Pitfalls in qPCR

Primer and probe design and synthesis
Fluorophore – quencher combinations

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Steps in qPCR assay

• Set up experiment
  – statistical relevant # samples/experimental group
  – controls
• Design and synthesis primers and probes
• RNA extraction
  – quality of RNA
• Reverse Transcription reaction
  – one step or two step reaction
• qPCR reaction
  – singleplex or multiplex
• Data analysis
PCR efficiency

- High PCR efficiency
  - high accuracy
  - high reproducibility

- PCR efficiency influenced by
  - length of amplicon
  - GC content of amplicon
  - secondary structures in primers, probes, amplicons
  - concentration reaction components
  - PCR inhibitors/PCR enhancers
  - quality RNA/cDNA
PCR efficiency

- Easiest way to determine PCR efficiency: standard curve with $R^2$ close to 1.00 and slope close to -3.32

Exponential amplification = $10^{(-1/slope)}$

Efficiency = $10^{(-1/slope)} - 1$
PCR efficiency

- 100% PCR efficiency
  - every PCR cycle amount of DNA is doubled
  - 2x dilution curve $\Delta Ct$ of 1 between every dilution
  - 10x dilution curve $\Delta Ct$ of 3.2 between every dilution

- Variation coefficient ($R^2$)
  - indication how well data points lie on one straight line
  - low $R^2$ indication for pipetting mistakes, inaccurate way of working, diluting out inhibitory factors
Why do you need a good design?

• Well-designed primers and probes are a prerequisite for successful RT qPCR in terms of
  – high PCR efficiencies
  – specific PCR products
  – no co-amplification of genomic DNA
  – no amplification of pseudogenes
  – most sensitive results
Design makes the difference!

- Comparison between two different primer-probe sets for 18S rRNA using same reaction components and experimental conditions
Design guidelines for primers

• Primers
  – length
    • 18-30 bases
  – GC content
    • 30-80% (ideally 40-60%)
  – Tm
    • 63-67°C (ideally 64°C), so that Tannealing is 58-62°C (ideally 59°C)
    • ΔTm forward primer and reverse primer < 4°C
  – avoid mismatches between primers and target, especially towards the 3’ end of the primer
  – avoid runs of identical nucleotides, especially of 3 or more Gs or Cs at the 3’ end
  – avoid 3’ end T (allows mismatching)
  – avoid complementarity within the primers to avoid hairpins (check using a software)
  – avoid complementarity between the primers, especially at 2 or more bases at the 3’ ends of the primers to avoid primer-dimers (check using a software)
  – design intron spanning or flanking primers to avoid co-amplification of genomic DNA (only possible in multiple exon genes, in single exon genes perform DNase I treatment of samples with RNase free DNase (Vandesompele, 2002))

# Positions of exons and introns can be found in NCBI LocusLink databases (www.ncbi.nlm.nih.gov/LocusLink/)
Intron spanning/flanking primers

- Intron spanning primers

![Diagram showing the use of intron spanning/flanking primers](image)

- gDNA
  - Exon 1 → Exon 2 → Exon 3
  - Forward
  - No amplification

- cDNA
  - Exon 1 → Exon 2 → Exon 3
  - Forward → Reverse
  - Amplification
Intron spanning/flanking primers

- Intron flanking primers

- Diagram showing:
  - Forward and reverse primers for Exon 1, Exon 2, and Exon 3
  - gDNA leading to Large amplicon*
  - cDNA leading to Small amplicon*

* Can be detected via melt curve
Design guidelines for probes

- **5’ Exonuclease probes**
  - **length**
    - 18-30 bases (>30 bases required, use internal quencher on dT around 20th base)
    - Optimal: 20
    - Lengths over 30 bases are possible, but it is recommended to position the quencher not at the 3’ end, but internally 18-25 bases from the 5’ end
  - **GC content**
    - 30-80%
  - **Tm**
    - Tm of the probe must be 8-10°C higher than the Tm of the primers (8°C for genotyping, 10°C for expression profiling)
  - select the strand that gives the probe more Cs than Gs
  - place probe as close as possible to primers without overlapping them
  - avoid mismatched between probe and target
  - avoid runs of identical nucleotides, especially of 4 or more Gs
  - avoid 5’ end G (quenches the fluorophore)
  - avoid complementarity of the probe with either of the primers (check using a software)
  - for multiplex assays: for genotyping
    - position the polymorphism in the center of the probe
    - adjust the probe length so that both probes have the same Tm
Design guidelines for amplicons

- **Amplicon**
  - length for SYBR® green I assays:
    - 80-150 bp
    - shorter amplicons will give higher PCR efficiencies
    - longer amplicons will give a higher \( \Delta R_{n} \) as more SYBR® green I is incorporated
  - length for 5’ exonuclease probe assays:
    - 80-120 bp
    - shorter amplicons will give higher PCR efficiencies
    - shorter amplicons will give more efficient 5’ nuclease reactions
  - GC content
    - 30-80% (ideally 40-60%)
  - avoid secondary structures in the amplicon (check with Mfold: www.bioinfo.rpi.edu/applications/mfold/)
  - check if generate amplicon is unique by submitting primers (and probe) to a BLAST search (www.ncbi.nlm.nih.gov/BLAST/)
Frequent pitfalls

- I do already have existing primers of a normal PCR, but cannot find a good probe to fit them. What should I do?
  - Although it is disappointing to hear, it is best to do the design from scratch. The criteria for primers are less stringent as for probes.

- I have used a design software to design my primers and probes, but it do not get them to work properly
  - A design software is not a 100% guarantee to get a good primer/probe set, but is a good tool to make your life easier
  - Especially with SYBR® green I assays; try several primer sets as \textit{in silico} differs from experimental
Frequent pitfalls

- The design software that I use can not find a suggestion, although the sequence I have inserted is more than 500 bases long
  - It is not always possible to design a primer/probe set for a specific sequence due to GC/AT rich sequences, repeats or secondary structures
  - In most cases you can already see the most homogenous part of your sequence by eye. This is the best part to design your primers and probe on
  - Sometimes you can force the software to design a primer-probe set by changing the parameters like amplicon length, primer length, Tm’s or GC content
Frequent pitfalls

• I took the first suggestion in the list of Primer Express®, but the primer/probe set does not lead to good results
  – The first suggestion in the list of Primer Express® is the shortest amplicon, not the best primer/probe set

• With the recommended temperature profile I obtain an amplicon, but the detection does not function due to the probe, which is not binding
  – Each software uses its own method of calculating the Tm and there can be a difference between the calculated and experimental temperature
  – If the probe does not bind to the amplicon then the annealing temperature is too high in comparison to the Tm of the probe
  – Check Tm using several softwares.
    If Tm’s differ > 3°C check Tm experimentally
Predicted vs. experimental Tm

Source: ABI User Bulletin 6 ABI PRISM® Sequence Detection System
Probe too long or Tm too low?

- AT rich sequence: long probes required to reach correct Tm
- SNP detection: short probes required to increase specificity
- With LNA bases length probes can be decreased or Tm can be increased

Conformation change from B helix to A helix due to LNAs
Example probe assay

- “Jump” in dilution series caused by secondary structure in primer
Example SYBR® green I assay

- Ct’s of all dilutions around same point due to primer dimers
Choice of fluorophore and quencher is part of a good design

• Well-chosen fluorophores and quenchers are a prerequisite for successful RT qPCR in terms of
  – maximal fluorescence
  – minimal back ground
  – maximal signal-to-noise ration
  – maximal sensitivity
Design guidelines for fluorophores and quenchers

- **Fluorophores**
  - choose fluorophore that fits your real-time thermocycler
  - choose fluorophore with high level of fluorescence (weak fluorescence: JOE, TAMRA)
  - choose fluorophore with narrow spectrum and one emission maximum
  - avoid fluorophores that require manual coupling (i.e. ROX)
  - multiplex qPCR:
    - choose fluorophores that are spectrally well separated

- **Quenchers**
  - choose quencher that fits fluorophore (emission spectrum of fluorophore must have substantial overlap with absorption spectrum quencher)
  - take the fluorophore quencher combination with highest signal-to-noise ratio to obtain maximal sensitivity
  - preferably take dark quenchers like BHQ1, 2 or 3 (are also very robust in synthesis)
  - only in case of singleplex go for FAM-TAMRA as this is the most cost-effective combination
  - multiplex qPCR
    - avoid the use of TAMRA. If you must use TAMRA, use it on all probes (click on TAMRA as quencher in plate set up software)
    - preferably use dark quenchers to avoid lost of sensitivity (click on None as quencher plate set up software)
Fluorophore has to fit real-time thermocycler

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Fluorophore has to fit real-time thermocycler

- Dragon Fly Orange: a new alternative to NED
  - Almost identical emission maxima to Ned
  - Robust Delta Rn and identical Ct values
  - Detect simultaneously up to 3 targets
Quencher has to fit fluorophore
Quencher has to fit fluorophore
High signal-to-noise ratio

BHQ1 leads to the highest signal-to-noise ratios and is superior to TAMRA and NFQ (EDQ)
TAMRA or dark quenchers?

![Graphs comparing fluorescent intensity vs. wavelength for FAM, TAMRA, and ROX](image)

- Top graph: FAM, TAMRA, ROX compared.
- Bottom graph: FAM, ROX compared.
Synthesis probes

- Probes are synthesized starting from 3’ end (quencher)
- Labelling process 5’ end
  - manual coupling via activated fluorophores and a C-6 spacer
    - very pure oligos, but low yield
    - 5’ and internal labelling (only on dT residu) possible
  - automatic coupling via labelled phosphoamidites
    - high purity in combination with high yield
    - only 5’end labelling possible
Useful software and websites

- **Design primers**
  - any primer design software (freeware on web)
  - Oligo® 6.0 (MedProbe for Europe)
  - Primer 3.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)

- **Design Taqman® probes**
  - Primer Express® (Applera)
  - BeaconDesigner® (Premier Biosoft Inc.)

- **Design Molecular Beacons**
  - BeaconDesigner® (Premier Biosoft Inc.)

- **Design Scorpion primers**
  - Scorpio (DNA software)

- **Verification of design**
  - Mfold (www.bioinfo.rpi.edu/applications/mfold/)
  - BLAST (www.ncbi.nlm.nih.gov/BLAST/)

- **A software is just a tool to help you, not a guarantee for the perfect design!**
Useful sources of information

- Available on www.eurogentec.com
- Frequently asked questions for RT qPCR and qPCR
- Troubleshooting guide for RT qPCR and qPCR
- Your one-stop-shop real-time PCR supplier (in your conference bag)