Experiment (8): Quantitative reverse transcription PCR (RT-qPCR)

Aim:

- To have a basic knowledge about using RT-qPCR to evaluate the gene expression.

Introduction:

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Using PCR, specific sequences can be amplified. In such traditional PCR (endpoint), detection of the amplicon is performed at the end of the reaction after the last PCR cycle, using gel electrophoresis. In real-time quantitative PCR, a fluorescent reporter dyes used to combine the amplification and detection steps of the PCR reaction. The assay relies on measuring the increase in fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle, and thus gives a quantitative information on the starting quantity of the amplified target. The reaction is characterized/identified by the PCR cycle at which fluorescence first rises above a defined or threshold background fluorescence, a parameter known as the threshold cycle (Ct).

Generally, RT-qPCR is a combination of three steps: (i) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, (ii) the amplification of the cDNA using the PCR and (iii) the detection and quantification of amplification products in real time.

PAUSE AND THINK ➔ What are the reaction components of RT-qPCR ?

RT-qPCR applications

The most common application of real-time PCR is the quantification of mRNA (gene expression). However, qualitative detection could be used.

RT-qPCR types:

The RT-qPCR assay can be performed either as a one-tube single RT and PCR enzyme method or a separate RT and PCR enzyme technique using one or a two tubes. **In One-step qRT-PCR,** combines the first-strand cDNA synthesis reaction and real-time PCR reaction in the same tube, simplifying reaction setup and reducing the possibility of contamination. **In Two-step RT-qPCR,** the reverse transcription of RNA into cDNA is done using a reverse transcriptase (RT).

RT-qPCR quantification methods:

Absolute quantification: describes a real-time PCR experiment in which samples of known quantity are serially diluted and then amplified to generate a standard curve. Unknown samples are then quantified by comparison with this curve.

Relative quantification: in which the expression of a gene of interest in one sample (i.e., treated) is compared to expression of the same gene in another sample (i.e., untreated). The results are expressed as fold change (increase or decrease). A normalizer gene (such as β-actin) is used as a control for experimental variability in this type of quantification.

RT-qPCR chemistries (detection systems):

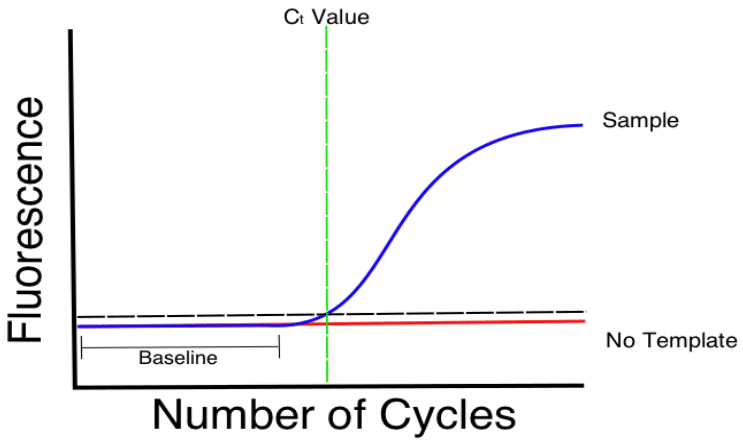
I. Principle of SYBR-green based assay:

The double-steands DNA- intercalation agent (DNA-binding dyes) such as SYBR Green 1. The SYBR Green 1 is only fluorescing when intercalated into dsDNA. The intensity of the fluorescence signal is therefore dependent on the quantity of dsDNA present in the reaction. The main disadvantage of this method is that it is not specific since the dye binds to all dsDNAs formed during the PCR reaction (i.e., nonspecific PCR products and primer-dimers).

II. Principle of fluorogenic 5' nuclease assay (TaqMan® probe assay):

A probe is used in the real-time quantitative TaqMan assay. The probe is a specific sequence which has a fluorescent reporter dye linked to its 5′ end and a non-fluorescent quencher at its 3′ end. The TaqMan probe anneals downstream the target sequence from one of the primer sites and is cleaved by the 5′ nuclease activity of the Taq polymerase during the PCR extension phase. Whilst the probe is intact, the quencher absorbs the fluorescence emitted by the reporter dye. Cleavage of the probe by Taq polymerase during PCR will cause the separation of the reporter and quencher dyes, thereby allowing the reporter's fluorescent signal to be liberated. With each cycle additional reporter dye molecules are cleaved from their respective probes, leading to an increase in fluorescence intensity proportional to the amount of amplicon produced.

RT-qPCR amplification curve:



Used terms:

1. **Baseline**: The baseline of the real-time PCR reaction refers to the little change in fluorescent signal during the initial cycles of PCR (background or the "noise" of the reaction).
2. **Threshold:** The threshold of the real-time PCR reaction is the level of signal that reflects a statistically significant increase over the calculated baseline signal.
3. **C_t (threshold cycle):** The threshold cycle (Ct) is the cycle number at which the fluorescent signal of the reaction crosses the threshold.

Steps of evaluation gene expression by RT-qPCR:

Step 1: RNA Extraction from Tissue/ Cell line or blood

According to the manufacturer's instruction of RNeasy® Mini Kit (Qiagen, DE).

Step 2: Reverse transcription to convert RNA to cDNA

According to the manufacturer's instruction of High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, US).

Step 3: Determination of cDNA using real time PCR

According to Power SYBR® Green PCR Master Mix and RT-PCR Protocol (PN 4367218) (Applied Biosystems, US).

Step 4: Data analysis

References:

1. Nolan, T., R.E. Hands, and S.A. Bustin, *Quantification of mRNA using real-time RT-PCR.* Nat Protoc, 2006. **1**(3): p. 1559-82.
2. https://www.thermofisher.com/content/dam/LifeTech/global/Forms/PDF/real-time-pcr-handbook.pdf
3. Arya, M., et al., *Basic principles of real-time quantitative PCR.* Expert Rev Mol Diagn, 2005. **5**(2): p. 209-19.