Real Time PCR

Aim:

Demonstration of Real-Time PCR through experimental samples.

Introduction:

Real-time quantitative PCR allows the sensitive, specific and reproducible quantitation of nucleic acids. It monitors the amplification of a targeted DNA molecule during the PCR (i.e. in real time), not at its end, as in conventional PCR. Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. Using PCR, specific sequences within a DNA or cDNA template can be copied, or "amplified", many thousand- to a million-fold using sequence specific oligonucleotides, heat stable DNA polymerase, and thermal cycling. The PCR is the cyclic reaction based on the rapid change in temperature during each step. During PCR, our gene of interest is amplified as well as quantified. Real-time quantitative PCR is the reliable detection and measurement of products generated during each cycle of the PCR process, which are directly proportional to the amount of template prior to the start of the PCR process.

Principle:

It is based on detection and qunatification of fluorescent repoter as the reaction progresses. Real-time detection of PCR products is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequence specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle. Fluorescent reporters used in real time PCR include double-stranded DNA (dsDNA)- binding dyes, or dye molecules attached to PCR primers or probes that hybridize with PCR product during amplification. The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with fluorescent dye scanning capability. By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction.

Requirements:

1. Template (cDNA)
2. SYBR green master mix
3. Gene specific primers (Forward primer Fp and Reverse primer Rp)
4. Nuclease free water
5. Optical PCR tubes

6. Ice bucket with ice

7. DNase/RNase free tips

8. Micropipettes

Important Instructions:

1. Before starting the experiment the entire procedure has to be read carefully.

2. Always wear gloves while performing the experiment.

3. Keep all the PCR components in ice throughout the procedure

4. SYBR green is a fluorescent dye therefore, it should be added in the dark.

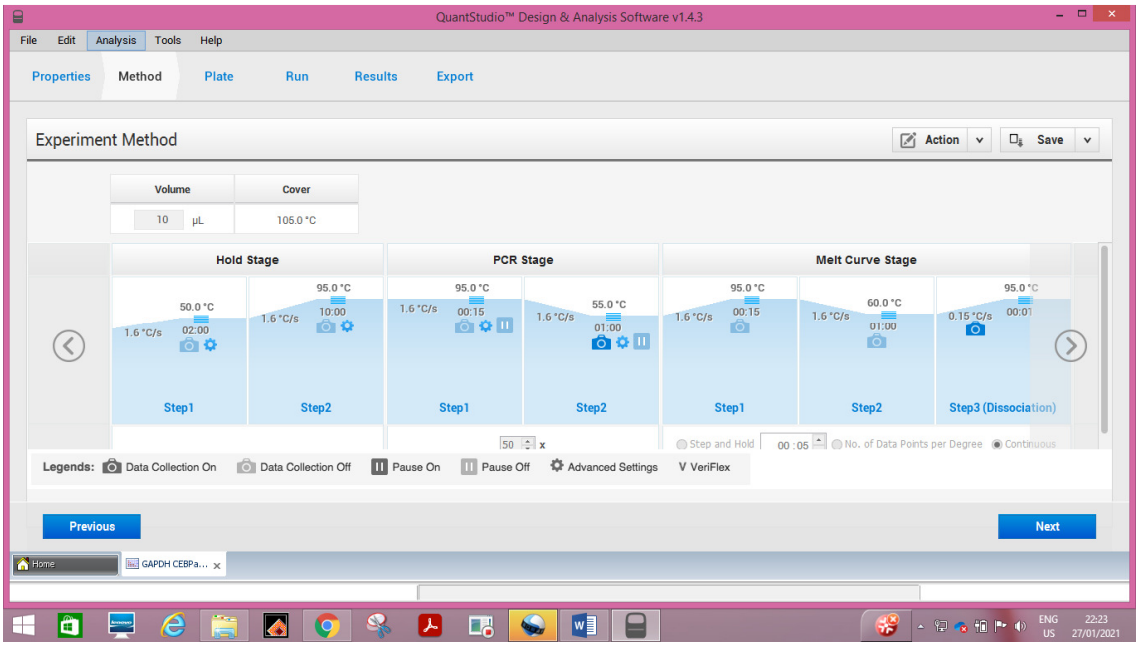
5. Always prepare master mix to avoid pipetting error as well as variations.

Procedure:

1. Prepare the master mix as per the given table:

S.N.	Components	Volume (µl) for 1X
1.	SYBR green master mix	5.0
2.	Forward primer (Fp)	0.5
3.	Reverse Primer (Rp)	0.5
4.	cDNA	2.0
5.	Nuclease free water	2.0
		Total = 10 ul

2. Set the reaction as per the following method:



3. Run the experiment

4. Save the data and analyze the data through $2^{(-\Delta\Delta CT)}$ relative quantification method.

The formula for the calculation of fold change is as follows:

$$\Delta\Delta CT = (C_{T\ GOI}-C_{T\ HG})_{Treated}- (C_{T\ GOI}-C_{T\ HG})_{Control}$$

$$\textbf{Fold Change} = [2^{(-\Delta\Delta CT)}]$$

Where,

$C_{T=}$ Threshold cycle

HG= Housekeeping gene

GOI= Gene of interest