BHARATHIDASAN UNIVERSITY

Tiruchirappalli- 620024,

Tamil Nadu, India

Programme: M.Sc., Biomedical Science

Course Code: 18BMS59C17

Course Title: Immune & Molecular Diagnostics

Unit-IV

Basic Molecular Methods

Dr. R. POORNIMA

Guest Faculty

Department of Biomedical Science

Unit IV:

Basic Molecular Methods: Types of mutations- PCR based mutation detection methods- for known & unknown mutations- ASP, ASOP, TTGE, DGGE, Heteroduplexing method, SSCP & sequencing- Discuss: Primer designing for PCR- Collection, processing and storage of sample- RNA extraction- cDNA preparation, RT-PCR- Principle, methods & Applications- Real time PCR- Principle, methods & Applications. Types of dyes (SYBR Green) and probes (Taq-Man). DNA finger printing- micro (STRs) and minisatellites (VNTRs)- principle and applications.

PRESENTATION: 2

Primer Designing for PCR

- **Primer Design** is a critical step in the Polymerase Chain Reaction (PCR) process, as primers determine the specificity and efficiency of the amplification.
- Proper design ensures successful amplification of the target sequence while minimizing non-specific products.

PCR Primer Design

Forward primer	5' GCTAAATGTTCAGGCTGTGG 3'
Reverse primer	5' GGAATCAAACGGAATGACCG 3'

Top tips for primer design:

- ✔ Length: ~20 nucleotides
- ✔ GC content: ~50%
- ✔ GC clamp: primers end in at least two G or C nucleotides
- ✔ No complementary regions between primer pairs
- ✔ Melting temperature (T_m): ~55-65°C

Considerations for Primer Design:

1. **Length:** Primers are typically 18-25 nucleotides long, providing a balance between specificity and efficient binding.
2. **Melting Temperature (Tm):** The optimal Tm for primers is generally between 55-65°C, ensuring stable annealing during the PCR cycle.

3. GC Content: A GC content of 40-60% is ideal for stable primer binding without excessive secondary structures.

4. Specificity: Primers should be unique to the target sequence to prevent non-specific amplification.

5. Avoiding Secondary Structures: Primer sequences should avoid internal hairpins, dimers, or self-complementarity, which can reduce PCR efficiency.

Collection, Processing, and Storage of Samples

Proper handling of samples is essential for reliable PCR results.

Collection:

- **Sterile Techniques:** Use sterile equipment and techniques to avoid contamination.
- **Appropriate Containers:** Collect samples in appropriate containers to maintain integrity.

Processing:

- **Sample Preparation:** Lyse cells or tissues to release nucleic acids.
- **Filtration/Centrifugation:** Remove debris to obtain a clear lysate.

Storage:

- **Short-term:** Store samples at 4°C for a few hours.
- **Long-term:** Store at -20°C or -80°C to prevent nucleic acid degradation.

RNA Extraction

- RNA extraction is a laboratory technique that isolates RNA molecules from biological samples.
- It’s a key step in many molecular techniques, such as quantitative polymerase chain reaction, microarray assays, and Northern blotting
- It’s a crucial step for studies involving gene expression.

Steps in RNA Extraction:

1.Lysis:

Use a detergent-based buffer to lyse cells and release RNA.

2. Phase Separation:

It is a process that separates a solution into three phases after centrifugation. Add phenol-chloroform to separate RNA from DNA and proteins.

3. Precipitation:

It is a cost effective method for purifying RNA and concentrating samples. It involves using a salt solution to neutralize the charge on the RNA’s sugar backbone, making it less hydrophilic and less soluble in water. Ethanol is added to create an electrostatic attraction that causes the RNA to drop out of the solution. Use isopropanol or ethanol to precipitate RNA from the aqueous phase.

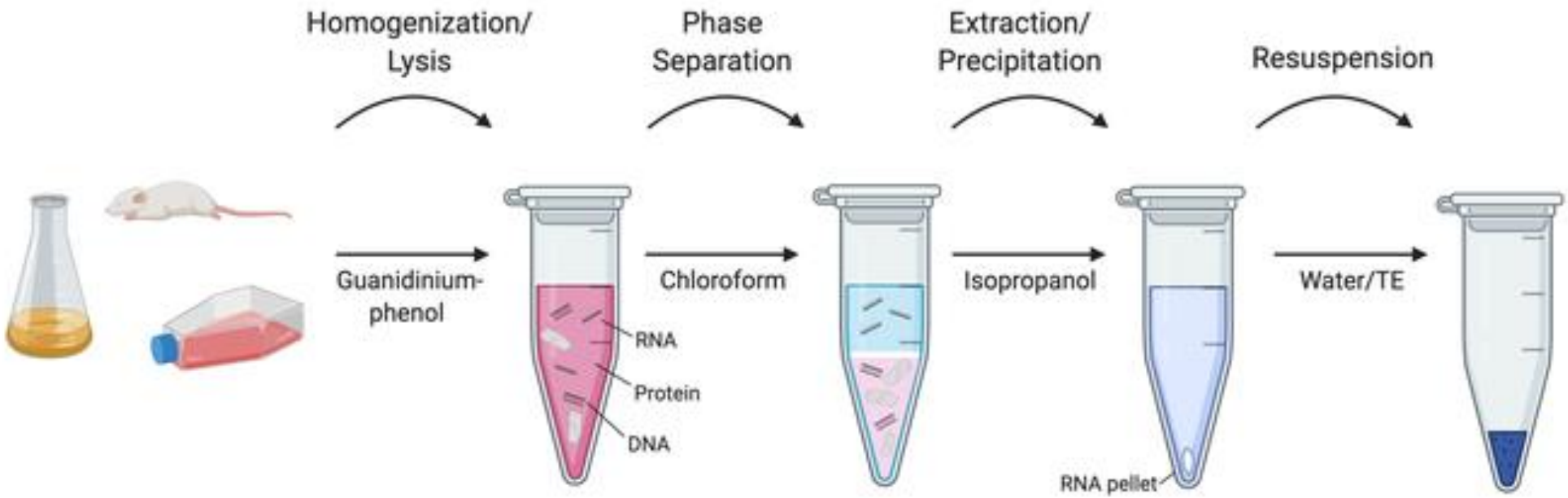
4. Washing:

Removes contaminants like proteins from the RNA pellet. This is typically done with 70- 80% ethanol, which solubilizes salts but not nucleic acid.

5. Resuspension:

It prepares RNA pellet for analysis by placing it in a buffer or water. Wash RNA with ethanol and resuspend in RNase-free water.

RNA Extraction



cDNA Preparation and RT-PCR

- **cDNA Preparation** or cDNA synthesis is a process that involves creating complementary DNA (cDNA) from an RNA template through reverse transcription which is necessary for RT-PCR.

Steps in cDNA Preparation:

1. **RNA Template:** Use purified RNA as the template.
2. **Reverse Transcription:** Use reverse transcriptase to synthesize cDNA from RNA.
3. **Primer Selection:** Use oligo-dT primers, random hexamers, or gene-specific primers based on the application.

Steps in cDNA Preparation:

1. Prepare the sample:

Prepare the RNA sample for cDNA synthesis. This can include removing genomic DNA and selecting a transcriptase.

2. Prepare the reaction mixture:

Combine the RNA sample with other components, scuh as primers, dNTP mix, and RNAseOut.

3. Incubate:

Incubate the reaction at different temperatures for different amounts of time

4. Inactivate the enzyme:

Inactivate the enzyme at a higher temperature for a set amount of time.

5. Dilute and store:

Dilute the reaction with water and store the cDNA product at a low temperature.

Reverse Transcription PCR (RT-PCR)

Reverse Transcription PCR (RT-PCR) is a technique to amplify cDNA, enabling the study of gene expression.

Principle of RT-PCR:

- RT-PCR involves converting RNA into cDNA, which is then amplified using PCR.

Methods:

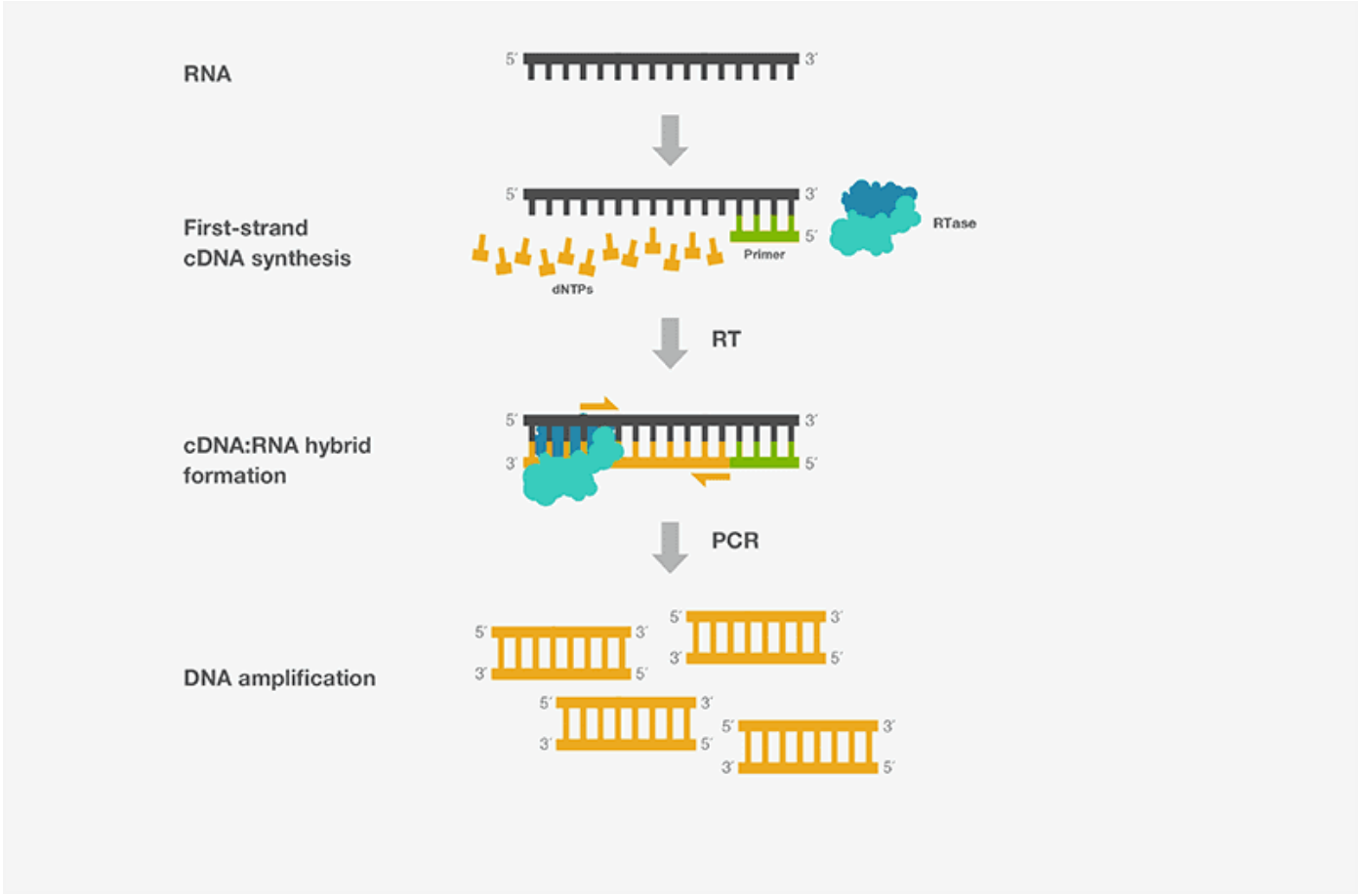
1. **One-Step RT-PCR:** Combines reverse transcription and PCR in a single tube.

2. **Two-Step RT-PCR:** Separates the reverse transcription and PCR steps, allowing greater flexibility in reaction conditions.

Applications:

- **Gene Expression Analysis:** Quantifying mRNA levels of specific genes.
- **Disease Diagnosis:** Detecting viral RNA in infections (e.g., SARS-CoV-2).

Reverse Transcription PCR (RT-PCR)



Real-Time PCR (qPCR)

Real-Time PCR also known as quantitative PCR is laboratory technique used to amplify and simultaneously quantify a targeted DNA molecule. It is a powerful tool in molecular biology for detecting and measuring the amount of DNA or RNA in a sample. It is a quantitative PCR technique that monitors the amplification of DNA in real-time using fluorescent dyes or probes.

Principle of Real-Time PCR:

As the DNA is amplified, a fluorescent signal increases proportionally, allowing quantification.

Methods:

1. **SYBR Green:** A dye that intercalates with double-stranded DNA, emitting fluorescence upon binding.
2. **TaqMan Probes:** Use a fluorophore and quencher; fluorescence increases as the probe is cleaved during amplification.

Process:

1. **DNA/RNA Extraction**: The nucleic acid is extracted from the sample (e.g., blood, tissue, or cells).
2. **Reverse Transcription (if RNA)**: RNA is converted to complementary DNA (cDNA) using reverse transcriptase.
3. **PCR Amplification**: Specific primers and a DNA polymerase enzyme are used to amplify the target DNA sequence.
4. **Real-Time Detection**: Fluorescent dyes or probes are used to monitor the amplification process in real-time. The fluorescence increases as the DNA is amplified, allowing for quantification.

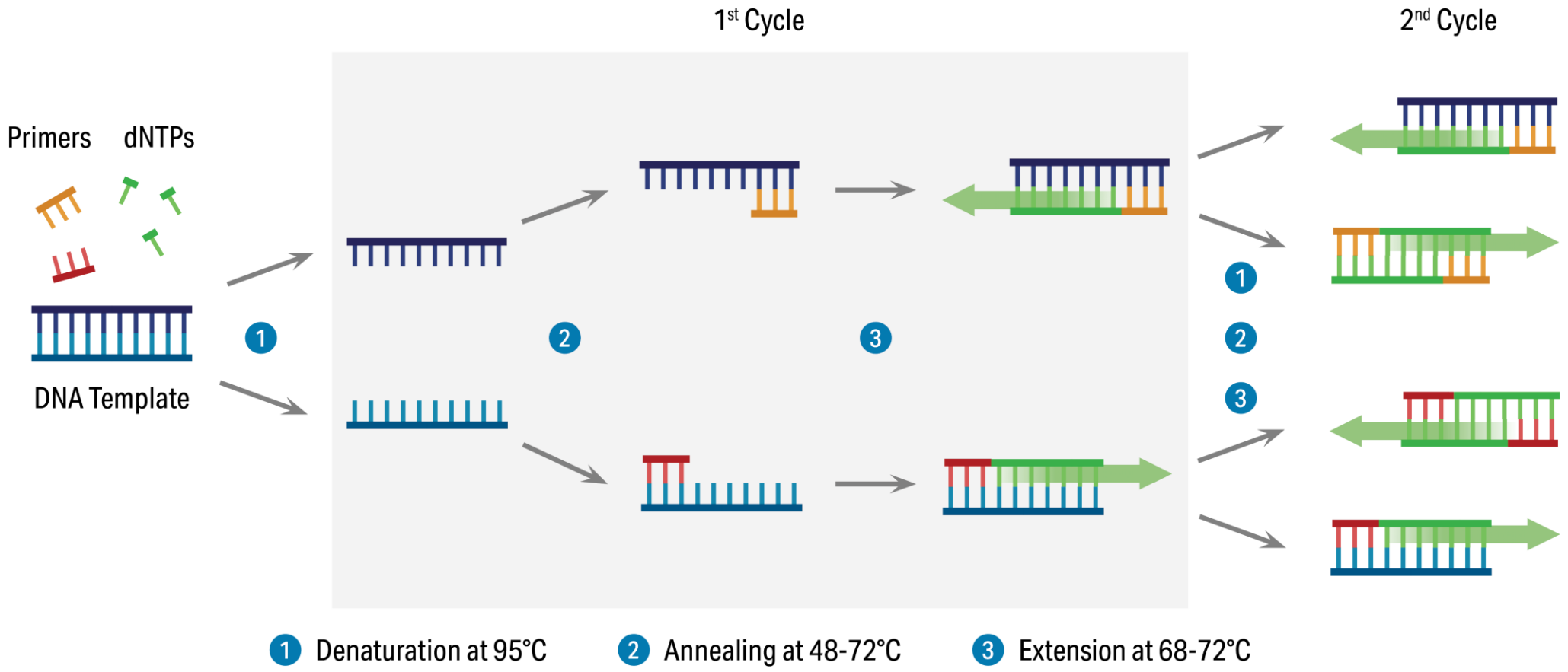
Key Features:

- **Sensitivity**: qPCR is highly sensitive, capable of detecting even small amounts of DNA or RNA.
- **Specificity**: The use of specific primers ensures that only the target sequence is amplified.
- **Quantification**: The technique provides quantitative data, indicating the amount of the target DNA or RNA present in the sample.
- **Speed**: Results can be obtained quickly, often within a few hours.

Applications:

- **Medical Diagnostics**: Detecting and quantifying pathogens (e.g., viruses, bacteria) in clinical samples.
- **Gene Expression Analysis**: Measuring the expression levels of genes in different tissues or conditions.
- **Genetic Testing**: Identifying genetic mutations or variations.
- **Forensic Science**: Analyzing DNA samples from crime scenes.
- **Food Safety**: Detecting foodborne pathogens or GMOs.

Real-Time PCR (qPCR)



Types of Dyes and Probes

Dyes:

1. SYBR Green:

1. Binds to double-stranded DNA.
2. Emits fluorescence upon binding.
3. Advantages: Cost-effective and easy to use.
4. Disadvantages: Binds non-specifically to any double-stranded DNA, including primer-dimers.

Probes:

1.TaqMan Probes:

1. Sequence-specific probes with a fluorescent reporter and quencher.
2. Advantages: High specificity, reducing false positives.
3. Disadvantages: More expensive and requires specific design for each target.

2.Molecular Beacons:

1. Hairpin-shaped probes that fluoresce upon hybridization with the target.
2. Useful in detecting mutations and SNPs.

3.Scorpion Probes:

1. Combines a primer and probe in one molecule for increased efficiency.

Conclusion:

- Primer design, sample handling, RNA extraction, and PCR techniques are fundamental aspects of molecular biology.
- RT-PCR and Real-Time PCR are powerful methods for detecting and quantifying nucleic acids, with applications spanning research, diagnostics, and personalized medicine.
- The choice of dyes and probes like SYBR Green and TaqMan enhances the specificity and sensitivity of these assays, making them indispensable tools in modern science.

ACKNOWLEDGEMENT

- The presentation is being used for educational and non-commercial purposes.
- Thanks are due to all the original contributors and entities whose pictures were used to create this presentation.