Polymerase Chain Reaction: Application and Practical Primer Probe Design
“qRT-PCR”
Enzyme based DNA amplification

- **Thermal Polymerase** derived from a thermophilic bacterium
- DNA dependant DNA polymerase with exonuclease activity
- Extends a partially double stranded molecule with a 5’ over-hang from the 3’ end to its full length
- In PCR such a structure is formed by annealing a single stranded primer to a template molecule
- It’s the balance between stability and instability of the complex is critical to the ability to undergo repeated rounds of denaturation, annealing, initiation and extension
- Reaction based on transition between three states
  - Denatured (template becomes single stranded)
  - Primers Annealed (formation of initiating template)
  - Native state at the optimal extension temperature for enzyme activity
- The product normally has blunt ends and lacks 5’ phosphate groups
Polymerase Chain Reaction (PCR)
Quantitative real-time PCR (qPCR)

The basic PCR technique does not have a quantitative output and cannot be used to inform template copy number.

We therefore use modifications of this technique to provide measurable output during the exponential phase of the amplification in “real-time”.
There are a number of different qPCR technologies

- Taqman Probes, Molecular Beacons etc along with Sybr Green
- These techniques utilize fluorescent detection of the amplification
- They are fundamentally different and as such the probes and primers have different design criteria
- Reliant upon detection of the accumulated amplicon during amplification.
- All of these technologies can be used for quantifying the amount of a target DNA molecule
  - If the target is an RNA the first step requires the conversion of the RNA/mRNA into cDNA using reverse transcriptase
  - The second requires the quantification of the copy number of a specific cDNA/DNA present in a complex mixture using the particular detection method involving PCR
Sybr Green is an intercalating fluorescent dye that binds in the minor grove of DNA.

In the bound conformation it is fluorescent and upon excitation at the appropriate wavelength emits light.

Thus, it nonspecifically detects helical DNA produced during amplification through a reversible molecular interaction.

The down side of this is that the quality of the data is particularly dependant upon the specificity of the PCR reaction, since non-specific amplification or primer dimmer formation cannot be differentiated from target amplification.

A method employed to test for non-specific amplification products is to construct a melting curve and identify multiple products with different melting temperatures.
Polymerase Chain Reaction (PCR)
Cycling typically used in combination with intercalating dyes

- Denature 95°C
- Cycle 30-40times
- Anneal below the Tm of the primer eg 55°C
- Extend from the 3’ end of the primer 72°C

Template, Primers, Enzyme & Reactants
• Taqman relies upon formation of an amplification fork, generated between the target and probe formed due to displacement of the 5’ end of the probe and the consequent degradation of the probe by the polymerase during elongation.

• This releases the 5’ “reporter” dye preventing forster resonance energy transfer (FRET) essentially the transfer the energy to the 3’ “quencher” following excitation by light.
Polymerase Chain Reaction (PCR)
Cycling typically used in combination with Taqman probes

Denature 95°C

Template, Primers, Enzyme & Reactants

Cycle 30-40times

Anneal & Extend from the 3’ end of the primer at the Tm of the primer eg 60°C
Since probe based reporters e.g. Taqman Probes are dependent upon the formation of a sequence specific dimer between the amplicon and probe they are less prone to non-specific target amplification errors and primer dimer products.

Probe based methods use fluorescent dyes and quenchers with narrow excitation and emission spectra and may thus be used in multiplexed reactions, to detect multiple targets simultaneously in a single tube by adopting different fluors for each target probe.

In practice rarely in diagnostics are more than two targets multiplexed and most usually the second target is used as an internal reference or internal controls:
  - for normalizing the signal
  - to correct for loading errors
  - to provide positive controls
Polymerase Chain Reaction (PCR)
Quantitative PCR (qPCR)

- Amplification is a process resulting in the doubling of the quantity of template at each round of amplification (each cycle)
  - $2, 4, 8, 16, 64 \ldots \ldots 2^n$ cycles
  - If the amplification is unlimited then 30 cycle will result in $2^{30}=1.07e^9$ molecules from each of the initial template molecules
  - Therefore the initial concentration of the template will determine the amount of product after a given number of cycles

- Typically amplification is plotted on as the cycle number vs the change in fluorescence from the start of the amplification to the current cycle ($\Delta Rn$)
  - Since cycle number is equivalent to a doubling of the target it is in reality a $\log_2$ scale of the relative amount of target

Serial dilution of template produces a series of curves shifted to the right
Polymerase Chain Reaction (PCR)
Quantitative PCR (qPCR)

• In practice the amplification is limited by some aspect of the reaction prior to completion and a threshold is applied equating an arbitrary ΔRn value with a cycle.

• The readout is therefore a cycle at which the amplification crosses the threshold called a threshold cycle (Ct-value).

• The initial concentration of a molecule can be calculated using either:
  • A standard curve constructed from a serial dilution of known concentration (absolute determination).
  Or
  • A relative comparison comparing each sample to one or more control/reference samples (relative determination).
Polymerase Chain Reaction (PCR)
Absolute Quantitation

- The crossing point of the amplification is determined and is looked up on a standard curve determining an absolute value.
  - Requires a standard to be constructed with reliable known concentrations.
  - Encounters error due to loading/dilution of the standard and pre-processing by the RT.
Polymerase Chain Reaction (PCR)
Relative Quantitation

• The crossing point of the amplification is determined for all treatments and a value is determined relative to a specific treatment
  • Produces a relative change or fold change compared to a specific sample such as an untreated control
  • At its most simplest this requires a straight forward calculation
    \[ \Delta C_t = C_{t_{\text{ref}}} - C_{t_{\text{test}}} \]

Since \( \Delta C_t \) is \( \log_2 \) value
Then an anti log is calculated to determine fold change
And fold change = \( 2^{-\Delta C_t} \)
This can be done in an excel spreadsheet
Quantitative Real Time Polymerase Chain Reaction (qPCR)

Strategy to design primers for diagnostics

Rational design

Prior knowledge of genomic structure content

Acquire all Genomic Sequences

Computational design

Identify potential target regions

Subdivide genome

Select DNA segments

Blast analysis to identify unique regions

Select DNA segments to design primers/probe sequence

Select primers/probe sequences
On basis of absence of common similarity to other DNA segments

design primers/probes

Validation by empirical testing

Subdivide genome

Reduce these to minimum subset of sequences representative of all known isolates
Polymerase Chain Reaction (PCR)
The basics of Primer Design

Primers are annealed at a temperature at or just below or at their melting temperature Hence

• Normally primers are designed to have a specified matched melting temperature (Tm)
• Lack Self Complementarity, if present this may result in internal structure would have an adverse effect on the amplification efficiency
• Lack Primer-Primer complementarity (3’ end complementarity should be avoided, this results in the amplification of primer dimer products in preference to amplicon)
• The primer binding site should be unique within the target molecules
• The combination of primer binding sites should be unique within the population of molecules being used. Primers can be searched against database entries using BLAST or some similar program

In practice primer design is done using software

• Many different packages are used but one of the most commonly used non-commercial package is called Primer3 with a web interface

• Primer3 is a very versatile package for designing oligonucleotides for various applications including real time PCR
Polymerase Chain Reaction (PCR)  
Primer Design around a large target region

>AB072389 Human herpesvirus 1 UL30 gene for DNA polymerase
…………….ggccgtcgcgcgcttggcgggtatta acatcacccgcaccatctacgacggccag
  cagatcgcgtttacgtgctctgctgtcgctggaccgaagggctttattctgccggacatccaggggcgatttaggggcgccgggggggaggcgcccaagcgtccggccgcagcccgggaggacg
  aggagcggccagaggctactctgcgttgctcagcagctgccctccgagggccgacgcagttggtgaggacg
…………

Non-Target sequence
Target region
DNA/RNA Segment

- The design options allows forcing the software to include specific sections within the amplicon using target region….. []
- Including or excluding specific regions to design primers within is achieved using { } or < >
Polymerase Chain Reaction (PCR)  
Primer Design around a large target region

- forced to include sections within the amplicon - target region..... []
- excluded region < >
Polymerase Chain Reaction (PCR)
Primer Design – test for primers for target specificity: megablast
Polymerase Chain Reaction (PCR)

Primer Design – test for primer specificity: blast

Only common matches for both primers are to target gene CD40.
Polymerase Chain Reaction (PCR)  
Quantitative PCR (qPCR)

**Target choice**

- **Amplicon size:**
  - critical choice in the design criteria and should aim to be **70-120 bp** for Taqman chemistry and a **max of 200-400bp** for SYBR. As the amplicon size increases amplification becomes increasingly inefficient and non-linear.
  - This limitation means that many PCR primer pairs chosen for other purposes are often inappropriate for use in qPCR

- **DNA contamination with non-target nucleic acids:**
  - A diagnostic specifically aims to discriminate but with poorly designed primers/probes this is a common cause of quantitive and qualatative errors in qPCR,
Primer Design

- Primers should be 15-30 bp in length
- Primer pairs should have a theoretical Tm within 2 °C of each other
- Annealing temperature for primers is typically 56-60°C. Taqman primers should usually have a Tm of 59°C.
- Minimise primer intra and intermolecular structure ie folding on itself or primer dimerisation.

Taqman Probe Design

- 20-30 bp in length, Tm 10°C higher than primers (65-70 °C). The Tm difference between probe and primers is critical for both Taqman Probes
  - the actual length of a Taqman probe is therefore a function of G/C content and required Tm (69°C)
- G/C 20-80%.
- Other criteria
  - Should not have more G’s than C’s
  - Avoid runs of 3+ of the same nucleotide (not always possible), but G’s are especially important.
  - No Guanidines at the 1st or 2nd position at the 5’ end, these act as quenchers and since the nucleolytic degradation by the polymerase leaves the 5’ reporter dye attached to the first base and sometimes subsequent bases, such a molecule would provide a poor readout
- When the probe and primers anneal to the target, the 5’ end of probe should be as near to the 3’ end of the primer on same strand (max of 10-12). This tends to lead to more efficient displacement and degradation of the probe.
Primer/Probe Design

- Again this can be carried out using numerous software packages including Primer3

- Optimal internal Oligo Tm is set to be 69/70°C, Primer Tm is set to 59/60°C

- Target region is defined to lay within the target region preventing

- Exclude regions are used to force primer design to minimise the size of the amplicon or avoid specific sequence and put the primers adjacent to the probe

- Internal hybridisation probe is selected
Primer/Probe Output

- manually check primers
  - Absence of runs of similar nucleotide at 3' end
  - Matching Tm

- manually check Internal oligo/probe
  - More Cs than Gs
  - No Runs of Gs and Cs
  - Absence of 5'G

- Excluded regions

- Specificity can be again checked using BLAST comparison via primer3Manager
References further reading

**General Text books in the library**
- Fundamental Concepts of Bioinformatics Krane D.E. and Raymer M.L.
- Understanding Bioinformatics Zvelebil M. and Boum J.O.

- Real-Time PCR technical tutorial produced by Margaret Hunt at University of South Carolina
  - [http://www.med.sc.edu:85/pcr/realtime-home.htm](http://www.med.sc.edu:85/pcr/realtime-home.htm)