



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
 - ...

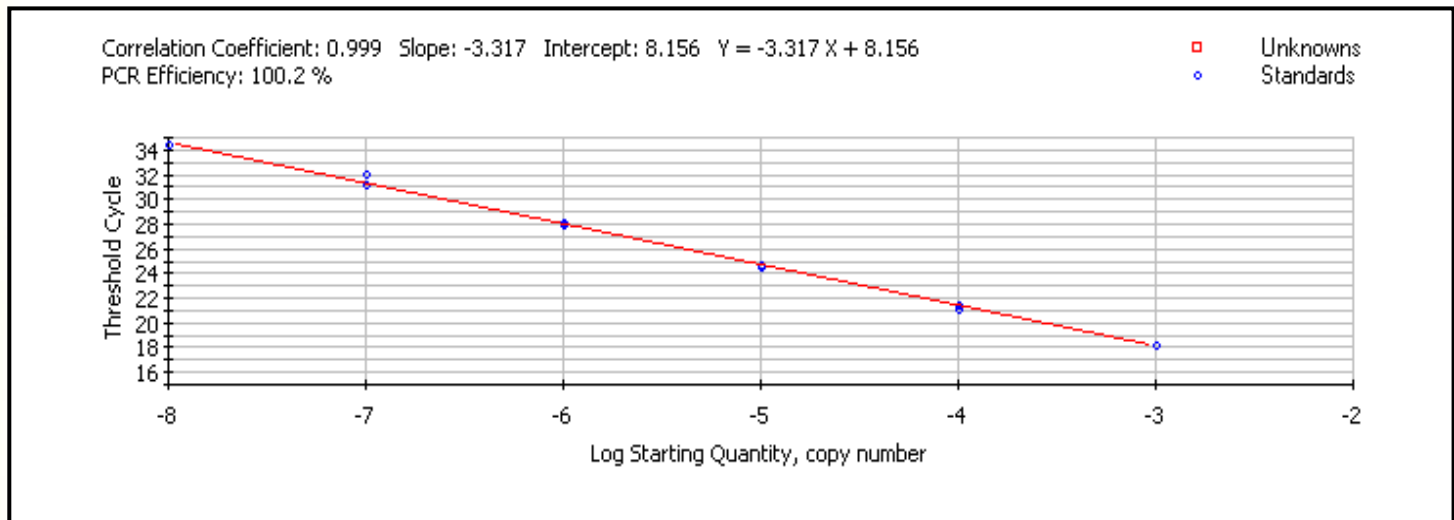




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PCR efficiency

- Easiest way to determine PCR efficiency: standard curve with R^2 close to 1,00 and slope close to -3,32



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

Efficiency = $10^{(-1/\text{slope})} - 1$



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PCR efficiency

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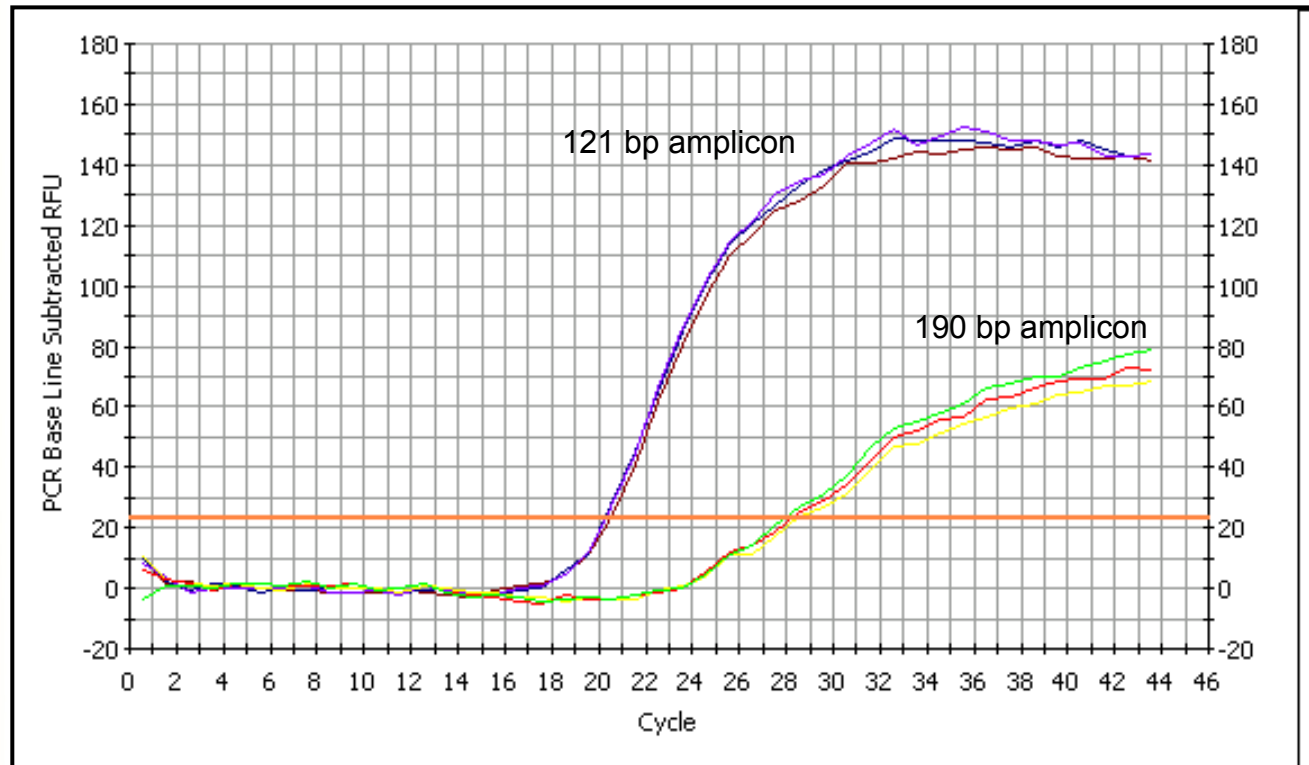




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Design makes the difference!

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



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Design guidelines for primers

- **Primers**
 - length
 - 18-30 bases
 - GC content
 - 30-80% (ideally 40-60%)
 - T_m
 - 63-67°C (ideally 64°C), so that $T_{annealing}$ is 58-62°C (ideally 59°C)
 - ΔT_m 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/)

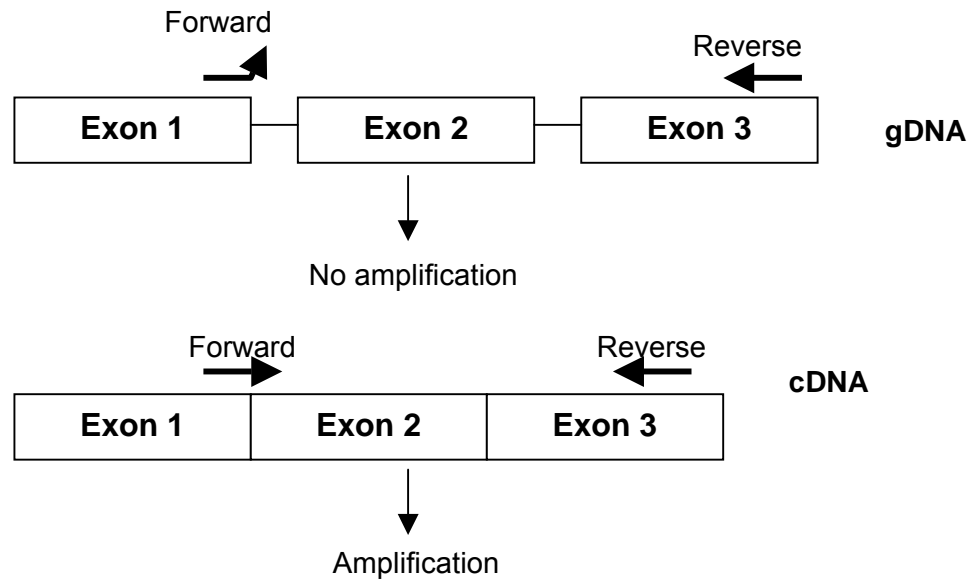




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Intron spanning/flanking primers

- Intron spanning primers

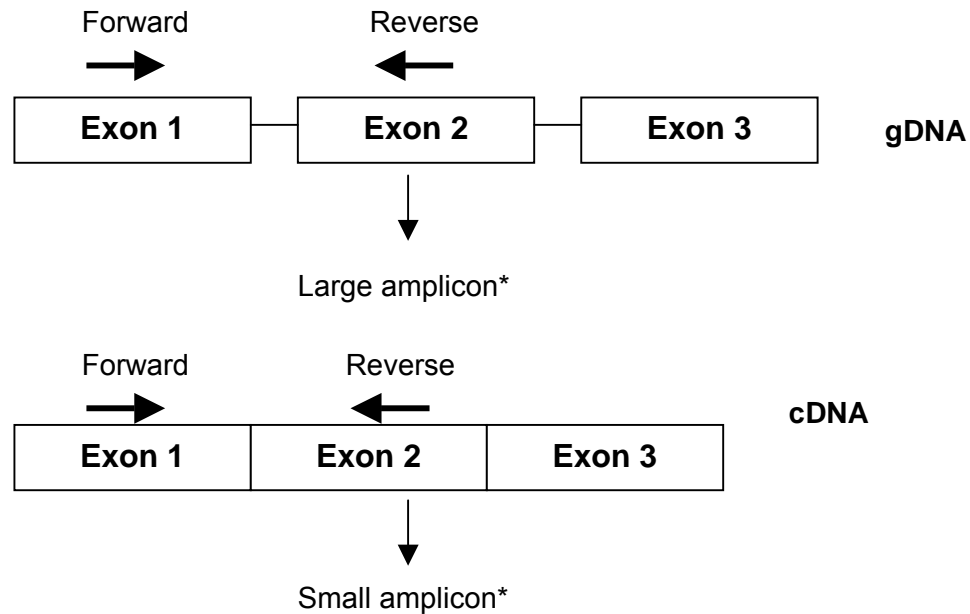




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Intron spanning/flanking primers

- Intron flanking primers



* Can be detected via melt curve





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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%
 - T_m
 - T_m of the probe must be 8-10°C higher than the T_m 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 mismatches 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 T_m





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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 ΔR_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/)





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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



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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 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, T_m 's or GC content





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Frequent pitfalls

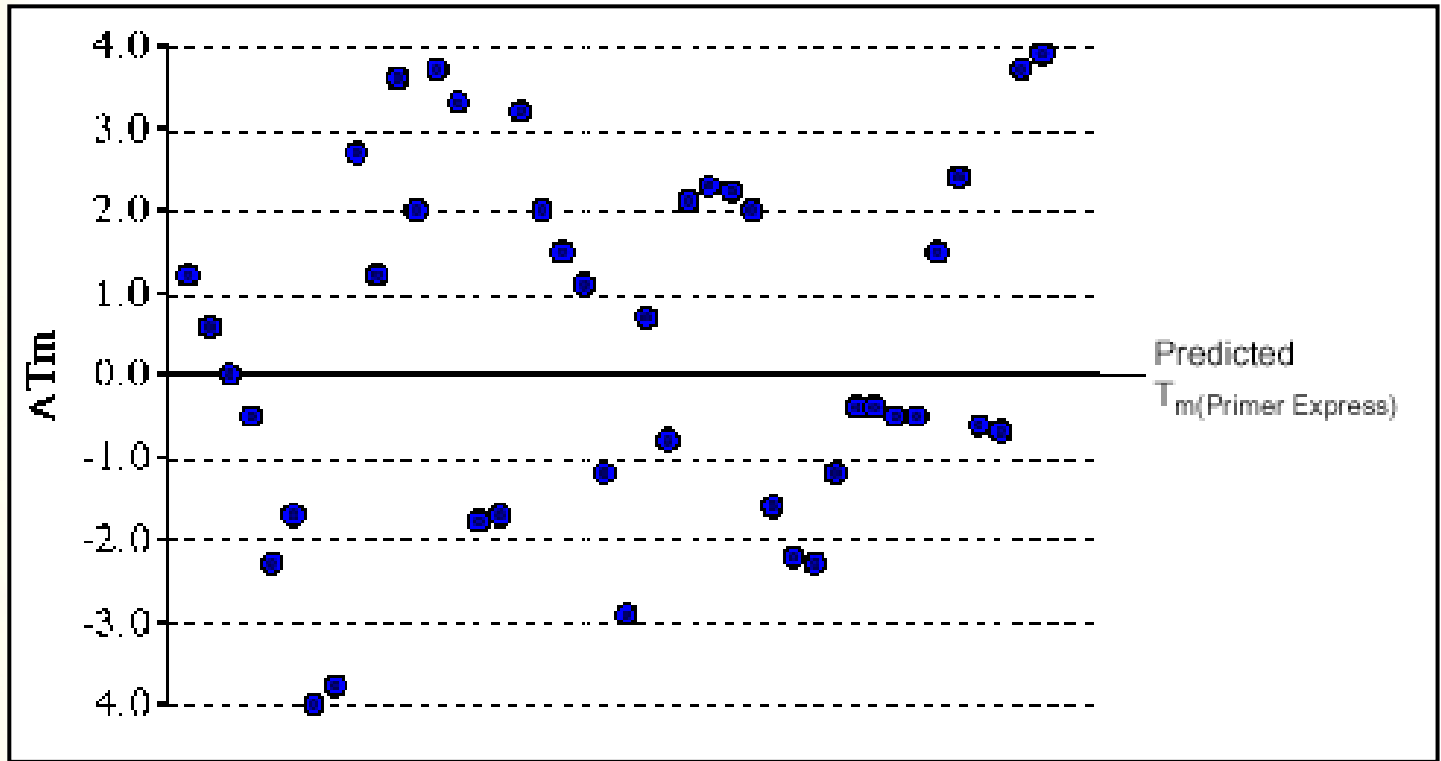
- I took the first suggestion in the list of Primer Express[®], but the primer/probe set does not lead to good results
 - The first suggestion in the list of Primer Express[®] is the shortest amplicon, not the best primer/probe set
- With the recommended temperature profile I obtain an amplicon, but the detection does not function due to the probe, which is not binding
 - Each software uses its own method of calculating the T_m 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 T_m of the probe
 - Check T_m using several softwares.
If T_m 's differ $> 3^\circ\text{C}$ check T_m experimentally





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Predicted vs. experimental T_m



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



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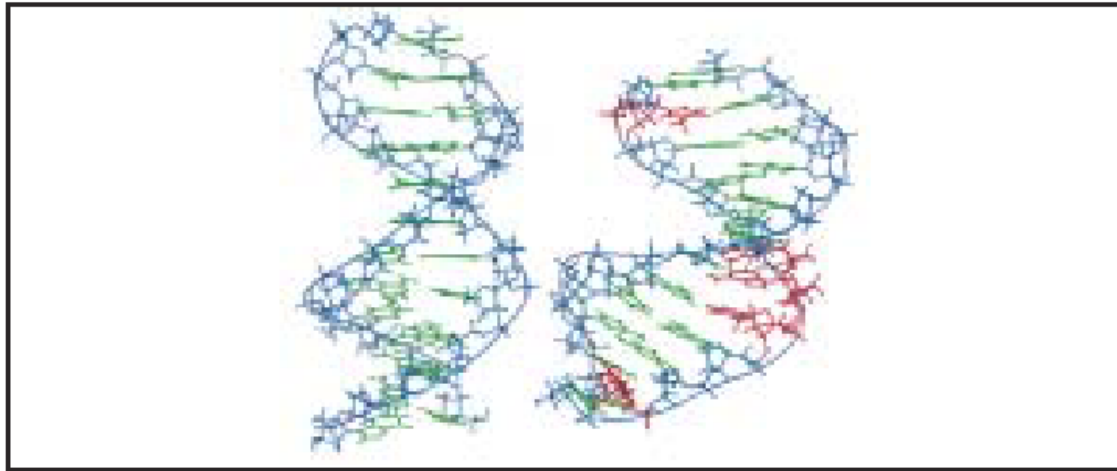




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Probe too long or T_m too low?

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



Conformation change from B helix to A helix due to LNAs



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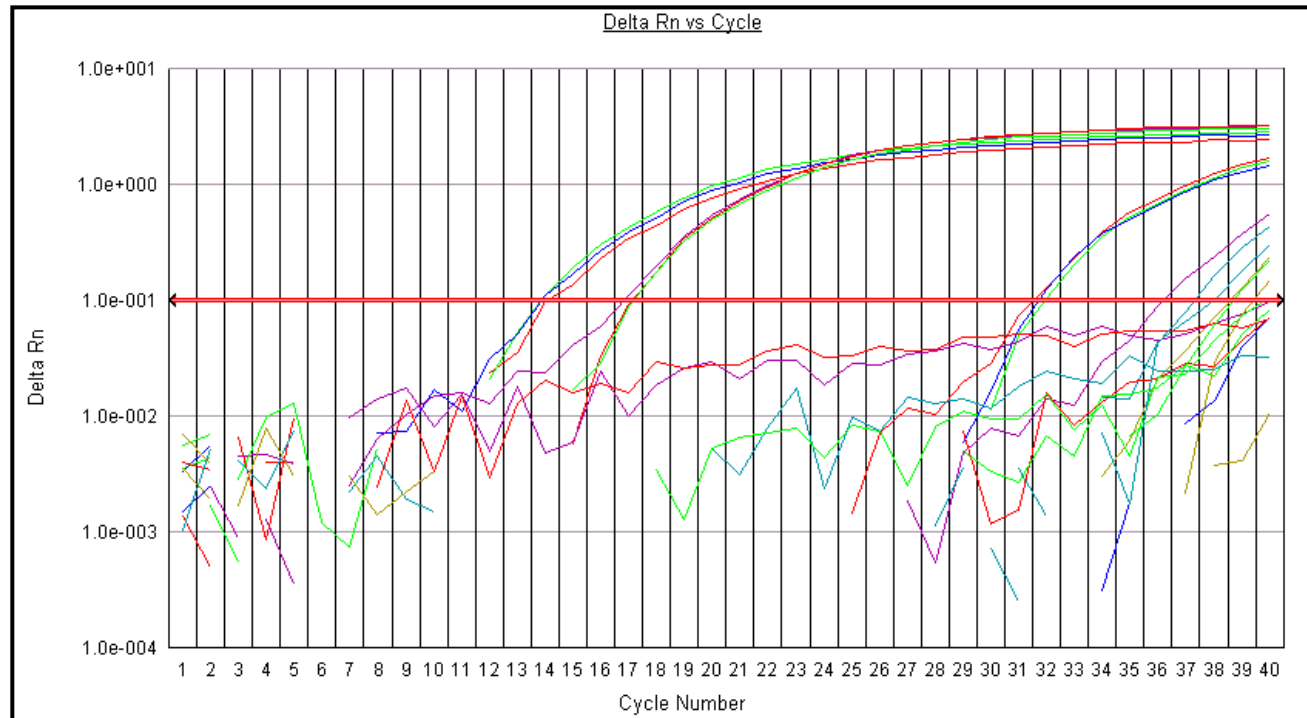




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Example probe assay

- “Jump” in dilution series caused by secondary structure in primer/probe



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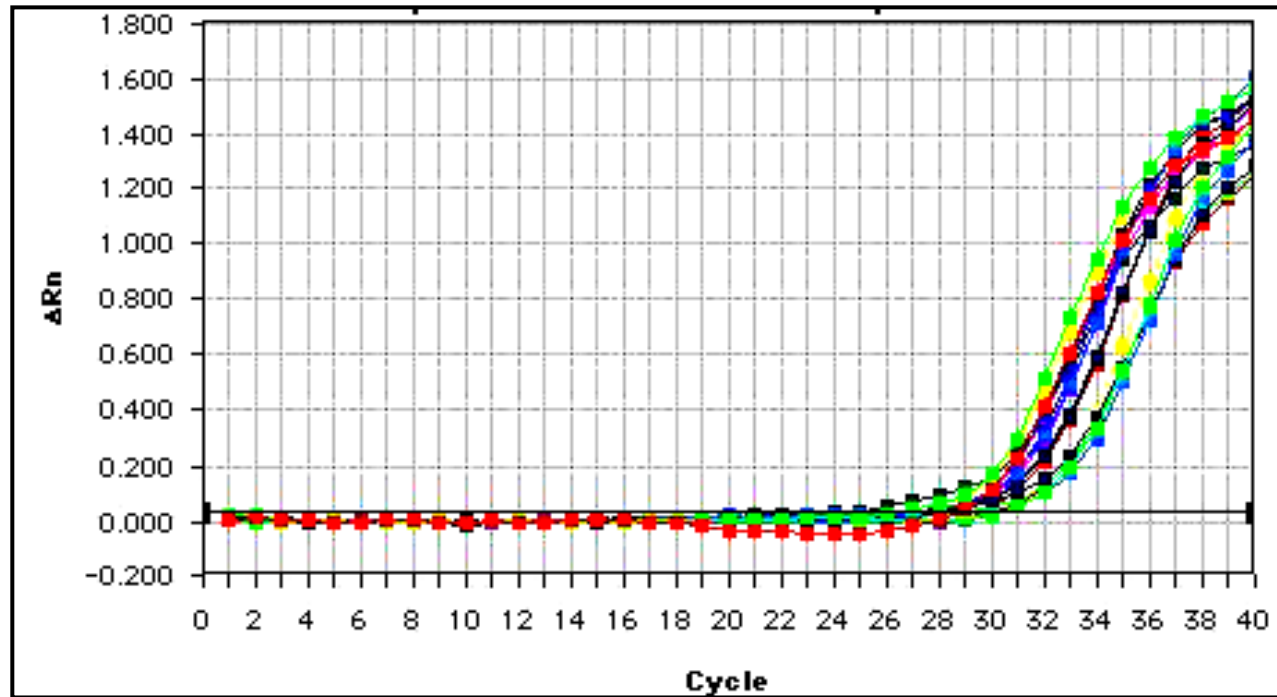




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Example SYBR[®] green I assay

- Ct's of all dilutions around same point due to primer dimers



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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



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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)





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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 | MAXROX/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 | | | | | |

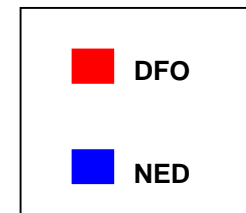
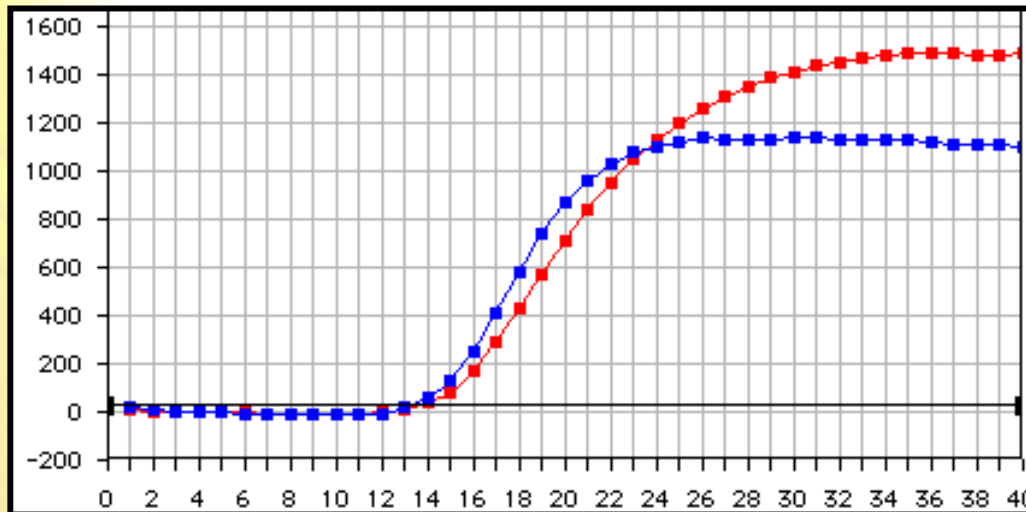




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Fluorophore has to fit real-time thermocycler

- 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



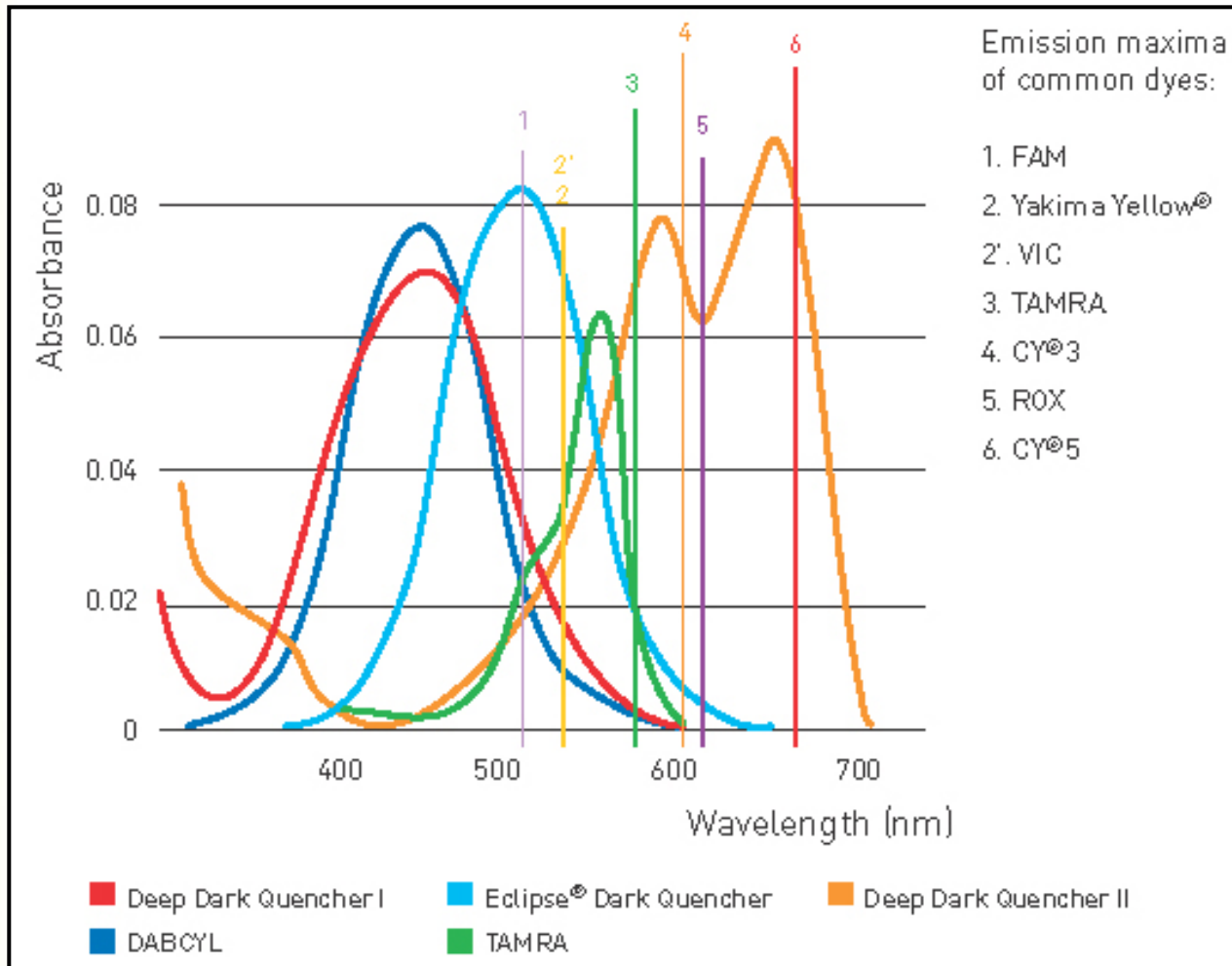
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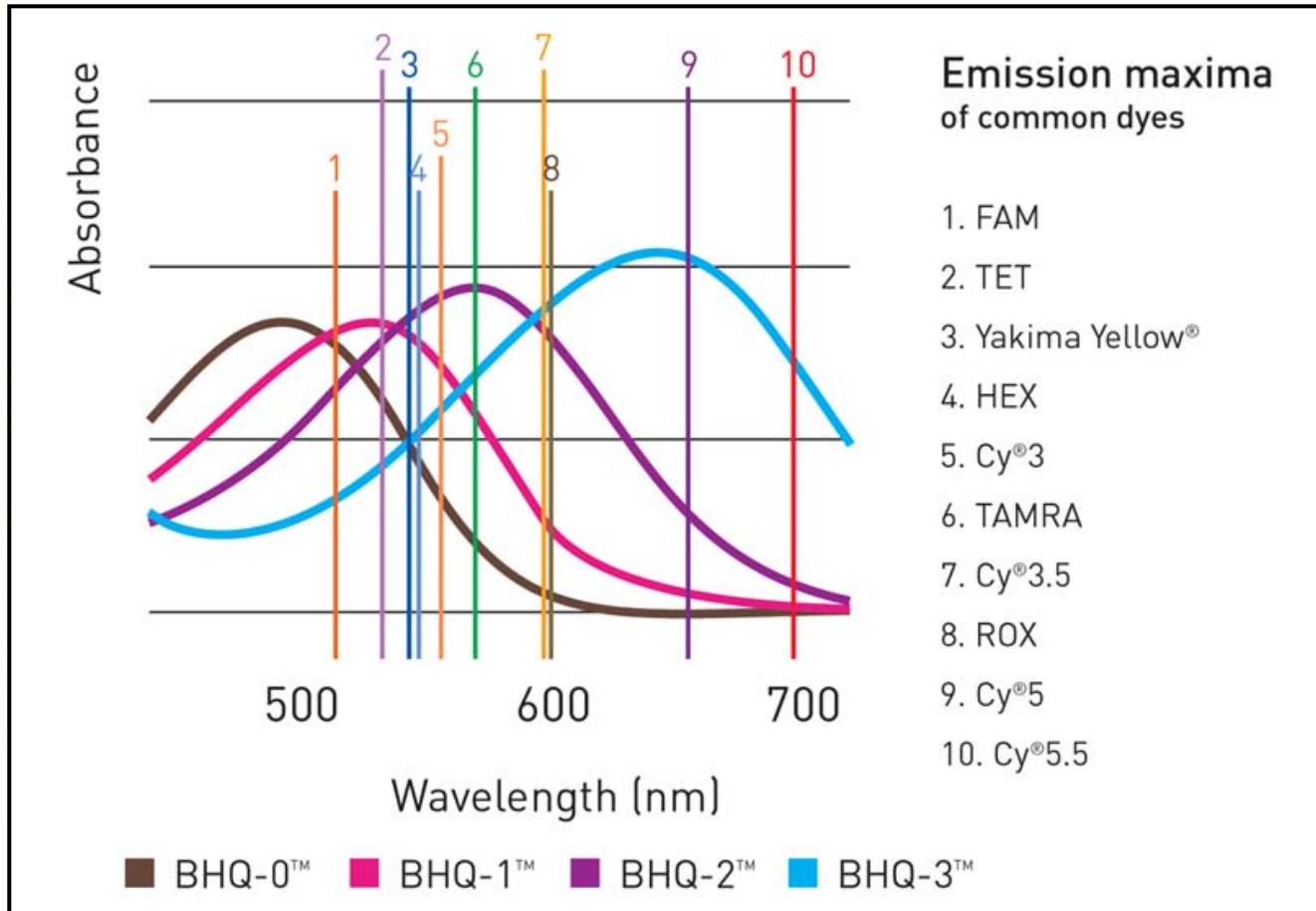
Quencher has to fit fluorophore





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Quencher has to fit fluorophore



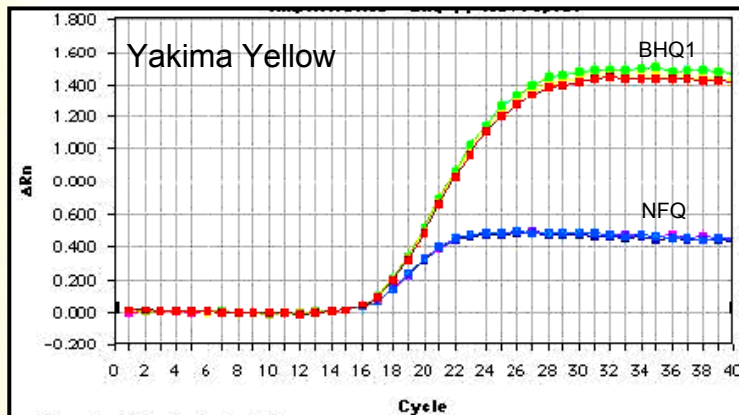
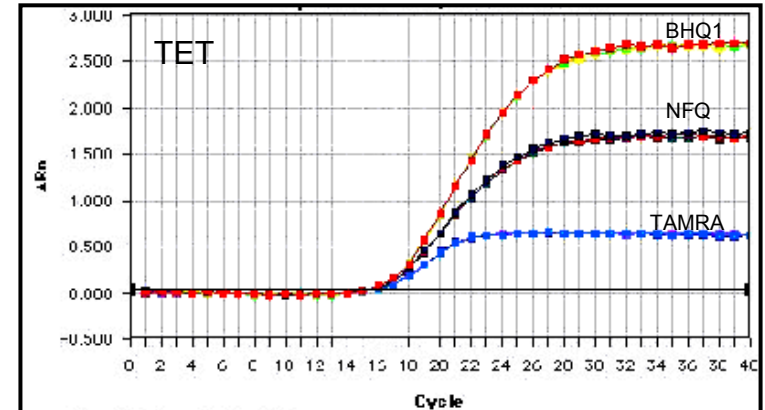
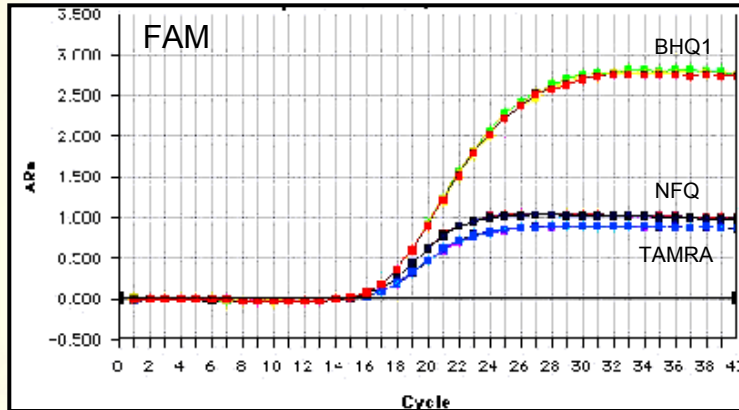
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High signal-to-noise ratio



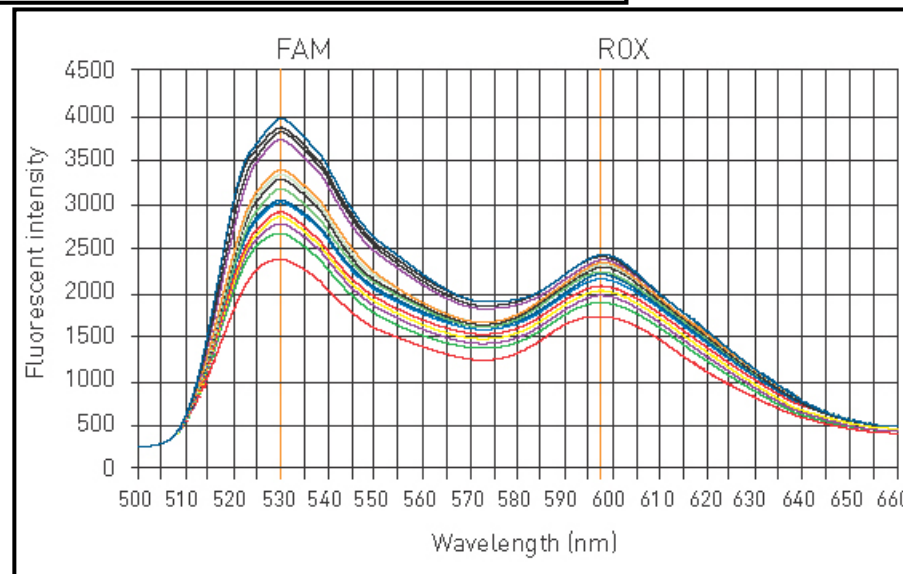
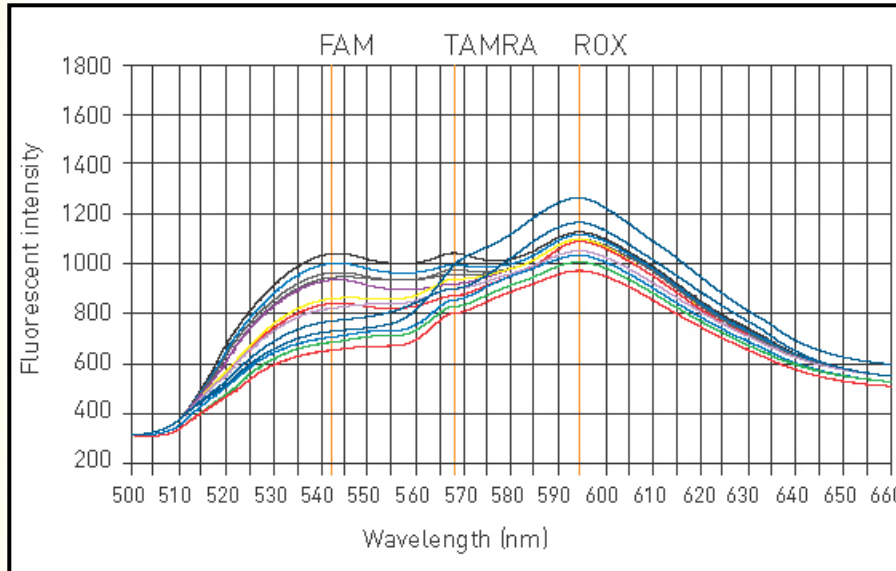
BHQ1 leads to the highest signal-to-noise ratios and is superior to TAMRA and NFQ (EDQ)





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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 (ROX, TAMRA...)
 - very pure oligos, but low yield
 - 5' and internal labelling (only on dT residu) possible
 - automatic coupling via labelled phosphoramidites (FAM, Yakima Yellow,...)
 - 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[®] (Applied)
 - 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!**








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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



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