
- T4 DNA ligase has been used to joint blunt-ended DNA molecules

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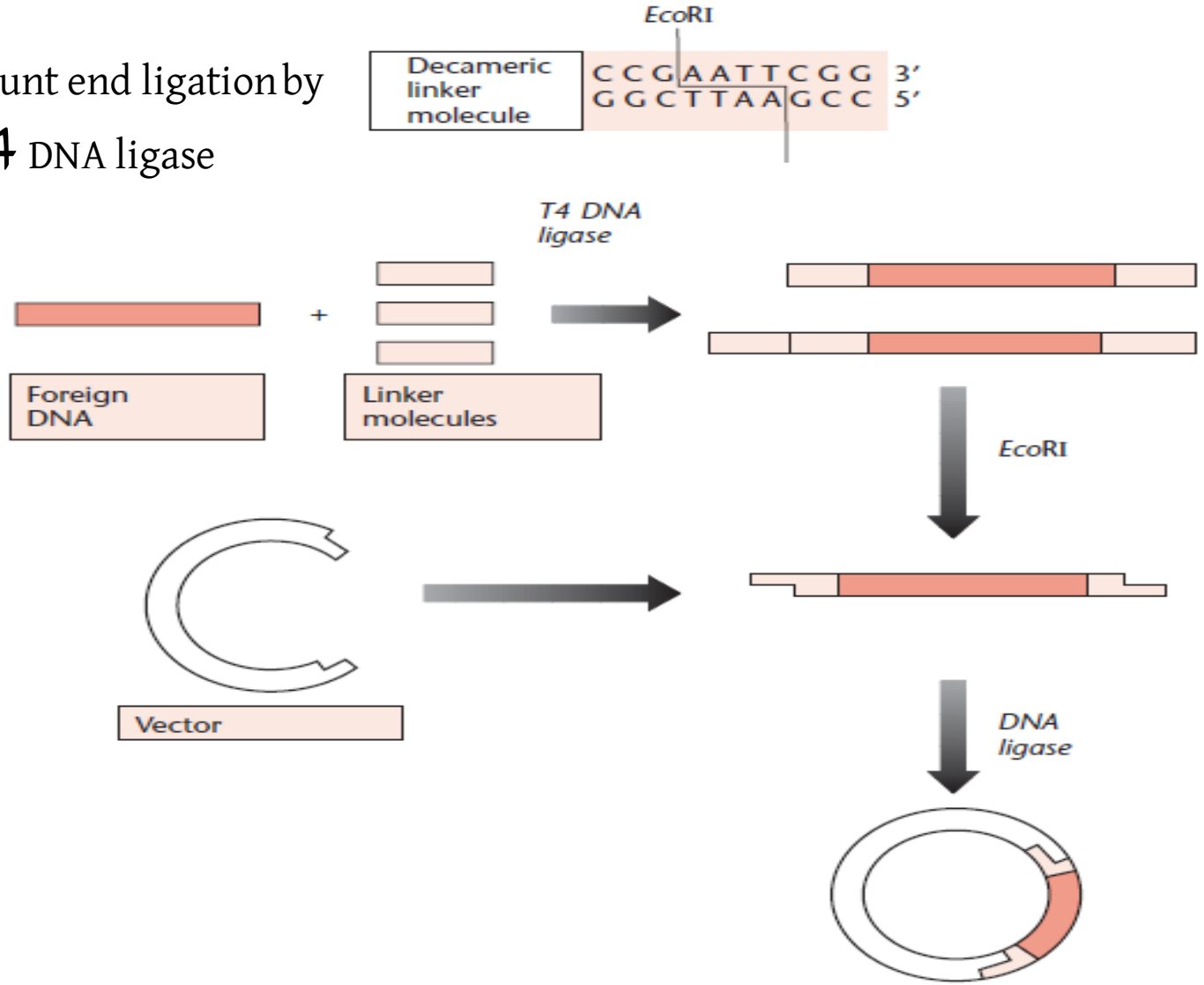
Blunt end ligation by T4 DNA ligase



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Joining DNA molecules

Adaptors

- Chemically synthesized adaptor molecules which have a preformed cohesive end can be used to insert blunt ended foreign DNA into vector

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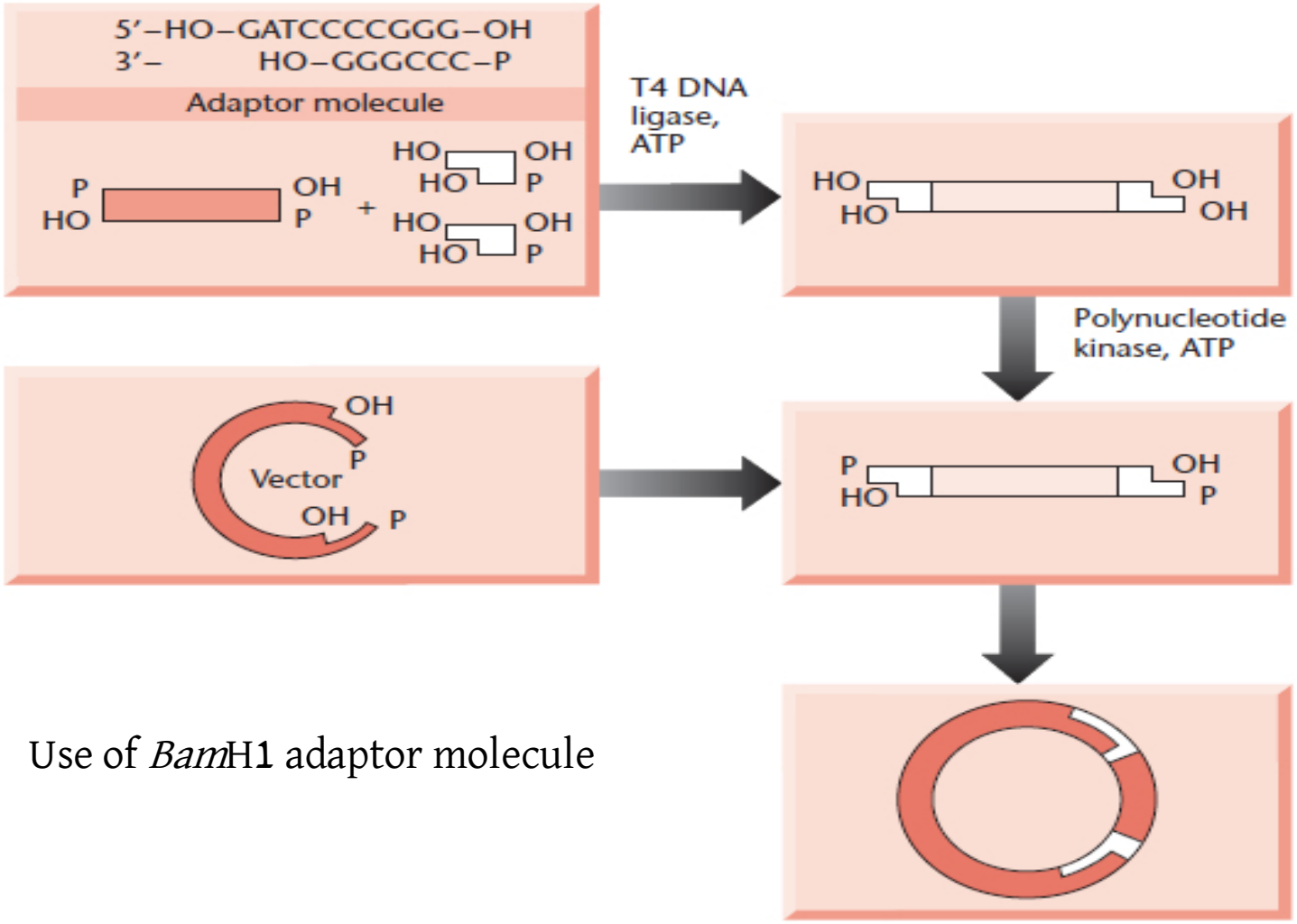


Use of *Bam*H1 adaptor molecule

Joining DNA molecules

Adaptors

- The main difference between linker and adaptor is that former having blunt ends while later have one cohesive end

Regards : Zarva Chaudhary & Chaudhary Moazzam

Essentials of Genetics

SOUTHERN BLOTTING

Regards : Zarva Chaudhary & Chaudhary Moazzam

Southern Blotting

SOUTHERN BLOTTING

- The Southern blot is used to detect the presence of a particular piece of DNA in a sample by a molecular probe.

Regards : Zarva Chaudhary & Chaudhary Moazzam

Southern Blotting

SOUTHERN BLOTTING

- Southern Blotting is named after its inventor, the British Biologist Edwin Southern (1975).

Regards : Zarva Chaudhary & Chaudhary Moazzam

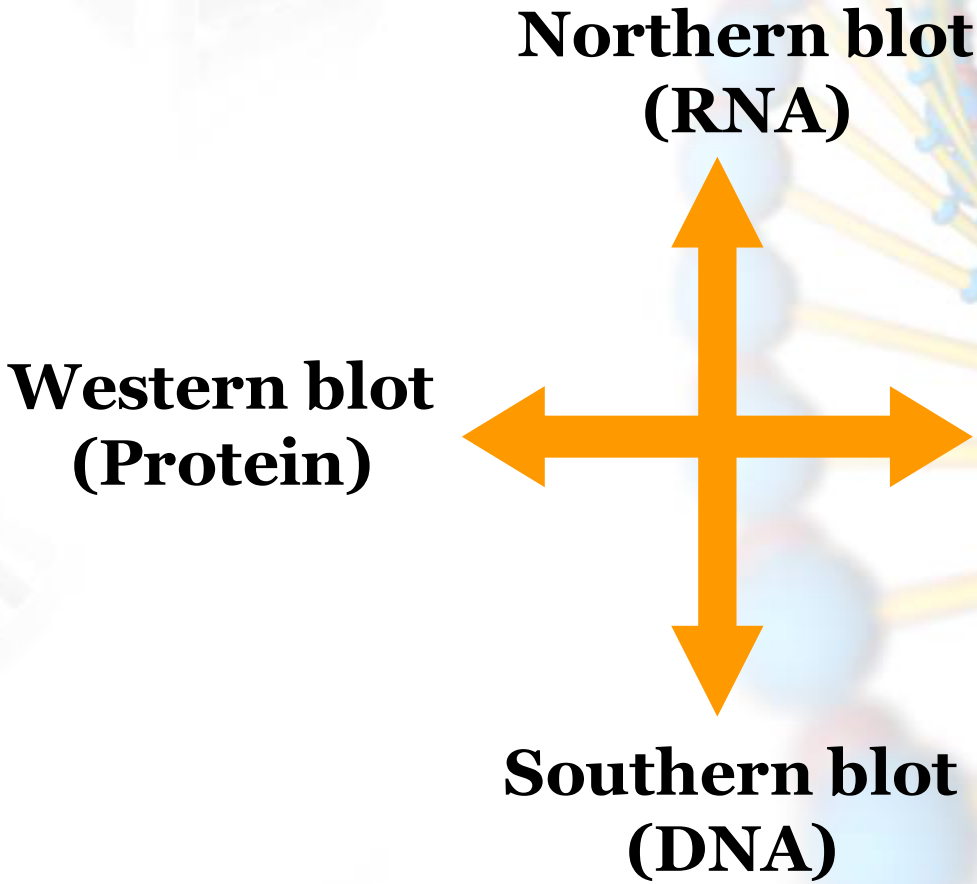
Southern Blotting

OTHER BLOTTING METHODS

- Other blotting methods with similar principles, but using protein or RNA, have been named in reference to Edwin Southern's name.

Southern Blotting

BACKGROUND



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Southern Blotting

SOUTHERN BLOTTING

- Identify DNA sequence (gene) of interest.
- Identified DNA may be a small piece of DNA or a mutation.

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Essentials of Genetics

SOUTHERN BLOTTING PROCEDURE

Regards : Zarva Chaudhary & Chaudhary Moazzam

Southern Blotting - Procedure

PROCEDURE

- DNA is extracted from cells, leukocytes.
- DNA is cleaved into many fragments by restriction enzyme (e.g, BamH1, EcoR1 etc)
- The resulting fragments are separated on the basis of size by electrophoresis.
- The DNA fragments are denatured and transferred to nitrocellulose membrane for analysis.

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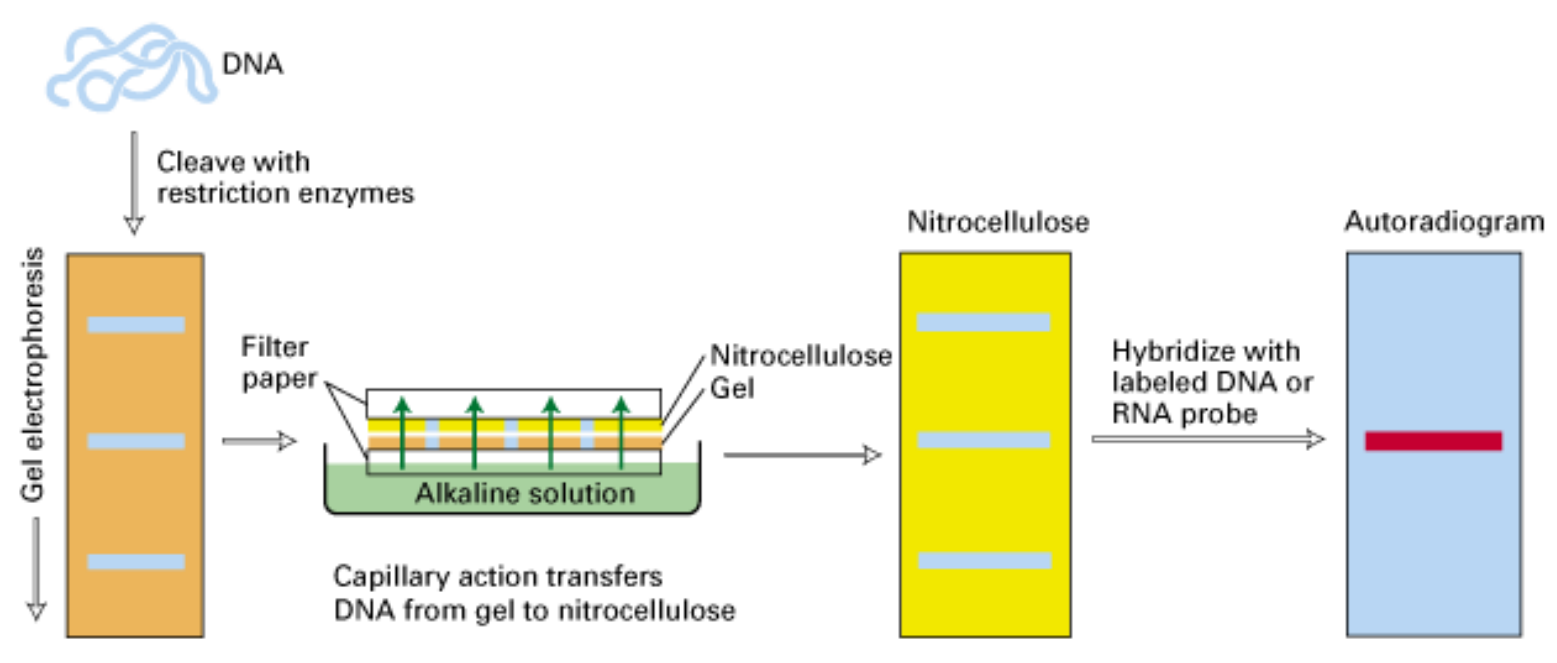
Southern Blotting - Procedure

PROCEDURE

- The labeled probe is added to the blocked membrane in buffer and incubated for several hours to allow the probe molecules to find their targets.
- Blot is incubated with wash buffers containing NaCl and detergent to wash away excess probe.
- Radioactive probes enable autoradiographic detection.

Southern Blotting - Procedure

PROCEDURE



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Southern Blotting - Procedure

CONCLUSION

- Southern blot is used to detect the presence of a particular piece of DNA in a sample by a molecular probe.

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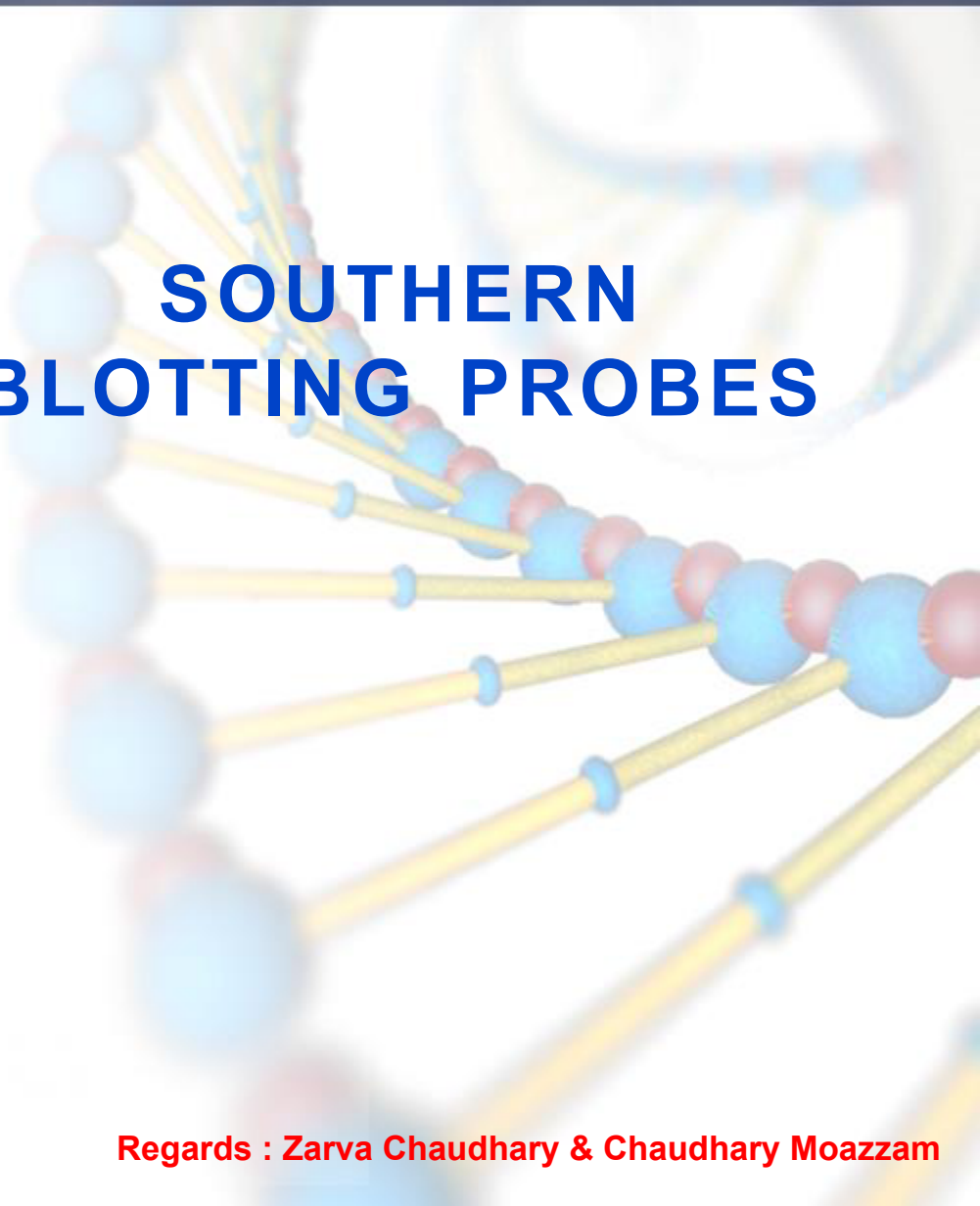
SOUTHERN BLOTTING PROBES



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Southern Blotting - Probes

PROBES

- Labeled material to detect a target.
- For DNA: 20-30 nucleotides, complementary to a region in the gene or DNA.

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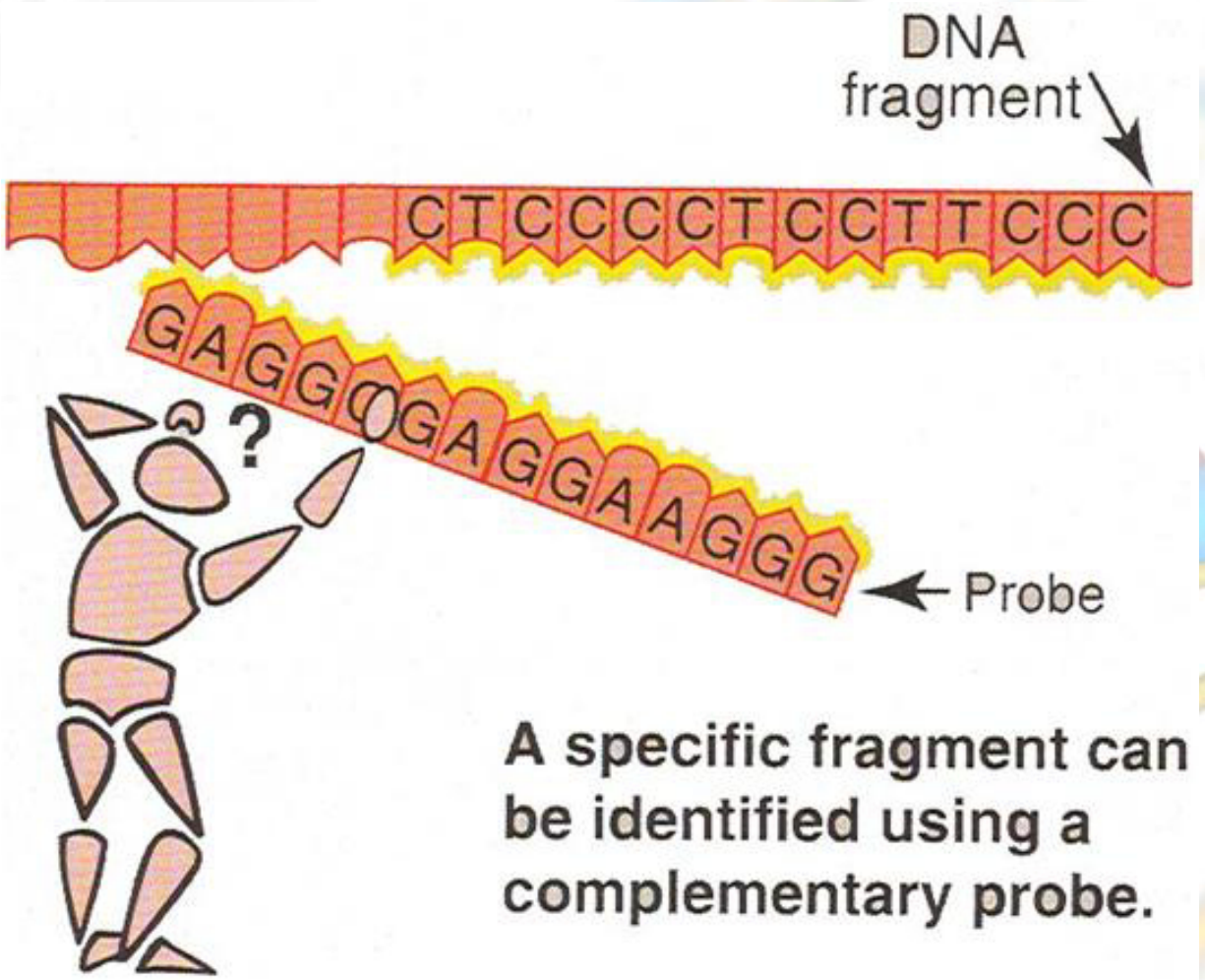
Southern Blotting - Probes



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Southern Blotting - Probes

RADIOACTIVE PROBE - P32

- Sensitive
- Relatively cheap
- Hazardous
- Radioactive waste disposal regulations should be followed

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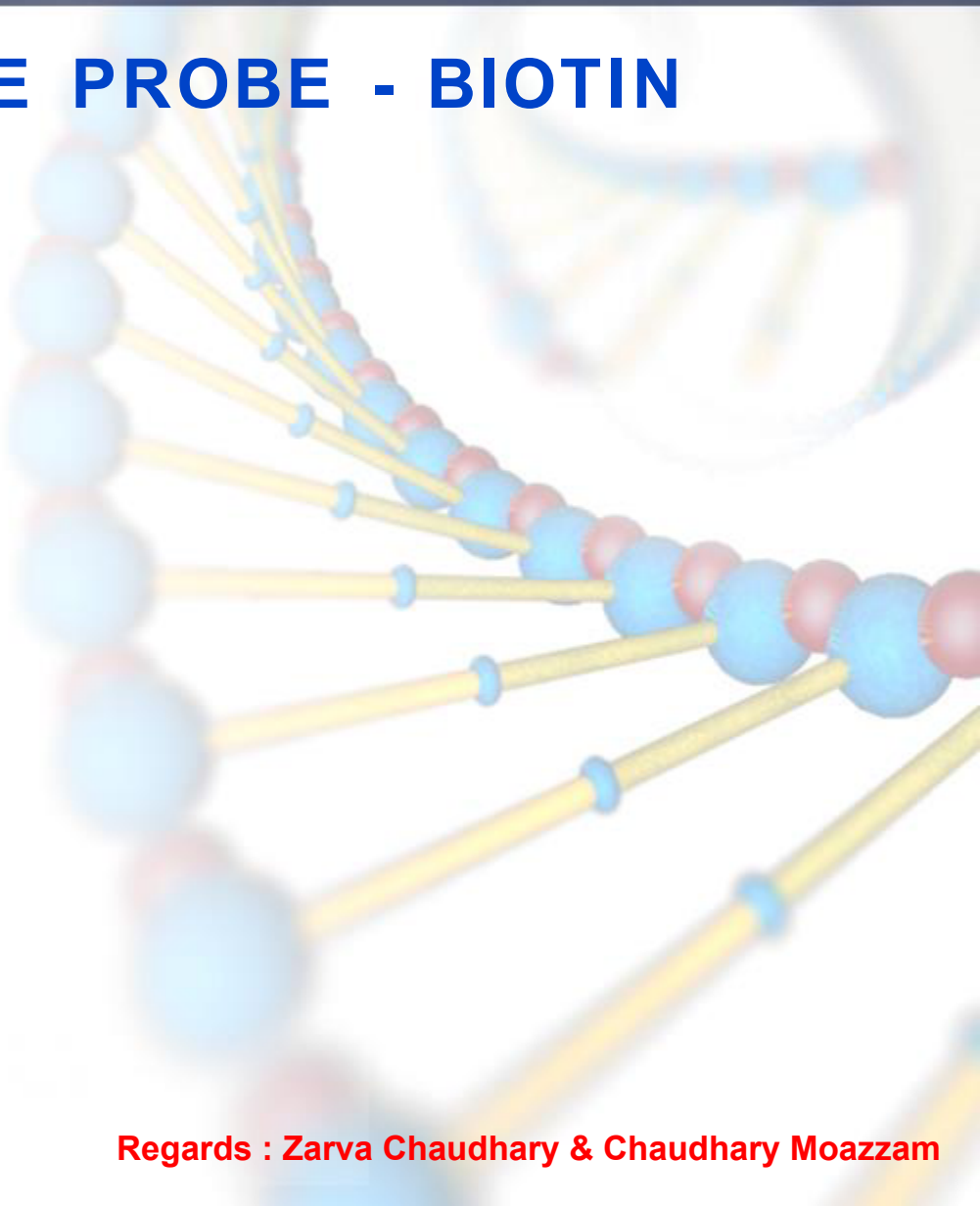
Southern Blotting - Probes

NON-RADIOACTIVE PROBE - BIOTIN

- Sensitive
- Relatively expensive



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Southern Blotting - Probes

HYBRIDIZATION OF PROBES

- The binding between single stranded labeled probe to a complementary nucleotide sequence on the target DNA.

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Southern Blotting - Probes

PROBES

- Labeled material to detect complementary region in the gene or DNA.

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Essentials of Genetics

TRANSFER METHODS IN SOUTHERN BLOTTING

Regards : Zarva Chaudhary & Chaudhary Moazzam

Transfer Methods - Southern

TRANSFER METHODS OF DNA TO MEMBRANE

- Upward capillary transfer
- Downward capillary transfer
- Simultaneous transfer to two membranes
- Electrophoretic transfer
- Vacuum transfer

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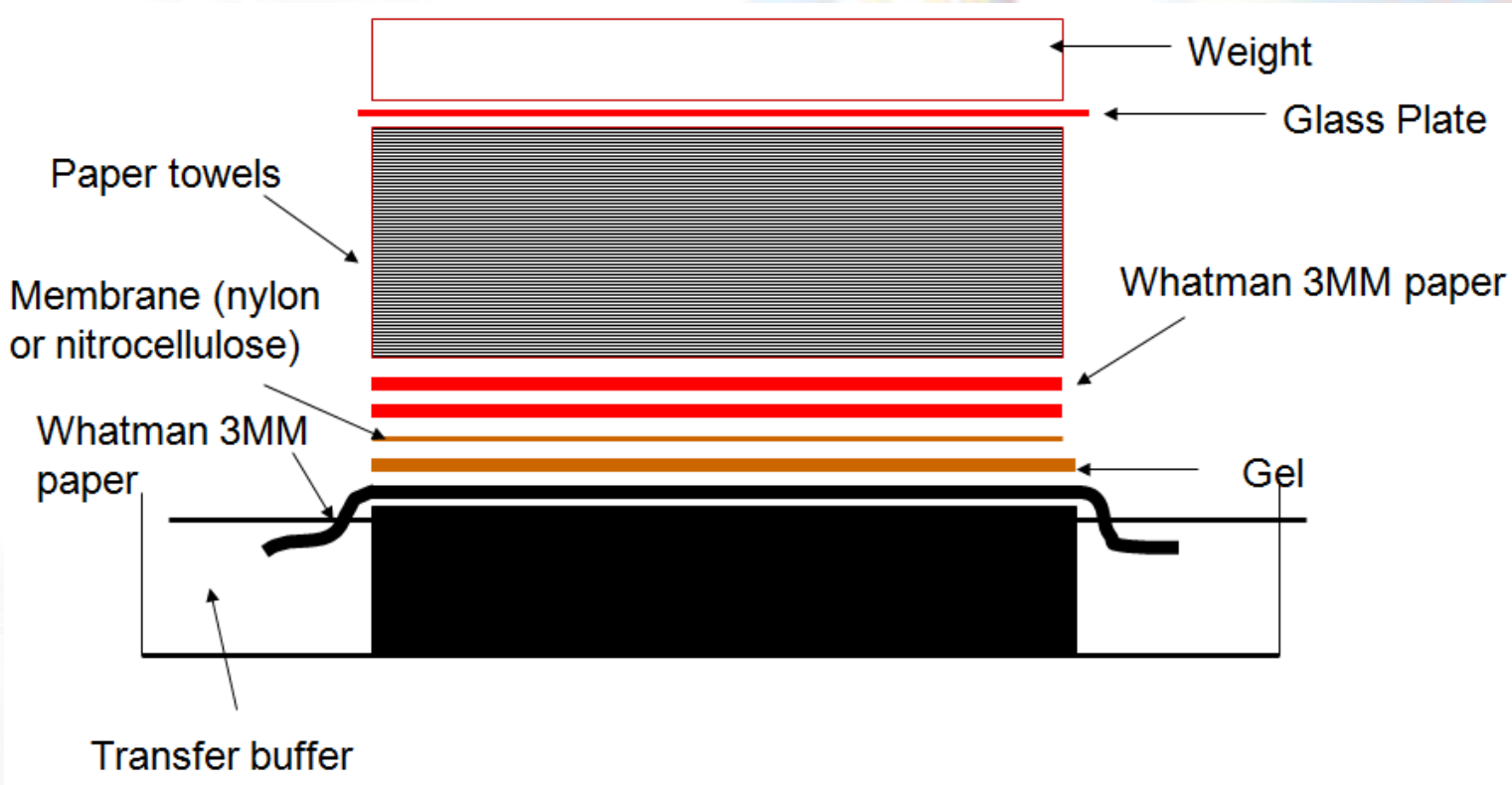
Transfer Methods - Southern

UPWARD TRANSFER



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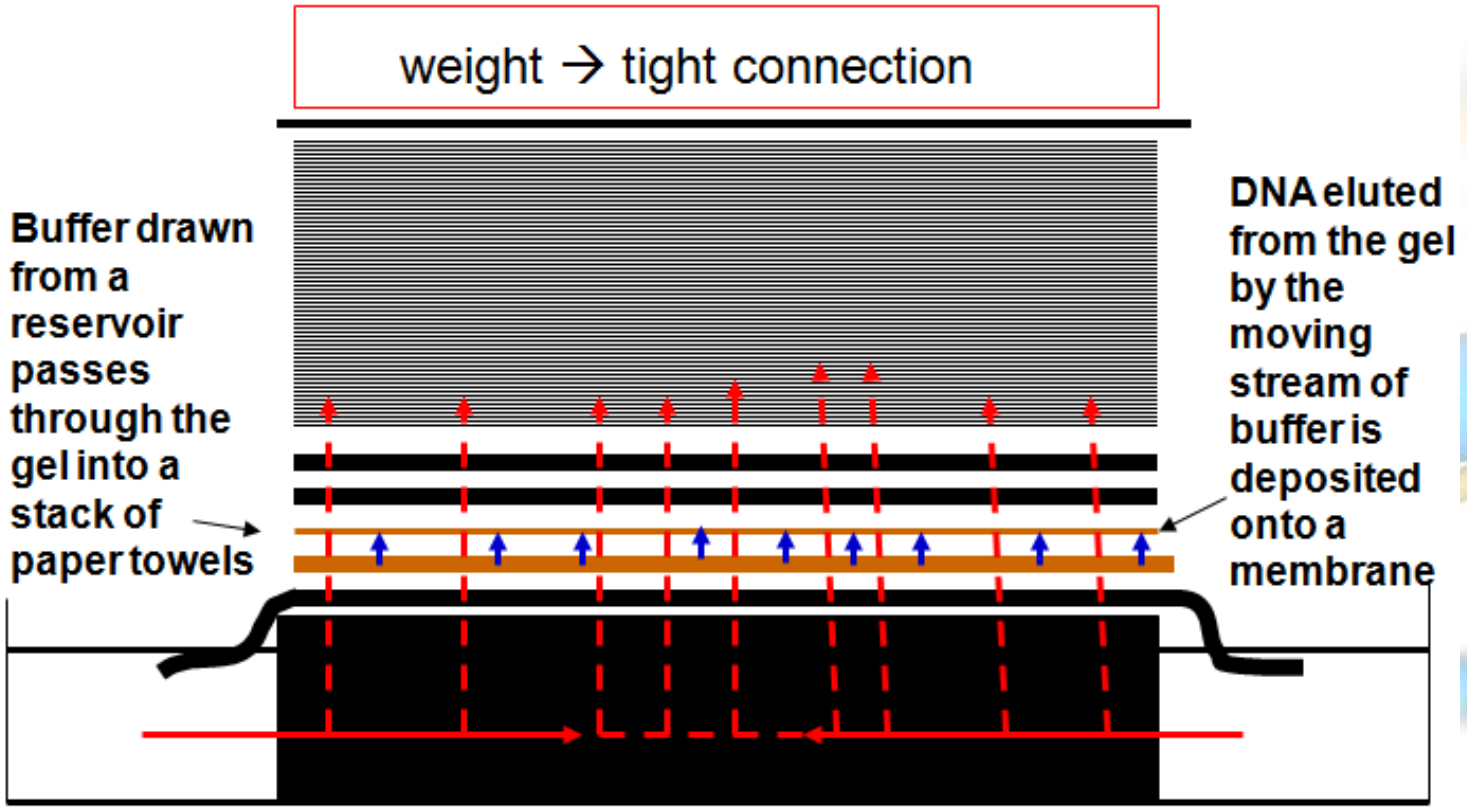
Transfer Methods - Southern

UPWARD TRANSFER OF DNA TO MEMBRANE



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Transfer Methods - Southern

TRANSFER METHODS

- Upward capillary transfer
- Downward capillary transfer
- Simultaneous transfer to two membranes
- Electrophoretic transfer
- Vacuum transfer

Regards : Zarva Chaudhary & Chaudhary Moazzam

Essentials of Genetics

MUTATIONS IDENTIFICATION BY SOUTHERN BLOTTING

Regards : Zarva Chaudhary & Chaudhary Moazzam

Mutations Identification

MUTATION IDENTIFICATION

- The presence of a mutation affecting a restriction site causes the pattern of bands to differ from those seen in a normal gene.
- A change in one nucleotide may alter the nucleotide sequence so that the restriction endonuclease fails to recognize and cleave at that site.

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Mutations Identification

MUTATION IDENTIFICATION

- Digestion of genomic DNA to DNA fragments.
- Size-separation of the fragments.
- In situ denaturation of the DNA fragments.
- Transfer of denatured DNA fragments into a solid support (nylon or nitrocellulose).
- Hybridization of the immobilized DNA to a labeled probe (DNA, RNA).
- Detection of the bands complementary to the probe (e.g. by autoradiography).

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Mutations Identification

MUTATION IDENTIFICATION

- Estimation of the size & number of the bands generated after digestion of the genomic DNA will be different.

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Mutations Identification

MUTATION IDENTIFICATION

- Mutations can be identified by Southern blotting.

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NORTHERN BLOTTING

Regards : Zarva Chaudhary & Chaudhary Moazzam

Northern Blotting

NORTHERN BLOTTING

- Northern blot is a method used for detection of a specific RNA sequence in RNA samples.

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Northern Blotting

PROCEDURE

- Isolation of intact mRNA.
- Separation of RNA according to size (through a denaturing agarose gel.
- Transfer of the RNA to a solid support.
- Fixation of the RNA.
- Hybridization of the immobilized RNA to probes complementary to the sequences of interest.

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Northern Blotting

PROCEDURE

- Removal of probe molecules that are nonspecifically bound to the solid matrix.
- Detection, capture and analysis of an image of the specifically bound probe molecules.

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Northern Blotting

NORTHERN BLOTTING

- Northern blot is a method used for detection of a specific RNA sequence in RNA samples.

Regards : Zarva Chaudhary & Chaudhary Moazzam

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NORTHERN BLOT APPLICATIONS

Regards : Zarva Chaudhary & Chaudhary Moazzam

Northern Blot - Applications

APPLICATIONS

- Study of gene expression in eukaryotic cells.
- To measure the amount & size of RNAs transcribed from eukaryotic genes.
- To estimate the abundance of RNAs.

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Northern Blot - Applications

APPLICATIONS

- To equalize the amounts of RNA loaded into lanes of gels.
- Use of housekeeping gene (endogenous constitutively-expressed gene).
- Normalizing samples according to their content of mRNAs of the housekeeping gene.

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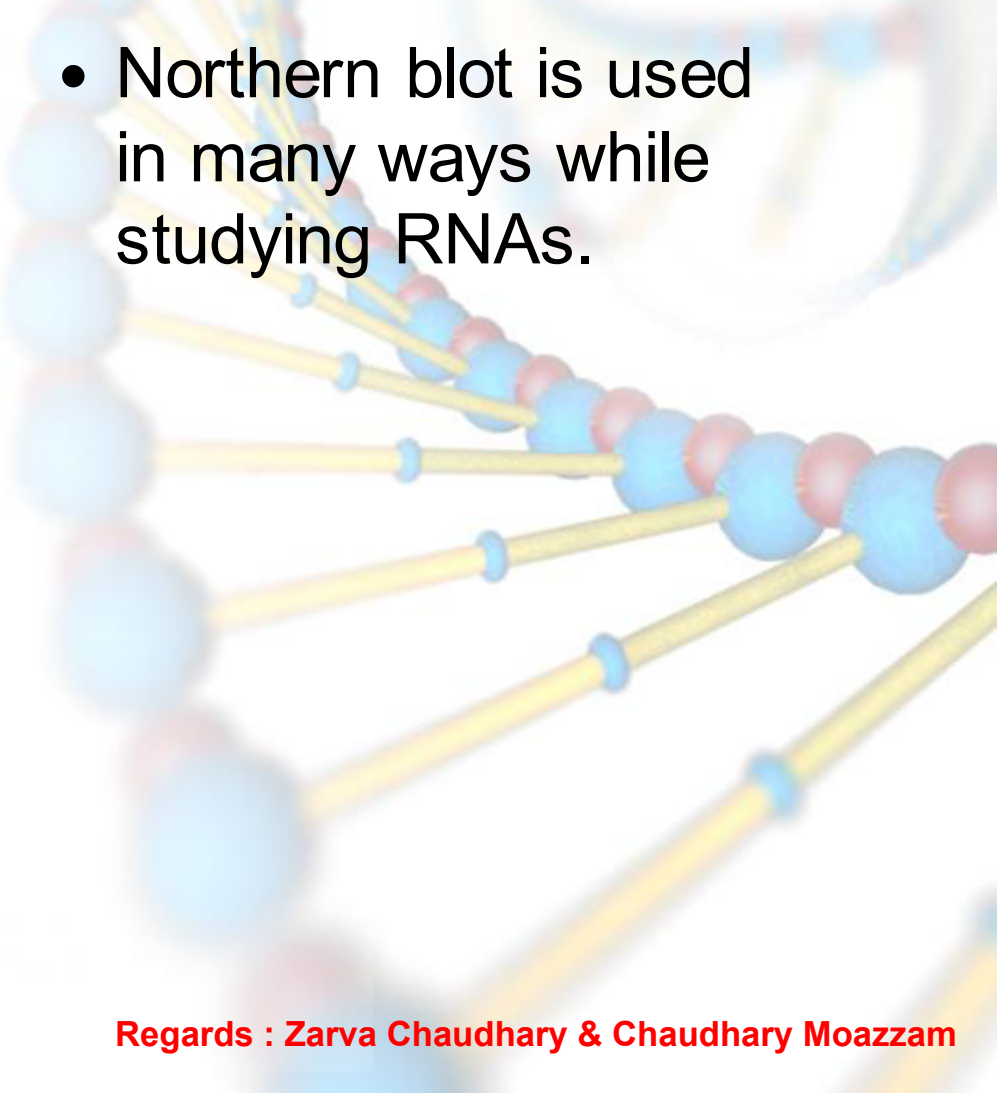
Northern Blot - Applications

APPLICATIONS

- Northern blot is used in many ways while studying RNAs.



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Essentials of Genetics

WESTERN BLOTTING

Regards : Zarva Chaudhary & Chaudhary Moazzam

Western Blotting

WESTERN BLOTTING

- A technique used to detect the presence of a specific protein in a complex protein mixture.

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Western Blotting

WESTERN BLOTTING

- To determine the molecular weight of a protein.
- To measure relative amounts (quantitation) of the protein present in complex mixtures of proteins that are not radiolabeled.

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Western Blotting

WESTERN BLOTTING

- Western blots have become one of the most common analytical tools for the detection of viral proteins.
- Characterization of monoclonal and polyclonal antibody preparations and in determining the specificity of the immune response to viral antigens.

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Western Blotting

WESTERN BLOTTING

- A technique used to detect the presence of a specific protein in a complex protein mixture.

Regards : Zarva Chaudhary & Chaudhary Moazzam

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WESTERN BLOTTING PROCEDURE

Regards : Zarva Chaudhary & Chaudhary Moazzam

Western Blotting - Procedure

PROCEDURE

1- Sample preparation

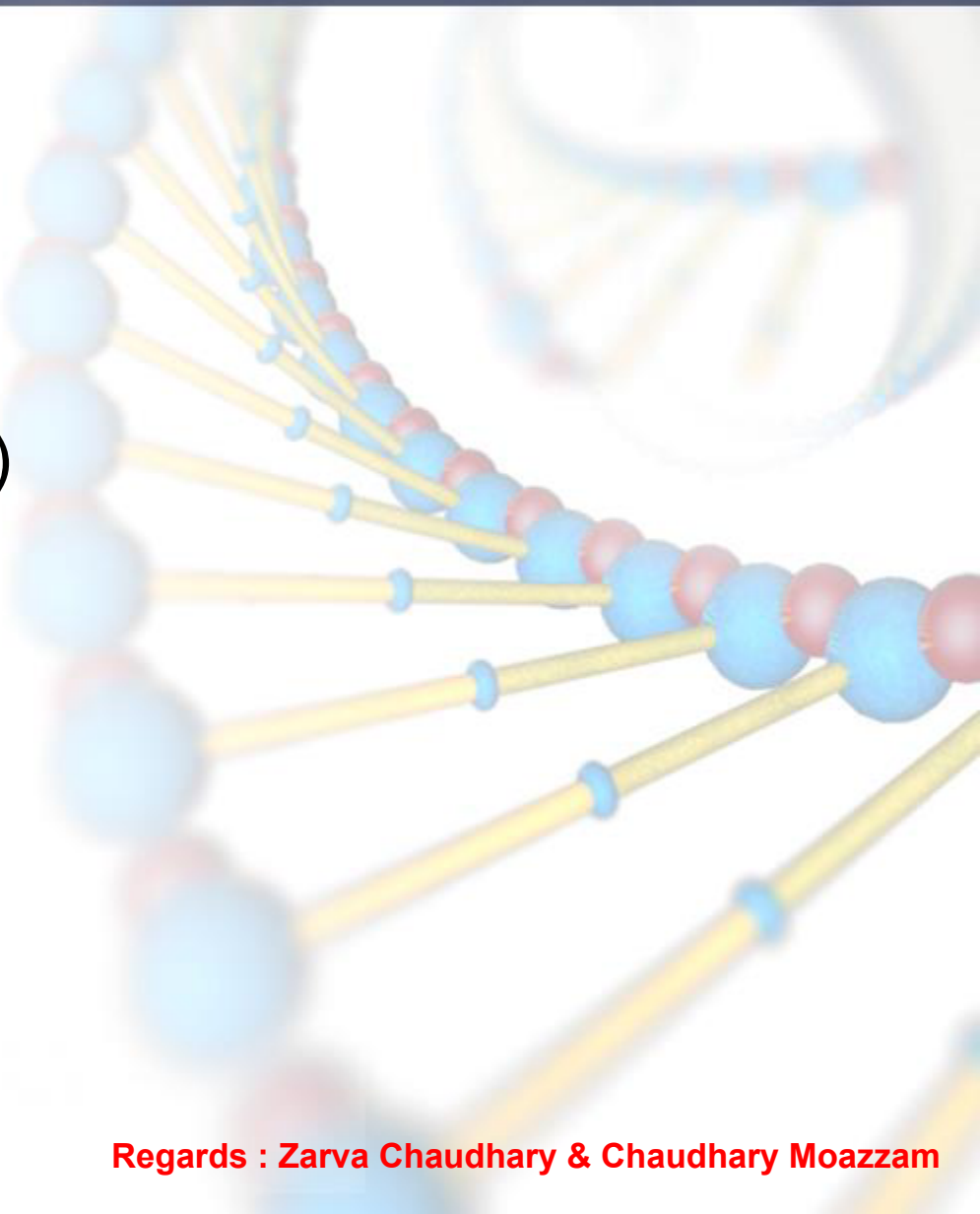
2- Gel Electrophoresis

3- Blotting (or transfer)

4- Blocking

5- Antibody probing

6- Detection



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Western Blotting - Procedure

PROCEDURE

1- The choice of extraction method depends primarily on the sample and whether the analysis is targeting all the proteins in a cell or only a component from a particular subcellular fraction.

2- Samples are loaded into separate wells. A protein marker is also loaded. The separated protein mixtures are transferred to a solid support for further analysis.

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Western Blotting - Procedure

PROCEDURE

3- Transfer can be done in wet or semi-dry conditions. Semi-dry transfer is generally faster. Wet transfer is recommended for large proteins, >100 kD.

4- Blocking is a very important step in the immunodetection phase of Western blotting because it prevents non-specific binding of antibody to the blotting membrane.

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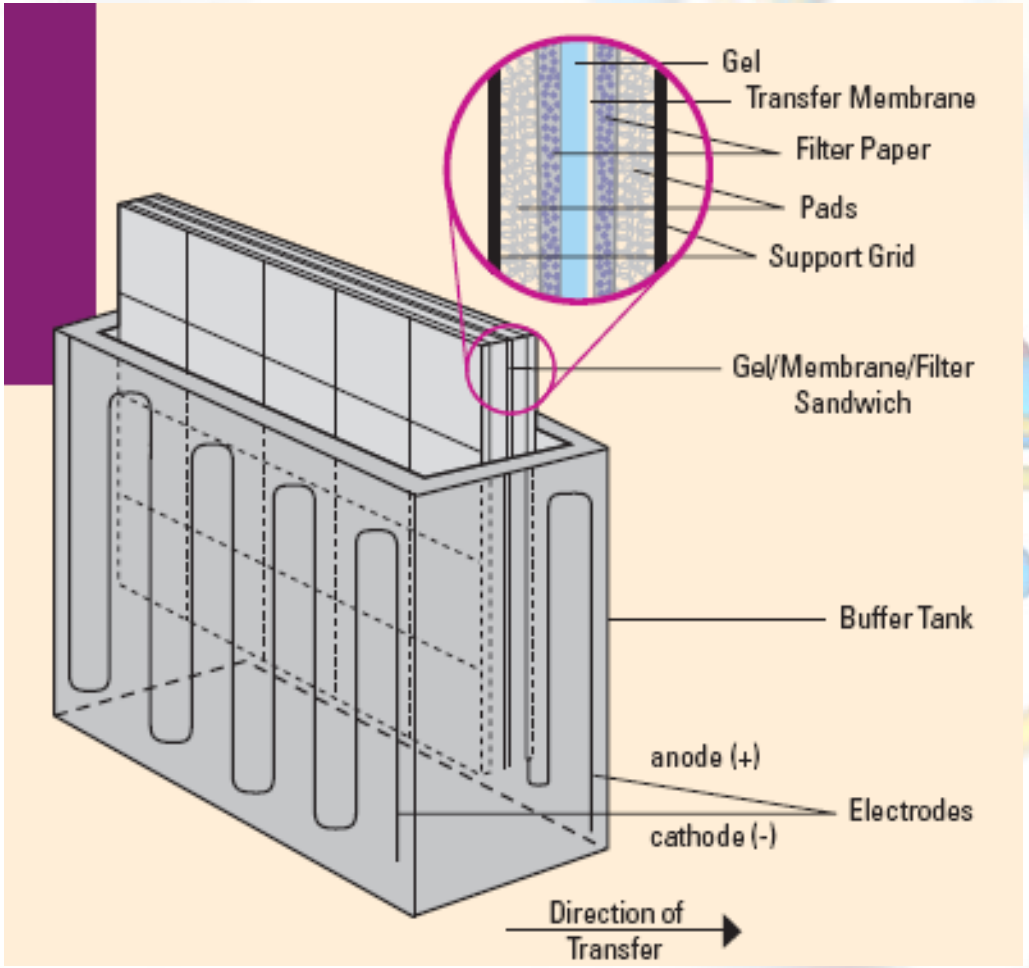
Western Blotting - Procedure

ELECTROPHORETIC TRANSFER



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Western Blotting - Procedure

PROCEDURE

Protein of interest is detected and localized using a specific antibody. Western blotting protocols utilize a non-labeled primary antibody directed against the target protein.

A species-specific, labeled secondary antibody directed against the constant region of the primary antibody is then used. The most common antibody label used in Western blots is HRP.

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Western Blotting - Procedure

PROCEDURE

The signal is detected when HRP is exposed to a substrate solution in the final step of the immunodetection procedure

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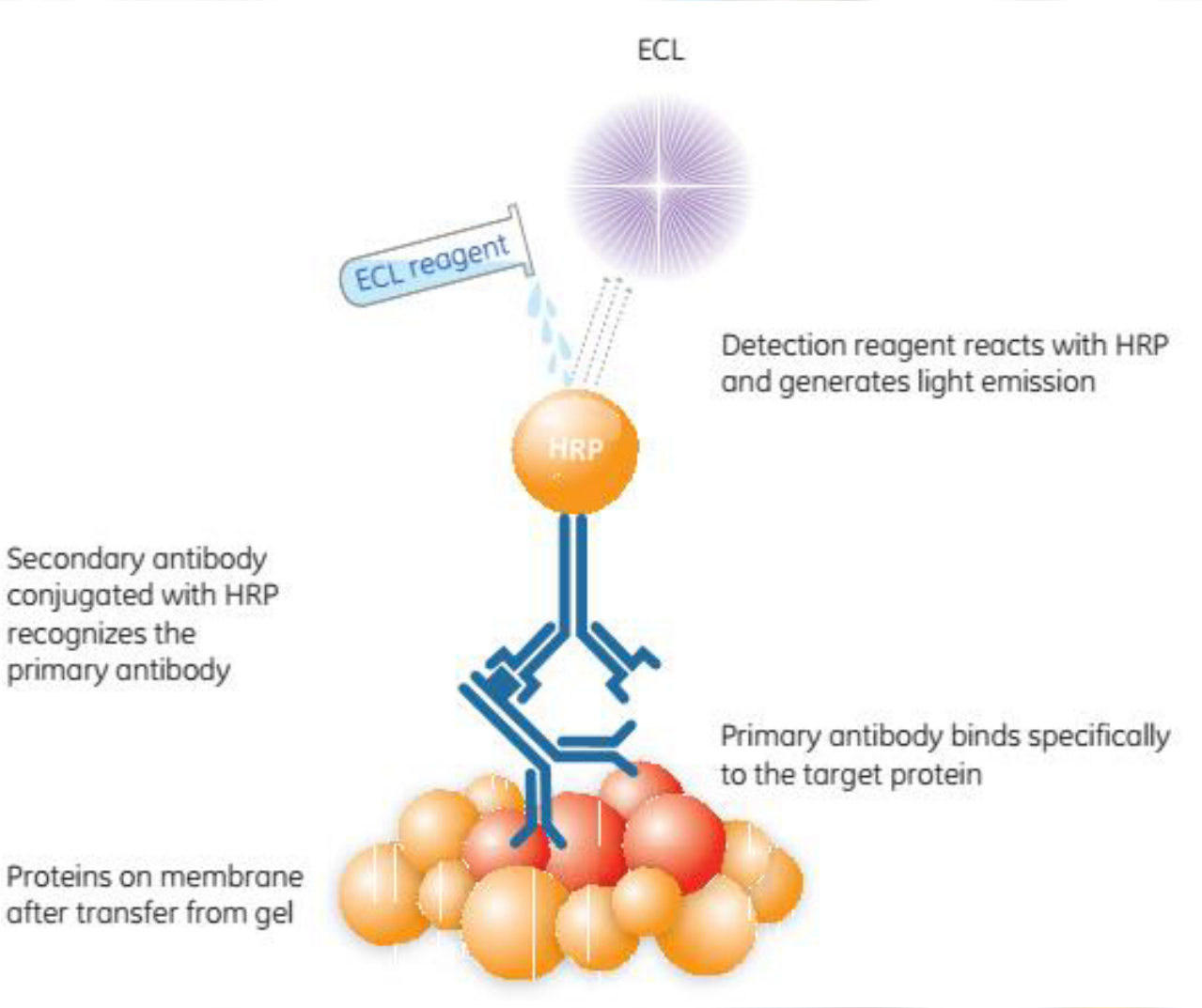
Western Blotting - Procedure



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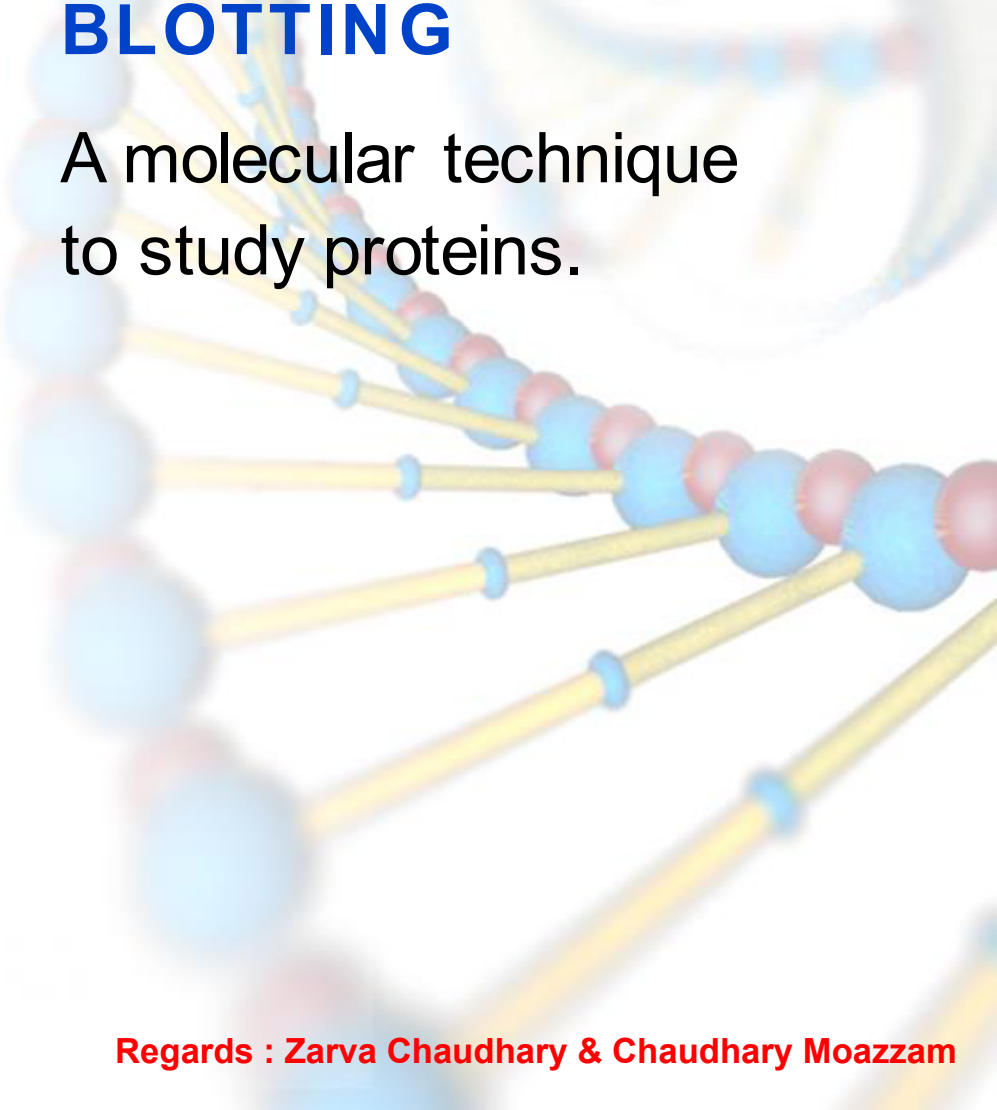
Western Blotting - Procedure

WESTERN BLOTTING

A molecular technique to study proteins.



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Applications of western blotting

Regards : Zarva Chaudhary & Chaudhary Moazzam

- Analysis Of IgG Fractions purified from human plasma.
- Diagnosis of HIV by ELISA, involves the western blotting technique.
- Western blotting technique is also used to Detect Some Forms Of Lyme Disease.
- Western blotting technique is used in Definitive Test For BSE, which is commonly know as Mad cow disease.
- Confirmatory Test For Hepatitis-B involves western blotting technique.
- Western blotting test is used in the Analysis Of Biomarkers such as hormones, growth factors & cytokines.
- This technique is also employed in The Gene Expression Studies

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Limitations in western blotting

- very delicate and time consuming process. A minute imbalance at any level of the procedure can skew the results of the entire process.
- Incorrect labeling of the protein can happen due to the reaction of secondary antibody.
- Cause erroneous in bands or no bands due to insufficient transfer.
- Well trained technicians are required for this technique.
- Primary antibody availability is crucial.
- It is just a semi-quantitative at best. Only an approx. estimation & not a precise measurement of molecular weight of the protein is possible

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Conclusion

- Western blotting technique is simply a way to identify unknown proteins on a polyacrylamide gel.
- It is sometimes called as protein blotting or immunoblotting.
- It is a widely used analytical technique in the fields of molecular biology, immunogenetics, and other biochemistry disciplines.
- Western blotting technique is also used in the field of medical diagnostics. i.e., in the analysis of various kinds of diseases.
- Apart from the limitations of western blotting, it is more helpful now a days. Hence, The discovery of Western blotting technique has become boon in the field of science & technology

Regards : Zarva Chaudhary & Chaudhary Moazzam

Difference between different blotting technique

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Northern vs Southern vs Western Blotting

Type of Molecule Detected	
Northern Blotting	Northern blotting detects a specific RNA sequence from an RNA sample.
Southern Blotting	Southern blotting detects a specific DNA sequence from a DNA sample.
Western Blotting	Western blotting detects a specific protein from a protein sample.
Type of Gel	
Northern Blotting	This uses Agarose/formaldehyde gel.
Southern Blotting	This uses an Agarose gel.
Western Blotting	This uses Polyacrylamide gel.
Blotting Method	
Northern Blotting	This is a capillary transfer.
Southern Blotting	This is a capillary transfer.
Western Blotting	This is an electric transfer.

Regards : Zarva Chaudhary & Chaudhary Moazzam

Northern vs Southern vs Western Blotting

Probes Used	
Northern Blotting	cDNA or RNA probes labeled radioactively or nonradioactively.
Southern Blotting	DNA probes are labeled radioactively or non-radioactively.
Western Blotting	Primary antibodies are used as probes.
Detection System	
Northern Blotting	This is done using an autoradiograph, or detection of light or color change.
Southern Blotting	This is done using an autoradiograph, detection of light or color change.
Western Blotting	This is done using the detection of light or color change.

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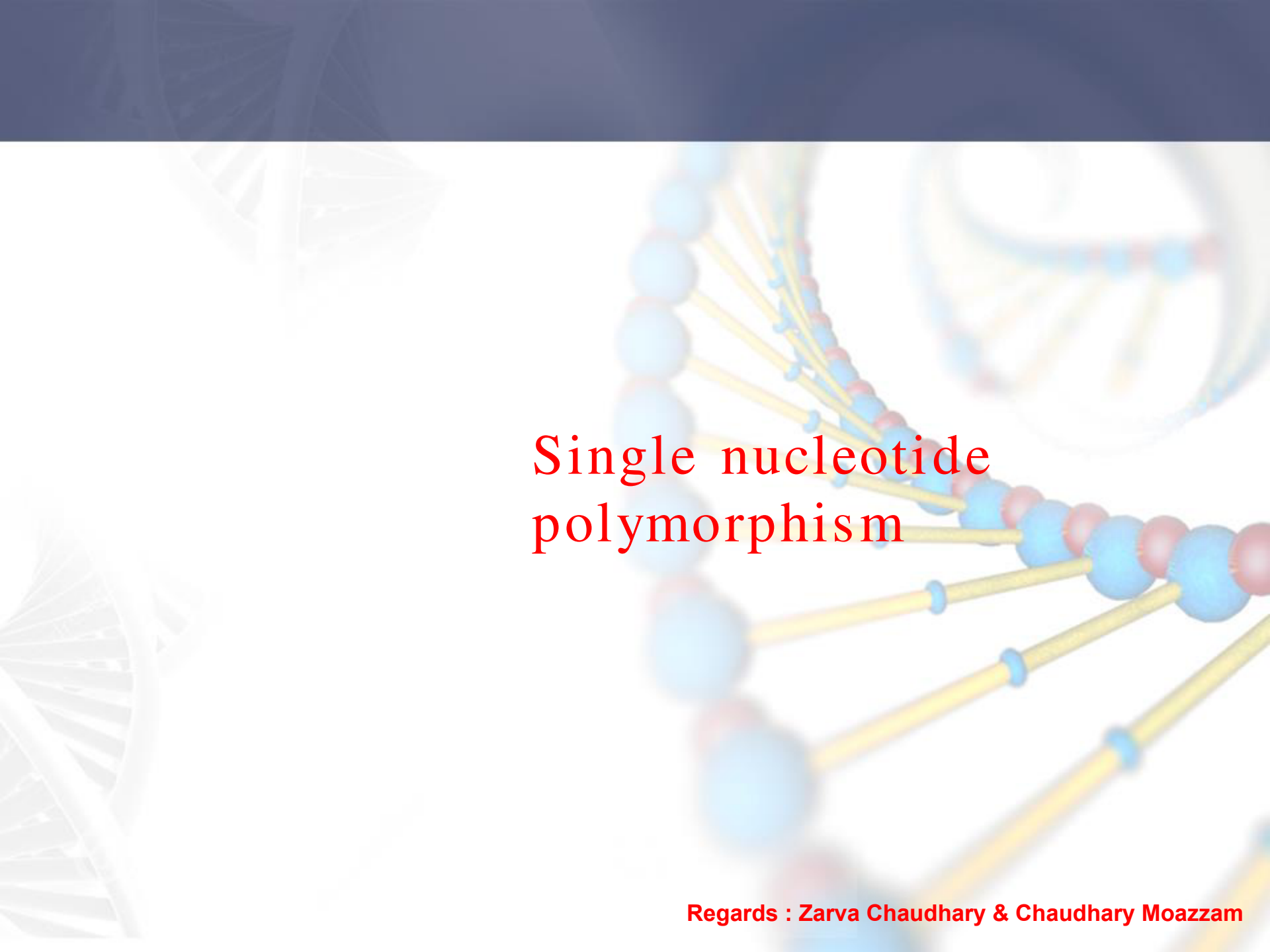
Single nucleotide polymorphism



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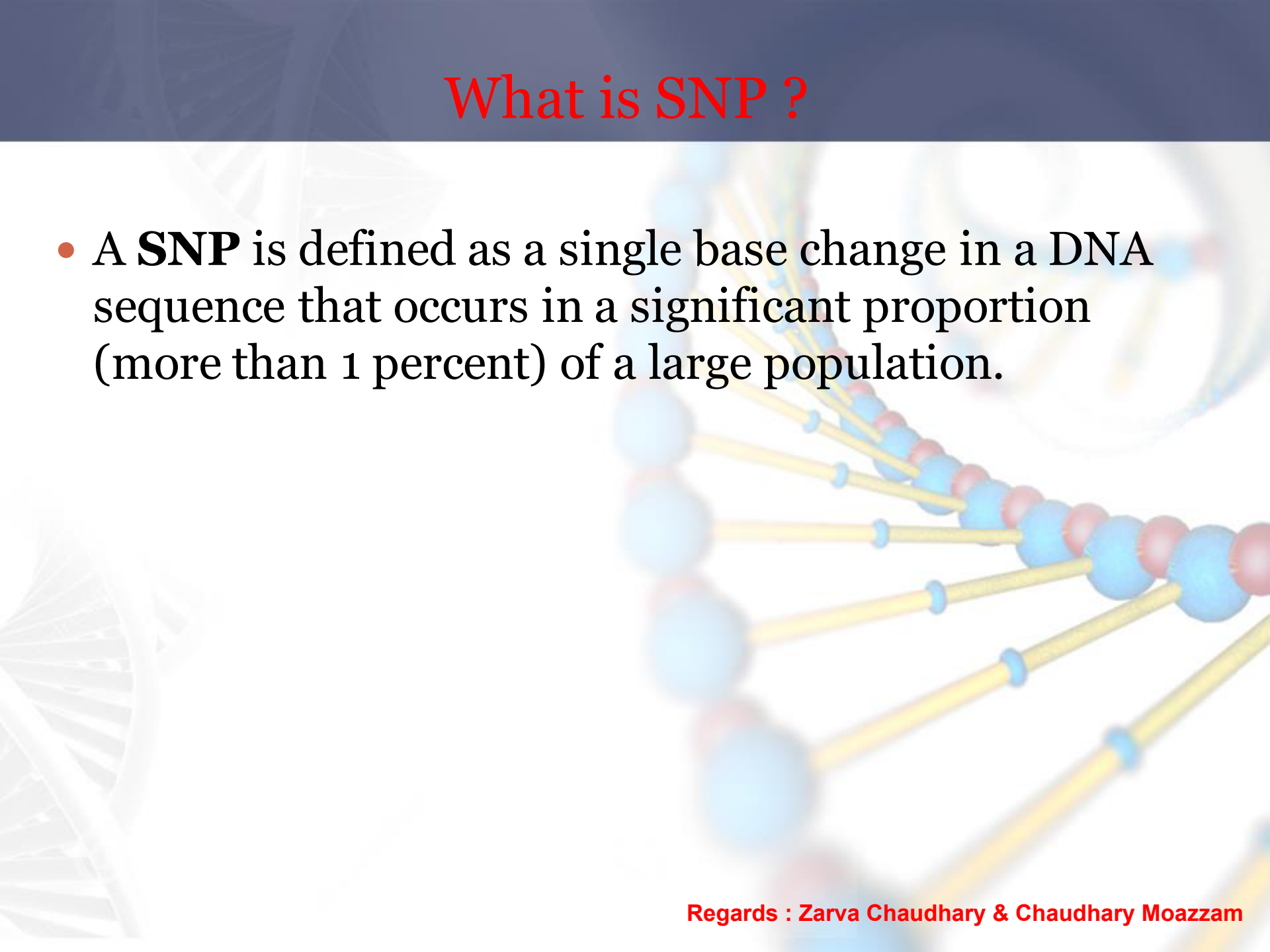
What is SNP ?

- A **SNP** is defined as a single base change in a DNA sequence that occurs in a significant proportion (more than 1 percent) of a large population.



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Some Facts

- In human beings, 99.9 percent bases are same.
- Remaining 0.1 percent makes a person unique.
  - Different attributes / characteristics / traits
    - how a person looks,
    - diseases he or she develops.

- These variations can be:
  - Harmless (change in phenotype)
  - Harmful (diabetes, cancer, heart disease, Huntington's disease, and hemophilia)
  - Latent (variations found in coding and regulatory regions, are not harmful on their own, and the change in each gene only becomes apparent under certain conditions e.g. susceptibility to lung cancer)

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- SNPs are found in
  - coding and (mostly) noncoding regions.
- Occur with a very high frequency
  - about 1 in 1000 bases to 1 in 100 to 300 bases.
- The abundance of SNPs and the ease with which they can be measured make these genetic variations significant.
- SNPs close to particular gene acts as a marker for that gene.
- SNPs in coding regions may alter the protein structure made by that coding region.

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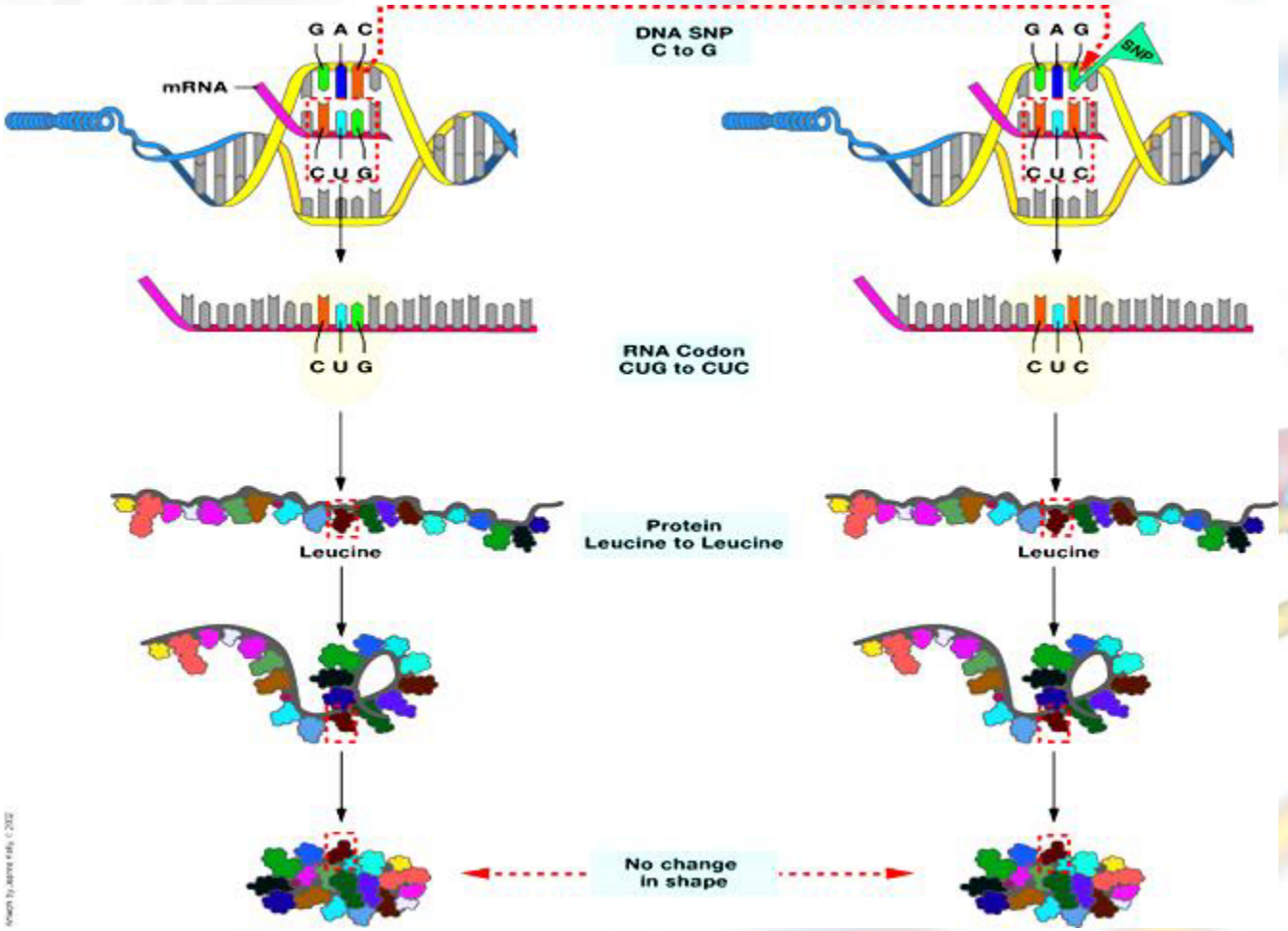
SNPs may / may not alter protein structure



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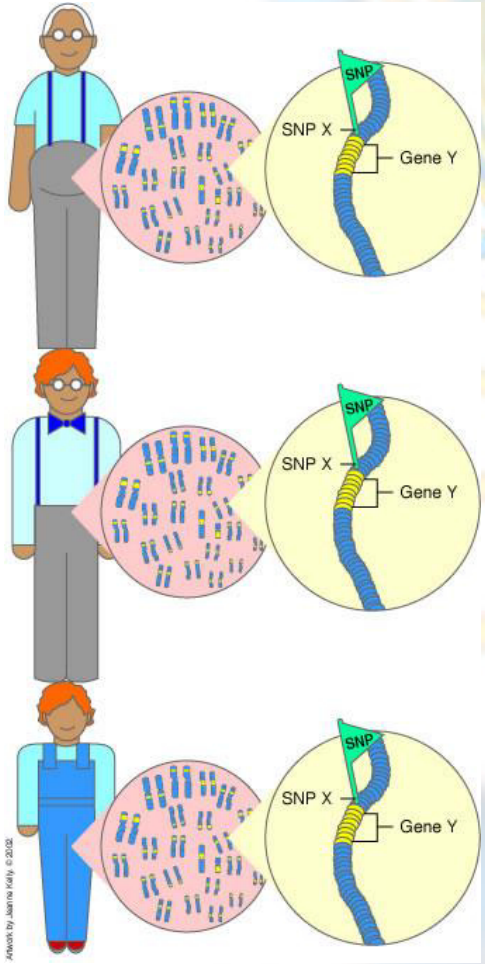
SNPs act as gene markers



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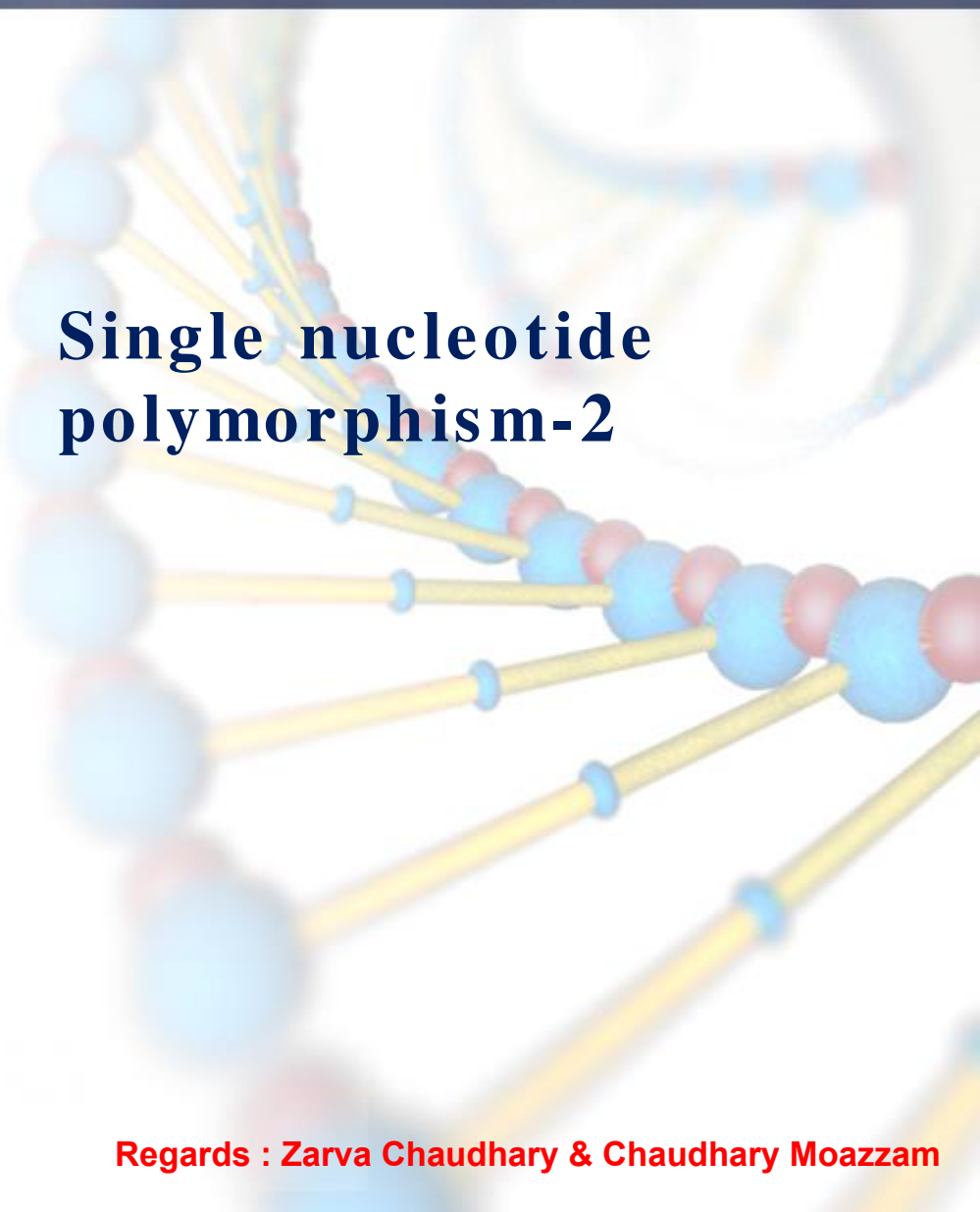
Single nucleotide polymorphism-2



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SNP maps

- Sequence genomes of a large number of people
- Compare the base sequences to discover SNPs.
- Generate a single map of the human genome containing all possible SNPs => SNP maps

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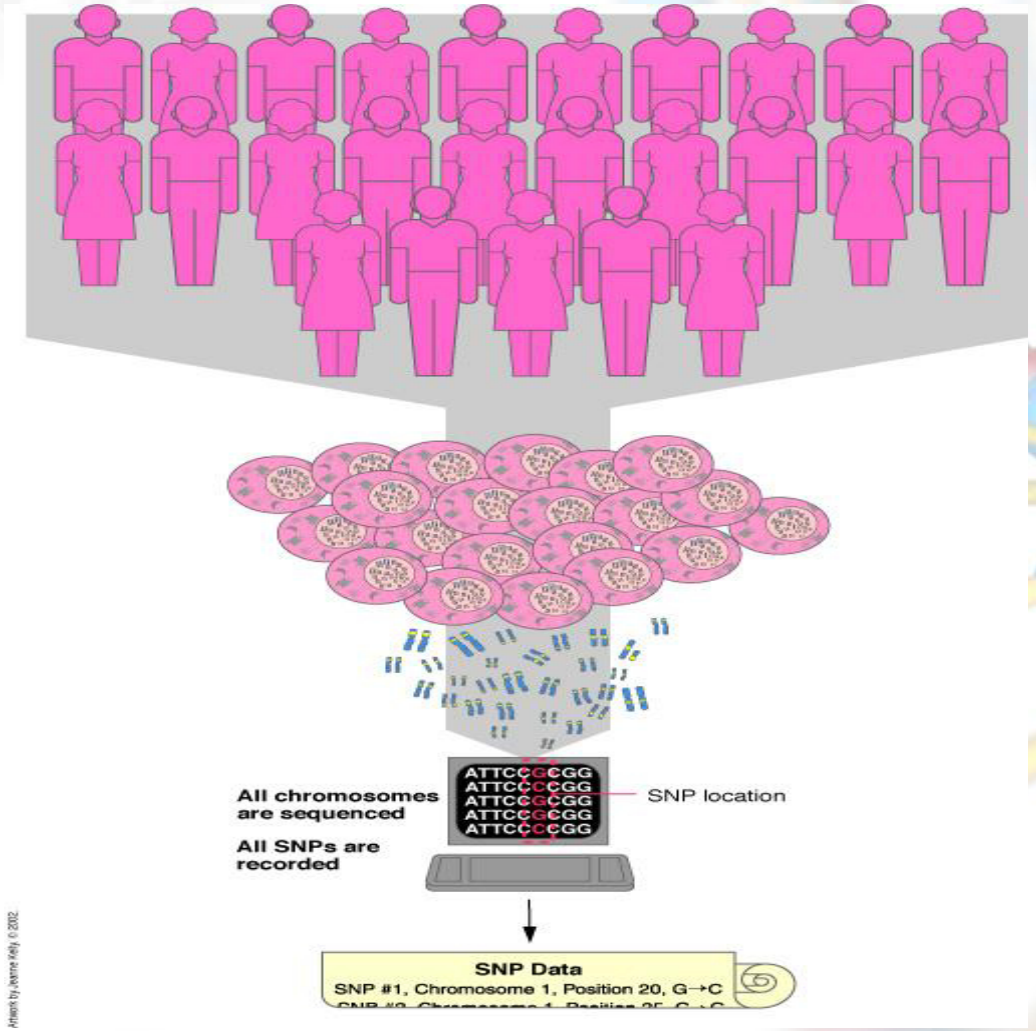
SNP Maps



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SNP Profiles

- Genome of each individual contains distinct SNP pattern.
- People can be grouped based on the SNP profile.
- SNPs Profiles important for identifying response to Drug Therapy.
- Correlations might emerge between certain SNP profiles and specific responses to treatment.

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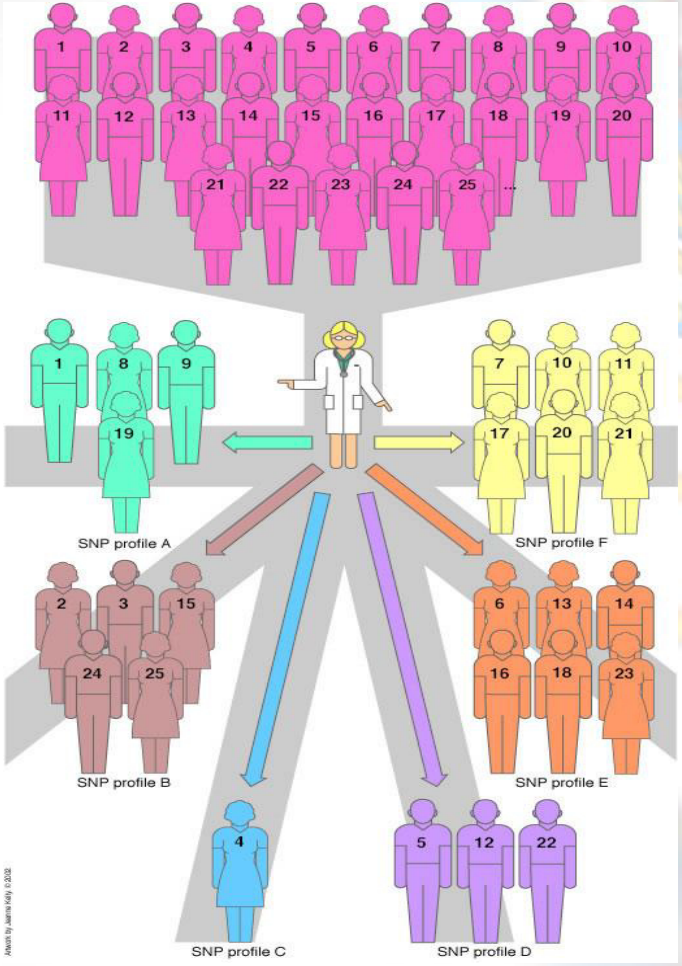
SNP Profiles



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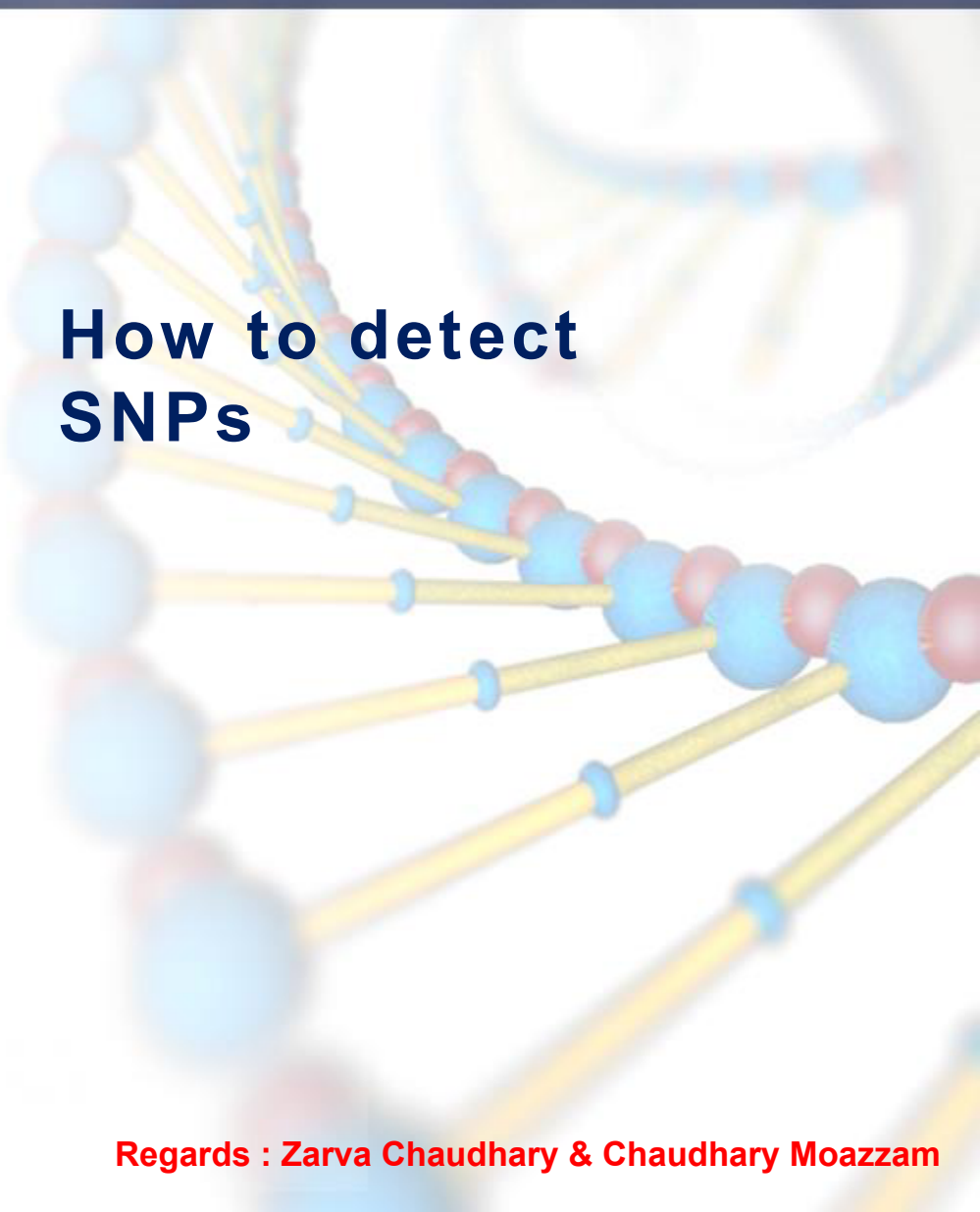
How to detect SNPs



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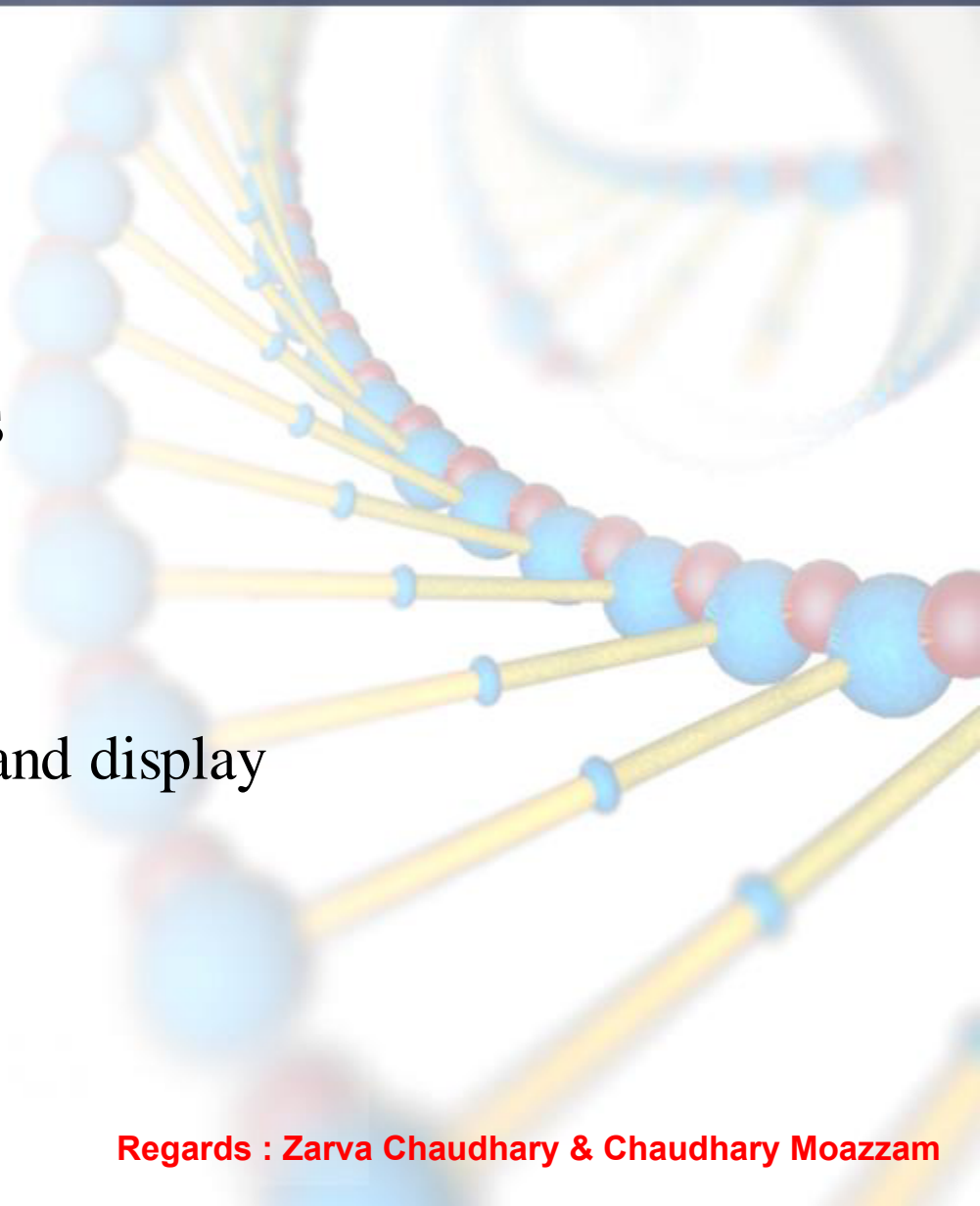
Techniques to detect known Polymorphisms

- Hybridization Techniques
  - Micro arrays
  - Real time PCR
- Enzyme based Techniques
  - Nucleotide extension
  - Cleavage
  - Ligation
  - Reaction product detection and display



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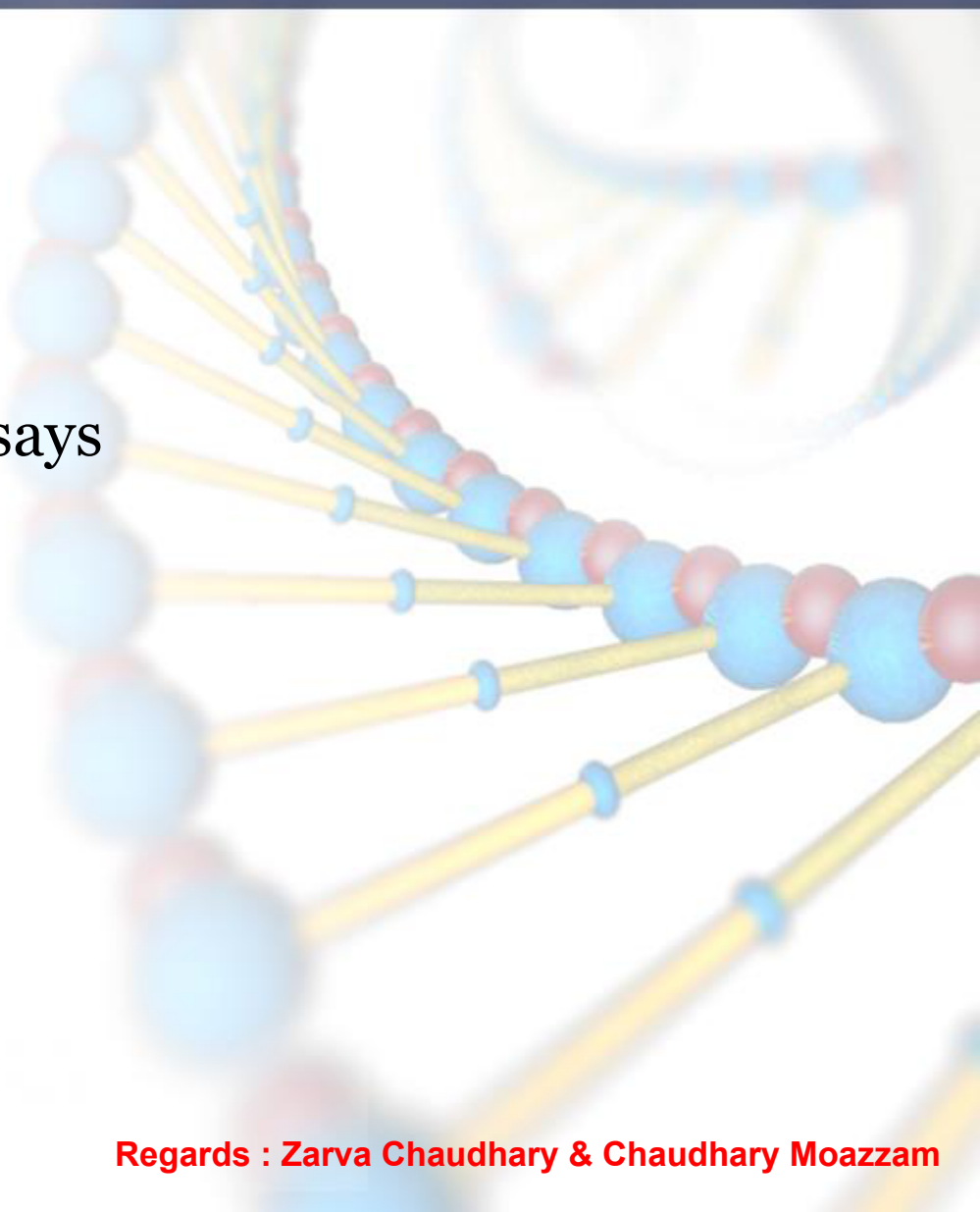
Techniques to detect unknown Polymorphisms

- Direct Sequencing
- Microarray
- Cleavage / Ligation
- Electrophoretic mobility assays



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Direct Sequencing

- Sanger dideoxysequencing can detect any type of unknown polymorphism and its position, when the majority of DNA contains that polymorphism.
- Misses polymorphisms and mutations when the DNA is heterozygous
- limited utility for analysis of solid tumors or pooled samples of DNA due to low sensitivity
- Once a sample is known to contain a polymorphism in a specific region, direct sequencing is particularly useful for identifying a polymorphism and its specific position.
- Even if the identity of the polymorphism cannot be discerned in the first pass, multiple sequencing attempts have proven quite successful in elucidating sequence and position information.

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SNP Screening

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SNP Screening

- Two different screening strategies
  - Many SNPs in a few individuals
  - A few SNPs in many individuals
- Different strategies will require different tools
- Important in determining markers for complex genetic states

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- SNP genotyping methods for detecting genes contributing to susceptibility or resistance to multifactorial diseases, adverse drug reactions:

- => case-control association analysis

- case GCCGTTGAC….
- ….GCCATTGAC….

- Control ...GCCATTGAC….
- ….GCCATTGAC….

-

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SIGNIFICANCE OF SNPs

- IN DISEASE DIAGNOSIS
- IN FINDING PREDISPOSITION TO DISEASES
- IN DRUG DISCOVERY & DEVELOPMENT
- IN DRUG RESPONSES
- INVESTIGATION OF MIGRATION PATTERNS

ALL THESE ASPECT WILL HELP TO LOOK FOR MEDICATION & DIAGNOSIS AT INDIVIDUAL LEVEL

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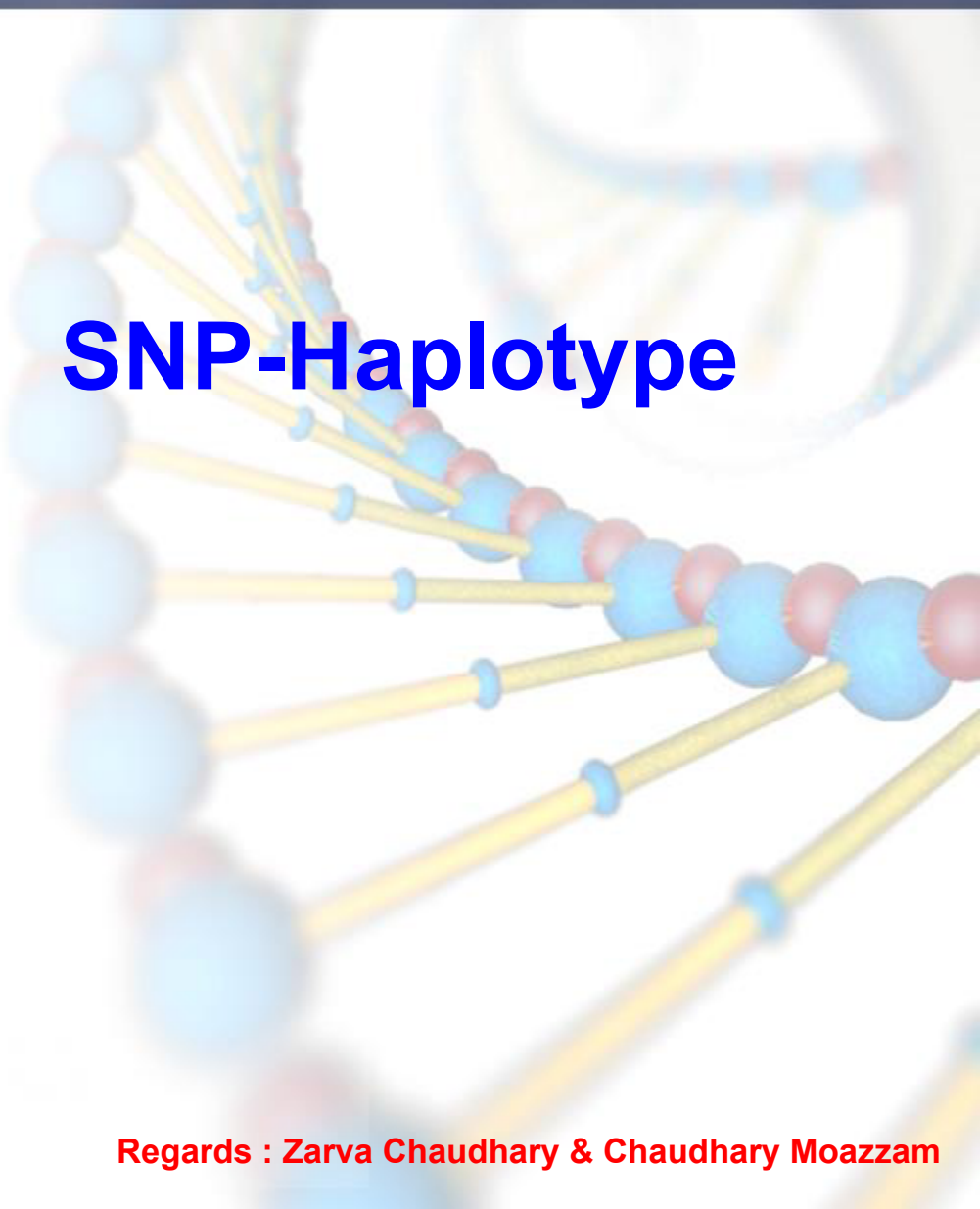
SNP-Haplotype



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HAPLOTYPE

- A set of closely linked genetic markers present
- on one chromosome which tend to be inherited
- together (not easily separable by recombination)

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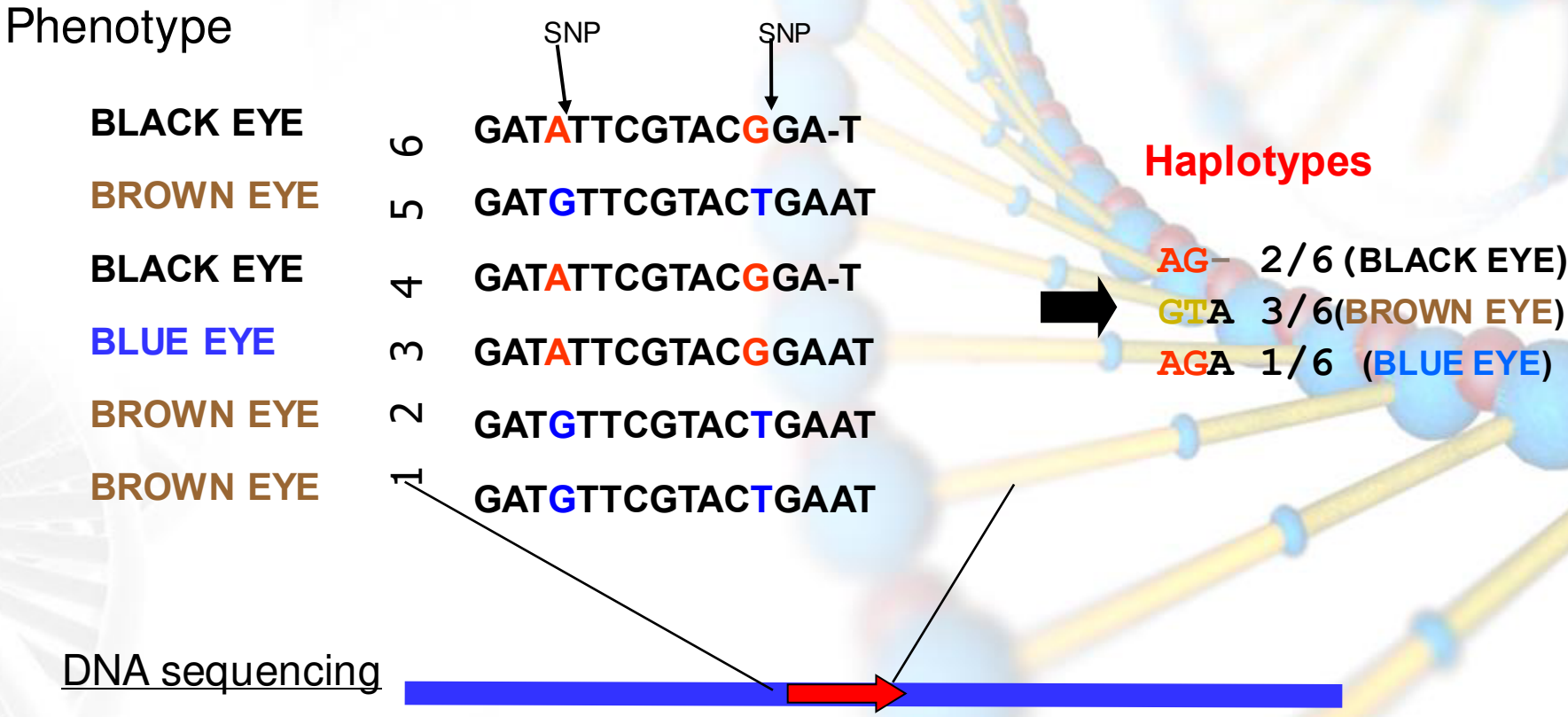
SNP-Haplotype



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HAPLOTYPE CORRELATION WITH PHENOTYPE

- **The "Haplotype centric" approach combines the information of adjacent SNPs into composite multilocus haplotypes**
- **Haplotypes are not only more informative but also capture the regional LD information, which is assumed to be robust and powerful**
- **Association of haplotype frequencies with the presence of desired phenotypic frequencies in the population will help in utilizing the maximum potential of SNP as a marker.**

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Some important SNP database Resources

- 1. dbSNP (http://www.ncbi.nlm.nih.gov/SNP/)
- LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/list.cgi)
- 2. TSC (http://snp.cshl.org/)
- 3. SNPper (http://snpper.chip.org/bio/)
- 4. JSNP (http://snp.ims.u-tokyo.ac.jp/search.html)
- 5. GeneSNPs (http://www.genome.utah.edu/genesnps/)
- 6. HGVbase (http://hgvbase.cgb.ki.se/)
- 7. PolyPhen (http://dove.embl-heidelberg.de/PolyPhen/)
- OMIM (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM)
- 8. Human SNP database
- (http://www-genome.wi.mit.edu/snp/human/)

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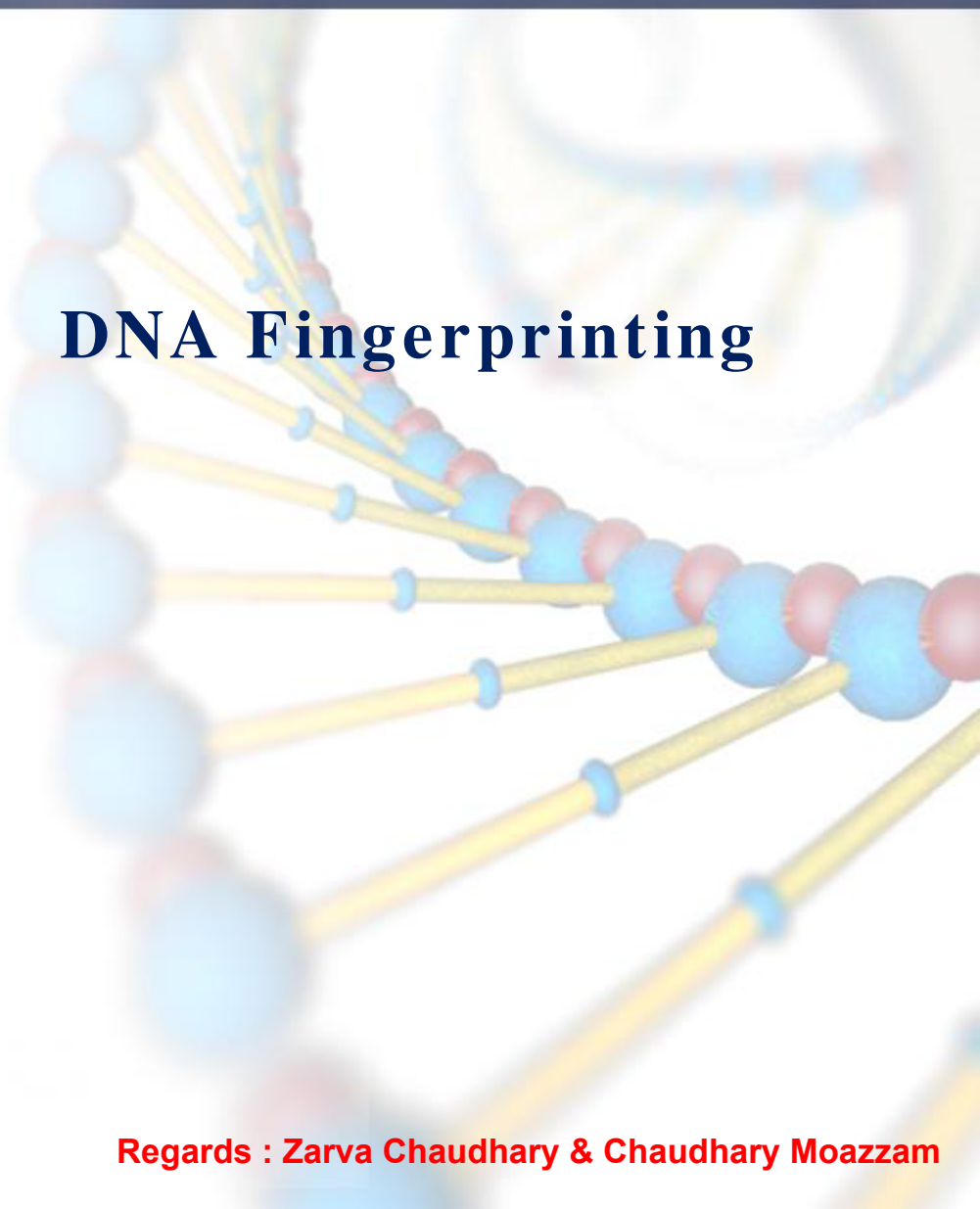
DNA Fingerprinting



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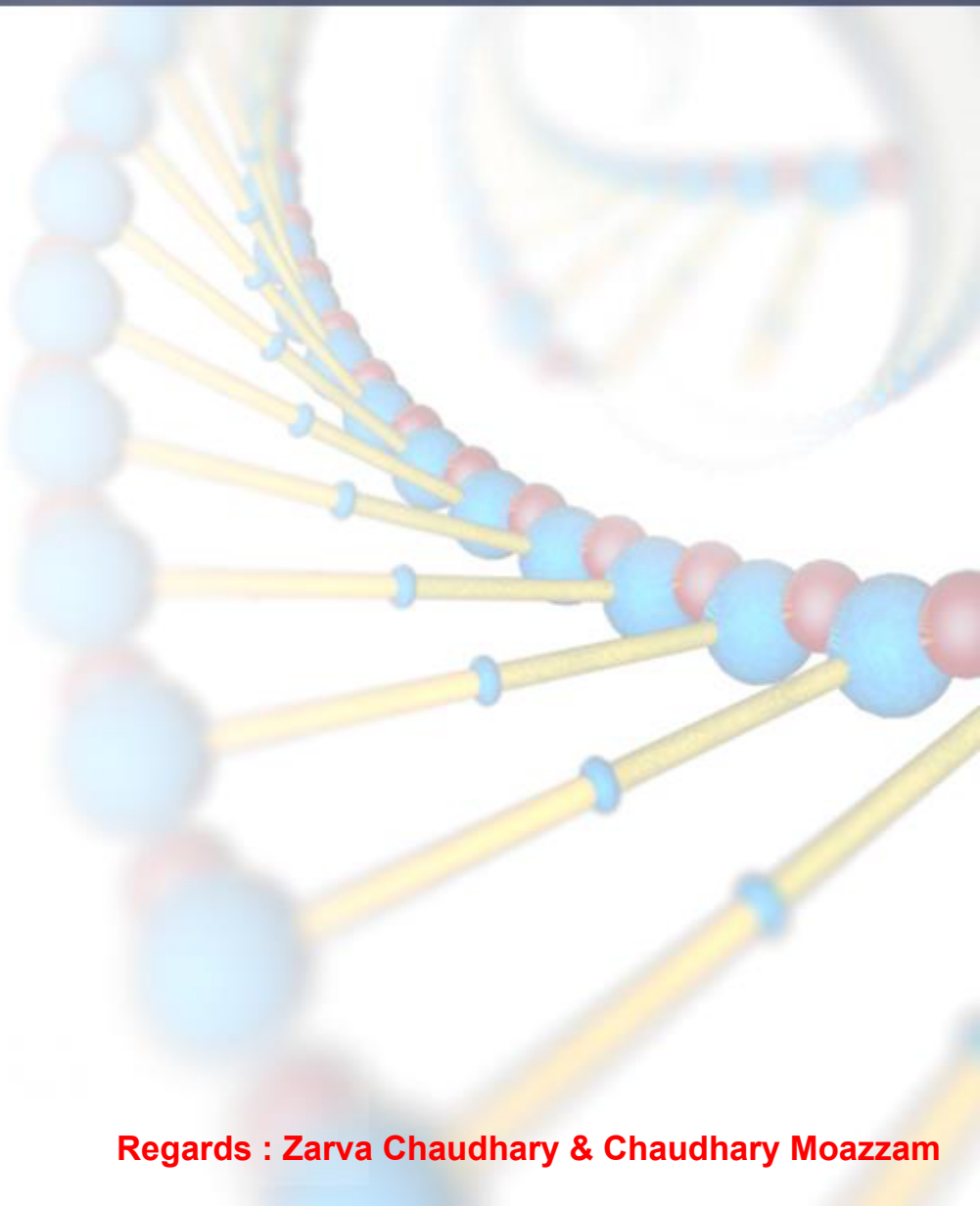
- **Synonyms…**
- DNA profiling.
- DNA testing.
- DNA typing.
- Genetic fingerprinting



Introduction

- The process of DNA fingerprinting was developed by Professor Alec Jeffreys at Leicester University in 1984 as a form of genetic analysis. • It was first used in the law courts of England in 1987 to convict a man in a rape case. • It has now been used successfully in many crime and paternity cases in worldwide.

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Although 99.9% of human DNA sequences are the same in every person, enough of the DNA is different to distinguish one individual from another, unless they are monozygotic twins.

- DNA profiling uses repetitive sequences that are highly variable, called variable number tandem repeats (VNTRs), particularly short tandem repeats (STRs). VNTR loci are very similar between closely related humans.
- The analysis of variable number of tandem repeats (VNTRs), to detect the degree of relatedness to another sequence of oligonucleotides , making them ideal for DNA fingerprinting.

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Variable Number Tandem Repeats

- A Variable Number Tandem Repeat (or VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat.
- These can be found on many chromosomes, and often show variations in length between individuals.
- Each variant acts as an inherited allele, allowing them to be used for personal or parental identification.
-

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- There are two principal families of VNTRs:
  - Microsatellites.
  - Minisatellites.

Microsatellites, also known as Simple Sequence Repeats (SSRs) or short tandem repeats (STRs), are repeating sequences of 2-6 base pairs of DNA. •

A minisatellites (also referred as VNTR) is a section of DNA that consists of a short series of bases 10–60 base pairs. •

Their analysis is useful in genetics and biology research, forensics, and DNA fingerprinting.

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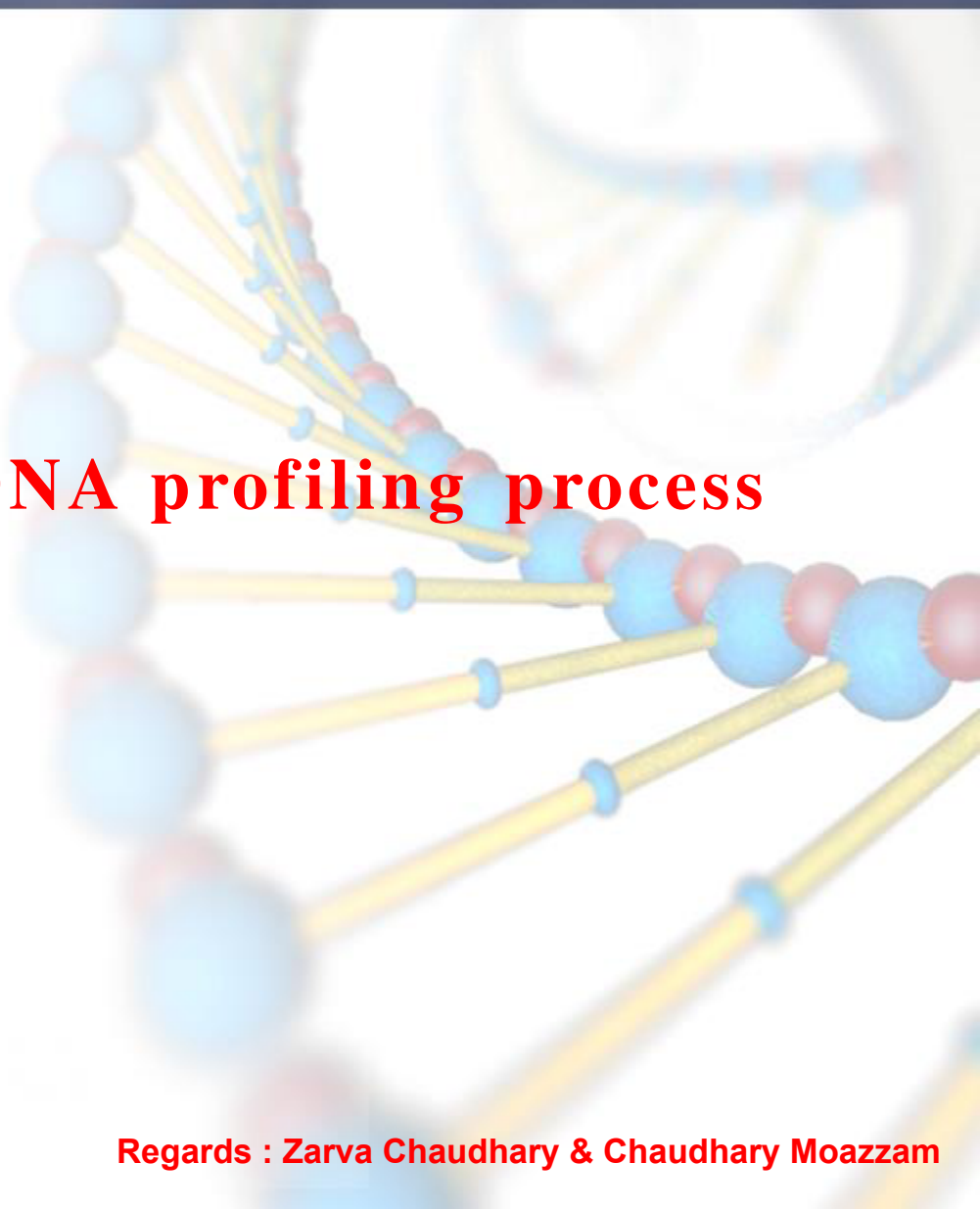
DNA profiling process



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- The process begins with a sample of an individu al's DNA (typically called a "reference sample").
  • The most desirable method of collecting a referenc e sample is the use of a buccal swab, as this reduces t he possibility of contamination. •
  When this is not available may need to be used to collect a sample of blood, saliva, semen, or othe r appropriate fluid or tissue from personal items (e.g. toothbrush, razor, etc.) or from stored samp les (eg: banked sperm or biopsy tissue).

Procedure

- 1) Isolation of DNA :

- • DNA must be recovered from the cells or tissues of the body.
- • Only a small amount of tissue, like blood, hair, or skin, is needed.
- • For example, the amount of DNA found at the root of one hair is usually sufficient.

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- 2) Cutting, sizing, and sorting :

• Special enzymes called restriction enzymes are used to cut the DNA at specific sites.

• For example, an enzyme called EcoR1, found in bacteria, will cut DNA only when the sequence 5'..GAATTC..3' occurs.

• The DNA pieces are sorted according to size by a sieving technique called electrophoresis.

• The DNA pieces are passed through a gel agarose. This technique is the DNA equivalent of screening sand through progressively finer mesh screens to determine particle sizes.

- 3) Transfer of DNA to nylon :
- The distribution of DNA pieces is transferred to a nylon sheet by placing the sheet on the gel and soaking them overnight.
- 4) Probing :
- Adding radioactive or colored probes to the nylon sheet produces a pattern called the DNA fingerprint.
- • Each probe typically sticks in only one or two specific places on the nylon sheet.
- 5) DNA fingerprint :
- The final DNA fingerprint is built by using several probes (5-10 or more) simultaneously. It resembles the bar codes used by grocery store scanners.

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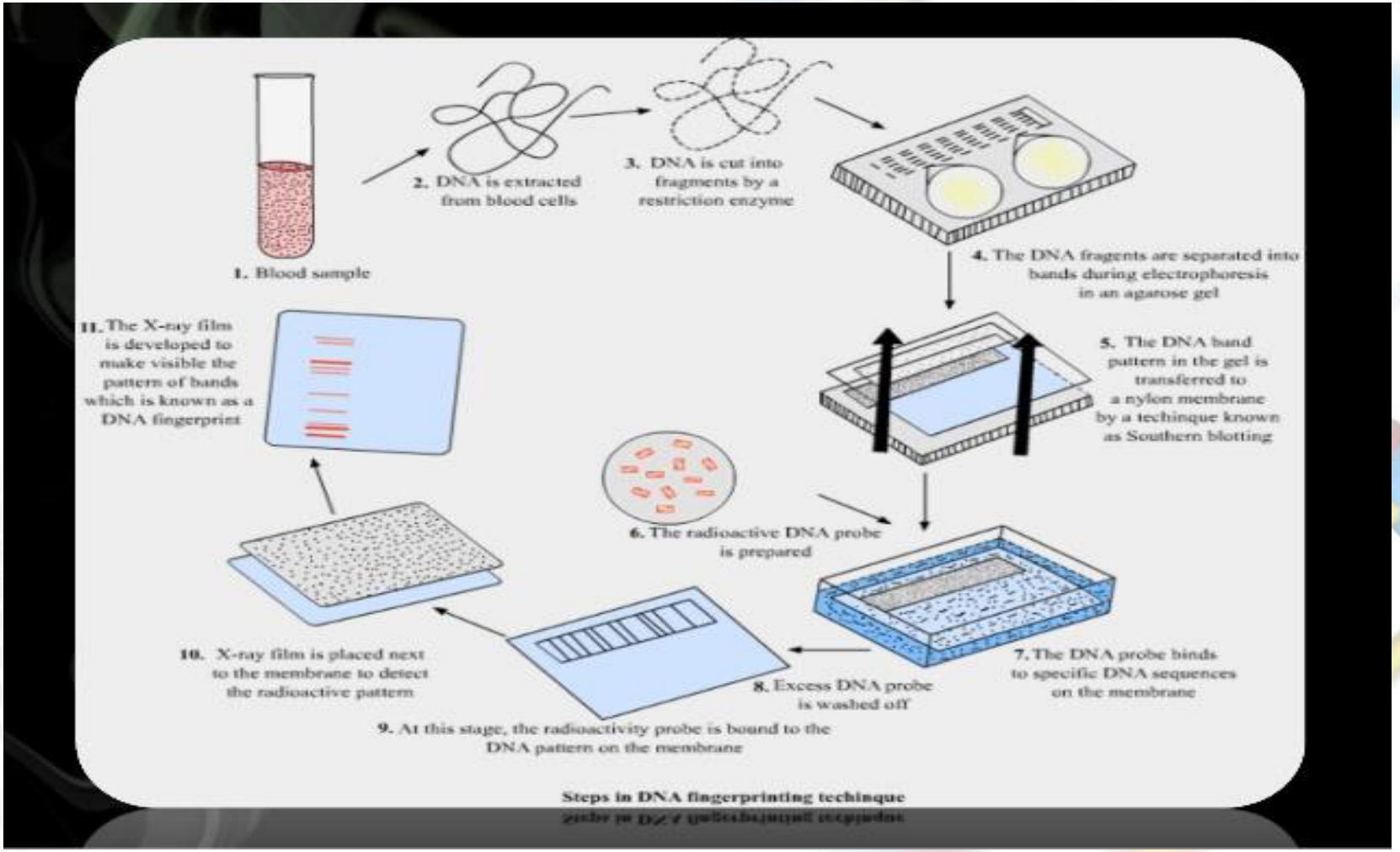

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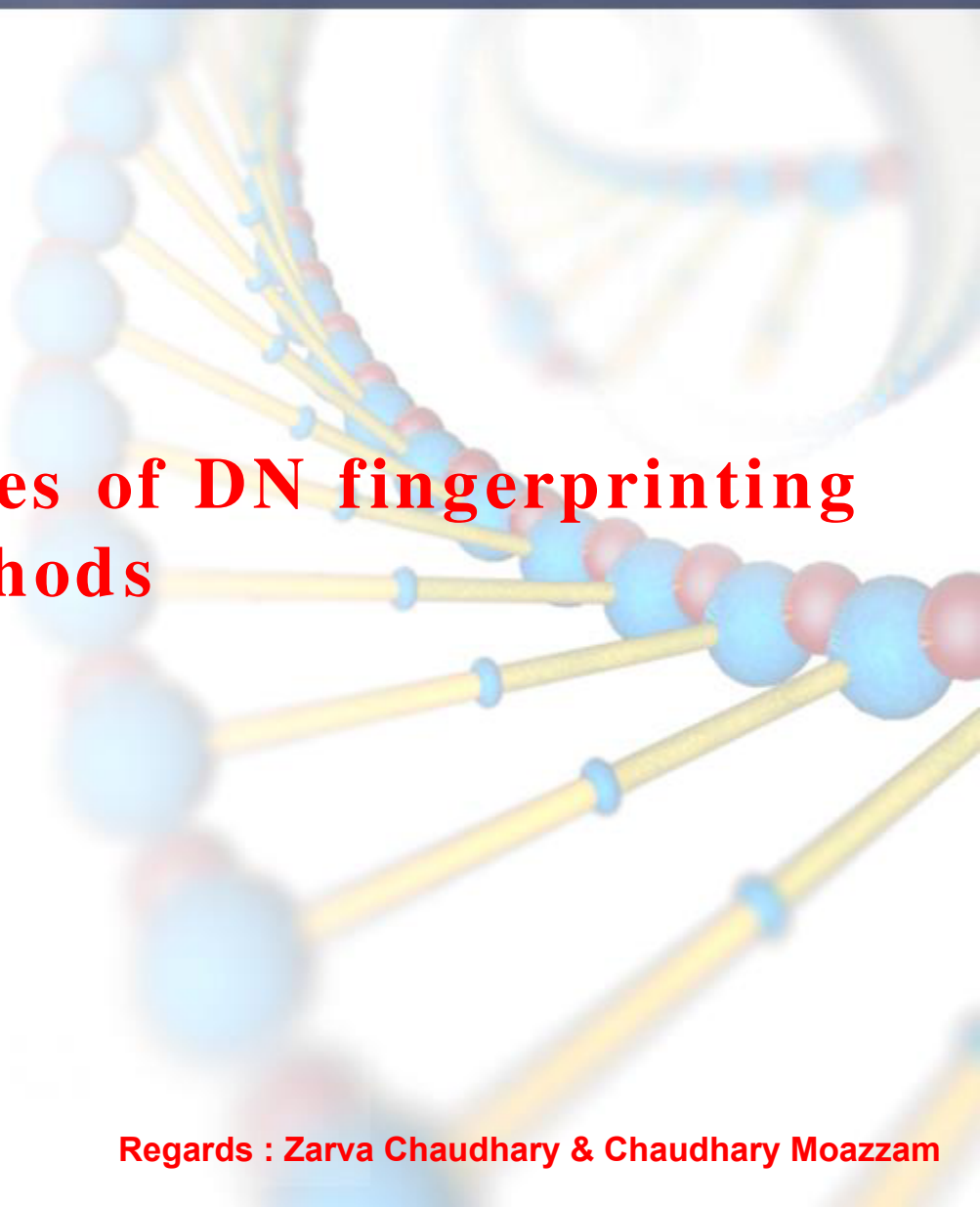
Types of DN fingerprinting methods



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- Types of DNA fingerprinting methods are…
- Electrophoresis.
- Polymerase chain reaction (PCR).
- Restriction fragment length polymorphism (RFLP).
- Random Amplified Polymorphic DNA(RAPD).
- Amplified fragment length polymorphism (AFLP).

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Electrophoresis

- Electrophoresis is a separations technique that is based on the mobility of ions in an electric field. Positively charged ions migrate towards a negative electrode and negatively-charged ions migrate toward a positive electrode. Ions have different migration rates depending on their total charge, size, and shape, and can therefore be separated.

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Polymerase chain reaction…

chain reaction (PCR) was developed

- The polymerase by Karry Mullis of the Cetus Corporation in 1983.
- In this process, the DNA sample is denatured into the separate individual strands.
- Specific DNA primers are used to hybridize to two corresponding nearby sites on opposite DNA strands in such a fashion that the normal enzymatic extension of the active terminal of each primer (that is, the 3' end) leads toward the other primer.
- In this fashion, two new copies of the sequence of interest are generated.

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- Repeated denaturation, hybridization, and extension in this fashion produce an exponentially growing number of copies of the DNA of interest.
- • The PCR analysis amplified isolated regions on the strands of the DNA under examination.

Restriction fragment length polymorphism...

- • RFLP analyzes the length of the strands of the DNA molecules with repeating base pair patterns.
- • The basic technique for detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest.
- • The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis, and transferred to a membrane via the Southern blot procedure.

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- Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which are complementary to the probe.
- • An RFLP occurs when the length of a detected fragment varies between individuals. Each fragment length is considered an allele, and can be used in genetic analysis.

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Types of DN fingerprinting methods-2

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Random Amplified Polymorphic DNA

- Random Amplified Polymorphic DNA... of PCR reaction, but the segments of DNA
- • It is a type that are amplified at random.
- • RAPD creates several arbitrary, short primers (8–12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify.
- • By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction.
- • RAPD does not require any specific knowledge of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence.

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ADVANTAGES OF RAPD

- It requires no DNA probes and sequence information for the design of specific primers.
- It involves no blotting or hybridization steps, hence, it is quick, simple and efficient.
- It requires only small amounts of DNA (about 10 ng per reaction) and the procedure can be automated.
- High number of fragments.
- Arbitrary primers are easily purchased.
- Unit costs per assay are low compared to other marker technologies.

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DISADVANTAGES OF RAPD…

- 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).
- Co -dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely.
- PCR is an enzymatic reaction, therefore, the quality and concentration of template DNA, concentrations of PCR compo-nents, and the PCR cycling conditions may greatly influence the outcome. Thus, the RAPD technique is notoriously labo-ratory-dependent and needs carefully developed laboratory protocols to be repro-ducible.

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- 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.
- • Lack of a prior knowledge on the identity of the amplification products.
- • Problems with reproducibility (sensitive to changes in the quality of DNA, PCR components and PCR conditions).
- •Problems of co-migration. Gel electrophoresis can separate DNA quantitatively, cannot separate equal-sized fragments qualitatively (i.e. according to base sequence).

Amplified fragment length polymorphism... RFLP

- This technique was also faster than analysis and used PCR to amplify DNA samples. It relied on variable number tandem repeat (VNTR) polymorphisms to distinguish various alleles, which were separated on a polyacrylamide gel.
- By using the PCR analysis to amplify the minisatellite loci of the human cell, this method proved quicker in recovery than the RFLP.
- However, due to the use of gel in its analysis phase, there are issues of bunching of the VTRN's, causing misidentifications in the process.

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Applications of Fingerprinting

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Applications…

- 1) Diagnosis and Developing cures for inherited disorders :

• DNA fingerprinting is used to diagnose inherited disorders in both prenatal and newborn babies in hospitals around the world.

• These disorders may include cystic fibrosis, hemophilia, Huntington's disease, familial Alzheimer's, sickle cell anemia, thalassemia, and many others.

• Early detection of such disorders enables the medical staff to prepare themselves and the parents for proper treatment of the child.

• In some programs, genetic counselors use DNA fingerprint information to help prospective parents

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- In other programs, prospective parents use DNA fingerprint information in their decisions concerning affected pregnancies.
- 2)Biological Evidence to Identify Criminals:
- • Where fingerprints are not available but biological specimens are available like blood or semen stains, hair, or items of clothing at the scene of the crime then these items may prove to be valuable sources of DNA of the criminal.
- • Since the year 1987, innumerable cases have been solved with the help of DNA fingerprint evidence.

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- 3) Paternity disputes :
- • Another important use of DNA fingerprints in the court system is to establish paternity in custody and child support litigation. In these applications, DNA fingerprints bring an unprecedented, nearly perfect accuracy to the determination.
- 4) Personal Identification :
- • DNA maybe the best way to identify a person as all body tissues and organs contain the same DNA type. The specimen required also is very small. In fact the US army has been doing DNA fingerprinting of all its soldiers and has a huge databank.

Methods in Molecular Biology
BIO 203

LABORATORY MANUAL

DEPARTEMENT OF BIOLOGY
VIRTUAL UNIVERSITY OF PAKISTAN

PREPARATION OF REAGENTS

The following general instructions are applicable in the preparation of all reagents.

Use graduated cylinders or pipettes closest to the volume being measured for preparing liquid reagents.

Store all reagents in sterile containers unless otherwise noted. Label all reagents with name of reagent, date prepared, initials of scientist that prepared reagent, lot number, and expiration date. Record each preparation in the lab's reagent logbook.

1M Tris-HCl [Tris(Hydroxymethyl)aminomethan] pH8

Tris base	121.1g
H_2O	to 800ml

Adjust to desired pH with concentrated HCl.
Mix and add H_2O to 1 Liter.

Store at room temperature

0.5 M EDTA (Ehylenediamine Tetraacetic Acid) pH 8.0

$Na_2EDTA.2H2O$	186.1g
H_2O	to 700ml

Adjust pH to 8.0 with 10M NaOH (almost 50ml)
Mix and add H_2O to 1 Liter.

Store at room temperature

10M NaOH

NaOH	400 g
H_2O	to 1 Liter

Store at room

temperature **10 mg/ml Ethidium Bromide**

Ethidium Bromide	0.2 g
H_2O	to 20ml

Mix well and store at $4^{o}C$ in dark.

TE (Tris 10 mM-EDTA 2mM) pH 8.0 (Lysis Buffer)

1M Tris-HCl ph 8.0	10 ml
0.5 M EDTA pH 8.0	4 ml
H_2O	to 1 Liter

Store at room temperatur

Low TE (Tris 10 mM-EDTA 0.2 mM) pH 8.0 for DNA storage

1M Tris-HCl pH 8.0	10 ml
0.5 M EDTA pH 8.0	0.4 ml
H_2O	to 1 Lite

Store at room temperature

Proteinase K (10mg/ml)

Proteinase K	100 mg lyophilized powder
Ultra-pure H_2O	to 10 ml

Aliquot and store at approximately -20□C.

CAUTION: Powder and solutions of Proteinase K can be irritating to mucous membranes.

SDS 10% w/v

Sodium dodecyl sulfate 100g H_2O to 700ml Heat to approximately 65^{o}C to dissolve.

Bring to a final volume of 1.0 L with ultra pure water.
Store at room temperature.

CAUTION: SDS can be irritating to mucous membranes. Wear safety glasses, mask and gloves when handling

TEN buffer (10mM Tris, 2mM EDTA, 400 mM NaCl)

1 M Tris-HCl ph 8.0	10 ml
5M NaCl	80 ml
0.5M EDTA	4 ml
H_2O	to 1 Liter

Store at room temperature.

50x TAE (Tris-Acetate-EDTA) Electrophoresis Stock buffer

Tris base	242g
Glaciall acetic acid	57.1 ml
0.5 M EDTA pH 8.0	100ml
H_2O to 1 Liter	

Store at room temperature

1x TAE (Tris 40mM, Acetate 20mM, EDTA 2mM) Electrophoresis working buffer

50x TAE 10 ml H_2O to 500 ml The pH of diluted buffer is 8.3.

Store at room temperature.

10x TBE (Tris 90mM-Borate 90mM-EDTA 2mM) Electrophoresis buffer

Tris base	108g
Boric Acid	55g
0.5M EDTA pH 8.0	40 ml H_2O to 1 Liter

Store at room temperature

2x Gel Loading Dye

2% Bromophenol blue	0.25 ml
2% Xylene cyanol	0.25 ml
Glycol	7ml
H_2O	10ml

Store at room temperature

5M Sodium Chloride

Sodium Chloride	292.2 g
H_2O	to 1 Liter

Store at room temperature.

6M Sodium Chloride

Sodium Chloride 351g

H_2O to 1 Liter

Store at room temperature.

Sources and Recovery of DNA

Sources of DNA

Purified DNA is required for a variety of molecular biology applications. DNA can be purified from any living organism and its living parts

Origin of Samples:

1. Human tissues i.e histological samples, prenatal samples, postmortem harvesting.
2. Blood, (EDTA).
3. Hair, (follicle part of the hair to be specific.
4. Rodent tissues, as rats are the most common lab mammals used in labs.
5. Leaf.
6. Bacteria, Bacterial cultures.
7. Yeast, yeast cultures.
8. Fungi.
9. Insect, i.e *Drosophila melanogaster*
10. Stool.
11. Body fluids, i.e semen.
12. Spores.
13. Soil.
14. Clinical samples (e.g. biopsy samples, fine needle aspirates).
15. Forensic samples (e.g. dried blood spots, buccal swabs finger prints).

Recovery of the DNA

Recovery of the DNA is called DNA extraction. DNA extraction involves many different methods. We will be discussing all those different methods in the next practical. Here in this practical we discuss only the basic steps involved in all DNA extraction methods.

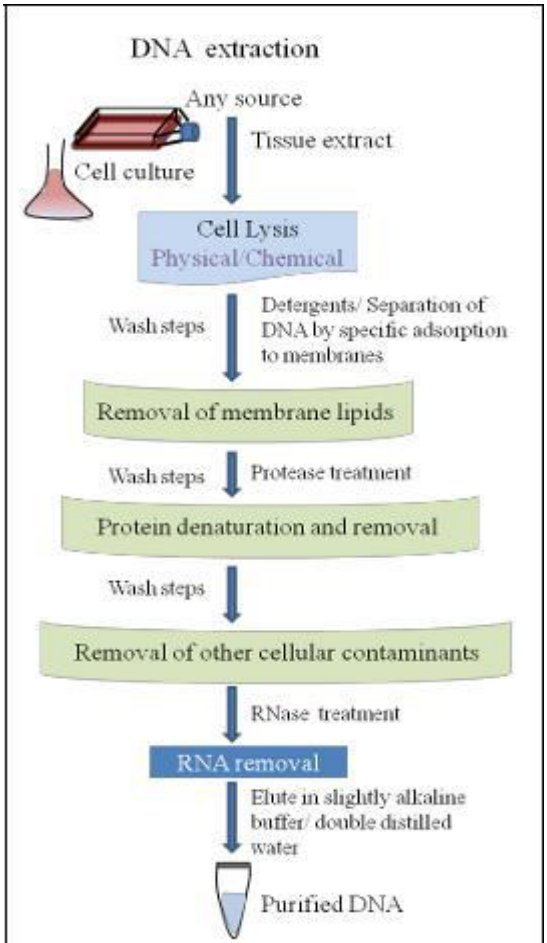

Figure 1. Basic steps involved in all DNA extraction methods.

Cell lysis:

Cell lysis is breakdown of the cellular structure so that long strands of DNA can come out from the entanglements of the cellular structures. It can be chemical or physical or combination of the both depending upon the origin of the sample. Cell lysis is achieved by using detergent which destabilized the plasma membrane. To break the cell wall of plants and bacterial samples, the physical force is utilized which may be in the form of sonication. The chemical reagents such as lysozyme, EDTA, lysozyme and EDTA combined, and other detergents are used for the lysis.

Removal of membrane lipids:

After lysis the membrane lipids are removed and DNA is proceeded to remove other impurities. Once membrane is solubilized by the detergents, it is removed when DNA is washed.

Protein denaturation and removal:

Protease is an enzyme which is used commonly to denature proteins in molecular biology experiments. Once proteins are denatured the soluble components of the proteins will be washed out in the subsequent washings of the DNA.

Removal of other cellular components:

The other cellular components are removed from the DNA by frequent washing steps.

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RNA denaturation and removal:

RNA is important contaminant of the DNA and to have a controlled experiment DNA must be free of any impurity including RNA. RNA is denatured using RNase, which is an RNA digesting enzyme.

DNA elution and storage:

DNA is eluted in an alkaline buffer solution or in double distilled water. Purified DNA is stored at -20°C preferably.

Methods of DNA extraction and purification

The basic criteria a method of DNA isolation from any sample type should meet include

1. Efficient extraction
2. Sufficient amount of DNA extracted for downstream processes
3. Removal of contaminants
4. Quality and purity of DNA

Based on these criteria following different methods are used for the extraction of the DNA.

Organic method

INTRODUCTION

Of all the methods of DNA extraction, the organic method (also known as the phenol-chloroform method has been the longest in use. This is because it is the most effective at extracting the large amounts of high molecular weight DNA that were required for the RFLPs that created the first DNA fingerprints in the 1980s. This protocol describes the standard method for nucleic acid purification by extraction first with phenol: chloroform and then with chloroform to remove any remaining phenol.

PRINCIPLE:

Organic extractions or Phenol–chloroform extraction is a liquid- liquid extraction technique in biochemistry and molecular biology for purifying nucleic acids and eliminating proteins. In brief, aqueous samples are mixed with equal volumes of a phenol: chloroform mixture. After mixing, the mixture is centrifuged and two distinct phases are formed, because the phenol: chloroform mixture is immiscible with water. The aqueous phase is on top because it is less dense than the organic phase (phenol: chloroform). The proteins will partition into the lower organic phase while the nucleic acids (as well as other contaminants such as salts, sugars, etc.) remain in the upper aqueous phase. The upper aqueous phase is pipetted off and care is taken to avoid pipetting any of the organic phase or material at the interface. This procedure is often performed multiple times to increase the purity of the DNA.

MATERIALS

Reagents

Chloroform

- Ethanol
- Ether (optional)

- Nucleic acid solution to be purified
- Phenol: Chloroform (1:1)
- Tris EDTA (pH 7.8) (optional)
- 3 M sodium acetate pH 5.2 or 5 M ammonium acetate
- 100% ethanol

Equipment

- Automatic pipette fitted with a disposable tip
- Pipettes, large-bore (optional)
- Polypropylene tube
- Rotating wheel (optional)

METHOD

1. Transfer the nucleic acid sample to a polypropylene tube and add an equal volume of phenol: chloroform.

(After mixing and centrifugation this will result in to two phases, lower organic phase and upper aqueous phase. The DNA is in the aqueous phase and will be extracted from it later. The nucleic acid will tend to partition into the organic phase if the phenol has not been adequately equilibrated to a pH of 7.8-8.0.)

2. Mix the contents of the tube until an emulsion forms.
3. Centrifuge the mixture at 80% of the maximum speed that the tubes can bear for 1 minute at room temperature. If the organic and aqueous phases are not well separated, centrifuge again for a longer time.
4. Use a pipette to transfer the aqueous phase to a fresh tube. For small volumes (<200µL), use an automatic pipettor fitted with a disposable tip. Discard the interface and organic phase.
5. Repeat Steps 1-4 until no protein is visible at the interface of the organic and aqueous phases.
6. Add an equal volume of chloroform and repeat Steps 2-4.
7. To recover DNA measure the volume of the aqueous phase.

8. Add 1/10 volume of sodium acetate, pH 5.2, (final concentration of 0.3 M.
9. Mix well and add 2 to 2.5 volumes of cold 100% ethanol (calculated after salt addition).
10. Mix well.
11. Place on ice or at -20 degrees C for >20 minutes.
12. Spin a maximum speed in a microfuge 10-15 min.
13. Carefully decant supernatant.
14. Add 1 ml 70% ethanol. Mix. Spin briefly. Carefully decant supernatant.
15. Air dry or briefly vacuum dry pellet.
16. Resuspended pellet in the appropriate volume of Tris EDTA buffer or double distilled water.
17. Proceed with quantification and intended use after storage.

ADVANTAGES AND DISADVANTAGES:

Its other main advantage is the fact that it can be used on a wide range of samples. However, this method does also have some disadvantages including being very labor intensive, being easily contaminated and exposing the scientist carrying out the extraction to dangerous chemicals.

READINGS:

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http://www.med.upenn.edu/lamitinalab/documents/EthanolPrecipitationofDNA.

http://bitesizebio.com/384/the-basics-how-phenol-extraction-works/

http://physiology.med.cornell.edu/faculty/mason/lab/zumbo/files/PHENOL-CHLOROFORM.pdf

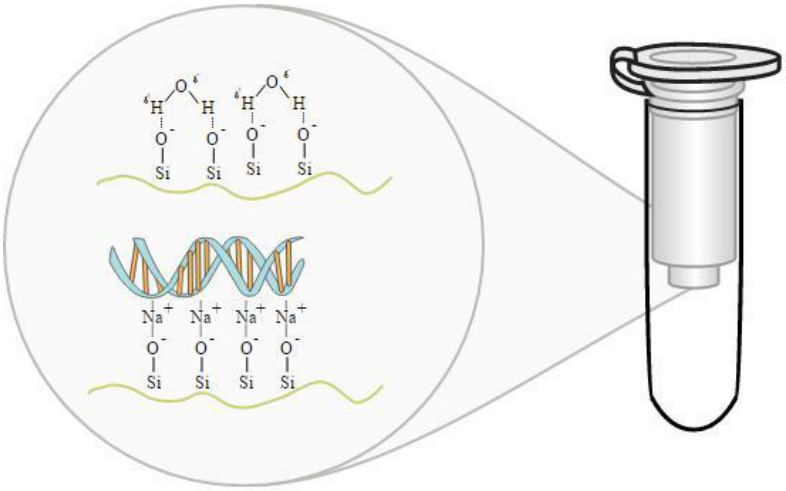
Silica Adsorption Method:

INTRODUCTION:

Silica DNA extraction methods have become the staple for many labs as it is quick reliable and produces very high quality DNA.

PRINCIPLE:

DNA separation by silica adsorption is a method of DNA separation that is based on DNA molecules binding to silica surfaces in the presence of certain salts and under certain pH conditions. Although the mechanism is not fully understood, one possible explanation involves reduction of the silica's surface's negative charge due to the high ionic strength of the buffer. This decrease in surface charge leads to a decrease in the electrostatic repulsion between the negatively charged DNA and the



Figure, 2: Silica in a spin column with water and with DNA sample in specific buffer

negatively charged silica. Meanwhile, the buffer also reduces the activity of water by formatting hydrated ions. This leads to the silica surface and DNA becoming dehydrated. These conditions lead to an energetically favorable situation for DNA to adsorb to the silica surface.

MATERIALS:

Kits are available which work with the said principle.

METHODS:

There are two basic steps:

Binding and washing:

The sample is run through a micro-channel, DNA binds to the channel, and all other molecules remain in the buffer solution. The channel is washed free of impurities.

Elution:

The silica is then dried and DNA is eluted using water or a low salt buffer. An elution buffer removes the DNA from channel walls, and the DNA is collected at the end of the channel.

ADVANTAGES & DISADVANTGES:

It is a quick, reliable method which produces high quality DNA. It is an expensive method and if chewing gum is the source of DNA, the process can be interfered by chewing gum.

Non-organic method:

INTRODUCTION:

In this method no organic solvents are used that's why it is termed as non-organic method.

PRINCIPLE:

Addition of Proteinase K to nucleic acid preparations rapidly inactivates nucleases that might otherwise degrade the DNA or RNA during purification. It is highly suited to this application since the enzyme is active in the presence of chemicals that denature proteins, such as SDS and urea, chelating agents such as EDTA, sulfhydryl reagents, as well as trypsin or chymotrypsin inhibitors. Proteinase K is used for the destruction of proteins in cell lysates (tissue, cell culture cells) and for the release of nucleic acids, since it very effectively inactivates DNases and RNases.

MATERIALS

Solutions/reagents:

- Digestion Buffer (10mM NaCl, 10mM TRIS (pH 8.0), 10mM EDTA (pH 8.0), 0.5% SDS)
- Proteinase K (20mg/ml)
- Sodium Acetate pH 5.2 (3M)
- Ice-cold 98% ethanol
- Ice-cold 70% ethanol
- 1X TE
- Water
- Tissue

Equipment:

- Incubator
- Centrifuge
- Sterile 1.5-ml micro-centrifuge tubes

METHODS:

Tissue Digestion

- Measure out Digestion Buffer into sterile 1.5-ml microcentrifuge tube.
- Add 0.005 volumes Proteinase K.
- That is, for each ml of Digestion Buffer, add 5 µl of ProteinaseK.
- Homogenize tissue in solution.

- Incubate at 55°C for 1 - 12 hours (overnight).
- Vortex the mixture for a few seconds.
- Centrifuge at maximum speed for 2 minutes at 4°C and aspirate out the top layer.
- Transfer top aqueous layer into sterile 1.5-ml microcentrifuge tube.
- Discard bottom layer.

Precipitation of Protein and Cell Debris

- Add 0.1 volume Sodium Acetate pH 5.2 to sterile 1.5-ml microcentrifuge tube.
- Close the tube tightly and gently mix the contents by inverting the tube.
- Incubate at -20°C for 15 minutes.
- Centrifuge at maximum speed for 20 minutes at 4°C and aspirate out the top layer.
- Transfer top aqueous layer into sterile 1.5-ml microcentrifuge tube.
- Discard bottom layer.
- Be careful not to transfer any of the white solid (cell debris and SDS) into the fresh tube.

Precipitation of Nucleic Acids

- Add 2 volumes ice-cold 98% ethanol to sterile 1.5-ml microcentrifuge tube.
- Close the tube tightly and gently mix the contents by inverting the tube.
- Incubate at -20°C for 15 minutes.
- Centrifuge at maximum speed for 20 minutes at 4°C, gently aspirate out the supernatant and discard it.
- Add 1 ml of ice-cold 98% ethanol.
- Vortex the mixture for a few seconds.
- Centrifuge at maximum speed for 5 minutes at 4°C, gently aspirate out the supernatant and discard it.
- Add 1 ml of ice-cold 70% ethanol.
- Vortex the mixture for a few seconds.
- Centrifuge at maximum speed for 5 minutes at 4°C, gently aspirate out the supernatant and discard it.
- (Optional) Add 1 ml of ice-cold 70% ethanol.
- Vortex the mixture for a few seconds.
- Centrifuge at maximum speed for 5 minutes at 4°C, gently aspirate out the supernatant and discard it.
- Dry the pellet in air.
- Option 1: Add 10 µl of 1X TE (or) Option 2: Add 10 µl of water.
- Resuspend the pellet by vortexing/by shaking vigorously.
- Ensure to dry the pelleted DNA completely before attempting to resuspend.

Protocol No 5

HOTSHOT Method of DNA Extraction

Requirements

- Fins of Zebra fish
- Micropipette and pipette tips
- Scalpel
- Micro centrifuge tubes
- Mortar and pestle
- Centrifuge
- Water bath
- 4^{o}c freezer
- -20^{o}c freezer

Reagents

- 50mM NaOH solution
- 1M Tris-Cl

Procedure

- Pluck out 20-100 mg of fish fin using a sterile scalpel and a forceps.
- Homogenize the fins using sterile mortar and pestle.
- Aliquot 100 µl of 50 mM NaOH in to a microfuge tube.
- Transfer the homogenized fins of zebra fish into this micro centrifuge tube.
- Incubate at 95°C for 20min in a water bath.
- After the incubation, the tubes are allowed to cool at 4°C.

Regards : Zarva Chaudhary & Chaudhary Moazzam

- Add 1/10th volume of 1 M Tris-HCl (pH 8.0) for neutralizing the basic solution.
- Centrifuge the sample at 5000rpm for 10min to pellet the debris. The supernatant obtained is collected in afresh micro centrifuge tube (1-5µl of this supernatant can be used immediately per 25 µL of PCR reaction mixture).
- Store the sample for at least 3 months at 4°C or longer at -20°C freezer.

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Chelex™ method

INTRODUCTION:

Chelex is one of the oldest methods of DNA extraction and utilizes a chelating resin.

PRINCIPLE

Chelex resin is often used for DNA extraction in preparation for PCR. The Chelex™ protects the sample from DNAases that might remain active after the boiling and could subsequently destroy the DNA. DNAases are enzymes, which naturally occur in all body tissues; they cut DNA into small fragments, rendering it unsuitable for PCR. Magnesium ions are essential cofactors for DNases. Chelex™ resin binds with cations including Mg2+. By binding with magnesium ions, the Chelex™ resin renders DNases inoperable, thus protecting DNA from their action.

MATARIALS

- Chelex 300 µL
- Heating Block
- ddH2O
- Sterile Forceps
- Vortex
- Centrifuge tubes

METHODS

1. Remove premade tubes filled with 300µL 10% Chelex from refrigerator. You will need for each sample, plus 1 extra for a control. Handle the container with gloves and shake out the number you need into your gloved hand. Do not put extra tubes back into jar.

2. Label each Chelex tube to correspond to your sample listed on your Chelex worksheet. Label the tubes on the cap. Put as much information as you can manage, but keep labels legible. Adding initials and a date is always a good idea. Avoid labeling on the side of tube as this writing can be washed off during the incubation stage.

3. Turn on heating block. Set to 95°C. Fill holes with ddH2O water.

4. Dip forceps into ethanol, then wave forceps through flame of an alcohol burner to ignite. When you are certain that the flame on the forceps has extinguished, repeat 2 times.

5. Using the sterile forceps, remove a small piece of tissue from your sample; uncap the tube of Chelex, place sample in the appropriately labeled tube and close lid. The piece of tissue should be big enough to be visible, but not so big as to be easily visible. Imagine cutting a 0.2mm section of a standard staple. This is plenty big. Too much tissue may inhibit your reactions. The piece of tissue should be about as big as a period.

6. Repeat (step 5) with each sample in a new Chelex tube, being sure to sterilize forceps 3 times between samples (step 4). When finished, make a negative Chelex control by dipping your sterilized forceps into a tube of Chelex slurry. (It may be necessary to wipe excess tissue from forceps with a Kim wipe prior to flame sterilization)

7. When finished with all tubes, vortex samples in Chelex slurry for 10-15 seconds. Be sure lids are snapped on tightly before beginning

8. Spin samples briefly (10-15 sec) at high speed in a microcentrifuge. This step is to ensure that the sample is inside the slurry of Chelex.

9. Incubate samples for 20 minutes at 95°C. The block temperature may drop slightly when doing this step. This drop is normal. Check tubes while incubating to ensure that lids have not popped off.

10. Vortex samples again for 10-15 seconds (Be careful as steam may pop lid off of centrifuge tube. Hold lids down).

11. Spin tubes again at high speed in microcentrifuge to ensure that all contents are in the bottom of the microcentrifuge tube.

12. Samples are ready to use (or not, see below). ONLY USE SUPERNATE FOR PCR REACTIONS. CHELEX BEAD WILL INACTIVATE TAQ!

ADVANTAGES & DISADVANTAGES

This is an effective method of DNA extraction. Its advantages are that it is cheap, quick, has a low contamination rate and does not use any dangerous chemicals. However, it's disadvantages include being inefficient for use on blood samples, producing low purity DNA samples and being unsuitable for restriction fragment length polymorphism DNA profiling.

READINGS

https://www.ncbi.nlm.nih.gov/pubmed/1867860

https://www.eeb.ucla.edu/Faculty/Barber/Web%20Protocols/Protocol2.pdf

http://www.fastbleep.com/biology-notes/41/122/1216

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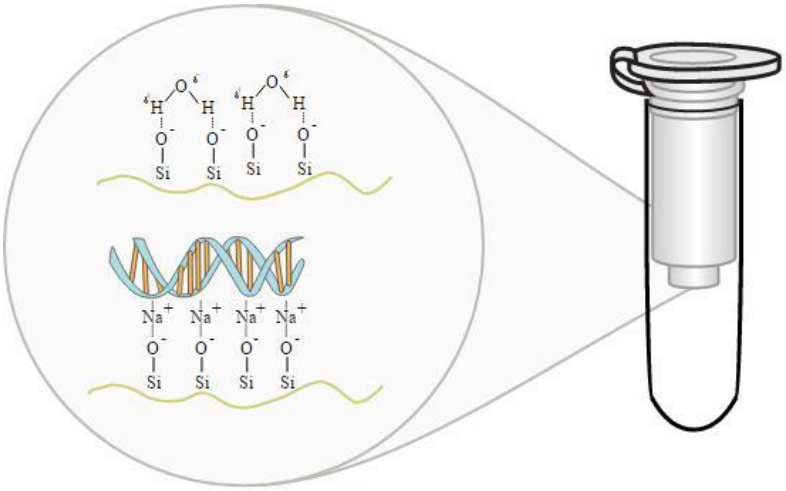

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READINGS:

http://www.ncbi.nlm.nih.gov/pubmed/9986822

http://bitesizebio.com/13516/how-dna-extraction-rna-miniprep-kits-work/

http://herpesvirus.tripod.com/research/protoDNA.htm

Regards : Zarva Chaudhary & Chaudhary Moazzam

FTA method

INTRODUCTION:

The FTA is an acronym for fast technology analysis. The FTA paper extraction method was initially used as a method of DNA collection in forensic science but due to the ease of the process has become a popular method of extraction.

Its Basic methodology is that

1. Sample source (usually blood) is dropped onto the paper and as it dries the cells are lysed and the DNA becomes trapped within the matrix of the paper.
2. The paper is punched to create discs, which are washed in a test tube.
3. The discs are then washed with a solvent and added to the PCR mix

PRINCIPLE

Biological samples, such as blood and saliva, adhere to the paper through the mechanism of entanglement, while the mixture of chemicals lyses cells and denatures proteins. Because nucleases are inactivated, the DNA is essentially stable when the sample is properly dried and stored. Nucleic acid damage from nucleases, oxidation, ultraviolet light (UV) damage, microbes, and fungus is reduced when samples are stored on the FTA card

MATARIALS

- FTA Purification Reagent
- TE Buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8.0)
- Solution 1: 0.1N NaOH, 0.3mM EDTA, pH 13.0
- Solution 2: 0.1M Tris-HCL, pH 7.0

METHODS

Procedure for wash protocol

1. Take a 6 mm punch using a regular single- hole paper punch from a dry spot and place in a 1.5 mL microtube. To ensure that there is no cross-contamination, rinse the cutting end of the punch with ethanol and let it dry between samples.
2. Add 1000 µl of FTA Purification Reagent to microtube and flash vortex 5 times or manually to mix.
3. Incubate for 5 minutes at room temperature (tube may be given moderate manual mixing or vortex if desired).
4. Remove and discard all spent FTA Purification Reagent using a pipette.
5. Repeat steps 2-4 twice, for a total of three (3) washes with FTA Purification Reagent.
6. Add 1000 µl of TE Buffer.

7. Incubate for 5 minutes at room temperature.
8. Remove and discard all spent TE Buffer with a pipette.
9. Repeat steps 6-8 twice for a total of three (3) washes with TE Buffer (punch should look white or pale with most of the blood removed).

Procedure for pH treatment

Time scheme: High pH for 5 minutes, neutralized for 10 minutes

1. Add 140 µl of Solution 1 to a 6 mm punch washed as above.
2. Incubate 5 minutes at 65^{o}C (deviation from FTA company protocol which incubates at room temperature).
3. Add 260 µl of Solution 2 and flash vortex 5 times to mix.
4. Incubate 10 minutes at room temperature.
5. Flash vortex 10 times.
6. Remove punch and squeeze to recover maximum volume of elute (can use a clean pipette tip to remove punch).

Elute contains gDNA in TE (66mM Tris-HCL, 0.1nm EDTA). Use 0.5µl for a 25µl PCR reaction.

ADVANTAGES & DISADVANTAGES

A marketable advantage of the FTA® technology is that samples spotted on treated cards may be stored at room temperature. The chemicals on the FTA cards enhance the preservation of the DNA and inactivate many dangerous pathogens that may be found in liquid blood samples or dried biological stains. Because the cards are small in size (approximately 3.5" x 5"), they are easily packaged, shipped, and stored for data basing .its other advantages include being easily repeated, easily automated and the added bonus that there is no need to quantify DNA extracted by FTA before PCR. However, due to the smaller nature of the "discs" of DNA obtained by this method, static electricity often causes them to jump out of their set location, leading to contamination.

READINGS

http://www.fastbleep.com/biology-notes/41/122/1216

https://www.promega.com/~/media/files/resources/application%20notes/genetic%20identity/an102%20extraction%20and%20isolation%20of%20dna%20from%20blood%20cards%20and%20buccal%20swabs%20in%20a%2096%20well%20format.pdf?la=en

https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1392818611307/litdoc28982222_20140311044843.pdf

Method of RNA purification from blood

INTRODUCTION:

RNA extraction is the purification of RNA from biological samples. This procedure is complicated by the ubiquitous presence of ribonuclease enzymes in cells and tissues, which can rapidly degrade RNA. Several methods are used in molecular biology to isolate RNA from samples; the most common of these is TRIzol extraction.

Note:

This protocol assumes the investigator is beginning this with one full Yellow-Top (type A) BD Vacutainer tube of human blood (equals roughly 8 ml) to yield approximately 30 µg of RNA.

Additional Note:

RNA is very easily degraded by ever-present RNAses. Therefore, all of the tubes and solutions in this protocol must be RNAse-free (autoclaving does NOT inactivate RNAses). One cannot overemphasize the need for a clean work environment when working with RNA.

PRINCIPLE:

Guanidinium isothiocyanate (powerful protein denaturant)

Inactivation of RNases

Acidic phenol/chloroform

Partitioning of RNA into aqueous supernatant for separation

Note: low pH is crucial since at neutral pH DNA not RNA partitions into the aqueous phase.

MATERIALS:

10x RBC Lysis Buffer

- 10.0 g $KHCO_3$
- 2.0 ml 0.5 M EDTA
- 89.9 g NH_4Cl

Dissolve the above in approximately 800 ml ddH_2O and adjust pH to 7.3. QS To 1 liter and mix thoroughly. This solution is stable for 6 months at 2 – 8° C in a tightly closed bottle.

1x RBC Lysis Buffer

Simply dilute the 10x stock solution 1:10 with ddH_2O. This is Stable for 1 week at room temperature.

TRIzol Reagent OR RNA STAT-60 Reagent

TRIzol Reagent Invitrogen Life Technologies: Cat No. 15596018
or
RNA STAT-60 Reagent Tel-Test: Cat No. CS-111

Other Reagents Needed

- Phosphate Buffered Saline (PBS)
- Isopropanol (2-propanol)
- Ethanol
- RNAse-free water
- RNAse-Away (a cleaning solution that neutralizes RNAses on bench tops, pipettor, centrifuges, and other equipment.

METHODS:

1) Transfer contents of tube into a 50 ml polypropylene conical centrifuge tube.
2) Bring volume to 45 ml with RBC Lysis Buffer (recipe follows protocol).
3) Let stand at room temperature for 10 minutes.
4) Pellet cells at 600 x g (approx. 1,400 rpm) for 10 minutes in a room temp centrifuge (program#3).
5) Carefully decant supernatant.
6) Gently resuspend the pellet in 1 ml of RBC Lysis Buffer and transfer to a 1.5 ml microcentrifuge tube. – Let stand for 5 minutes.
7) Pellet cells for 2 minutes by centrifuging in a microfuge at room temperature at 3000 rpm.
8) Carefully aspirate the supernatant.
9) Resuspend the pellet in 1 ml of sterile DPBS.
10) Pellet cells as in step 7.
11) Carefully aspirate the supernatant.
12) Add 1200 µl of TRIzol solution to each tube and resuspend the cells. Note: for a full 8 ml blood tube, the 1200 µl TRIzol solution can be split into 2, 600 µl aliquots and frozen at -80 C until further processing.
13) Add 0.2 ml of Chloroform ($CHCl_3$) and vortex each tube for 15 seconds, ONE AT A TIME.
14) Centrifuge the samples at 13,000 rpm for 10 minutes at 4°C.
15) Remove the upper phase and transfer to a clean microcentrifuge tube. Be careful not to remove any of the white interface when collecting the upper phase of the extraction
16) For the future collection of micro RNA (miRNA), carefully remove ~20% of the volume of the upper phase from step 16 and place into another clean, labeled, 1.5ml microfuge tube. Store this aliquot at -80 C until further processing.
17) To the remaining upper phase from step 16, add an equal volume of cold isopropanol and invert to mix.
18) The samples can be placed in a -20°C freezer to precipitate.
19) Samples are centrifuged at 13,000 rpm for 10 minutes at 4°C.

Note: you may be able to see a small white pellet of RNA at the bottom of the tube after this step.

20) Carefully decant the supernatant, and rinse the pellet with 0.5 ml of ice-cold 75% ethanol. The 75% EtOH should be prepared RNase-free and stored at -20 C.

21) Centrifuge the samples at 13,000 rpm for 10 minutes at 4°C.

22) Decant the supernatant.

23) Using a pipettor, carefully remove all of the remaining liquid in the bottom of the tube.

24) Allow the pellet to dry for 5 to 10 minutes to remove any remaining ethanol.

25) Dissolve the RNA pellet by adding 20 µl of RNAse-free H2O to each sample.

26) RNA should be quantitated within 2 hours of elution. It can be kept at 4°C until that time; it can also be held temporarily at -20°C until permanent storage at -80°C. Repeated freeze-thaws are to be avoided, so RNA should be aliquoted for transfer as soon as possible after quantitation.

Figure 3: Phases of TRIzol

ADVANTAGES & DISADVANTAGES:

- TRIzol or tri (name depends on manufacturer) combines phenol and guanidine isothiocyanate and thereby some of the advantages of the above two
- Removes protein and DNA but depends on pipetting skills (disturbing the phases leads to contamination)
- RNA is protected by the reagent during the extraction procedure
- Phenol and chloroform are potentially harmful reagents (handle under the hood)

READINGS:

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https://www.lerner.ccf.org/gmi/gmb/documents/RNA%20Isolation.pdf
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http://www.nature.com/nprot/journal/v1/n2/full/nprot.2006.83.html
https://www.ncbi.nlm.nih.gov/pubmed/16028681
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http://openwetware.org/wiki/RNA_extraction#TRIzol_or_tri_followed_by_chloroform_and_precipitation

Affinity purification of total RNA.

INTRODUCTION:

High-quality mRNA is needed for a number of molecular biology techniques, including cDNA library construction. Not surprisingly, numerous mRNA extraction kits are now commercially available.

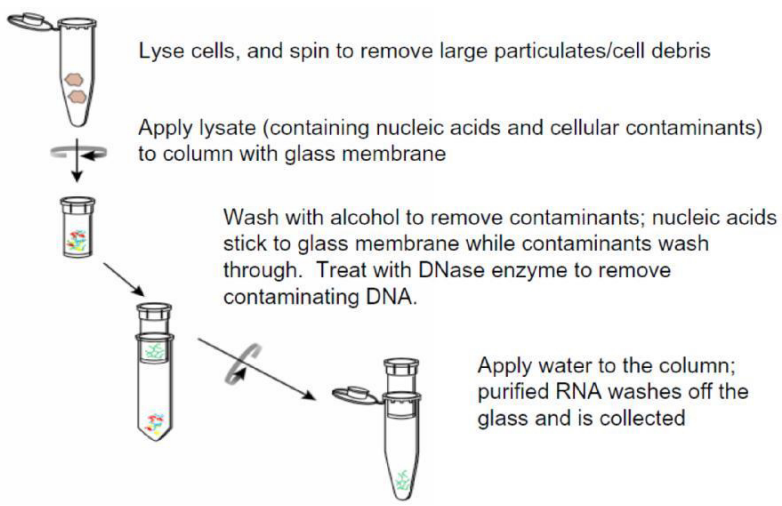

Figure 4: **Affinity purification of total RNA.**

PRINCIPLE:

The affinity selection of polyadenylated mRNA using oligodeoxythymidylate (Oligo (dT)).

MATERIALS

- All materials used in this procedure should be sterile and of molecular biology grade.
- All Tris-containing solutions are prepared using RNase-free water and autoclaved.
- All other solutions, unless otherwise stated, should be treated directly with diethyl pyro carbonate (DEPC) and autoclaved. DEPC is an efficient, nonspecific inhibitor of RNase activity. It is, however, a carcinogen and should be handled in a fume hood with extreme care. Hands are a major source of RNase activity. Because of this, gloves should be worn for all procedures.
- RNase-free water: Add 0.1% DEPC to water. Allow to stand overnight at 37°C and autoclave to destroy residual DEPC activity. All solutions except Tris, which inactivates DEPC, can be treated in the same way.
- SDS (sodium dodecyl sulphate): SDS is dangerous if inhaled and should be weighed in a fume hood. A 10% stock solution is normally prepared. This solution is unstable if autoclaved, however any residual RNase activity can be destroyed by heating the solution at 65°C for 2 h.
- Oligodeoxythymidylate-cellulose (oligo (dT)): Oligo (dT) cellulose is available commercially.
- Although the binding capacity of oligo(dT) cellulose varies between different suppliers, a general rule is to use 25 mg of oligo(dT) for each 1 mg of total RNA. Suspend oligo (dT) cellulose in loading buffer at a concentration of 5 mg per 1 mL loading buffer.
- Oligo (dT) is insoluble and should be resuspended by gentle tapping or inversion. Do not put it in a vortex. It can be stored either dry at 4°C or in suspended in loading buffer at – 20°C.

- RNase-free glass wool and Pasteur pipets: Wrap both the glass wool and pipets in aluminium foil and bake at 200°C for 2–4 h to remove any RNase activity.
- 5 M NaCl: Store at room temperature.
- 3 M Sodium acetate pH6: Store at room temperature.
- Absolute alcohol: Store at –20°C.
- 70% ethanol: Prepare this solution using DEPC-treated water. Store at 4°C.
- Loading buffer: 0.5 M NaCl in 0.5% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 Store at room temperature.
- Elution buffer: 1 mM EDTA, 10 mM Tris-HCl, pH 7.5. The buffer can be stored at room temperature but should be preheated to 65°C prior to use.
- Recycling buffer: 0.1 M NaOH, which should be prepared immediately before use and used fresh.

METHODS

Preparing an Oligo (dT) Column

Oligo (dT) columns are available commercially or can be prepared by using a 1–3 mL syringe. Preparing your own columns is both easy and cheap.

1. Remove the plunger from the syringe and plug the base with glass wool.
2. Add oligo (dT) cellulose to the syringe using a sterile RNase-free Pasteur pipet. The oligo (dT) cellulose will collect, as a column, above the glass wool. The loading buffer will escape through the glass wool and can be discarded. To ensure that the oligo (dT) cellulose is packed and free from air locks, add 3 volumes of loading buffer using a pipette and allow the solution to run through the column. The column is now ready for immediate use and should not be allowed to run dry.

Isolation of Poly (A+) RNA:

1. Resuspend the RNA pellet in loading buffer or, if the buffer is in solution, add 1/10th volume of 5 M NaCl (see Note 1).
2. Heat denature the RNA and immediately load it onto the column and apply 3 volume of loading buffer.
3. Reapply the eluate to the column.
4. Wash with 3 volume of loading buffer (see Note 4). Discard eluate.
5. Recover the bound poly (A+) mRNA by adding 3 volume elution buffer. Collect the mRNA in a sterile tube on ice (see Note 5).
6. The mRNA is precipitated by adding 1/10th volume of 3 M sodium acetate and 2 volume of ice-cold absolute ethanol. An overnight precipitation at –20°C maximizes the precipitation of RNA.
7. Centrifuge at 15,000g for 15 min to pellet the RNA. Discard the supernatant.
8. Wash the RNA pellet in ice-cold 70% ethanol. Centrifuge at 15,000g for 5 min to repellet the RNA which may have been disturbed by washing. Discard the supernatant.

Isolation of mRNA:

9. Dry the RNA pellet. Once it is dry, resuspend it in DEPC-treated water.
10. Assess the purity and integrity of mRNA.

ADVANTGES & DISADVANTAGES:

- Eliminates need for organic solvents
- Compatible with a variety of sample types (tissue, tissue culture cells, white blood cells, plant cells, bacteria, yeast, etc.)
- DNase treatment eliminates contaminating genomic DNA
- Excellent RNA purity and integrity
- Less amount of RNA obtained.
- Expensive

READINGS

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http://www.sabiosciences.com/newsletter/RNA.html

http://www.gelifesciences.com/webapp/wcs/stores/servlet/CategoryDisplay?categoryId=11763&catalogId=10101&productId=&top=Y&storeId=11765&langId=-1

http://www.thermofisher.com/pk/en/home/life-science/dna-rna-purification-analysis/rna-extraction/rna-types/mrna-extraction.html

PCR and its types:

(Nested, Multiplex, Reverse transcriptase, Real time, hot start, Asymmetric, Long, Allele specific, colony, In-situ, Inverse, AFLP PCR).

INTRODUCTION:

The polymerase chain reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Developed in 1983 by Kary Mullis, PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and DNA paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.

PRINCIPLE

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers, the short DNA fragments containing sequences complementary to the target region along with a DNA polymerase, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations .

MATERIALS

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase (an enzyme originally isolated from the bacterium Thermus aquaticus). This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. Following reagents are required for PCR

- DNA template that contains the DNA region (target) to amplify

- Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target

- Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C

- Deoxynucleoside triphosphates (dNTPs, sometimes called "deoxynucleotide

triphosphates"; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand

- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase

- Bivalent cations, magnesium or manganese ions; generally Mg2+ is used, but Mn2+ can

be used for PCR-mediated DNA mutagenesis, as higher Mn2+ concentration increases the error rate during DNA synthesis

- Monovalent cation potassium ions

METHODS

The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample through a defined series of temperature steps. In the first step, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

Typically, PCR consists of a series of 20–40 repeated temperature changes, called cycles, with
each cycle commonly consisting of 2–3 discrete temperature steps, usually three (Figure below).
The cycling is often preceded by a single temperature step at a high temperature (>90 °C), and
followed by one hold at the end for final product extension or brief storage. The temperatures
used and the length of time they are applied in each cycle depend on a variety of parameters.
These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs
in the reaction,and the melting temperature (Tm) of the primers

Initializationstep:

This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes.

Denaturation step:

This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing step:

The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should only bind to a perfectly complementary part of the template. If the temperature is too low, the primer could bind imperfectly. If it is too high, the primer might not bind. Typically the annealing temperature is about 3–5 °C below the Tm of the primers used.

Stable DNA–DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation. It is very vital to determine the annealing temperature in PCR. This is because in PCR, efficiency and specificity are affected by the annealing temperature. An incorrect annealing temperature will cause an error in the test.

Extension/elongation step:

The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to amplify. As a rule-of-thumb, at its optimum temperature, the DNA polymerase polymerizes a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

Final elongation

This single step is occasionally performed at a temperature of 70–74 °C (this is the temperature needed for optimal activity for most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold:

This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction

Figure 5: Polymerase Chain Reaction

ADVANTGES & DISADVATAGES

Selective DNA isolation, Amplification and quantification of DNA, Disease diagnosis,

DNA polymerase is prone to error, which in turn causes mutations in the PCR fragments that are made. Additionally, the specificity of the PCR fragments can mutate to the template DNA, due to nonspecific binding of primers. Furthermore, prior information on the sequence is necessary in order to generate the primers

PCR VARIATIONS:

Nested PCR:

It Increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.

Reverse Transcription PCR (RT-PCR):

It is useful for amplifying DNA from RNA. Reverse transcriptase reverse

transcribes RNA into cDNA, which is then amplified by PCR. RT-PCR is widely used

in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene. The 5' end of a gene (corresponding to the transcription start site) is typically identified by RACE-PCR (Rapid Amplification of cDNA Ends).

Quantitative PCR (qPCR):

It is used to measure the quantity of a target sequence (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA, or RNA. Quantitative PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. Quantitative PCR has a very high degree of precision. Quantitative PCR methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. It is also sometimes abbreviated to RT-PCR (real-time PCR) but this abbreviation should be used only for reverse transcription PCR. qPCR is the appropriate contractions for quantitative PCR (real-time PCR).

Hot start PCR:

A technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 95 °C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that dissociate only after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.

Asymmetric PCR:

Preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in sequencing and hybridization probing where amplification of only one of the two

complementary strands is required. PCR is carried out as usual, but with a great excess of the primer for the strand targeted for amplification. Because of the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required. A recent modification on this process, known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature (Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

Nanoparticle-Assisted PCR (nanoPCR):

In recent years, it has been reported that some nanoparticles (NPs) can enhance the efficiency of PCR (thus being called nanoPCR), and some even perform better than the original PCR enhancers. It was also found that quantum dots (QDs) can improve PCR specificity and efficiency. Single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) are efficient in enhancing the amplification of long PCR. Carbon nanopowder (CNP) was reported be able to improve the efficiency of repeated PCR and long PCR. ZnO, TiO2, and Ag NPs were also found to increase PCR yield. Importantly, already known data has indicated that non-metallic NPs retained acceptable amplification fidelity. Given that many NPs are capable of enhancing PCR efficiency, it is clear that there is likely to be great potential for nanoPCR technology improvements and product development.

Allele-specific PCR:

A diagnostic or cloning technique based on single-nucleotide variations (SNVs not to be confused with SNPs) (single-base differences in a patient). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNV (base pair buffer around SNV usually incorporated). PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

Inverse PCR:

It is commonly used to identify the flanking sequences around genomic inserts. It involves a series of DNA digestions and self-ligation, resulting in known sequences at either end of the unknown sequence.

Colony PCR:

Colony PCR is a method used to screen for plasmids containing a desired insert directly from bacterial colonies without the need for culturing or plasmid purification steps.

In situ PCR:

In situ PCR is a method in which the polymerase chain reaction actually takes place in the cell on a slide, and the product can be visualized in the same way as in traditional in situ hybridization.

AFLP-PCR:

AFLP-PCR uses restriction enzymes to digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments is then selected to be amplified. This selection is achieved by using primers complementary to the adaptor sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments. The amplified fragments are separated and visualized on denaturing polyacrylamide gels, either through autoradiography or fluorescence methodologies, or via automated capillary sequencing instruments.
READINGS

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http://link.springer.com/protocol/10.1385%2F1-59259-384-4%3A3
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http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1993/mullis-lecture.html
http://www.ncbi.nlm.nih.gov/tools/epcr/

Multiple x-PCR:

It consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.

Real Time PCR

Introduction to Real Time PCR

As the name suggests, real time PCR is a technique used to monitor the progress of a PCR reaction in real time. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes). Real time PCR facilitates the monitoring of the reaction as it progresses. One can start with minimal amounts of nucleic acid and quantify the end product accurately. Moreover, there is no need for the post PCR processing which saves the resources and the time. These advantages of the fluorescence based real time PCR technique have completely revolutionized the approach to PCR-based quantification of DNA and RNA. Real time PCR assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation. Real time PCR is also referred to as real time RT PCR which has the additional cycle of reverse transcription that leads to formation of a DNA molecule from a RNA molecule. This is done because RNA is less stable as compared to DNA

Real Time PCR procedure

In a real time PCR protocol, a fluorescent reporter molecule is used to monitor the PCR as it progresses. The fluorescence emitted by the reporter molecule manifolds as the PCR product accumulates with each cycle of amplification. Based on the molecule used for the detection, the real time PCR techniques can be categorically placed under two heads:

1. Non-specific Detection using DNA Binding Dyes

2. Specific Detection Target Specific Probes

Non-specific Detection using DNA Binding Dyes

In real time PCR, DNA binding dyes are used as fluorescent reporters to monitor the real time PCR reaction. The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase. If a graph is drawn between the log of the starting amount of template and the corresponding increase the fluorescence of the reporter dye fluorescence during real time PCR, a linear relationship is observed.

SYBR Green is the most widely used double-strand DNA-specific dye reported for real time PCR. SYBR Green binds to the minor groove of the DNA double helix. In the solution , the unbound dye exhibits very little fluorescence. This fluorescence is substantially enhanced when the dye is bound to double stranded DNA. SYBR Green remains stable under PCR conditions and the optical filter of the thermocycler can be affixed to harmonize the excitation and emission wavelengths. Ethidium bromide can also be used for detection but its carcinogenic nature renders its use restrictive.

Although these double-stranded DNA-binding dyes provide the simplest and cheapest option for real time PCR, the principal drawback to intercalation based detection of PCR product accumulation is that both specific and nonspecific products generate signal.

Specific Detection using Target Specific Probes

Specific detection of real time PCR is done with some oligonucleotide probes labeled with both a reporter fluorescent dye and a quencher dye. Probes based on different chemistries are available for real time detection, these include:

a. Molecular Beacons

b. TaqMan Probes

c. FRET Hybridization Probes

d. Scorpion Primers

Real Time PCR Applications Include

1 . Quantitative mRNA expression studies.

2 . DNA copy number measurements in genomic or viral DNAs.

3 . Allelic discrimination assays or SNP genotyping.

4 . Verification of microarray results.

5 . Drug therapy efficacy.

6 . DNA damage measurement.

Quantitative PCR (qPCR)

Real-Time PCR Real-Time PCR, also referred to as Quantitative PCR (or qPCR), was developed as a precise, efficient and rapid method for nucleic acid detection. This technique is based on traditional Polymerase Chain Reaction (PCR) technology with a few improvements:

Real-Time PCR combines nucleic acid amplification and detection in one step

It can use smaller amounts of starting material than traditional PCR

It is possible to quantify the product based on fluorescent detection

There is no post-amplification process

The detection of template DNA (or RNA if a reverse transcription step is added prior to amplification) is live and based on when the PCR product is amplified above the threshold of background or the cycle threshold number (Ct number). Far different from the former end-point method of standard PCR techniques, Real-Time PCR can be quantitative because the PCR product is detected using fluorescent dyes in real time. Thus, Real-Time PCR earned the alternate and distinctive name of Quantitative PCR or qPCR.

Key Applications of Real-Time PCR:

qPCR has been used for a wide number of applications over the years but there some uses for the technique that have been particularly popular. These include:

Gene Expression (mRNA) Analysis

microRNA and Non-Coding RNA analysis

Genetic Variation

Mutation Detection

SNP Analysis

Genotyping/Allelic Discrimination

Real Time PCR VS Traditional PCR

Regards : Zarva Chaudhary & Chaudhary Moazzam

Real time PCR allows for the detection of PCR product during the early phases of the reaction. This ability of measuring the reaction kinetics in the early phases of PCR provide a distinct advantage over traditional PCR detection. Traditional methods use gel electrophoresis for the detection of PCR amplification in the final phase or at end-point of the PCR reaction.

Limitations of End-point PCR

In a PCR reaction as the reaction progresses, the reagents are being consumed as a result of amplification. Now the PCR product is no longer being doubled at each cycle due to this reagent constraint. This depletion will occur at different rates for each replicate. Thus, the samples begin to diverge in their quantities. This diminished amplification is the linear phase of the reaction. The plateau for each tube will differ due to the different reaction kinetics for each sample. It is in this phase where traditional PCR takes its measurement, also known as the end-point. This End-Point Detection has some problems such as low resolution, poor precision, low sensitivity and the need for post PCR processing. Also, the results of this detection are not expressed in numbers and there is no scope for automation.

Real Time PCR Primer and Probe Design with AlleleID & Beacon Designer™

Beacon Designer™ is a comprehensive real time PCR primer and probe design tool for designing single template and multiplex assays. Real time PCR chemistries supported include Molecular beacons, TaqMan, FRET, Scorpions and SYBR Green. It designs molecular beacons for standard and NASBA assays and designs LNA spiked Taqman® probes as well. AlleleID supports all the major features of Beacon Designer™ except NASBA® assay, LNA™ spiked Taqman probe design and multiplex assay design. TaqMan MGB probe design is unique to AlleleID (whereas regular TaqMan probes based assays can be designed using both AlleleID and Beacon Designer™. AlleleID is written for advance real time PCR applications such as oligo design for species identification and taxa discrimination. It includes a multiple sequence alignment module for identifying conserved and species specific regions. AlleleID also includes support for real time PCR primer design over exon junctions for selective amplification of cDNA.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

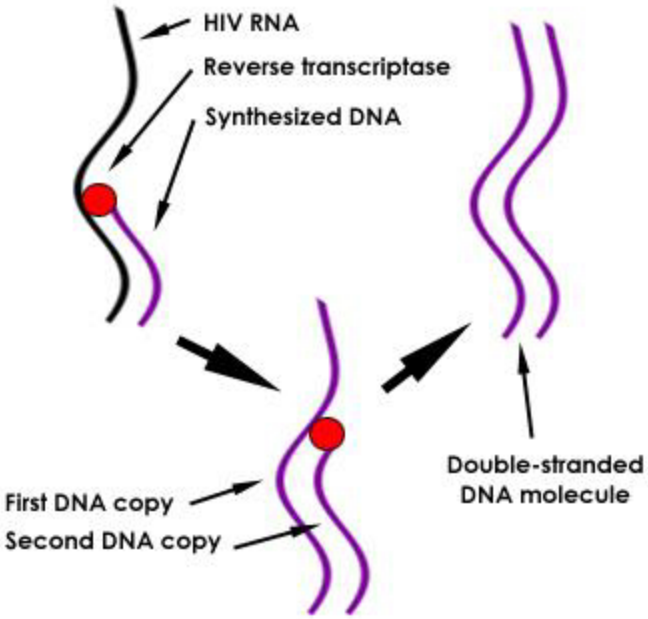
Reverse transcriptase PCR (RT-PCR) was developed to amplify RNA targets (RNA viruses such as HIV, HCV, and influenza are key examples). Essentially, the method entails an initial step of transcribing a portion of the RNA genome into complementary DNA (cDNA) which is then amplified through PCR.

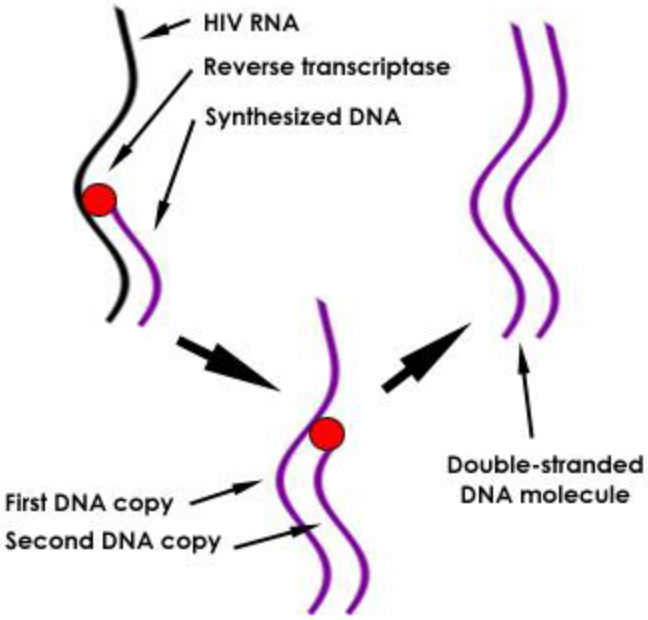
PCR depends on the Taq Polymerase enzyme; RNA is not an efficient substrate for this enzyme. This is why the target of interest (if present) is first transcribed into complementary DNA (cDNA), which can then be amplified.

RT-PCR Process

- After RNA is released from cellular material through extraction, an aliquot of the extracted sample is added to a reaction mixture which contains reverse transcriptase enzyme, primers specific for the target of interest, and nucleotides.
- If the target is present, primers anneal to the RNA strand.
- Reverse transcriptase enzyme synthesizes a complementary DNA strand, extending from the primer.
- The temperature is raised to 95^{o} C, and the RNA/DNA strands are denatured.
- The temperatures are lowered, allowing primers to anneal to the newly formed cDNA.
- Polymerase enzyme synthesizes a new DNA strand, extending from the primer.
- Multiple cycles geometrically increase the number of copies of DNA.

RT-PCR can be performed as one or two step procedures. In a one-step procedure, the reverse transcriptase is performed in the same reaction tube as the polymerase chain reaction. In a two-step procedure, transcription of the RNA to cDNA is performed first. Transcription occurs between 40^{o} C and 50^{o} C, depending on the properties of the reverse transcriptase enzyme utilized; products of that reaction are then amplified in a separate reaction.





Analysis Of proteins by SDS

SDS gel preparation:

To make 12% SDS-PAGE gel, the resolving gel components for 20 ml were added as follows.

a) **Resolving Gel:**

Component	Amount(mL)
Aqua dest.	8.6
40 % acrylamide mix	6
Tris (1.5 M, pH 8.8)	5

- 200µL of 10% SDS solution was added
- 20µL of APS (25%) was added
- 20µL of TEMED was added.
- This gel mixture was added to the Gel apparatus after careful mixing and avoiding froth. Isopropanol was added on top of the gel to remove the bubbles and even the upper front of the gel.

b) **Stacking Gel:**

The resolving gel is allowed to polymerize and then stacking gel mixture is added to at the top after removing isopropanol layer.

For 5 ml of stacking gel

Component	Amount(mL)
Aqua dest.	3.645
40 % acrylamide mix	0.625
Tris (1.0, pH 6.8)	0.630

- 50µL of 10% SDS solution was added
- 05µL of APS (25%) was added
- 05µL of TEMED was added.
- This gel mixture was added to the Gel apparatus after careful mixing and avoiding froth.
- Combs were placed avoiding entrapment of any air bubbles.

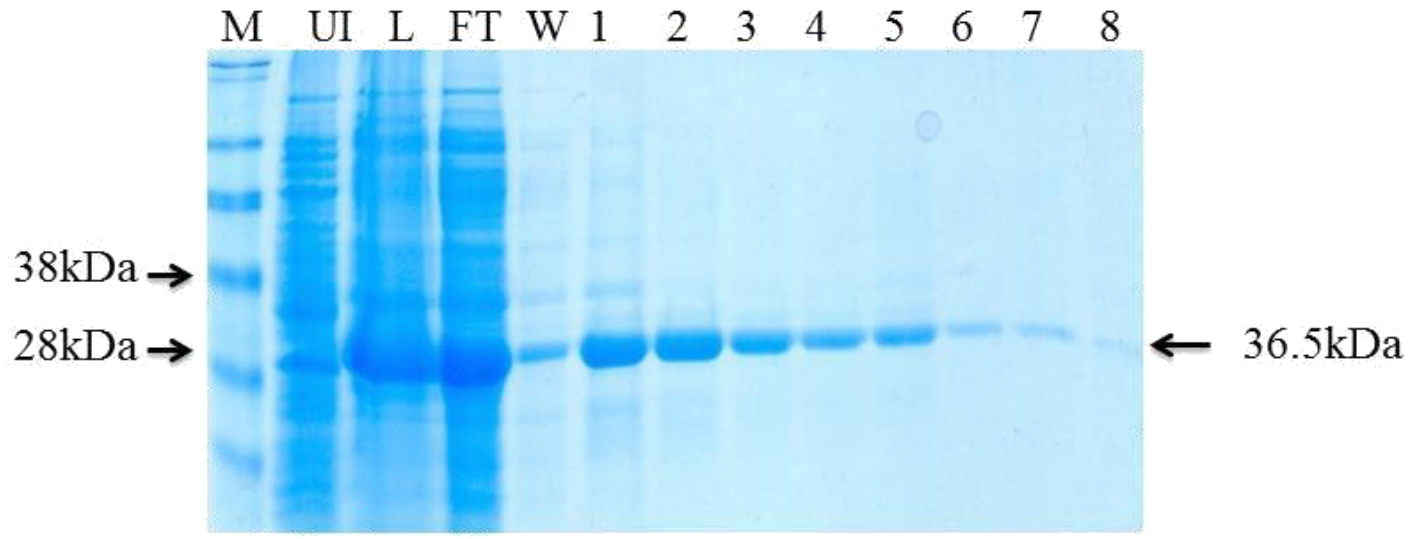


Figure 1: Purification of His -tagged PROTEIN (326 aa) by Ni-NTA affinity purification. Protein expression induced by 0.5 mM IPTG for 3 hours was used for affinity purification using Ni-NTA beads. Uninduced was collected before induction, load was lysate sample before addition of Ni-NTA beads. Flow-through is lysate which passed through the column, Wash is wash buffer passed through the column, while rest are the eluted fractions. All the protein samples were boiled in SDS loading buffer and resolved on SDS-PAGE where expected 36 kDa protein is visible in eluted fractions (1-5). M: protein ladder UI: uninduced; L: Lysate/load; FT: Flow through; W: Wash; 1-4: serial elutions with 100mM imidazole; 5-6: serial elutions with 250mM imidazole and 7-8: serial elutions with 400mM imidazole. Stained with colloidal coomasie stain.

Comparing plasmids of different molecular weights using molecular weights marker

Principle:

Plasmid DNA isolation

- A single white bacterial colony of the transformant will be inoculated into 5 mL of LB medium containing 100 µg/mL ampicillin in a test tube.
- The culture will be incubated overnight at 37^{o}C in shaking incubator.
- Three mL of culture will be poured into new microfuge tube and centrifuge at 12000 rpm for 1 min and supernatant will be discarded.
- Pellet will be resuspended into 100 µL of ice-cold solution I by vigorous shaking
- Then 200 µL of freshly prepared solution II will be added. Contents will be mixed by inverting the tube gently 4-5 times
- Then tube will be incubated on ice for 5 min.
- Finally 150 µL of Ice-cold solution III will be added. The tube will be inverted gently several times to disperse the solution III through the viscous bacterial lysate.
- Tube will be stored on ice for 3 -5 min and centrifuge at 12000 rpm for 5 min at 4^{o}C.
- Supernatant will be transferred to fresh tube and equal volume of phenol:choloroform (1:1) will be added and centrifuge at12000 rpm for 5 min.
- Supernatunt will be transferred to fresh tube.
- DNA will be precipitated with 2 volume of absolute ethanol and store at -20˚C for half an hour. Centrifugation will be done at 12000 rpm for 10 min at 4^{o}C.
- Supernatunt will be discarded and the tubes will be allowed to stand on paper towel in an inverted position to allow all the solution to dry away.
- Pellet will be washed with 500 µL of 70% ethanol.
- Supernatant will be removed and pellet allow to air dry .
- Eighty µL of water and 1 µL of RNase will be added and left for 30 min at 37ºC and then stored at –20°C.
- The isolated plasmid will be analyzed on 1% agarose gel electrophoresis.

Restriction analysis

- The presence of insert in pTZ57R/T will be confirmed by restriction digestion of the recombinant plasmid.
- Initially the plasmid will be restricted with suitable restriction enzyme using 2X Tango buffer.

- The reaction mixtures each of 15 µL will be prepared as mentioned in below table 8.1 and then incubated at 37^{o}C for 4 h.
- Electrophoresis will be done with 1% agarose gel to visualize the required product under UV light.

Table 8.1: Restriction analysis

Reagents	**Sample Amylase**
10X Yellow-Tango buffer	3 µL
*Eco*RI	1 µL
*Hin*dIII	1 µL
DNA	10 µL
Total	15 µL

Agarose Gel Electrophoresis

Principle

Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones (Sambrook & Russel 2001). In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest. Extension of the technique includes excising the desired ―band‖ from a stained gel viewed with a UV transilluminator (Sharp et al.,1973)

Equipment Required

 An electrophoresis chamber and power supply

 Gel casting trays, which are available in a variety of sizes and composed of UV- transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.

 Sample combs, around which molten agarose is poured to form sample wells in the gel.

 Transilluminator (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels. NOTE: always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.

 Pipettes ---- covering 1 to 100 ul range

Reagent Required

 Electrophoresis buffer, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).

 DNA sizing standard/ladder

 Loading buffer, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.

 Ethidium bromide, a fluorescent dye used for staining nucleic acids. *NOTE: Ethidium bromide is a known mutagen and should be handled as a hazardous chemical – wear gloves while handling.*

Procedure:

To pour a gel, agarose powder is mixed with electrophoresis buffer to the desired

concentration, then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (final concentration 0.5 ug/ml) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

After the gel has solidified, the comb is removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis

and just covered with buffer. Samples containing DNA mixed with loading buffer are then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied. You can confirm that current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored red.

The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively. When adequate migration has occurred, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA or RNA, the gel is placed on an ultraviolet trans-illuminator. Be aware that DNA will diffuse within the gel over time, and examination or photography should take place shortly after cessation of electrophoresis.

Migration of DNA Fragments in Agarose

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the log10 of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the log10 of either their molecular weights or number of base pairs, a roughly straight line will appear.

Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass. Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized. Additionally, most preparations of uncut plasmid contain at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles. The image to the right shows an ethidium-stained gel with uncut plasmid in the left lane and the same plasmid linearized at a single site in the right lane.

Several additional factors have important effects on the mobility of DNA fragments in agarose gels, and can be used to your advantage in optimizing separation of DNA fragments. Chief among these factors are:

Agarose Concentration: By using gels with different concentrations of agarose, one can resolve different sizes of DNA fragments. Higher concentrations of agarose facilitate separation of small DNAs, while low agarose concentrations allow resolution of larger DNAs.

Voltage: As the voltage applied to a gel is increased, larger fragments migrate proportionally faster than small fragments. For that reason, the best resolution of fragments larger than about 2 kb is attained by applying no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).

Electrophoresis Buffer: Several different buffers have been recommended for electrophoresis of DNA. The most commonly used for duplex DNA are TAE (Tris-acetate- EDTA) and TBE (Tris-borate-EDTA). DNA fragments will migrate at somewhat different rates in these two buffers due to differences in ionic strength. Buffers not only establish a pH, but provide ions to support conductivity. If you mistakenly use water instead of buffer, there will be essentially no migration of DNA in the gel! Conversely, if you use concentrated buffer (e.g. a 10X stock solution), enough heat may be generated in the gel to melt it.

Effects of Ethidium Bromide: Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels, as shown by all the images on this page. As described above, it can be incorporated into agarose gels, or added to samples of DNA before loading to enable visualization of the fragments within the gel. As might be expected, binding of ethidium bromide to DNA alters its mass and rigidity, and therefore its mobility.

Short Tandem Repeats (STRs)

The human genome is full of repeated DNA sequences. These repeated sequences come in various sizes and are classified according to the length of the core repeat units, the number of contiguous repeat units, and/or the overall length of the repeat region. DNA regions with short repeat units (usually 2-6 bp in length) are called Short Tandem Repeats (STR). STRs are found surrounding the chromosomal centromere (the structural center of the chromosomes). STRs have proven to have several benefits that make them especially suitable for human identification.

STRs have become popular DNA markers because they are easily amplified by polymerase chain reaction (PCR) without the problem of differential amplification; that is, the PCR products for STRs are generally similar in amount, making analysis easier. An individual inherits one copy of an STR from each parent, which may or may not have similar repeat sizes. The number of repeats in STR markers can be highly variable among individuals, which make these STRs effective for human identification purposes.

For human identification purposes, it is important to have DNA markers that exhibit the highest possible variation in order to discriminate between samples. It is often challenging to obtain PCR amplification products from forensic samples because either the DNA in those samples is degraded, or mixed, such as in a sexual assault case.

The smaller size of STR alleles make STR markers better candidates for use in forensic applications, in which degraded DNA is common. PCR amplification of degraded DNA samples can be better accomplished with smaller target product sizes.

Because of their smaller size, STR alleles can also be separated from other chromosomal locations more easilyto ensure closely linked loci are not chosen. Closely linked loci do not follow the predictable pattern of random distribution in the population, making statistical analysis difficult.

STR alleles also have lower mutation rates, which makes the data more stable and predictable.

Because of these characteristics, STRs with higher power of discrimination are chosen for human identification in forensic cases on a regular basis. It is used to identify victim, perpetrator, missing persons, and others.

Plasmid Isolation

Introduction

The term ‘plasmid’ was coined by Joshua Lederberg in 1592. Originally evolved from bacteria, plasmids are extrachromosomal genetic elements present in most species of Archae, Eukarya and Eubacteria that can replicate independently. Plasmids are circular double stranded DNA molecule that are distinct from the cells chromosomal DNA.

The structure and function of a bacterial cell is directed by the genetic material contained within the chromosomal DNA. In some cases plasmids are generally not essential for the survival of the host bacterium. Although not essential, plasmids contribute significantly to bacterial genetic diversity and plasticity by encoding functions that might not be specified by the bacterial chromosomal DNA. Plasmids specify traits that allow the host to persist in environments that would otherwise be either lethal or restrictive for growth. For example antibiotic resistance and protein expression. Antibiotic resistance genes are often encoded by the plasmid, which allows the bacteria to persist in an antibiotic containing environment, thereby providing the bacterium with a competitive advantage over antibiotic-sensitive species. As a tool, plasmids can be modified to express the protein of interest (e.g., production of human insulin using recombinant DNA technology).

Plasmids have served as invaluable model systems for the study of processes such as DNA replication, segregation, conjugation, and evolution. Plasmids have been pivotal to modern recombinant DNA technology as a tool in gene-cloning and as a vehicle for gene-expression.

Characteristics of Plasmid

Plasmids present in the bacterium differ in their physical properties such as in size (kbp), geometry and copy number.

Plasmid Size

Plasmids range in size from 1 kbp (kilo base pair) to 1000 (kilo base pair) megaplasmids that are many hundred base pairs in size.

Plasmid Geometry

Although most plasmids possess a circular geometry, there are now many examples of plasmids that are linear in a variety of bacteria. Plasmid DNA may appear in one of the five conformations nicked open circular DNA which has one strand cut, relaxed circular DNA is fully intact with both strands uncut, but has been enzymatically relaxed, linear DNA has free ends, supercoiled DNA is fully intact with both strands uncut, and supercoiled denatured DNA is like super coiled DNA, but has unpaired regions that make it slightly less compact.

Plasmid Copy Numbers

Copy number refers to the average or expected number of copies per host cell. Plasmids are either low, medium or high copy number. Knowing which category plasmid falls under is very important when starting out an experiment. If working with a low-copy number plasmid which is associated with a low yield and might therefore be required to set up more cultures. On the other hand, if a poor yield is obtained from a high copy plasmid, troubleshooting is required. In bacterium with high copy number plasmids, during cell division the plasmids get segregate randomly in the daughter cells, whereas bacterium with low copy numbers, during cell division and partition the plasmids divided equally in the daughter cells. An advantage of high copy number is the greater stability of the plasmid when random partitioning (i.e. partitioning of plasmids into daughter cells) occurs at cell division.

Plasmid Isolation

The isolation of plasmid DNA from bacteria is a crucial technique in molecular biology and is an essential step in many procedures such as cloning, DNA sequencing, transfection, and gene therapy. These manipulations require the isolation of high purity plasmid DNA. The purified plasmid DNA can be used for immediate use in all molecular biology procedures such as digestion with restriction enzymes, cloning, PCR, transfection, in vitro translation, blotting and sequencing.

Alkaline lysis is a method used in molecular biology, to isolate plasmid DNA or other cell components such as proteins by breaking the cells open. Bacteria containing the plasmid of interest is first grown, and then allowed to lyse with an alkaline lysis buffer consisting of a detergent sodium dodecyl sulfate (SDS) and a strong base sodium hydroxide. The detergent cleaves the phospholipid bilayer of membrane and the alkali denatures the proteins which are involved in maintaining the structure of the cell membrane. Through a series of steps involving agitation, precipitation, centrifugation, and the removal of supernatant, cellular debris is removed and the plasmid is isolated and purified.

Principle

Purification of plasmid DNA from bacterial DNA using is based on the differential denaturation of chromosomal and plasmid DNA using alkaline lysis in order to separate the two. The basic steps of plamid isolation are disruption of the cellular structure to create a lysate, separation of the plasmid from the chromosomal DNA, cell debris and other insoluble material. Bacteria are lysed with a lysis buffer solution containing sodium dodecyl sulfate (SDS) and sodium hydroxide. During this step disruption of most cells is done, chromosomal as well as plasmid DNA are denatured and the resulting lysate is cleared by centrifugation, filtration or magnetic clearing. Subsequent neutralization with potassium acetate allows only the covalently closed plasmid DNA to reanneal and to stay solubilized. Most of the chromosomal DNA and proteins precipitate in a complex formed with potassium and SDS, which is removed by centrifugation.

The bacteria is resuspended in a resuspension buffer (50mM Tris-Cl, 10 mM EDTA, 100 µg/ ml RNase A, pH 8.0) and then treated by 1% SDS (w/v) / alkaline lysis buffer (200mM NaOH) to liberate the plasmid DNA from the E. coli host cells. Neutralization buffer (3.0 M potassium acetate, pH 5.0) neutralizes the resulting lysate and creates appropriate conditions for binding of plasmid DNA to the silica membrane column. Precipitated protein, genomic DNA, and cell debris are then pelleted by a centrifugation step and the supernatant is loaded onto a column. Contamination like salts, metabolites, and soluble macromolecular cellular components are removed by simple washing with ethanolic wash buffer (1.0 M NaCl, 50mM MOPS, pH 7.0, isopropanol (v/v) 15 %). Pure plasmid DNA is finally eluted under low ionic strength conditions with slightly alkaline buffer (5 mM Tris / HCl, pH 8.5).

Culture Media

Yield and quality of plasmid DNA highly depend on the type of culture media used. Most plasmid purification are optimized with cultures grown in standard Luria Bertani (LB) medium. For LB medium preparation dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 800 ml distilled water. Adjust the pH to 7.0 with 1 N NaOH. Adjust the volume to 1 liter by adding distilled water and sterilize by autoclaving. The cell culture should be incubated at 37 °C with constant shaking (200–250 rpm) preferably 12–16 h overnight. Usually an OD of 3–6 can be achieved. Alternatively, rich media like 2 x YT (Yeast / Tryptone), TB (Terrific Broth), or CircleGrow can be used.

Also cares needs to taken, as overgrowing a culture might lead to a higher percentage of dead or starving cells and the resulting plasmid DNA might be partially degraded or contaminated with chromosomal DNA. To find the optimal culture conditions, the culture medium and incubation times have to be optimized for each host strain / plasmid construct combination individually.

Lysate & Neutralization

Lysis formulas may vary depending on whether you want to extract DNA/RNA/Plasmid. All methods of lysing bacteria will yield plasmid solutions contaminated with chromosomal DNA and RNA. Centrifugation removes the vast majority of chromosomal DNA (it will form a pellet, while plasmid DNA remains soluble), and treatment with RNase will eliminate contaminating RNA.

Generally speaking, lysis buffers contain a high concentration of chaotropic salts. Chaotropes have two important roles in nucleic acid extraction. Firstly, they destabilize hydrogen bonds, van der Waals forces and hydrophobic interactions, leading to destabilization of proteins, including nucleases. Secondly, they disrupt the association of nucleic acids with water, thereby providing optimal conditions for their transfer to silica.

Separation and removal of the plasmids from the bacterial cell is brought about by resuspension of 1-5 mL of culture in a resuspension buffer (50mM Tris-Cl, 10 mM EDTA, 100 µg/ ml RNase A, pH 8.0) and pellet cells in a microcentrifuge at 11000 x g for 30 s.

Lysate is achieved by adding 250 µL of lysis buffer with neutralization buffer, as it aids in complete precipitation of SDS, protein, and genomic DNA. Incomplete neutralization leads to reduced yield. However, released plasmid DNA is very vulnerable at this point and shaking too much or too strongly will damage the DNA.

Binding and Washing in Silica Membrane

After centrifuging the lysate through silica membrane, the desired nucleic acids should be bound to the column and impurities such as protein and polysaccharides should be in the flow-through. While, plant samples will likely contain polysaccharides and pigments, while for blood samples, the membrane may be slightly brown or yellow in color. The wash steps will remove such impurities. There are typically two wash steps, although it varies depending on sample type. The first wash will often include a low concentration of chaotropic salts to remove residual proteins and pigments. This is always followed with an ethanol wash to remove the salts.

Columns contain a silica resin that selectively binds to DNA/RNA. The DNA of interest can be isolated by virtue of its ability to bind silica in the presence of high concentrations of chaotropic salts. These salts are then removed with an alcohol based wash and the DNA is eluted using a low-ionic-strength solution such as TE buffer or water. The binding of DNA to silica seems to be driven by dehydration and hydrogen bond formation, which competes against weak electrostatic repulsion. Hence, a high concentration of salt will help drive DNA adsorption onto silica, and a low concentration will release the DNA.

Elution

The elution buffer volume and method can be adapted to the subsequent downstream application to achieve higher yield and / or concentration that the standard method. Elution buffer is used to wash away unbound proteins at first and at a greater concentration it releases the desired protein from the ligand. It is important that the elution buffer works quickly without changing the function or activity of the desired protein. For maximal DNA elution, allow the buffer to stand in the membrane for a few minutes before centrifugation. Elution Buffer AE (5 mM Tris/HCl, pH 8.5) can be replaced by TE buffer or water as well. Using a weakly buffered slightly alkaline buffer containing no EDTA is preferred especially if the plasmid DNA is intended for sequencing reactions.

Analytical gel analysis

Removing and saving aliquots during the purification procedure is recommended.

If the plasmid DNA is of low yield or low in quality, the samples can be analyzed by agarose gel electrophoresis to determine at what stage of the purification procedure the problem occurred.

Procedure

Harvest Bacterial and Resuspended Cells

Choose a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approximately 8 h at 37°C with vigorous shaking (approx. 300 rpm).

Dilute the starter culture 1/500 to 1/1000 into 3 ml selective LB medium. Grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm).

Harvest the bacterial cells by centrifugation at 6000 x g for 15 min and remove as much of the supernatant as possible. Resuspend the bacterial pellet in 0.1-0.5 ml of resuspension buffer (50mM Tris-Cl, 10 mM EDTA, 100 µg/ ml RNase A, pH 8.0). The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

Cell Lysis

Add 0.25 ml of lysis buffer, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min. Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min.

Neutralization

Add 0.3 ml of neutralization buffer, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for 5 min. Precipitation is enhanced by using chilled neutralization buffer and incubating on ice. After addition of neutralization buffer, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and KDS. The lysate should be mixed thoroughly to ensure even potassium dodecyl sulphate precipitation. If the mixture still appears viscous, more mixing is required to completely neutralize the solution. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

Load Lysate on Column

Before loading the column, carefully remove the supernatant and then transfer it to a collection tube containing the column and centrifuge at 13,000 rpm for 1 minute.

Discard the flow-through liquid and remove supernatant containing plasmid DNA promptly. After centrifugation, the supernatants should be clear.

If the supernatant is not clear, a second, shorter centrifugation should be carried out to avoid applying any suspended or particulate material to the column. Suspended material (which causes the sample to appear turbid) will clog the column and reduce or eliminate flow.

Bind and Wash

Add 0.7 ml of wash buffer to the column placed in the collection tube and centrifuge for 10 minutes at 13000 rpm for 1 minute. Equilibrate by applying 1 ml equilibration buffer (750 mM NaCl, 50 Mm MOPS, ph 7.0, 15 % isopropanol) and allow the column to empty by gravity flow. Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer.

Apply the supernatant from step 6 to the column and allow it to enter the resin by gravity flow.

Plasmid Elution

Elute DNA with 0.8 ml elution buffer (1.23 M NAcL, 50 mm Tris-Cl, pH 8.5, 15 %v isopropanol) Collect the elute in a 1.5 ml or 2 ml microcentrifuge tube.

Precipitate DNA by adding 0.7 volumes (0.56 ml per 0.8 ml of elution volume) of room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥10,000 rpm for 30 min in a microcentrifuge. Carefully decant the supernatant. All solutions should be at room temperature in order to minimize salt precipitation.

Wash DNA pellet with 1 ml of 70% ethanol and centrifuge at 10,000 rpm for 10 min.

Carefully decant the supernatant without disturbing the pellet.

The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10mM Tris-Cl, pH 8.5). Redissolve the DNA pellet by rinsing the walls to recover all the DNA.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm and quantitative analysis on an agarose gel. To quantitate the nucleic acid concentration, dilute the plasmid DNA 1 : 100 or 1 : 50 (depending on the plasmid copy number) in TE buffer and measure the absorbance (optical density) at 260 nm (A260) and 280 nm (A280). Use TE buffer as the blank. This measurement permits the direct calculation of the nucleic acid concentration using the formula

$$[\text{DNA}]\ (\mu g/mL) = A260 \times \text{Dilution factor} \times 50$$

where 50 is the extinction coefficient of DNA. The ratio A260/A280 provides a reasonable estimate of the purity of the preparation.

Introduction to Phylogenetic Analysis

Irit Orr

Subjects of this lecture

1 Introducing some of the terminology of phylogenetics.

2 Introducing some of the most commonly used methods for phylogenetic analysis.

3 Explain how to construct phylogenetic trees.

- Taxonomy - is the science of classification of organisms.
- Phylogeny - is the evolution of a genetically related group of organisms.
- Or: a study of relationships between collection of "things" (genes, proteins, organs..) that are derived from a common ancestor.

Phylogenetics - WHY?

- Find evolutionary ties between organisms.

(Analyze changes occuring in different organisms during evolution).

- Find (understand) relationships between an ancestral sequence and it descendants.

(Evolution of family of sequences)

- Estimate time of divergence between a group of organisms that share a common ancestor.

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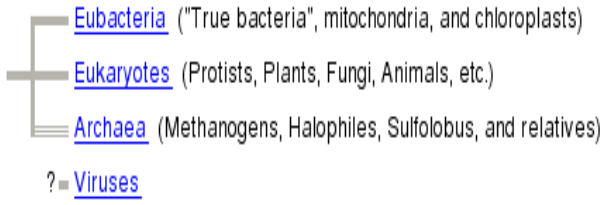
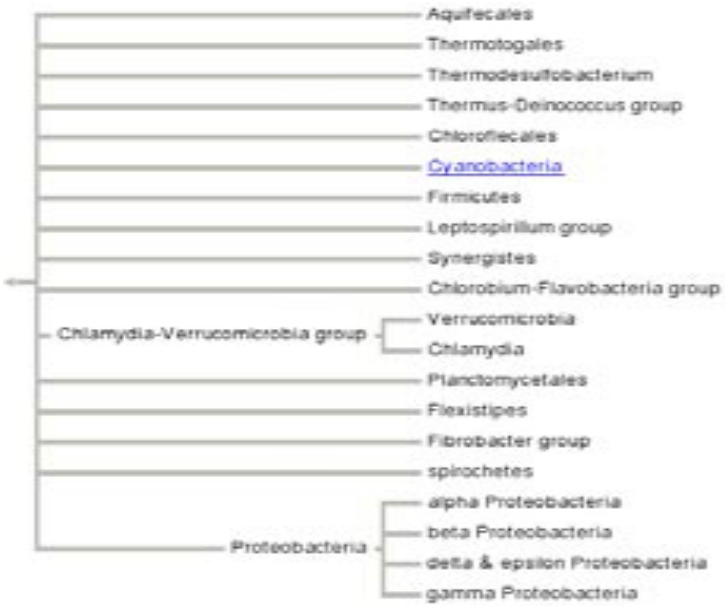


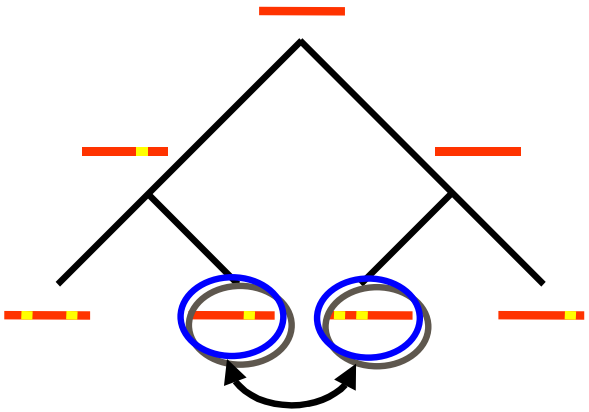
Basic tree
Of Life

Eukaryote tree



Eubacteria tree

Similar sequences, common ancestor...



... common ancestor, similar function

From a common ancestor sequence, two DNA sequences are diverged.

Each of these two sequences start to accumulate nucleotide substitutions.

The number of these mutations are used in molecular evolution analysis.

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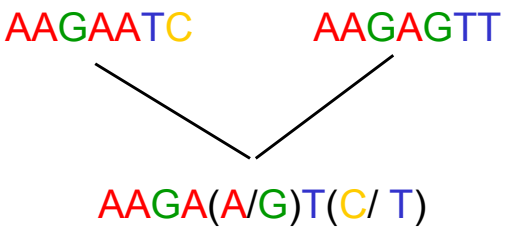
How we calculate the Degree of Divergence

- If two sequences of length N differ from each other at n sites, then their degree of divergence is:

  n/N or n/N*100%.

Relationships of Phylogenetic Analysis and Sequences Analysis

When 2 sequences found in 2 organisms are very similar, we assume that they have derived from one ancestor.



The sequences alignment reveal which positions are conserved from the ancestor sequence.

Relationships of Phylogenetic Analysis and Sequences Analysis

- The progressive multiple alignment of a group of sequences, first aligns the most similar pair.
- Then it adds the more distant pairs.
- The alignment is influenced by the "most similar" pairs and arranged accordingly, but….it does not always correctly represent the evolutionary history of the occured changes.
- Not all phylogenetic methods work this way.

Relationships of Phylogenetic Analysis and Sequences Analysis

- Most phylogenetic methods assume that each position in a sequence can change independently from the other positions.
- Gaps in alignments represent mutations in sequences such as: insertion, deletion, genetic rearrangments.
- Gaps are treated in various ways by the phylogenetic methods. Most of them ignore gaps.

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Relationships of Phylogenetic Analysis and Sequences Analysis

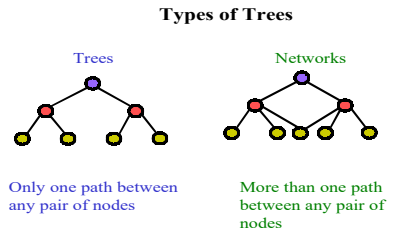
- Another approach to treat gaps is by using sequences similarity scores as the base for the phylogenetic analysis, instead of using the alignment itself, and trying to decide what happened at each position.
- The similarity scores based on scoring matrices (with gaps scores) are used by the DISTANCE methods.

What is a phylogenetic tree?

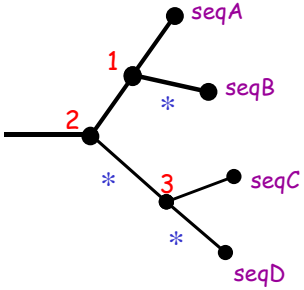
- An illustration of the evolutionary relationships among a group of organisms.
- Dendrogram is another name for a phylogenetic tree.
- A tree is composed of nodes and branches. One branch connects any two adjacent nodes. Nodes represent the taxonomic units. (sequences)

What is a phylogenetic tree?

- E.G: 2 very similar sequences will be neighbors on the outer branches and will be connected by a common internal branch.



Rooted Phylogenetic Tree



Leaves = Outer branches
Represent the taxa (sequences)

Nodes = 1 2 3
Represent the relationships
Among the taxa (sequences)
e.g Node 1 represent the ancestor
seq from which seqA and seqB derived.

Branches *
The length of the branch represent
the # of changes that occurred in the
seqs prior to the next level of separation.

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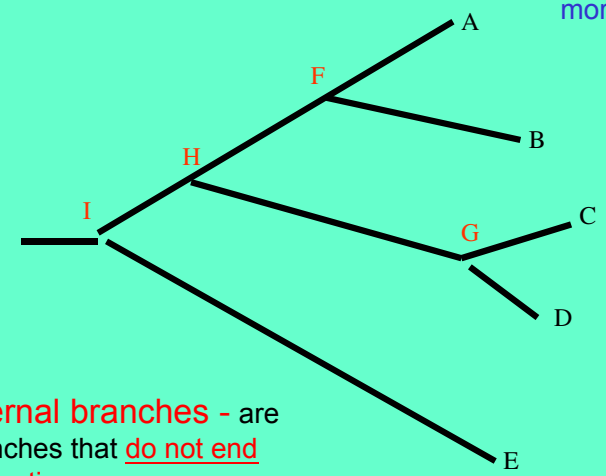
External branches - are branches that end with a tip.
(FA,FB,GC,GD,IE)
more recent diversions



Internal branches - are branches that do not end with a tip.
(IH,HF,HG)
more ancient diversions

In a phylogenetic tree...

- ✓ Each NODE represents a speciation event in evolution. Beyond this point any sequence changes that occurred are specific for each branch (specie).
- ✓ The BRANCH connects 2 NODES of the tree. The length of each BRANCH between one NODE to the next, represents the # of changes that occurred until the next separation (speciation).

In a phylogenetic tree...

- ✓ NOTE: The amount of evolutionary time that passed from the separation of the 2 sequences is not known. The phylogenetic analysis can only estimate the # of changes that occurred from the time of separation.
- ✓ After the branching event, one taxon (sequence) can undergo more mutations then the other taxon.
- ✓ Topology of a tree is the branching pattern of a tree.

Tree structure

- ♠ Terminal nodes - represent the data (e.g sequences) under comparison (A,B,C,D,E), also known as OTUs, (Operational Taxonomic Units).
- ♠ Internal nodes - represent inferred ancestral units (usually without empirical data), also known as HTUs, (Hypothetical Taxonomic Units).

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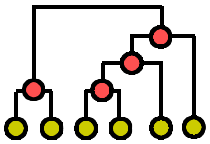
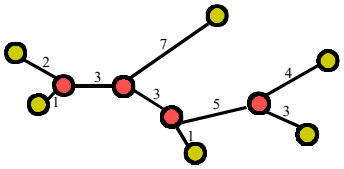
Slide taken from Dr. Itai Yanai

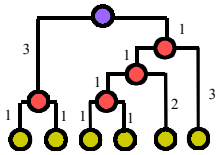
Different kinds of trees can be used to depict different aspects of evolutionary history



1. Cladogram:
   simply shows relative recency of common ancestry



2. Additive trees:
   a cladogram with branch lengths, also called phylograms and metric trees



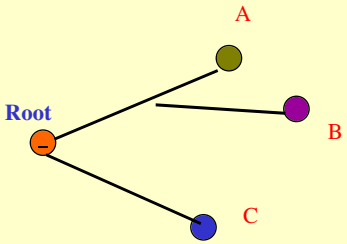
3. Ultrametric trees:
   (dendograms) special kind of additive tree in which the tips of the trees are all equidistant from the root

The Molecular Clock Hypothesis

- All the mutations occur in the same rate in all the tree branches.
- The rate of the mutations is the same for all positions along the sequence.
- The Molecular Clock Hypothesis is most suitable for closely related species.

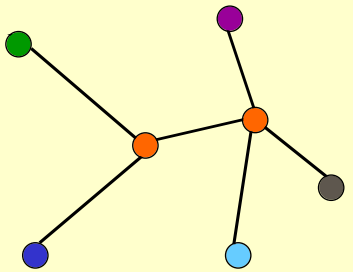
Rooted Tree = Cladogram

- A phylogenetic tree that all the "objects" on it share a known common ancestor (the root).
- There exists a particular root node.
- The paths from the root to the nodes correspond to evolutionary time.



Unrooted Tree = Phenogram

A phylogenetic tree where all the "objects" on it are related descendants - but there is not enough information to specify the common ancestor (root).



- The path between nodes of the tree do not specify an evolutionary time.

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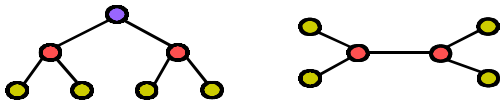
Types of Trees

Rooted vs. Unrooted



		Branches	Nodes
Rooted	Interior	M – 2	M – 1
	Total	2M – 2	2M – 1
Unrooted	Interior	M – 3	M – 2
	Total	2M – 3	2M – 2

- M is the number of OTU's

Rooted versus Unrooted

- The number of tree topologies of rooted tree is much higher than that of the unrooted tree for the same number of OTUs.
- Therefore, the error of the unrooted tree topology is smaller than that of the rooted tree.

Slide taken from Dr. Itai Yanai

The number of rooted and unrooted trees:

Number of OTU's	Possible Number of	
	Rooted trees	Unrooted trees
2	1	1
3	3	1
4	15	3
5	105	15
6	945	105
7	10395	945
8	135135	10395
9	2027025	135135
10	34459425	2027025

OTU – Operational Taxonomical Unit

- Orthologs - genes related by speciation events. Meaning same genes in different species.
- Paralogs - genes related by duplication events. Meaning duplicated genes in the same species.

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Selecting sequences for phylogenetic analysis

What *type* of sequence to use, Protein or DNA?

The rate of mutation is assumed to be the same in both coding and non-coding regions.

However, there is a difference in the substitution rate.

Selecting sequences for phylogenetic analysis

- Non-coding DNA regions have more substitution than coding regions.
- Proteins are much more conserved since they "need" to conserve their function.

So it is better to use sequences that mutate slowly (proteins) than DNA. However, if the genes are very small, or they mutate slowly, we can use them for building the trees.

Known Problems of Multiple Alignments

- Important sites could be misaligned by the software used for the sequence alignment. That will effect the significance of the site - and the tree.
  - For example: ATG as start codon, or specific amino acids in functional domains.
- Gaps - Are treated differently by different alignment programs and should play no part in building trees.

Alignment of a coding region should be compared with the alignment of their protein sequences, to be sure about the placement of gaps.

```
T  Y  R  R  S  R          ACA  TAC  AGG  CGA
                             T    Y    R    R
T  Y  R  R  S  R          ACA  TAC  AGG  CGA
                             T    Y    R    R
T  Y  R  -  S  R          ACA  TAC  AGG  ---
                             T    Y    R    -
T  Y  R  -  S  R          ACA  TAC  ---  CGA
                             T    Y    -    R
T  Y  R  R  S  R          ACA  TAC  AGG  CGA
                             T    Y    R    R
```

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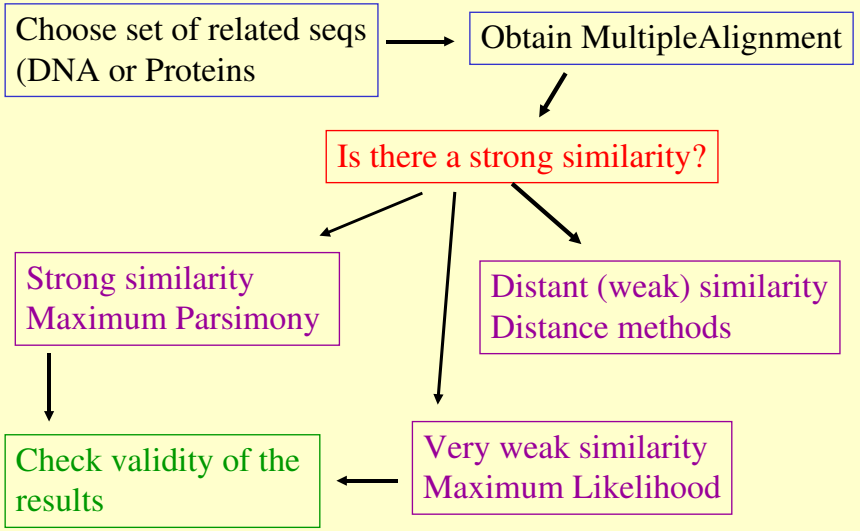
Known Problems of Multiple Alignments

- ♥ Low complexity regions - effect the multiple alignment because they create random bias for various regions of the alignment.
- ♥ Low complexity regions should be removed from the alignment before building the tree.
- ✳ If you delete these regions you need to consider the affect of the deletions on the branch lengths of the whole tree.

Selecting sequences for phylogenetic analysis

- ‡ Sequences that are being compared belong together (orthologs).
- ‡ If no ancestral sequence is available you may use an "outgroup" as a reference to measure distances. In such a case, for an outgroup you need to choose a close relative to the group being compared.
  - ‡ For example: if the group is of mammalian sequences then the outgroup should be a sequence from birds and not plants.

How to choose a phylogenetic method?

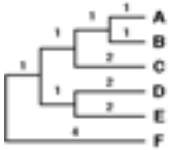


Taken from Dr.Itai Yanai

Given a multiple alignment, how do we construct the tree?

```
A - GCTTGTCCGTTACGAT
B - ACTTGTCTGTTACGAT
C - ACTTGTCCGAAACGAT
D - ACTTGACCGTTTCCTT
E - AGATGACCGTTTCGAT
F - ACTACACCCTTATGAG
```

?



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Building Phylogenetic Trees

Main methods:

- Distances matrix methods
  - Neighbour Joining, UPGMA
- Character based methods:
  - Parsimony methods
  - Maximum Likelihood method
- Validation method:
  - Bootstrapping
  - Jack Knife

Statistical Methods

✓ Bootstrapping Analysis –

Is a method for testing how good a dataset fits a evolutionary model.

This method can check the branch arrangement (topology) of a phylogenetic tree.

In Bootstrapping, the program re-samples columns in a multiple aligned group of sequences, and creates many new alignments, (with replacement the original dataset).

These new sets represent the population.

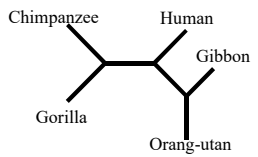
Statistical Methods

- The process is done at least 100 times.
- Phylogenetic trees are generated from all the sets.
- Part of the results will show the # of times a particular branch point occurred out of all the trees that were built.

The higher the # - the more valid the branching point.

Taken from Dr. Itai Yanai

Given the following tree, estimate the confidence of the two internal branches



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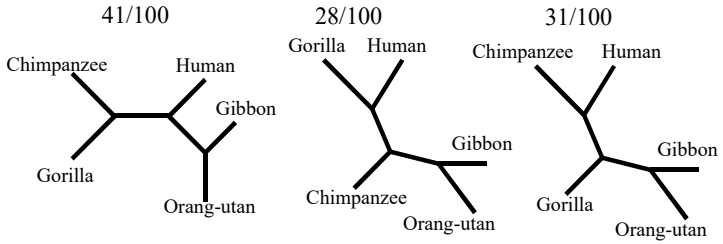
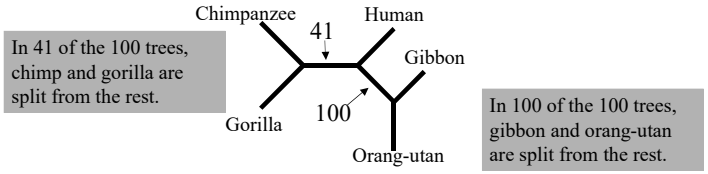
Taken from Dr. Itai Yanai

Estimating Confidence from the Resamplings

1. Of the 100 trees:



2. Upon the original tree we superimpose bootstrap values:



Statistical Methods

- Bootstrap values between 90-100 are considered statistically significant

Character Based Methods

All Character Based Methods assume that each character substitution is independent of its neighbors.

- Maximum Parsimony (minimum evolution) - in this method one tree will be given (built) with the fewest changes required to explain (tree) the differences observed in the data.

Character Based Methods

Q: How do you find the minimum # of changes needed to explain the data in a given tree?

A: The answer will be to construct a set of possible ways to get from one set to the other, and choose the "best". (for example: Maximum Parsimony)

```
CCGCCACGA
  P   P   R
CGGCCACGA
  R   P   R
```

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Character Based Methods - Maximum Parsimony

- Not all sites are informative in parsimony.
- Informative site, is a site that has at least 2 characters, each appearing at least in 2 of the sequences of the dataset.

Maximum Parsimony

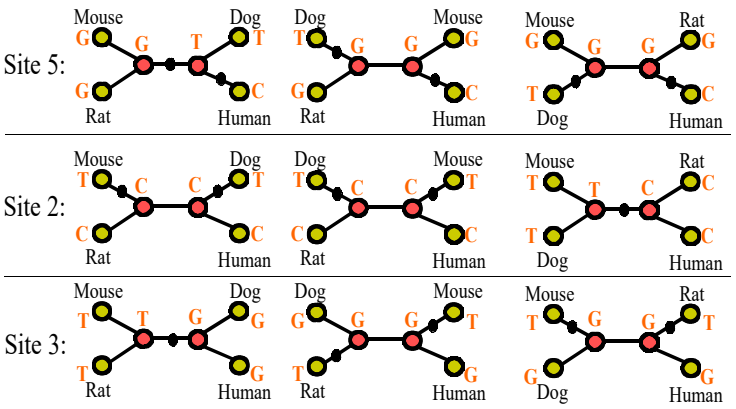
Start by classifying the sites:

```
                  12345678901234567890
Mouse             CTTCGTTGGATCAGTTTGATA
Rat               CCTCGTTGGATCATTTTGATA
Dog               CTGCTTTGGATCAGTTTGAAC
Human             CCGCCTTGGATCAGTTTGAAC
-------------------------------------
Invariant     *   * ******** *****
Variant        ** *         *     **
-------------------------------------
Informative   **                  **
Non-inform.      *         *
```

Taken from Dr. Itai Yanai

```
                  12345678901234567890
Mouse             CTTCGTTGGATCAGTTTGATA
Rat               CCTCGTTGGATCATTTTGATA
Dog               CTGCTTTGGATCAGTTTGAAC
Human             CCGCCTTGGATCAGTTTGAAC
                   ** *
```

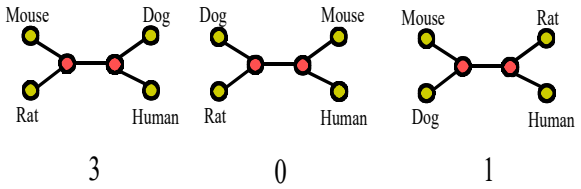
Taken from Dr. Itai Yanai



Maximum Parsimony

```
                  12345678901234567890
Mouse             CTTCGTTGGATCAGTTTGATA
Rat               CCTCGTTGGATCATTTTGATA
Dog               CTGCTTTGGATCAGTTTGAAC
Human             CCGCCTTGGATCAGTTTGAAC
Informative    **                  **
```

Taken from Dr. Itai Yanai



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Character Based Methods - Maximum Parsimony

∞ The Maximum Parsimony method is good for similar sequences, a sequences group with small amount of variations

Maximum Parsimony methods do not give the branch lengths only the branch order.

For larger set it is recommended to use the "branch and bound" method instead Of Maximum Parsimony.

Maximum Parsimony Methods are Available…

➢ For DNA in Programs:

paup, molphy,phylo_win

In the Phylip package:

DNAPars, DNAPenny, etc..

➢ For Protein in Programs:

paup, molphy,phylo_win

In the Phylip package:

PROTPars

Character Based Methods - Maximum Likelihood

- Basic idea of Maximum Likelihood method is building a tree based on mathemaical model.
- This method find a tree based on probability calculations that best accounts for the large amount of variations of the data (sequences) set.
- Maximum Likelihood method (like the Maximum Parsimony method) performs its analysis on each position of the multiple alignment.

  This is why this method is very heavy on CPU.

Character Based Methods - Maximum Likelihood

- Maximum Likelihood method – using a tree model for nucleotide substitutions, it will try to find the most likely tree (out of all the trees of the given dataset).
- The Maximum Likelihood methods are very slow and cpu consuming.
- Maximum Likelihood methods can be found in phylip, paup or puzzle.

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Maximum Likelihood method

- Are available in the Programs:

paup or puzzle

In phylip package in programs:

DNAML and DNAMLK

Character Based Methods

- The Maximum Likelihood methods are very slow and cpu consuming (computer expensive).
- Maximum Likelihood methods can be found in phylip, paup or puzzle.

Distances Matrix Methods

- Distance methods assume a molecular clock, meaning that all mutations are neutral and therefore they happen at a random clocklike rate.
- This assumption is not true for several reasons:
  - Different environmental conditions affect mutation rates.
  - This assumption ignores selection issues which are different with different time periods.

Distances Matrix Methods

- Distance - the number of substitutions per site per time period.
- Evolutionary distance are calculated based on one of DNA evolutionary models.
- Neighbors – pairs of sequences that have the smallest number of substitutions between them.
- On a phylogenetic tree, neighbors are joined by a node (common ancestor).

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Distances Matrix Methods

- Distance methods vary in the way they construct the trees.
- Distance methods try to place the correct positions of all the neighbors, and find the correct branches lengths.
- *Distance based clustering methods*:
  - Neighbor-Joining (unrooted tree)
  - UPGMA (rooted tree)

Distance method steps

1. Multiple alignments - based on all against all pairwise comparisons.
2. Building distance matrix of all the compared sequences (all pair of OTUs).
3. Disregard of the actual sequences.
4. Constructing a guide tree by clustering the distances. Iteratively build the relations (branches and internal nodes) between all OTUs.

Distance method steps

Construction of a distance tree using clustering with the Unweighted Pair Group Method with Arithmatic Mean (UPGMA)

First, construct a distance matrix:

```
A - GCTTGTCCGTTACGAT
B - ACTTGTCTGTTACGAT
C - ACTTGTCCGAAACGAT
D - ACTTGACCGTTTCCTT
E - AGATGACCGTTTCGAT
F - ACTACACCCTTATGAG
```

→

	A	B	C	D	E
B	2				
C	4	4			
D	6	6	6		
E	6	6	6	4	
F	8	8	8	8	8

From http://www.icp.ucl.ac.be/~opperd/private/upgma.html

Distances Matrix Methods

- Distances matrix methods can be found in the following Programs:

  Clustalw, Phylo_win, Paup

  In the GCG software package:

  Paupsearch, distances

  In the Phylip package:

  DNADist, PROTDist, Fitch, Kitch, Neighbor

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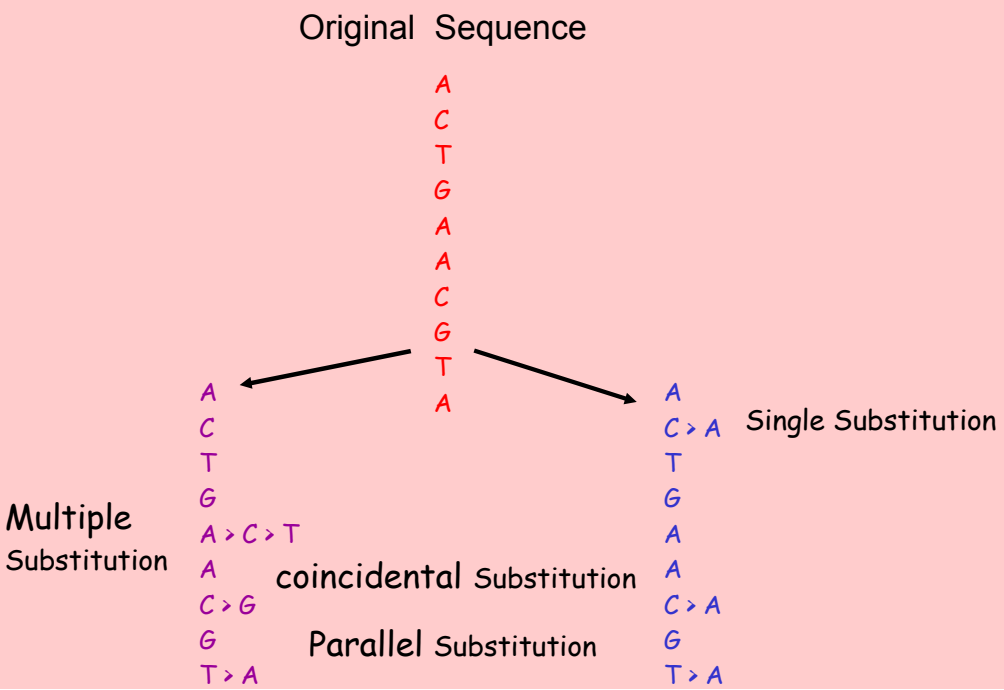
Mutations as data source for evolutionary analysis

- Mutation - an error in DNA replication or DNA repair.
- Only mutations that occur in germline cells play a rule in evolution. However, in some organisms there is no distinction between germline or somatic mutation.
- Only mutations that were fixed in the population are called substitutions.

Correction of Distances between DNA sequences

- In order to detect changes in DNA sequences we compare them to each other.
- We assume that each observed change in similar sequences, represent a "single mutation event".
- The greater the number of changes, the more possible types of mutations.



Mutations - Substitutions

Q: What do we measure by sequence alignment?

A: Substitutions in the aligned sequences.

- The rate of substitution in regions that evolve under no constraints are assumed to be equal to the mutation rate.

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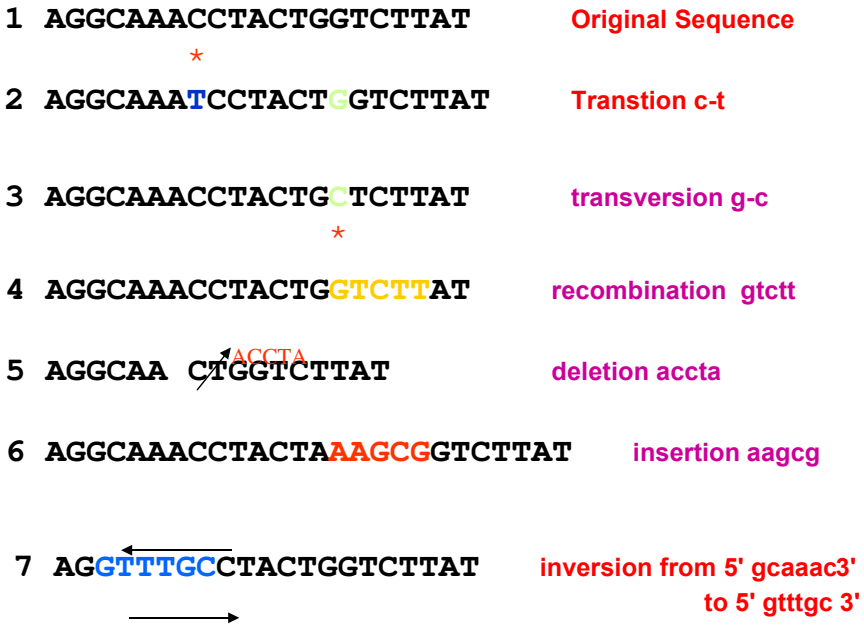
Mutations - Substitutions

- Point mutation - mutation in a single nucleotide.
- Segmental mutation - mutation in several adjacent nucleotides.
- Substitution mutation - replacement of one nucleotide with another.
- Recombination - exchange of a sequence with another.

Mutations

- ♣Deletion - removal of one or more nucleotides from the DNA.
- ♣Insertion - addition of one or more nucleotides to the DNA.
- ♣Inversion - rotation by 180 of a double-stranded DNA segment comprising 2 or more base-pairs.



Substitution Mutations

- Transition - a change between purines (A,G) or between pyrimidines (T,C).
- Transversion - a change between purines (A,G) to pyrimidines (T,C).
- *Substitution mutations usually arise from mispairing of bases during replication.*

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Let's assume that..

A mutation of a sense codon to another sense codon, occur in equal frequency for all the codons.

If so we can now compute the expected proportion of different types of substitution mutations from the genetic code.

Where do the mutations occur?

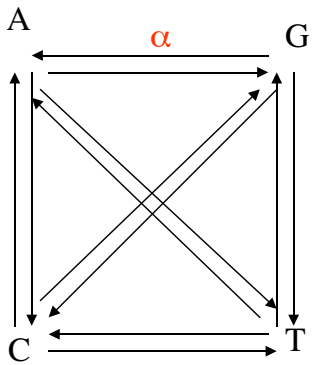
- Synonymous substitutions mostly occur at the *3rd* position of the codon.
- Nonsynonymous substitutions mostly occur at the *2nd* position of the codon.
- Any substitution in the *2nd* position is nonsynonymous

Correction of Distances between DNA sequences

- There are several evolutionary models used to correct for the likelihood of multiple mutations and reversions in DNA sequences.
- These evolutionary models use a normalized distance measurement that is the average degree of change per length of aligned sequences.

Jukes & Cantor one-parameter model

This model assumes that substitutions between the 4 bases occur with equal frequency. Meaning no bias in the direction of the change.



α Is the rate of substitutions In each of the 3 directions For one base.

α (Is the one parameter).

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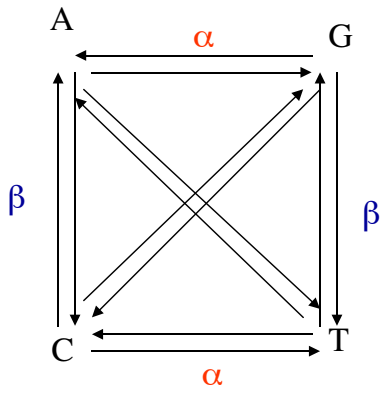
Kimura two-parameter model

This model assumes that transitions (A - G or T - C) occur more often than transversions (purine -pyrimidine).



α Is the rate of transitional Substitutions.

β Is the rate of transversional substitutions.

- These evolutionary models improve the distance calculations between the sequences.
- These evolutionary models have less effect in phylogenetic predictions of closely related sequences.
- These evolutionary models have better effect with distant related sequences.

DNA Evolution Models

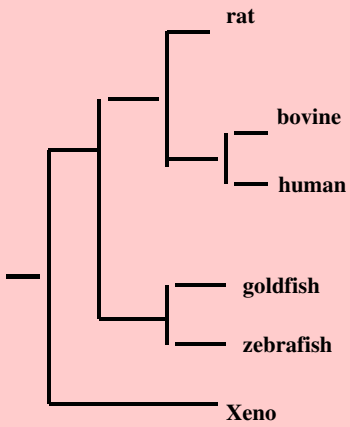
① A basic process in DNA sequence evolution is the substitution of one nucleotide with another.

① This process is slow and can not be observed directly.

① The study of DNA changes is used to estimate the rate of evolution, and the evolution history of organisms.

How to read the tree?

Start at the base and follow The progression of the branch points (nodes)



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How to draw Trees? (Building trees software)

* Unrooted trees should be plotted using the DRAWGRAM program (phylip), or similar.

* Rooted trees should be plotted using the DRAWTREE program (phylip), or similar.

* On a PC use the TreeView program

A Tip...

- For DNA sequences use the Kimura's model in the building trees programs.

- For PROTEINS the differences lie with the scoring (substitution) matrices used. For more distant sequences you should use BLOSUM with lower # (i.e., for distant proteins use blosum45 and for similar proteins use blosum60).

Known problems of Phylogenetic Analysis

- Order of the input data (sequences) -
  The order of the input sequences effects the tree construction. You can "correct" this effect in some of the programs (like phylip), using the Jumble option. (J in phylip set to 10).

- The number of possible trees is huge for large datasets. Often it is not possible to construct all trees, but can guarantee only "a good" tree not the "best tree".

Known problems of Phylogenetic Analysis

- The definition of "best tree" is ambiguous.
  It might mean the most likely tree, or a tree with the fewest changes, or a tree best fit to a known model, etc..

The trees that result from various methods differ from each other. Never the less, in order to compare trees, one need to assume some evolutionary model so that the trees may be tested.

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Known problems of Phylogenetic Analysis

- The amount of data used for the tree construction is not always "informative". For example, if we compare proteins that are 100% similar in a site, we do not have information to infer to phylogenetic relationships between these proteins.
- Population effects are often to be considered, especially if we have a lot of variety (large # of alleles for one protein).

How many trees to build?

- For each dataset it is recommended to build more than one tree. Build a tree using a distance method and if possible also use a character-based method, like maximum parsimony.
- The core of the tree should be similar in both methods, otherwise you may suspect that your tree is incorrect.

Regards : Zarva Chaudhary & Chaudhary Moazzam