Understanding qRT PCR:

Basic principles and applications

Introduction

The polymerase chain reaction (PCR) is an *in vitro* molecular biology technique, used to replicate (or amplify) a specific region or segment of DNA billion-fold in just a few hours. Further development of reverse transcription coupled PCR i.e. reverse transcriptase PCR (RT PCR) opened up tremendous scope for the development of molecular diagnostic methods for pathogens with RNA genomes (RNA viruses and retroviruses) as well as basic biological science research involving all types of RNA entities (including messenger RNA (mRNA) transfer RNA (tRNA), ribosomal RNA (rRNA), micro-RNA (miRNA) molecules, ribozymes etc) in living cells. RNA is a crucial intermediate in flow of information (DNA makes RNA makes protein). More recently, among the few changes forced by the SARS CoV-2 pandemic the installation of real time thermal cyclers in molecular diagnostic labs across the world has led the medical fraternity to turn to RT PCR based diagnosis of infectious disease above culture based assays.

Going back in history, the discovery of the thermophilic bacteria by Thomas D. Brock and Hudson, named as ***T**hermus **aq**uaticus,* published in the *Journal of Microbiology* in 1969 led to the isolation of thermostable DNA polymerase enzyme, *Taq* DNA polymerase (in 1976). This laid the foundation stone for conceptualization of polymerase chain reaction (PCR) by Karry Mullis in1983 and allowed for the first time, specific detection and production of large amounts of DNA. Similarly, the discovery of **reverse transcriptase (RT)** an RNA dependent DNA polymerase enzyme in retroviruses (tumor viruses with RNA genome) simultaneously by David Baltimore (at MIT, Cambridge, USA) and Howard Temin (at UW, Madison, USA) in 1976 further revolutionized molecular biology and paved way for detection of retroviruses based on reverse transcription of natural RNA template followed by PCR to detect and quantify the cDNA product.

The earlier version of PCR thermal cyclers was designed to perform DNA amplification. The instrument comprises of the thermal block with slots into which the micro-tube or 96 well plate is inserted. In a very precise and pre-programmed manner, the machine raises and lowers the temperature of the block so that the reaction solution is first heated above the melting point of the DNA (usually 95-98^{0}C, 1-3 minutes), which allows the strands (of the DNA double helix) to separate, and is referred to as the denaturation step. The temperature is then lowered (50-60^{0}C, 15-45 seconds) to allow the specific primers to bind to their complementary site in the target DNA segments, a process called hybridization or annealing step. The temperature is raised again (72^{0}C, 30 seconds per kilobase of expected amplicon length), at which time the DNA polymerase is able to extend the primers by adding nucleotides to the developing DNA strand. The cycles of denaturation, annealing and extension are repeated several times (nearly 20-40 times) and with each repetition of these three steps, the number of copied DNA molecules is doubled. The subsequent detection of PCR amplicons was performed by gel electrophoresis. This labor-intensive end-point detection involved limitations such as and post-PCR processing, inaccurate quantification, poor sensitivity as well as risk of carryover contamination.

Higuchi *et al.* in 1992 described the idea of “real-time PCR” where inclusion of a fluorescent reporter system made it possible to continuously monitor the accumulation of PCR amplicons in “real-time” along side the amplification. The real-time PCR thermal cyclers were thereafter developed and this refinement of the technology was shown to be more sensitive and specific, offering the possibility of quantification, and since no post-PCR processing is performed, the risk of carryover contamination is reduced. The principle behind qPCR is to monitor the accumulation of PCR amplicons in “real time” by measuring the change in emission of fluorescence from either fluorescent DNA-binding dyes or target-specific fluorescently labeled primers or probes added to the PCR. The different primer and probe technologies will be discussed in the next sections.

I. Basic principles:

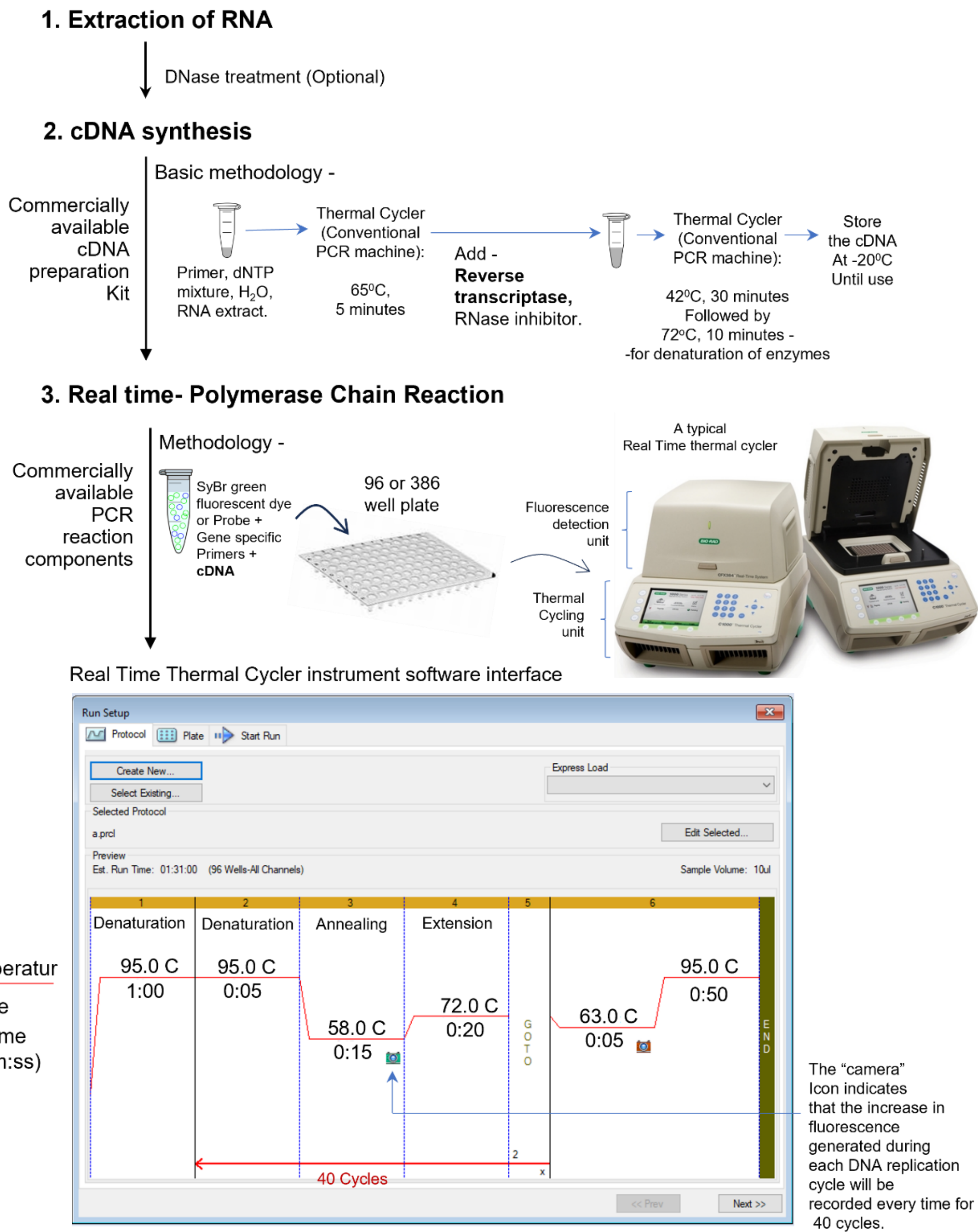
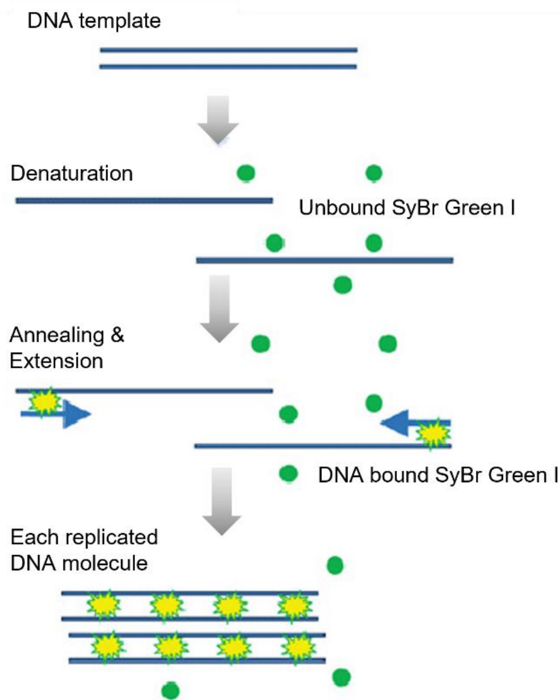
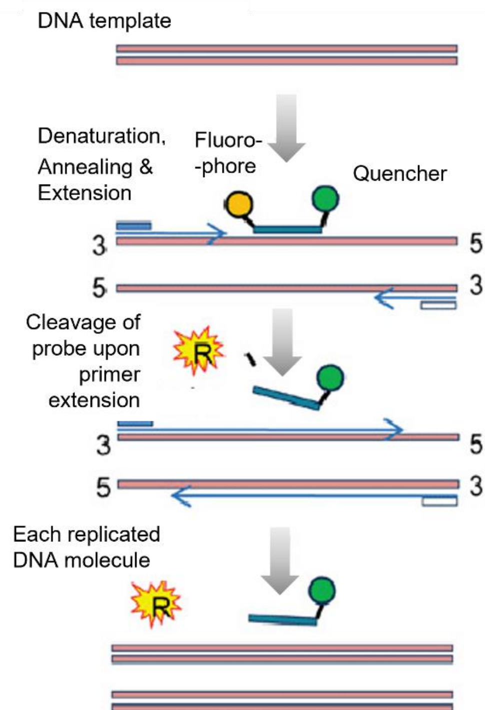


Figure 1: A typical work flow demonstrating setting up of quantitative RT PCR assay.

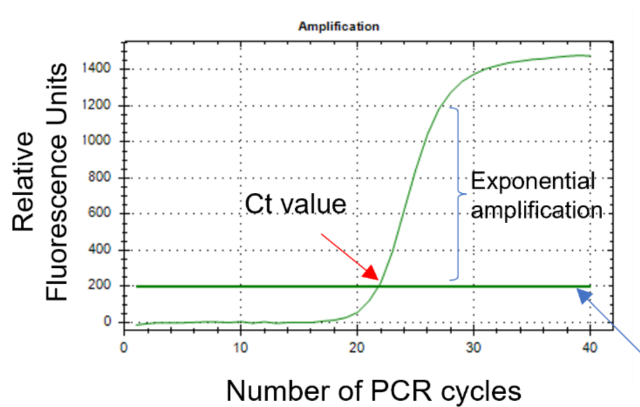
The fluorescent reporter system employed to monitor the accumulation of PCR amplicons in “real-time” is either Fluorescent dye based (SyBr Green, Eva Green etc) of Fluorescent probe based as described below.





The Real time PCR (qPCR as well as qRT PCR) results are visualized in an amplification plot. Fluorescence is represented on the Y axis, whereas the number of PCR cycles is plotted along the X axis.

2(a) **A typical amplification curve :**



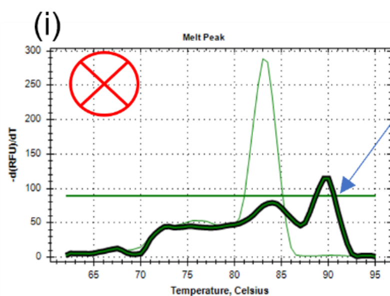
Each sigmoid curve represents increase in fluorescence associated with amplification of DNA per PCR cycle for each sample

The PCR **C**ycle number at which the fluorescence generated by each amplified DNA sample crosses the **t**hreshold is referred to as the “Ct” value

Threshold fluorescence level set by the instrument

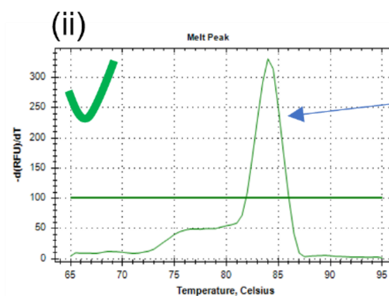
2(b) Melt peak :

(i)



Multiple peaks in the Melt curve Indicates Multiple Non-spesific PCR products

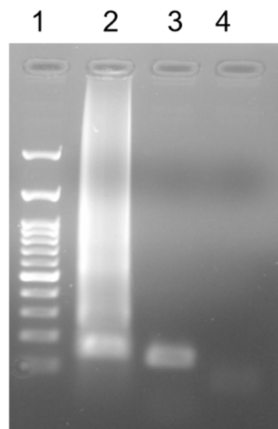
(ii)



Single peak in the Melt curve Indicates spesific Amplification of single PCR amplicon.

2(c) RT PCR products run on 1% agarose gel. The quality of the RT PCR amplified product can be further analysed by running the amplified products on agarose gel:

1 2 3 4



1: 100bp DNA ladder

2: Smear represents non specific amplification indicating the designed primers are not working and are giving spurious amplification.

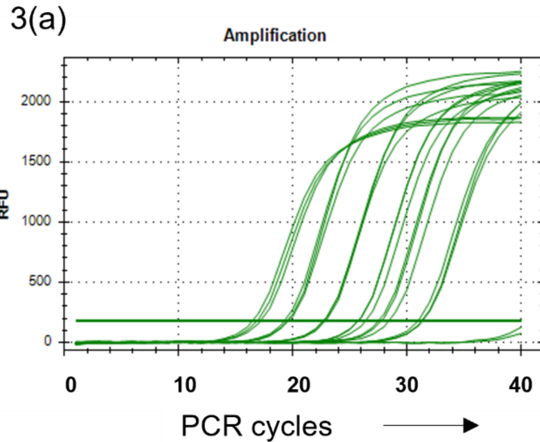
3: PCR amplified product of 120bp

4: No amplification, only primer dimer visible.

Results & data analysis

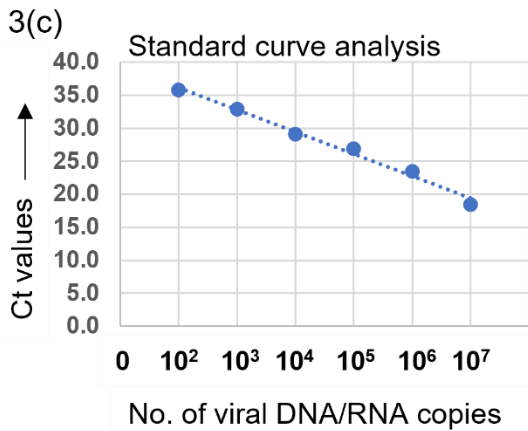
Absolute quantitation:

Example: RT PCR with precisely defined and serially diluted concentrations of target viral gene (eg. synthesized RNA copy of SARS CoV-2 RdRp gene):



3(b)

Number of viral DNA/RNA copies	Ct (a)	Ct (b)	Ct (c)	Avg Ct
10^7	16.29	16.17	16.15	**16.64**
10^6	18.5	18.3	18.5	**19.41**
10^5	23.3	23.6	23.3	**22.81**
10^4	27.0	26.9	26.9	**25.77**
10^3	29.1	29.2	29.0	**27.53**
10^2	33.0	32.9	32.8	**31.17**
0	No Ct	No Ct	No Ct	**0**



Equation: (y= mx+x)

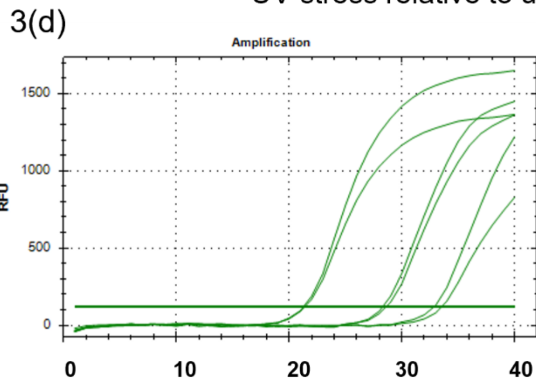
$$Y = -3.3537x + 39.494$$

R^2= 0.9886

Here: Y= Ct Value; x = Viral load

Relative quantitation:

Example: RT PCR to assess TP53 mRNA expression levels in cells exposed to UV stress relative to unexposed cells.



	Ct (TP53)	Ct (18s rRNA)	Δ Ct	ΔΔ Ct
Cells (+ UV)	29.1	21.5	7.6	-3.3
Cells (No UV)	32.2	21.3	10.9	0

Fold change in TP53 mRNA levels in UV treated cells relative to untreated cells is ($2^{-\Delta\Delta Ct}$) = 9.84

II. Applications

1. Many clinically important viruses have genomes composed of RNA, RT-PCR is useful for detecting such viruses. RT-PCR has also been used for the detection of the viral causes of meningitis and 2. meningoencephalitis, such as enteroviruses and the West Nile virus. RT-PCR is being used for the detection of viruses including Dengue virus, Hantavirus, Human metapneumovirus and Severe acute respiratory syndrome (SARS, eg. SARS CoV-2)

2. Quantitative RT-PCR assays are also commonly used for the detection of HIV and HCV **viral load** (amount of these viruses present in the blood of a patient) testing. Viral load data are important for monitoring the response of the individual patient to therapy. For instance, after appropriate antiretroviral therapy, patient infected with HIV virus should demonstrate an increase in CD4 count and a decrease in HIV viral load.

3. RT-PCR may also be used **to detect other microorganisms** (bacteria, parasites, and fungi) by targeting their rRNA. This approach is better than detection of DNA, as the presence of RNA is more likely associated with the presence of **viable** organisms.

4. Quantitative real-time PCR (qPCR) has been widely used in recent environmental microbial ecology studies as a tool for detecting and quantifying microorganisms of interest, which aids in better understandings of the complexity of wastewater microbial communities.