

## Frequently Asked Questions

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### **1 - What can be the cause that the qPCR reaction, which has been working well with specific probe system, is no longer working?**

The probe may have bleached if it has been left in the light for some time.

Although the reaction is working the fluorophore is no longer reporting the result. Dye labeled oligonucleotides should be stored in aliquots in the dark at -20°C. Freeze thawing should be avoided. No more than 5 freeze -thaw cycles are recommended.

### **2 - What can cause a very high background level when working with probes?**

Probes can be degraded separating the fluorophore from quencher, leading to a high background level of the fluorophore. When aliquoting the oligonucleotides, sterile tubes and tips must be used to avoid contamination with DNases.

### **3 - How can a high background level be avoided when working with TAMRA, ROX, CY3 or CY5 as fluorophore in a double dye oligo?**

The commonly used quenchers TAMRA, DABCYL and Methyl Red are not suitable to quench TAMRA, ROX, CY3 or CY5. For these long-wave emitting dyes ElleQuencher should be used. This quencher is available from Eurogentec

### **4 - What can cause a SYBR Green I™ reaction, which has been working well, stop working?**

SYBR Green I™ is unstable if diluted in a watery solution and also if it is not kept in the dark. Therefore Eurogentec offers kits that contain DMSO to stabilize the SYBR Green I™. Kits should be stored in the dark.

### **5 - What can cause “No Template” Controls to give a positive result?**

The master mix may be contaminated with DNA template or PCR product from a previous PCR. Clean working practices should be used to avoid DNA template contamination. To avoid contamination from previous PCRs an UNG step should be introduced providing dUTP has been used in the dNTP mix.

All Eurogentec Mastermixes contain UNG, it can be ordered separately for the Core kits.

### **6 - What can cause “No Template” Controls to give a positive result in the presence of UNG?**

There can be an excess of the probe and the positive result is an artifact of this. Using a no amplification control where the Taq polymerase is left out can assess this. A positive result will then suggest an artifact and different reagent concentrations can be used.

### **7 - How can the performance of allelic discrimination be increased?**

The probe design might be bad or the mismatch created is particularly stable. Try redesigning the primers/probe. It may be worth trying to place the primer over the mutation rather than the probe if a design is particularly difficult. A melt curve can help to indicate at which temperature the best discrimination is obtained. Eurogentec offers a design service for qPCR probes.

### **8 - How can a low signal/background ratio for Molecular Beacons be explained?**

When designing Molecular Beacons, pay attention to the folding of the probe. It might fold into alternate conformations, which are not well quenched. Change the stem or loop sequence, or both to avoid this.

If the salt concentration of the buffer is too low (below 1 mM MgCl<sub>2</sub>) the probe does also not fold correctly.

Choose Eurogentec's Molecular Beacons and we will help you with the design.

### **9 - How can a low signal/background ratio for probes be explained?**

The most likely reason is contamination by either free fluorophores or oligonucleotides that contain the fluorophore but not the quencher. The fluorophores can be removed by HPLC.

All Eurogentec probes for real time PCR are HPLC purified.

### **10 - Which fluorophores can be combined in a multiplex double dye oligo assay?**

It is dependent on the equipment which dyes can be combined. The combination that works best on most qPCR Thermalcyclers is FAM-DarkQuencher with YakimaYellow-DarkQuencher. Both are available from Eurogentec.

### **11 - Can Yakima Yellow, as fluorophore, be combined with TAMRA, as quencher, on a double dye oligo?**

As the emission spectrum of Yakima Yellow and absorption spectrum of TAMRA show little overlap it is advisable to combine Yakima Yellow with DarkQuencher, with which it shows a good overlap.

### **12 - Can a FAM-TAMRA double dye oligo be combined with a YakimaYellow-DarkQuencher double dye oligo?**

It is possible to combine a FAM-TAMRA with a YakimaYellow-DarkQuencher double dye oligo. However, most qPCR Thermalcycler correct for the background fluorescence of TAMRA, in case TAMRA is used as quencher. This means that the fluorescent signal of the FAM-TAMRA probe is measured correctly, but that the YakimaYellow-DarkQuencher signal is overcorrected and therefore gives a decrease in sensitivity.

Therefore it is recommended to use dark quenchers when performing multiplex real time PCRs.

**13 - Which fluorescent dye should I use for my target and controls?**

Mostly FAM is used for the target gene and Yakima Yellow for the control gene, when performing a multiplex real time PCR. Eurogentec does offer endogenous controls with both FAM and Yakima Yellow.

**14 - What is the difference between a Core kit and a Mastermix?**

A core kit is a 10x kit that contains all components in separate tubes, which gives a maximum in flexibility. The mastermixes are 2x kits, with all components premixed. These guarantee a high reproducibility and ease of use. Eurogentec offers both types of kits.

**15 - What is the difference between a Mastermix and a Mastermix Plus?**

The Mastermix and Mastermix Plus kits contain exactly the same components, only the packaging size is bigger. The Mastermix for SYBR Green I™ comes with a separate tube of SYBR Green I™, where this is already included in the Mastermix Plus for SYBR Green I™.

**16 - Why do qPCR kits contain dUTP (and UNG)?**

qPCR kits contain dUTP and UNG to prevent carry over from previous experiments.

The kits contain normal dNTPs, mixed with dUTPs. The dUTPs will be incorporated instead of dTTPs during the qPCR. UNG (Uracil-N-glycosylase) is an enzyme that cleaves dsDNA, containing dUTP, into small fragments. When the qPCR reaction is started with an initial incubation with UNG, no contamination through carry over from previous qPCRs will be seen, as that DNA will be degraded.

**17 - What is the advantage of working with SYBR Green I™?**

SYBR Green I™ is a inexpensive, universal dye which binds to all dsDNA. It can be easily used in combination with a simply primer pair to detect PCR products in real time. This dye is mainly very attractive for researchers analysis lots of different genes.

**18 - What is the advantage of working with a probe system?**

A probe system is always specific (except Amplifluor probes) and therefore does only detect the gene of interest. If you have a difficult to optimize PCR it will not show you any primer dimmers or unspecific products. With a probe system it is also possible to distinguish between similar sequences with small differences like Snips or mutations.

**19 - Which probe systems can be used for expression profiling?**

All probe systems can be used for expression profiling. The double dye oligos are the most user friendly as they are easy to design, optimize and use.

Eurogentec offers double dye oligos that fit each qPCR Thermocycler that does exist.

**20 - Which probe systems can be used for allelic discrimination?**

For allelic discrimination double dye oligos, molecular beacons, scorpions and hybridization probes and the most commonly used systems. The molecular beacons and scorpions have the advantage that they can be monitored at an optimal temperature, to make the distinguishing power as big as possible.

The hybridization probes can be used together with melt curves and the double dye oligos stay the easiest to use.

**21 - Why do the qPCR and RT qPCR kits contain HotGoldStar, a hot start Taq polymerase?**

Unspecific products can we seen very well in real time PCR, as the method is more sensitive than gel analysis. To avoid the amplification of unspecific products and the forming of primer dimmers during the initial heating phase of the PCR a hot start Taq polymerase is used, which becomes active after heating at 95°C for 10 minutes.

**22 - Why do the Eurogentec RT qPCR kits contain EuroScript, a MmuLV reverse transcriptase?**

In real time PCR it is recommended to use small amplicons, up till 250 bases.

Therefore it is unnecessary to use reverse transcriptases that can transcribe long fragments, as they are costly and often difficult to produce.

**23 - What is the difference between a one step and a two step RT qPCR reaction?**

In a one step RT qPCR reaction the RT reaction and the qPCR reaction are done in one and the same tube. The buffer is a combination of a RT and PCR buffer in which both enzymes do work. The one step RT qPCR reaction is a closed tube assay, so contamination can be avoided. It saves time and is easy in handling.

In a two step RT qPCR the RT reaction and the qPCR reaction are done in separate tubes. It gives a more flexible way of working in that the cDNA can be used for more than one qPCR reaction and can be archived, so that it eliminates the need to continually isolate RNA.

Eurogentec offers both one and two step RT qPCR kits.

**24 - Does Eurogentec helps design for real time probes?**

Eurogentec is a specialist in the field of real time PCR. Eurogentec offers a design service and can even do complete real time projects.

For more information contact us through [info.usa@eurogentec.com](mailto:info.usa@eurogentec.com)

**25 - How do typical cycling conditions for a double dye oligo on a 96 well real time Thermocycler look like?**

For most double dye oligos, which have been designed using the Primer Express Software, used in combination with the qPCR Mastermix the following cycling conditions can be used:

50°C 120s	UNG hydrolysis step
95°C 600s	inactivation UNG, activation HotGoldStar
95°C 15s	denaturation
60°C 60s	annealing-elongation

#### **26 - Why do certain qPCR kits contain ROX passive reference?**

All kits meant for the ABI Prism 5700, 7000, 7700 and 7900 contain a passive ROX reference as these systems require a constant ROX level to be able to calculate the fluorescent signal correctly.

#### **26 - How to optimize a qPCR assay?**

If the primers or primers and double dye oligo have been designed using Primer Express and are used in combination with the qPCR Mastermix or qPCR Mastermix for SYBR green I the following matrices can be used to define the correct primer concentration and the correct probe concentration. First define the optimum for the primers and then determine the best concentration for the probe that fits to the primer optimum.

	forward			
reverse	50 nM	300 nM	900 nM	
50nM	50/50	300/50	900/50	
300 nM	50/300	300/300	900/300	
900 nM	50/900	300/900	900/900	

	probe			
	50 nM	125 nM	250 nM	
opt. primers	50/opt	125/opt	250/opt	

#### **27 - In which concentration should I use my probes in qPCR?**

See how to optimize a qPCR assay.

#### **28 - In which concentration should I use my primers in qPCR?**

See how to optimize a qPCR assay.

#### **29 - How to select a good endogenous control?**

A good endogenous control is not up regulated by the effect that needs studying, so the condition, drug, therapy, medium etcetera. It should be expressed in the same range and there should preferably be no similar genes or pseudogenes of this gene. Cytoskeletal molecules are often a good choice.

#### **30 - Is a probe assay more sensitive than a SYBR Green ITM assay?**

A probe assay and a SYBR Green ITM assay are equally sensitive. In cases of difficult to optimize PCR's the SYBR Green I<sup>TM</sup> assay might be less beneficial as it show the total fluorescent signal of primer dimmers, unspecific product and desired product.

#### **31 - Are DNA or RNA standards preferred in qPCR?**

DNA standards are preferred when starting from genomic DNA. RNA standards are preferred when starting from RNA because then the efficiency of the RT reaction can also be measured.

#### **32 - In which case is one step RT qPCR recommended?**

A one step RT qPCR is recommended when doing HTS, where the experiment should be as easy and straight forward as possible and when doing experiments where all sources of contamination should be excluded.

#### **33 - Should DNA and qPCR Mastermix be mixed after setting up a qPCR experiment?**

Spinning the 96 well plate or tubes will most of the time do, but the results are more reproducible if the plate or tubes are mixed on a shaker.

#### **34 - Which dyes are preferably used on double dye oligos?**

The choice of the dyes is dependent on the qPCR Thermocycler used. Eurogentec recommends to take dyes with as few spectral overlap as possible when doing multiplex qPCR, so for example FAM-DarkQuencher and YakimaYellow-DarkQuencher. In single plex FAM-TAMRA will be the best choice.

#### **35 - Should anything be changed in the software when analyzing double dye oligos with quenchers other than TAMRA?**

When analyzing fluorescent signals from double dye oligos labeled dark quenchers the background correction for TAMRA should be turned off.

**36 - Can genomic DNA be amplified in expression profiling when using the endogenous control kits?**

The primers that are used in Eurogentec's endogenous control kits are exon-exon spanning and can only amplify genes from cDNA and not from genomic DNA.

**37 - In which buffer should I resuspend qPCR probes?**

As fluorophores are sensitive to hydrolysis, Eurogentec recommends resuspending qPCR probes in TE instead of just water. If the qPCR probes are dissolved in an acid solution the fluorophores can hydrolyze and will give a low fluorescence signal.

**38 - How should I store qPCR probes?**

After dissolving qPCR probes they should be aliquoted stored at -20°C in the dark and undergo not more than 5 freeze-thaw cycles.

**39 - What is UNG and how does it work?**

UNG stands for Uracil-N-glycosylase. It is an enzyme, which hydrolyses uracil-glycosidic bonds in DNA containing dUTP, therefore degrading the DNA into small fragments. In this way contamination from previous qPCR reactions can be avoided.

**40 - How many reactions can be done with one batch of double dye oligo?**

One batch of double dye oligo, produced in 0,2 mmol scale, will give a final yield of minimal 10 nmol. This is on average sufficient for 2000 qPCR reactions of 50 ml.

**41 - Is there a difference in PCR efficiency when using dUTP instead of dTTP?**

dUTP is not naturally present in DNA and therefore not preferred by Taq polymerase. The Taq polymerase will incorporate the dUTP, but not with the same efficiency as normal nucleotides. Therefore the efficiency of the reaction is reduced compared to a reaction in which dATP, dCTP, dGTP and dTTP are used.

Eurogentec offers kits that contain no dUTPs. However, these kits have to be used very carefully, as there will be no possibility to digest the PCR products with UNG.

**42 - Why does the one step RT qPCR Mastermixes not contain UNG?**

In the one step RT qPCR Mastermix RNA is transcribed into cDNA. dUTPs are incorporated into the cDNA during this process at 48°C. Because UNG works optimal at 52°C it will immediately destroy the cDNA again. It is not possible to do the UNG reaction before the RT reaction, as the UNG is heat stable and will not lose all its activity after 10 minutes inactivation at 95°C. Therefore the one step RT qPCR Mastermixes do not contain UNG. They do, however, contain dUTP to avoid contamination of future qPCR reactions.

**43 - What is the difference between endpoint analysis and real time analysis?**

qPCR can be done both as endpoint analysis and real time analysis. In endpoint analysis the fluorescence is measured when the PCR is complete. In real time analysis the fluorescence is measured in each cycle of the PCR. It is only possible to quantify in real time, as the final yield of PCR products does not stand in direct relation to the initial amount of DNA, due to limiting factors at the end of the PCR. Therefore endpoint analysis is often used for allelic discrimination and real time analysis for quantification.

**44 - Can YakimaYellow used with VIC settings?**

As YakimaYellow and VIC are very similar molecules it is no problem to use YakimaYellow double dye oligos with VIC settings. As YakimaYellow is normally combined with DarkQuencher, the background correction for TAMRA should be turned off.

**45 - How should the qPCR and RT qPCR kits be stored?**

All qPCR kits should be stored at -20°C and are guaranteed stable for 1 year. Mastermixes can be stored for 1 month at 4°C. One step RT qPCR kits should be stored at -20°C and are guaranteed stable for ½ year. Two step RT qPCR kits should be stored preferably at -70°C and are guaranteed stable for ½ year.

**46 - What is absolute quantitation?**

Absolute quantitation is a method in which unknown samples are quantitated by comparing them to a standard curve.

**47 - What is relative quantitation?**

Relative quantitation is a method in which unknown samples are compared to reference samples to determine the increase or decrease of gene expression.

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