Advance Molecular Techniques

Lecture 1

1- Advance Molecular techniques

Molecular diagnostics laboratory techniques continue to be of critical importance to public health worldwide. Molecular genetic testing facilitate the detection and characterization of disease, as well as monitoring of the drug response, and assist in the identification of genetic modifiers and disease susceptibility. A wide range of molecular-based tests is available to assess DNA variation and changes in gene expression. However, there are major hurdles to overcome before the implementation of these tests in clinical laboratories, such as which test to employ, the choice of technology and equipment, and issues such as cost-effectiveness, accuracy, reproducibility and personnel training. At present, PCR-based testing predominates; however, alternative technologies aimed at exploring genome complexity without PCR are anticipated to gain momentum in the coming years. Furthermore, development of integrated chip devices ("lab-on-a-chip") should facilitate genetic readouts from single cells and molecules. Together with proteomic-based testing, these advances will improve molecular diagnostics and will present additional challenges for implementing such technology in public or private research units, hospitals, clinics, and pharmaceutical industries.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The Polymerase Chain Reaction (PCR) has been invented in 1983 by Kary Mullis (Nobel Price in 1993). Three years after its invention, there was an incredible expansion of use the *Taq* polymerase, a polymerase that resists high temperatures. In 1991, the first hydrolysis probe was used in combination with the technique. In 1992, the technique was again improved by the use of Ethidium Bromide (EtBr),. The kinetics of fluorescence accumulation during thermocycling was directly related to the starting number of DNA copies. This was the starting point of Real-Time qPCR. Today, 25 years after Mullis's discovery, both PCR and qPCR are widely used technologies.

The principle, and aim, of the PCR technology is to specifically increase a target from an undetectable amount of starting material. In classical PCR, at the end of the amplification, the product can be run on a gel for detection of this specific product. In Real-Time PCR, this step can be avoided since the technology combines the DNA amplification with the immediate detection of the products in a single tube. The homogeneous format is highly beneficial as it removes the significant contamination risk caused by opening tubes for post-PCR manipulation. It is also less time consuming than gel based analysis and can give a quantitative result.

Current detection methods are based on changes in fluorescence, which are proportional to the increase of target. Fluorescence is monitored during each PCR cycle providing an amplification plot, allowing the user to follow the reaction in real time. (Figure 1).

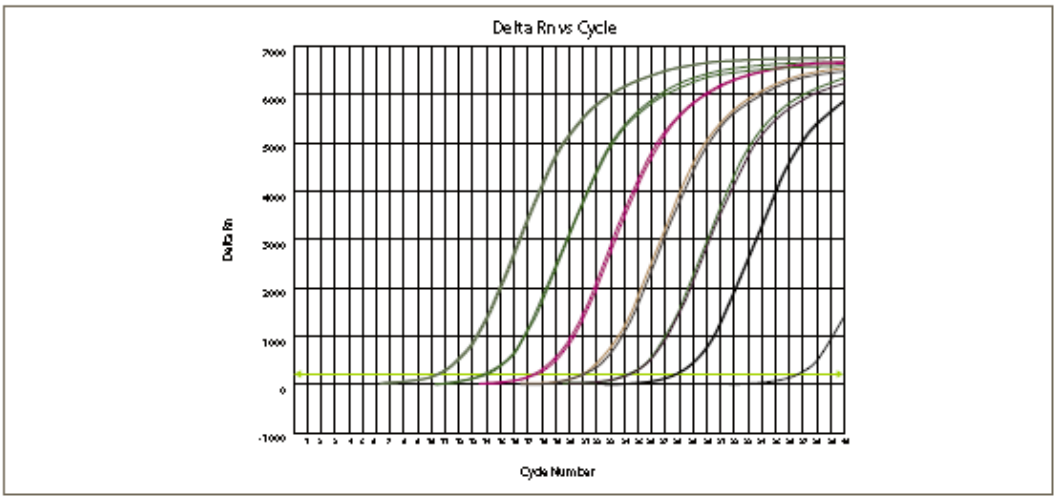


Figure 1. Amplification plot of 10 x serial diluted cDNA, using SYBR® Green detection method, on an ABI 7500 thermocycler.

Variability in qPCR is often related to steps upstream to the qPCR step itself. Sample extraction, quantity of sample, or efficiency of the reverse transcription (RT) are some of the many parameters that can influence the results of your qPCR assay. That's why it is important to also consider the quality of these steps prior to performing your qPCR assay

THE GOAL OF REAL-TIME PCR

The basic goal of real-time PCR is to precisely distinguish and measure specific nucleic acid sequences in a sample even if there is only a very small quantity. Real-time PCR amplifies a specific target sequence in a sample then monitors the amplification progress using fluorescent technology. During amplification, how quickly the fluorescent signal reaches a threshold level correlates with the amount of original target sequence, thereby enabling quantification.

WHAT IS PCR?

At its core, real-time PCR technology utilizes conventional PCR. PCR is a procedure by which DNA can be copied and amplified. As shown in Fig. 2, PCR exploits DNA polymerases to amplify specific pieces of DNA using short, sequence-specific oligonucleotides added to the reaction to act as primers. The first and most commonly used of these enzymes is *Taq* DNA polymerase (from *Thermus aquaticus*), whereas *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is used widely because of its higher fidelity when copying DNA. Although these enzymes are subtly different, they both have two basic capabilities that make them useful for PCR: *1*)they can generate new strands of DNA using a DNA template and primers, and *2*) they are heat resistant. The latter attribute is necessary because after each round of DNA copying, the resulting double-stranded DNA (dsDNA) must be "melted" into single strands by high temperatures within the reaction tube (95°C). The reaction is then cooled to allow the oligonucleotide primers to anneal to the new single-stranded template DNA and direct the DNA polymerase enzyme to initiate elongation by adding single complementary nucleotides to create a new complete strand of DNA. Thus dsDNA is created. This new dsDNA must then be melted apart before the next cycle of copying can occur. Therefore, if the reaction works with perfect efficiency, there will be twice as much specific dsDNA after each cycle of PCR.

Quantitative PCR

In quantitative PCR, a specific or non-specific detection chemistry allows the quantification of the amplified product. The amount detected at a certain point of the run is directly related to the initial amount of target in the sample. The most common applications of quantitative PCR are gene expression analysis, pathogen detection/quantification and microRNA quantification. Quantitative PCR software uses the exponential phase of PCR for quantification. During the exponential phase, the amount of target is assumed to be doubling every cycle and no bias is expected due to limiting reagents. Analysis takes the Ct (cycle number) value, at the point when the signal is detected above the background and the amplification is in exponential phase. The more abundant the template sample, the quicker this point is reached, thus giving earlier Ct values.

Qualitative PCR

In qualitative PCR, the goal is to detect the presence or absence of a certain sequence. It could be for virus sub-typing and bacterial species identification for example. It can also be used for allelic discrimination between wild type and mutant, between different SNPs (Single Nucleotide Polymorphisms) or between different splicing forms. In this case, different fluorophores can be used for the two alleles, and the ratio of the fluorophores signals correlates to the related amount of one form compared to the other one. Specific detection methods such as Double-Dye probe systems are more often used for these applications, and probes can be used to detect single base mutations or small deletions.

THE CHEMISTRIES OF REAL-TIME PCR

The key to real-time PCR is the ability to monitor the progress of DNA amplification in real time. This is accomplished by specific chemistries and instrumentation. Generally, chemistries consist of special fluorescent probes in the PCR. Several types of probes exist, including DNA-binding dyes like EtBr or SYBR green , hydrolysis probes , hybridization probes, molecular beacons, sunrise and scorpion primers, and peptide nucleic acid (PNA) light-up probes. Each type of probe has its own unique characteristics, but the strategy for each is simple. SYBR green binds to the minor groove of dsDNA, emitting 1,000-fold greater fluorescence than when it is free in solution. Therefore, the greater the amount of dsDNA present in the reaction tube, the greater the amount of DNA binding and fluorescent signal from SYBR green. If there are two or more peaks, it suggests that more than one amplified sequence was obtained, and the amplification was not specific for a single DNA target. Hydrolysis probes (also called 5'-nuclease probes because the 5'-exonuclease activity of DNA polymerase cleaves the probe) offer an alternative approach to the problem of specificity. These are likely the most widely used fluorogenic probe format and are exemplified by TaqMan probes. In terms of structure, hydrolysis probes are sequencespecific fluorophore-labeled DNA oligonucleotides. One fluorophore is termed the quencher and the other is the reporter. When the quencher and reporter are in close proximity, that is, they are both attached to the same short oligonucleotide, the quencher absorbs the signal from the reporter. This is an example of fluorescence resonance energy transfer (also called Fo¨rster transfer) in which energy is transferred from a “donor” (the reporter) to an “acceptor” (the quencher) fluorophore. During amplification, the oligonucleotide is broken apart by the action of DNA polymerase (5'-nuclease activity) and the reporter and quencher separate, allowing the reporter’s energy and fluorescent signal to be liberated. Thus destruction or hydrolysis of the

oligonucleotide results in an increase of reporter signal and corresponds with the specific amplification of DNA. Examples of common quencher fluorophores include TAMRA, DABCYL, and BHQ, whereas reporters are more numerous (e.g., FAM, VIC, NED, etc). Hydrolysis probes afford similar precision as SYBR green , but they give greater insurance regarding the specificity because only sequence-specific amplification is measured. In addition, hydrolysis probes allow for simple identification of point mutations within the amplicon using melting curve analysis

COMMON APPLICATIONS FOR REAL-TIME PCR.

Relative and absolute quantitation of gene expression.

To evaluate gene expression, RNA must first be isolated from the samples to be studied. After isolation, RNA is linearly converted to cDNA, which is used for real-time PCR. Amplification curves are graphed by the software to help determine the "cycle time" at which fluorescence reaches a threshold level. This CT value is inversely proportional to the amount of specific nucleic acid sequence in the original sample. Both relative and absolute quantitation of gene expression utilize the CT value to quantitate cDNA and thereby determine gene expression. In a perfectly efficient PCR, the amount of amplified product doubles each cycle. Therefore, a difference of 1 between sample CTs means that the sample with the lower CT value had double the target sequence of the other sample; a change in CT of 2 means a fourfold difference; a change in CT of 3 means an eightfold difference, and so on.

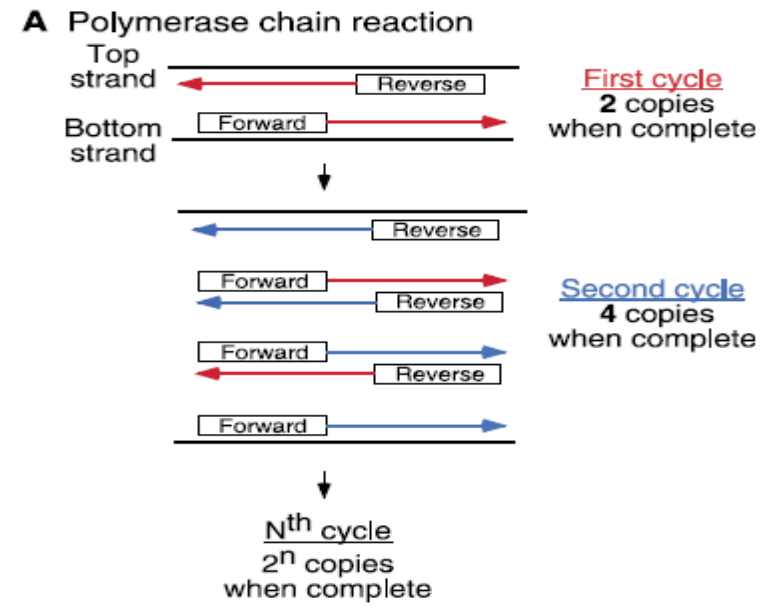
Relative quantitation measures changes in the steady-state levels of a gene of interest relative to an invariate control gene. Housekeeping genes (e.g., cyclophilin, glyceraldehyde-3-phosphate dehydrogenase, ribosomal protein 36B4, 18S rRNA, transferrin receptor, etc.) that are not expected to change under the experimental conditions serve as a convenient internal standard.

Counting bacterial, viral, or fungal loads.

Real-time PCR can distinguish specific sequences from a complex mixture of DNA. Because of this, it is useful for determining the presence and quantity of pathogen-specific or other unique sequences within a sample.

Identification of mutations (or single nucleotide polymorphisms) by melting curve analysis.

Real-time PCR is ideally suited for the analysis of mutations, including single nucleotide polymorphisms (SNPs), often replacing other techniques such as sequencing, single-strand conformation polymorphism assays, and restriction fragment-length polymorphism analysis.



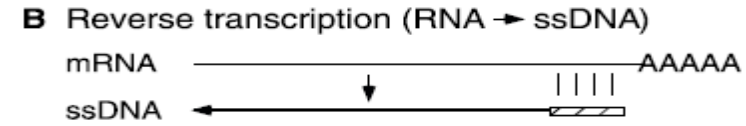


Fig. 2. Enzyme reactions that make real-time PCR possible. *A*: PCR is depicted. High temperatures are used to "melt" double-stranded (ds)DNA into its top and bottom strands. This mixture is cooled in the presence of sequence-specific primers (denoted as forward and reverse) that anneal to their targets, and an optimal temperature is then applied to allow elongation of complementary DNA (arrows) by the action of DNA polymerase to complete a cycle. This is repeated numerous times, and, if no reagents are limiting, 2^n copies of the desired DNA fragment can be obtained. *B*: because DNA polymerase does not utilize RNA as a template, the conversion of RNA to DNA can be achieved using the enzyme reverse transcriptase. *ss*DNA, single-stranded DNA.

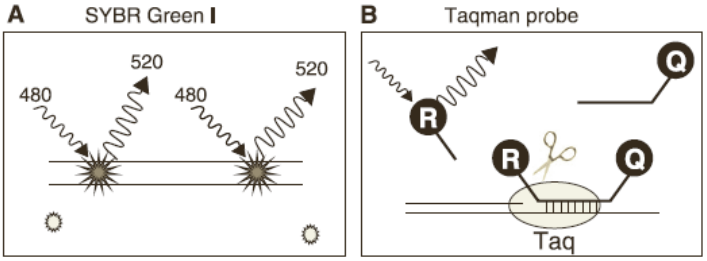


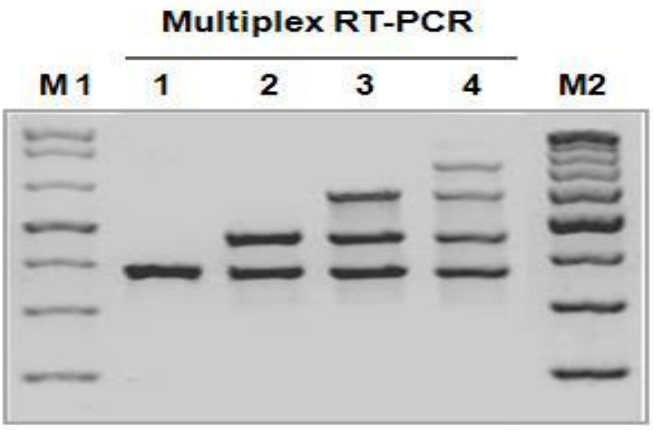
Fig. 4. Real-time PCR chemistries. *A*: SYBR green I fluoresces (absorbing light of 480-nm wavelength and emitting light of 520-nm wavelength) when associated with dsDNA. *B*: other detection formats often utilize compatible fluorphores. Shown in this example is the Taqman probe, which contains a reporter fluorophore (R) that emits at a wavelength absorbed by the quencher fluorophore (Q). During PCR amplification, the DNA polymerase (*Taq*) cleaves the probe, thus liberating the reporter from the quencher and allowing for measurable fluorescence.

Other types of Polymerase Chain Reaction (PCR)

Multiplex-PCR:

It is a special type of PCR used for detection of multiple pathogens by using Multiple primers sets each one targets a particular pathogen.

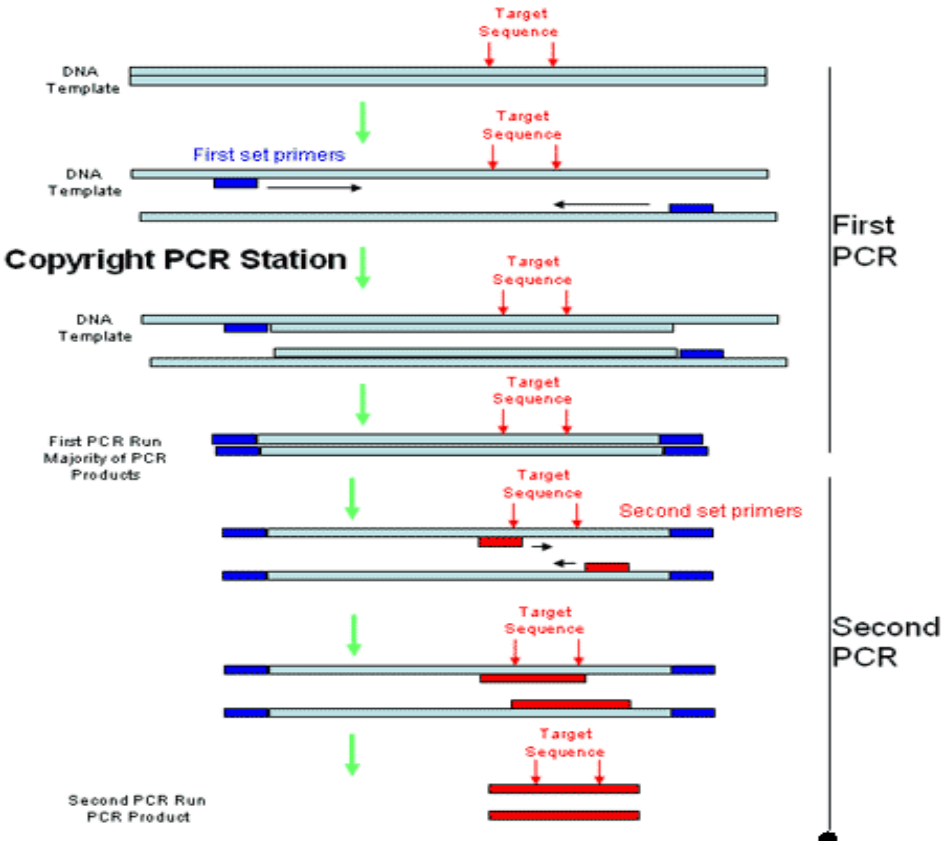
Uses: permits the simultaneous analysis of multiple targets in a single sample.



Nested-PCR

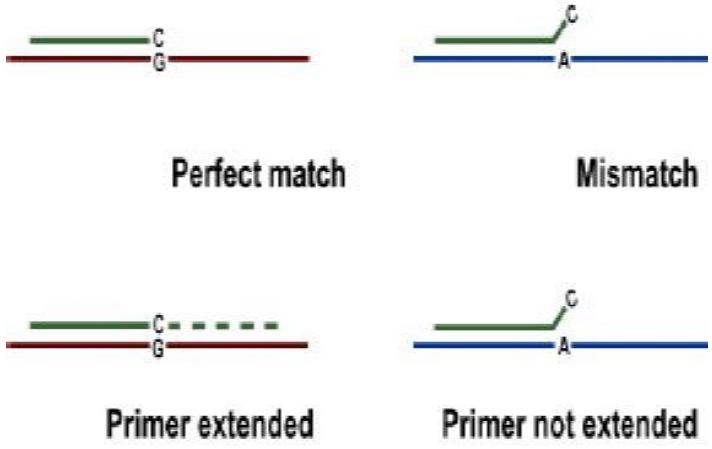
This type of PCR is used to increase the specificity of DNA amplification. **Two sets** of primers are used in two successive reactions. In the first PCR, one pair of primers is used to generate DNA products, which will be the target for the second reaction. Using two different primers whose binding sites are located (nested) within the first set, thus increasing specificity. Nested PCR is often more successful in specifically amplifying long DNA products than conventional PCR.

Uses: Detection of pathogens that occur with very few amount.



ALLELE-SPECIFIC PCR

A diagnostic or cloning technique which is based on single-nucleotide polymorphisms (SNPs) (single-base differences in DNA). It requires prior knowledge of a DNA sequence, including differences between alleles, and 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, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence

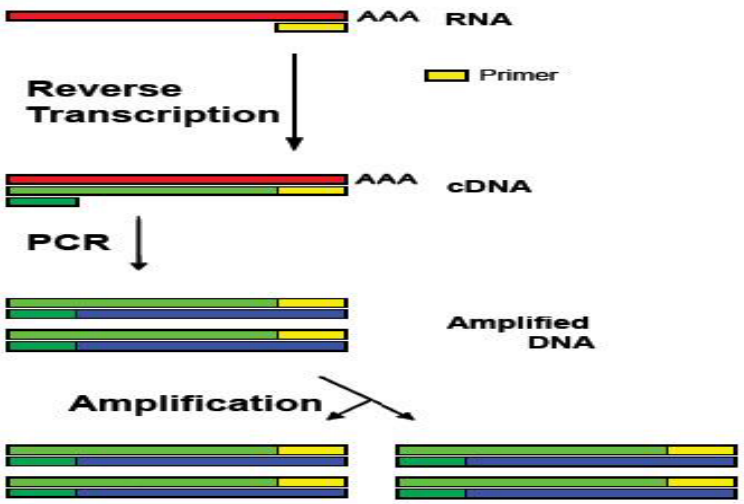


RT-PCR (Reverse Transcription PCR, Real Time - PCR)

This type of PCR is used to reverse-transcribe and amplify RNA to cDNA. PCR is preceded by a reaction using reverse transcriptase, an enzyme that converts RNA into cDNA. The two reactions may be combined in a tube.

Uses: 1-Detection of RNA virus like (HCV).

2-Detection of other M.O. through targeting of their Ribosomal RNA.



Uses or Application of PCR:

Medical applications:

1.The first application of PCR was for genetic testing, where a sample of DNA is analyzed for the presence of genetic disease mutations.

2.PCR analysis is also essential to preimplantation genetic diagnosis, where individual cells of a developing embryo are tested for mutations.

3.PCR can also be used as part of a sensitive test for tissue typing, vital to organ transplantation.

4.Many forms of cancer involve alterations to oncogenes. By using PCR-based tests to study these mutations, therapy regimens can sometimes be individually customized to a patient.

Infectious disease applications:

1.Characterization and detection of infectious disease organisms by using the PCR assays: For example: Human Immunodeficiency Virus (HIV), Tuberculosis (T.B) and Brucellosis.

Forensic applications:

1.The development of PCR-based genetic fingerprinting protocols has seen widespread application in forensics:
The genetic fingerprinting can uniquely discriminate any one person from the entire population of the world. Minute samples of DNA can be isolated from a crime scene, and compared to that from suspects, or from a DNA database of earlier evidence or convicts.

2.Less discriminating forms of DNA fingerprinting can help in parental testing, where an individual is matched with their close relatives. DNA from unidentified human remains can be tested, and compared with that from possible parents. Similar testing can be used to confirm the biological parents of an adopted (or kidnapped) child. The actual biological father of a newborn can also be confirmed (or ruled out).