**Molecular Diagnostics in Microbiology**

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**Abstract:** By developing quicker and more precise methods for diagnosing infectious diseases, molecular and genomic approaches have completely transformed microbiology. Traditional techniques, such as biochemical testing and microbe culture, take much time and could miss antibiotic-resistant strains. On the other hand, molecular and genomic approaches, Polymerase chain reaction, and sequencing of the entire genome (PCR) based procedures offer quick and accurate identification of pathogens, early-stage illnesses, and strains of bacteria resistant to bacteria. The potential for targeted medicines, high sensitivity and specificity, and personalized medicine are benefits of these methods. "However, there are obstacles to using molecular and genomic approaches because of their cost, equipment, knowledge, and data analysis. Concerns about the use of genetic data and patient privacy also arise from an ethical and legal standpoint. The extensive use of molecular and genomic techniques, however, is crucial for the future of microbiology since it can help identify types of bacteria that are resistant to antibiotics and improve patient outcomes. The full potential of molecular and genomic approaches in microbiology requires further development, teaching, and investigation of ethical aspects.

**Keywords:** Molecular technique, genomic methods, PCR, microbiology, antibiotic-resistant, data analysis

1. **Introduction**: Infectious illnesses are the world's largest cause of death and morbidity. The number of infections brought on by diverse pathogens, such as bacteria, viruses, parasites, and fungi, is always rising. Contributing variables include aging and illnesses including cancer and immunosuppression. Antibiotic, antiviral, antimycotic, and antiparasitic drugs have helped reduce these infectious agents' prevalence. Still, their widespread use has also resulted in the emergence of multi-drug resistant (MDR) pathogens that, if not identified and controlled in time, could spread over wide geographic areas. Regarding diagnosis, standard clinical and microbiological techniques including cultures, serology, and microscopy continue to be the preferred methods and are also reasonably priced.

The standard microbiological cultures, however, are not diagnostic procedures that can reliably identify the infection on their own. Since diagnostic science is the foundation of medical judgment, it is vital to the healthcare system. Diagnostic tests provide valuable information on many aspects of medical care, such as health administration, preventative measures, detection, diagnosis, and treatment. They also help determine the best antibiotics to treat infections and inform individualized treatment plans for different diseases (1). Among the primary diagnostic subfields are molecular diagnostics, immunology, hematology, microbiology, and clinical chemistry. Due to its ability to provide thorough insights into both diagnosis and treatment approaches, molecular diagnostics has garnered a lot of attention lately. Significant changes have occurred in this sector, revolutionizing healthcare by thoroughly comprehending different illness states (2).

Despite cultivable bacteria, many patients with symptoms and indicators typical of infectious diseases do not exhibit an organism in cultures. Molecular biology techniques are frequently utilized in diagnostic medical microbiology research centers for the above reasons. Clinical microbiology laboratories employ various methodologies to assist clinical decision-making about infection diagnosis, treatment, and prognosis. Clinical samples taken from cases are used in the gold standard to detect the pathogen directly. Several techniques are employed, including culture, electron microscopy, and for infectious agents the process of polymerase chain reaction (PCR) genome detection. Molecular methods are now used more and more in clinical microbiology diagnoses.

Molecular biology is the branch of biology that studies how genes are translated into RNA, how those RNAs are translated into proteins, and how those proteins affect cellular function. The main characteristics impacting the use of molecular testing in therapeutic settings will be the subject of this chapter. There are descriptions of the most popular methods. DNA, RNA, and proteins are among the molecular components of cells that may be identified, isolated, and altered thanks to techniques developed by molecular biologists. Our knowledge of the molecular mechanisms behind both normal and abnormal speech and swallowing has improved due to recent developments in basic research, genomics, and proteomics.

Numerous infectious diseases are being diagnosed using molecular and genomic methods [3]. The human papilloma virus (HIV), liver infection, and influenza are among the viral illnesses that are commonly detected using polymerase chain reaction-based diagnostic methods [4]. The diagnosis of bacterial infections including gonorrhoea, chlamydia, and tuberculosis can also be done with these techniques [5]. Whole-genome sequencing is another technique that is being used in medical microbiology at a rapid pace. This method involves sequencing all of the DNA present in a microbe sample, allowing for a comprehensive analysis of each bacterium [6]. Whole-genome sequencing can help track the spread of infectious diseases and identify their source when outbreaks happen [7].

Given that the number of basic science papers about voice alone has increased by almost 80% in the last seven years (Benninger, in press), it is more important than ever to comprehend fundamental biological processes to grasp the more specialized literature. The majority of molecular techniques used to identify bacteria rely on some form of DNA analysis, whether it be amplification or sequencing. PCR, real-time PCR, and RAPDPCR are examples of basic DNA amplification techniques. More complex methods based on targeted gene and whole-genome sequencing, restriction fragment analysis, and mass spectrometry are also included. There has also been research on techniques that rely on unique protein signatures, including matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

1. **Polymerase Chain Reaction (PCR**): Kary Mullis created PCR in the 1980s and was awarded the Nobel Prize in 1994 (8). Since its description, this method has brought about a true revolution in biological research, bringing basic biological processes into accord in practical fields such as plant and animal genetic enhancements and diagnosis (9). A polymerase chain reaction, or PCR for short, is an experimental technique that may rapidly produce (amplify) thousands to multi-billion copies of a certain DNA sequence for further analysis. PCR can employ source DNA from a variety of tissues and creatures, including microbes, skin, hair, saliva, and peripheral blood. Only small amounts of DNA are needed for PCR to generate enough copies of DNA for examination using conventional laboratory methods. This makes PCR a sensitive assay.

All PCR assays require DNA polymerase, nucleotides, primers, and template DNA. The crucial enzyme DNA polymerase is responsible for joining disparate nucleotides to produce the PCR result. DNA comprises four bases: adenine, thymine, cytosine, and guanine (A, T, C, and G). We refer to these as nucleotides. The DNA polymerase uses them as building blocks to create the final PCR result. The primers in the procedure specify the exact DNA fragment that must be amplified. Primers are short segments of DNA with a particular sequence that complements the target DNA that must be located and amplified. For the DNA polymerase, this offers an extension point on which to construct. Following their combination in a 96-well plate or test tube, the materials are placed in a device that permits three straightforward cycles of DNA amplification. This machine is essentially a thermal cycler. It has a pierced heat block into which the plates or test tubes holding the PCR reaction mixture are inserted. The machine raises the block's temperature in precise, pre-programmed increments (10). The initial step in denaturation is to heat the reaction solution above the melting temperatures of the target DNA's two complementary strands. After that, the temperature is lowered to allow for hybridization—also known as annealing—where the specific primers bind to the target DNA segments. Annealing occurs only when the primers and target DNA contain complimentary sequences, such as A binding to G. Once the temperature is raised again, the DNA polymerase can lengthen the primers by adding nucleotides to the expanding DNA strand. Every time these three processes are repeated, the number of DNA molecules that are replicated doubles.

**PCR components:** Several parts and reagents are needed for a basic PCR setup (11), including:

1. **DNA template:** Template DNA is the sample DNA containing the selected amino acid sequence that has to be amplified. Only DNA plasmid DNA, complementary DNA (cDNA), and genomic DNA (gDNA) can be used as the template. RNAs are used as starting materials for the reverse transcriptase polymerase chain reaction (RT-PCR), however before amplification, they are mostly transformed into complementary DNA (cDNA). It is required that the template DNA have an absorbance ratio of approximately 1.8 and be extremely pure. 30 μg to 50 μg is the optimal amount, however, 0.1 to 200 μg can be utilized.
2. **DNA polymerase:** The most popular and well-known DNA polymerase utilized in PCR since its inception is Taq DNA polymerase, which is an enzyme that was isolated from the bacteria Thermus aquaticus. Taq DNA polymerase is thermally stable and maintains its activity even after several cycles of heating and cooling. The most efficient reaction occurs between 72°C and 78°C, integrating around 60 bases per second, and it is stable up to 95°C. DNA polymerases are essentially enzymes that use the template strand to sequentially assemble nucleotides to create complementary DNA strands. One to two Taq polymerase units in a 50 L reaction mixture is sufficient for amplification. The *Pfu enzyme* from *Pyrococcus furiosus* and the Vent enzyme from *Thermococcus litoralis* are the two other thermostable DNA polymerase enzymes that are currently accessible.
3. **Primers:** Primer sequences are brief single-stranded oligonucleotides the sequences that complement the target amino acid set in the base sequence DNA, and they are produced artificially. These are brief sequences that serve as the building blocks for DNA synthesis and range in length from 15 to 30 bases. They anneal in a single-stranded template DNA strand at their complementary positions. This primer's 3' OH-end is subsequently extended by the DNA polymerase enzyme to create a new complementary strand.For a PCR reaction, 10–12 pMol of each primer is usually enough. There are two varieties of PCR primers: forward and reverse primers. The forward primers are complementary to the antisense strand (the template strand from the 3' to 5' position), which results in the amplification of the antisense strand. 5' primers are another name for them. The reverse primers are complementary to the sense strand (the template strand from 5' to 3' direction), which results in amplification of the sense strand. 3' primers are another name for them.
4. **Nucleotides (dNTPs or deoxynucleotide triphosphates):** New DNA strands are produced using deoxynucleotide triphosphates (dNTPs), which are artificially generated nucleotides. The four different dNTPs used in the PCR are deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), and deoxycytidine triphosphate (dCTP). These four dNTPs are added to the matured primer one after the other by the DNA polymerase enzyme, which then produces a new DNA strand complementary to the template strand.
5. **PCR Buffers and Other Chemicals:** A buffer system with a pH of 8.0 to 9.5 that is based on Tris and HCl must be used for the entire procedure. The most widely used buffer system is 10X buffer with extra MgCl2. Dimethyl Sulfoxide (DMSO), ammonium sulphate ((NH4)2SO4), polyethylene glycon (PEG), potassium chloride (KCl), magnesium chloride (MgCl2), tetramethyl ammonium chloride, N, N, N-trimethyl glycine, Common components of buffers used for PCR include tris-HCl, ethylenediaminetetraacetic acid (EDTA), 7-deaza-2′-deoxyguanosine 5'-triphosphate, glycerol, formamide, serum albumin, and so on. The buffer system stops inhibition and the creation of secondary structures while improving the reaction's efficiency and selectivity.
6. **Thermocycler:** The thermocycler, also called a PCR apparatus, is essentially an electronic radiator that controls the temperature as necessary for each PCR step. During the decomposition phase, this apparatus increases the temperature, decreases it during the annealing phase, and then raises it once more during the elongation phase. This cycle of temperature increases and decreases is based on the user's pre-programmed setup or instructions before operation.

**Steps of PCR**: Any kind of PCR involves the three fundamental processes of denaturation, annealing, and elongation. Together with these three primary stages, the preparation stage at the beginning and the PCR product analysis step at the end are also essential. Three steps can be used to summarise the entire process: product analysis, amplification, and pre-preparation.

**Pre-preparation:** This is the initial step before the polymerase chain reaction takes place within the thermocycler. To increase the size of the target DNA or RNA section, a reaction mixture must be prepared and loaded onto a thermocycler that has been preprogrammed. After being extracted from the sample, the DNA or RNA is kept (pre-extracted nucleic acids might be used). Everything is set up, precautions are taken, the area used to prepare all of the chemicals is brought to operating temperature, the PCR reaction is cleaned, the sample is taken out of storage or extracted, and the PCR reaction mixture is made; after programming the thermocycler, the reaction mixture is put into it.

**Amplification:** In PCR, the primary reaction process takes place. Denaturation, annealing, and elongation are all part of the amplification stage, and they happen sequentially and cyclically for a predetermined number of cycles that the user has preprogrammed.

1. **Denaturation:** In the initial step of the amplification process, thermally denatured double-stranded genetic material is transformed into two single-stranded DNA templates. About 30 to 90 seconds are spentraising thetemperature to roughly 94°C (90 to 95°C). At this temperature, the heat energy breaks the unstable hydrogen bonds holding the two segments of DNA together, forcing them to separate.

**dsDNA →   2 ssDNA templates**

1. **Annealing:** The annealing stage, in which the ssDNA templates are annealed at their complementary locations by the primer comes after denaturation. The forward primer anneals at the complementary site of the antisense strand of the template DNA, whereas the reverse primer anneals at the complementary site of the sense strand. The temperature must be lowered to between 55°C and 70°C for annealing to take place; the annealing temperature varies depending on the primer's GC material. In the majority of PCR procedures, annealing takes only 30 to 60 seconds.

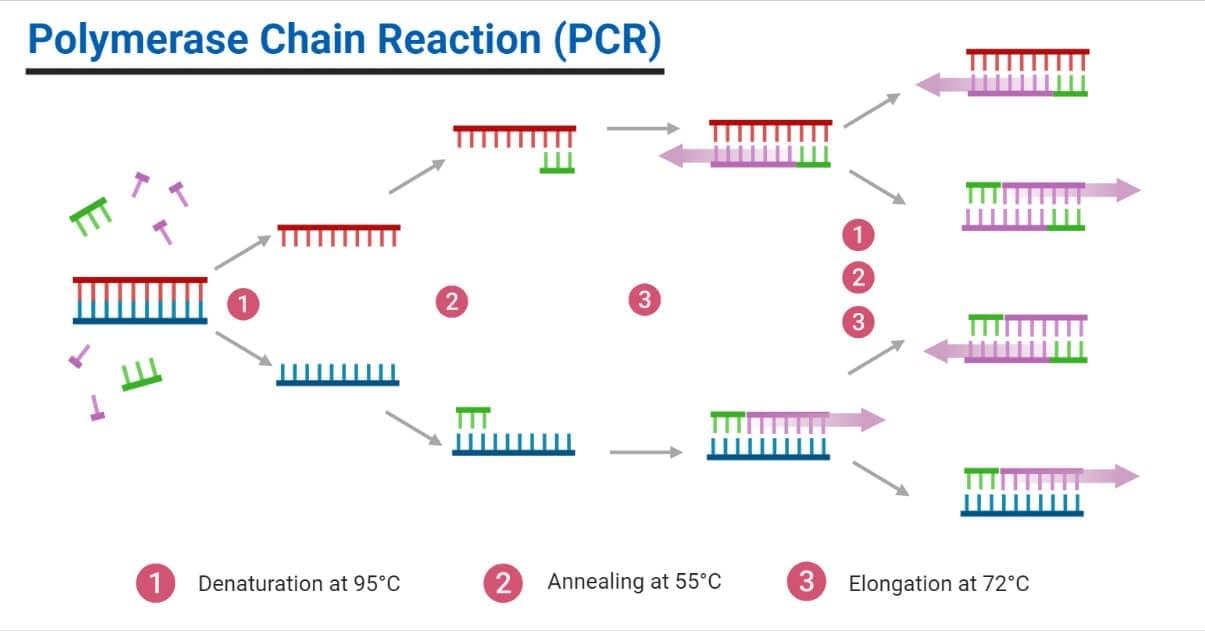
**ssDNA + Forward and reverse primers → ssDNA with annealed primers**

1. **Elongation:** In the final step of the amplification reaction, the temperature has been increased to 72°C. This triggers the Taq DNA polymerase enzyme to start creating new DNA strands in the 5' to 3' arrangement. To produce a new complementary strand, the DNA synthesizer enzyme moves nucleic acids across the product of the reaction mixture to the 3' OH-end of the annealed primer. The duration of extension depends on the length of the amino acid sequence in the specimen and the DNA enzyme activity.

Typically, elongation occurs at 1 kbps every 0.5-10 minutes. After elongation, two new double-stranded molecules will be produced from an individual double-stranded template at the beginning of the reaction.

**2 ssDNA with annealed primers + dNTPs → 2 new ds DNAs**

1. **Product Analysis:** This stage occurs after the PCR is finished and involves analyzing the reaction mixture to make sure the intended amplification was accomplished. Agarose gel electrophoresis is typically used to detect amplified DNAs or RNAs. However, no further step is necessary with certain PCR types, such as real-time PCR.



**Fig 1:** Steps of PCR

**Quantitative PCR:** Beyond just detecting DNA, qRT-PCR, or statistical real-time, provides data. It displays the quantity of any specific gene or genetic material found in the specimen. While the PCR product is being synthesized, Real-time quantification and identification are made possible by a quantitative reverse transcription.(12). Two popular techniques for identifying and measuring the product are (1) sequence-specific DNA probes made up of fluorescently labeled reports and (2) fluorescent dyes that non-specifically intercalate with double-stranded DNA. These only allow for detection once the probe and its complementary DNA target have hybridized. Reverse transcription, or the conversion of messenger RNA into cDNA, can be used in conjunction with real-time PCR. qPCR is then used to quantify the cDNA (13). There are problems with end-point PCR or analysis after the final PCR cycle, but these can be avoided by measuring the target gene during exponential amplification. Tumor analysis is a perfect example of PCR application. This method can isolate and amplify the DNA of proto-oncogenes or tumor suppressor genes. Quantitative PCR can then be used to measure the quantity of the recovered specific gene. However, quantitative PCR may measure any combination of proteins, mRNAs, and DNA and analyze individual cells. (14).

**Application of PCR**: PCR is already widely used, and new applications are constantly being developed. PCR has a lot of advantages. Initially, it produces results fast and is simple to learn and use (15). A particular product could be produced in millions to billions of copies using this extremely sensitive method for analysis, cloning, and sequencing. Although one advantage of quantitative real-time is its capacity to measure the synthesized product, this is also true for qRT-PCR. As a result, it can be used to analyze variations in the expression levels of genes in tumors, microbes, and other disease states. Even while PCR is a useful method, it has drawbacks. The highly sensitive nature of PCR means that even minute amounts of DNA might contaminate the sample, leading to inaccurate results (15,16). Additionally, some prior sequence data is required to construct primers for PCR. As a result, only the presence or absence of a known gene or microbe can be determined using PCR. The possibility that PCR primers will anneal non-specifically to sequences that are comparable but distinct from the target DNA is another disadvantage. The DNA polymerase can also introduce incorrect nucleotides into the PCR sequence, albeit this is extremely rare. The use of PCR is becoming more and more common and is greatly simplifying DNA sequencing.

* With its exceptional sensitivity, PCR can amplify minuscule amounts of DNA. Tiny levels of particular bacteria and viruses can therefore be found in tissues using the right primers, which makes PCR a vital tool for medical diagnostics.
* These days, PCR is a great tool for describing DNA samples that are crucial to medicine. RFLPs, for instance, are quickly taking the place of screening for human genetic disorders.
* PCR has become a vital component of forensic medicine because of its high sensitivity. The DNA from a single human hair or a little drop of blood recovered from a crime scene can even be amplified using PCR to allow for comprehensive characterization.

1. **Real-time PCR:** The molecular biology laboratory technique known as the real-time polymerase chain (real-time PCR, or qPCR when used quantitatively) is based on the polymerase chain reaction (PCR). It tracks a targeted DNA molecule's amplification in real-time, during the PCR, as opposed to after the conclusion, as is the case with traditional PCR. Both quantitative and semi-quantitative methods can be applied to real-time PCR. There are two common methods for detecting PCR products in real-time PCR: non-specific fluorescent dyes, which intercalate with any double-stranded DNA, and sequence-specific DNA probes, which are composed of oligonucleotides labeled with a fluorescent reporter and permit detection only after the probe has hybridized with its complementary sequence.

The MIQE standards, which stand for Minimum Information for Publication of Quantitative Real-Time PCR Experiments, indicate that reverse transcription–qPCR should be referred to as RT-qPCR, and the acronym for the quantitative real-time PCR technique must be qPCR. (17). The convention that the name "RT-PCR" refers to reverse transcription polymerase chain reaction rather than real-time PCR is not always followed by publications. (18). These days, DNA microarray and quantitative PCR are used to evaluate gene expression. Northern blot, RNase protection test, and differential display were the older techniques used to quantify mRNA abundance.

Northern blotting is frequently used to visualize the amount of a gene's mRNA transcript in a sample, hence estimating the gene's expression level. With this technique, a particular complementary biological or genetic probe to a specific gene is employed to probe the purified RNA after it has been categorized by agarose gel electrophoresis and moved to a solid matrix (such as a nylon membrane). Despite its continued usage in evaluating gene expression, this method only yields partially qualitative or descriptive data on mRNA levels and requires comparatively large volumes of RNA (19). Catalytic efficiency the integrity of the genome, and a host of additional variables might lead to estimation mistakes that arise from differences in the quantification technique. As a result, certain standardization systems often referred to as normalization methods have been created. To measure the overall level of gene expression, some have been devised.

However, the most popular ones are designed to measure the particular gene under investigation using a gene that regulates it is selected because of its almost steady level of expressiveness. These genes are commonly selected from household genes because constitutive gene expression is usually required for their involvement in basic cellular survival. (20,21). This makes it possible for researchers to compare the way the relevant genes are expressed without knowing the exact amount of expression by reporting a ratio of the genes' expression to that of the chosen normalizer. Presenting a ratio of the genes' expression to that of the selected normaliser enables researchers to compare the expression of the genes of interest without knowing the precise level of expression.

**Steps of RT-PCR:** Both PCR amplification and reverse transcription can be carried out in two steps in a single tube or with two different reactions. In both instances, cDNA is created via reverse transcription of RNA and subsequently utilized as the template for PCR amplification. Sequence-specific and non-sequence-specific primers (a mix of oligo-dT or random hexamers) are both available for chromosomal synthesis.

1. **Non-sequence-specific primers:**

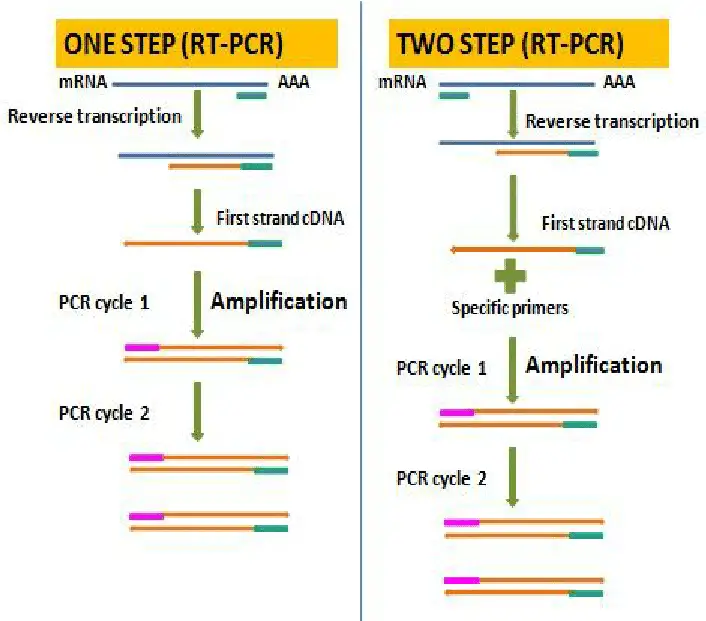
* **The random hexamers:**  A mixture of every possible pairing of six nucleotide sequences, known as random hexamers, can haphazardly bind to mRNA and start reverse transcription of the whole RNA pool.
* **Oligo-dT primers:** Since oligo-dT primers and the poly-A tail of mRNA molecules complement one other, cDNA can only be synthesized from mRNA molecules.

1. **Sequence-specific primers:**

* Sequence-specific primers are the most restricted since they are designed to bind exclusively to the mRNA molecules of interest. Conversely, reverse transcription is a target-specific process.

**One-step RT-PCR:** The same reaction buffer solution is used in the same reacting vessel for both PCR and cDNA synthesis. cDNA synthesis and target-specific amplification are directed by gene-specific primers. Some of the key advantages of a one-step reaction are low sample handling, shorter bench times, and closed-tube reactions that reduce the risk of cross-contamination and pipetting errors. One-step RT-PCR efficiency is affected by the availability and quality of RNA samples. Since the cDNA synthesis product cannot be maintained after one-step RT-PCR, additional aliquots of the original RNA sample or samples are required to repeat reactions or assess the expression of other genes.

**Two-step RT-PC:** In two-step RT-PCR, cDNA synthesis is carried out using random hexamers, oligo-dT primers, and/or gene-specific primers to create a mixture of cDNA molecules. Amplification of the resultant cDNAs is done using specific primers. cDNA is created in a single reaction in two-step RT-PCR, and a sample of the cDNA is then utilized in a different PCR test. Longer hands-on time, more pipetting manipulations, and additional open-tube processes are needed for this, which could increase unpredictability and contamination risk. A single RNA/cDNA sample can be used to quantify the expression of many genes or the residual cDNA can be stored for subsequent use.

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**Fig 2:** One-step and two-step methods of RT-PCR.

**Application of RT-PCR:** Numerous clinical specialties make extensive use of PCR or reverse transcription For instance, in clinical oncology, RT-PCR has been utilized to demonstrate graft versus lymphoma effects, monitor minimal residual illness, and discover and quantify chromosomal translocations(22-24). Additional uses include the detection of pertinent single nucleotide polymorphisms and predictive genetic testing. Quantification of gene expression is a significant application area for real-time reverse transcriptase RT-PCR assays. RT-PCR can be used to identify clinically relevant viruses because many of their genomes are composed of RNA. The West Nile virus and enteroviruses, which are the viral culprits of meningitis and meningoencephalitis, have also been identified using RT-PCR. With RT-PCR, the following viruses are being identified:

1. Dengue virus
2. Hantavirus
3. Viruses that infect humans
4. Severe acute respiratory syndrome (SARS)

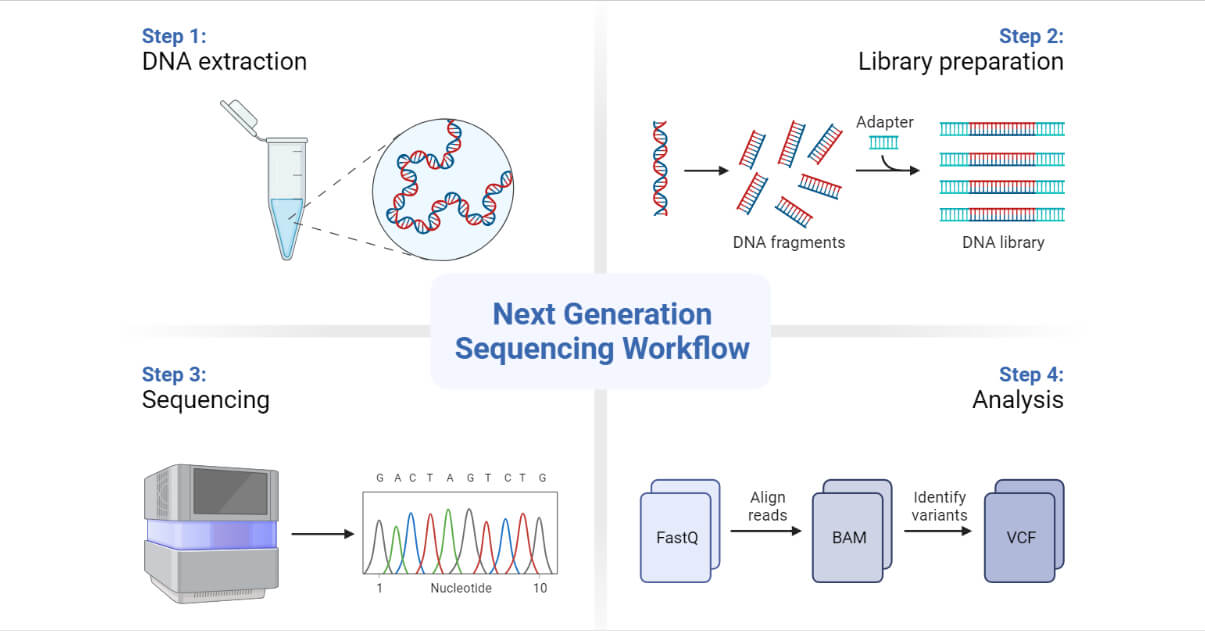
It is common practice to utilize quantitative RT-PCR assays to test for HIV and HCV viral load, or the amount of these viruses in a patient's blood. In addition to bacteria, parasites, and fungi, RT-PCR can also be used to identify other microorganisms by concentrating on their rRNA. This approach works better than DNA detection because the presence of RNA is more often associated with the existence of live creatures.

RT-PCR techniques have several limitations, just as other diagnostics. Certain chemicals found in biological samples can block RT-PCR. RNA must be used in an additional enzymatic step, which can lead to mistakes. Given that RNA is far more labile than DNA, isolation must be done carefully to guarantee the integrity of the RNA as well as the elimination of genomic DNA, contaminating nucleases, and reverse transcriptase or PCR inhibitors. Although this can occur with any sample source, clinical samples are particularly problematic since irregularities in sample size, collection, storage, and transportation can result in RNA templates of varying quality. Because there are various reverse transcriptase enzymes with distinct properties and different classes of oligonucleotides can be used to prime RT, the conversion of RNA to cDNA during the RT reaction is also susceptible to variability. However, the biggest current drawback of RT-PCR is most likely human error rather than a flaw in the technology itself: poor assay design, inaccurate data analysis, or erroneous conclusions. RT-PCR primer sets must be designed and validated by stringent criteria to ensure the specificity and accuracy of the results. When designing an assay to detect pathogens in microbiology, it is important to take into account the possibility of false positives or negatives, visually inspect amplification and melting curves, and double-check the accuracy of independent calculations based on these curves. Naturally, conclusions based on data derived from RT-PCR are best utilized when the biological context is well understood.

1. **Next-Generation Sequencing:** The next-generation sequencing, or NGS, is a novel technology used to find mutations and variations in DNA and RNA. NGS allows for the rapid sequencing of complete genomes or hundreds of thousands of chromosomes This method combines the benefits of different sequencing matrices, unique sequencing chemicals, and bioinformatics technology. This combination allows for the relatively quick sequencing of the complete genome or of various DNA or RNA sequence lengths with significant pairs (25). It is a novel sequencing method that comes after Sanger sequencing has been developed. Numerous significant sequencing steps are involved in NGS. There has been a significant growth in the desire for sequencing methods that are more affordable and faster since the first human genetic material was sequenced.

The development of next-generation sequencing technology (NGS), or second-generation sequencing techniques, has been fuelled by this need. Significantly simultaneous sequencing, made possible by NGS technology, allows thousands of genetic material segments from just one specimen to be sequenced simultaneously. Thanks to high-throughput sequencing made possible by methodology for enormously parallel sequencing, it takes approximately a day to analyze an entire genome. In the past decade, several NGS platforms have been created for high-throughput, cost-effective sequencing. These days, two of the most popular platforms in research and diagnostic laboratories are the Life Technologies Ion Torrent Personal Genome Machine (PGM) and the Illumina MiSeq. The introduction of this and other NGS systems has made sequencing more accessible to more labs, resulting in a significant increase in clinical evaluation and nucleic acid sequencing research. Genetic material Next-generation sequencing (includes, for example, library preparation, bioinformatics analysis, variant/mutation annotation and interpretation, DNA fragmentation, and significantly simultaneous sequencing.

1. **DNA fragmentation:**  The targeted genetic material is split up into multiple short segments, usually between 100 and 300 bp, during the chromatin segmentation procedures. There are numerous ways to accomplish this. DNA can be broken down by mechanical methods, enzyme digestion, and other methods (26). For instance, DNA can be broken up into short fragments by sonication. Specific supplementary probes of different designs are used to retrieve the brief portions relevant to the specified genetic material (27,28). This method is commonly referred to as a transformation capture assay. An additional method is polymerase chain reaction (PCR) amplification. This method amplifies the specially selected genetic segments by PCR using a significant amount of template pairs. Short portions of the specific genetic material are used in the PCR products. This technique is commonly referred to as amplicon assay (29,30). Obtaining the library ready is the next stage using the DNA segments.
2. **Library Preparation:** DNA segments are changed throughout the library preparation process to provide each DNA sample with a sample-specific index, such as sample identification, that helps identify the patient from whom the genome sequencing was performed. Furthermore, this process can introduce the sequencing adaptors to the segments of the genome.This change makes it possible for the organizing primers to bind any DNA segments, enabling subsequent enormously simultaneous processing.
3. **Sequencing and Imaging:** Nucleotides are added gradually to the developing DNA strand, which is in addition to the suggested segment, in a process known as synthesis-based sequencing. A single nucleotide simultaneously can be incorporated more easily thanks to the sequencing platform. Each of these has a distinct fluorescent or luminous tag attached to it. A camera or sensor picks up the signal that a nucleotide emits during incorporation. The signal that is emitted depends on the kind of nucleotide (adenine [A], thymine [T], cytosine [C], or guanine [G]). It enables real-time sequence determination by the sequencing program. This high-throughput method makes it possible to quickly sequence vast amounts of DNA.
4. **Data Analysis:** The last step in the next-generation sequencing process is data analysis. The initial stage involves converting the raw signal data acquired during sequencing into nucleotide sequences using a computer. This method of communication known as base calling, entails figuring out the matching nucleotides by analyzing the fluorescent or luminescent signals. After being created during the sequencing process, the short sequences, or reads, are either assembled de novo if no reference genome is available or matched to one. When the last stage involves identifying variations between the sequenced DNA and variations in the corresponding genetic material, a procedure called variant calling, de novo assembly is employed, whereas alignment to a reference genome enables the precise reconstruction of the original DNA sequence. This method includes identifying insertions, deletions, singular nucleotide polymorphisms (SNPs), and other structural alterations. After that, annotation gives these variations a biological meaning, which helps us comprehend how they affect gene function and are linked to illnesses.



**Fig 3: Next-generation Sequencing Methodology**

**Applications:** Rapid advancements in numerous biological science-related domains are made possible by the seemingly limitless uses of NGS. Worldwide, infectious diseases continue to be a major contributor to human morbidity and mortality. The therapeutic approach can be aided by the quick and accurate diagnosis of aetiologic microbes. When employing culture technology, the variety of detectable microorganisms is comparatively limited (31,32), including laborious and imprecise identification methods including pathogen isolation, selective culture, and pathological inspection. It can take days or weeks for clinical specimens to get definitive results when it comes to growing harmful bacteria (33). To further encourage the clinical use of NGS, we provide a detailed summary of its applications in the identification of viruses, fungi, and bacteria (Tab.1).

* **Bacteria:** One important and effective technique in infectious disease epidemiology is NGS, which exhibits excellent resolution concerning bacterial genotypes (34). Gram-negative and gram-positive bacteria, anaerobes, and fungi are among the pathogenic microorganisms that appear differently in septic patients under different clinical conditions. About 50% of sepsis patients have culture-negative sepsis, in which the causing organisms are still unknown. Numerous studies have shown that certain microbes are associated with portions of their genomic RNA or DNA. Consequently, bacterial detection in sepsis specimens and genetic relatedness information can be obtained using NGS of cfRNA or cfDNA in pure plasma (35).
* **Fungi:** The methods used to examine human fungal communities may have an impact on the analysis and outcomes of fungal detection (36). Given the severity of the fungal disease and the dearth of reliable detection techniques for clinical fungal infections, NGS's use in fungal research diagnostics must be taken into consideration. When it comes to identifying fungal infections, NGS technology has several benefits. First, microbial disorders brought on by hostile environments and slowly proliferating microorganisms, such as fungi, can be treated with NGS technology (37). Furthermore, samples with low fungal burdens benefit from NGS (38). Second, NGS provides more precise fungi identification as well as significance and is more precise than alternative methods (39).
* **Viruses:** The identification of viruses using next-generation sequencing (NGS) has become more and more common. Furthermore, NGS provides a state-of-the-art instrument for extensive, large-scale genome sequencing of viruses, including coronavirus, hepatitis C virus, and Hantaan virus (HTNV). For monitoring, tracking, and controlling the risk of viral diseases, NGS has ushered in a new era of viral genomes. To identify and create preventive strategies for HTNV outbreaks, it is essential to isolate transmissible particles and do the sequencing of the entire genome. Twelve HTNVs were isolated from the lung tissues of field mice with striped patterns in areas where hemorrhagic fever with renal syndrome (HFRS) is extremely prevalent by Dong Hyun Song et al. HTNV isolates' genomic sequence was obtained using single-primer, sequence-independent amplification (SISPA) NGS. Based on the entire length of the prototype, the nucleotide sequences of the HTNV S, M, and L segments were covered to 99.4–100%, 97.5–100%, and 95.6–99.8%, respectively. HTNV 76–118. (40).

**Tab 1: The merit and demerit of DNA sequences for identifying various infections**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **The infectious agent** | **Merit** | **Demerit** | **Sequencing methodology** | **References** |
| Bacterium | * Deconstructed biological informatics. * The quantity of bacteria present and their target. * Lower cost | * Only the recognition of bacterial species * Specific microorganisms. * Particular species may still be missed because of primer incompatibility. * It might be more difficult to find rare variants of infectious genomes. | t-NGS (16S rRNA) | 41 |
| The fungus | * Lower cost * Increased sensitivity and vast amounts of knowledge. * Increased velocity. * Deconstructed biological informatics. * The majority of accessible fungi can be amplified. | * Certain organisms are still missed because of primer incompatibilities. * Particularly, certain fungal and parasitic groupings. * It might be more difficult to find rare copies of pathogen sequences. | t-NGS (18S rRNA gene/ITS gene sequence). | 42 |
| Viruses | * Increased data volume and sensitivity enable *de novo* assembly and even read-level detection. * The ability to identify the entire range of viruses, including unexpected and undiscovered ones. * Enabling the comprehensive detection of small variations, which is a definite benefit over direct (Sanger) sequencing * Pathogen detection without the need for amplification or references. | * Compilation and description of intricate and highly repetitive genomic areas * Rebuilding the whole "genuine" viral haplotype. | WGS; mNGS | 43,44,45 |

1. **Advantages of Molecular Techniques:** Molecular approaches could be used in several domains, such as biotechnology, plant genetic improvement, and the food industry, which uses microbes to safely create food (46, 47). In addition to being extremely valuable for investigating fundamental scientific issues, cellular biology techniques might be used for a variety of problems that impact the human condition overall. Applying molecular biology techniques regularly addresses applications such as the development of new protein-containing substances, the avoidance and management of illnesses, and the alteration of ecosystems and creatures to produce specific genetic characteristics.

These methods are developing increasingly prevalent a few would even introduce them because of their wide range of possibilities in the nowadays technologically focused world. As these methods have advanced, it is now feasible to identify illnesses and genetic mistakes more rapidly and efficiently for upcoming therapies. Furthermore, these molecular methods are also widely used in scientific research and are effective in identifying, genotyping, and cataloging medically significant bacteria and viruses with low genetic material and high specificity (48).

By allowing the examination of the changes in gene expression that take place as an organism matures, the application of these tools to the study of developmental biology has tremendously helped the cell theory, which maintains that all organisms are composed of countless distinct cells. The ability to separate, modify, and reinsert certain genes has provided strong evidence in favor of the chromosomal hypothesis of inheritance, which holds that the chromosomes in each cell control the physical properties of the cells. The isolation and sequence analysis of related genes from multiple species, as well as the examination of the presence of alleles in wild populations, have provided evidence in favor of the theory of evolution by natural selection, which holds that complex organisms become descended from more primitive organisms through a process of accumulation of changes in physical traits of cells.

Several new methods, including restriction fragment length polymorphism (RFLP) analysis, the polymerase chain reaction (PCR), and DNA fingerprinting, have been made possible by molecular biology and are increasingly being used to determine a person's genetic makeup. Because each individual (apart from identical twins) has a unique combination of genes, and because the DNA of an organism contains the genes that code for all of the physical characteristics of a specific individual, these analysis techniques are all based on the notion that a DNA sample taken from an individual can be used to predict physical traits or to identify the individual from a group.

1. **Limitations of Molecular Techniques:** The faster and more sensitive molecular diagnostic techniques can take the place of conventional immunoassays and culture techniques**.** However, only a small percentage of analyses have shifted to using rapid diagnostics, despite the undeniable benefits they offer to food safety. There are still some factors that prevent the widespread use of diagnostic tests that use the pathogen's genetic material rather than its phenotype. The high rate of false negative and false positive results is a significant contributing factor. The atmosphere, the laboratory, and even the equipment used to create the reaction mix can contain DNA. This may result in a false positive for an illness. It is more challenging to remove DNA contamination from surfaces and lab equipment than it is to remove contamination from living cells. If antagonists are present, the results could be falsely negative. The object to be investigated is often a complex matrix, such as cheese or salami, which may contain substances that can interfere with the enzyme's ability to function. Therefore, blockage of the enzyme may lead to a misleading negative result. Ad hoc research on nucleic acid extraction techniques is therefore required to remove inhibitors from specific food matrices.

It is also necessary to introduce positive controls, like the IPC described for the TaqMan PCR, to guarantee the validity of the test. When it comes to complex food products or processed or ground meat, the genomic DNA of the host organism itself can sometimes impede the detection process due to competition for the probe and primer annealing. This effect is not negligible even with very few host cells because the genomes of plants and vertebrates are usually 103–104 times larger than those of bacteria. In many cases, this problem can be fixed by simplifying DNA, for as by eliminating genomic repeating repetitions (47). Generally speaking, bacterial enrichment through culture is a required step due to the abundance of inhibitors or competitors (49,50).

A stomacher device that mechanically tears up the food matrix in a culturing broth is typically employed to achieve enrichment. After filtering, the homogenized sample is incubated at the proper growth temperature for one or two days. When dealing with bacteria and fungi that have thick cell walls, an aggressive strategy that may involve both chemical and enzymatic reagents is required for efficient wall lysis before the extraction of genomic DNA and the amplification reaction.

1. **Conclusion:** The evaluation of new advancements in molecular biology and biotechnology techniques is crucial for the development of new scientific research, quicker and more accurate diagnosis, the production of drugs and vaccines, and other procedures. Thus, these advancements help to improve public health. Microbiology is undergoing a revolution thanks to the application of molecular and genomic methods. The speed and precision of infectious illness diagnosis have increased thanks to these methods, which may benefit patient outcomes.

Additionally, molecular and genomic methods facilitate the detection of bacterial strains resistant to antibiotics, which iscrucial given the rise in antibiotic resistance. It is expected that molecular and genomic techniques will become more accessible and widely available in the future, notwithstanding implementation hurdles. In addition to continuing research and development, medical practitioners will need to get training in the application of these procedures. Lastly, the application of genomic and molecular methods in medical microbiology holds promise for improving patient outcomes globally and revolutionizing the diagnosis and treatment of infectious diseases.

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