Department of BSBE
Indian Institute Of Technology Guwahati

Nested PCR, inverse PCR, Real-time PCR.

Dr. Sanjukta Patra

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Genetic Engineering

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Variants of PCR

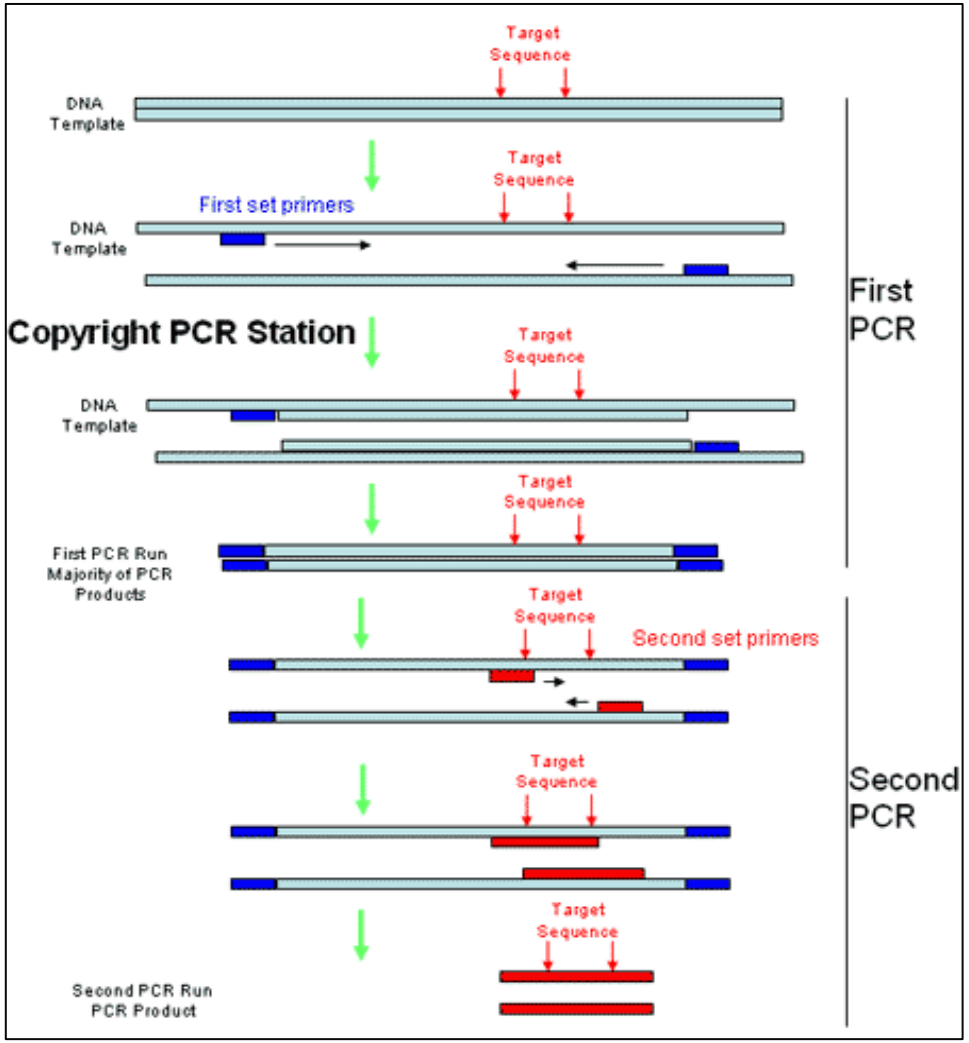
- Conventional PCR
- RT-PCR and qRT-PCR
- Quantitative PCR
- Multiplex PCR
- Nested PCR
- Hot-start PCR
- Inverse PCR
- RACE-PCR (*Rapid Amplification of cDNA Ends*)
- Touchdown PCR
- Assembly PCR
- Colony PCR
- Asymmetric PCR
- Allele-specific PCR
- Methylation-specific PCR
- LAMP assay

Nested-PCR

- 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.

Uses:

- Detection of pathogen marker that occur in very less amount.



NESTED PCR

- This PCR increases the ***specificity*** of DNA amplification, by reducing background due to non-specific amplification of DNA.
- Two sets (instead of one pair) of primers are used in two successive PCRs.
- In the first reaction, one pair of primers "outer pair" is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments.
- The product(s) are then used in a second PCR after the reaction is diluted with a set of second set "nested or internal" primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction.
- The specificity of PCR is determined by the specificity of the PCR primers.
- The second pair of primers (**nested primers**) bind within the first PCR product and produce a second PCR product that will be shorter than the firstone.
- The strategy is that if the wrong locus were amplified by mistake, the probability is very low that it would also be amplified a second time by a second pair of primers.

USES OF NESTED PCR

When a complete genome sequence is known, it is easier to be sure you will not amplify the wrong locus. But since very few of the world's genomes have been sequenced completely, nested primers will continue to be an important control for many experiments.

Inverse-PCR

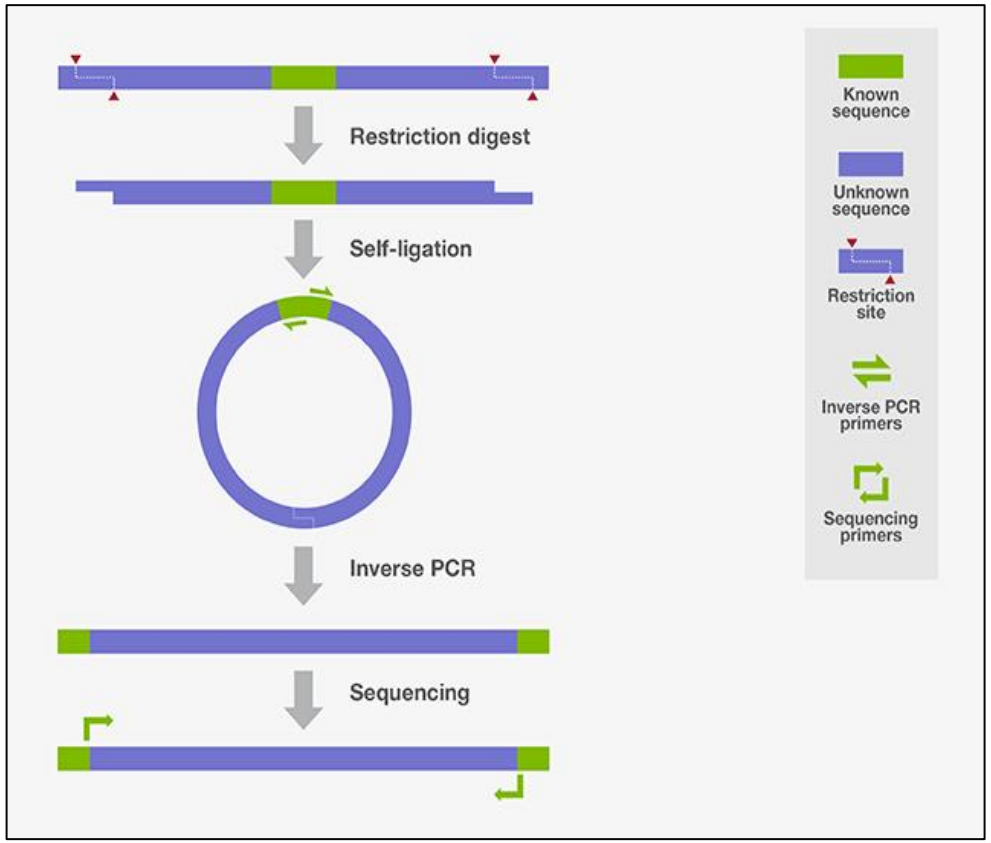
Ochman et al. (1988).

In this method amplification of DNA of unknown sequence is carried out from a known sequence.

Inverse PCR is useful for the determination of insert locations.

Various retroviruses and transposons and integrate into genomic DNA. Inverse can help locate them.

A **transposable element** (**TE, transposon**, or **jumping gene**) is a DNA sequence that can change its position within a genome.
Leading mutations, making genes non functional.
These known sequences can be targeted.



To identify the sites where they have entered, the known, "internal" viral or transposon sequences can be used to design primers that will amplify a small portion of the flanking, "external" genomic DNA.

The amplified product can then be sequenced and compared with DNA databases to locate the sequence which has been disrupted.

The inverse PCR method involves a series of restriction digests and ligation, resulting in a looped fragment that can be primed for PCR from a single section of known sequence.

Then, the DNA is amplified by the temperature-sensitive DNA polymerase:
A target region with an internal section of known sequence and unknown flanking regions is identified.

Genomic DNA is digested into fragments of a few kilobases by a usually low-moderate frequency (6-8 base) cutting restriction enzyme.

Under low DNA concentrations, self-ligation is induced to give a circular DNA product.
PCR is carried out as usual, with primers complementary to sections of the known internal sequence.

Finally the sequence is compared with the sequence available in the data base.

Inverse PCR Steps

- The inverse PCR method includes a series of digestions and self-ligations with the DNA being cut by a restriction endonuclease.
- This cut results in a known sequence at either end of unknown sequences.
- Target DNA is lightly cut into smaller fragments of several kilobases by restriction endonuclease digestion.
- Self-ligation is induced under low concentrations causing the phosphate backbone to reform. This gives a circular DNA ligation product.
- Target DNA is then restriction digested with a known endonuclease.
- This generates a cut within the known internal sequence generating a linear product with known terminal sequences. This can now be used for PCR (polymerase chain reaction).
- Standard PCR is conducted with primers complementary to the now known internal sequences.

USES OF INVERSE PCR

1. It is commonly used to identify the flanking sequences around genomic inserts.
2. Inverse PCR has numerous applications in molecular biology including the amplification and identification of sequences, flanking transposable elements, and the identification of genomic inserts.

RT-PCR (Reverse Transcription PCR)

The discovery of reverse transcriptase during the study of viral replication of genetic material led to the development of RT-PCR, which has since displaced northern blot as the method of choice for RNA detection and quantification.

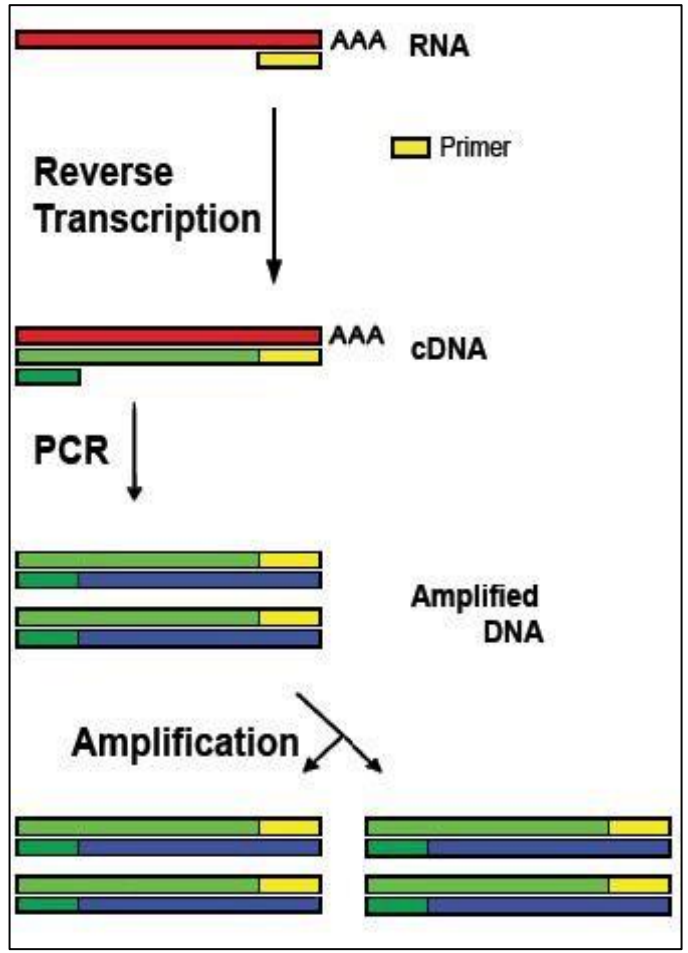
RT-PCR has risen to become the benchmark technology for the detection and/or comparison of RNA levels for several reasons:

(a) it does not require post PCR processing
(b) a wide range (>10^7-fold) of RNA abundance can be measured
(c) it provides insight into both qualitative and quantitative data

RT-PCR (Reverse Transcription PCR)

- Employed for amplification of RNA to cDNA using Reverse Transcriptase.
- Widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript.

Polymerase chain reaction	PCR
Reverse transcription polymerase chain reaction	RT-PCR
Real-time polymerase chain reaction	qPCR
RT-PCR / qPCR combined technique	qRT-PCR



One-step RT-PCR vs. two-step RT-PCR

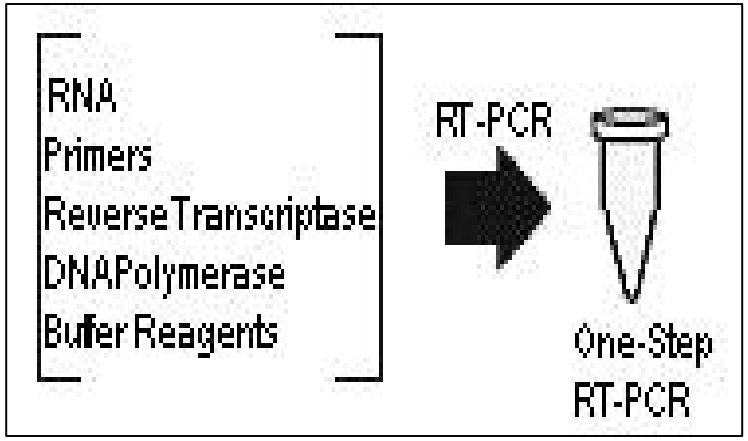
One-step approach - the entire reaction from cDNA synthesis to PCR amplification occurs in a single tube.

It minimizes experimental variation by containing all of the enzymatic reactions in a single environment.

However, the starting RNA templates are prone to degradation in the one-step approach.

The use of this approach is not recommended when repeated assays from the same sample is required.

One-step approach is reported to be less accurate compared to the two-step approach.



Two-step RT-PCR

Reverse transcriptase reaction and PCR amplification be performed in separate tubes.

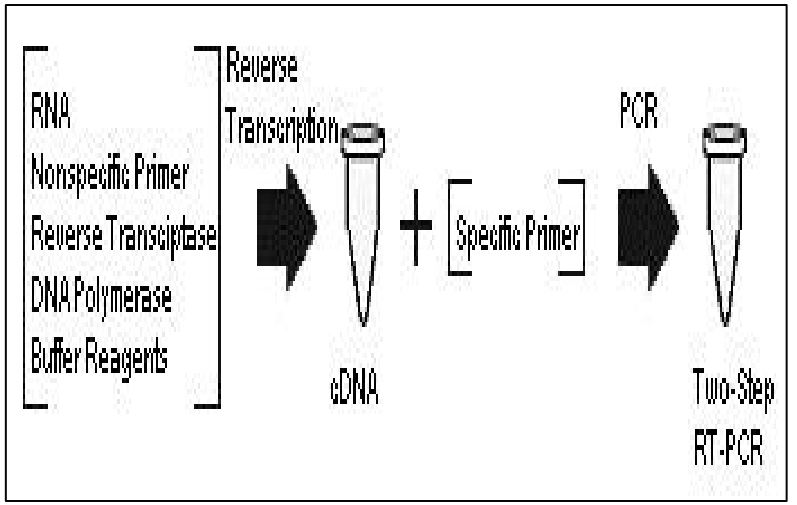
This method is more sensitive than the one-step method.

1. Combine template RNA, primer, dNTP mix, and nuclease-free water in a PCR tube. Add RNase inhibitor and reverse transcriptase to the PCR tube. Place PCR tube in thermal cycler for one cycle that includes annealing, extending and then inactivating reverse transcriptase (heat). Proceed directly to PCR or store on ice until PCR can be performed.

2. Add a master mix (containing buffer, dNTP mix, $MgCl_2$, Taq polymerase and nuclease-free water) to PCR tube. Add appropriate primer. Place PCR tubes in thermal cycler for 30 cycles of the amplification program, which includes three steps: (1) denaturation (2) annealing (3) elongation.

The RT-PCR products can then be analyzed with gel electrophoresis.

The disadvantage of the two-step approach is susceptibility to contamination due to more frequent sample handling.



Real-time PCR or quantitative PCR (qPCR)

Technique combining reverse transcription of RNA into DNA (complementary DNA or cDNA) and amplification of specific DNA targets using polymerase chain reaction (PCR).

- It is primarily used to measure the amount of a specific RNA -Achieved by monitoring the amplification reaction using fluorescence, **a technique called real-time PCR or quantitative PCR (qPCR).**

- Combined RT-PCR and qPCR are routinely used for analysis of gene expression and quantification of viral RNA in research and clinical settings.

- RT-PCR can be used without qPCR, for example to enable molecular cloning, sequencing or simple detection of RNA.
- Conversely, qPCR may be used without RT-PCR, for example to quantify the copy number of a specific piece of DNA.

- If the genomic DNA sequence of a gene is known, RT- PCR can be used to map the location of exons and introns in the gene.

Due to its simplicity, specificity and sensitivity, RT-PCR is used in a wide range of applications:

- Quantification of yeast cells in wine (ITS 2-26S, intergenic spacer (IGS) 1
- Diagnostic tools for detecting infectious agents such as the avian flu virus
- Detection of RNA virus.
- Detection of other microorganisms through targeting of their rRNA

Application

1.The exponential amplification via reverse transcription polymerase chain reaction provides for a highly sensitive technique in which a very low copy number of RNA molecules can be detected.

2.RT-PCR is widely used in the diagnosis of genetic diseases and, semiquantitatively, in the determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression.

3. **Gene insertion**

RT-PCR can also be very useful in the insertion of eukaryotic genes into prokaryotes.

Because most eukaryotic genes contain introns, which are present in the genome but not in the mature mRNA, the cDNA generated from a RT-PCR reaction is the exact (without regard to the error-prone nature of reverse transcriptases) DNA sequence that would be directly translated into protein after transcription.

Genetic disease diagnosis

RT-PCR can be used to diagnose genetic disease such as Lesch–Nyhan syndrome. This genetic disease is caused by a malfunction in the HPRT1 gene, which clinically leads to the fatal uric acid urinary stone and symptoms similar to gout.

Analyzing a pregnant mother and a fetus for mRNA expression levels of HPRT1 will reveal if the mother is a carrier and if the fetus will likely to develop Lesch–Nyhan syndrome.

Cancer detection

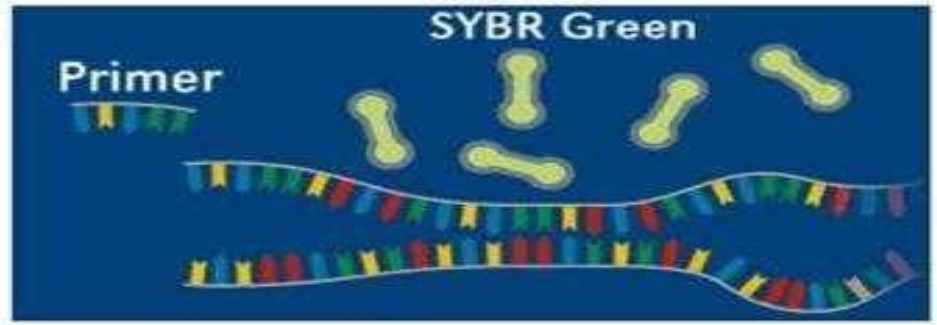
Scientists are working on ways to use RT-PCR in cancer detection to help improve prognosis, and monitor response to therapy.

Circulating tumor cells produce unique mRNA transcripts depending on the type of cancer. The goal is to determine which mRNA transcripts serve as the best biomarkers for a particular cancer cell type and then analyze its expression levels with RT-PCR.

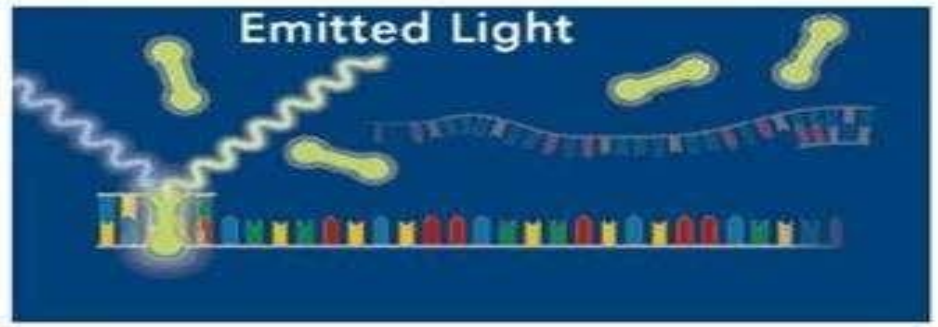
RT-PCR is commonly used in studying the genomes of viruses whose genomes are composed of RNA, such as Influenza virus A and retroviruses like HIV.

Quantitative real time PCR (Q-RT PCR)

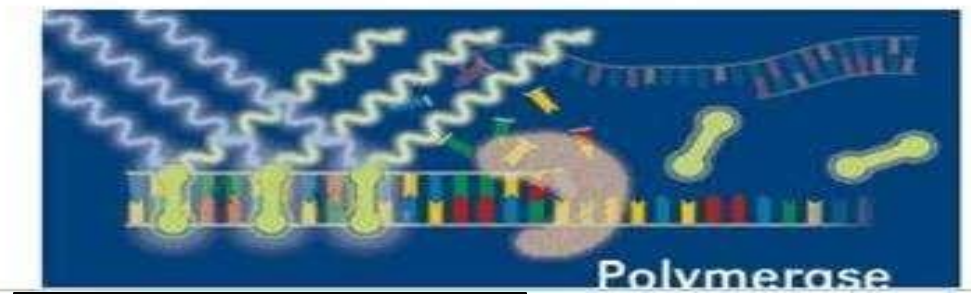
- It quantitatively measures starting amounts of DNA, cDNA or RNA.
- *Quantitative real-time PCR* has a very high degree of precision.
- QRT-PCR methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time.
  - Detection and quantitation of fluorescent reporter the signal of which increases in direct proportion to the amount of PCR product in a reaction
  - Does not measure the amount of end product but its production in real time
  - Used to measure the specific amount of target DNA (or RNA) in a sample.
  - By measuring amplification only within the phase of true exponential increase, the amount of measured product more accurately reflects the initial amount of target.
  - Special thermal cyclers are used that monitor the amount of product during the amplification.
- 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.



At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly fluoresce, producing a minimal background fluorescence signal which is subtracted during computer analysis.



After annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation.



Non specific method

Real-time PCR technique can be classified as specific or non-specific detection.

Non-specific detection: Real-time PCR with double-stranded DNA-binding dyes as reporters

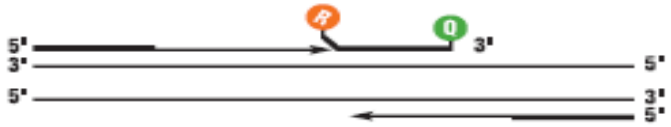
- A DNA-binding dye binds to all double-stranded (ds) DNA in PCR, increasing the fluorescence quantum yield of the dye.
- An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity measured at each cycle.
- However, dsDNA dyes such as SYBR Green will bind to all dsDNA PCR products, including nonspecific PCR products (such as Primer dimer).
- This can potentially interfere with, or prevent, accurate monitoring of the intended target sequence.
- In real-time PCR with dsDNA dyes the reaction is prepared as usual, with the addition of fluorescent dsDNA dye.
- Then the reaction is run in a real-time PCR instrument, and after each cycle, the intensity of fluorescence is measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product).
- This method has the advantage of only needing a pair of primers to carry out the amplification, which keeps costs down
- Multiple target sequences can be monitored in a tube by using different types of dyes.

Detection platforms in Q-RT PCR

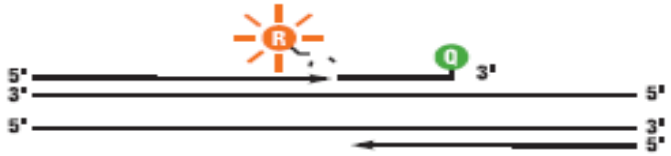
TAQMAN® PROBE-BASED ASSAY CHEMISTRY

1. **Polymerization:** A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan® probe, respectively.

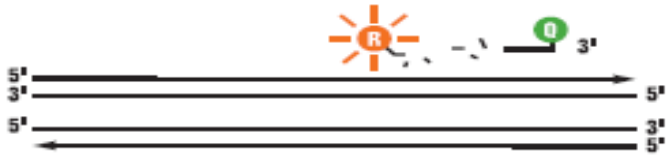
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



3. **Cleavage:** During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.



4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.

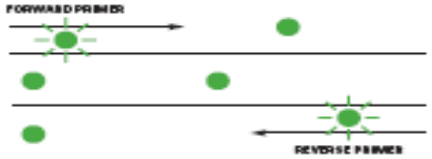


SYBR® GREEN I DYE ASSAY CHEMISTRY

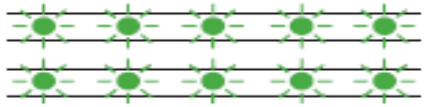
1. **Reaction setup:** The SYBR® Green I Dye fluoresces when bound to double-stranded DNA.

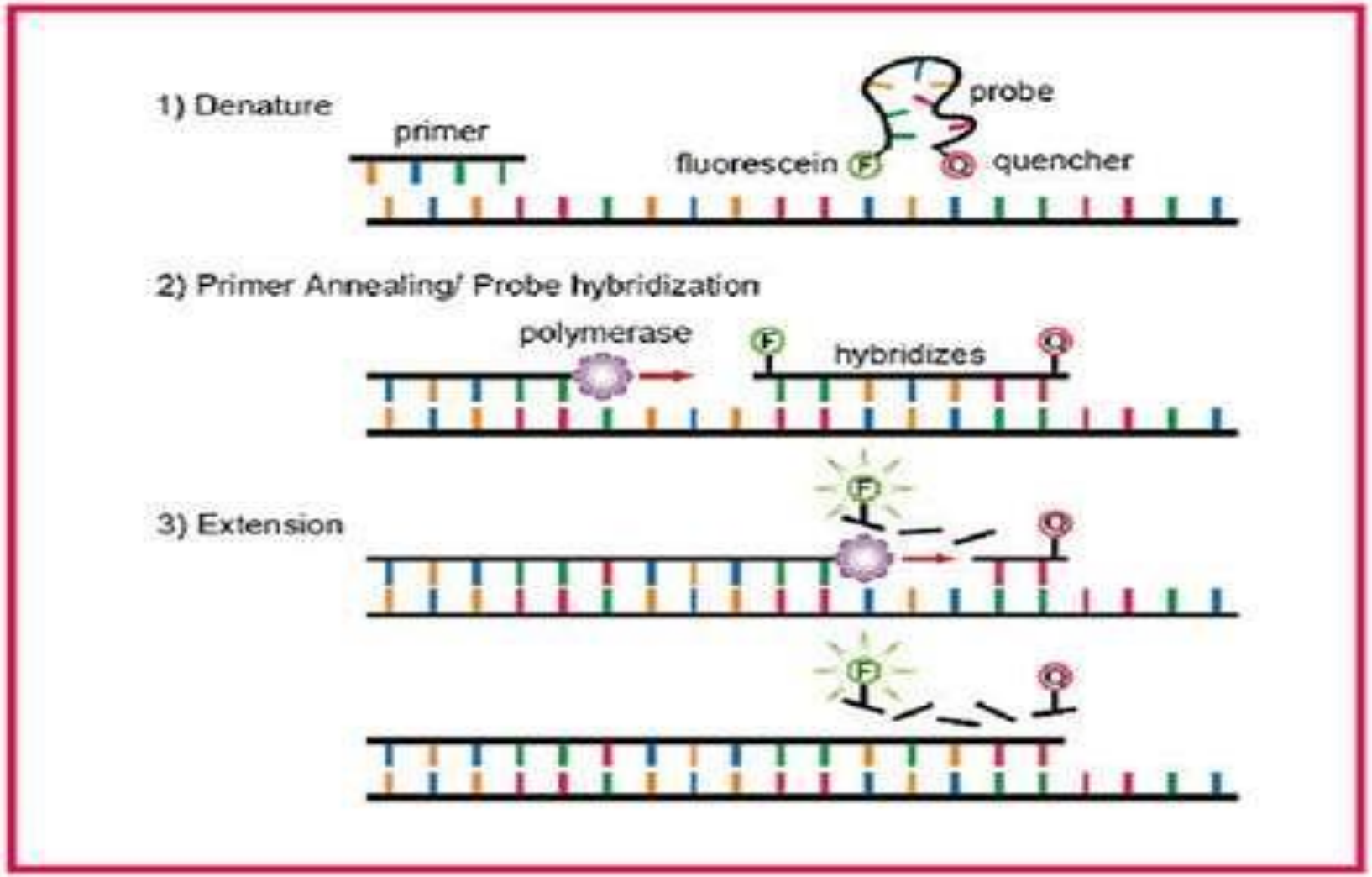
2. **Denaturation:** When the DNA is denatured, the SYBR® Green I Dye is released and the fluorescence is drastically reduced.

3. **Polymerization:** During extension, primers anneal and PCR product is generated.



4. **Polymerization completed:** When polymerization is complete, SYBR® Green I Dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the 7900HT system.





TaqMan® Probe Method

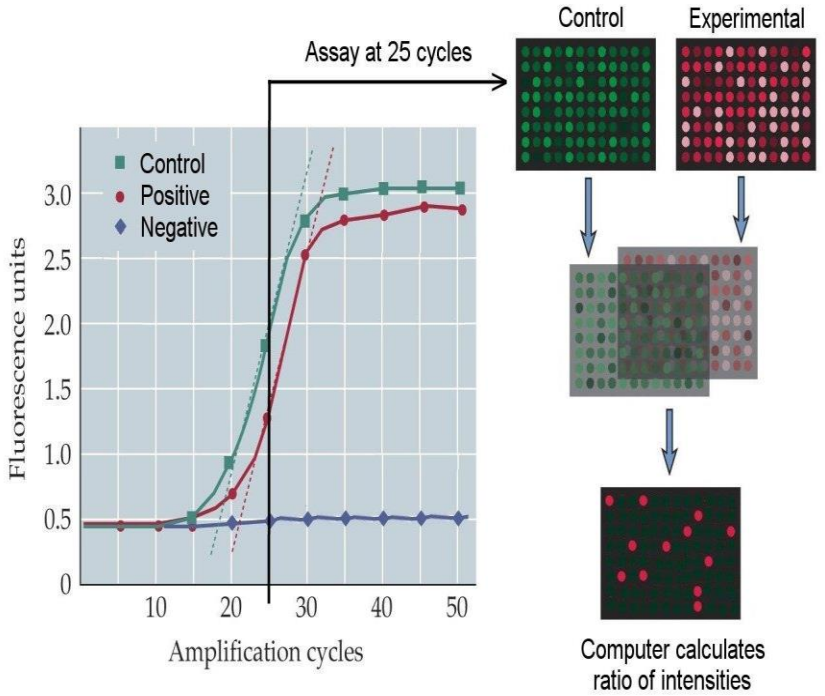
Specific method

Specific detection: fluorescent reporter probe method

- Fluorescent reporter probes detect only the DNA containing the sequence complementary to the probe; therefore, use of the reporter probe significantly increases specificity, and enables performing the technique even in the presence of other dsDNA.
- Using different-coloured labels, fluorescent probes can be used in multiplex assays for monitoring several target sequences in the same tube.
- The specificity of fluorescent reporter probes also prevents interference of measurements caused by primer dimers.
- The method relies on a DNA-based probe (molecular beacon) with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end of the probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence; breakdown of the probe by the 5' to 3' exonuclease activity of the Taq polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected after excitation with a laser.
- An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter.
- The PCR is prepared as usual and the reporter probe is added.
- As the reaction commences, during the annealing stage of the PCR both probe and primers anneal to the DNA target.
- Polymerisation of a new DNA strand is initiated from the primers, and once the polymerase reaches the probe, its 5'-3'-exonuclease degrades the probe, physically separating the fluorescent reporter from the quencher, resulting in an increase in fluorescence.
- Fluorescence is detected and measured in a real-time PCR machine, and its geometric increase corresponding to exponential increase of the product is used to determine the quantification cycle (C_q) in each reaction.

- Progress of **DNA** amplification during ***real time (RT-PCR)*** by measuring the release of fluorescent "flashes" during amplification.

- A computer measures the rate of "flashing" in 96 simultaneous **experimental PCR** reactions relative to a **control** reaction



Applications

There are numerous applications for quantitative polymerase chain reaction in the laboratory.

- Commonly used for both diagnostic and basic research.
- Uses of the technique in industry include the quantification of microbial load in foods or on vegetable matter.
- the detection of GMOs (Genetically modified organisms).
- the quantification and genotyping of human viral pathogens.

Quantification of gene expression

Quantifying gene expression by traditional DNA detection methods is unreliable. Detection of mRNA on a Northern blot or PCR products on a gel or Southern blot does not allow precise quantification. For example, over the 20–40 cycles of a typical PCR, the amount of DNA product reaches a plateau that is not directly correlated with the amount of target DNA in the initial PCR.

Real-time PCR can be used to quantify nucleic acids by two common methods: relative quantification and absolute quantification.

Absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards using a calibration curve.

It is therefore essential that the PCR of the sample and the standard have the same amplification efficiency.

Relative quantification is based on internal reference genes to determine fold-differences in expression of the target gene. The quantification is expressed as the change in expression levels of mRNA interpreted as complementary DNA (cDNA, generated by reverse transcription of mRNA).

Relative quantification is easier to carry out as it does not require a calibration curve as the amount of the studied gene is compared to the amount of a control reference gene.

As the units used to express the results of relative quantification are unimportant the results can be compared across a number of different RTqPCR.

The reason for using one or more housekeeping genes is to correct non-specific variation, such as the differences in the quantity and quality of RNA used, which can affect the efficiency of reverse transcription and therefore that of the whole PCR process.

However, the most crucial aspect of the process is that the reference gene must be stable.
The selection of these reference genes was traditionally carried out in molecular biology using qualitative or semi-quantitative studies such as the visual examination of RNA gels, Northern blot densitometry or semi-quantitative PCR (PCR mimics).

Now, in the genome era, it is possible to carry out a more detailed estimate for many organisms using transcriptomic technologies.

However, research has shown that amplification of the majority of reference genes used in quantifying the expression of mRNA varies according to experimental conditions.
It is therefore necessary to carry out an initial statistically sound methodological study in order to select the most suitable reference gene.
A number of statistical algorithms have been developed that can detect which gene or genes are most suitable for use under given conditions. Those like geNORM or BestKeeper can compare pairs or geometric means for a matrix of different reference genes and tissues.

Diagnostic uses

Diagnostic qualitative PCR is applied to rapidly detect nucleic acids that are diagnostic of, for example, infectious diseases, cancer and genetic abnormalities. The introduction of qualitative PCR assays to the clinical microbiology laboratory has significantly improved the diagnosis of infectious diseases, and is deployed as a tool to detect newly emerging diseases, such as new strains of flu, in diagnostic tests.

Microbiological uses

Quantitative PCR is also used by microbiologists working in the fields of food safety, food spoilage and fermentation and for the microbial risk assessment of water quality (drinking and recreational waters) and in public health protection.

Detection of phytopathogens

The agricultural industry is constantly striving to produce plant propagules or seedlings that are free of pathogens in order to prevent economic losses and safeguard health.
Systems have been developed that allow detection of small amounts of the DNA of *Phytophthora ramorum*, an oomycete that kills Oaks and other species, mixed in with the DNA of the host plant.

Discrimination between the DNA of the pathogen and the plant is based on the amplification of ITS sequences, spacers located in ribosomal RNA gene's coding area, which are characteristic for each taxon. Field-based versions of this technique have also been developed for identifying the same pathogen.

Detection of genetically modified organisms

- qPCR using reverse transcription (RT-qPCR) can be used to detect GMOs given its sensitivity and dynamic range in detecting DNA. Alternatives such as DNA or protein analysis are usually less sensitive. Specific primers are used that amplify not the transgene but the promoter, terminator or even intermediate sequences used during the process of engineering the vector. As the process of creating a transgenic plant normally leads to the insertion of more than one copy of the transgene its quantity is also commonly assessed. This is often carried out by relative quantification using a control gene from the treated species that is only present as a single copy.
- **Clinical quantification and genotyping**
- Viruses can be present in humans due to direct infection or co-infections which makes diagnosis difficult using classical techniques and can result in an incorrect prognosis and treatment. The use of qPCR allows both the quantification and genotyping (characterization of the strain, carried out using melting curves) of a virus such as the Hepatitis B virus.[

The degree of infection, quantified as the copies of the viral genome per unit of the patient's tissue, is relevant in many cases; for example, the probability that the type 1 herpes simplex virus reactivates is related to the number of infected neurons in the ganglia. This quantification is carried out either with reverse transcription or without it, as occurs if the virus becomes integrated in the human genome at any point in its cycle, such as happens in the case of HPV (human papillomavirus), where some of its variants are associated with the appearance of cervical cancer.