Essentials of Genetics

POLYMERASE CHAIN REACTION

Polymerase Chain Reaction

PCR

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

Polymerase Chain Reaction

PCR

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

Polymerase Chain Reaction

PCR - A CHAIN REACTION

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

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.

Polymerase Chain Reaction

CONCLUSION

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

Essentials of Genetics

STEPS OF PCR

Steps of PCR

THREE STEPS

- Denaturation
- Annealing
- Extension

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.

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.

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.

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.

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.

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.

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.

Essentials of Genetics

INGREDIENTS OF PCR

Ingredients of PCR

MAJOR INGREDIENTS

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

Ingredients of PCR

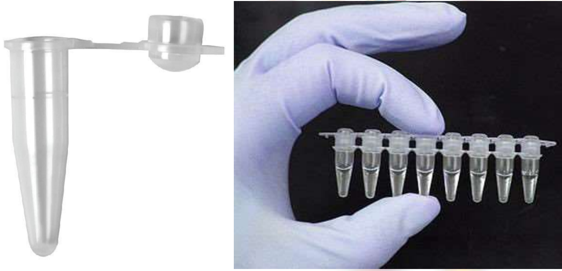
MAJOR INGREDIENTS

- Distilled water
- Deoxynucleotide triphosphates
- DNA polymerase

Ingredients of PCR

MICROFUGE TUBES

- These are small cylindrical plastic tubes with conical bottoms.



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.

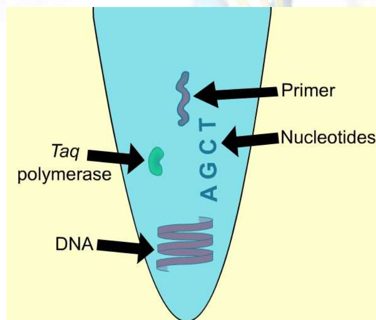
Ingredients of PCR

DNA POLYMERASES

- Polymerases
  - Taq polymerase
  - Pfu polymerase
  - Vent polymerase

Ingredients of PCR

INGREDIENTS IN PCR TUBE



Ingredients of PCR

TEMPLATE CAN BE DNA OR RNA

- Template DNA
- RNA in case of reverse transcriptase

Essentials of Genetics

PRIMERS FOR PCR

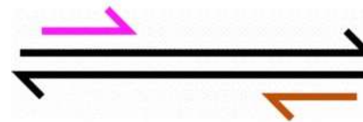
PRIMERS FOR PCR

PRIMERS

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

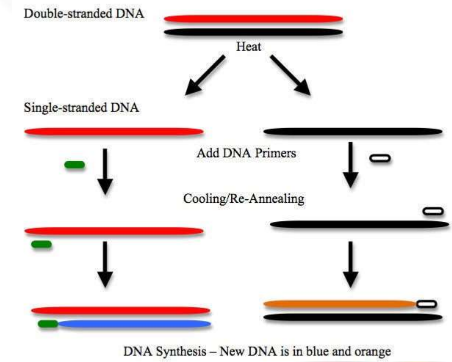
PRIMERS FOR PCR

PRIMERS



PRIMERS FOR PCR

PRIMERS



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.

PRIMERS FOR PCR

FEATURES OF PRIMERS

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

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

PRIMERS FOR PCR

FORMULA FOR CALCULATING MELTING TEMPERATURE OF PRIMERS

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

Essentials of Genetics

DNA POLYMERASES

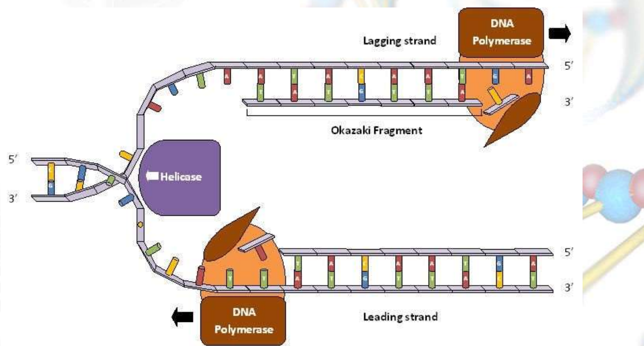
DNA Polymerases

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*

DNA Polymerases

POLYMERASES



DNA Polymerases

POLYMERASES

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

DNA Polymerases

DNA POLYMERASES

- Polymerases
  - Taq polymerase
  - Pfu polymerase
  - Vent polymerase

Essentials of Genetics

STANDARD PCR REACTION

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

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

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

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.

Essentials of Genetics

INTERPRETATION OF RESULTS

Interpretation of Results

INTERPRETATION

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

Interpretation of Results

PRIMERS DIMERS MAY REDUCE AMPLIFICATION

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

Interpretation of Results

BLANK REACTION

- Controls for contamination

  contains all reagents except DNA template.

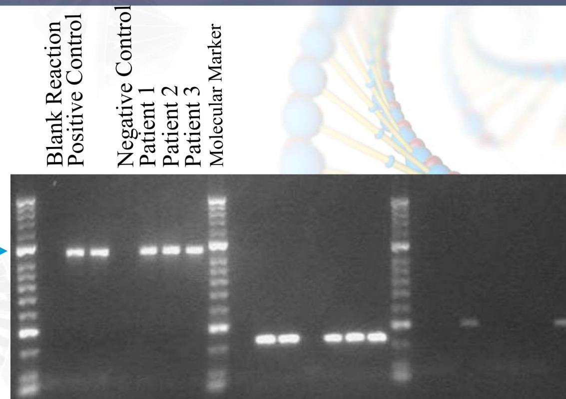
Interpretation of Results

NEGATIVE CONTROL

- Controls for specificity of the amplification reaction

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

Interpretation of Results



Interpretation of Results

CONCLUSION

- The PCR product should be of the expected size.

Essentials of Genetics

TYPES OF PCR

Types of PCR

TYPES

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

Types of PCR

TYPES

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

Types of PCR

TYPES

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

Essentials of Genetics

NESTED PCR

Nested PCR

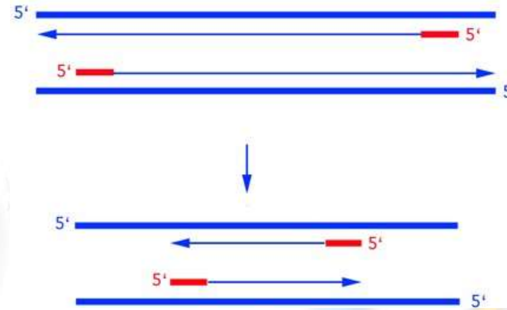
NESTED PCR

- 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.

NESTED PCR – SECOND PAIR OF PRIMERS

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

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.

SECOND PCR PRODUCT IS SPECIFIC

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

ADVANTAGE OF NESTED PCR

- Very low probability of nonspecific amplification.

MULTIPLEX PCR

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.

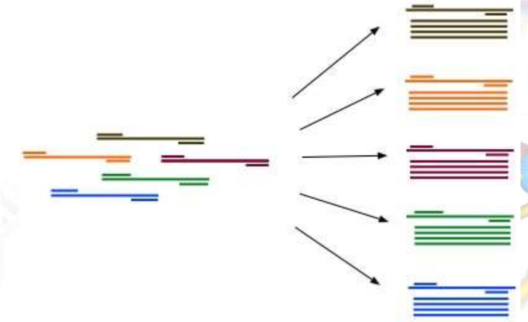
Multiplex PCR

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.

Multiplex PCR

MULTIPLEX PCR



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.

Multiplex PCR

ANNEALING TEMPERATURES BE OPTIMIZED

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

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.

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.

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.

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.

Essentials of Genetics

REVERSE TRANSCRIPTASE PCR

Reverse Transcriptase PCR

REVERSE TRANSCRIPTASE PCR

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

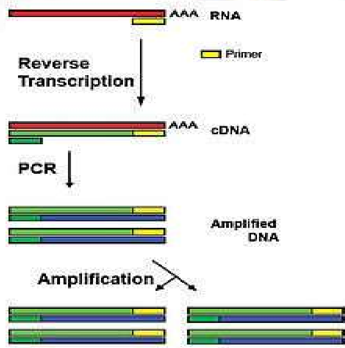
Reverse Transcriptase PCR

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 .

Reverse Transcriptase PCR

REVERSE TRANSCRIPTASE PCR



Reverse Transcriptase PCR

ENZYME REVERSE TRANSCRIPTASE

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

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.

Essentials of Genetics

REAL TIME PCR

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.

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.

Real Time PCR

REAL TIME PCR

Real Time PCR

REAL TIME PCR

- Quantitative real-time PCR is often confusingly known as RT-PCR (Real Time PCR) or RQPCR.
- 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.

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.

Essentials of Genetics

HOT START PCR

Hot Start PCR

HOT START PCR

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

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.

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 .

Essentials of Genetics

ASYMMETRIC PCR

Asymmetric PCR

ASYMMETRIC PCR

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

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.

Asymmetric PCR

ASYMMETRIC PCR

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

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.

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.

Essentials of Genetics

LONG PCR

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.

LONG PCR

MIXTURES OF POLYMERASES

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

LONG PCR

LONG PCR

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

Essentials of Genetics

ALLELE SPECIFIC PCR

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.

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.

Allele Specific PCR

AMPLIFICATION WITH SNP SPECIFIC PRIMER

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

Allele Specific PCR

ALLELE SPECIFIC PCR

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

Essentials of Genetics

COLONY PCR

Colony PCR

COLONY PCR

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

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.

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.

Colony PCR

COLONY PCR

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

Essentials of Genetics

IN SITU PCR

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 .

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.

Essentials of Genetics

INVERSE PCR

Inverse PCR

INVERSE PCR

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

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.

Inverse PCR

IDENTIFICATION OF SEQUENCES FLANKING TRANSPOSABLE ELEMENTS

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

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.

Essentials of Genetics

AFLP PCR

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.

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.

Essentials of Genetics

ASSEMBLY PCR

Assembly PCR

ASSEMBLY PCR

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

Assembly PCR

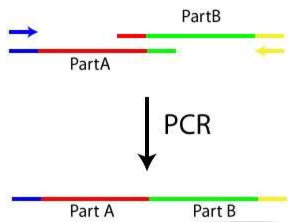
ASSEMBLY PCR - PRINCIPLE

- 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.

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.



Assembly PCR

ASSEMBLY PCR

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

Essentials of Genetics

SUICIDE PCR

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.

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.

Suicide PCR

SUICIDE PCR

Use of any primers combination only once in a PCR.

Essentials of Genetics

METHYLATION SPECIFIC PCR

Methylation Specific PCR

METHYLATION SPECIFIC PCR

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

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).

Methylation Specific PCR

MSP

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

Essentials of Genetics

INTER SEQUENCE SPECIFIC PCR

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.

InterSequence Specific PCR

PCR

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

Essentials of Genetics

LIGATION

MEDIATED PCR

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.

Ligation-mediated PCR

PCR

- DNA sequencing
- Genome walking
- DNA footprinting

Essentials of Genetics

WHOLE GENOME AMPLIFICATION PCR

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.

Whole Genome Amplification PCR

PCR

- Whole genomes can be amplified by WGA.

Essentials of Genetics

MINI-PRIMER PCR

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.

Mini Primer PCR

MINI PRIMER PCR

- PCR that can extend from short primers.

Essentials of Genetics

ADVANTAGES AND LIMITATIONS OF PCR

Advantages & Limitations

ADVANTAGES

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

Advantages & Limitations

LIMITATIONS

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

Essentials of Genetics

APPLICATIONS OF PCR

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

Applications of PCR

APPLICATIONS

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

Applications of PCR

APPLICATIONS

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

Applications of PCR

APPLICATIONS

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

Applications of PCR

CLINICAL MICROBIOLY

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

Applications of PCR

CLINICAL MICROBIOLY

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

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.

Polymerase chain reaction (PCR)

PCR-Gene cloning and expression

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

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

Polymerase chain reaction (PCR)

PCR-Forensic sciences

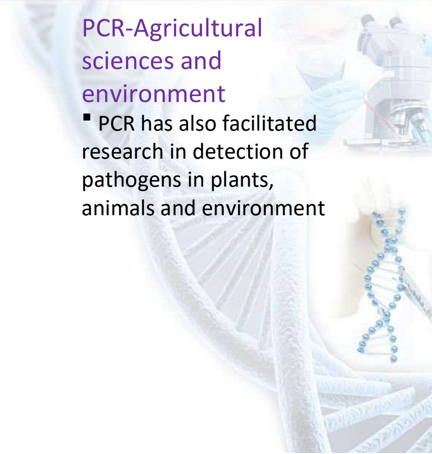
- Forensic science is the application of scientific procedures to solve criminal and legal matters
- Molecular methods are used to established the filiations of a person or to obtain evidence from minimal samples of saliva, semen or other tissues

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

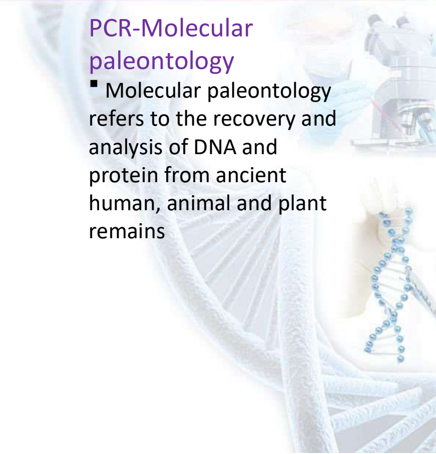
Polymerase chain reaction (PCR)



PCR-Agricultural sciences and environment

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

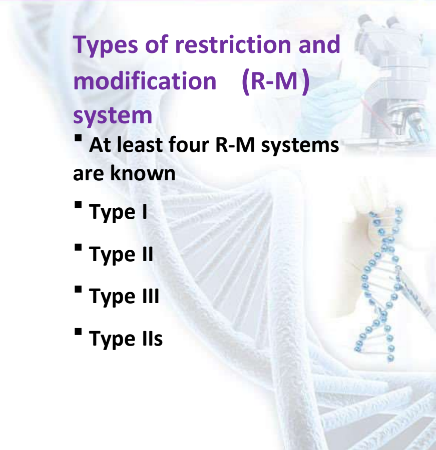
Polymerase chain reaction (PCR)



PCR-Molecular paleontology

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

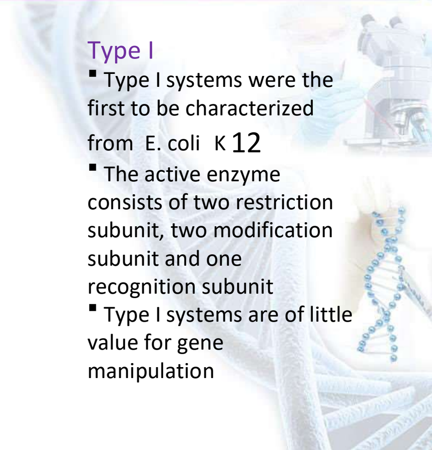
Cutting DNA molecules



Types of restriction and modification (R-M) system

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

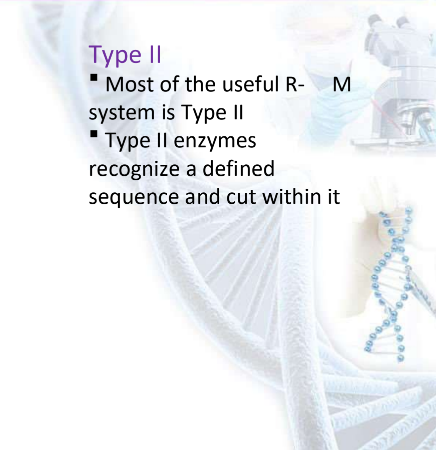
Cutting DNA molecules



Type I

- Type I systems were the first to be characterized from E. coli K 12
- 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

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

Cutting DNA molecules

Type III

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

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

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

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

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

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

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

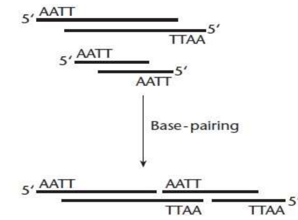
5′-GAATTC -3′
5′-CTTAAG -3′

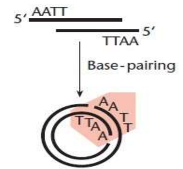
5′-G A A T T C-3′
3′-C T T A A G-5′

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 EcoR1





Cohesive fragments of DNA produced by digestion with EcoR1

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

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

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

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

Joining DNA molecules

DNA modifying enzymes

- Nucleases
- DNA Polymerase
- Reverse transcriptases
- DNA ligases

Joining DNA molecules

Nucleases

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

Joining DNA molecules

DNA Polymerase

- An enzyme that catalyzes template-dependent synthesis of DNA

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

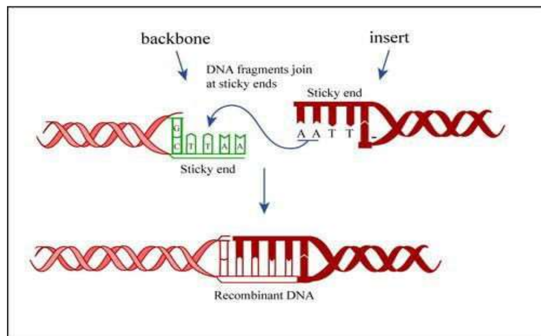
Joining DNA molecules

Methods of joining DNA fragments

- Mainly three methods are used for joining DNA in vitro

i). Joining covalently annealed cohesive ends by DNA ligase

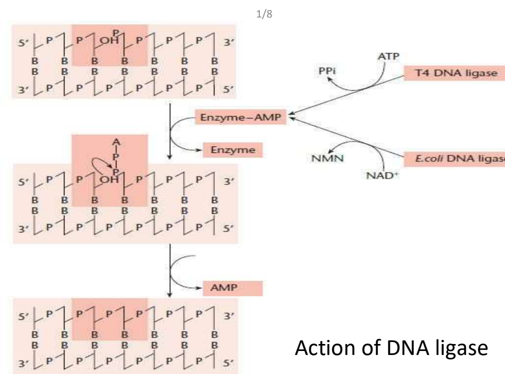
ii). Joining blunt-ended fragments by DNA ligase from phage T4 infected E. coli



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



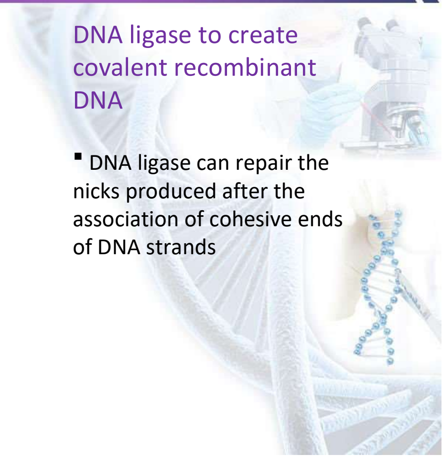
Action of DNA ligase

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

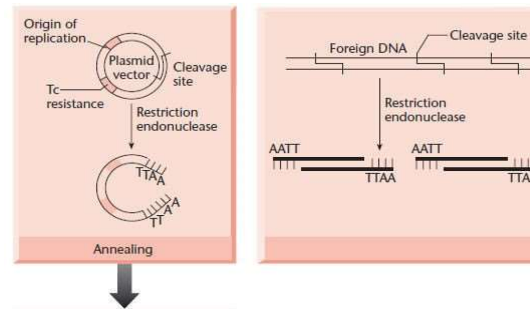
Joining DNA molecules

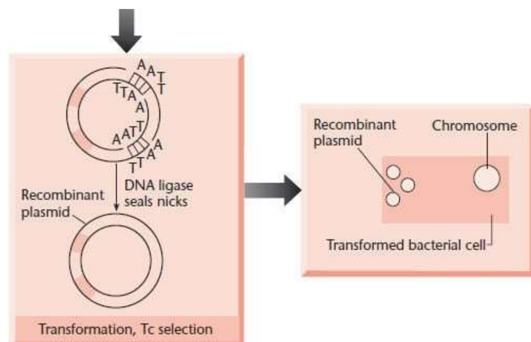


DNA ligase to create covalent recombinant DNA

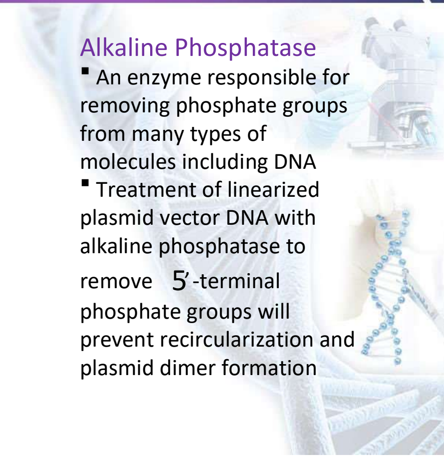
- DNA ligase can repair the nicks produced after the association of cohesive ends of DNA strands

Use of DNA ligase to create a covalent DNA recombinant





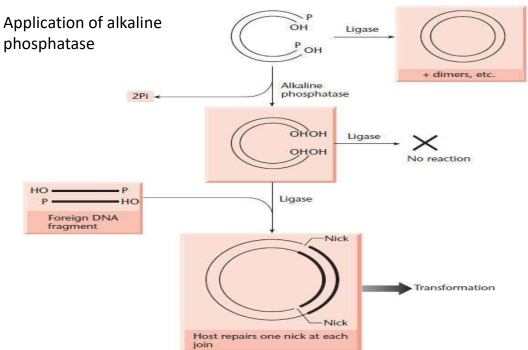
Joining DNA molecules



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

Application of alkaline phosphatase

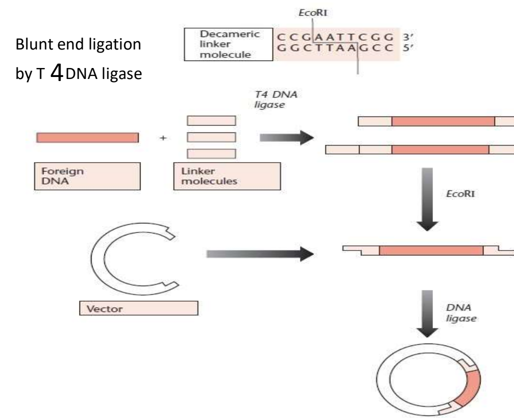


Joining DNA molecules

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

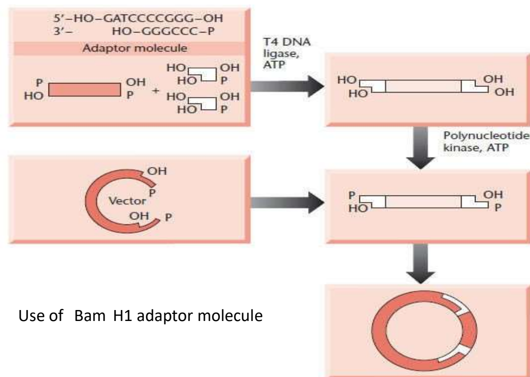
Blunt end ligation by T 4 DNA ligase



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



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

Essentials of Genetics

SOUTHERN BLOTTING

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.

Southern Blotting

SOUTHERN BLOTTING

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

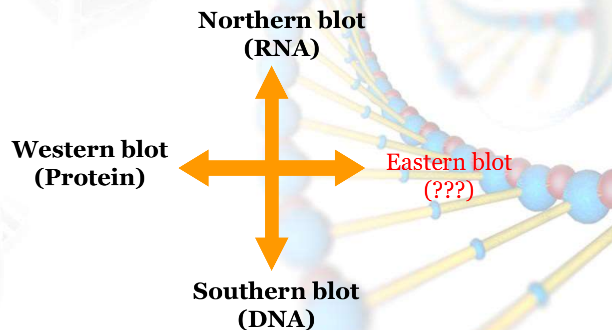
Southern Blotting

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



Southern Blotting

SOUTHERN BLOTTING

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

Essentials of Genetics

SOUTHERN BLOTTING PROCEDURE

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.

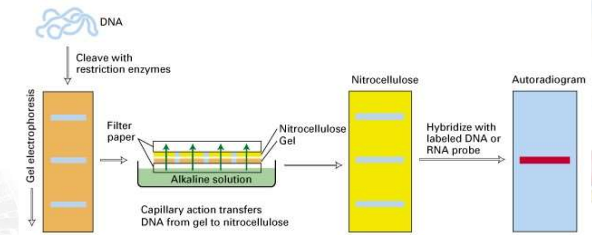
Southern Blotting - Procedure

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



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.

Essentials of Genetics

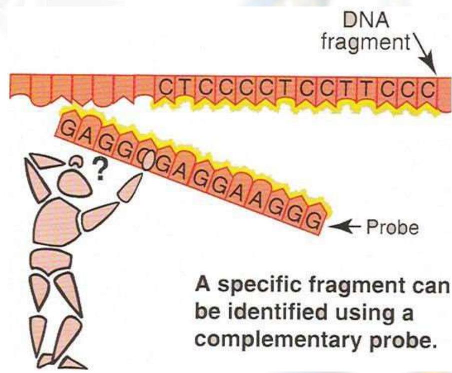
SOUTHERN BLOTTING PROBES

Southern Blotting - Probes

PROBES

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

Southern Blotting - Probes



Southern Blotting - Probes

RADIOACTIVE PROBE - P32

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

Southern Blotting - Probes

NON-RADIOACTIVE PROBE - BIOTIN

- Sensitive
- Relatively expensive

Southern Blotting - Probes

HYBRIDIZATION OF PROBES

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

Southern Blotting - Probes

PROBES

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

Essentials of Genetics

TRANSFER METHODS IN SOUTHERN BLOTTING

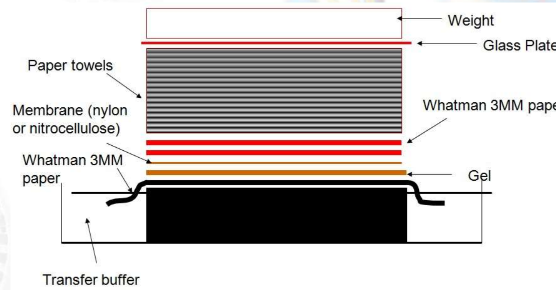
Transfer Methods - Southern

TRANSFER METHODS OF DNA TO MEMBRANE

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

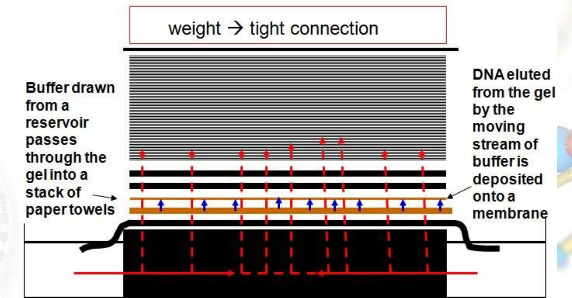
Transfer Methods - Southern

UPWARD TRANSFER



Transfer Methods - Southern

UPWARD TRANSFER OF DNA TO MEMBRANE



Transfer Methods - Southern

TRANSFER METHODS

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

Essentials of Genetics

MUTATIONS IDENTIFICATION BY SOUTHERN BLOTTING

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.

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).

Mutations Identification

MUTATION IDENTIFICATION

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

Mutations Identification

MUTATION IDENTIFICATION

- Mutations can be identified by Southern blotting.

Essentials of Genetics

NORTHERN BLOTTING

Northern Blotting

NORTHERN BLOTTING

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

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.

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.

Northern Blotting

NORTHERN BLOTTING

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

Essentials of Genetics

NORTHERN BLOT APPLICATIONS

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.

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.

Northern Blot - Applications

APPLICATIONS

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

Essentials of Genetics

WESTERN BLOTTING

Western Blotting

WESTERN BLOTTING

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

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.

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.

Western Blotting

WESTERN BLOTTING

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

Essentials of Genetics

WESTERN BLOTTING PROCEDURE

Western Blotting - Procedure

PROCEDURE

1- Sample preparation

2- Gel Electrophoresis

3- Blotting (or transfer)

4- Blocking

5- Antibody probing

6- Detection

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.

Western Blotting - Procedure

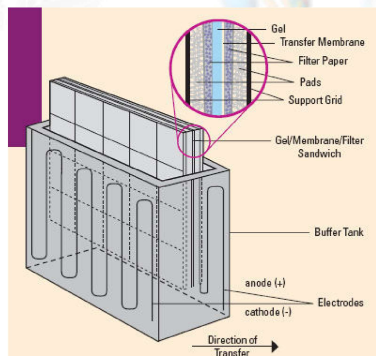
PROCEDURE

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.

Western Blotting - Procedure

ELECTROPHORETIC TRANSFER



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.

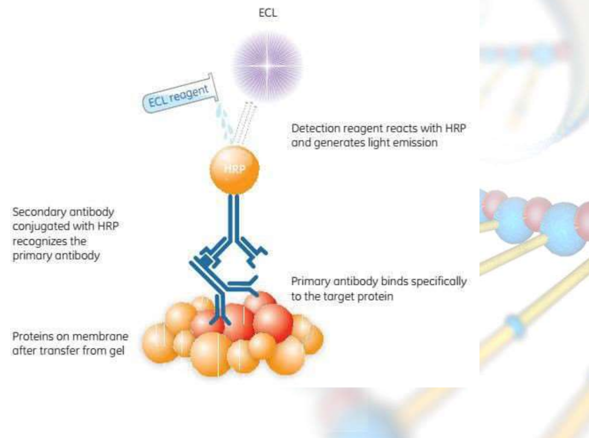
A species-specific, labeled secondary antibody directed against the constant region of the primary antibody is then used. The most common antibody label used in Western blots is HRP.

Western Blotting - Procedure

PROCEDURE

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

Western Blotting - Procedure



Applications of western blotting

- 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

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

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

- File compressed by Talha Waraich to facilitate students in printing using less pages.
- Actual work belongs to Virtual University of Pakistan & Credit goes to them.

- Good luck everyone for Semester Activities & I request you to pray for my health.
- Regards:
- Talha Waraich

Difference between different blotting technique

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.

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.
Detectio n 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.

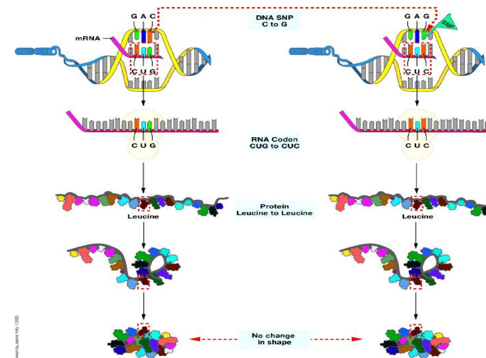
Single nucleotide polymorphism

What is SNP ?

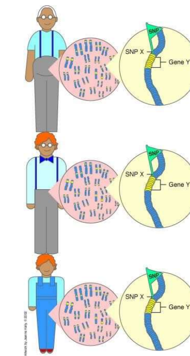
- A **SNP** is defined as a single base change in a DNA sequence that occurs in a significant proportion (more than 1 percent) of a large population.

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)

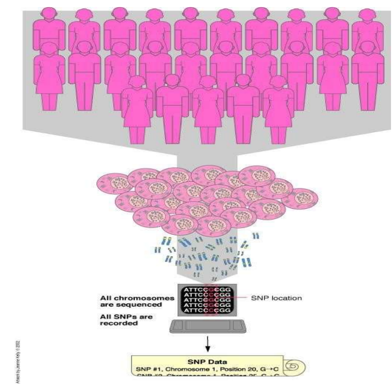


- SNPs are found in
  - coding and (mostly) noncoding regions.
- Occur with a very high frequency
  - about 1 in 1000 bases to 1 in 100 to 300 bases.
- The abundance of SNPs and the ease with which they can be measured make these genetic variations significant.
- SNPs close to particular gene acts as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

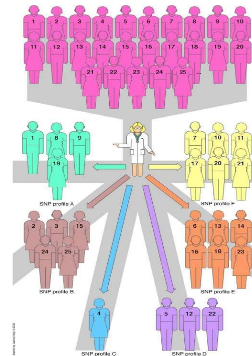


Single nucleotide polymorphism -2

- 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



- 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.



How to detect SNPs

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

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

- 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.

- **Two different screening strategies**
  - **- Many SNPs in a few individuals**
  - **- A few SNPs in many individuals**
- **Different strategies will require different tools**

- **Importantin determiningmarkers forcomplex genetic states**
  - **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… .

- **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

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

Phenotype SNPSNP

- 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.

- 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/)

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

- 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.

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.

- A Variable Number Tandem Repeat (or VNTR) is a location in a genome where a short nucleotide seq uence is organized as a tandem repeat.

- These can be found on many chromosomes, and often show variations in length between individu als.
- Each variant acts as an inherited allele, allowing t hem to be used for personal or parental identifica tion.
- There are two principal families of VNTRs:
  - Microsatellites.
  - Minisatellites.

Microsatellites, also known as Simple Sequence Repea ts (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 o f DNA that consists of a short series of bases 10–60 base pairs. •

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

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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).

- 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.

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.
- 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.

- • 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.
- 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.

- 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.

- 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.

- 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.
- 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).

- 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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- VU Biology Zone
- 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
- 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.
- 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.
- 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).

- 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.

- 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.