Swayam Course - Analytical Techniques

Week: 11, Module 28 PCR and Real Time PCR

Content Writer **Dr. Karthikeyan Pethusamy, Senior Resident, Department of Biochemistry, All India Institute of Medical sciences, New Delhi.**

Objectives:

- Difference between DNA replication in vivo and PCR
- Essential and optional components used in a PCR reaction
- Phases of PCR reaction
- Visualisation of PCR amplicons
- Advantages and Limitations of PCR
- Reverse-transcription PCR
- Applications of RT-PCR
- Real-time PCR (qPCR)
- Methods used in qPCR
- Melting curve analysis
- Variants of PCR

Introduction

Polymerase chain reaction (PCR) is the in vitro, enzymatic, DNA amplification technique to amplify a specific DNA segment of interest. Kary B Mullis was awarded the Noel prize in the year 1993 "for his invention of the PCR method".

In PCR, repetitive rounds of amplification of DNA is done using template DNA, specific primers and DNA dependent DNA polymerase enzyme. Products of the previous round act as template for the subsequent rounds, hence chain reaction

Let us compare in-vivo DNA replication & in vitro PCR reaction.

	DNA Replication	**PCR**
Nature of primer	RNA	DNA
Separation the two strands of DNA	Helicase	Heat
Name of enzyme that elongates the new strand of DNA	Thermolabile DNA polymerases (usually)	Thermostable DNA polymerases
Proof reading	+	±

1. Components of PCR

a. Template DNA
b. Pair of DNA Primers
c. Thermostable DNA polymerase
d. dNTPs
e. Buffer with divalent cation

Let us discuss every component briefly.

1.1. Template DNA

- 50-100 ng of pure DNA is needed.
- Should be free of proteins and lipids.

- DNA can be isolated from blood, tissues, cultured cells, hair follicle etc.
- Purity of the DNA is assessed by 260/280 ratio

1.2. Pair of Primers

Unlike RNA polymerase, DNA polymerase requires free 3' OH group to begin the polymerisation reaction. Primers are designed in such a way that they will anneal/hybridise to the region flanking the DNA sequence to be amplified.

1.2.1. Points to be considered while designing primer

- Length: 18-25 nucleotides – If the primer is too short, there will be non-specific hybridization. If the primer is too long, the melting temperature and annealing temperature will be very high.
- Melting Temperature: 55-65° C
- Annealing Temperature: Lower than the melting temperature
- GC Content: 40-60%
- Primer secondary structures due to self-complementarity: Avoided
- Repeating nucleotide sequences: Avoided

1.3. Thermostable DNA polymerases

Before the discovery of thermostable polymerases, PCR was done using the Klenow fragment (DNA polymerase I without 5'→ 3' exonuclease activity) of E. coli DNA polymerase. The problem with Klenow fragment is that it gets inactivated by high temperature.

Thermostable polymerases are derived from bacteria living in hot springs. Some of the thermostable polymerases and their origin are given below:

- *Taq (Thermus aquaticus)*
- *Pfu (Pyrococcus furiosus)*
- *Vent (Thermococcus litoralis)*
- *Tth (Thermococcus thermophiles)*

1.3.1. Taq Polymerase

Taq polymerase is a DNA dependent DNA polymerase derived from *Thermus aquaticus* with the half-life of 45 minutes at 95° C. Its extension rate is 2 to 5 kb/min and the processivity is 50-60 bases. The reason behind the thermostability of Taq polymerase is the increased hydrophobicity of the core of the enzyme and stabilization by electrostatic forces.

1.3.2. Pfu polymerase

Pfu is derived from *Pyrococcus furiosus*. It has 3' to 5' exonuclease activity (proof reading) which is absent in Taq polymerase. So, fidelity of the reaction is high.

1.3.3. Recombinant DNA polymerases

Most of the DNA polymerases used in PCR reactions currently are synthesised using recombinant DNA technology. The naturally derived (native) Taq polymerase does not contain proof-reading activity. Using recombinant technology, 3' to 5' exonuclease activity can be added to Taq polymerase to produce high-fidelity polymerases.

1.4. dNTPs (deoxynucleotide triphosphates)

Equimolar concentrations (200-250 μM each) of dATP, dCTP, dGTP, dTTP are used. dNTPs are the substrates for the DNA polymerase reaction. ddNTP (dideoxynucleotide triphosphates) are NOT used in PCR. ddNTPs are used in sanger's chain-termination method of DNA sequencing.

1.5. Buffer

Tris-Cl buffer is used to maintain the pH. The pH of the buffer at room temperature is 8.3 to 8.8. When incubated at 72 degrees (the temperature used for the extension phase of PCR), the pH of the reaction mixture decreases to around 7.2, the optimum pH of Taq Polymerase.

1.6. **Divalent cations**

All DNA polymerases require free divalent cations– usually Mg^{2+} for activity. These are usually provided in the buffer itself which is optimum DNA polymerase reactions.

1.7. Optional components of PCR

In addition to the above-mentioned components, the following optional components are also used in PCR reactions. It includes mainly co-solvents and additives. They are used to decrease the mispriming and increase the efficiency of amplification.

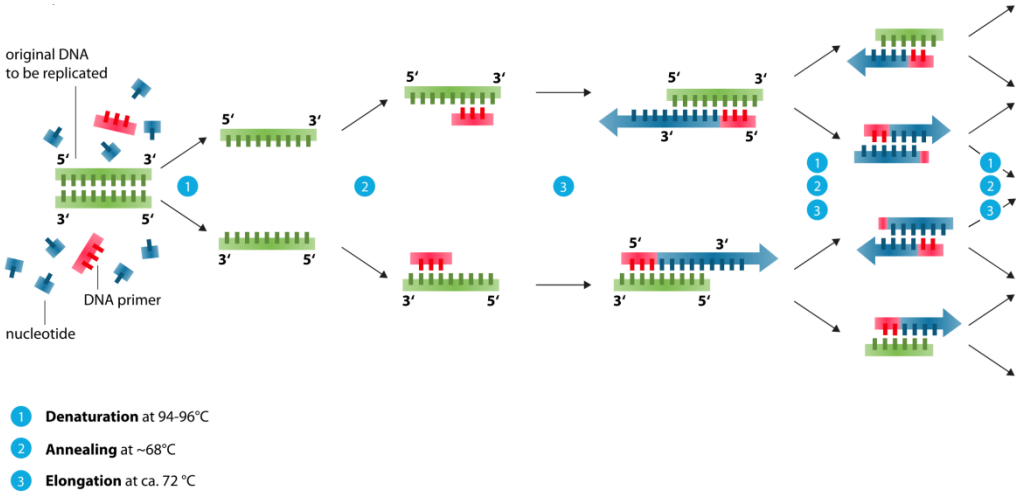
Optional component	Concentration	Use
Bovine serum albumin (BSA)	0.1 to 0.8 μg/μL	stabilizes Taq polymerase and overcome PCR inhibitors
DMSO	2-5% v/v, inhibitory at > 10% v/v	Denaturant - good at keeping GC rich template/primer strands from forming secondary structures
Glycerol	5-10% v/v	Increases apparent concentration of primer/template mix, and often increases PCR efficiency at high temperatures
Non-ionic detergents (Triton-X, Tween-20 or Nonidet P-40)	0.1–1%)	Stabilize Taq polymerase & suppress formation of 2º structure
Stringency enhancers (Formamide, Betaine, TMAC)	Concentrations used vary by type	Enhances yield and reduces non-specific priming

It should be noted that SDS should not be used as an optional component of PCR reaction since it decreases the activity of Taq polymerase by 10%.

2. PCR reaction

All these components (reaction mixture) are taken in a PCR tube and placed in the thermocycler (a.k.a PCR machine). Thermocycler can change the temperature of the reaction mixture rapidly.

When the reaction mixture is heated for 94° C, the hydrogen bond between the DNA strands break. When the thermocycler rapidly cooldowns to annealing temperature, the primers anneal to the template DNA. On raising the temperature to the optimum temperature of *taq* polymerase, i.e. 72°, elongation of the primer takes place. The cycle is repeated again and again up to 30 to 40 times.



Schematic drawing of a complete PCR cycle – notice the exponential amplification.
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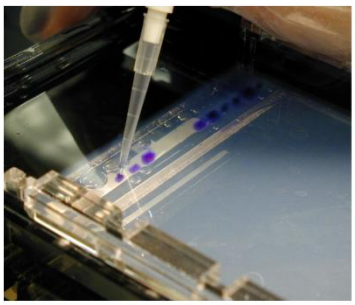
2.1. Phases of PCR

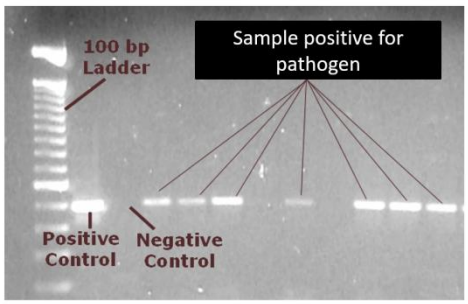
A PCR reaction consists of the following three phases.

1) **Exponential**: Exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). The reaction is very specific and precise.
2) **Linear**: The reaction components are being consumed, the reaction is slowing and components are starting to degrade.
3) **Plateau**: In due course of PCR reaction, the components get depleted and polymerase efficiency decreases. So, the reaction ceases and no more products are being made. If left long enough, the PCR products will begin to degrade.

2.2. Visualisation of PCR amplicons

The products produced by PCR amplification are known as amplicons. These are of a specific size based on the primer we have designed. So, we can do a gel electrophoresis to visualise the presence of a specific product size.





2.3. Advantages of PCR:

- PCR is exquisitely sensitive. A single DNA molecule can be amplified and detected.
- PCR can be made highly specific using the stringency of hybridization at relatively high temperature.
- The sequence of the target need not be known. Only the flanking sequence need to be known.
- Even targets larger than 10 kb can also be amplified by PCR using specific methods.

2.4. Limitations of PCR

In the beginning of the PCR reaction, copies of the target sequence are generated exponentially. Only during the exponential phase of the PCR reaction is it possible to extrapolate back to determine the starting quantity of the target sequence contained in the sample. Because of inhibitors of the polymerase reaction found in the

sample, reagent limitation, accumulation of pyrophosphate molecules, and self-annealing of the accumulating product, the PCR reaction eventually ceases to amplify target sequence at an exponential rate and a "plateau effect" occurs, making the end point quantification of PCR products unreliable. This is the attribute of PCR that makes Real-Time (Quantitative) PCR so necessary.

3. Reverse transcription PCR (RT-PCR)

RT-PCR is a 2-step process – reverse transcription followed by PCR.

The initial sample we take is RNA. Reverse transcription is the RNA directed DNA synthesis using the enzyme reverse transcriptases. The DNA created by the reverse transcription is complementary to the RNA and is known as complementary DNA (cDNA).

3.1. Components of reverse transcription/cDNA synthesis:

1. Template RNA
2. Reverse transcriptase
3. DNA primers
4. Buffer
5. dNTPs

3.1.1. Template RNA:

Template RNA should be checked for its integrity using RNA electrophoresis before reverse transcription and the amount of RNA needs to be quantified. Failure to do the integrity check before cDNA synthesis can lead to false estimation of RNA amount when there is a genomic contamination. Moreover, genomic DNA contamination in RNA will affect the downstream procedures like quantitative real-time PCR. The initial amount of RNA to be used for cDNA synthesis is

3.1.2. Reverse transcriptase:

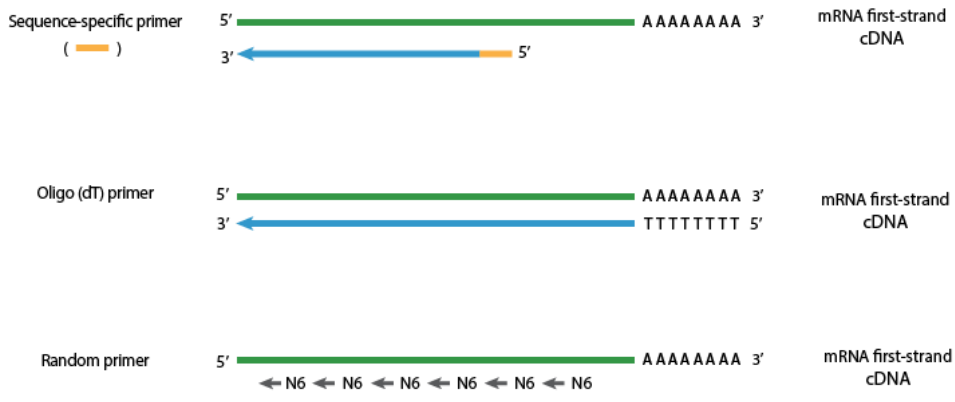
Reverse transcriptases are RNA dependent DNA polymerases present in retroviruses. Enzymes used for Reverse transcription is usually MMLV RT/AMV RT (Moloney strain of murine leukaemia virus reverse transcriptase/ Avian myeloblastosis virus). AMV reverse transcriptase was the first enzyme to be used for reverse transcription. The enzyme is a heterodimer with an optimal temperature of 42 to 48° C and it is less sensitive to inhibition by secondary structure. AMV RT enzyme contains RNAse H activity that degrades RNA in RNA:cDNA hybrids. This results in shorter cDNA fragments (<5 kb). So, when we want cDNA of >5kb length, AMV RT can’t be used.

MMLV RT is a popular alternative for AMV RT. It is monomeric enzyme and so it is easy to modify the properties of enzyme using recombinant DNA technology. MMLV RT is less thermostable than AMV RT. But because of its minimal RNAse H activity it can generate longer cDNA fragments (<7 kb) efficiently.

3.1.3. DNA primers:

Three types of DNA primers used for reverse transcription/cDNA synthesis:

1. Oligo dT primers
2. Random primers (random hexamers or random decamers)
3. Sequence-specific primers



3.1.3.1. Oligo dT primers:

Oligo dT refers to the linear sequence of 12 to 18 deoxythymidines. Oligo dT primers bind to the poly-A tail of mRNA. So, only mRNAs can be converted to cDNA when we use oligo dT primers. mRNAs not having poly A tail like histone mRNA can't be converted to cDNA by this method. The efficiency of reverse transcription by oligo dT priming is less when the mRNA is longer.

3.1.3.2. Random primers (random hexamer or decamer):

Random Hexamers are short oligo deoxyribonucleotides of random sequence [d(N)6] or [d(N)10] that anneal to random complementary sites on a target RNA. Using these primers, all types of mRNAs can be converted to cDNA. Longer RNAs can also be efficiently converted to cDNA.

3.1.3.3. Sequence-specific primers:

When there is a need to produce the cDNA of a specific gene (e.g. cDNA of insulin), sequence specific primers are used. Single step real-time PCR reactions also use sequence specific primers.

3.2. RT reaction

All the components of reverse transcription are kept in a water bath or dry heat block at 42° C for forty minutes. RT enzyme mixture present in the cDNA can interfere in the downstream processes. So, it is important to inactivate the enzyme by heating the reaction mixture to 92° C before storage.

cDNA produced by reverse transcription is the first strand of cDNA. That is why the commercially available kits for cDNA synthesis are known as "1st strand cDNA Synthesis Kit". When the first strand of cDNA is amplified by PCR, double-stranded cDNA is generated. Amplified DNA is detected by electrophoresis.

3.3. Applications of RT-PCR:

- Used in detection of RNA viruses
- To study mRNA expression levels in cells, tissues in a semiquantitative manner.
- To study for the presence of active infection, e.g. TB

4. Real-time PCR (qPCR)

Real-time PCR is used to quantify the amount of initial DNA in the reaction. Traditional PCR detects the DNA by electrophoresis at the end-point of the reaction. Real-time PCR generally uses a fluorescent dye to measure the amount of product as it is generated. This allows for data collection after each cycle of PCR instead of only at the end of the 20 to 40 cycles.

4.1. Three general methods used for the qPCR:

1. DNA-binding dyes: (SYBR Green, Syto 9, Eva green)
2. Hydrolysis probes: (Taq Man, Beacons, Scorpions)
3. Hybridization probes

We will limit our discussion only to dye-binding methods. PCR mixture is prepared along with double-stranded DNA binding dye (e.g. SYBR green). The dye shows increased fluorescence upon binding to the double stranded DNA. After each cycle, the level of fluorescence is measured. Fluorescence detectors capture the signals and convert them to graphical representation on the screen.

4.2. Controls used in qPCR

You might have learnt that in any analytical techniques, we need to use controls. Three types of controls are used in qPCR – Positive control, Negative control and minus RT control.

4.2.1. Negative control

This is also known no template control-NTC. Here, we don't add cDNA template. NTC is used to check reagent contamination. This is Must for every gene in every PCR.

4.2.2 Minus RT control

If there is a DNA contamination in the RNA, that is carried on to cDNA also. Presence of DNA in the cDNA can produce signal in the qPCR even in the absence of mRNA for the particular gene. To avoid this, during each cDNA synthesis reaction minus RT control is prepared.

4.2.3. Positive control

This is important for diagnostic applications. This ensures that the primer and reagents are working.

4.3. Role of passive reference dye

ROX is a passive reference dye used as a component of qPCR reaction. It is used for the normalization of fluorescence intensity of reporter dyes, such as SYBR Green I or TaqMan probes. It also allows to correct for pipetting errors, and instrumental drift such as change of lamp intensity output over time. Modern qPCR machines do not require the use of ROX dye as they have overcome the requirement of ROX.

4.4. Role of Housekeeping genes

During cDNA synthesis we take equal amount of mRNA for all the samples. But the efficiency of reverse transcriptase can vary from sample to sample. To normalise this, we should also study the expression of a house-keeping gene in addition to the gene of interest. The expression of house-keeping genes is constant over time.

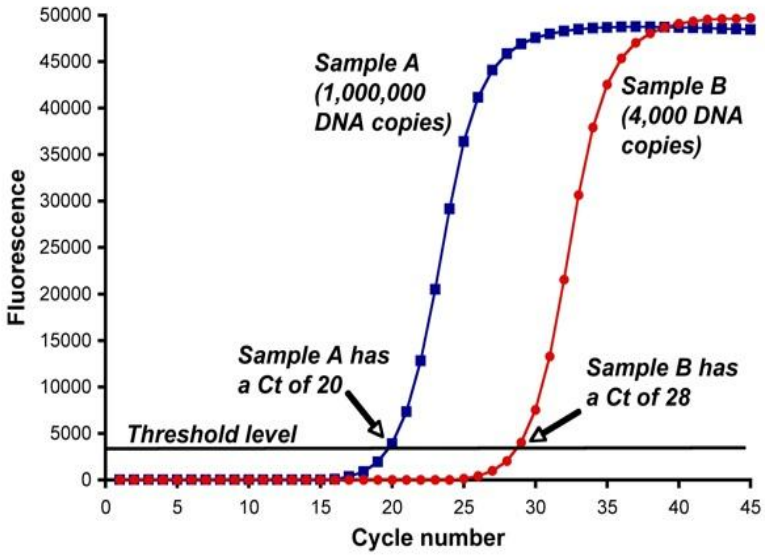
Reference gene used for normalisation	Physiological role
GAPDH (Glyceraldehyde 3 phosphate dehydrogenase)	Glycolytic enzyme with moonlighting functions
28S ribosomal protein	Subunit of ribosome with peptidyl transferase activity
TFRC (Transferrin receptor)	Intracellular iron uptake
HPRT (Hypoxanthine-guanine phosphoribosyl transferase)	Purine salvage pathway
TBP (Tata box binding protein)	Transcription factor

4.5. Analysis of Real-time Data

It is important to understand the following three terms used in qPCR.

1. **Baseline** is defined as PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument.
2. **Threshold**: is an arbitrary level of fluorescence chosen on the basis of the baseline variability. A signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (CT) for a sample. Threshold can be adjusted for each experiment so that it is in the region of exponential amplification across all plots.
3. **Threshold cycle (Ct)** is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. The CT is a basic principle of real time PCR and is an essential component in producing accurate and reproducible data.

   If the initial amount of cDNA (in turn mRNA) is less, it will take a long time to achieve the threshold fluorescence, and thus the Ct value will be higher. The initial amount of cDNA/mRNA is inversely proportional to the Ct value. For example when the threshold is set for a Real time PCR and sample A has a Ct value of 20 and sample B has a Ct value of 28, it means that sample A has 2^(Ct of B -Ct of A), that is 8 times, more starting template than sample B.



The original amount of cDNA (RNA) was higher in sample A compared to sample B. Sample A contains 2^8 fold higher amount of RNA.

Fold gene expression is usually calculated using the following method.

ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

ΔΔCt = ΔCt (treated sample) – ΔCt (untreated sample)

Fold gene expression = 2^-(ΔΔCt)

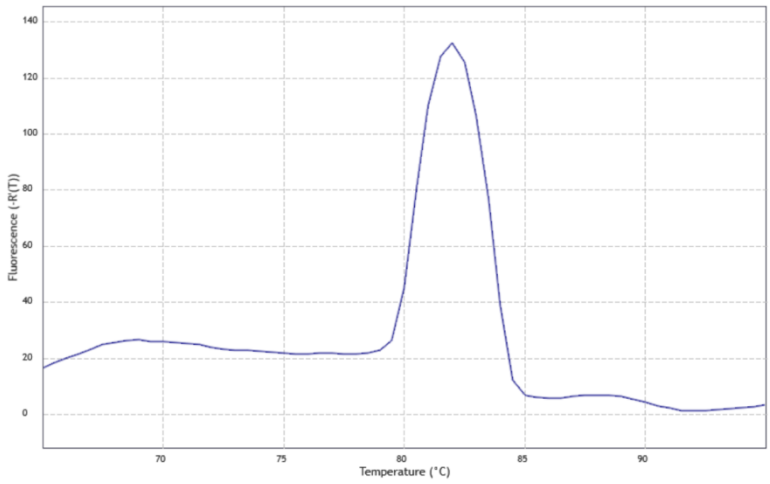
The major disadvantage of delta delta Ct method is that it presumes that the efficiency of the PCR reaction is 100%.

	Experimental Well 1	Experimental Well 2	Experimental Well 3	Control Well 1	Control Well 2	Control Well 3	Average Experimental Ct Value	Average Experimental Ct Value	Average Control Ct Value	Average Control Ct Value	ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
	Raw Ct Value	Raw Ct Value	Raw Ct Value	Raw Ct Value	Raw Ct Value	Raw Ct Value	TE	HE	TC	HC	ΔCTE	ΔCTC	ΔΔCt	2^-ΔΔCt
Housekeeping Gene	21.00	20.50	20.60	20.00	20.50	20.30	-	20.70	-	20.27	1.80	5.93	-4.13	17.54919968
Gene being Tested	23.00	22.00	22.50	26.00	26.20	26.40	22.50	-	26.20	-				

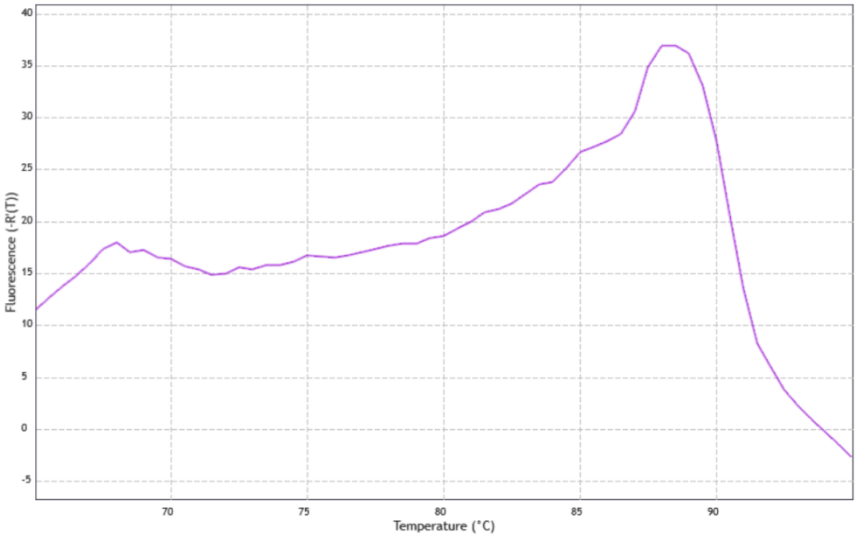
Example of fold change detection using the delta delta Ct method

4.6. Melting-curve analysis:

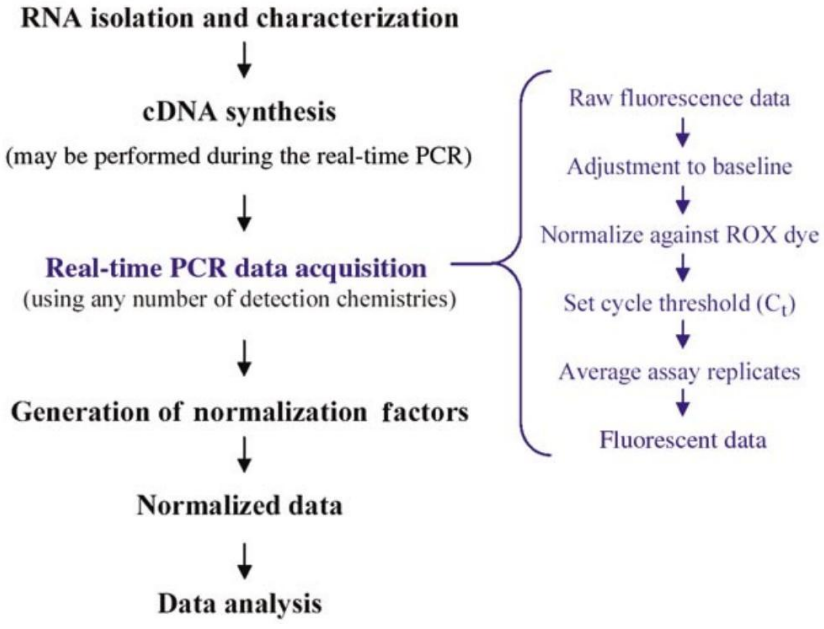
Each qPCR reactions should always be followed by melt-curve analysis to prove that genuine amplification is taking place. In melting curve analysis, the amplicons are subjected to a temperature above the melting temperature of the primer pair and the amount of fluorescence is measured. The temperature is increased incrementally. As we have already learnt, dyes like SYBR green can bind only to double-stranded DNA not to the single stranded DNA. So, as the temperature increases, the fluorescence will decrease. The change in slope of this curve is then plotted as a function of temperature to obtain the melt curve. The presence of a single peak indicates a single amplicon, that is the amplification is specific.



Melt-curve showing single peak denoting the specific amplification.



Melt-curve showing non-specific amplification.



Overview of Real time PCR

4.7. Absolute quantitation using qPCR

For most of the research purposes, relative quantification using the delta CT method is sufficient. But, absolute quantification is required in diagnostic applications like estimation of viral load in HIV positive individuals. Absolute quantitation requires a serially diluted standards of known concentrations to generate a standard curve. The standard curve produces a linear relationship between Ct and initial amounts of total RNA or cDNA. The concentration of unknown can be calculated by curve-fitting.

4.8. Advantages of Real-Time PCR compared to conventional PCR

- Amplification can be monitored in real time
- Less than two-fold change can also be detected.
- No post-run processing like electrophoresis is required.

5. Variants of PCR:

As PCR is versatile, there are so many variations of PCR available. Interested readers can further study these techniques in the literature.

1. Multiplex PCR
2. Nested PCR
3. Colony PCR
4. Digital PCR
5. Suicide PCR
6. Methylation-specific PCR (MS-PCR)
7. Amplification-refractory mutation system (ARMS)-PCR

6. Summary

- Polymerase chain reaction is an in vitro, enzymatic DNA amplification technique.
- Template DNA, Pair of DNA Primers, Thermostable DNA polymerase, dNTPs, Buffer with divalent cation are the components of PCR.
- Taq polymerase is a thermostable DNA polymerase.
- ddNTPs are used in Sanger's chain-termination method of DNA sequencing. ddNTPs are not used in PCR.
- Reverse transcriptase is an RNA-dependant DNA polymerase.
- Oligo dT primers, Random primers (random hexamers or decamers) and Sequence-specific primers are used for cDNA synthesis.
- SYBR green dye is a DNA-binding dye used in real-time PCR.