Reverse Transcriptase Polymerase Chain Reaction, RT-PCR

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Objectives of RT-PCR

- To amplify the specific segment of RNA, resulting in billions of copies of a single RNA segment.
- To diagnose certain infections, genes, and study gene expression.

Polymerase chain reaction (PCR)

- Polymerase chain reaction (PCR) is a temperature-dependent nucleic acid amplification technique used to amplify the DNA or RNA in vitro enzymatically.

- Developed by Kary Mullis and his associates at mid – 1980s, it is a very powerful and most important tool in modern biology – molecular biology and genetics.
- It combines the principle of nucleic acid hybridization with the principle of nucleic acid replication. Using this non-culture-based nucleic acid amplification technique, we can produce billions of copies of a single segment of DNA or RNA in a very short time.
- Since its development, several modifications have been made, and now there are different types of PCR techniques available for different purposes. Reverse transcriptase PCR and Quantitative PCR (qPCR) are the most commonly used PCR types.

Reverse Transcriptase Polymerase Chain Reaction, RT-PCR

- Reverse transcriptase polymerase chain reaction, RT-PCR, is a type of PCR technique that enzymatically amplifies the RNA in vitro.
- It is the only type of PCR that can amplify the RNA. It uses a reverse transcriptase enzyme in addition to the other basic components of the PCR.
- First, the sample RNA is converted to complementary DNA (cDNA) in reverse transcription, catalyzed by the reverse transcriptase enzyme. These cDNA molecules are then used as a template for amplification in the PCR process.
- RT-PCR is used to analyze the mRNA or micro RNA and study gene expression.

Principle of RT-PCR

- RT-PCR combines the reverse transcription process with the conventional PCR process. The sample RNA is first converted to double-stranded DNA (complementary DNA) by reverse transcriptase enzyme in the reverse transcription process.
- The cDNA can then be thermally broken down into two single-stranded DNA templates. In these ssDNA templates, primers can anneal to their complementary sequences based on the nucleic acid hybridization principle.
- DNA polymerase then elongates the primer by sequentially adding the nucleotides to the 3’ end and generates a dsDNA following the principle of DNA replication. These three processes, denaturation, annealing, and elongation, are repeated in a cyclic manner regulating the reaction temperature and resulting in millions of copies of the cDNA.

Requirements (Enzymes) of RT-PCR

- 1. Sample (RNA)
- 2. Reverse Transcriptase Enzyme
- 3. DNA Polymerase Enzyme
- 4. Primers : Three different types of primers are used in RT-PCR;

Random Primers, Oligo Primers and Sequence-specific Primers.

- 5. Deoxynucleotide Triphosphates(dNTPs)

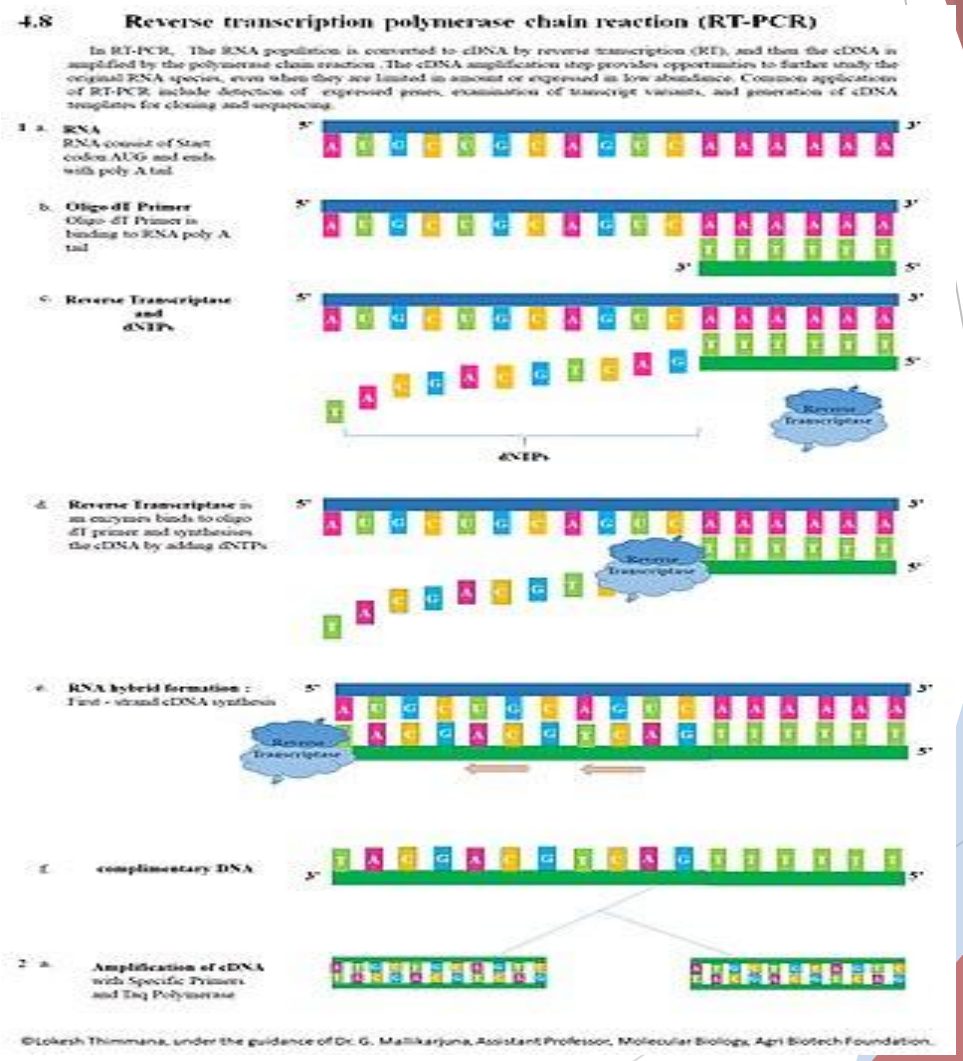
Deoxyadenosine triphosphate (dATP),

Deoxyguanosine triphosphate (dGTP),

Deoxythymidine triphosphate (dTTP), and

Deoxycytidine triphosphate (dCTP).

- 6. PCR Buffers and Other Chemicals
- 7. Thermocycler (PCR Machine)



Types of RT-PCR

Based on whether the reverse transcription and the amplification steps occur either in a single reaction (or tube) or in two separate reactions (or tubes), RT- PCR can be classified into two types:

1. One-Step RT-PCR

It is a type of RT – PCR where the reverse transcription and the amplification reactions occur in a single tube. All the required components are added in a single tube. First, reverse transcription occurs, forming cDNA, which is then amplified in a PCR process.

2. Two-Step RT-PCR

It is another type of RT – PCR where the reverse transcription and the amplification process occur in two separate tubes. In the first tube, a reverse transcription reaction takes place, yielding cDNA. These cDNAs are then transferred to another tube where the PCR mixture is added, and the cDNAs are amplified.

Advantages and Disadvantages

Advantages of One–Step RT – PCR over Two–Step RT – PCR

- It has a simple and easy handling setup.
- It has higher accuracy and specificity.
- It has a lesser chance of contamination.
- It is a cheaper and faster method.

Disadvantages of One-Step RT-PCR over Two-Step RT-PCR

- It detects fewer templates per reaction mixture due to using multiple chemicals in a single reaction tube.
- Due to lower template detection, it requires a larger template for starting.
- It does not permit the storage and further analysis of the cDNA formed during the reaction.
- There is a higher chance of primer – dimer and non–specific binding.
- The chance of reaction failure is comparatively high.

Advantages and Disadvantages

Advantages of Two-Step RT – PCR over One-Step RT – PCR

- It allows us to store cDNA formed by reverse transcription.
- It has higher efficiency, accuracy, and reliability and detects larger templates per reaction mixture.
- Comparatively, lower chance of reaction failure, non-specific binding, and primer–dimer bonding.

Disadvantages of Two–Step RT – PCR over One-Step RT – PCR

- There is a higher chance of contamination.
- It is a more complex and tedious process requiring more resources and a well-trained person.

Steps/Procedure of RT-PCR

The core procedure can be broadly classified into two phases; reverse transcription and amplification. The procedure also varies on one-step and two-step RT – PCR. But, the general steps involved in both of the types are the same and can be summarized into four stages;

- Preparatory stage,
- reverse transcription,
- amplification, and
- product analysis stage,

1. Preparatory Stage

- It is the initial stage where RNA extraction is done, and all the reaction mixture is prepared. First, all materials are arranged, safety measures are taken, the PCR reaction preparation area is cleaned, all the reagents are brought to working temperature, and the sample is extracted or brought from storage.

- In the one-step RT-PCR, sample RNA, reverse transcriptase enzyme, RNase H, primers, DNA polymerase, dNTPs, buffers, and all other components are added in a specified and pre-calculated amount in a single reaction tube. The tube is then loaded into a thermocycler for further processing.

- In the two-step, RT-PCR, sample RNA, reverse transcriptase, RNase H, primers, dNTPs, and other buffers and chemicals for reverse transcription are loaded in a tube. Then the tube is subjected to a specified temperature in a thermocycler where cDNAs are formed.

2. Reverse Transcription

- It is the primary step where the RNA is converted into cDNA, which then undergoes amplification.

- All the reaction mixture, including reverse transcriptase, RNase H, dNTPs mixture, primers, nuclease-free water, reverse transcription buffer, and other components in one-step RT-PCR and DNA polymerase and other amplification components in the two-step RT-PCR are added in a tube and subjected to a temperature of 40 – 50°C for 10 minutes to 30 minutes in a thermocycler. At this temperature, the primer will bind to the respective site of the RNA sample, and the reverse transcriptase enzyme will synthesize cDNA by adding the free dNTPs.

3. Amplification

- This step is similar to the amplification process of other PCR techniques for DNA amplification. In a one-step RT-PCR, the same reaction mixture is subjected to an amplification process. At the same time, in the two-step RT-PCR, the cDNA is isolated and placed in another tube where DNA polymerase, primers, PCR buffer, dNTPs, and other chemicals are added. Then the tube is placed in a thermocycler for amplification.

- The amplification step includes denaturation, annealing, and elongation occurring cyclically one after another for a certain number of cycles pre-programmed by the user.

4. Product Analysis Stage

- It is the final step where the reaction mixture subjected to PCR is analyzed to confirm that desired amplification is achieved. The gel electrophoresis method is mostly used for product analysis. In real-time RT-PCR, there is no need for this additional step.

Applications of RT-PCR

- Study Gene Expression
- The Traditional Northern Blot technique requires a larger mRNA sample to analyze and study the gene expression. However, using RT-PCR, we can amplify the minute mRNA sample and study the sequence of nucleotides, thus analyzing the gene expression. It is used in studying and identifying multidrug-resistant genes and their expressions in pathogens.

- Identification of Unknown Species
- RT-PCR is used to identify viruses like HIV, SARS viruses, dengue viruses, HCV, etc. Besides, other microorganisms and even higher organisms are identified by studying their rRNA and mRNA.

- Infectious Disease Diagnosis
- Diagnosis of different types of viral infection, bacterial infection, fungal and parasite infection, cancer cell, and genetic diseases are done using the RT-PCR technique in clinical laboratories.

- Gene Insertion and Gene Therapy Study
- RT-PCR is used to prepare cDNA from eukaryotic mRNA, which lacks introns and can be inserted into prokaryotes. RT-PCR is used in monitoring the result of gene insertion and gene therapy. These procedures are supposed to show particular gene expression and code for a particular protein, hence translating specific types of mRNA sequence. This specific mRNA sequence can be analyzed using RT-PCR.

- Study Mutation and Cancer Cells
- RT-PCR can detect and quantify tissue-specific mutant alleles. It can also detect any undesired changes in the mRNA sequence and unique mRNAs, which are produced only by the different types of cancer cells in our body.

- Tools of Genetic Engineering and Viral Study
- RT-PCR is used in genetic engineering for analyzing modified DNAs and their transcribed RNAs and amplifying target RNA.

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Thank you