

COMPLETE SLIDES

Methods in Molecular Biology

BIO203

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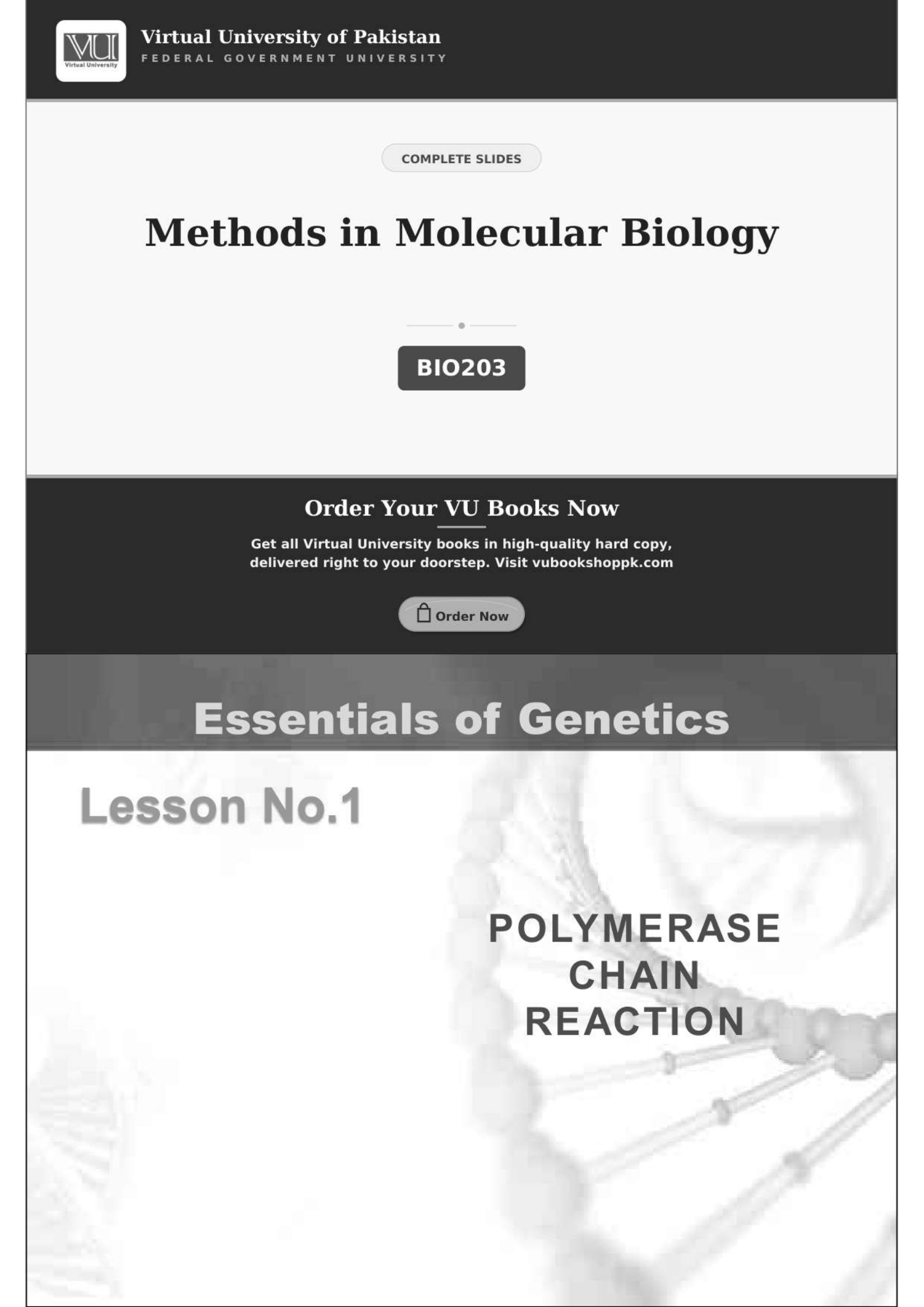
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Essentials of Genetics

Lesson No.1

**POLYMERASE
CHAIN
REACTION**

A faint, grayscale background image of a DNA double helix structure, showing the characteristic twisted ladder shape with spheres representing atoms and lines representing the sugar-phosphate backbone.

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

Lesson No.2

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

Lesson No.3

INGREDIENTS OF PCR

Ingredients of PCR

MAJOR INGREDIENTS

- Microfuge tube
- Thermal cycler
- DNA template
- Primers
- Buffer
- $MgCl_2$

Ingredients of PCR

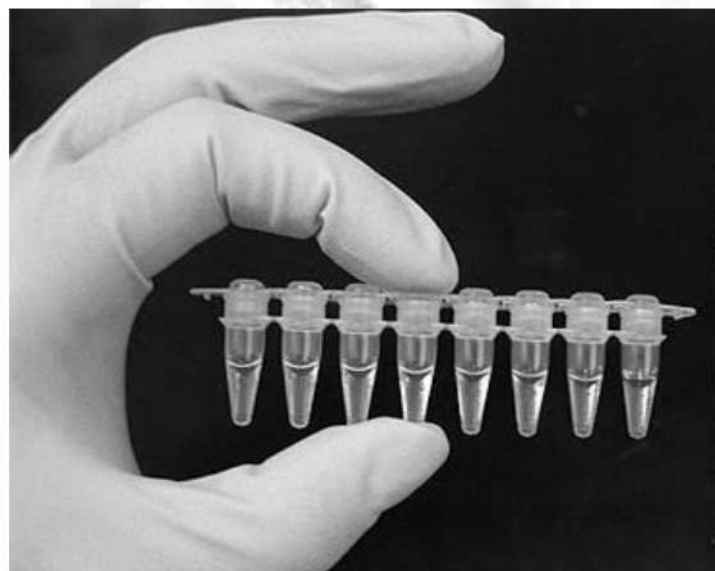
MAJOR INGREDIENTS

- 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.



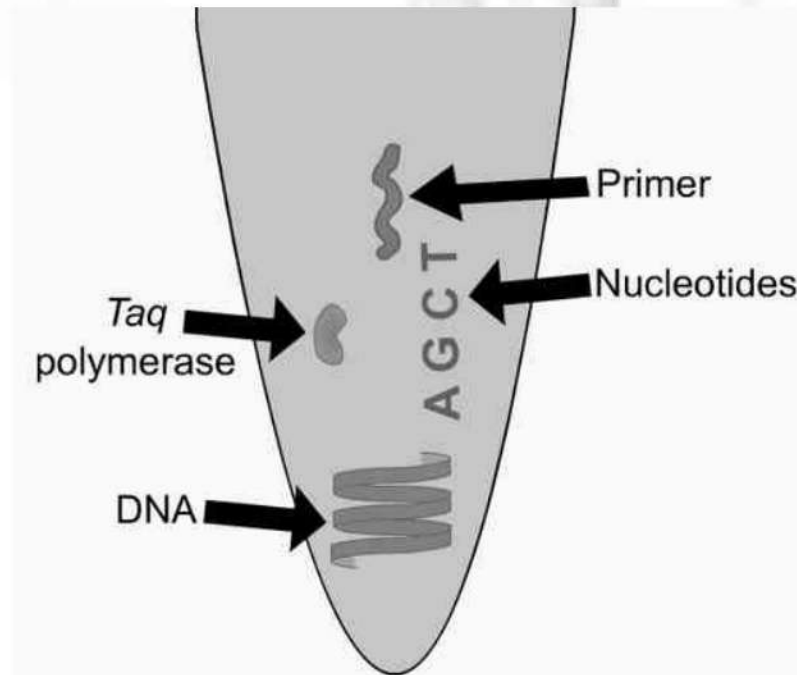
Ingredients of PCR

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

Lesson No.4

PRIMERS FOR PCR

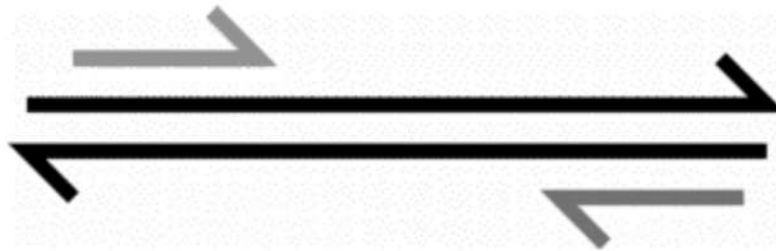
PRIMERS FOR PCR

PRIMERS

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

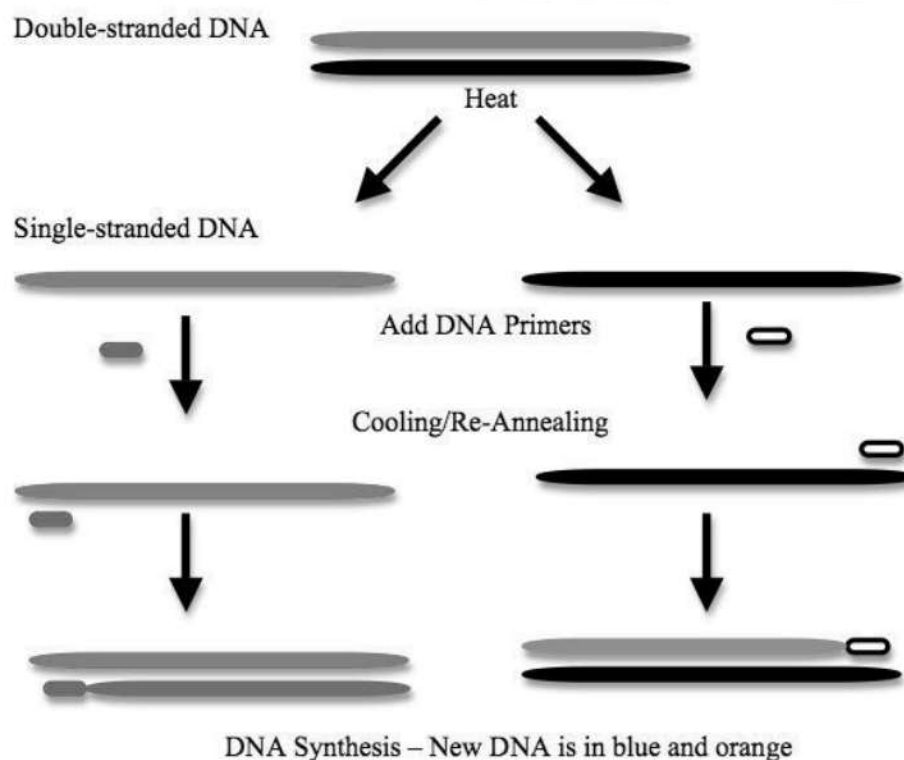
PRIMERS FOR PCR

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

Lesson No.5

DNA POLYMERASES

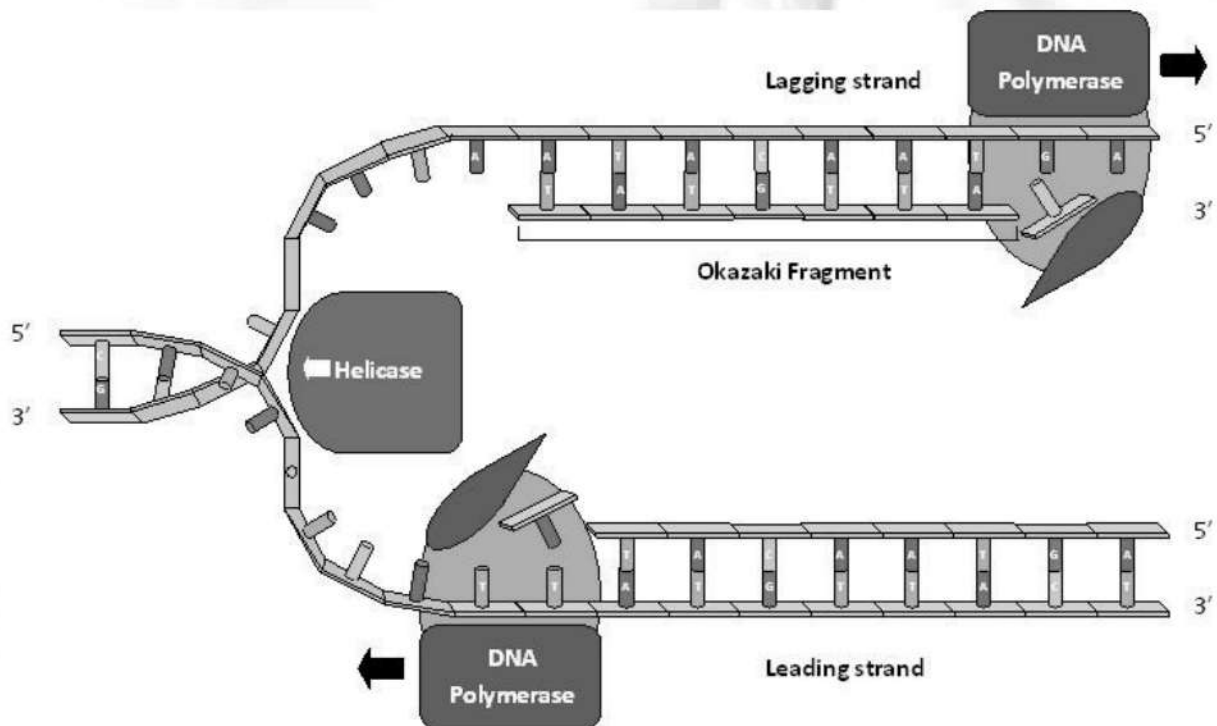
DNA Polymerases

POLYMERASES

Polymerase	Extension Rate (nt/sec)	Source
<i>Taq pol</i>	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

Lesson No.6

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
 - $90-96^\circ\text{C}$, 20-45 seconds
- Annealing temperature
 - Controls specificity of hybridization
 - $40-68^\circ\text{C}$, 20-30 seconds
- Extension temperature
 - Optimized for individual polymerases
 - $70-75^\circ\text{C}$, 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.

Essentials of Genetics

Lesson No.7

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

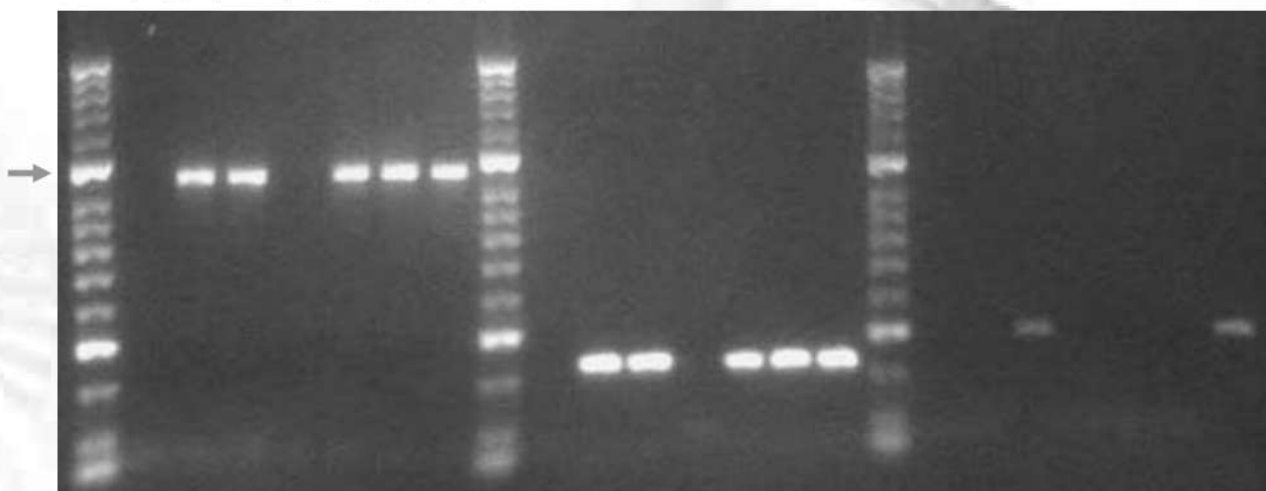
NEGATIVE CONTROL

- 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.

Essentials of Genetics

Lesson No.8

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

Lesson No.9

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.

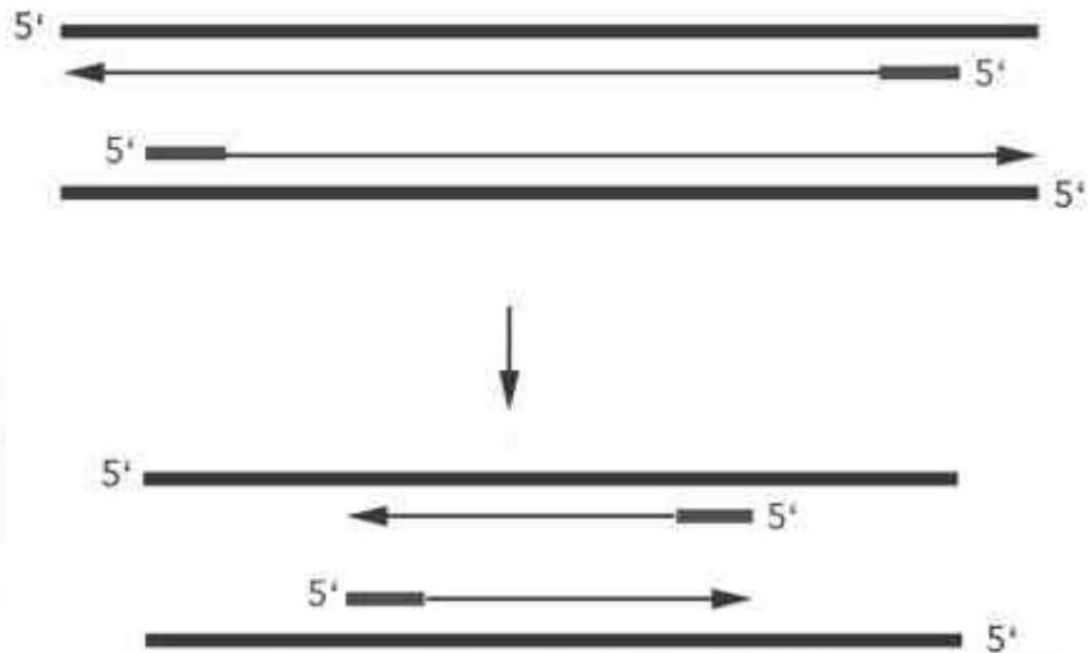
Nested PCR

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.

Essentials of Genetics

Lesson No.10

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.

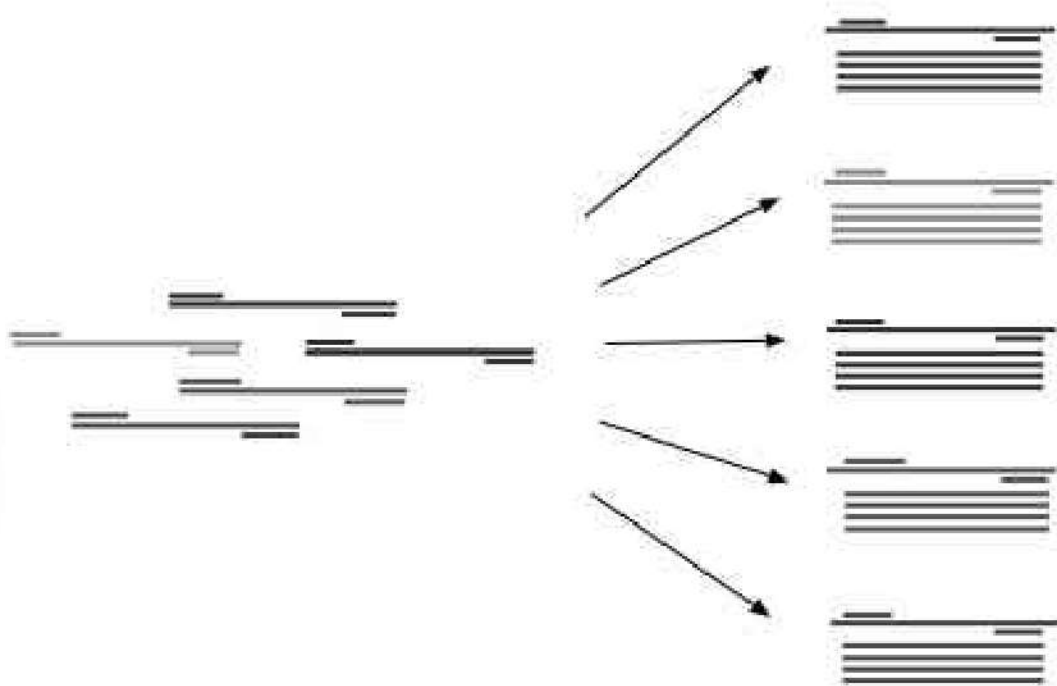
Multiplex PCR

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

Lesson No.11

REVERSE TRANSCRIPTASE PCR

Reverse Transcriptase PCR

REVERSE TRANSCRIPTASE PCR

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

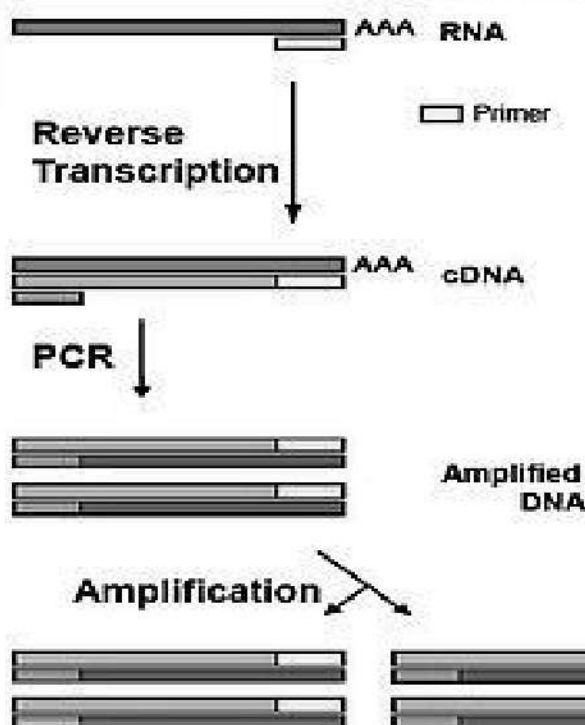
Reverse Transcriptase PCR

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

Lesson No.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 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

- 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

Lesson No.13

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

Lesson No.14

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

Lesson No.15

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

Lesson No.16

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.