Chapter 16

Setting up of quantitative real time PCR

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Real Time PCR is one of the PCR based assays to monitor the amplification of a particular gene /gene product in real time basis without any need for the post amplification process for visualizing DNA such as agarose gel electrophoresis, capillary electrophoresis etc. The fluorescent dye added to the reaction mixture allows the monitoring of the amplification staring from the first cycle of the PCR run and concomitantly the fluorescence is increased to 2 to 1000-fold as amplification progresses. Thus, based on the fluorescence, the DNA can be quantified over wide range of concentrations with the help of standard curves. Further, the data generated from the amplification process can easily be analyzed. The sensitivity and reliability of the result is significantly higher compared to conventional PCR. Real time PCR can be used for viral/bacterial quantification, gene/allele copy number, allelic discrimination assays (SNPs) gene expression, Methylation studies etc.

The real time detection of the nucleic acid amplification is achieved by nonspecific or sequence specific strategies. The nonspecific method uses intercalating dyes which can able to produce fluorescence while binding with ds DNA.The commonly used nonspecific dye in real-time PCR is asymmetric cyanine dye called SYBR Green I. This dye has higher affinity to ds DNA compared to that of ethidium bromide and the intensity of the bound dye is higher (magnitude of 1000 folds) than the free form of syber green. This enables an increase in fluorescence during amplification. However, once the melting of the double stranded DNA after polymerisation causes the denaturation of DNA and signal strength falls off due to the detachment of fluorescent dye. Other dyes of this category include, O-PRO-1, BEBO, YOYO -1. The major advantage of nonspecific dyes are less

expensive, and can be used with any pair of primers/target. The disadvantage is that it binds non-specifically to any ds DNA yielding signal from nonspecific products. However, this can be verified at the end with the help of melt curve analysis by subjecting the amplicon to a temperature range beyond its melting temperature.

The sequence specific strategies employ the use of either hydrolysis probes or hybridization probes. These probes are synthesised based on the sequences of the internal fragments of the two primers. The quantification of the PCR product is done by measuring the fluorescence signal strength based on either quenching or FRET mechanism.

Hydrolysis probes are the probes which are hydrolysed due to 5'-3' exonuclease activity of DNA polymerase during the elongation stage of the PCR cycle. TaqMan Probe is widely known hydrolysis probe for RT PCR application. It is nothing but a oligo sequence labelled with reporter dye in one end (5'end) and quencher dye at the other end. In intact the fluorescence emitted from the reporter dye is banned due to the presence of quencher dye in its close proximity. During PCR run, DNA is denatured and both primer and the probe annealed to the target DNA. However, the Taq polymerase has exonuclease activity will cleave the probe and the reporter and quencher dye get separated, thus allowing the fluorescence emission from the reporter dye when it excited with a suitable light source. As amplification progress, the signal strength gets increased enabling the quantification of DNA. The melting point of the probe should be 10 degrees higher than primer Tm as cleavage of the probe take place only during the elongation step of the PCR. In addition to TaqMan Probe, TaqMan MGB probes are also used. The Minor groove binder increase the melting temperature of the probe and it increase the duplex stability particularly for shorter probes. In case of hybridization probes the fluorescent signal is obtained due to the structure changes in the secondary structure of the probe during hybridization phases. The changes in the structure causes increase the distance between reporter and quencher dye preventing the fluorescence resonance energy transfer (FRET) from a reporter dye to

quencher dye. The probe in its intact form is a hair pin like structure and behaving non-fluorescence chromophore due to close proximity of both quencher and reporter dye. However, the conformation changes during hybridization demands separation of both dyes and the far distance among the dyes prevent the energy transfer through FRET mechanism. Thus, the increased fluorescent signal from the reporter dye enables the quantitative estimation of the DNA. . With both types of assays, the exponential increase in fluorescence is used to determine the cycle threshold (Ct) which is the number of PCR cycles at which significant exponential increase in fluorescence is detected. Using a standard curve for Ct values at different DNA concentrations, quantitation of target DNA in any sample can be made.

Protocol for setting up of Absolute Quantitative real time PCR

Material required

Genomic DNA of Known concentration (50ng/µl)

Primers and probes

2X Taqman universal master mix

DNAse free water

DNAse free TE

Plasticware

Real time instrument

Strategy:

Make standards of known concentration by serially diluting a stock concentration of genomic DNA of known concentration

Set up a real time PCR assay for the standards, unknown and negative control samples.

Plot a standard curve for the Ct values vs the log concentration of the standards from the standard curve determine the concentration of the unknown samples using Ct values.

Procedure:

1 Make 10-fold serial dilution for the genomic DNA to minimum of 4 standard concentrations.

2 Make master mix taking in to consideration about the replicates, NTC, standards, unknown samples

Components	Volume per reaction µl
2X TaqMan master mix	5.0
20X TaqMan Primer probe mix	0.5
Water	2.5
Total	8.0

3 Mix well and centrifuge briefly

4 Prepare the mix for each replicate by four time of 8 µl master mix (step2) with respective DNA/TE/NTC)

5 Disperse the master mix into four individual tubes for each DNA/TE/NTC. Centrifuge the tube briefly

6: Set up the experiment on the instrument using system software.