## Part I

## Wed. 29/03/2017

### Introduction to RT-qPCR

### Polymerase chain reaction …. Brief introduction

In principals, this reaction amplifies a target sequence of the gene in an entire biochemical reaction… Kary Mullis invented this technique in 1983. Any PCR reaction relies on using a thermostable DNA polymerase which is namely called Taq Polymerase or Taq and this abbreviation came from *Thermus aquaticus*. In presence of single- strand DNA template, Taq will add the ribonucleotides the growing strand. The way that the Taq works, it adds nucleotides to the 3' end of a short template to base pair with the Longer DNA template, I mean the original sequence we want to amplify. The short template. The primer, has a complementary sequence to the original target sequence and Taq will continues in adding the nucleotides to the 3' end.

***Do both of DNA strands starts the replication at the same time ????***

Answer is ***NO*** and from that fact the term leading and lagging DNA strands came???

The first step in DNA replication is making pair of replication forks opposite to each other and strands of the double helix are subject to unwinding with the help of helicase. The result from that separation are one DNA strand orients from 5' to 3' and the other from 3' to 5'. 5' to 3' strands starts to be replicated as a continuous leading strand as the orientation of DNA polymerase to work in the same direction ***BUT*** the 3 to 5' lagging stand has a different story to be replicated **….** While helicase continuing his work in separating the strands and enlarging the replication fork the DNA polymerase starts to copy small fragments from the lagging strand till the next template fragment comes and so on …. Then those fragments that is called Okazaki fragments will be ligated with each other by DNA ligase.

### The 3 main steps of PCR reaction are:

1- Denaturation, separating the strands of the double helix to 2 by heating up the reaction to 94 C.
2- Annealing, Allowing the primers to anneal to the template by cooling the reaction mixture to somewhere between 50-70.
3- Extending the growing strands by setting the temperature to be optimum for Taq 72 which allows it adding the nucleotides to the 3' end.

1 DNA strand

2 DNA strands

4 DNA strands

8 DNA strands

It is and exponential increase $2^1$ …$2^2$….. $2^3$… so on.

## Types of PCR

1- Conventional PCR
2- RT-PCR
3- RT-qPCR

## Conventional PCR

IT refers to the reverse transcription of the RNA to complementary DNA by reverse transcriptase and then starting the ordinary PCR by making copies of the target gene … BUT this technique only tells whether the segment is present or absent … MEANS there is no quantitation…

## Disadvantages of conventional PCR and how the RT-qPCR is different and more advantageous….

| PCR or Conventional PCR | Real Time quantitative PCR |
|---|---|
| Not possible | We can see the results of each cycle while the program is running |
| Ethidium bromide and UV light | It relies on using a dye gives a signal during the reaction and the more copies of the gene … the stronger signal that we get and earlier amplification |
| Result will be as a band in agarose gel | Results as amplification curves with relative or absolute quantity of the target gene |
| Time consuming and needs electrophoresis | Gives results quickly and at early stage of amplification |
| End point reaction in collecting data | Exponential data analysis |
| Imprecise | precise |
| Not very sensitive to tiny variations | Very sensitive test |

| | |
|---|---|
| Limited numbers | Easily to make replicates |
| Interferes with the small DNA fragments | Primer dimers will be detected with no interferences |
| | |

## *Lecture 2 Wed. 7/4/2017*

### *Types of Real Time PCR Assays*

One of the two main assays that are used in real time pcr is TaqMan assay which relies on using hydrolysis probes or TaqMan probes which designed to detect very specific sequences within the target gene. This technique was invented more than 25 years ago for diagnostic purposes at the beginning then for research application which was developed by applied biosystem. Not all Fluorescence dyes can detect the specific sequences in the target template and this is the main disadvantages that led the scientists to find more specific assays such as the TaqMan. So The qPCR assay was improved by developing fluorogenic-labeled probes depends on the activity of the nuclease of Taq DNA Polymerase to amplify only the specific amplification products.

Any probe can be constructed by attaching a fluorophore reporter dye covalently to the 5' of the oligonucleotide and a quencher dye at the 3' end ….. The ***FRET*** or the fluorescence resonance energy transfer phenomenon was employed to reduce the fluorescence that is emitted by the reporter dye with the growing primer strand… ***then how will the probe emit the fluorescence and would that interfere with the primer work???***

This technique is selective as it relies on the activities of Taq polymerase… The exonuclease activity will only degrade dsDNA that encounters while extending a DNA fragment.. It will degrade a secondary primer if bound to the same strand.

### *What are the steps??*

1- While the probe is intact , there will be equality between the reporter and the quencher dyes depending on FRET.
2- If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by 5' exonuclease activity of Taq DNA polymerase as this primer is extended
3- The cleavage results in:
   Increases the emitted fluorescence as it cleaves the reporter dye from the quencher dye.
   Displaces the probe from the target strand allowing primer extension to continue to the end of the target template. Thus inclusion of the probe reaction doesn't interfere with all PCR process. Further fluorescence cleavage with each cycle increases the intensity of the fluorescence which will be proportional to the amount of the amplicon of interest produced.

***There are two types of Taqman Probes***

1- TaqMan Probes with TAMRA dye as a quencher dye
2- TaqMan MGB Molecular Beacons probes which contains non-fluorescent quencher at 3' end then the detection will be more precise since the quencher dye doesn't fluoresce and in addition, there is a minor groove binder at the 3' which increases the Tm of the probe then we can choose shorter probes. Since the MGB probes exhibit greater differences in Tm values then this method is the best to discriminate between alleles.

***What are the advantages of TaqMan chemistry????***

1- Specific hybridization between the probe and the target
2- Probes can be labeled with two distinguishable reporter dyes to detect two distinct sequences in one reaction tubes
3- No further post-PCR processing which reduces the cost and efforts.

**BUT** preparing two different probes is required for different sequences.

**SYBR GREEN**

Is a very common fluorescent binding dye which can binds to the dsDNA and intensity of Fluorescence is increasing with each cycle. It is believed that this dye binds to the minor grooves of the dsDNA and upon binding the fluorescence increase over a hundred fold. BUT further accumulations will affect the PCR reaction. Inside the instrument the SYBR is monitored in channel F1.

***What is the advantage of using the SYBR green???***

It can be used for any dsDNA… so there is no need to design and optimize Probes but it is the same time a big disadvantage as it detects the specific and non-specific and even primer dimers will be amplified. Optimizing the PCR reaction will reduce probability to form primer dimer to a very low level of copies. The melting curve of the dissociation curve is indicative for the target product and gives an irregular and smaller melting curve of the primer dimer. Once the LightCycler detects the melting curve of the product it will separates the one of the primer dimers by emitting a fluorescence above the Tm of the primers but lower than Tm of the target.

### *SYBR GREEN REACTION.*

At the beginning of the reaction, the unbound SYBR gives only very low level of fluorescence ***BUT*** once it binds to the DNA fluorescence will be detected at high levels.

Later on and at early cycles the reaction mixture has, unbound SYBR, primers and DNA … The unbound SYBR will fluoresce very low level of fluorescent which will be considered as noise and will be chopped out during computer analysis.

Very few molecules of SYBR will bind to DNA when the primers annealed to the DNA…. This will give lots of fluorescence.

More and more SYBR molecules will bind to the newly synthesized DNA and continuous fluorescence will be measured… For the next heating cycle and upon the DNA is desaturated the dye molecules will released and the fluorescence will fall again.

## Part II

### *Quantitation*

In gene expression, there is very important aspect to compare the gene expression level at different samples or treatments, it is the quantitation. Quantitation presents clear evidences about epigenetics and how every gene is expressed quantitatively. There are two types of quantitation, relative or absolute. The relative measurement quantify the level of mRNA expression of the gene of interest GOI fold change normalized to house keeping genes. While, the absolute measurement displays the exact copy number of GOI using calibration curves. In fact, the second assay needs more efforts and further steps to optimize the conditions compared to the relative expression which is easier to be achieved it only compares the level of expression of the target gene against the reference genes.

**Absolute quantification**

This type of quantification needs high consistency and reproducibility over long time of storage and thus will give highly specific and precise results. There is wide range of calibration that could be between $10^1$ to $10^{10}$ starting molecules. The standards are either plasmid DNA molecules or genomic DBNA or any RT PCR product.

## *Factors that could affect the absolute quantitation*

1- Cloned DNA is more stable when it is stored for long time compared to the freshly prepared.
2- Longer recombinant DNA give consistent results better than the shorter ones as they are more resistant to the unspecific cleavage and proofreading activity of the polymerase. However, the shorter cloned DNA could give precise results due to the accurate information about their concentrations and lengths, in addition, their preparation doesn't consume that much time including purification, cloning, transformation, plasmid preparation, linearization and exact determination of standard concentration.
3- Significant variation within the absolute quantitation comes from the differences in preparing the standard curves and the unknown samples. Unknow samples are prepared by reverse transcription in many cases freshly while the standardmolecules have been made differently and stored for longer period.. so given the sensitivity of the qPCR assay then this would give rise inconsistencey… recommendation standard curves should be prepared for each sample.

Relative

Comparative quantitation

What is TaqStart Abs