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HIGHLIGHTED HANDOUT

MB504T

Methods in Molecular Biology (Theory)

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Essentials of Genetics

MODULE# 01

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.

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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.

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

MODULE# 02

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

MODULE# 03

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

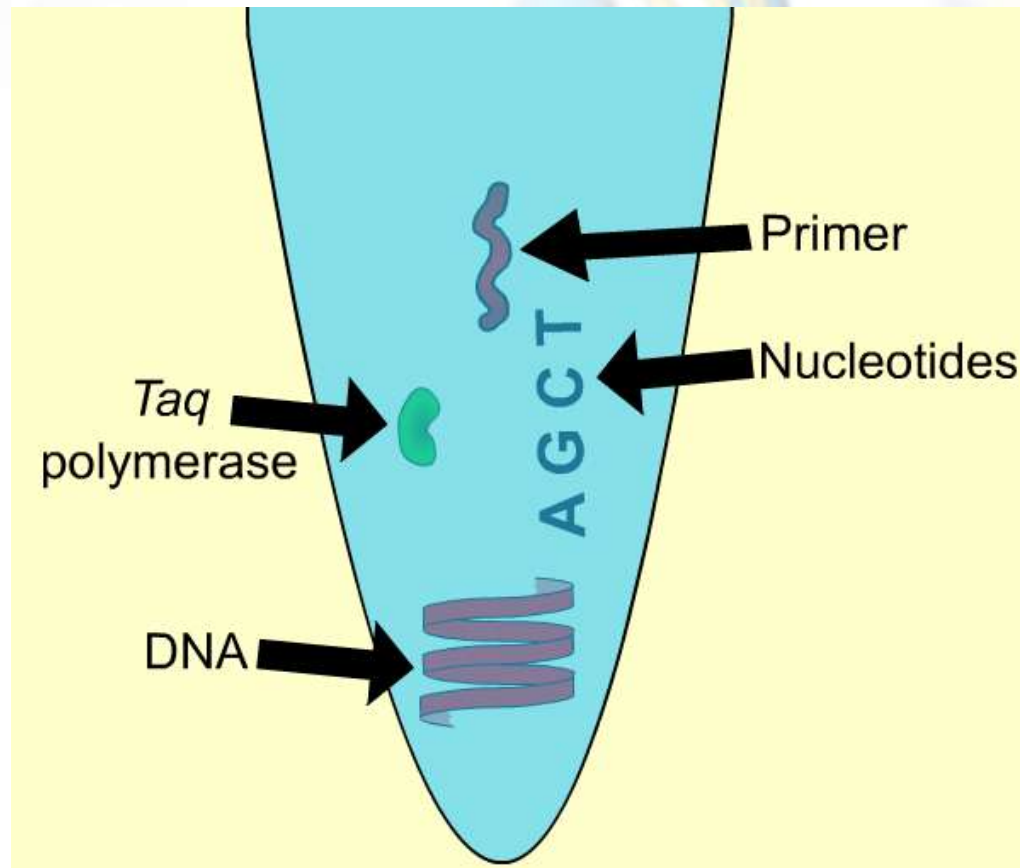
Taq polymerase

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

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MODULE# 04

PRIMERS FOR PCR

PRIMERS FOR PCR

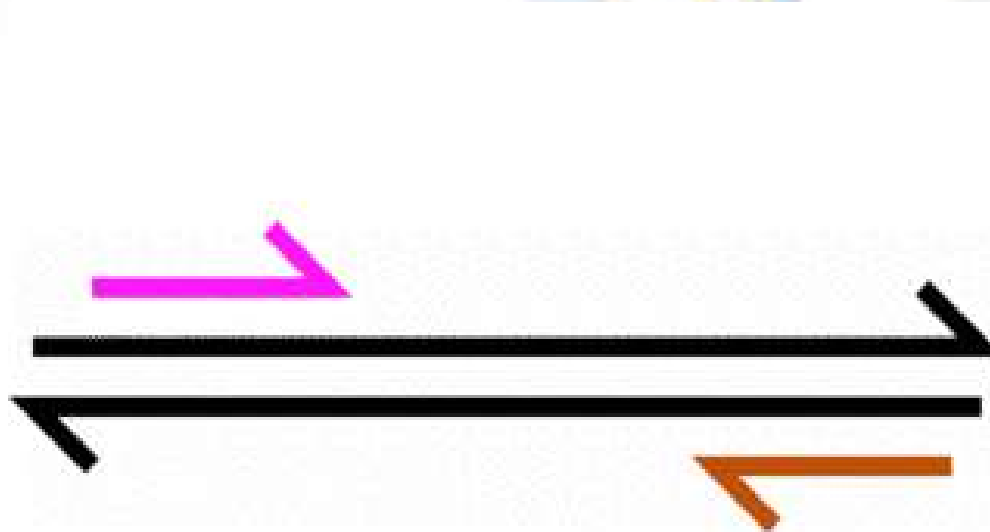
PRIMERS

Primers are single-stranded 18–30 bp long DNA fragments

Complementary to sequences flanking the region to be amplified.

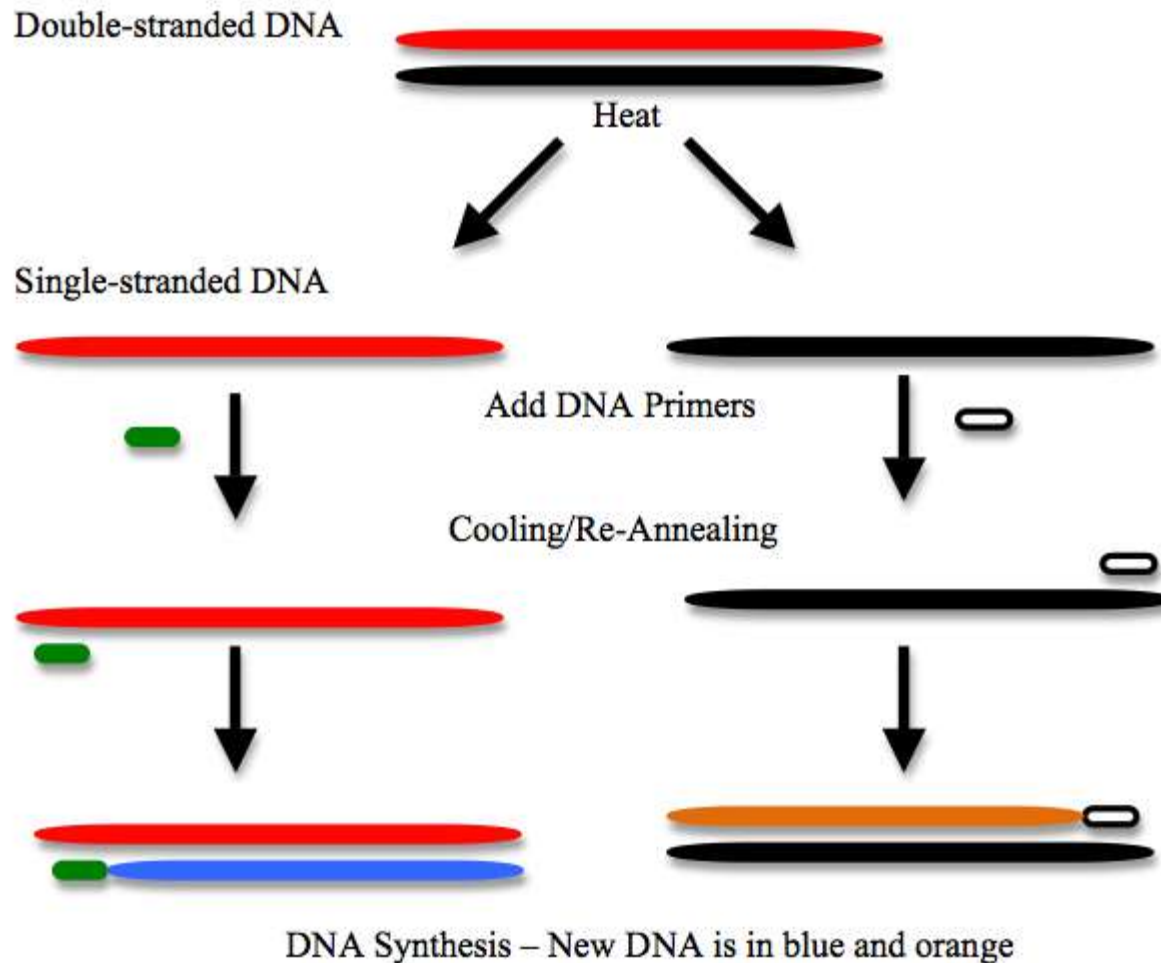
PRIMERS FOR PCR

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

$$T_m = 4(G+C) + 2(A+T)$$

Essentials of Genetics

MODULE# 05

**DNA
POLYMERASES**

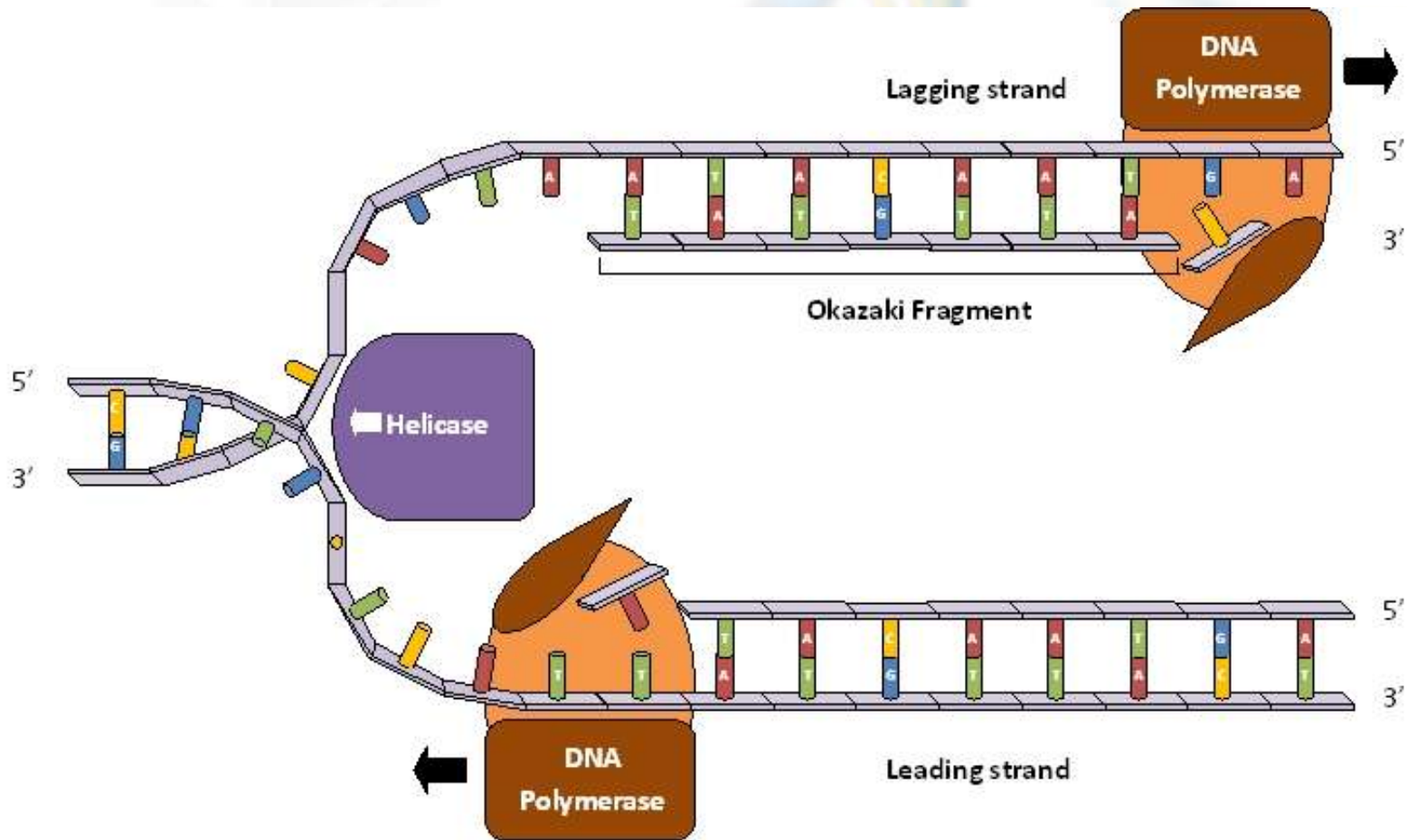
DNA Polymerases

POLYMERASES

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

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

MODULE# 06

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 annealing 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.

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MODULE# 07

INTERPRETATION OF RESULTS

Interpretation of Results

INTERPRETATION

The PCR product should be of the expected size.

Misprimers 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.

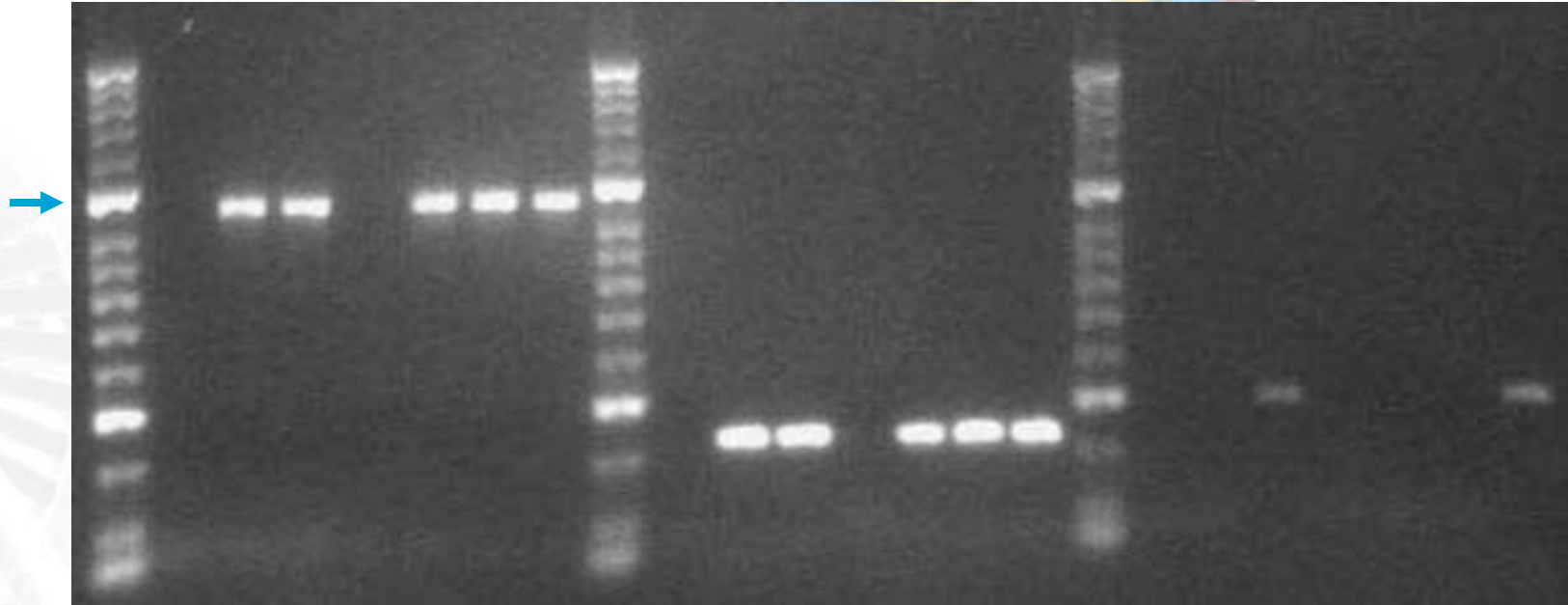
Interpretation of Results

NEGATIVE CONTROL

Controls for
specificity of the
amplification
reaction
contains all reagents
and a DNA template
lacking the target
sequence.

Interpretation of Results

Blank Reaction
Positive Control
Negative Control
Patient 1
Patient 2
Patient 3
Molecular Marker



Interpretation of Results

CONCLUSION

The PCR product should be of the expected size.

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MODULE# 08

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

MODULE# 09

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.

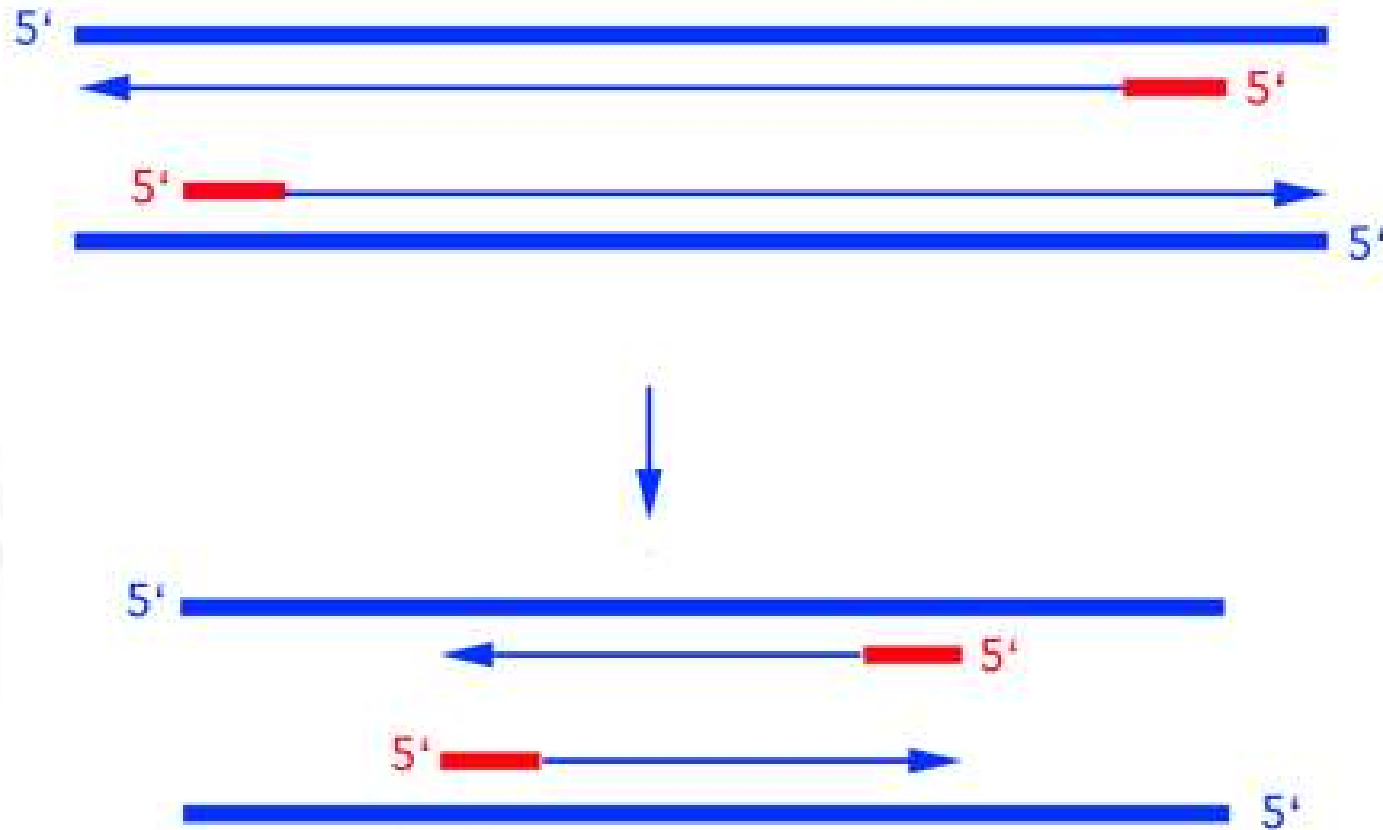
Nested PCR

NESTED PCR – SECOND PAIR OF PRIMERS

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

Nested PCR

NESTED PCR



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.

Nested PCR

**SECOND PCR
PRODUCT IS
SPECIFIC**

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

Nested PCR

ADVANTAGE OF NESTED PCR

Very low probability of nonspecific amplification.

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MODULE# 10

MULTIPLEX PCR

Multiplex PCR

MULTIPLY 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.

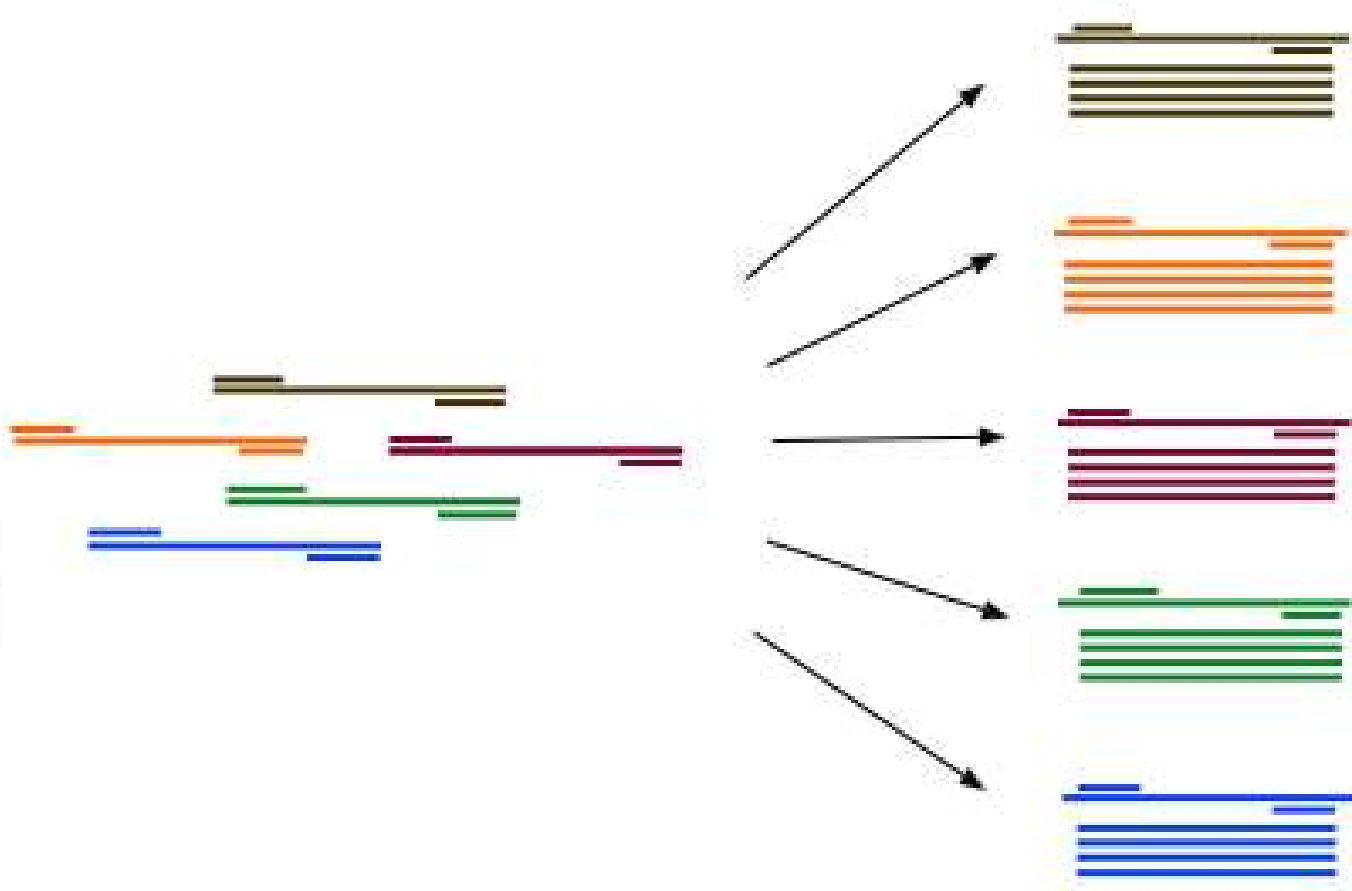
Multiplex PCR

**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

MULTIPLY 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

MULTIPLY 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

MODULE# 011

**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

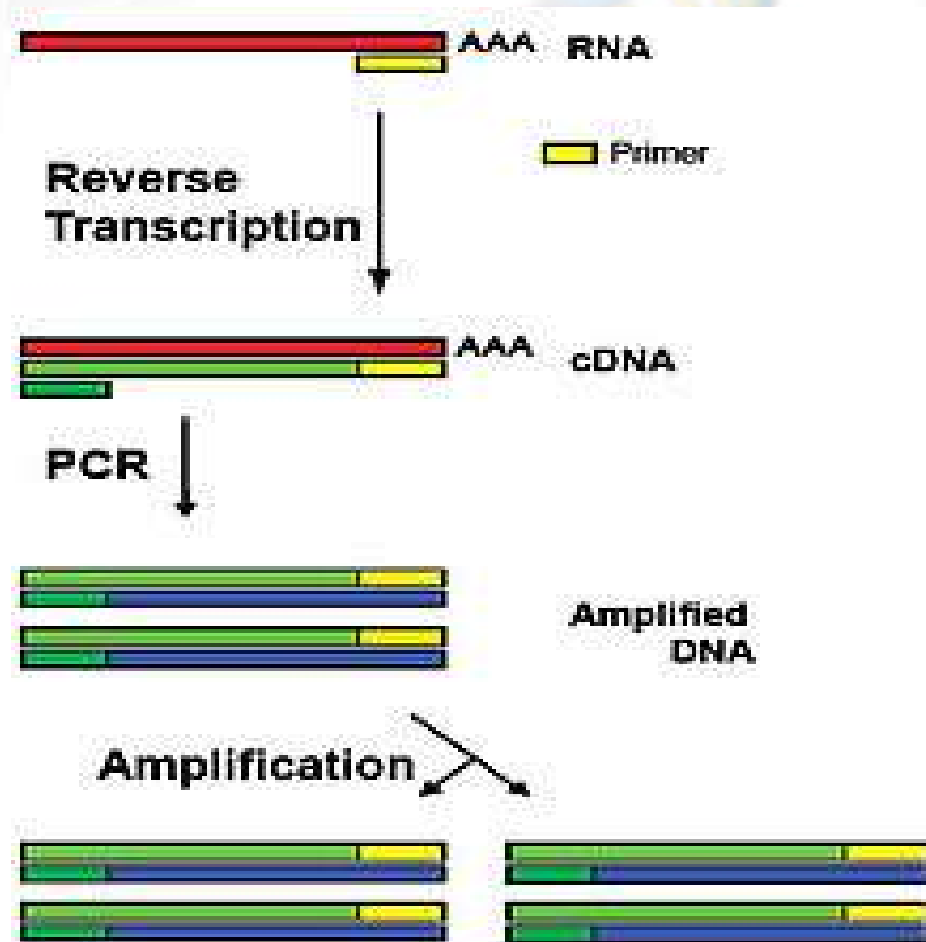
Synthesis of cDNA using oligo dT primers
(37°C) one hour.

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

MODULE# 12

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 cyclor 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

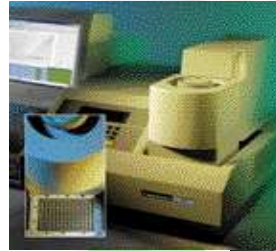
- Intuitive programming
- Fast and accurate performance
- Flexibility for multiple users
- Small footprint



iCycler
BioRad



LightCycler
Roche



5700
Applied Biosystems



7700
Applied Biosystems



FluorTracker
Stratagene



FluorImager
Molecular Dynamics

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.

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

MODULE# 013

**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

MODULE# 014

**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

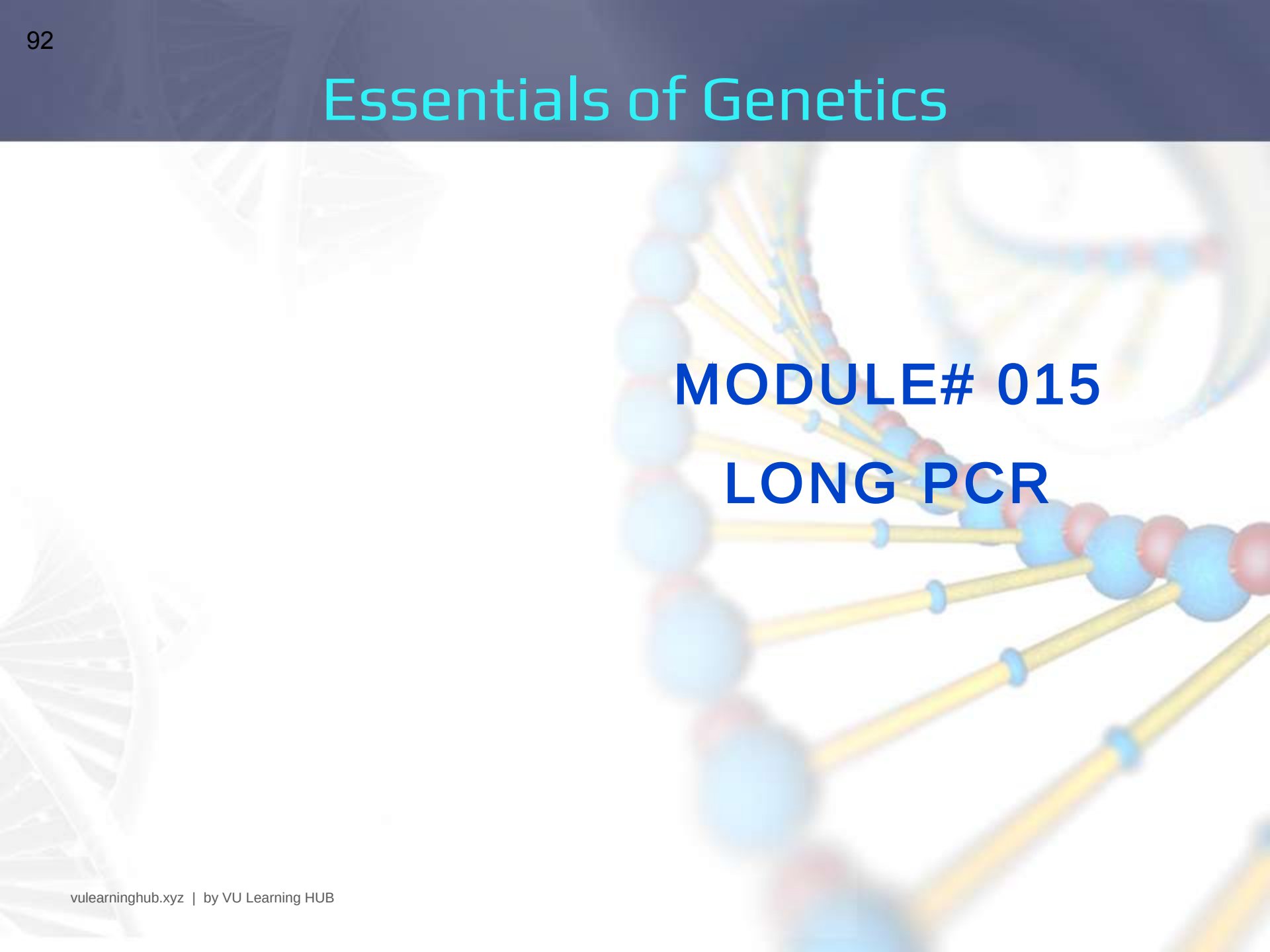
Due to the slow amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required.

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



MODULE# 015

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.

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Essentials of Genetics

MODULE# 016

**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

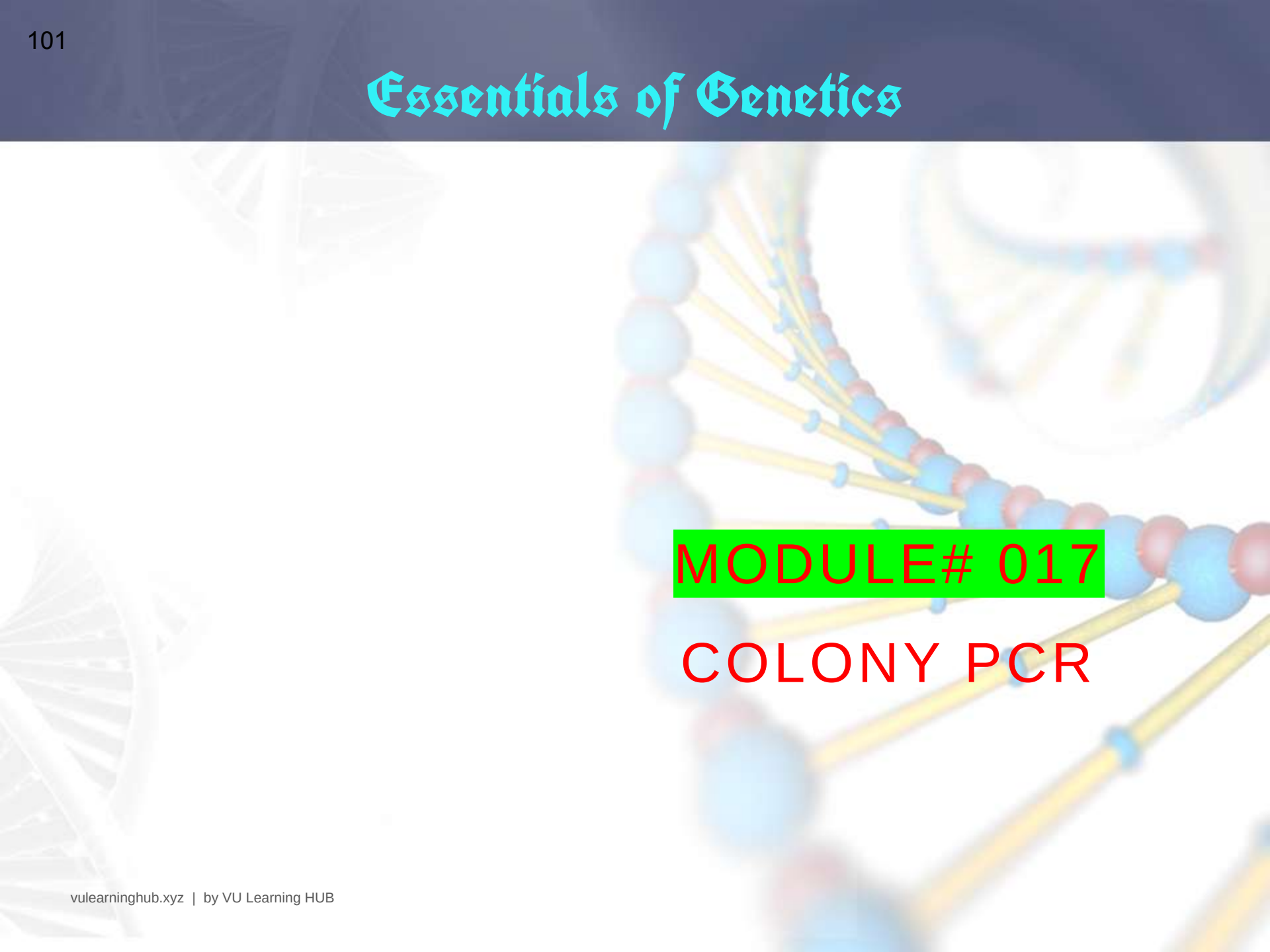
Successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

Allele Specific PCR

ALLELE SPECIFIC PCR

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

Essentials of Genetics



MODULE# 017

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 μ l of TE autoclaved dH₂O in a microfuge tube.

Heat the mix in a boiling water bath (90-100°C) for 2 minutes

Spin sample for 2 minutes high speed in centrifuge.

Colony PCR

COLONY PCR

Transfer 20 μ l of the supernatant into a new microfuge tube.

Take 1-2 μ l of the supernatant as template in a 25 μ l PCR standard PCR reaction.

Colony PCR

COLONY PCR

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

Essentials of Genetics

MODULE#018

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

MODULE# 019

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

MODULE# 020

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.

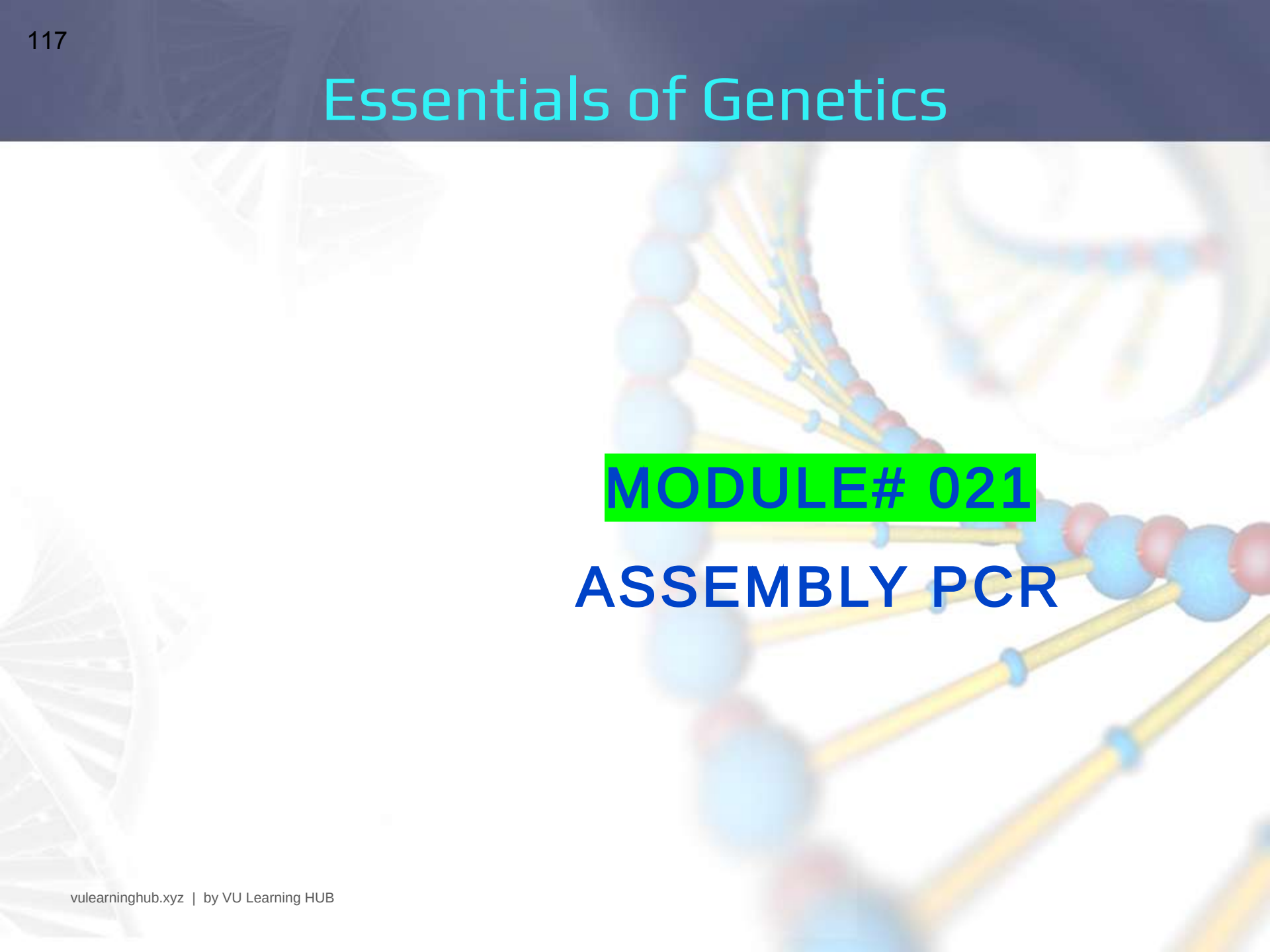
AFLP PCR

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.

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MODULE# 021

ASSEMBLY PCR

Assembly PCR

ASSEMBLY PCR

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

Assembly PCR

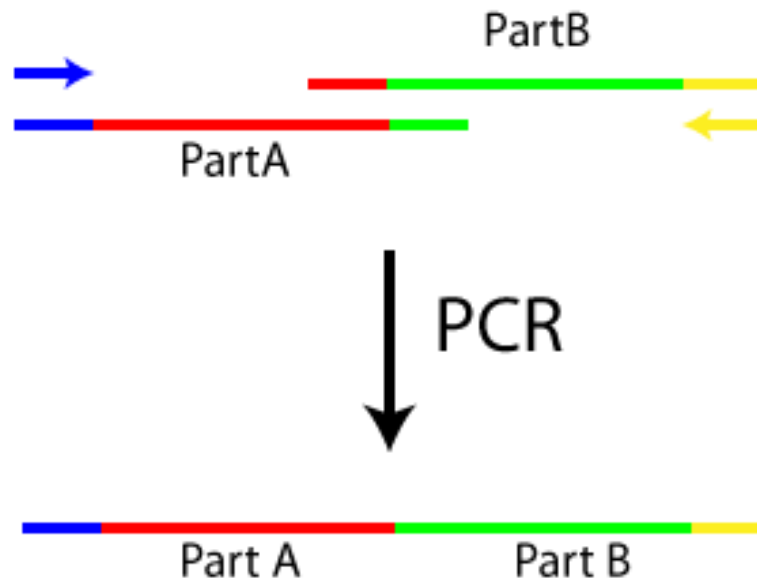
ASSEMBLY PCR - PRINCIPLE

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.

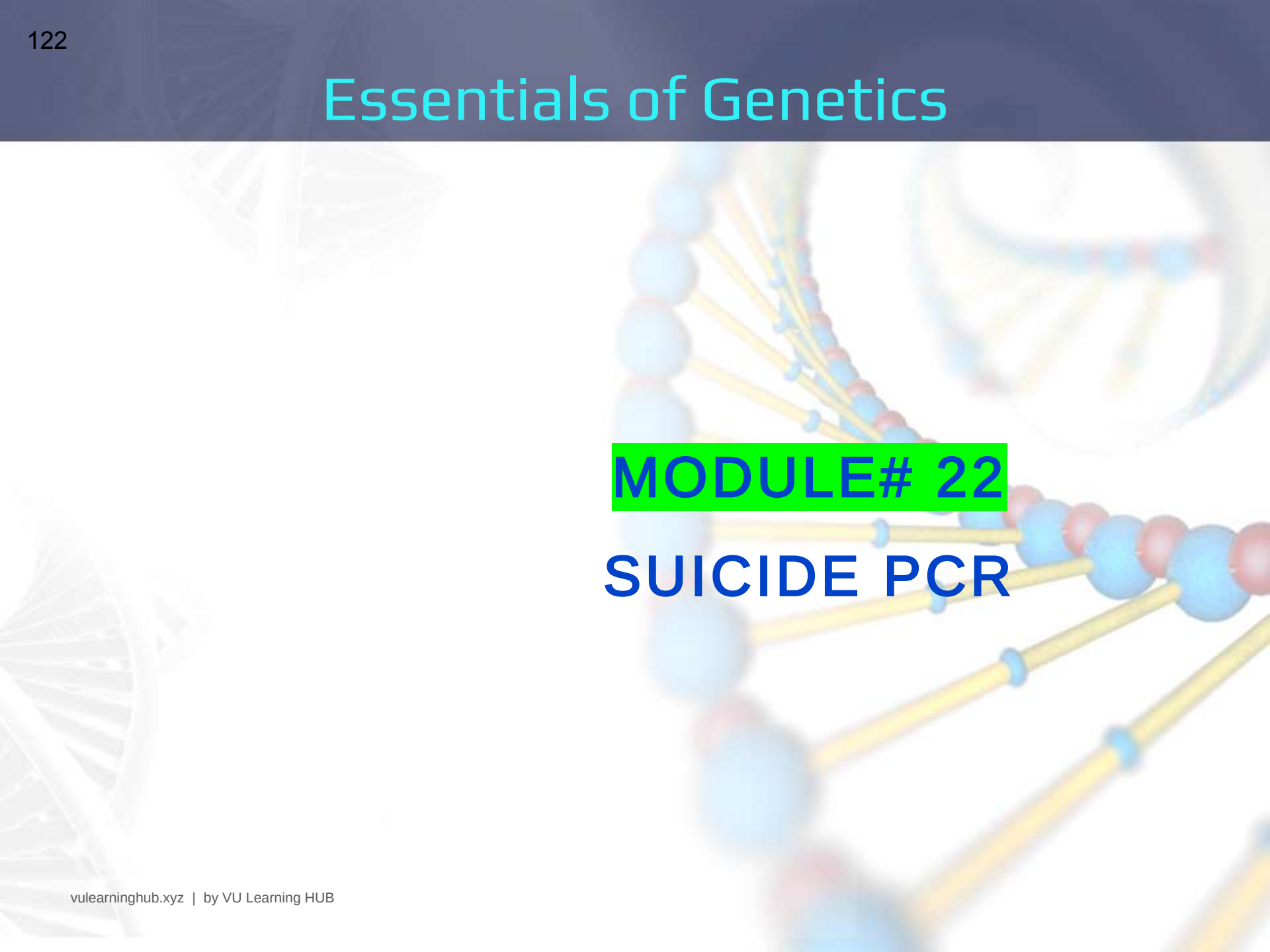


Assembly PCR

ASSEMBLY PCR

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

Essentials of Genetics



MODULE# 22

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

MODULE# 023

**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

MODULE# 24
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.

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MODULE# 25
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

MODULE# 26

**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

MODULE# 27

**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

MODULE# 28

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

MODULE# 29

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 MICROBIOLOGY

Identification of
bacteria:

Slow growing
bacteria

Bacterial antibiotic
resistance genes.

VIRUS: almost all
viruses identification
and viral load.

Applications of PCR

CLINICAL MICROBIOLOGY

FUNGUS:

Aspergillus spp

Candida spp

Plasmodium spp

Trypanosoma spp

Leishmania spp

Babesia spp

Polymerase chain reaction (PCR)

Module# 030

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

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§ BS ZOOLOGY 03336361244

Polymerase chain reaction (PCR)

Module# 31

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)

Module# 32

PCR-Forensic sciences

§ Forensic science is the application of scientific procedures to solve criminal and legal matters

§ Molecular methods are used to establish 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)

Module# 33

PCR-Agricultural sciences and environment

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

Polymerase chain reaction (PCR)

Module# 34

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

Module# 35

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 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

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

Module# 36

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 *EcoK*

§ 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
<i>SmaI</i>	<i>Serratia marcescens</i> , 1st enzyme	CCCGGG
<i>HaeIII</i>	<i>Haemophilus aegyptius</i> , 3rd enzyme	GGCC
<i>HindII</i>	<i>H. influenzae</i> , strain d, 2nd enzyme	GTPyPuAC
<i>HindIII</i>	<i>H. influenzae</i> , strain d, 3rd enzyme	AAGCTT
<i>HamHI</i>	<i>Bacillus amyloliquefaciens</i> , strain H, 1st enzyme	GGATCC

Cutting DNA molecules

Module# 37

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

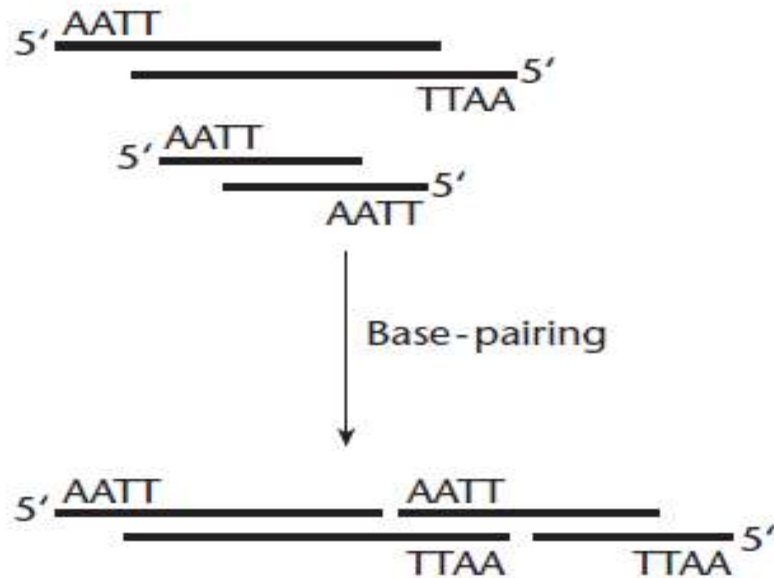
5'-A A T T C-3'

3'-C T T A A-5'

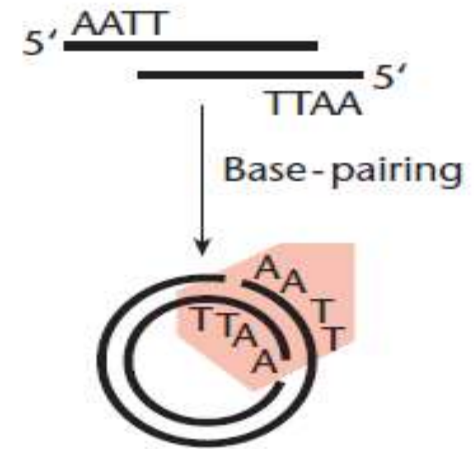
G-5'

Single stranded breaks by *EcoR* 1

Intermolecular association



Intramolecular association



Cohesive fragments of DNA produced by digestion with EcoR1

Cutting DNA molecules

Module# 38

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	Arabidopsi s	E. Coli	Human
<i>Apal</i>	GGGCCC	25000	15000	2000
<i>BamHI</i>	GGATCC	6000	5000	5000
<i>SpeI</i>	ACTAGT	8000	60000	10000

Cutting DNA molecules

Module# 39

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

Module# 40

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

Module# 41

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

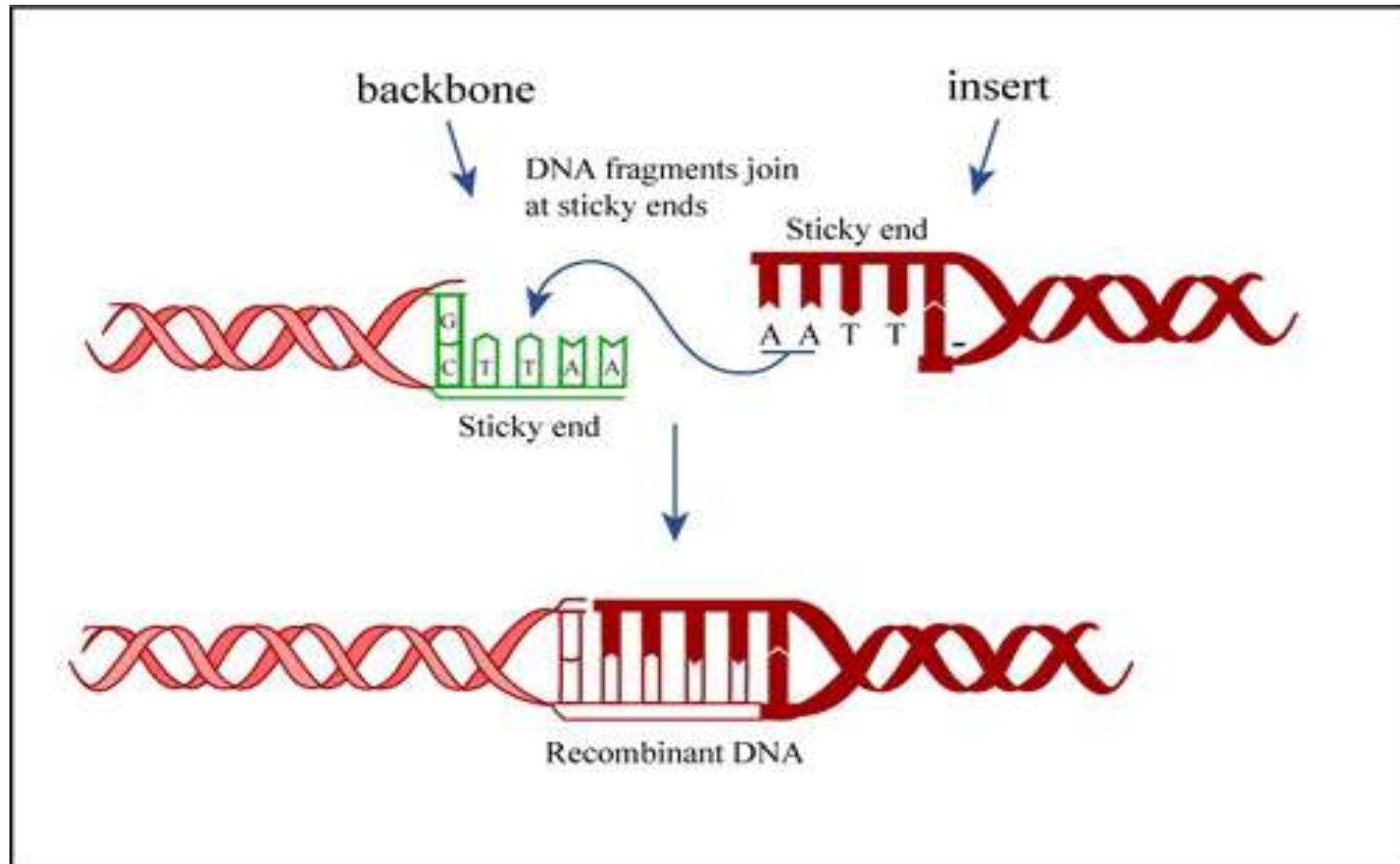
Methods of joining DNA fragments

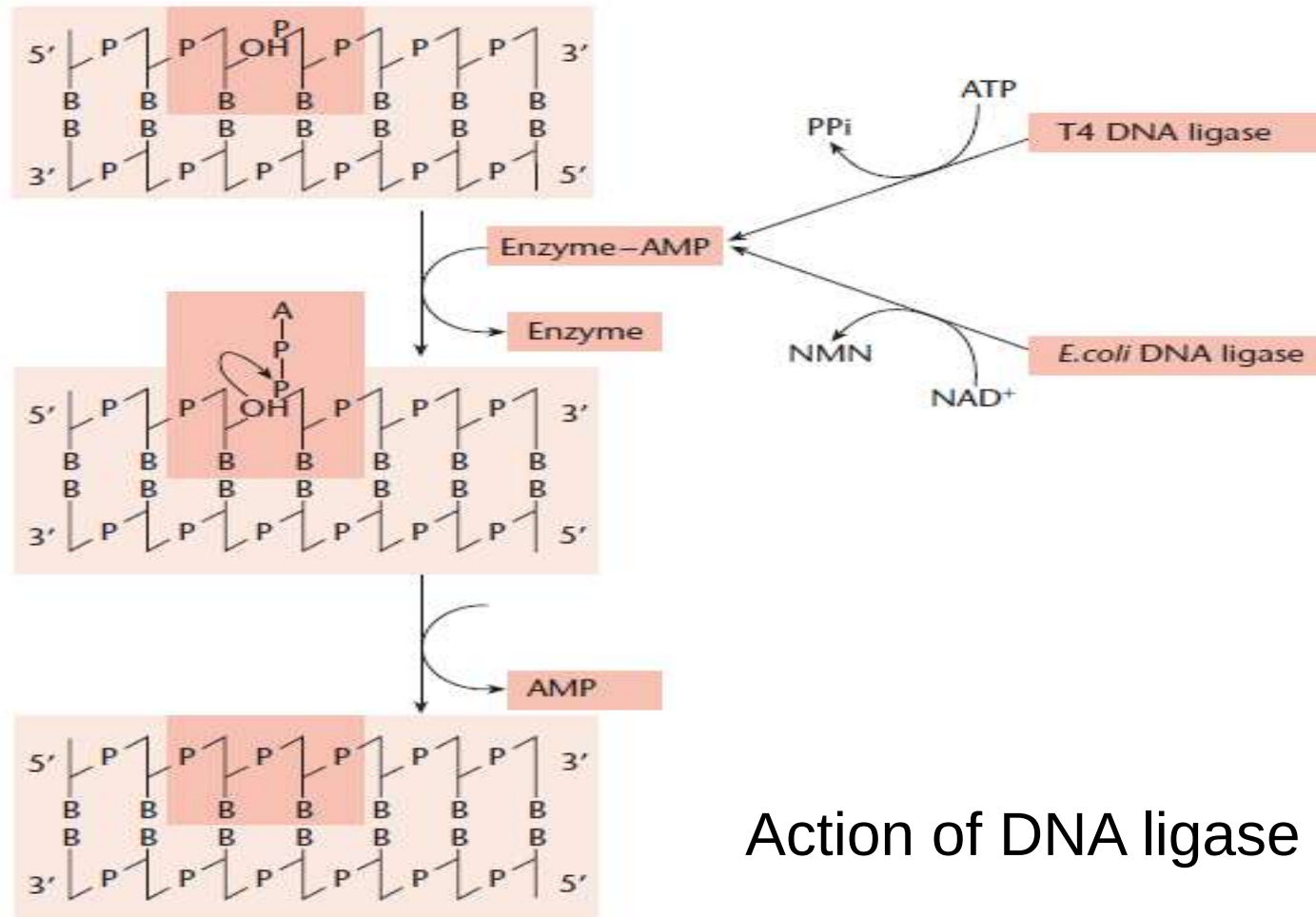
iii). The third utilizes the enzyme terminal deoxynucleotidyltransferase to synthesize homopolymeric 3' single stranded tails at the ends of fragments

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





Action of DNA ligase

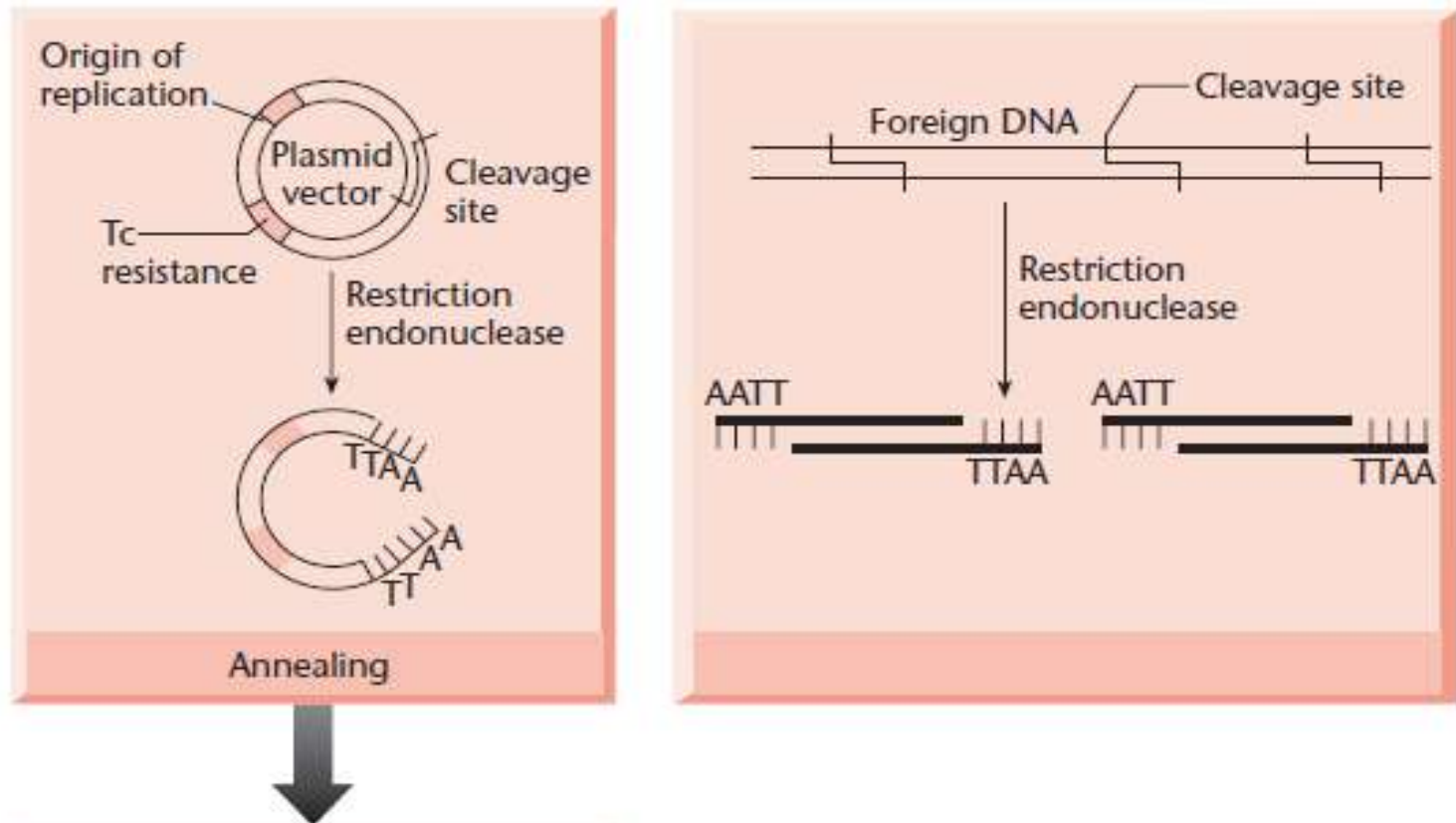
Joining DNA molecules

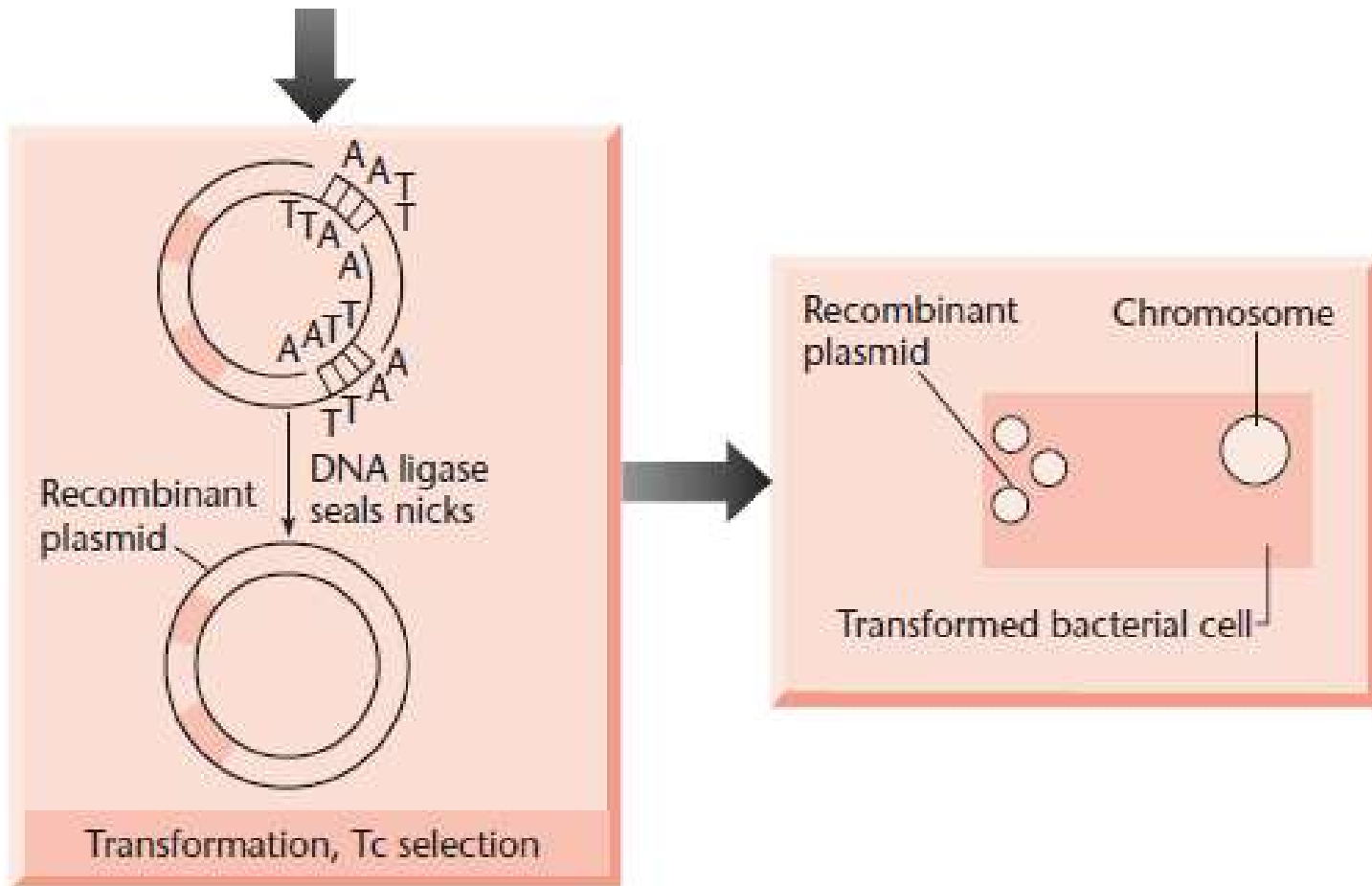
Module# 42

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

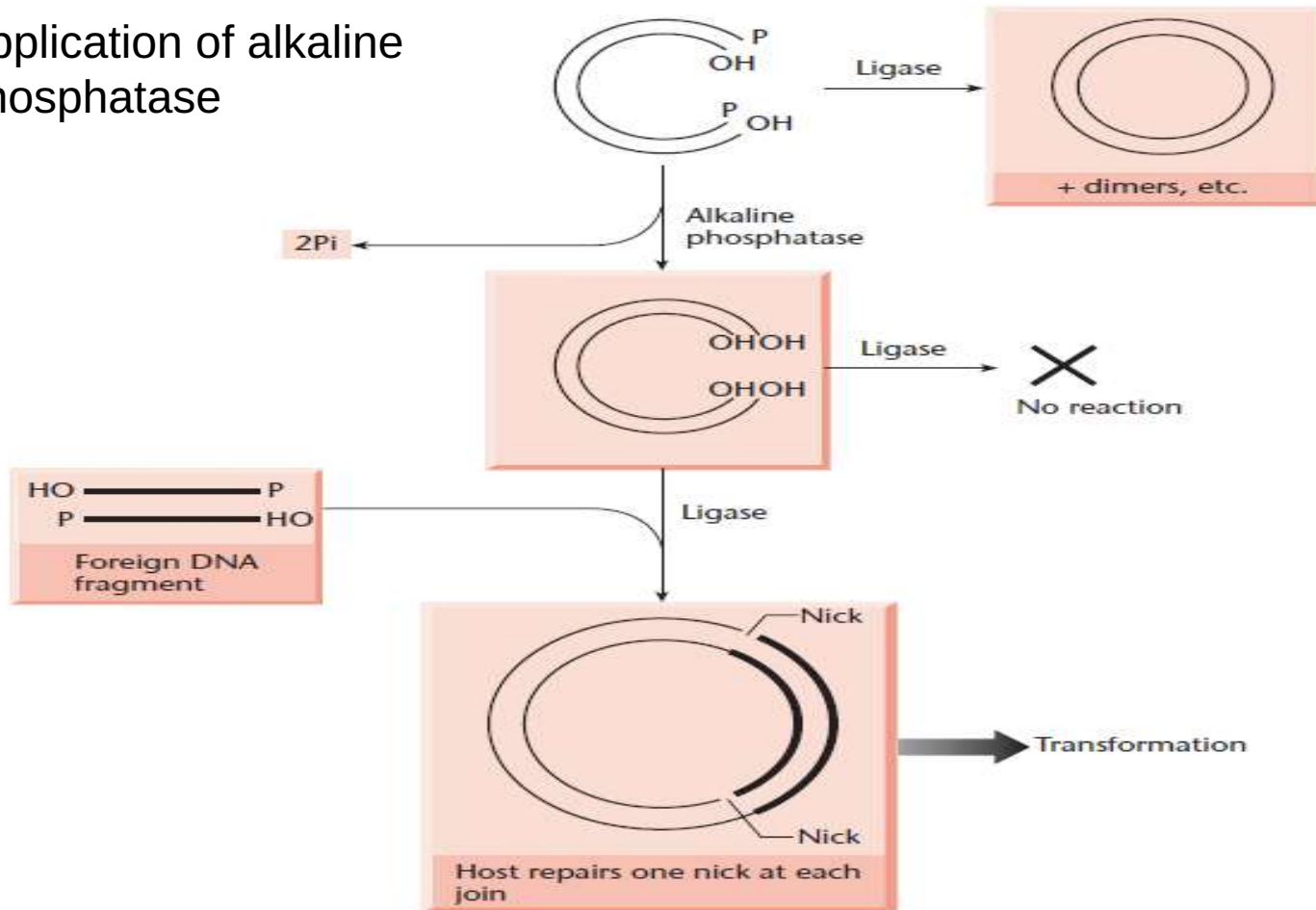
Module# 43

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

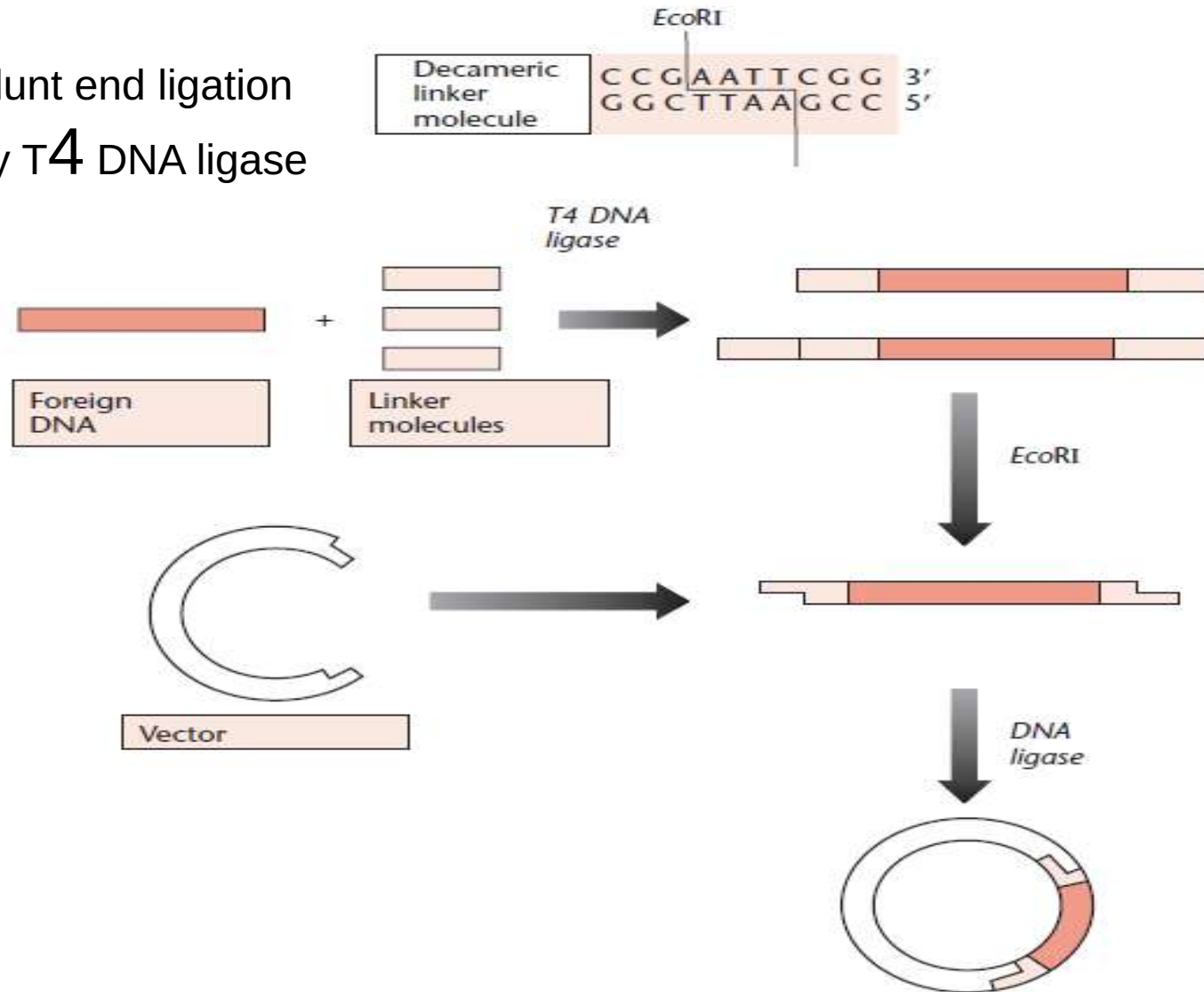
Module# 44

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 T4 DNA ligase

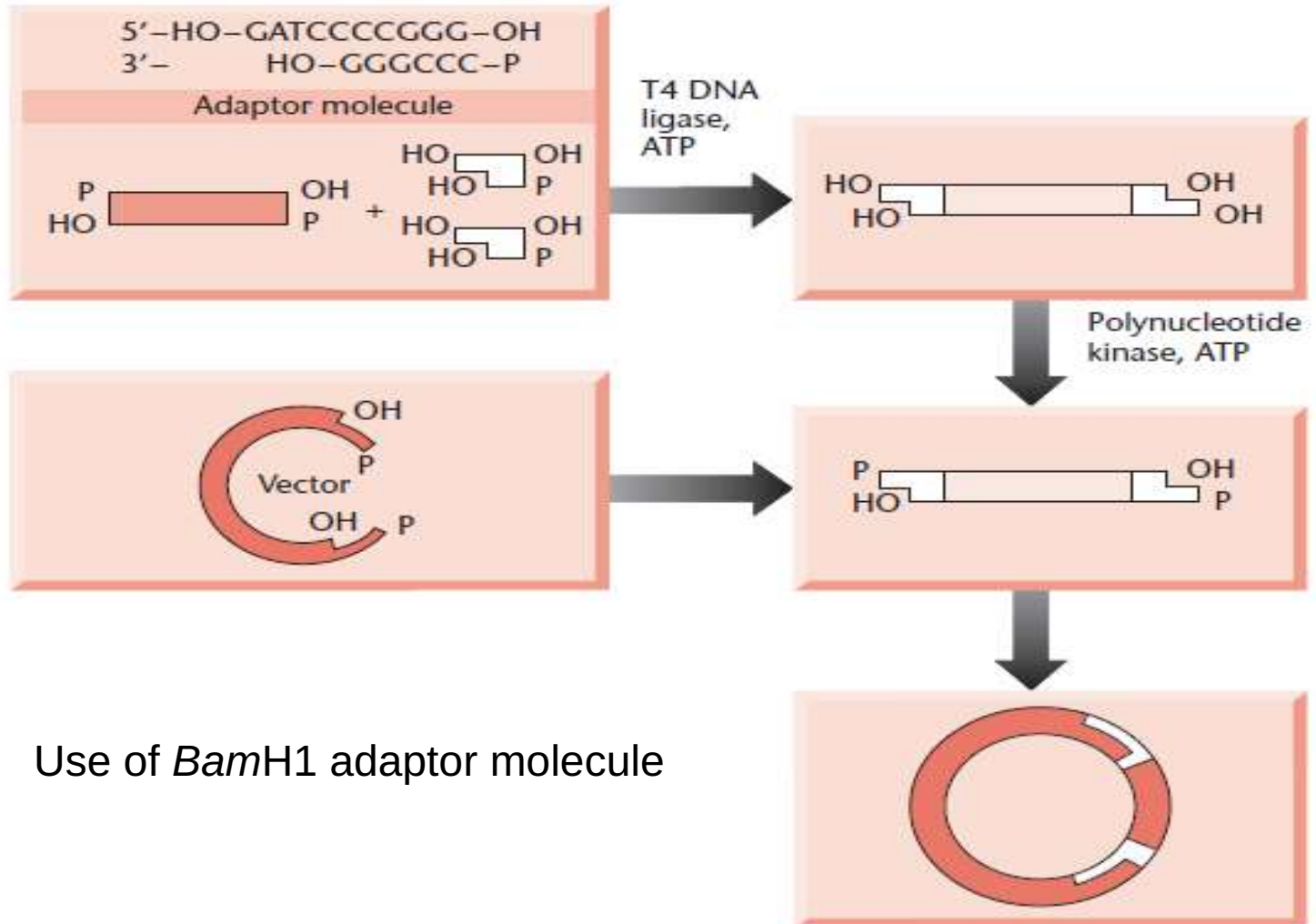


Joining DNA molecules

Module# 45

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.

§ Merged by BAQIR HUSSAIN
§ BS ZOOLOGY 03336361244

Essentials of Genetics

MODULE# 46

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).

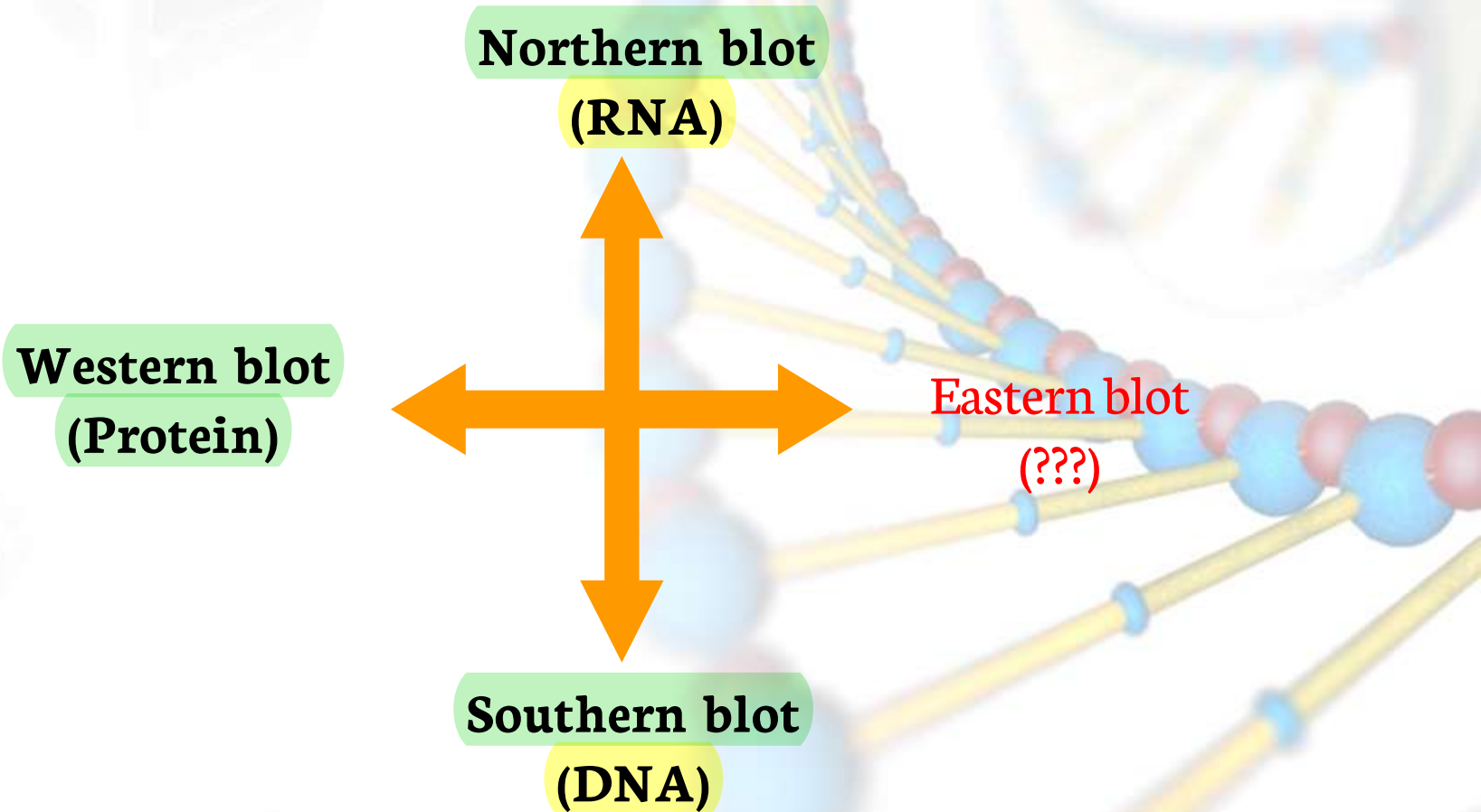
Southern Blotting

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

MODULE# 47

**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

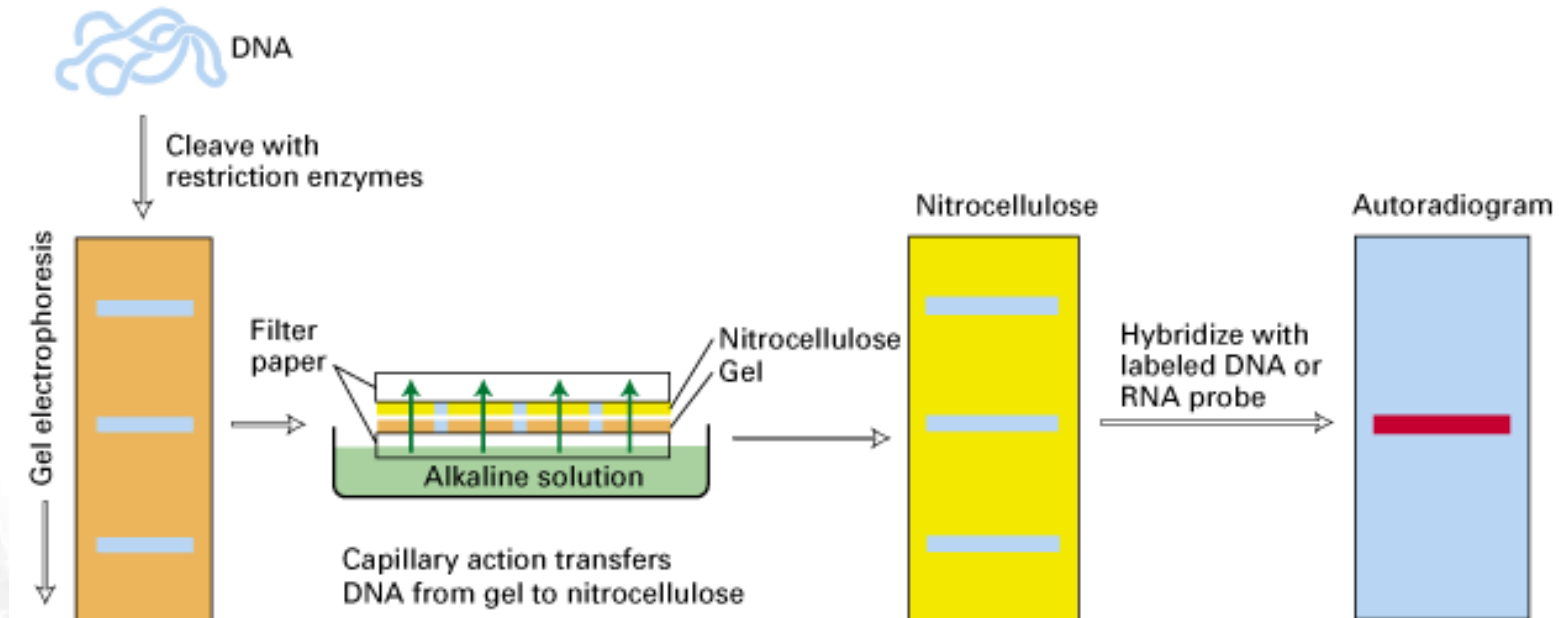
The labeled probe is added to the blocked membrane in buffer and incubated for several hours to allow the probe molecules to find their targets.

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

MODULE# 48

**SOUTHERN BLOTTING
PROBES**

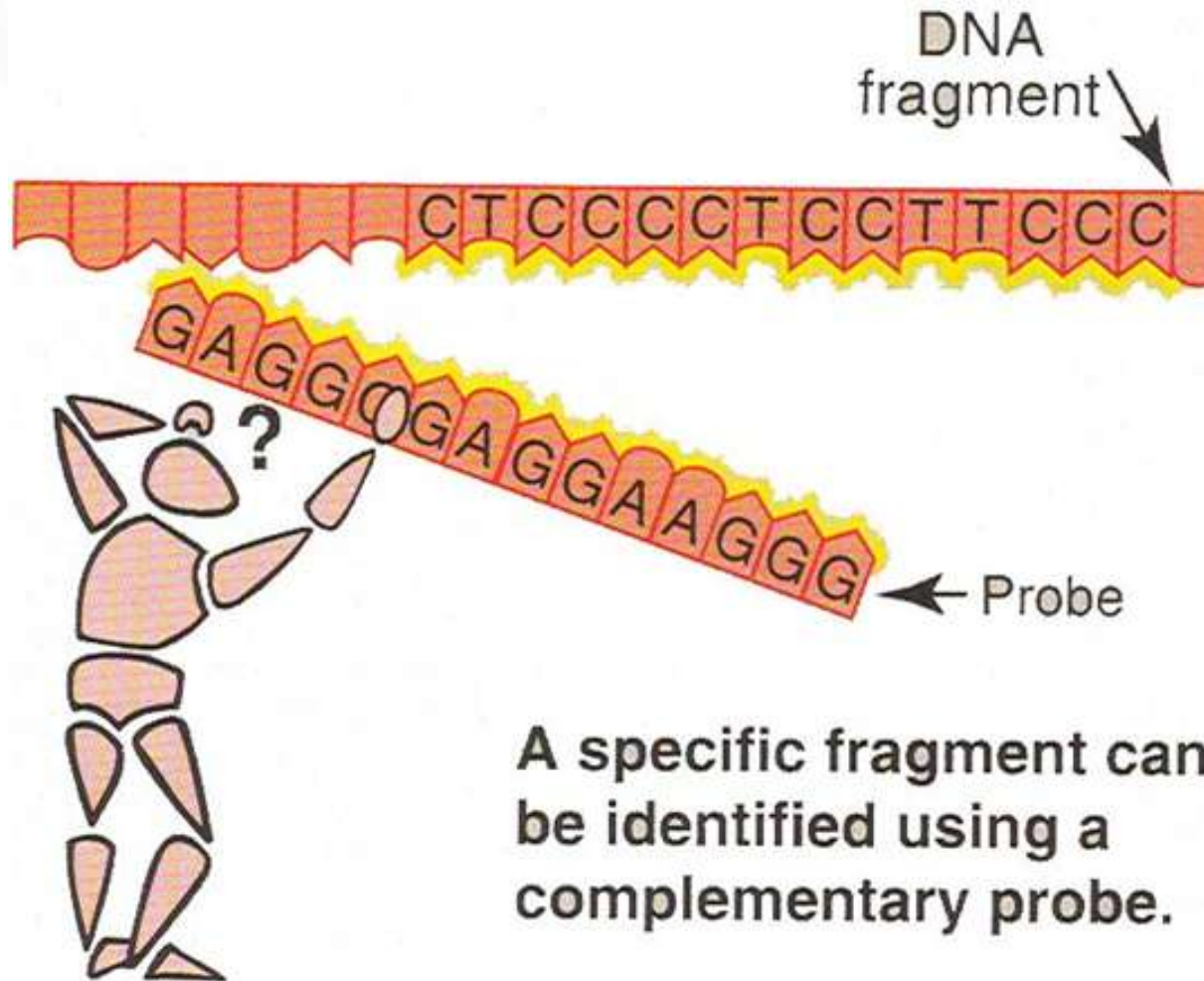
Southern Blotting - Probes

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

MODULE# 49

TRANSFER METHODS IN SOUTHERN BLOTTING

Transfer Methods - Southern

TRANSFER METHODS OF DNA TO MEMBRANE

Upward capillary transfer

Downward capillary transfer

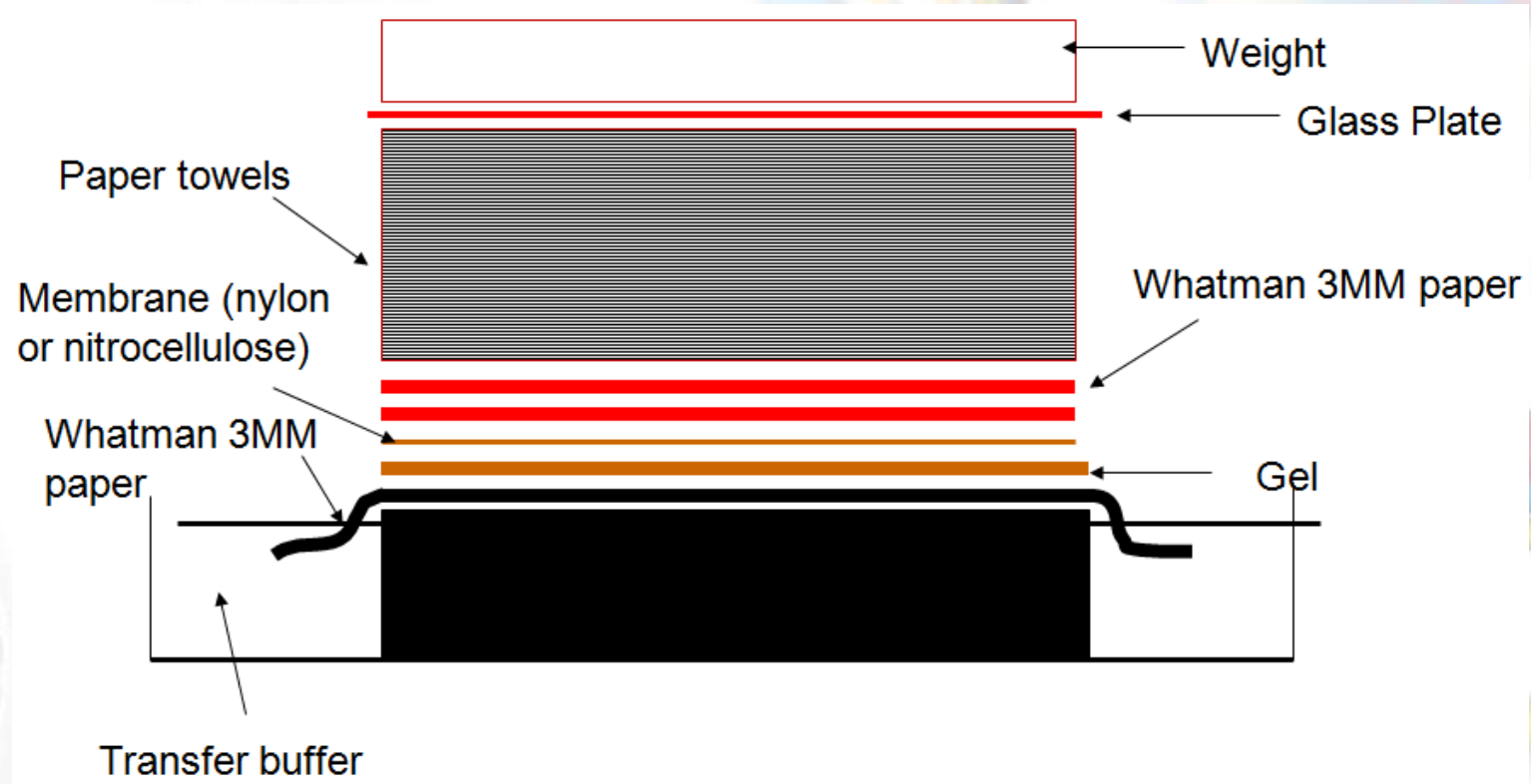
Simultaneous transfer to two membranes

Electrophoretic transfer

Vacuum transfer

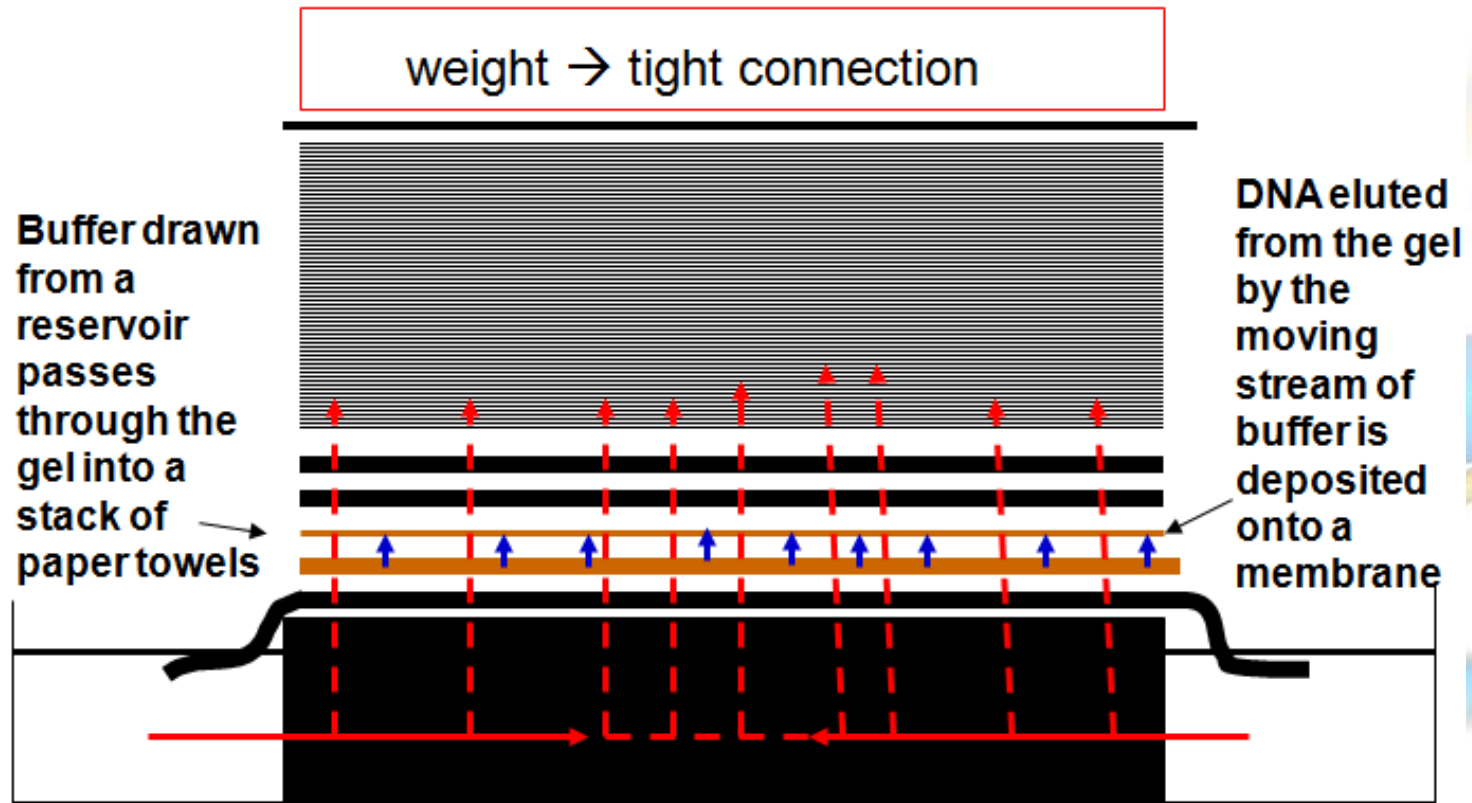
Transfer Methods - Southern

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

MODULE# 50

**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.

MERGED BY BAQIR
HUSSAIN BS ZOOLOGY
03336361244

Essentials of Genetics

MODULE# 51

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

MODULE# 52

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

MODULE# 53

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

MODULE# 54

**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

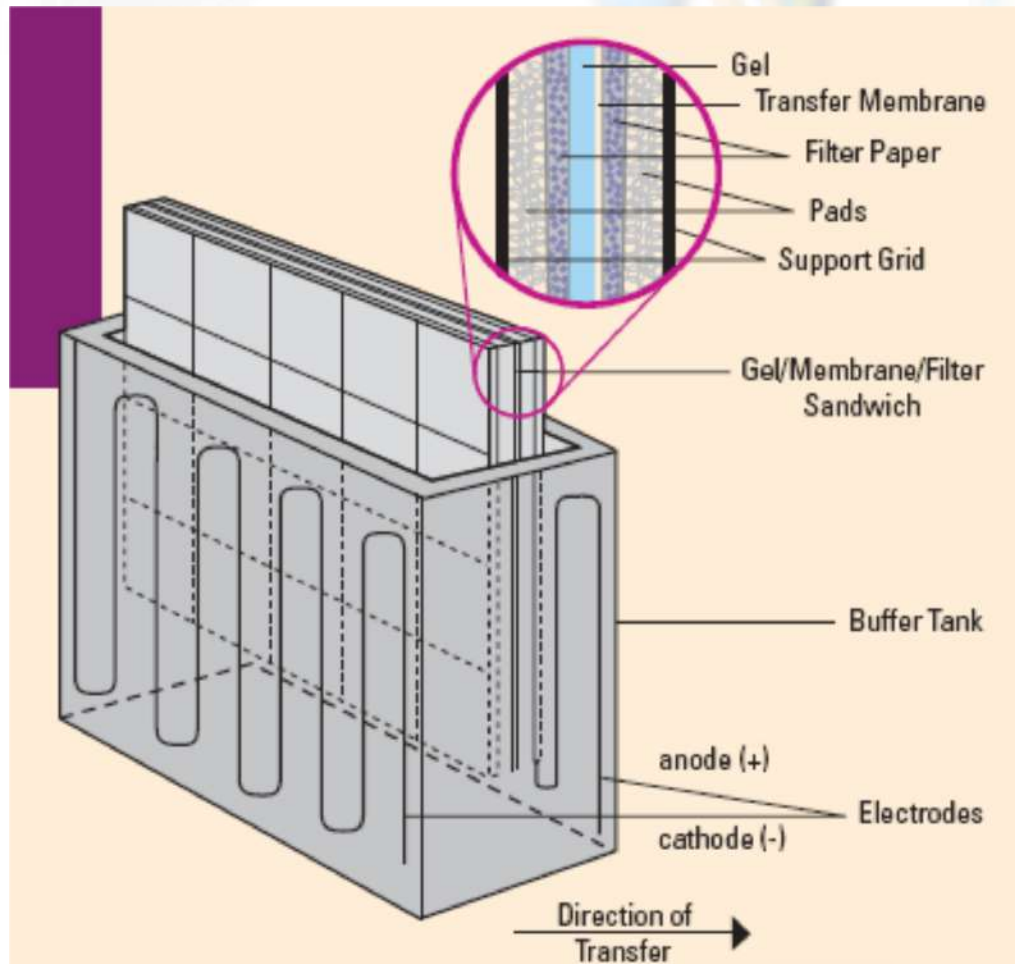
PROCEDURE

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.

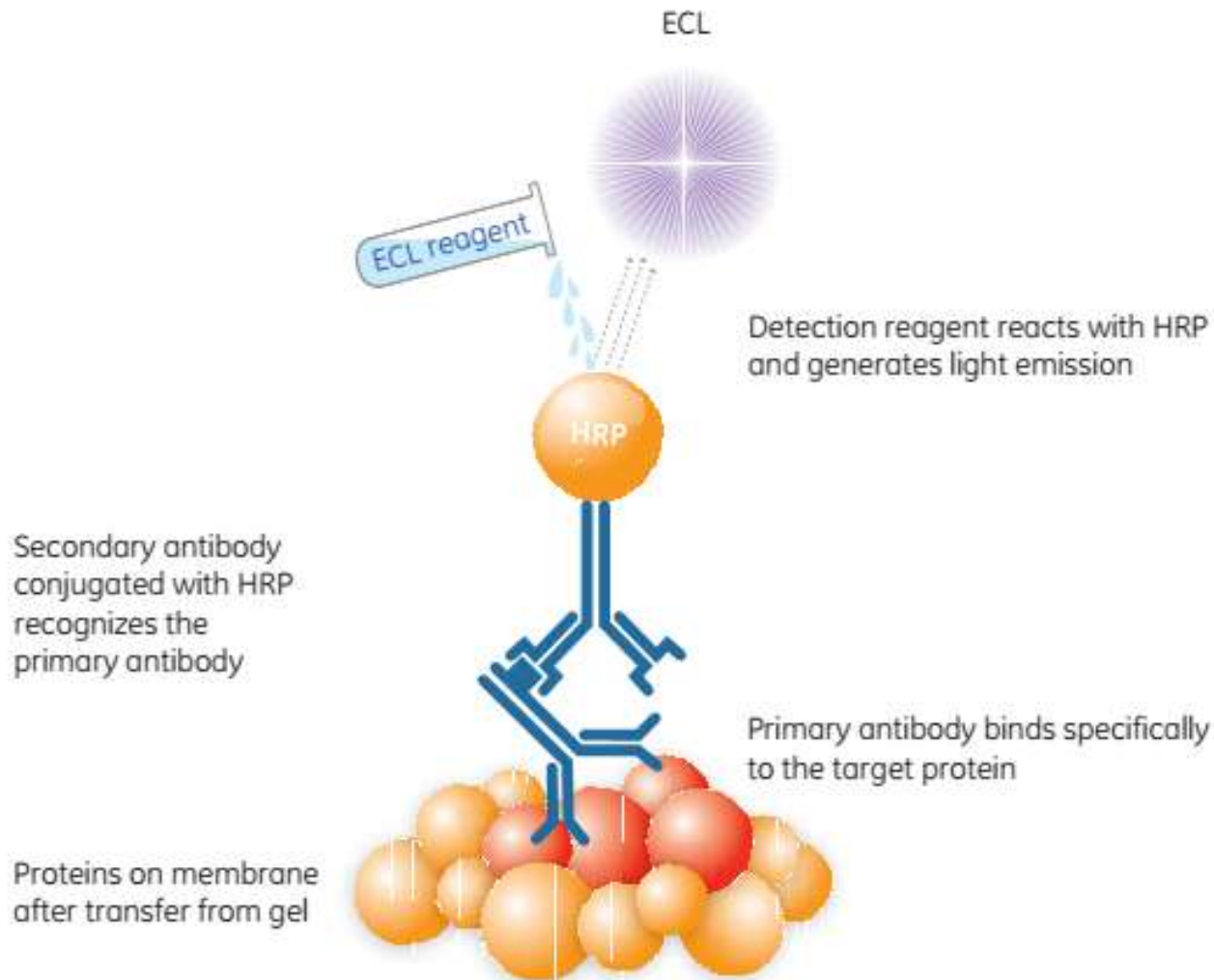
A species-specific, labeled secondary antibody directed against the constant region of the primary antibody is then used. The most common antibody label used in Western blots is **HRP**.

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



Western Blotting - Procedure

WESTERN BLOTTING

A molecular technique to study proteins.

The background of the slide features a stylized, semi-transparent illustration of DNA double helix structures. The helices are composed of yellow rods representing the sugar-phosphate backbones, with blue and red spheres representing the nitrogenous base pairs. The structures are arranged in a way that they appear to be floating and overlapping, creating a sense of depth and scientific focus.

Module# 55

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



Module# 56

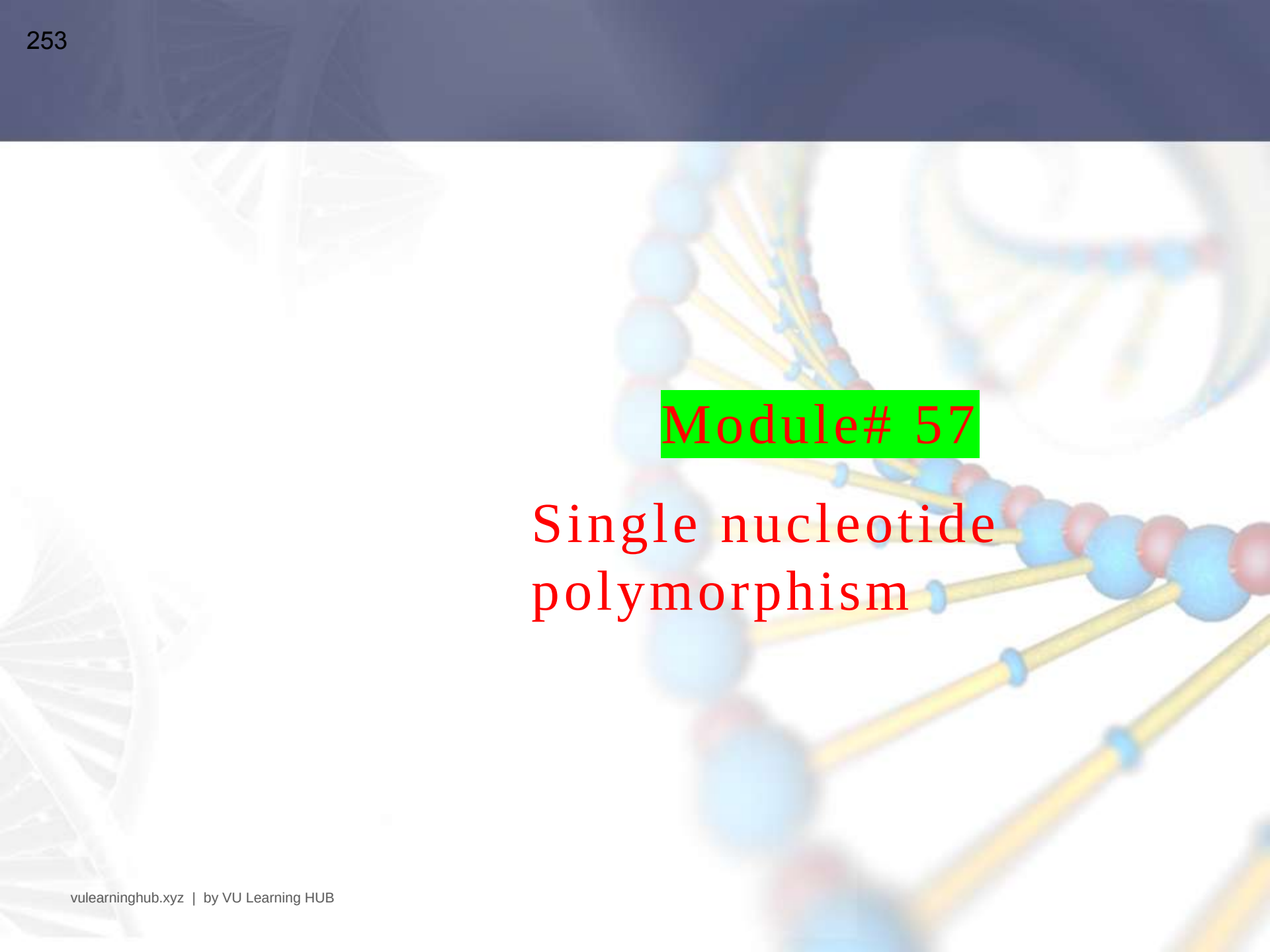
Difference between different blotting technique

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.
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.



Module# 57

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.

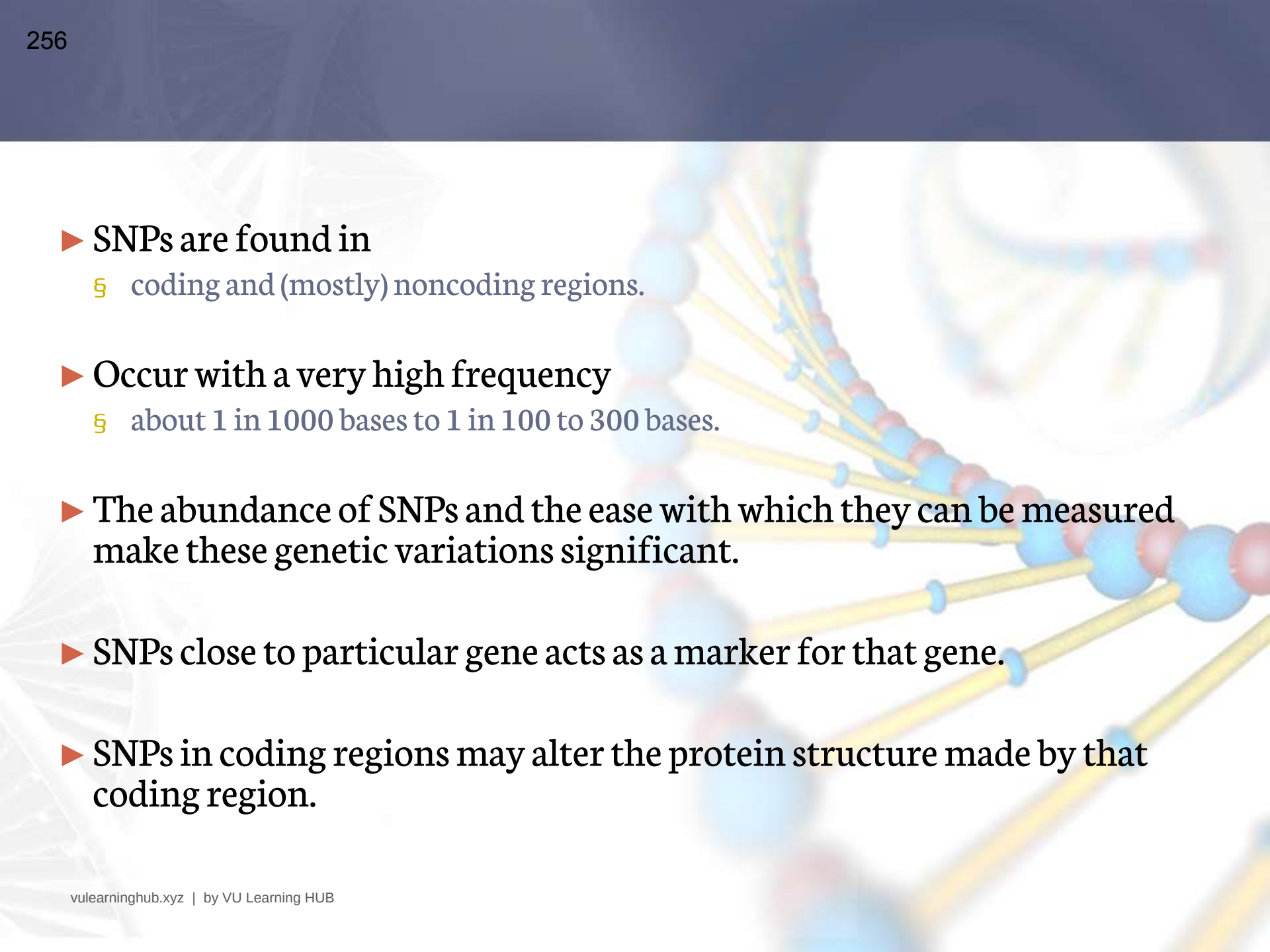
Different attributes / characteristics / traits
how a person looks,
diseases he or she develops.

These variations can be:

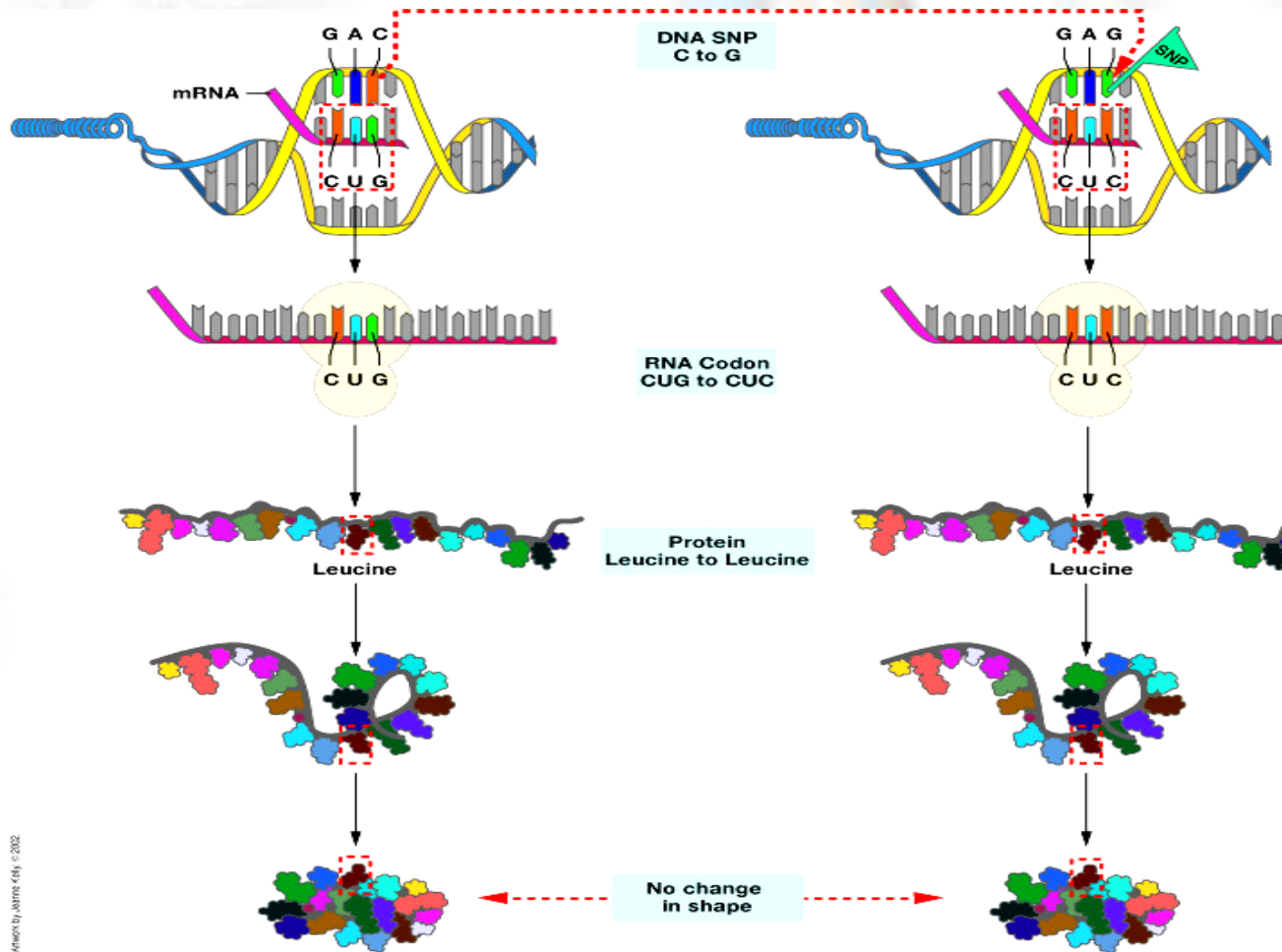
Harmless (change in phenotype)

Harmful (diabetes, cancer, heart disease, Huntington's disease, and hemophilia)

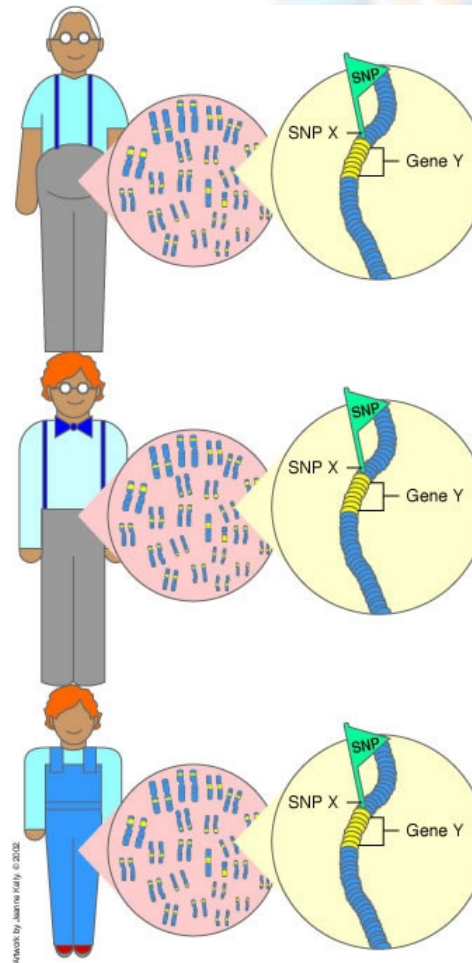
Latent (variations found in coding and regulatory regions, are not harmful on their own, and the change in each gene only becomes apparent under certain conditions e.g. susceptibility to lung cancer)

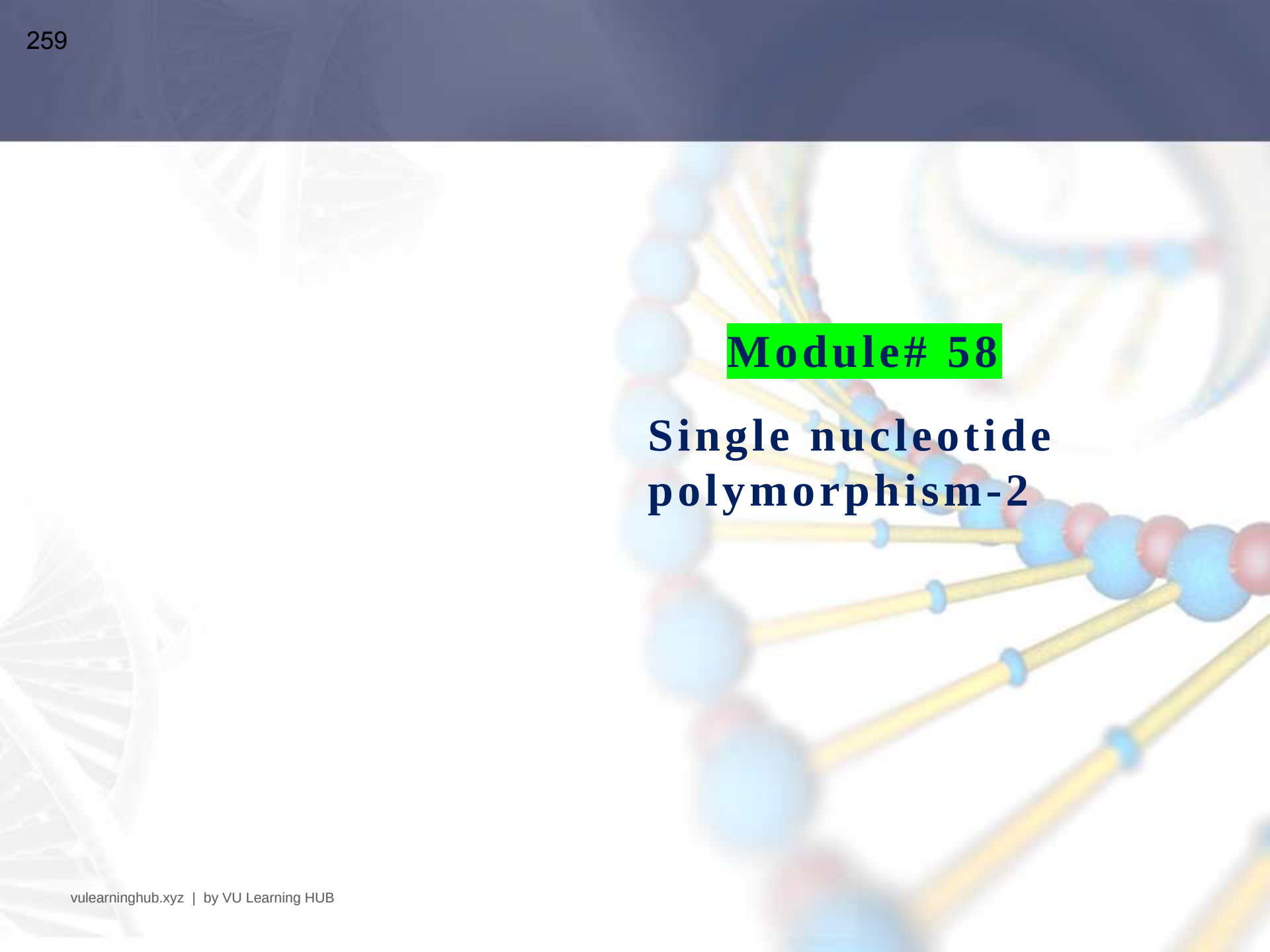
- 
- ▶ SNPs are found in
 - § coding and (mostly) noncoding regions.
 - ▶ Occur with a very high frequency
 - § about 1 in 1000 bases to 1 in 100 to 300 bases.
 - ▶ The abundance of SNPs and the ease with which they can be measured make these genetic variations significant.
 - ▶ SNPs close to particular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

SNPs may / may not alter protein structure



SNPs act as gene markers



The background of the slide features a soft-focus image of DNA double helix structures. One helix is prominent in the foreground on the right, showing blue and red spheres connected by yellow rods. Another helix is visible in the background on the left, rendered in a lighter, semi-transparent style.

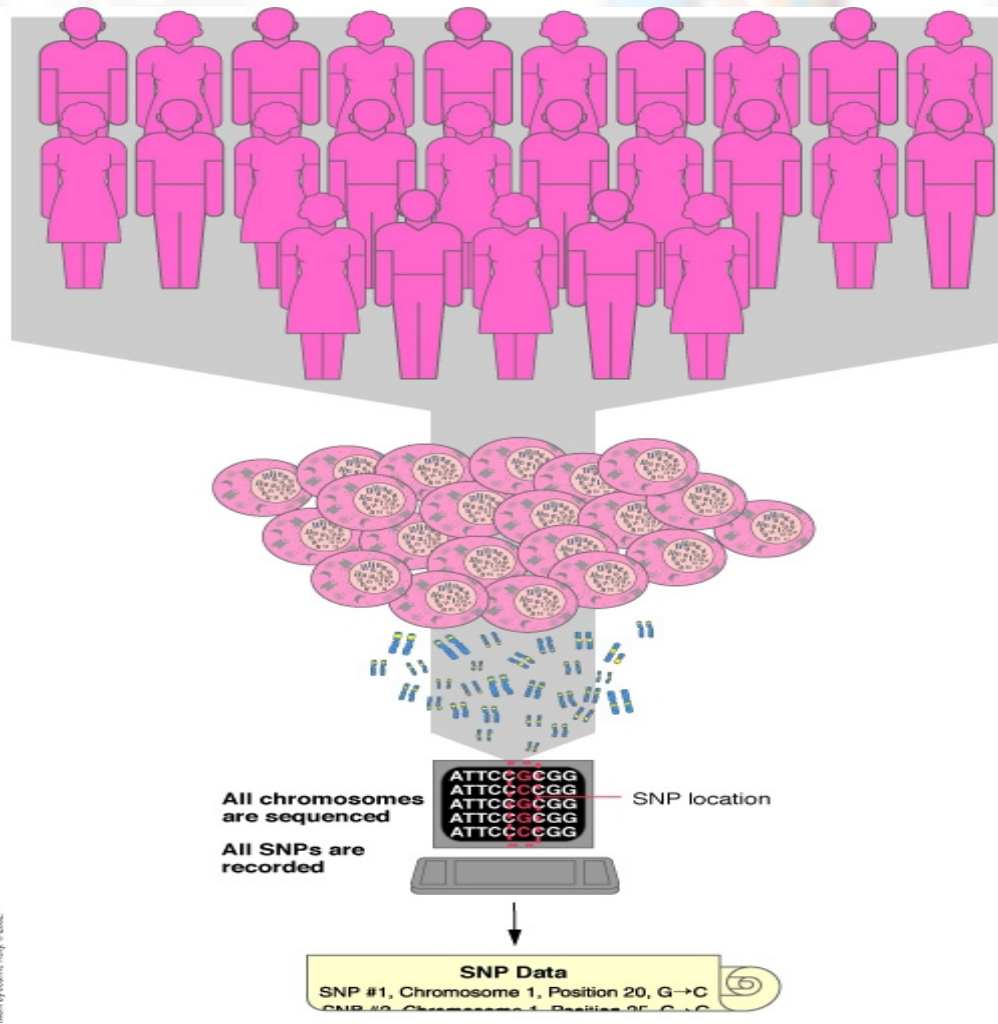
Module# 58

Single nucleotide polymorphism-2

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

SNP Maps



SNP Profiles

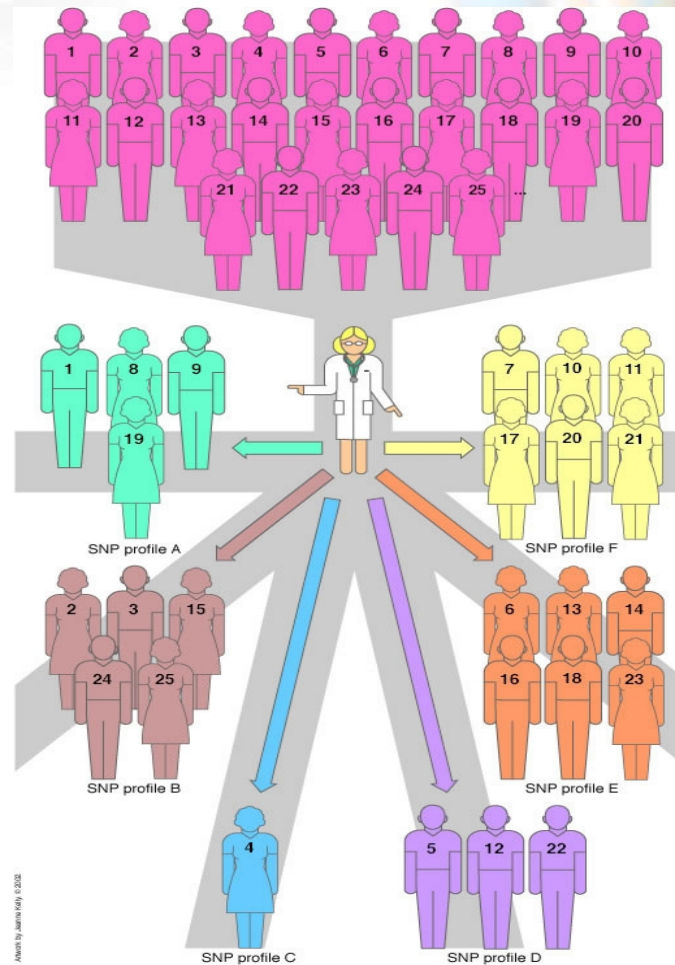
Genome of each individual contains distinct SNP pattern.

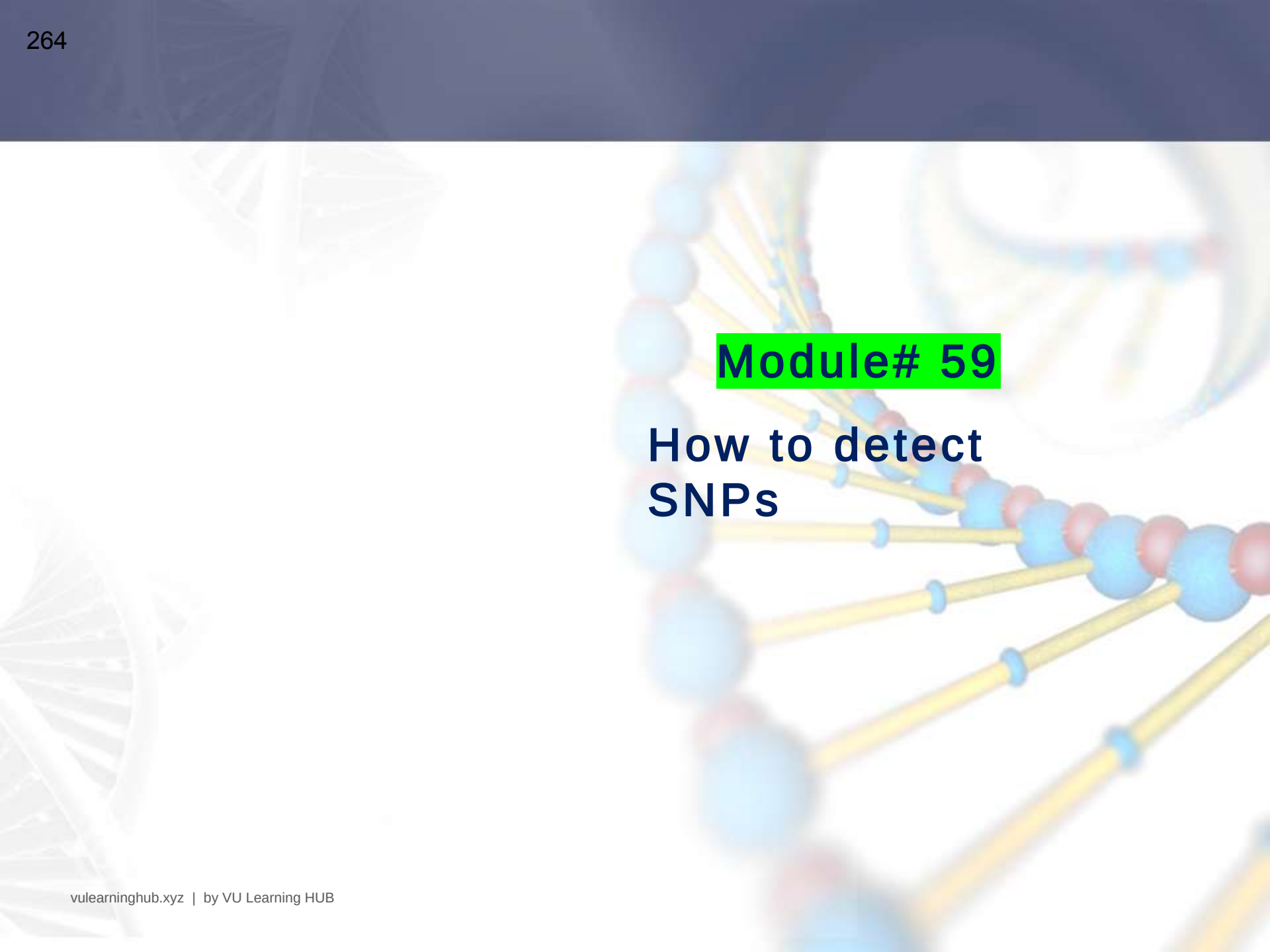
People can be grouped based on the SNP profile.

SNPs Profiles important for identifying response to Drug Therapy.

Correlations might emerge between certain SNP profiles and specific responses to treatment.

SNP Profiles



The background of the slide features a stylized, semi-transparent DNA double helix. The structure is composed of yellow rods representing the sugar-phosphate backbone, with blue and red spheres representing the nitrogenous bases. The helix is shown in a perspective view, winding across the frame. The text is overlaid on this background.

Module# 59

How to detect SNPs

Techniques to detect known Polymorphisms

Hybridization Techniques

- Micro arrays

- Real time PCR

Enzyme based Techniques

- Nucleotide extension

- Cleavage

- Ligation

- Reaction product detection and display

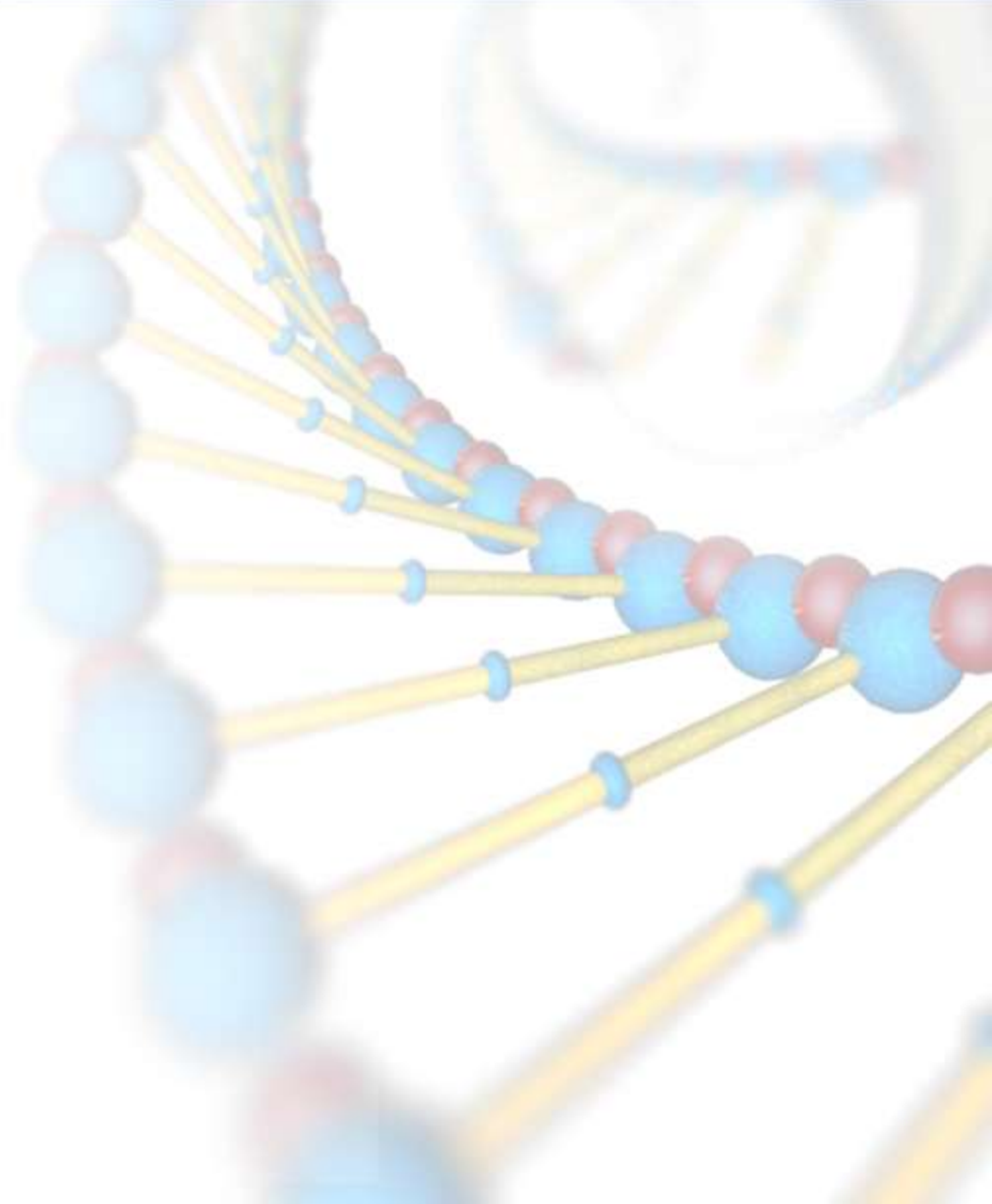
Techniques to detect unknown Polymorphisms

Direct Sequencing

Microarray

Cleavage / Ligation

Electrophoretic mobility assays



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.

Module# 60
SNP Screening

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

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

=> case-control association analysis

case GCCGTTGAC...
 ... GCCATTGAC... .



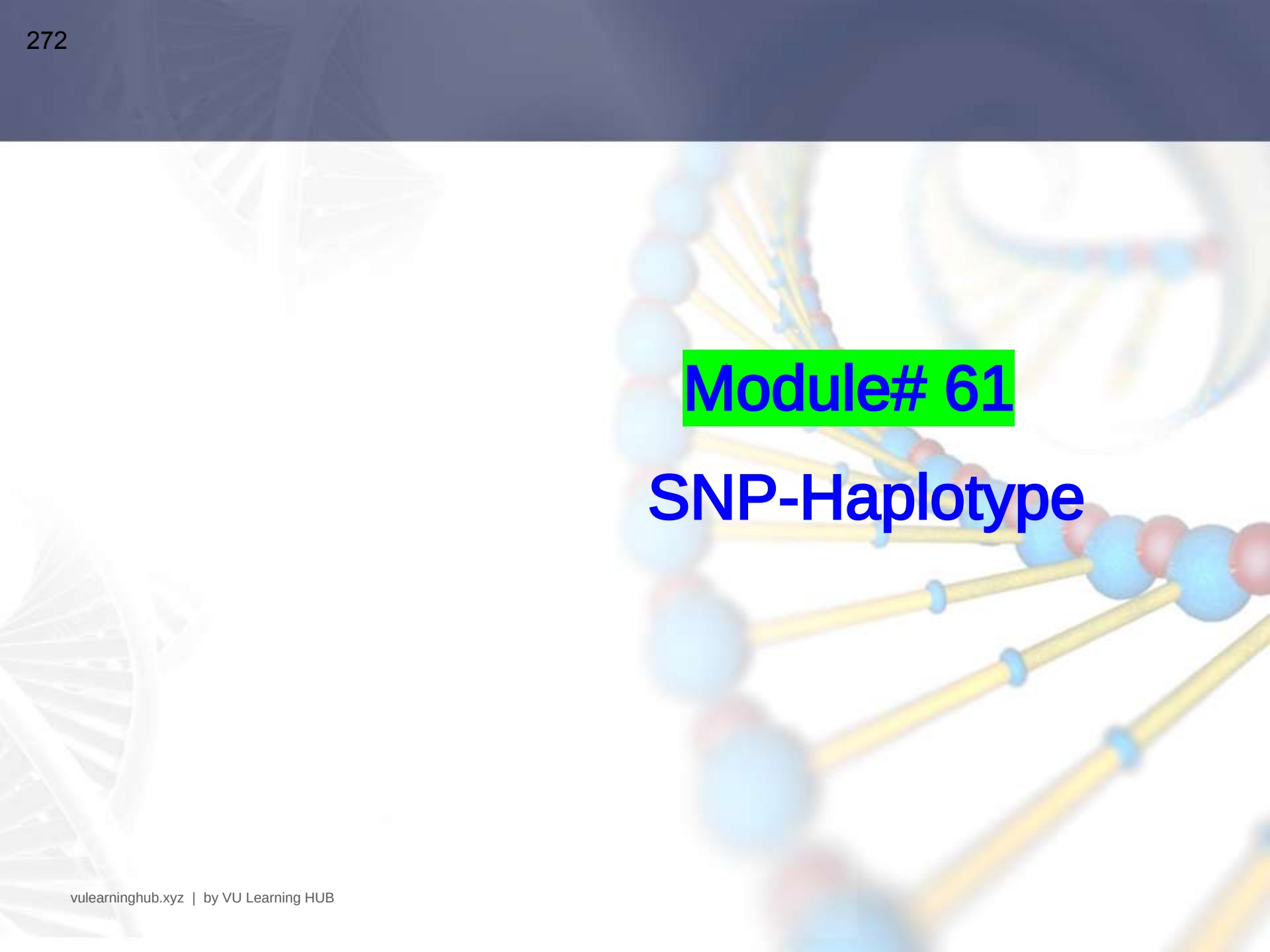
Control ...GCCATTGAC...
 ... GCCATTGAC... .



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



Module# 61

SNP-Haplotype

HAPLOTYPE

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

SNP-Haplotype

Phenotype

BLACK EYE

BROWN EYE

BLACK EYE

BLUE EYE

BROWN EYE

BROWN EYE

SNP

SNP

GAT**A**TTTCGTAC**G**GA-TGAT**G**TTTCGTACT**T**GAATGAT**A**TTTCGTAC**G**GA-TGAT**A**TTTCGTAC**G**GAATGAT**G**TTTCGTACT**T**GAATGAT**G**TTTCGTACT**T**GAAT6
5
4
3
2
1

Haplotypes

AG - 2/6 (BLACK EYE)**GTA** 3/6 (BROWN EYE)**AGA** 1/6 (BLUE EYE)

DNA sequencing



- ❑ 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.

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/>)

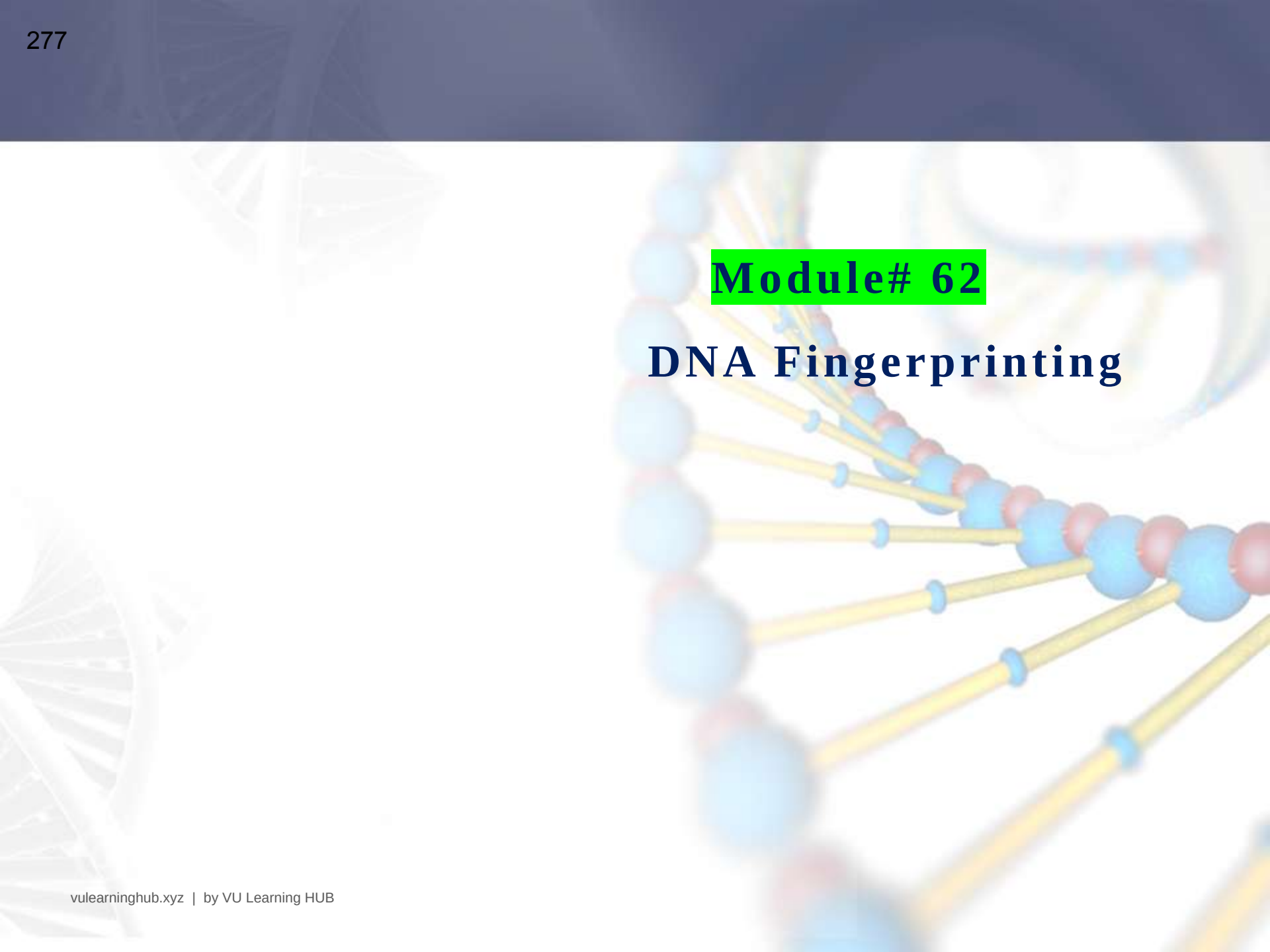
6. HGVbase (<http://hgibase.cgb.ki.se/>)

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/>)

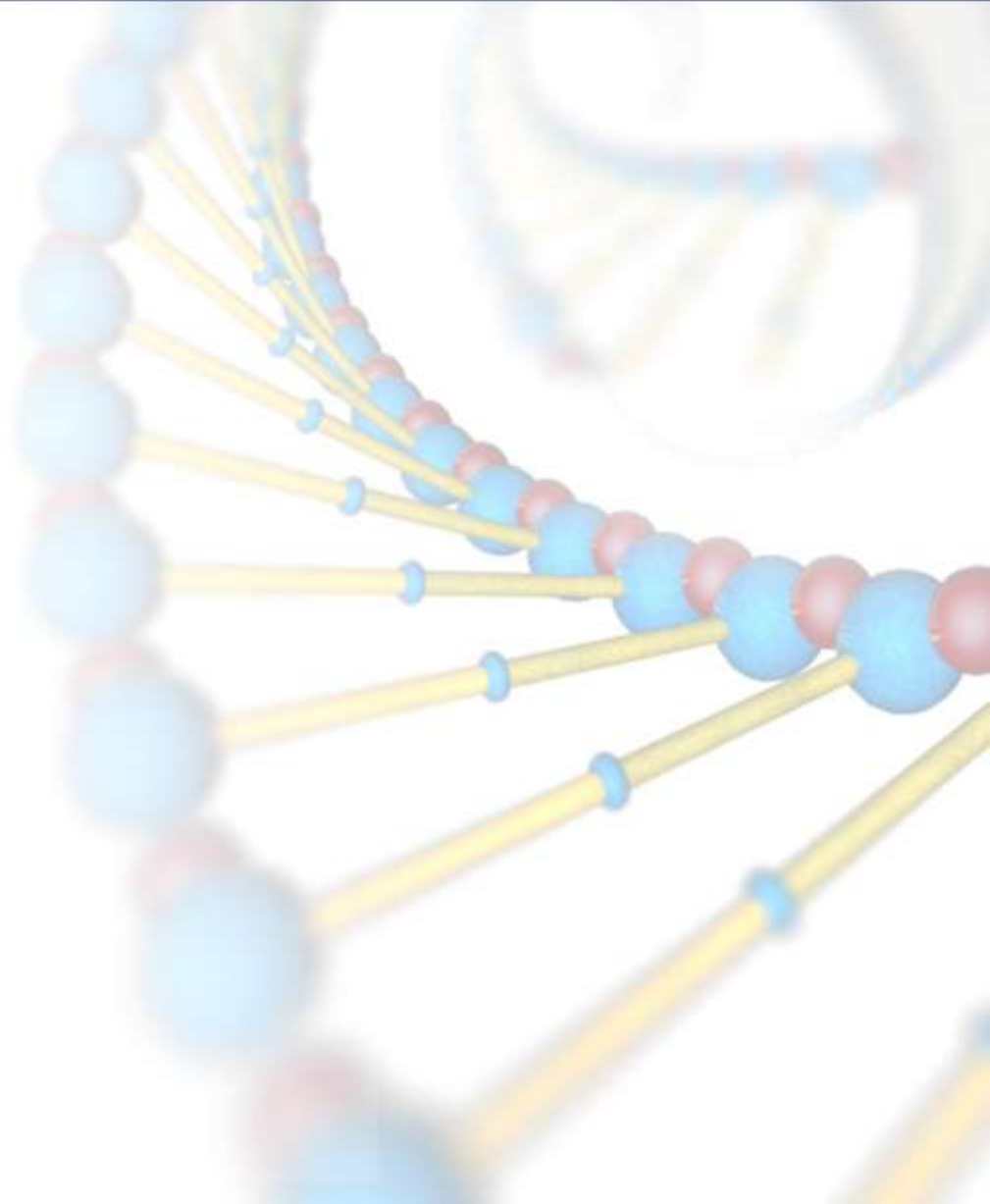


Module# 62

DNA Fingerprinting

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.



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.

Variable Number Tandem Repeats

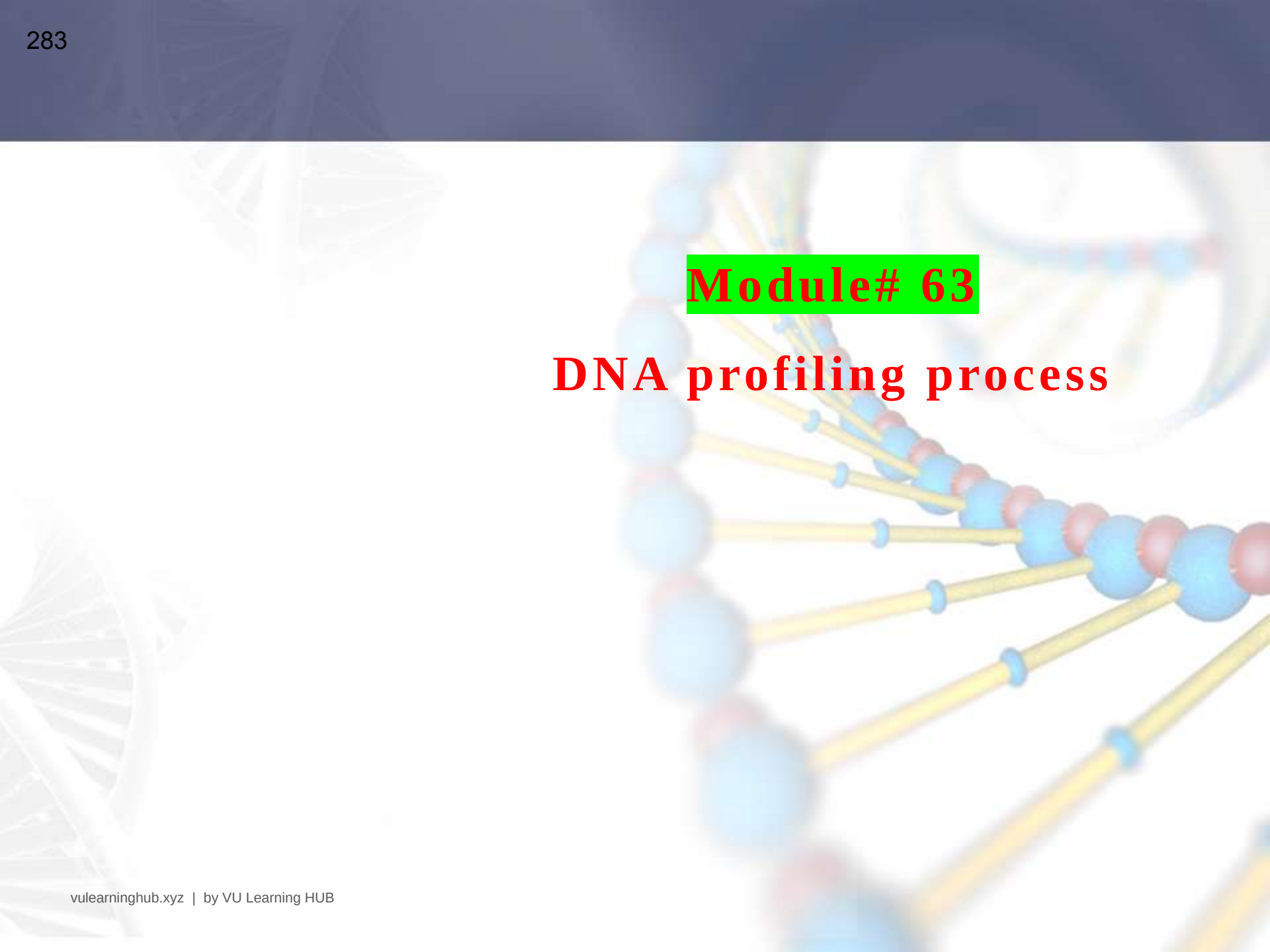
A Variable Number Tandem Repeat (or VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat.

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.

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.



Module# 63

DNA profiling process

The process begins with a sample of an individual's DNA (typically called a "reference sample"). •

The most desirable method of collecting a reference sample is the use of a buccal swab, as this reduces the possibility of contamination. • When this is not available may need to be used to collect a sample of blood, saliva, semen, or other appropriate fluid or tissue from personal items (e.g. toothbrush, razor, etc.) or from stored samples (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.

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.

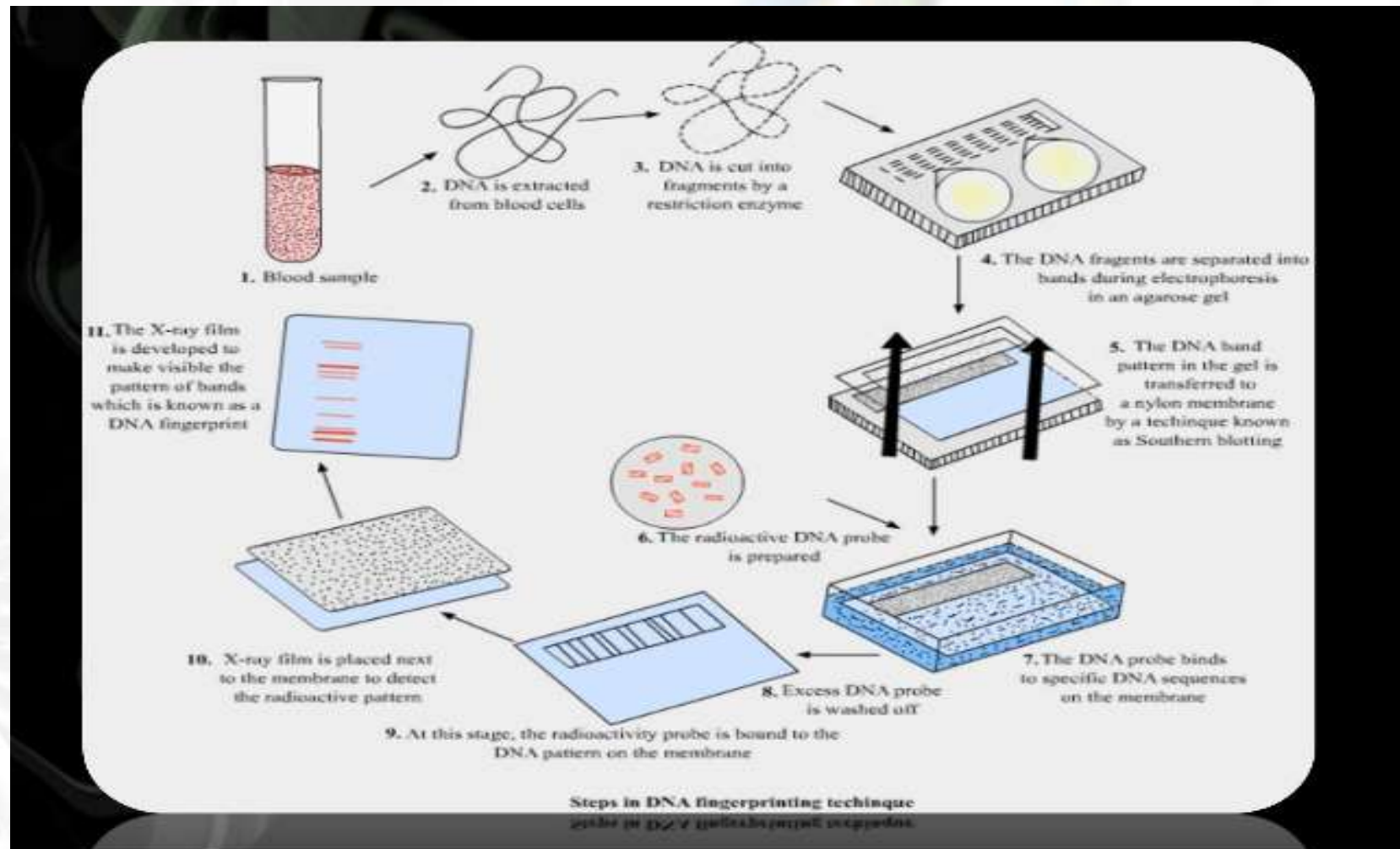
4) Probing :

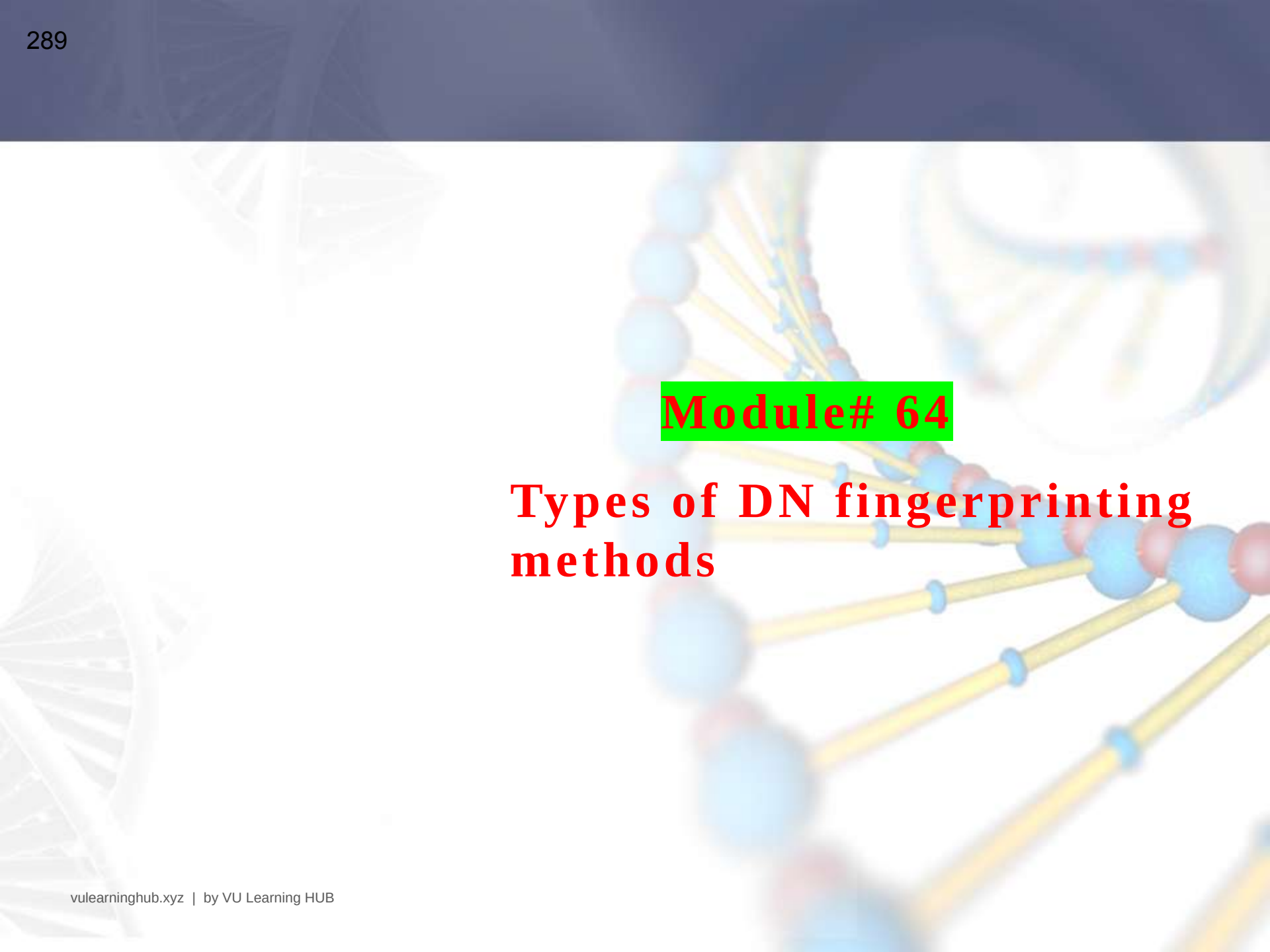
Adding radioactive or colored probes to the nylon sheet produces a pattern called the DNA fingerprint.

- 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.





Module# 64

Types of DN fingerprinting methods

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

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.

Polymerase chain reaction...

chain reaction (PCR) was developed

- The polymerase by Kary 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.

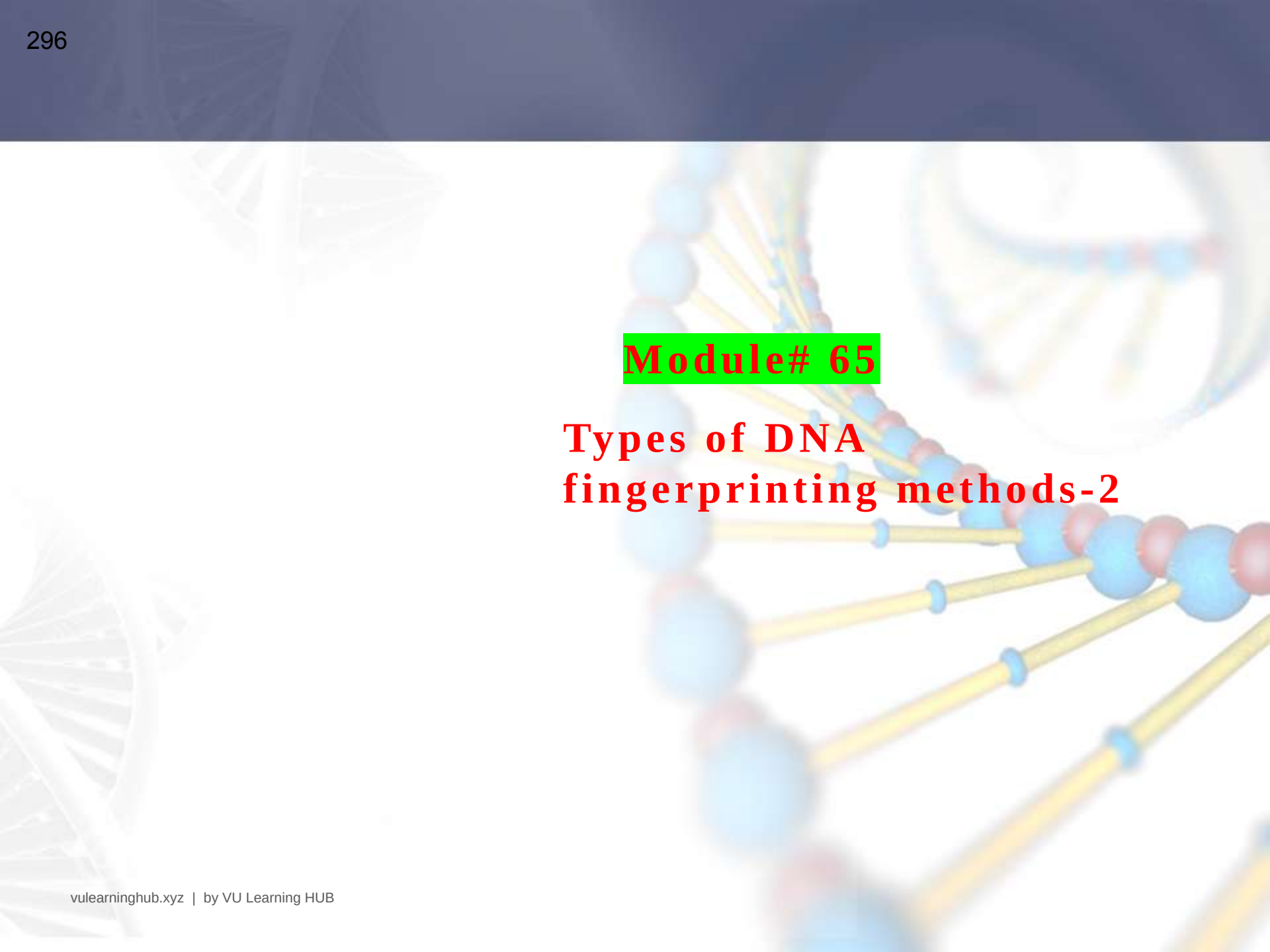
- The PCR analysis amplified isolated regions on the strands of the DNA under examination.

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.

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.



Module# 65

Types of DNA fingerprinting methods-2

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.

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.

DISADVANTAGES OF RAPD...

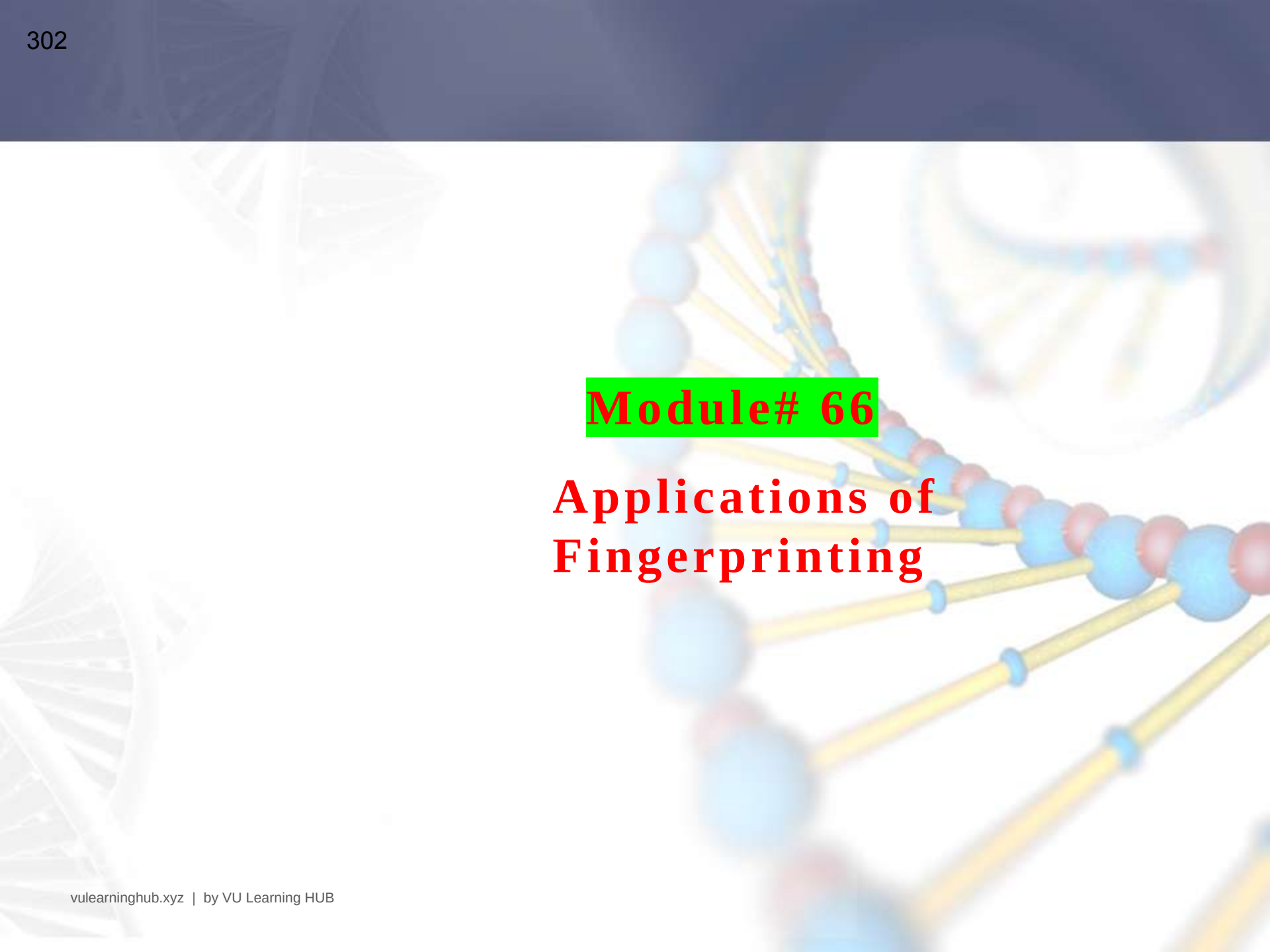
- Nearly all RAPD markers are dominant, i.e. it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies).
- Co -dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are detected only rarely.
- PCR is an enzymatic reaction, therefore, the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions may greatly influence the outcome. Thus, the RAPD technique is notoriously laboratory-dependent and needs carefully developed laboratory protocols to be reproducible.

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.



Module# 66

Applications of Fingerprinting

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

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.