Experiment (7): Quantitative reverse transcription PCR (RT-qPCR)

Aim:

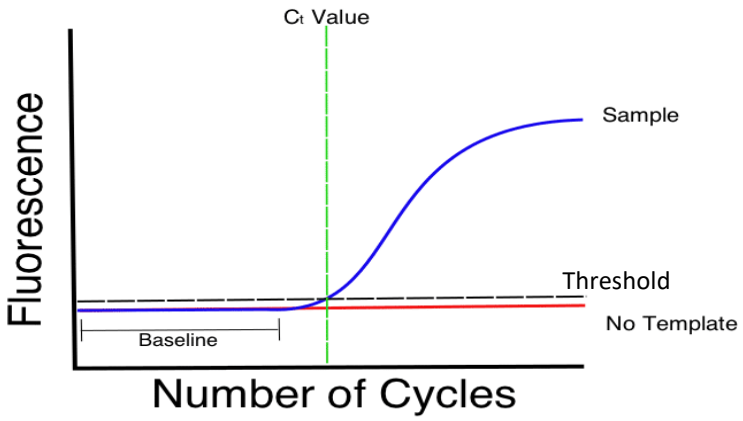
- To have a basic knowledge about using RT-qPCR to evaluate the gene expression.

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 allow a PCR reaction to be visualized "in real time" as the reaction progresses by combine the amplification and detection steps in 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).*

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

RT-qPCR amplification curve:



Used terms:

1. **Baseline**: Refer to the little change in fluorescent signal during the initial cycles of PCR (background or the "noise" of the reaction).

2. **Threshold:** Is the level of signal that reflects a statistically significant increase over the calculated baseline signal.

3. **C_t (threshold cycle):** Is the cycle number at which the fluorescent signal of the reaction crosses the threshold.

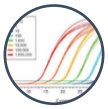
RT-qPCR applications

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

Evaluating gene expression by RT-qPCR steps:

RNA Extraction from Tissue/ Cell line or blood
(Tissue must be :Fresh, Stored in RNlater, or Liqued nitrogen)

Reverse transcription to convert RNA to cDNA
(Very important Step, RNA is very unstable)



Determination of cDNA using real time PCR (flurosenec)

Data analysis

RT-qPCR types:

The RT-qPCR assay can be performed by two methods regarding the revers transcription step and the real-time PCR reaction step:

1. One-step RT-qPCR: Combines the synthesis of the cDNA step by reverse transcriptase in the same tube as the real- time PCR reaction (one tube-one step). This method simplifying reaction setup and reducing the possibility of contamination. Gene-specific primers (GSP) are required.

2. Two-step RT-qPCR: Creating cDNA first by means of a separate reverse transcription reaction and then adding the synthesized cDNA to the real-time PCR reaction tube (two tubes-two steps). Random primers or a mixture of oligo(dT) and random primers is usually used.

- PAUSE AND THINK ➔ What is the advantages and disadvantages of each type?

RT-qPCR quantification methods:

Absolute quantification: Determines actual copy numbers of target. This done by 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. This method is the most difficult form of quantitation.

Relative/comparative 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 a calibrator sample (i.e., untreated). The results are expressed as fold change (increase or decrease). A normalizer gene (such as β-actin or GAPDH) is used as a control for experimental variability in this type of quantification. This method applied to most gene expression studies.

RT-qPCR chemistries (detection systems):

I. Principle of SYBR®-green based assay:

Based on using a double-strands DNA- intercalation agent (dsDNA-binding dyes) such as SYBR Green 1, which only fluorescing when it intercalated into dsDNA. The intensity of the fluorescence signal is therefore proportional to the quantity of dsDNA present in the reaction (cDNA amount) and measured at each cycle. 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., contamination, nonspecific PCR products and primer-dimers).

- PAUSE AND THINK ➔ How can we overcome the non-specificity?

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. This assay is very specific since it relies on both primer specificity and hybridization specificity (probe).

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.