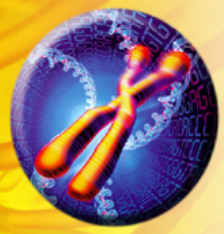

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Pitfalls of primer and probe design and synthesis

Mary Span
Senior Product Manager
Eurogentec S.A.

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Steps in qPCR assay

- Set up experiment
  - statistical relevant # samples/experimental group
  - controls
- **Design 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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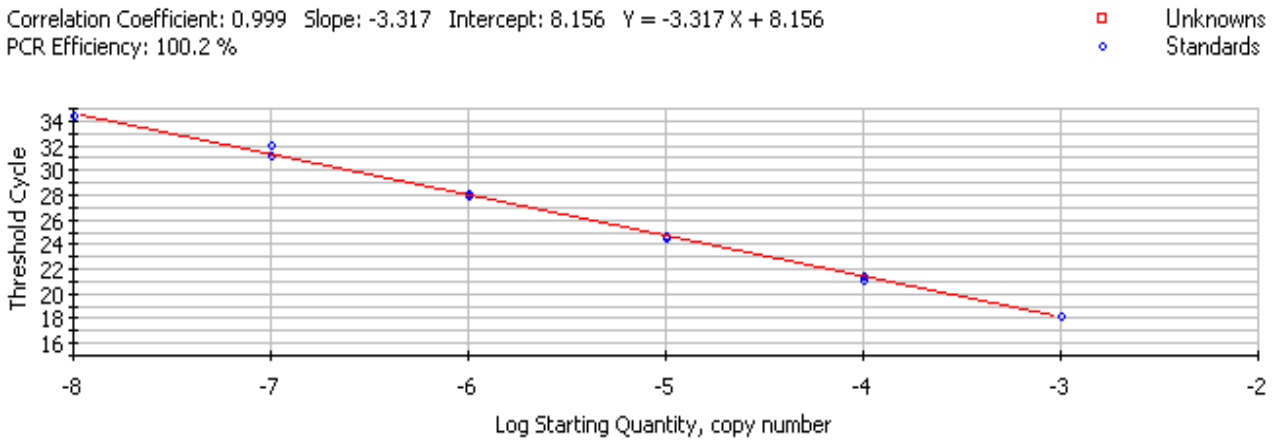
PCR efficiency

- Easiest way to determine PCR efficiency: standard curve with R^2 close to 1,00 and intercept 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 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 primer and probe 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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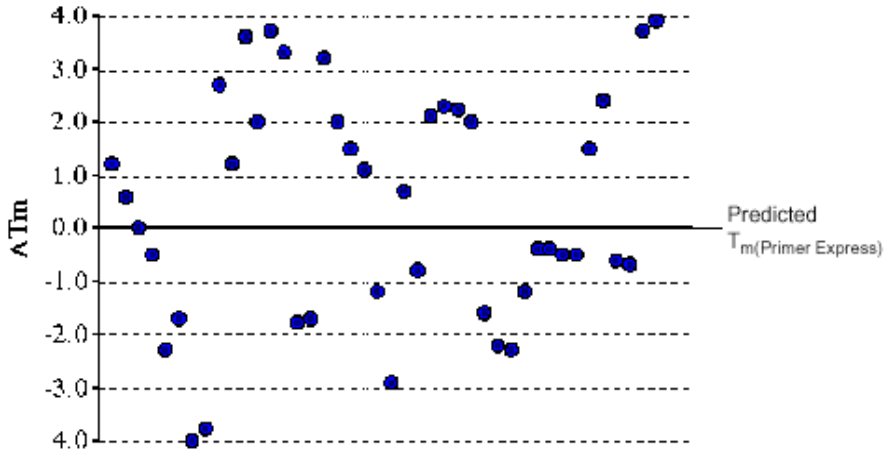
Remarks primer and probe design

- Try to make primers fit the probe and not the probe fit the primers
- Do not expect that the primers you used before for your normal PCR will be the ideal pair to which a probe can be designed
- Using 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
- It is not always possible to design a primer/probe set for a specific sequence
- The first suggestion in the list of Primer Express® is the shorted amplicon, not the best primers and probe

Remarks primer and probe design

- Check Tm using several softwares
- If Tm differs by more than 3ºC check Tm experimentally



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

Design guidelines for SYBR® green I assays

- **Primers**
  - length
    - 18-30 bases
  - GC content
    - 30-80% (ideally 40-60%)
  - Tm
    - 63-67ºC (ideally 64ºC), so that Tannealing 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)

Design guidelines for SYBR® green I assays

- **Amplicon**
  - length
    - 80-150 bp
    - shorter amplicons will give higher PCR efficiencies
    - longer amplicons will give a higher ΔRn as more SYBR® green I is incorporated
  - GC content
    - 30-80% (ideally 40-60%)
  - avoid secundary structures in the amplicon (check with Mfold: www.bioinfo.rpi.edu/applications/mfold/)

Design guidelines for 5’ exonuclease assays

- **Probes**
  - length
    - 18-30 bases
    - 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 (8°C for genotyping, 10°C for expression profiling) higher than the Tm of the primers
  - 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)
  - 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 5’ exonuclease assays

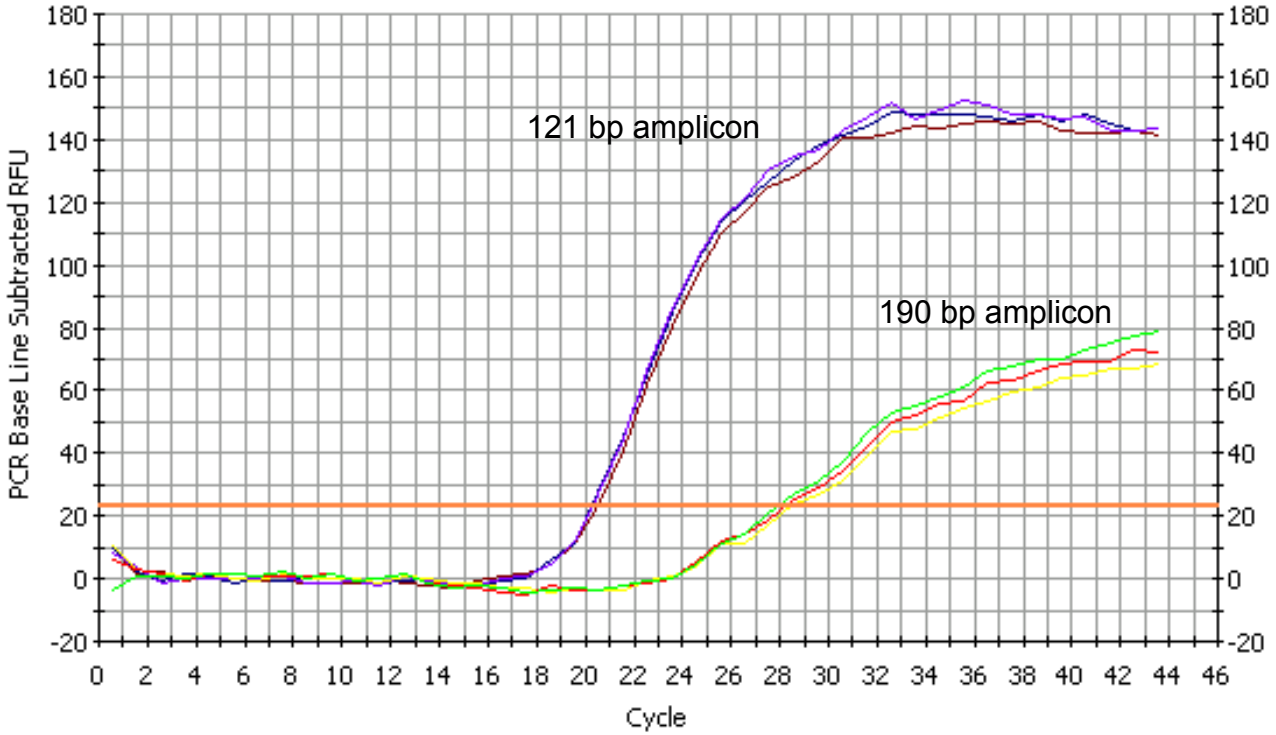
- **Primers**
  - length
    - 18-30 bases
  - GC content
    - 30-80% (ideally 40-60%)
  - Tm
    - 63-67ºC (ideally 64ºC), so that Tannealing is 58-62ºC (ideally 59ºC)
    - ΔTm forward primer and reverse primer < 4ºC
  - avoid mismatches between primers and target, especially towards the 3’ end of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

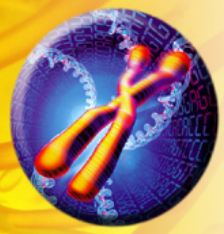
Design guidelines for 5’ exonuclease assays

- **Amplicon**
  - length
    - 80-120 bp optimal (up to 1000 bp possible with adjusted reaction times)
    - shorter amplicons will give higher PCR efficiencies and 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/)

Example design 18S rRNA

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





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Guidelines for fluorophores and quenchers

- 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	ROX			
ABI Prism® 7700	FAM	VIC/YY/JOE/TET	NED/TAMRA	ROX			
ABI Prism® 7900	FAM	VIC/YY/JOE/TET	NED/TAMRA	ROX			
ABI Prism® 7300	FAM	VIC/YY/JOE	NED/TAMRA	ROX			
ABI Prism® 7500	FAM	VIC/YY/JOE	NED/TAMRA/Cy3	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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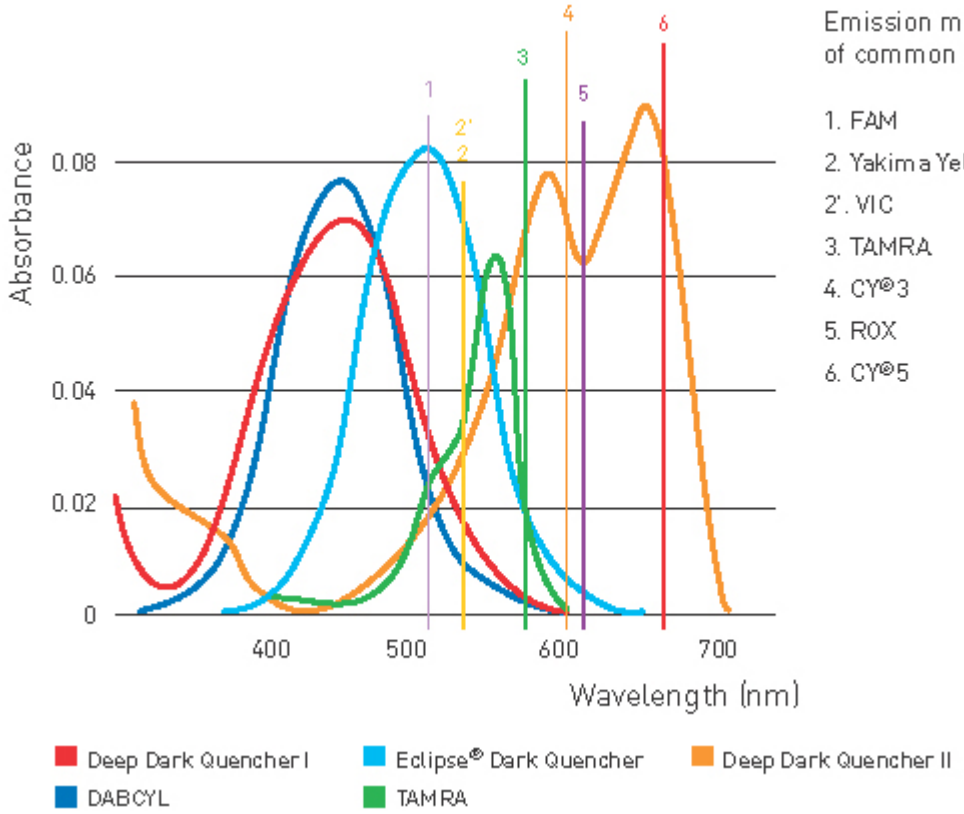
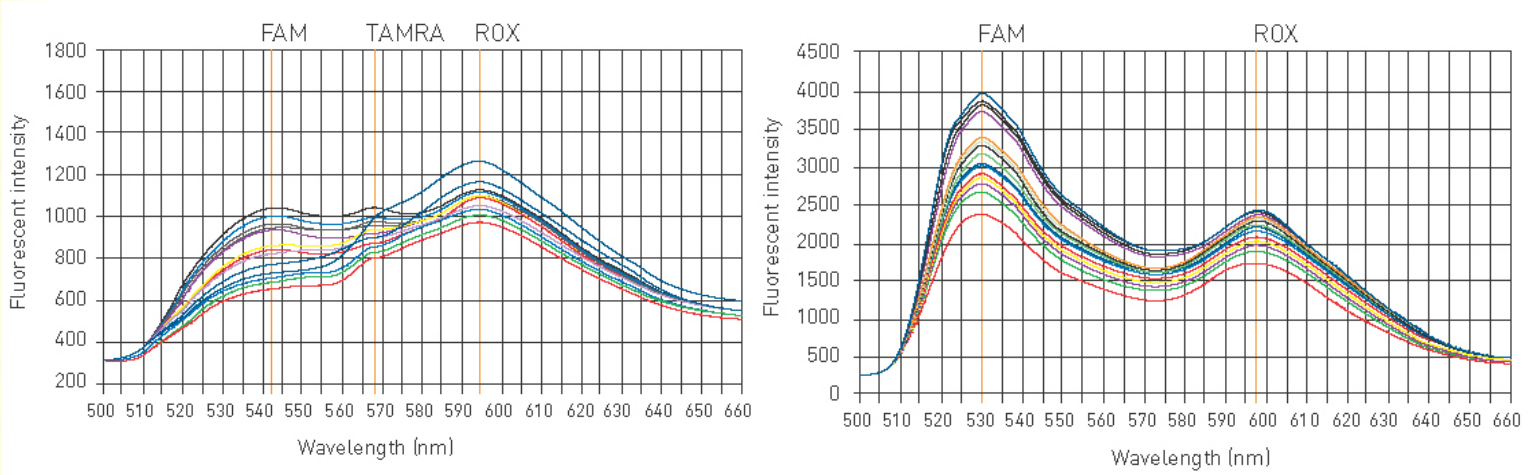
Guidelines for fluorophores and quenchers

- Quencher has to fit fluorophore



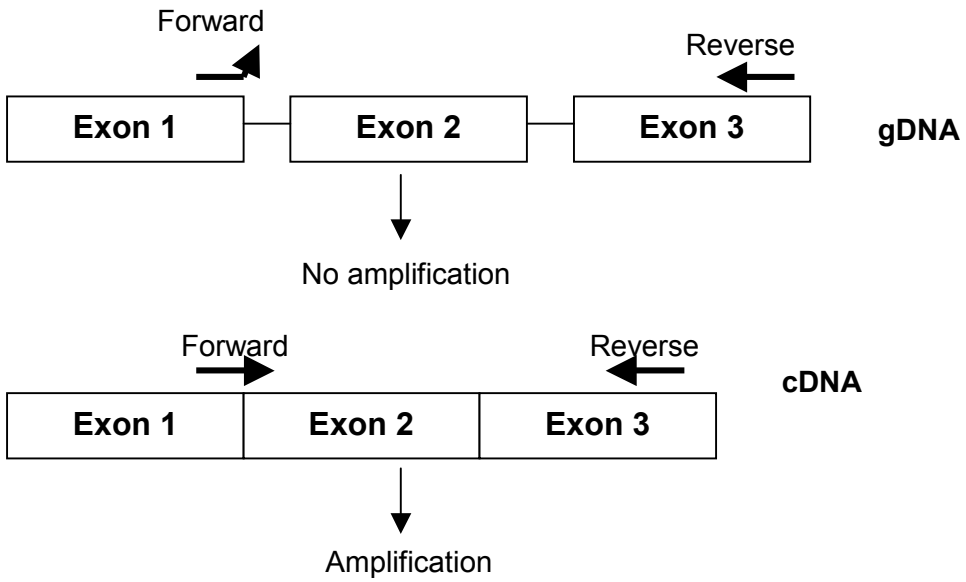
Guidelines for fluorophores and quenchers

- Singleplex or multiplex - TAMRA or DDQ1?



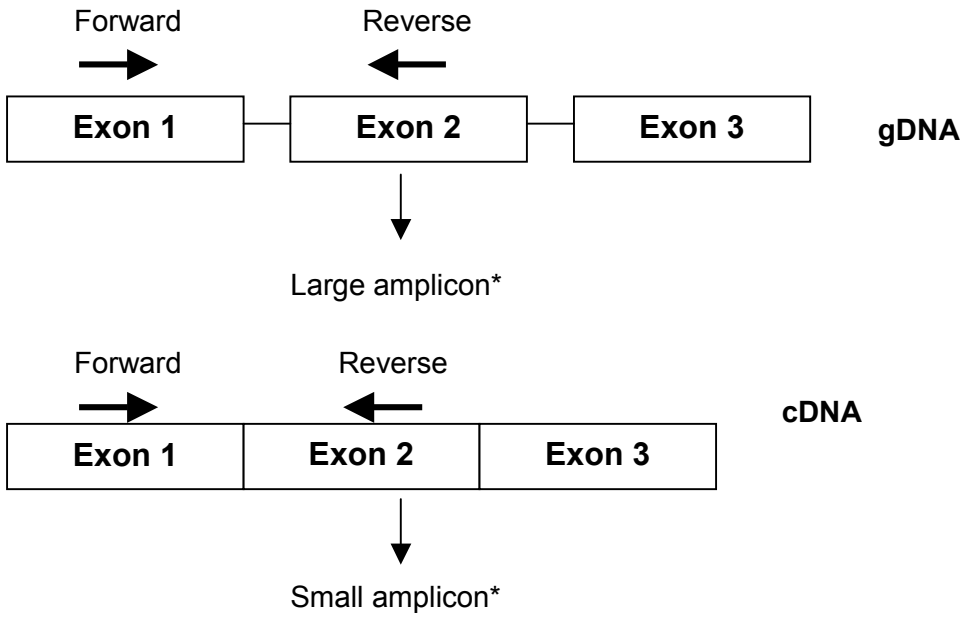
How to avoid or detect co-amplification of genomic DNA?

- Intron spanning primers

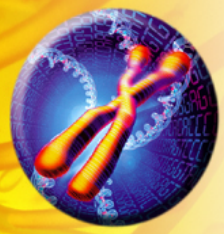


How to avoid or detect co-amplification of genomic DNA?

- Intron flanking primers



* Can be detected via melt curve



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How to avoid or detect co-amplification of genomic DNA?

- DNase I treatment of samples with RNase free DNase (Vandesompele, 2002) especially in cases of single exon genes

- Positions of exons and introns can be found in NCBI LocusLink databases (www.ncbi.nlm.nih.gov/LocusLink/)

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How to avoid co-amplification of other genes?

- Check if primers are unique and specific by submitting your primers to a BLAST search (www.ncbi.nlm.nih.gov/BLAST/)

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How to avoid primer-dimers or primer-probe dimers?

- Avoid complementarity between the primers, especially at 2 or more bases at the 3’ ends of the primers (check using a software)

- Avoid complementarity of the probe with either of the primers (check using a software)

Recommended softwares

- Design of primers and probes
  - any primer design software/Oligo® 6.0/Primer 3.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)
  - Primer Express® (TaqMan ® probes)
  - BeaconDesigner® (Molecular Beacons)
  - Scorpio (Scorpions® primers)
- Verification of design
  - Mfold
  - BLAST

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

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Additional information

- Your one-stop-shop real-time PCR supplier
- Troubleshooting guide for RT qPCR and qPCR
- Frequently asked questions for RT qPCR and qPCR
- This documentation is also available on www.eurogentec.com

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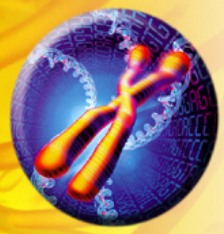

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