GENOMICS

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

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PCR efficiency

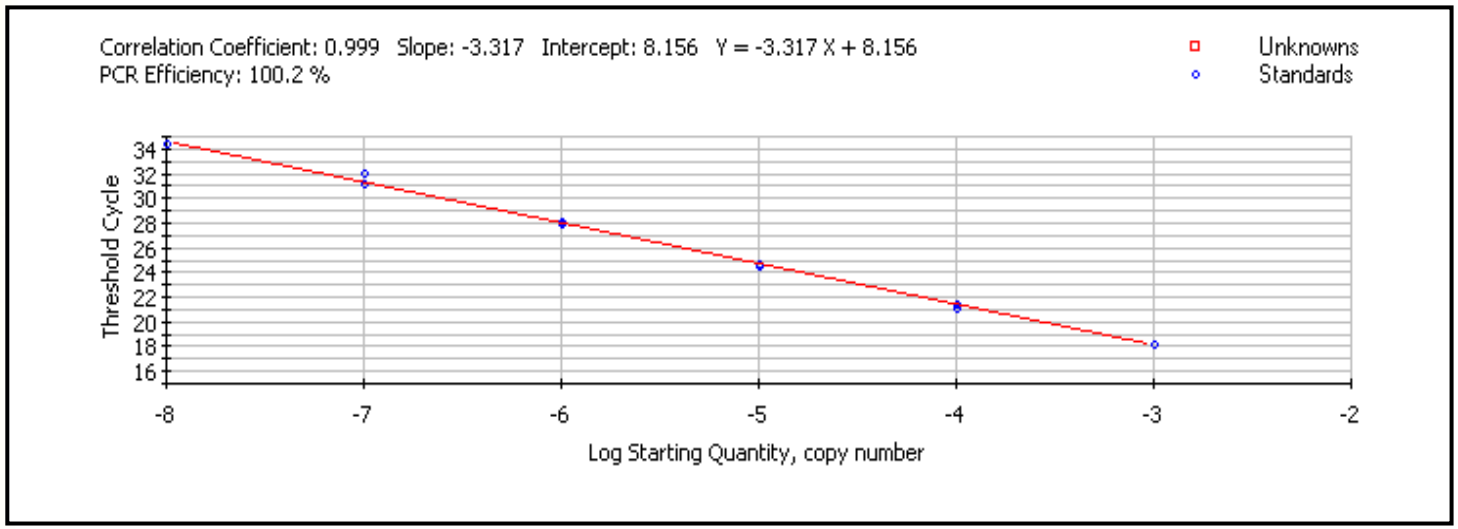
- High PCR efficiency
  - high accuracy
  - high reproducibility
- PCR efficiency influenced by
  - length of amplicon
  - GC content of amplicon
  - secondary structures in primers, probes, amplicons
  - concentration reaction components
  - PCR inhibitors/PCR enhancers
  - quality RNA/cDNA



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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

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PCR efficiency

- 100% PCR efficiency
  - every PCR cycle amount of DNA is doubled
  - 2x dilution curve ΔCt of 1 between every dilution
  - 10x dilution curve ΔCt 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



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Why do you need a good design?

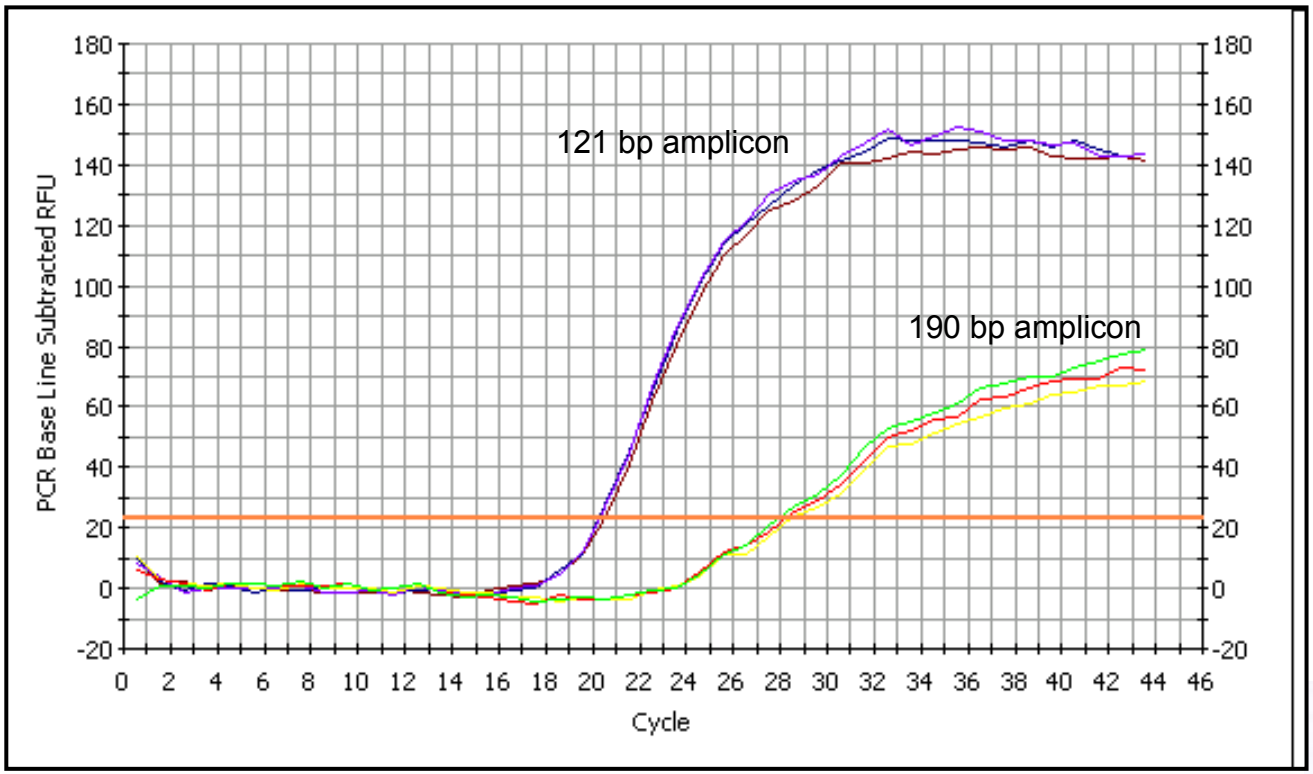
- Well-designed primers and probes are a prerequisite for successful RT qPCR in terms of
  - high PCR efficiencies
  - specific PCR products
  - no co-amplification of genomic DNA
  - no amplification of pseudogenes
  - most sensitive results

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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

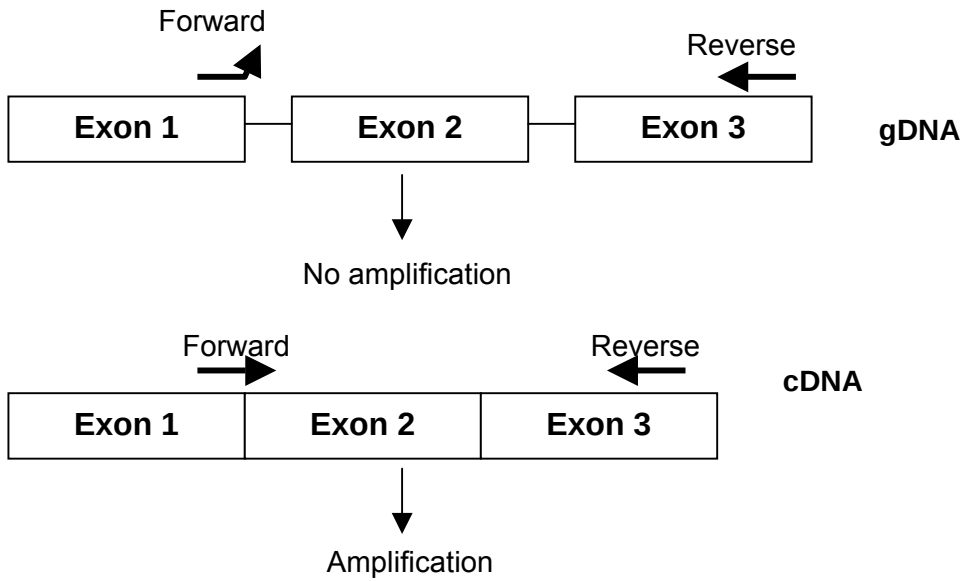
- **Primers**
  - length
    - 18-30 bases
  - GC content
    - 30-80% (ideally 40-60%)
  - Tm
    - 63-67ºC (ideally 64ºC), so that $T_{annealing}$ is 58-62ºC (ideally 59ºC)
    - ΔTm forward primer and reverse primer < 4ºC
  - avoid mismatches between primers and target, especially towards the 3' end 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/)



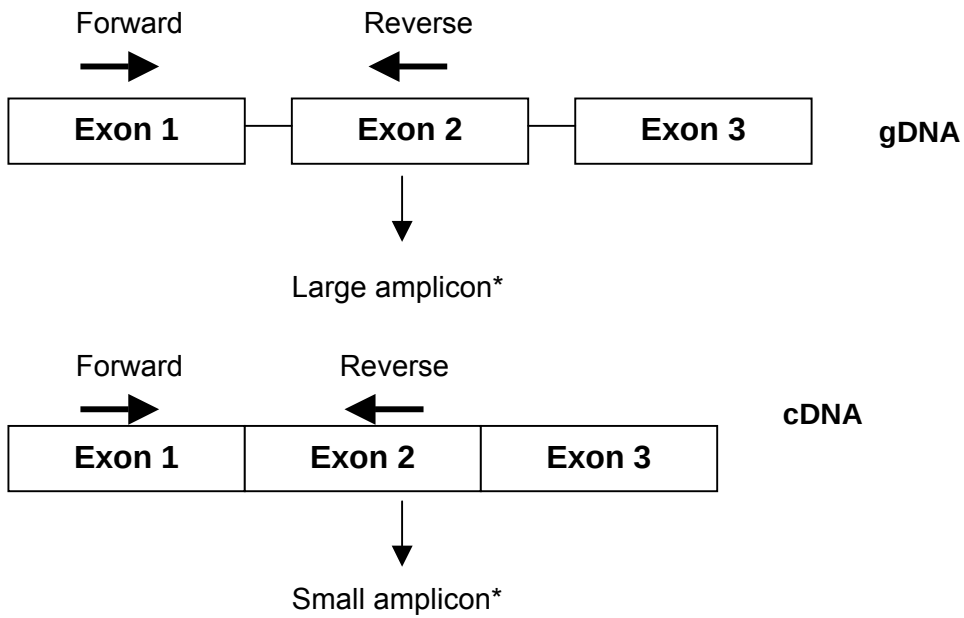
Intron spanning/flanking primers

- Intron spanning primers



Intron spanning/flanking primers

- Intron flanking primers



* 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 ΔRn 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 secundary 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 can not 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 *in silico* differs from experimental



Frequent pitfalls

- The design software that I use can not find a suggestion, although the sequence if 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 secundary 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 ampilicon length, primer length, Tm's or GC content



Frequent pitfalls

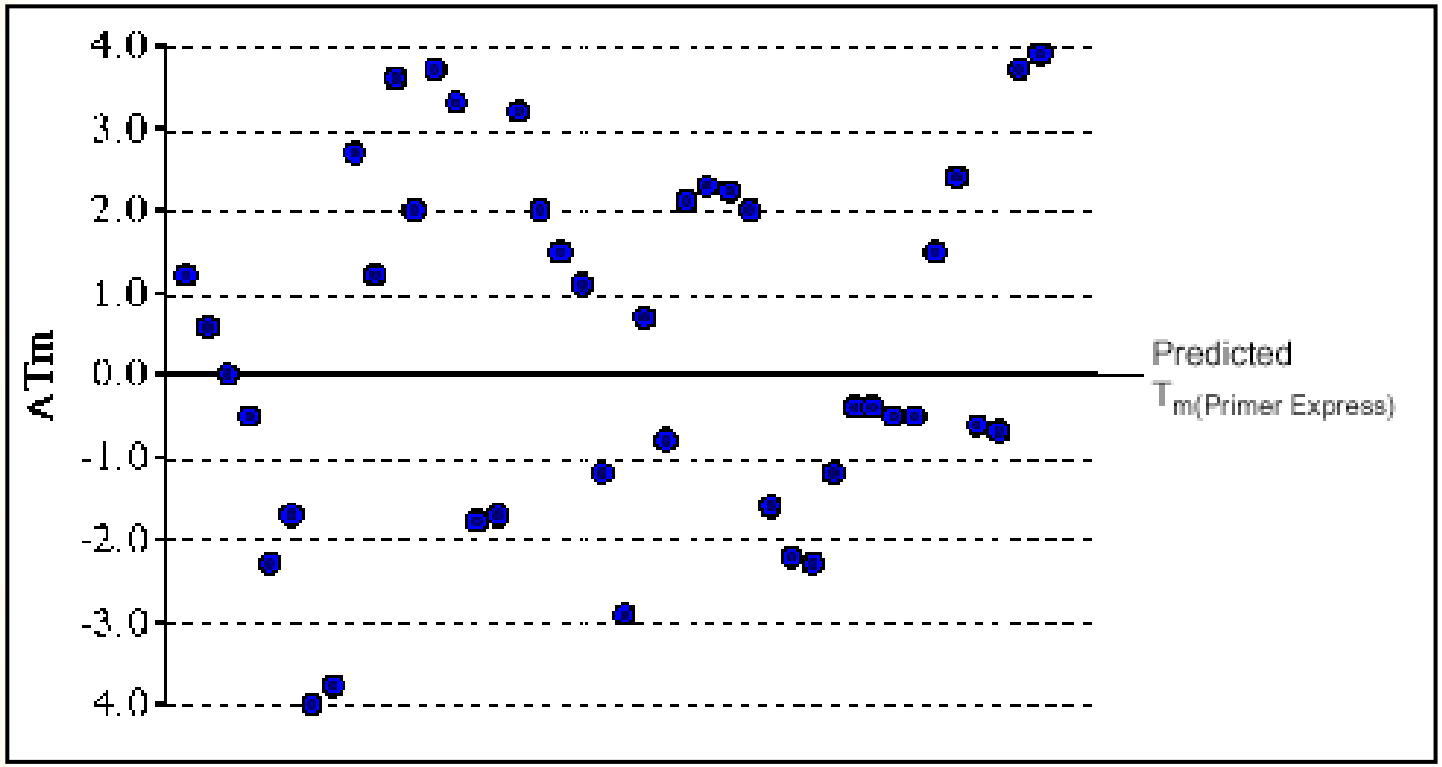
- I took the first suggestion in the list of Primer Express®, but the primer/probe set does not lead to good results
  - The first suggestion in the list of Primer Express® is the shortest amplicon, not the best primer/probe set

- 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 > 3ºC check Tm experimentally



Predicted vs. experimental Tm

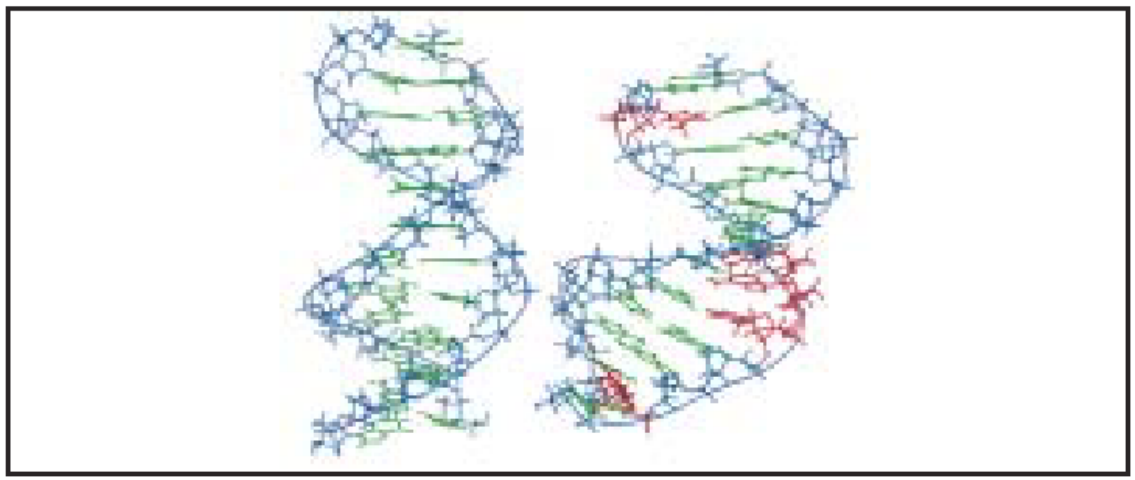


Source: ABI User Bulletin 6 ABI PRISM® Sequence Detection System

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

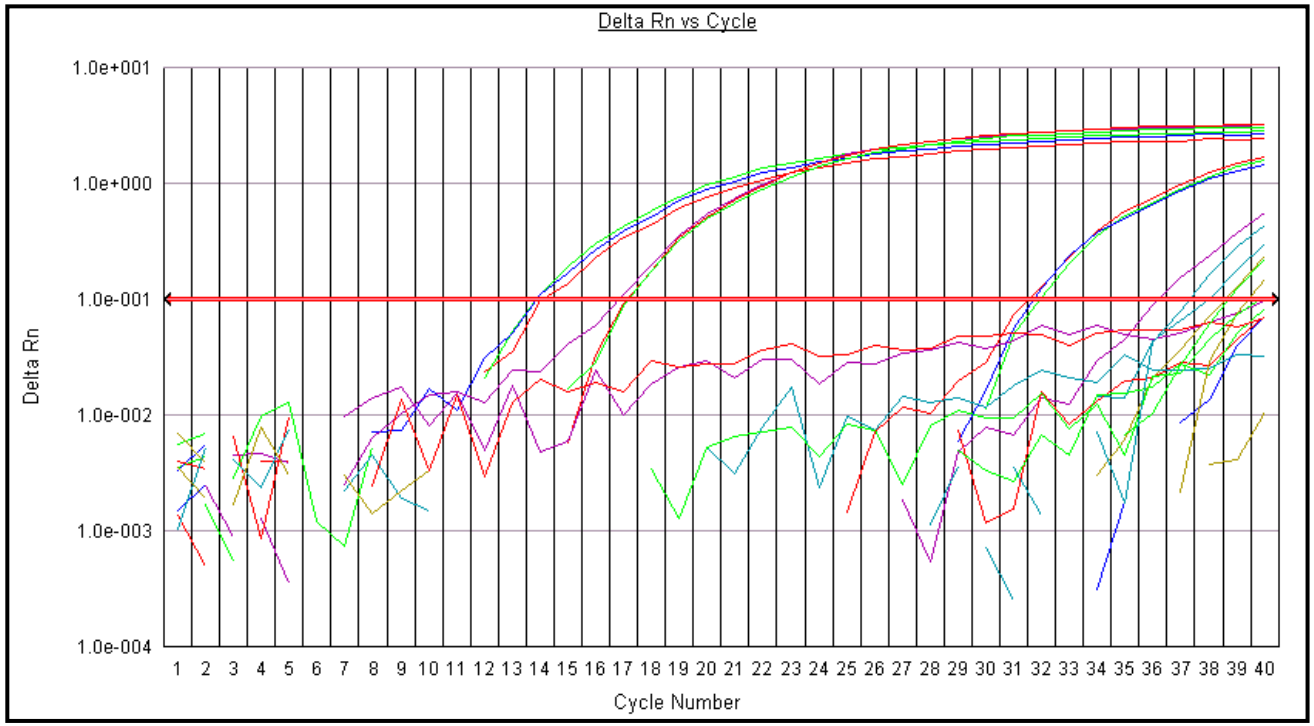
- With LNA bases length probes can be decreased or Tm can be increased



Conformation change from B helix to A helix due to LNAs

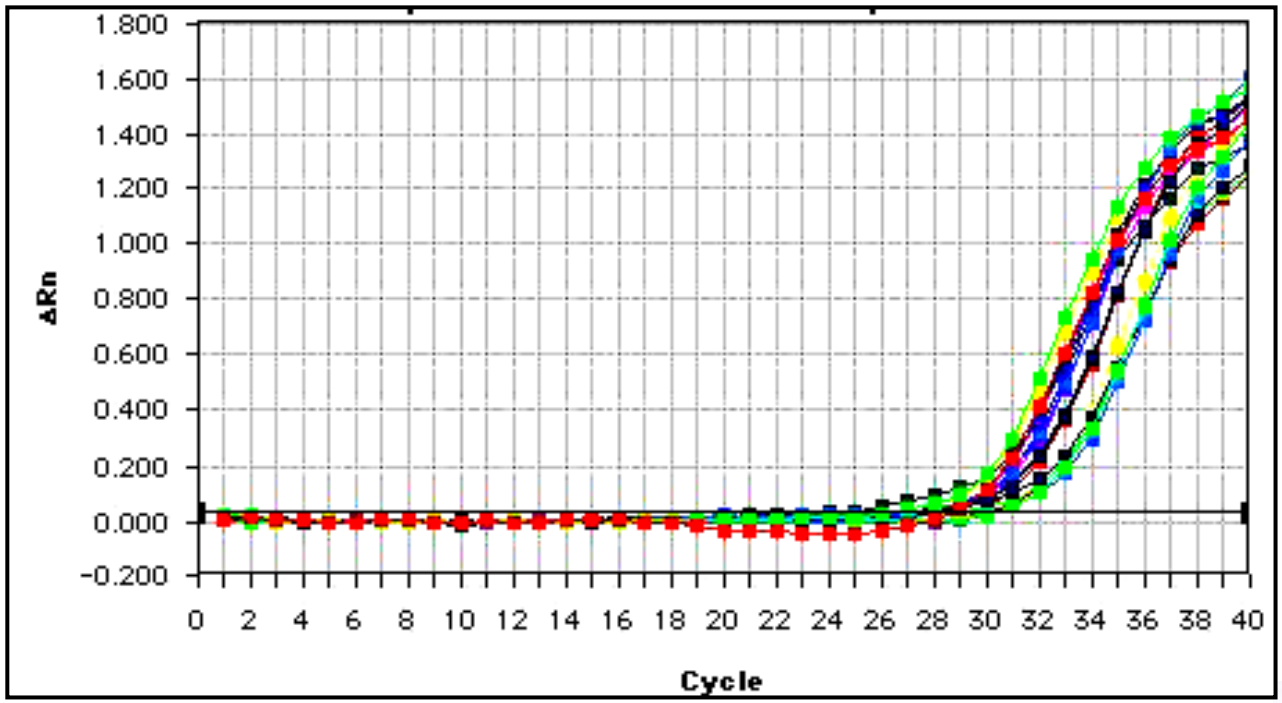
Example probe assay

- “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 seperated
- **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)



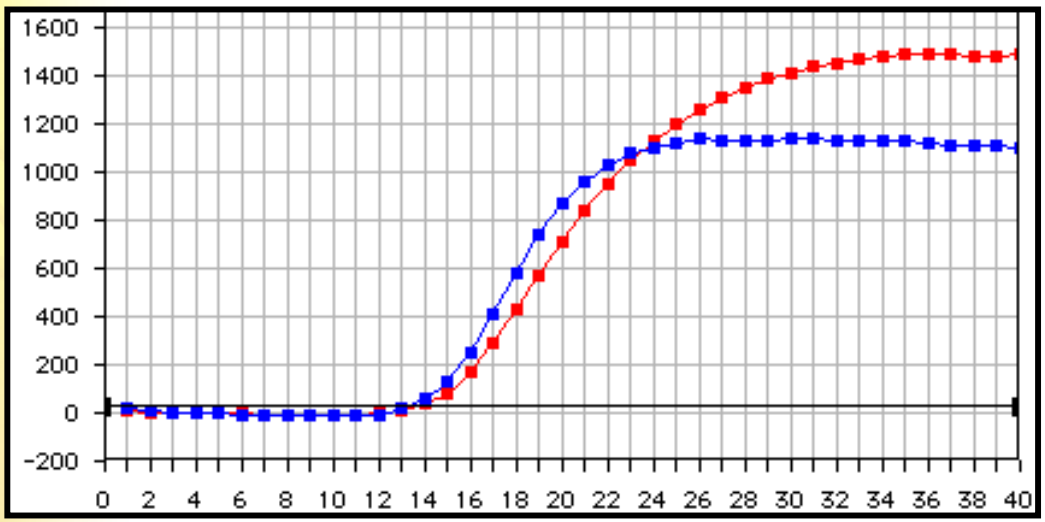
Fluorophore has to fit real-time thermocycler

Thermocycler	Dye1	Dye2	Dye3	Dye4	Dye5	Dye6	Dye7
GeneAmp® 5700	FAM						
ABI Prism® 7000	FAM	VIC/YY/JOE	NED/TAMRA/DFO	ROX			
ABI Prism® 7700	FAM	VIC/YY/JOE/TET	NED/TAMRA/DFO	ROX			
ABI Prism® 7900	FAM	VIC/YY/JOE/TET	NED/TAMRA/DFO	ROX			
ABI Prism® 7300	FAM	VIC/YY/JOE	NED/TAMRA/DFO	ROX			
ABI Prism® 7500	FAM	VIC/YY/JOE	NED/TAMRA/Cy3/DF	ROX/TR	Cy5		
i-cycler IQ®	FAM	VIC/HEX/TET/Cy3/YY	Cy3/TAMRA	ROX/TR	Cy5		
Mx3000P®	FAM	TET/YY	HEX/JOE/VIC/YY	TAMRA	Cy3	TR/ROX	Cy5/Alexa 350
Mx4000®	FAM	TET/YY	HEX/JOE/VIC/YY	TAMRA	Cy3	TR/ROX	Cy5
Rotorgene 2000	FAM	TET/JOE/VIC/YY	ROX/TAMRA/Cy3/TR	Cy5			
Rotorgene 3000	FAM	TET/JOE/VIC/YY	MAX/ROX/Cy3/TR	Cy5			
DNA Engine Opticon® 1	FAM						
DNA Engine Opticon® 2	FAM	TET/HEX/VIC/YY/TAMRA					
Chromo 4	FAM	TET/JOE/VIC/YY	ROX/TR	Cy5			
Smartcycler® 1	FAM	TET/JOE/VIC/YY	TAMRA/Cy3/Alexa	ROX/TR			
Smartcycler® 2	FAM	TET/Cy3/YY	ROX/TR	Cy5			
Lightcycler®	FAM	LC Red 640/ROX	LC Red 705/Cy5				
Lightcycler® 2.0	FAM	HEX/VIC/YY	LC Red 610	LC Red 640	LC Red 670	LC Red 705	
Quantica®	FAM	TET/HEX/VIC/YY/TAMRA					

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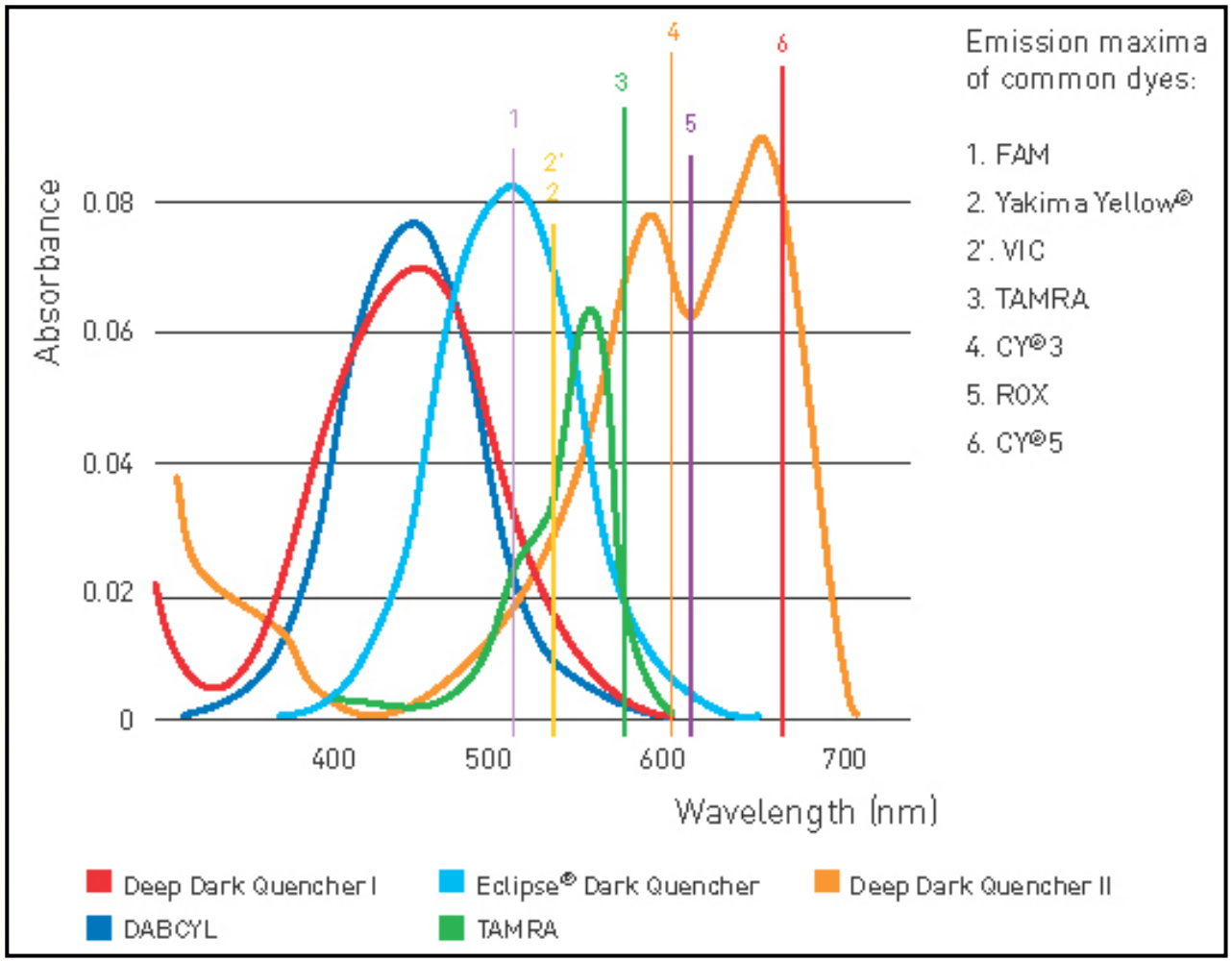
Fluorophore has to fit real-time thermocycler

- 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

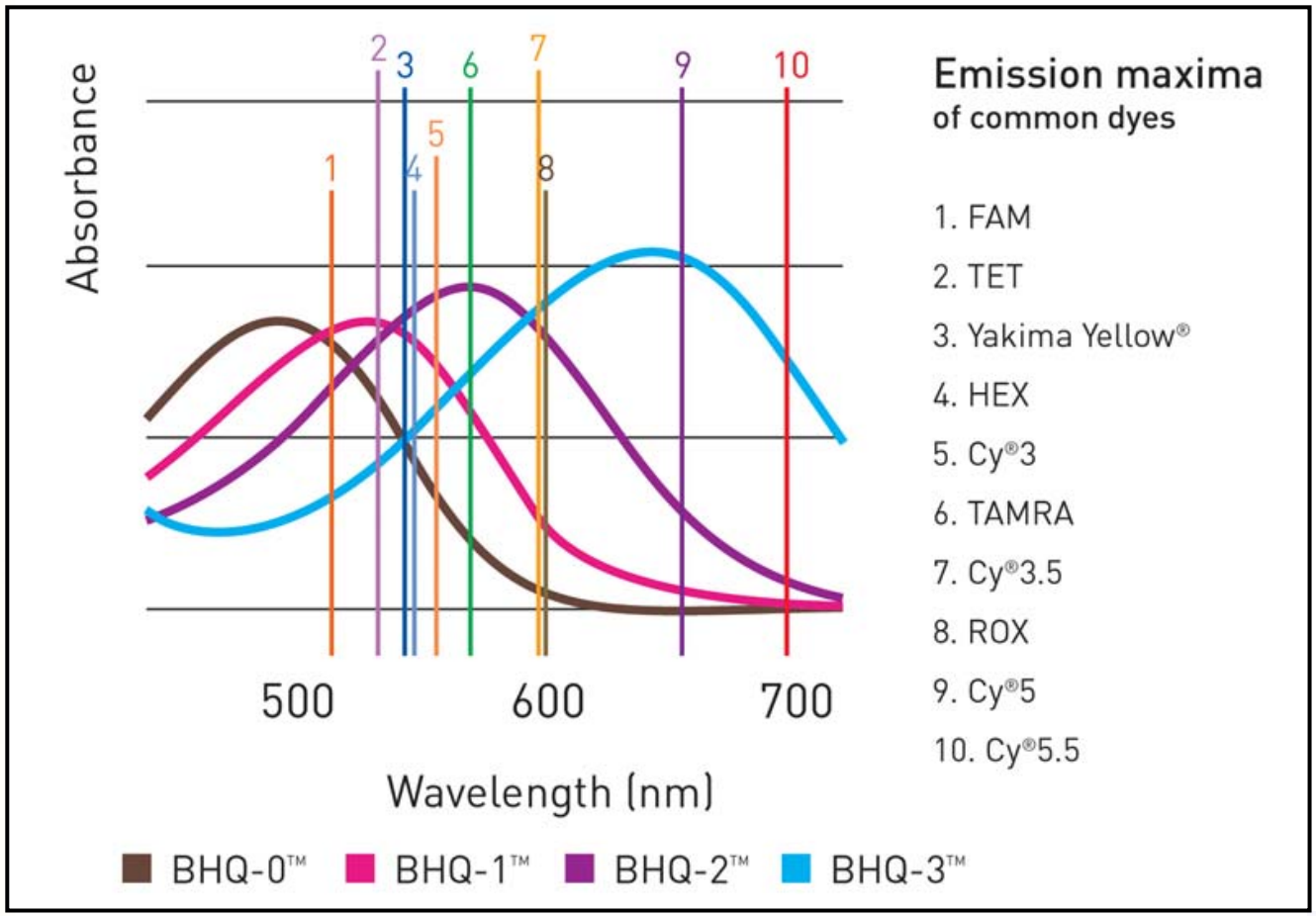


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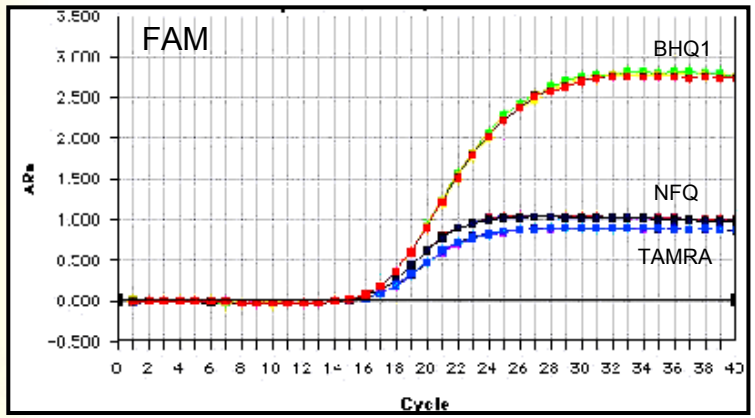
Quencher has to fit fluorophore

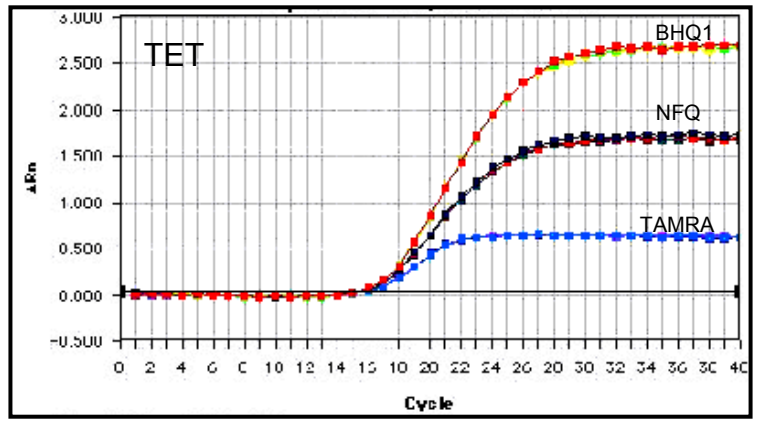


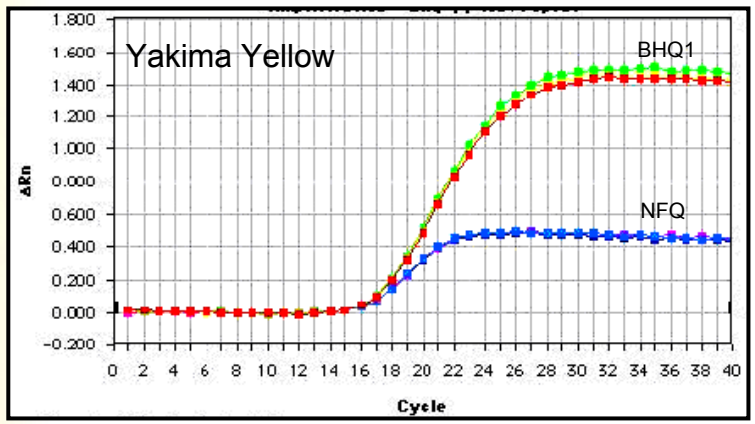
Quencher has to fit fluorophore



High signal-to-noise ratio

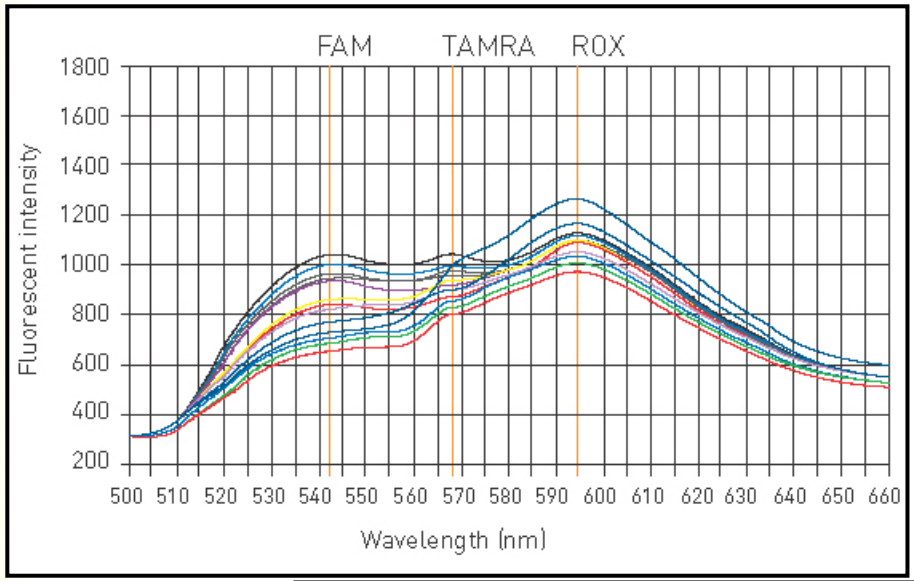


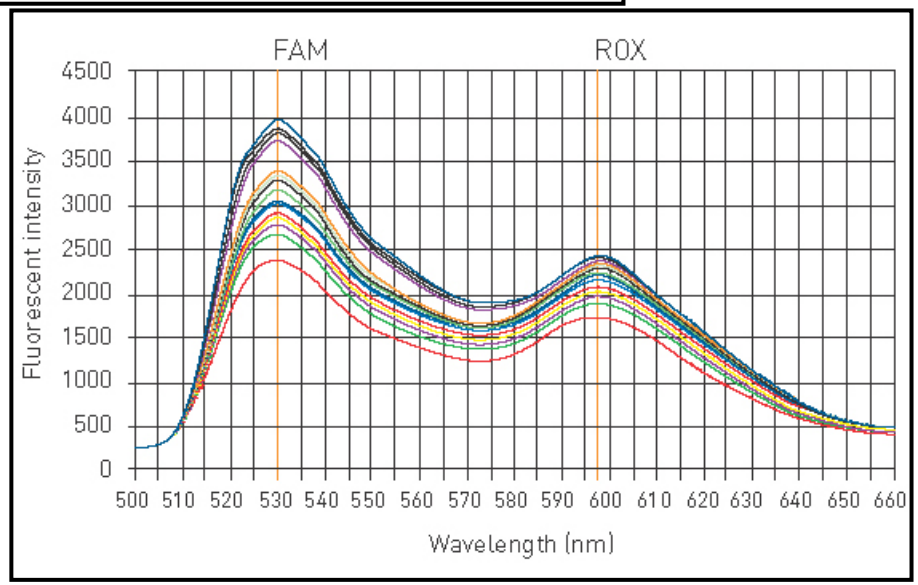




BHQ1 leads to the highest signal-to-noise ratios and is superior to TAMRA and NFQ (EDQ)

TAMRA or dark quenchers?





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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



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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)

