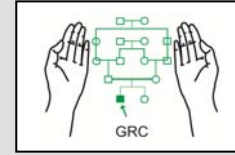


Quantitative PCR (Q-PCR)

Maj Gen (R) Suhaib Ahmed, HI (M)



In an ideal PCR the number of DNA molecules should double with each cycle. In practice the doubling of DNA molecules is achieved only in the initial cycles. In the latter cycles the reaction is gradually slowed down due to consumption and degradation of the reaction ingredients. A typical PCR may be divided into three phases (Fig 6.1):

1. Exponential: a short lived phase in which the DNA molecules double with each cycle. This is the most efficient phase of reaction.
2. Linear: as the reaction components are consumed the doubling of DNA is progressively slowed down and it may take more than one cycle for the DNA molecules to double.
3. Plateau: when the reaction components are consumed to a large extent the doubling of DNA molecules also comes to a halt. If the reaction is allowed to proceed indefinitely the number of amplified DNA may actually decrease due to their degradation. This is the most inefficient phase of the reaction.

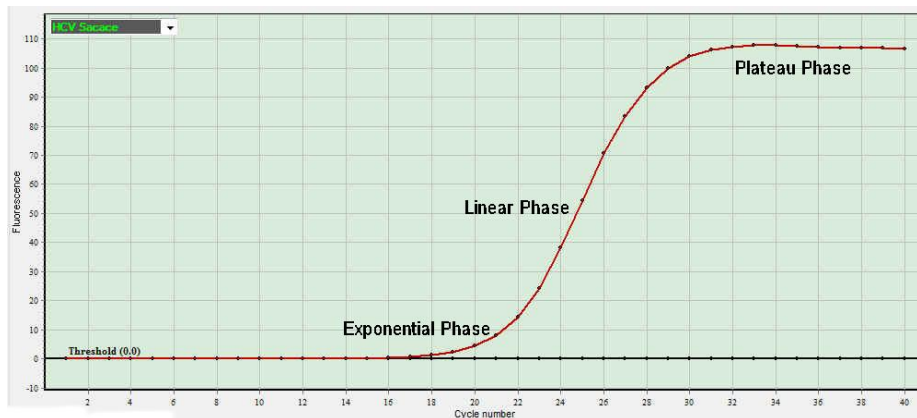
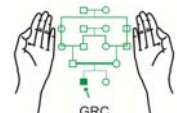


Fig. 6.1. Real Time PCR plot showing the three phases (1) Exponential, (2) Linear and (3) Plateau phase.

In a conventional PCR the amplified DNA is usually examined by gel electrophoresis at end of the PCR when reaction is usually in the “plateau phase”. The endpoint analysis by gel electrophoresis is unsuitable for quantitative assessment of target DNA because in the plateau phase the difference between two or more DNA targets with different quantities would give more or less the same result.

In a real time PCR the amplification is monitored (visualized) after each cycle and the result is plotted on a linear or a logarithmic scale. This method provides a window for monitoring the amplification in each phase of the reaction. The quantitative assessment of



DNA is best made when the reaction is in the “exponential phase”. The cycle number at which the amplification first appears is dependent on the quantity of target DNA. The amplification appears earlier when the target DNA is more and it appears late with decreasing concentration of the target DNA molecules. This makes real time PCR an ideal tool for accurate quantitative estimation of DNA.

Real time monitoring of amplification

SYBR green method

SYBR green is a fluorescent dye that binds to minor groove of double stranded DNA. It gives little fluorescence when in solution but emits a strong fluorescent signal after binding with double-stranded DNA. SYBR green can be used for the real time monitoring of PCR. As the amplified double stranded DNA accumulates in the PCR tube fluorescence of the SYBR green also increases. The main drawback of SYBR green is that it also binds to primer dimers and the DNA that is non-specifically amplified. The SYBR green method is also unsuitable for analysis of multiplex PCR products. The latter, however, may be overcome by using the option of melting curve analysis.

Molecular probe methods

The amplification of DNA is best monitored by specific methods employing fluorescent labeled short sequences of DNA complementary to the target (probe). Several types of molecular probes have been developed for real time monitoring of PCR.

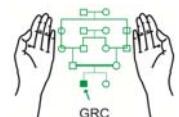
The fluorescence emitted in the molecular probe methods is directly proportional to the amount of amplification. These methods are highly specific unless the target has cross homology with another sequence in the genome.

Fluorescence Resonance Energy Transfer (FRET) Probe method

In the FRET probe method the target is amplified by the usual pair of primers. The FRET probes comprise a pair that anneals to the inner region of the target in a head to tail configuration. The upstream probe has a fluorescent dye (donor) at the 3' end whereas the downstream probe has another fluorescent dye (acceptor) attached at the 5' end. If the target DNA is present it is amplified by the two primers and the two FRET probes also anneal to the target. The head to tail configuration of the FRET probes ensures that the fluorescent dye at the 3' end of the upstream probe comes in close proximity to the fluorescent dye at the 5' end of the downstream probe. On excitation by the light of appropriate wavelength the energy from the upstream probe (λ_1) is transferred to the downstream probe (λ_2). The fluorescent dye on the downstream probe after absorbing energy emits this in the form of light of yet another wavelength (λ_3). The latter is measured by the instrument and is directly proportional to the amount of amplification.

Molecular Beacon method

Target DNA is amplified by the usual pair of primers. The molecular beacon probe is designed to be complementary to the inner region of the target DNA. At each end of the probe 5-7 nucleotides, complementary to each other, are inserted to ensure a hairpin structure (stem) of the probe (Fig. 6.2). At each end of the probe a fluorescent reporter dye and a quencher are also attached. In the non-annealed probe the reporter and the



quencher are very close to each other and the fluorescence from the reporter dye is quenched. As a result of annealing of the probe to its target the hairpin structure opens up and separates the reporter from the quencher. The net result is that on excitation by the light of appropriate wavelength the reporter dye starts emitting fluorescence. The latter is directly proportional to the amount of amplification.

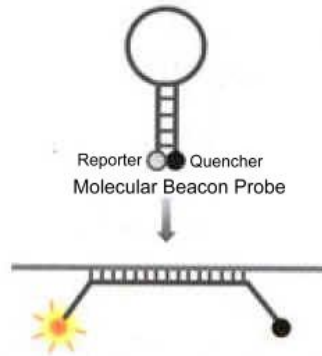


Fig. 6.2. Molecular Beacon probe in the un-annealed and the annealed state.

TaqMan® probe method

TaqMan® probe is a short sequence of DNA complementary to the internal region of the PCR target DNA. The 5' end of the probe is labeled with a fluorescent dye (reporter) while its 3' end is labeled with a fluorescence quencher (suppressor). The quencher may be a high energy fluorescent dye like rhodamine (TAMRA) or a non-fluorescent chemical quencher e.g. DABCYL and black hole quenchers (BHQ). In the intact probe the reporter dye and the quencher are in close proximity to each other. This suppresses the fluorescence of the reporter dye.

In the TaqMan® method of real time PCR the target DNA is amplified by the usual pair of primers. The reaction mixture also contains the TaqMan® probe that emits no fluorescence when it is not annealed to its target. If the target DNA is absent there is no amplification and the probe does not emit any fluorescence. When the target DNA is present it would be amplified. During amplification the probe, being complementary to the internal region of the target, also anneals to the target. The Taq polymerase used in PCR has 5' to 3' exonuclease activity which removes the probe from the target in a stepwise manner. First the reporter dye is removed then the probe itself and finally the quencher is also removed (Fig 6.3). As a result the reporter dye is relieved from the quencher and starts emitting fluorescence on excitation by the light of appropriate wavelength. The net increase in fluorescence from the reaction is directly proportional to the amount of probe annealed to the target and hence the amplification.

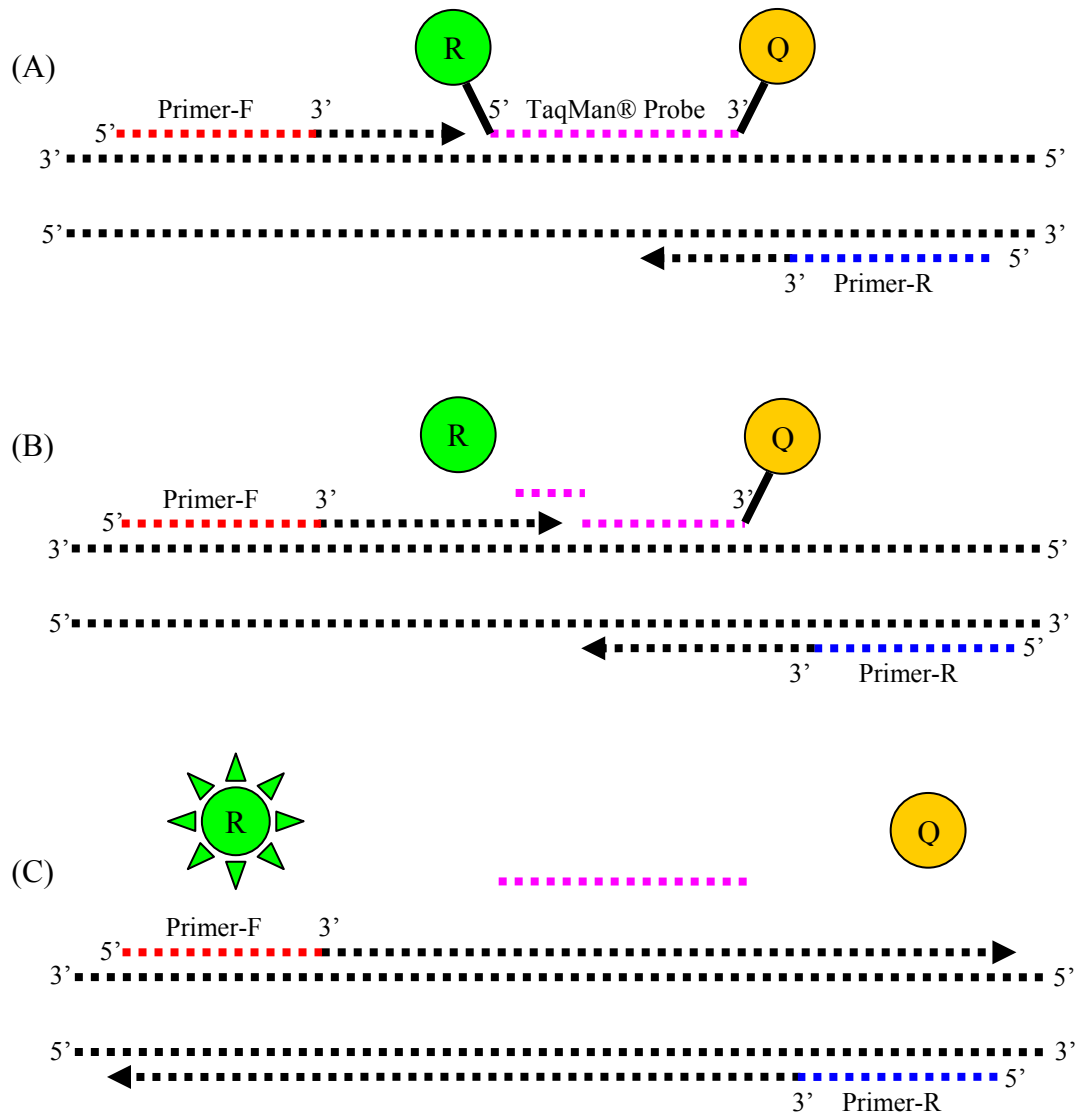
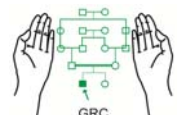


Fig. 6.3. Real time PCR by TaqMan® probe method. (A) The target is amplified by primers F & R. TaqMan® probe is annealed to the target and there is no fluorescence from the reporter (R) because of the close proximity to the quencher (Q). (B) As the primer extension takes place and reaches the 5' end of the probe, the reporter (R) is released due to the 5' to 3' exonuclease activity of Taq polymerase. (C) Continued extension of the new strand removes the probe and finally the quencher (Q). As the distance between the reporter and the quencher increases the reporter dye gives a bright fluorescence on excitation that is measured by the instrument. The quantity of fluorescence is directly proportional to the amount of target and the amplification.

TaqMan® probe designing

The TaqMan® probe is designed while keeping the following parameters in mind:

1. T_m of the probe should be 10°C higher than that of the primers.



2. Runs of identical nucleotides especially “Gs” should be avoided.
3. The G+C content should be 30-80%.
4. There should be more Cs than Gs.
5. There should be no G at the 5' end.

Primers for TaqMan® assay

In addition to the general requirements of primer designing (Chapter 3) the primer pair used with the TaqMan® probe should have the following additional properties:

1. Should have no runs of four or more Gs (any nucleotide).
2. Should have no more than two G+C at the 3' end.
3. Should have no G at the 5' end (A or C is preferred).
4. The amplicon size should range between 50-150bp (max 400).
5. Preferably should span the exon-exon junctions in cDNA.

The TaqMan® probes are best designed by computer software specially made for this purpose. Many commercial software are available for this purpose. Some real time PCR machine vendors also provide the software with the machine. The sequence of interest is uploaded in the FASTA format or it can be cut and pasted in the software window. The software offers several design options and provides a list of possible primers and the probe combinations. The best combination is chosen for a particular application.

Choice of reporter and quencher

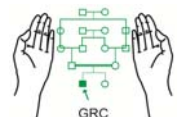
The reporter and the quencher pair for a probe should be compatible with each other chemically as well as for the wavelength of fluorescent light. Table 6.1 gives a list of the reporter dyes with the wavelengths of incident and emerging light. An appropriate dye can be chosen depending on the capability of the machine on which the probe would be used.

The quencher may be fluorescent e.g. TAMRA or chemical e.g. DABSYL and black hole quenchers (BHQ). TAMRA as a quencher has a drawback of giving high background fluorescence. DABCYL also has a limitation of having poor spectral overlap between itself and the reporter. Black hole quenchers (BHQ1, BHQ2 and BHQ3) have been developed to overcome these problems. Table 6.1 also provides a list of compatible quenchers for the relevant reporter dyes.

When choosing probes for a multiplex real time PCR the reporter dyes should be selected so that their excitation wavelengths are clearly separate with minimal overlap.

Background fluorescence subtraction

In a PCR done by fluorescent dyes some degree of background fluorescence is emitted even when there is no amplification. Subtraction of the background fluorescence in a real time PCR may be difficult because the noise has tendency to either increase or decrease with the reaction cycles. Various options for background subtraction include subtraction of minimal background noise, or an average background noise or the assumption of



various background trends. Some real time PCR machines use addition of a passive reference dye like ROX in the reaction mixture. It does not participate in the 5' nuclease reaction but it serves as an internal reference for background fluorescence emission.

Table 6.1. Wavelengths of the incident and the emergent light of some commonly used reporters and the quenchers for TaqMan® probes.

| Reporter dye | Incident light wavelength | Emergent light wavelength | Compatible quenchers |
|----------------|---------------------------|---------------------------|----------------------|
| FAM/SYBR Green | 492 | 516 | TAMRA, DABCYL, BHQ1 |
| TET | 517 | 538 | TAMRA, DABCYL, BHQ1 |
| HEX/JOE/VIC | 535 | 555 | TAMRA, DABCYL, BHQ1 |
| Cy3 | 545 | 568 | BHQ2 |
| TAMRA | 556 | 580 | BHQ2 |
| ROX/Texas Red | 585 | 610 | BHQ2 |
| Cy5 | 635 | 665 | BHQ3 |

SYBR green method

SYBR green non-specifically binds to double stranded DNA and is frequently used in real-time PCR reactions. It fluoresces very brightly when it is bound to double stranded DNA. The non-specific binding of SYBR green is a disadvantage in real time PCR as the dye could also bind to the non-specific PCR amplified products or the primer dimers. The problem can be overcome by a special procedure called “melting curve analysis”.

Another disadvantage of the SYBR green method is that the larger amplified products give a stronger signal. The SYBR green method is also unsuitable for multiplex PCR unless it is combined with melting curve analysis.

The SYBR green method may be used after extensive optimization in detection of single amplicons with no non-specific amplification and primer dimer formation.

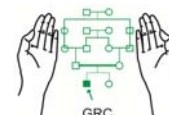
Melting curve analysis

The real time PCR machines using SYBR green based methods also provide option for melting curve analysis. At the end of the PCR the temperature of the heating block is raised in steps of 1°C. At each step the T_m of the reaction products is measured by the amount of fluorescence emitted. At the end of the analysis the software plots the T_m readings against the temperature. The presence of a single or more than one amplified products can be identified by their respective T_ms.

Real time PCR machine

The hardware

The instrument consists of a thermal cycler, light source, interchangeable light filters and sensitive light recording device. The light source is either halogen lamp or LED and the



recording device may be CCD camera, photodiode, or photomultiplier tube. The machines vary in the capability of detecting the number of colours. A basic machine can detect two colours while more advanced machines can detect up to five or more colours. Detection of more than two colours is useful for simultaneous detection of greater number of probes with different fluorochromes in a single reaction tube for example multiplex PCR.

The software

The instrument software provides an interface for the thermal cycling parameters and the choice of dye (fluorochrome) and quencher. As the run starts the real time graphic record of amplification after each cycle is displayed on the screen. At the end of the run the amplification record can be seen on linear or logarithmic scale.

Threshold cycle (Ct)

In a real time PCR recording the cycle number at which the first significant increase in fluorescence is seen is called “Cycle-threshold” or “Ct”. It correlates indirectly with the starting amount of template DNA. Higher the amount of template DNA smaller is the Ct (Fig. 6.4).

Rn

Rn is an indicator of the amount of fluorescence generated in a PCR. The amount of fluorescence (Rn) of a test reaction and a non target control (NTC) are called Rn^+ and Rn^- respectively. A difference between the Rn^+ and R^- is called ΔRn (Fig. 6.4).

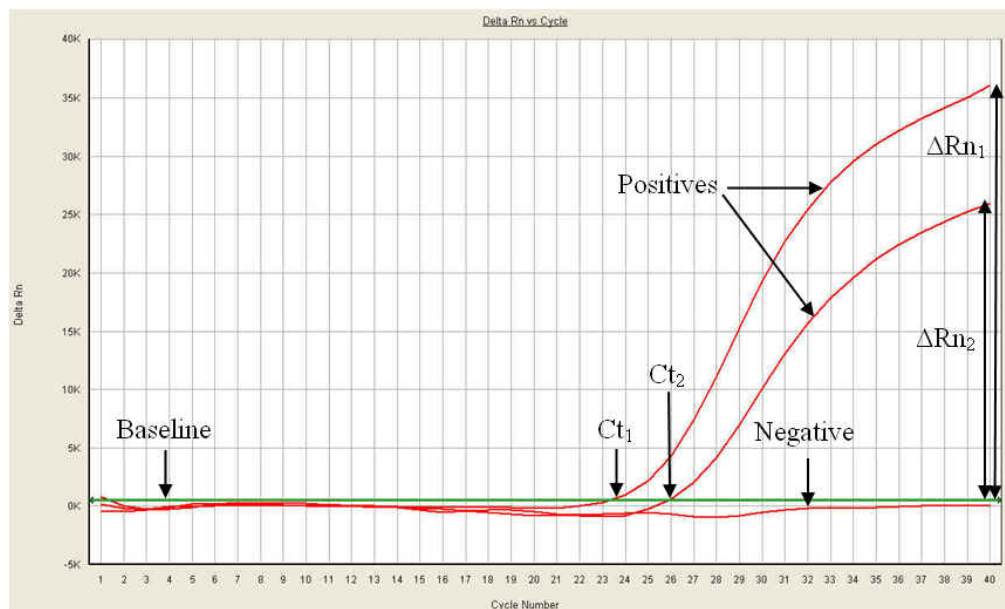
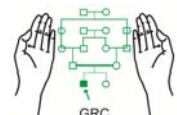


Fig. 6.4. Real time PCR plot on a linear scale. The cycle numbers are shown on X-axis while the amount of fluorescence is shown on Y-axis. The Ct_1 (23.6) and Ct_2 (26.0) indicate that there is greater quantity of target DNA in the sample 1 than in the sample 2. This is also reflected in the ΔRn values of the two samples.



Linear versus logarithmic recording

In PCR one molecule of DNA is doubled with every cycle. In a real time PCR our main interest is in knowing the first detectable change in the amount of fluorescence (increase in DNA). If the result of real time PCR is plotted on a linear scale it becomes difficult to appreciate the smaller change in fluorescent signal in the earlier cycles. However, if the results are plotted on a log scale the change in the earlier cycles becomes more pronounced and easy to recognize. The software of the real time PCR machines provides option for displaying the results in linear as well as log scale.

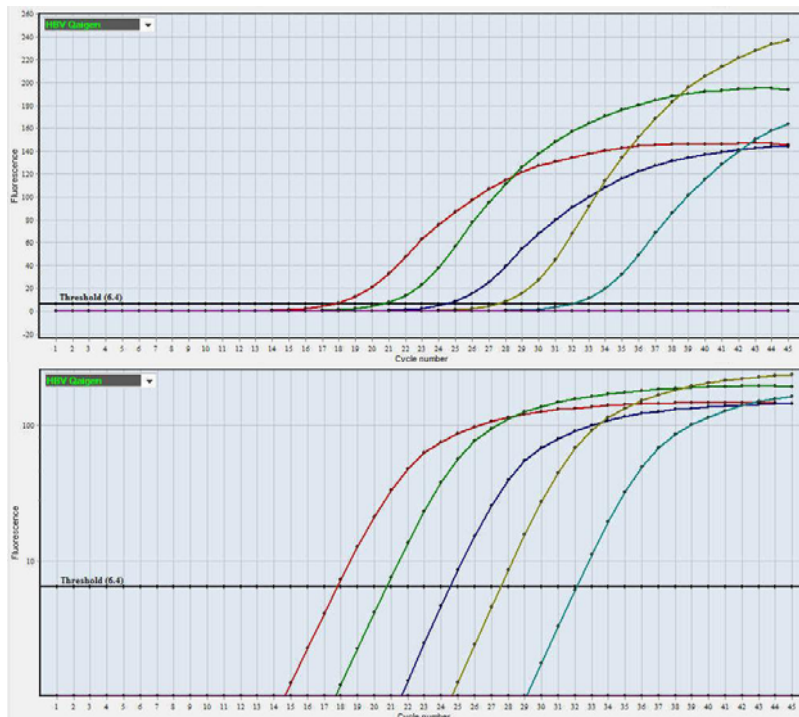
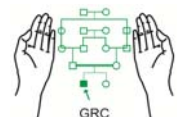


Fig 6.5. Real time PCR recording of the same batch of standard as seen on linear and log scales.

Real time PCR applications

The best use of real time PCR is in quantification of DNA and RNA. The quantification may be absolute, relative or comparative. In absolute quantification accurately quantified standards are used to make a standard curve. In relative quantification serial dilutions of a calibrator are used to make a standard curve. In comparative quantification the calibrator is used only once and the calculations are made mathematically.

Another common use of real time PCR is in detection of pathogens like viruses or bacteria. Here the major advantage of real time PCR is in elimination of end point processing which is a major source of false positives due to carry over of amplified product from one sample to another. An additional advantage is in the reduction of steps in analysis and hence less chances of errors.



Real time PCR may also be used in multiplex allelic discrimination and single nucleotide polymorphism (SNP) assays. Multiple TaqMan® probes with different fluorescent dyes can be used for different alleles. In SYBR green based methods melting curve analysis can be used to detect multiple amplified fragments.

PCR efficiency

Efficiency of PCR means the rate at which the amplified product is generated. The PCR efficiency is highest in the initial “exponential phase” when the number of DNA molecules is doubling with each cycle. The efficiency of PCR decreases in the latter “linear” and “plateau” phases. The reaction efficiency decreases because the reaction components are being consumed and degraded.

In a real time PCR the efficiency is usually gauged by the number of cycles in which a given amount of DNA is increased tenfold. In 100% efficient PCR tenfold increase in the target DNA is achieved in 3.32 cycles. The efficiency of PCR is usually measured from the slope of a tenfold diluted standard curve ($E = 10^{-1/\text{slope}}$). A steep slope indicates low efficiency whereas a gradual slope indicates a more efficient PCR.

The efficiency of PCR is dependent on a large number of variables including the reaction conditions and the PCR machine. Composition of the PCR mix is a major determinant of PCR efficiency. Several additives that enhance PCR (Chapter 3) can improve PCR efficiency. PCR efficiency between 90-110% is considered acceptable. A higher efficiency of PCR is more likely to detect smaller amounts of the target and vice versa.

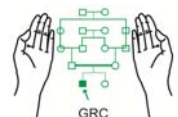
Coefficient of correlation (R^2)

In a real time PCR there is a linear correlation between the Ct value and the concentration of DNA. Under perfect conditions the Ct values of ten fold dilution of DNA standard when plotted against the concentration should show a straight line (100% correlation or $R^2=1.0$). However, inconsistencies in carrying out PCR, notably the pipetting errors, may cause varying degrees of deviation from the straight line. This may result in R^2 values below 1.0.

Absolute quantification of DNA by real time PCR

Absolute quantification of DNA is done by running tenfold diluted DNA standards of known concentration (Fig 6.6). The DNA concentration of each dilution is plotted against its Ct value. The plot is also known as “standard curve”.

Fig 6.6. shows the real time plot of a tenfold diluted HBV DNA standard. The sample with highest concentration of DNA has the lowest Ct. The real time PCR software is used to plot the DNA concentration of each standard dilution against its Ct value (Fig 6.7). The samples with unknown concentration of DNA are also run in a similar way as the tenfold diluted DNA is run. The real time PCR software automatically calculates the DNA concentration of the unknown samples with the help of the standard curve.



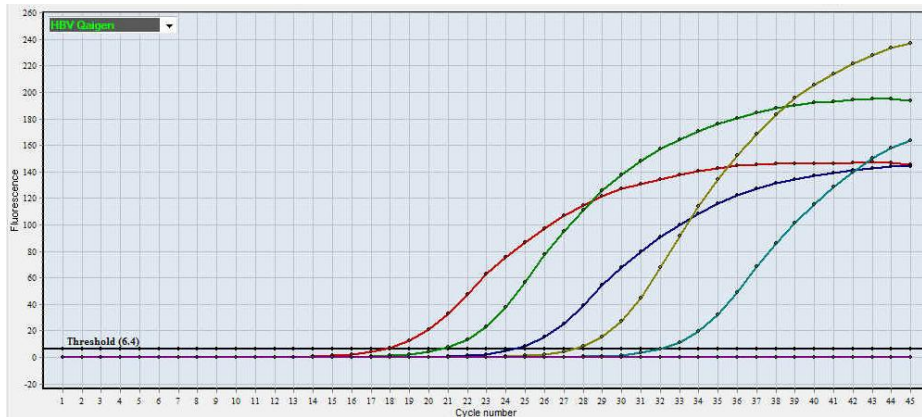


Fig. 6.6. Real time PCR plot of 10 fold diluted HBV Standard DNA. The Ct of the first dilution is the lowest and it increases with each dilution.

Quantitative analysis

| Number of the hole | Identifier of the tube | Ct, Fam | Ct, Hex | Concentration, copies/ml |
|--------------------|------------------------|---------|---------|--------------------------|
| A1 | QS-1 (HBV Qaigen) | 17.7 | | 1.0E+07 |
| A2 | QS-2 (HBV Qaigen) | 20.7 | | 1.0E+06 |
| A3 | QS-3 (HBV Qaigen) | 24.4 | | 100,000 |
| A4 | QS-4 (HBV Qaigen) | 27.5 | | 10,000 |
| A5 | QS-5 (HBV Qaigen) | 32.1 | | 1,000 |
| A6 | C_- (HBV Qaigen) | | | |

* Manual(Threshold) analysis method (B,F) Threshold_FAM = 6.4 Threshold_HEX = 0.0

$$Y = 42.211 - 3.549 * X$$

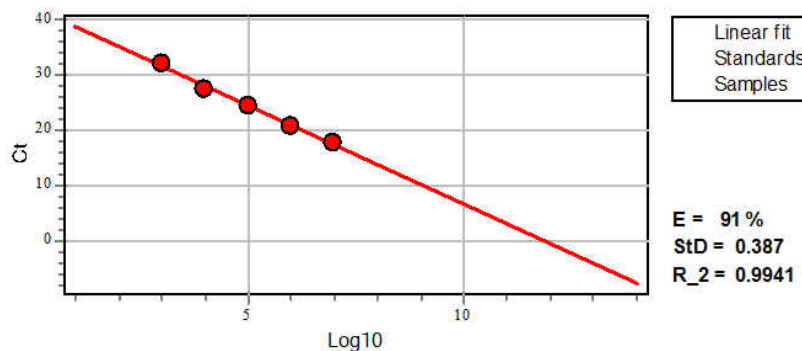
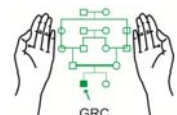


Fig. 6.7. Standard curve plot between the DNA concentration (x-axis) and the corresponding Ct value (y-axis) of the 10 fold DNA dilutions (blue dots) and unknown DNA samples (red dots) run on the Rotor Gene 6000 machine. The inside window also displays the related PCR statistics.

Relative Quantification of DNA by real time PCR

The amount of target DNA in a sample can also be calculated from the difference in the Ct values of a target DNA and an internal reference (internal control DNA) or a DNA



standard with known concentration. The relative quantification is subject to the quality of the RNA or DNA in the target and the reference material. Ideally the quality of RNA or DNA of the two should be similar. The target and the reference RNA or DNA can be run in the same or two different reaction tubes. The difference in the Ct value between the unknown (target) and the standard (reference) is called ΔCt . A major drawback of the relative quantification by using a single standard is that it does not take into account the PCR efficiency that is calculated from the slope of the standard curve. Since PCR efficiency is higher in the earlier than in the later cycles a single standard is unlikely to give accurate results in the very high or the very low range.

Example:

$$\Delta\text{Ct} = \text{Ct target} - \text{Ct reference (standard)}$$

The amount of target is given by the mathematical formula $2^{-\Delta\text{Ct}}$

For example at ΔCt of +2.0 the amount of target would be:

$$2^{2.0} = 4.0$$

Or the target sample has four times (fold) DNA or RNA as compared to that present in the reference (standard).

At ΔCt of -2.0 the amount of target would be:

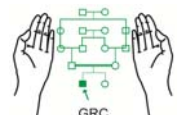
$$2^{-2.0} = 0.25$$

Or the target sample has 0.25 (1/4) fold DNA or RNA as compared to that present in the reference (standard).

A scientific calculator or Excel spreadsheet can be used to calculate complex values of ΔCt like 2.7 or -3.4 etc.

DNA quantification by real time PCR versus gel electrophoresis

Fig. 6.8 shows the real time PCR plot of a serially diluted DNA sample. All dilutions have clearly distinct Ct values that correlate with the amount of DNA in the sample. It is also seen that after the 30th cycles the reaction enters a plateau phase. If gel electrophoresis is done on the same samples at the end of 35 cycles they would show bands of only slightly differing intensities. This is why real time PCR is considered superior to gel electrophoresis for DNA quantification by PCR.



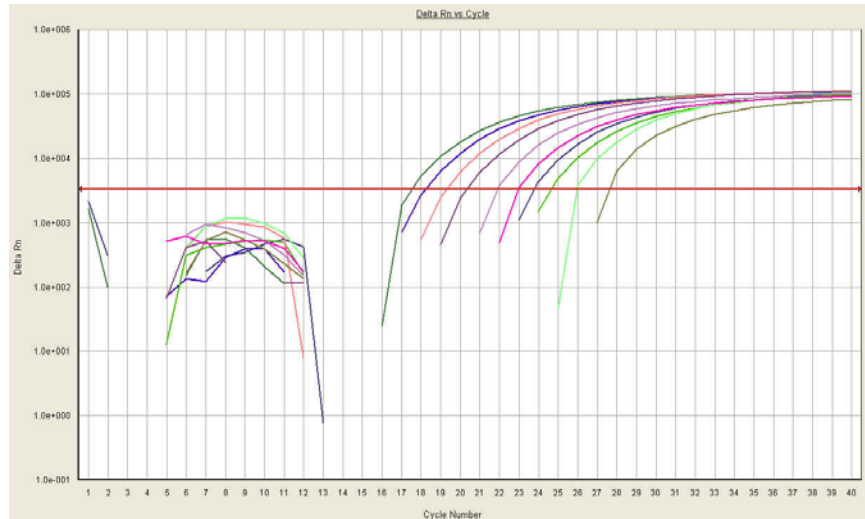


Fig. 6.8. Real time plot of a serially diluted sample of DNA. All dilutions have clearly distinct Ct values that correlate with the amount of DNA in the sample. After the 30th cycles the reaction has entered a plateau phase. If gel electrophoresis is done on the same samples at the end of 35 cycles they would show bands of only slightly differing intensities.

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