

Frequently Asked Questions qPCR kits & RT qPCR kits

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1. General information on Real-Time PCR kits

What is the advantage of working with SYBR® green I?

SYBR® green I is an inexpensive, universal dye which binds to all dsDNA. It can be easily used in combination with a simple primer pair to detect PCR products in Real-Time. This dye is mainly very attractive for researchers analysis lots of different genes. However it is important to do a good primer design to avoid primer dimers, which will also be detected by SYBR® green I.

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 optimise PCR it will not show you any primer dimers or aspecific products.

With a probe system it is also possible to distinguish between similar sequences with small differences like SNPs or mutations.

In general, probe assays need less optimisation than SYBR® green I assays.

Is a probe assay more sensitive than a SYBR® green I assay?

A probe assay and a SYBR® green I assay can be equally sensitive. In cases of difficult to optimize PCRs the SYBR® green I assay might be less beneficial as it shows the total fluorescent signal of primer dimers, aspecific product and wanted product.

Can I use a SYBR® green I kit for a probe assay?

This is not possible because the buffer for SYBR® green I kits and the buffer for probe kits are different. The kits for SYBR® green I applications contain special stabilizers to prevent the SYBR® green I from degradation and do differ in pH and salt concentration.

How should the qPCR and RT qPCR kits be stored?

All qPCR kits should be stored at -20°C and are guaranteed stable for 2 years. 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 $\frac{1}{2}$ year. Two step RT qPCR kits should be stored preferably at -70°C and are guaranteed stable for $\frac{1}{2}$ year.

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. In average Core kits give a bit more sensitive results

Eurogentec offers both types of kits.

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 as it is already prediluted in the Mastermix Plus for SYBR® green I.

Should the DNA and the qPCR Mastermix be mixed before thermocycling?

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 magnetic shaker.

What is the difference between an 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 a 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 pipeting steps and 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.

Further more it allows the use of oligo d(T) and random nonamer primers in the RT step and specific primers in the PCR step. This will increase the specificity and sensitivity of the assay.

Eurogentec offers both one and two step RT qPCR kits.

In which case is a one step RT qPCR recommended?

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

Why is a Two step RT qPCR kit more efficient than a One step RT qPCR kit?

When performing a RT it is possible to use three different primer types:

- Oligo d(T) primers, which bind to the poly A tail of the RNA and then only transcribe RNA. This will avoid contamination with genomic DNA. As the poly A tail is located at the beginning of the gene it will also lead to more full transcripts.
- Random nonamers, which bind anywhere in the genome and allow the reverse transcriptase to fill up the gaps, will leads to high yields.
- Specific primers, which bind to the gene of interest, and will therefore give specific products.

The combination of oligo d(T) primers and random nonamers will give the highest yields and the longest transcripts, whereas specific primers transcribe only specific RNA but reduce the yield.

With an One step RT qPCR kit it is only possible to add specific primers, as it should be avoided that oligo d(T) primers and random nonamers participate in the PCR reaction, giving many aspecific products. As a RT reaction is performed at 40-50°C, the primers can bind with mismatches to the RNA and therefore transcribe unwanted sequences, which then also will be amplified in consecutive PCR, leading to aspecific PCR products. This disadvantage is inherent to the method.

In a Two step kit the oligo d(T) primers and the random nonamers are included in the kit and will give ride to cDNA. Then two specific primers have to be selected and this will amplify the exact sequence.

Why do the qPCR and RT qPCR kits contain HotGoldStar, a hotstart *Taq* polymerase?

Aspecific products influence the results of a Real-Time PCR in a negative way leading to wrong quantitation. To avoid the amplification of aspecific products and accumulation of primer dimers during the initial heating phase of the PCR a hotstart *Taq* polymerase is used, which only becomes active after heating at 95°C for 10 minutes.

Why do the Eurogentec RT qPCR kits contain EuroScript, a MmLV reverse transcriptase?

In real time PCR it is recommended to use small amplicons, without secondary structures.

Therefore it is unnecessary to use reverse transcriptases that can transcribe long fragments, or can lead to over secondary structures, which are in general costly. Furthermore is MMLV Reverse Transcriptase an enzyme, which gives high yields and longer transcripts, which hen again leads to more sensitive PCRs, as more full copies of cDNA are available?

Why do qPCR kits contain dUTP (and UNG)?

qPCR kits contain dUTP and UNG to prevent carry over from previous qPCRs. 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, which hydrolyzes the release of free uracil from uracil-containing DNA. 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, so it cannot participate anymore in the PCR process.

Why does an initial step at 50°C during 2 min and a second step at 95°C during 10 min have to be performed when using UNG for carry over prevention?

When performing an assay with UNG, a first step at 50°C during 2min and a second step at 95°C during 10min has to be added before performing a normal qPCR:

50°C during 2min
95°C during 10min

94°C during 15s
55°C-65°C during 30s
72°C during 60s
repeat during 35 cycles

Hold at 50°C for ever

The fist step is needed to activate the UNG enzyme, to allow it to degrade U containing ds DNA.

The second step at 95°C will deactivate the UNG and will activate the HotGoldStar.

Why do 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 newly transcribed cDNA. 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.

Why do certain qPCR kits contain the ROX passive reference?

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

Can an “ABI” kit with a ROX passive reference be used on a iCycler iQ?

A kit with a ROX passive reference can be used on a iCycler iQ. It will perform as well as a kit without a ROX passive reference. But, it will block the channel, in which ROX and Texas Red can be detected.

What should I do to use a ROX or a No ROX qPCR Core kit or MasterMix for SYBR® green I correctly on a iCycler iQ?

To enable a correct determination of the well factor, fluorescein additive should be added to the kits, when setting up the reactions.

How do I avoid amplification of genomic DNA, which can be a contamination factor in my isolated RNA in RT qPCR?

By designing exon-exon spanning primers you will only amplify cDNA, as the primers do not bind to gDNA. In this way your amount of PCR products is directly related to the initial amount of cDNA, which is again directly related to the initial amount of RNA.

2. Theory behind Real-Time PCR

What can cause a qPCR reaction, which has been working well with specific probe system, to stop 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. The probe may have degraded. Although the PCR is working, the probe no longer binds to the PCR products.

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.

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.

How to optimize a qPCR assay?

If the 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

In which concentration should I use my probes in qPCR?

See how to optimize a qPCR assay.

In which concentration should I use my primers in qPCR?

See how to optimize a qPCR assay.

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.

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.

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 the *Taq* polymerase. The *Taq* polymerase will incorporate dUTP, but not with the same efficiency as normal nucleotides. Therefore the efficiency of the reaction is reduced compared to a reaction in which on 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.

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 and enzymes are often a good choice.

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 (ΔR_n) 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 is not proportional 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 is used for quantification.

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. An additional detection step can be programmed in the PCR protocol.

Eurogentec offers a design service for qPCR probes.

What is absolute quantitation?

Absolute quantitation is a method in which unknown samples are quantitated by comparing them to a standard curve, based on absolute amounts of DNA.

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. The out come is presented as an index, not an absolute amount.

3. Probes...

In which buffer should qPCR probes be resuspended?

As fluorophores are sensitive to hydrolysis, Eurogentec recommends resuspending qPCR probes in TE 0.01 instead of just water. If the qPCR probes are desolved in an acidic solution the fluorophores can hydrolyze and will give a lower signal to noise ratio.

How should qPCR probes be stored?

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

How many reactions can be done with one synthesis of Double-Dye Oligo?

One batch of Double-Dye Oligo, produced in 0,2 μmol scale, will give a final yield of minimal 10 nmol. This is on average sufficient for 2000 qPCR reactions of 50 μl .

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.

How can a high background level be avoided when working with TAMRA, ROX, CY3 or CY5 as fluorophore using 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.

How can a low signal to noise 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, mass spectrometry controlled and HPLC controlled.

How can a low signal to noise 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_2) the probe does also not fold correctly.

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

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, require minimal optimisation and are easy to use.

Eurogentec offers Double-Dye Oligos that fit each qPCR cyclers.

Which probe systems can be used for allelic discrimination?

For allelic discrimination Double-Dye Oligos, Molecular Beacons, Scorpions® and hybridisation 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 hybridisation probes can be used together with melt curves and the Double-Dye Oligos are the easiest to use.

How do typical cycling conditions for a Real-Time PCR using 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	} X 40
60°C 60s	annealing-elongation	

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

In most cases 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™.

Which dyes are preferably used on Double-Dye Oligos?

The choice of the dyes is dependent on the Real-Time thermocycler used. Eurogentec recommends to take dyes with as few spectral overlap as possible when doing multiplex qPCR, so for example FAM-Eclipse® Dark Quencher and YakimaYellow™-Eclipse® Dark Quencher. In single PCR FAM-TAMRA will be the best choice.

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 cyclers is FAM-Eclipse® Dark Quencher with Yakima Yellow™-Eclipse® Dark Quencher. Both are available from Eurogentec.

Can a FAM-TAMRA Double-Dye Oligo be combined with a Yakima Yellow™-Eclipse® Dark Quencher Double-Dye Oligo?

It is possible to combine a FAM-TAMRA with a Yakima Yellow™- Eclipse® Dark Quencher Double-Dye Oligo. However, most Real-Time thermocycler correct for the background fluorescence of TAMRA, incase TAMRA is used as quencher. This means that the fluorescent signal of the FAM-TAMRA probe is measured correctly, but that the Yakima Yellow™-Eclipse® Dark Quencher signal is overcorrected and therefore gives to a lower sensitivity.

Therefore it is recommended to use dark quenchers when performing multiplex Real Time PCRs, so lost of sensitivity can be reduced to a minimum.

Can Yakima Yellow™, as fluorophore, be combined with TAMRA, as quencher, on a Double-Dye Oligo?

Yakima Yellow™ cannot be combined with TAMRA, as these two molecules will lead to incompatible synthesis steps.

Can Yakima Yellow™ be used with VIC settings?

As Yakima Yellow™ and VIC are very similar molecules. Yakima Yellow™ Double-Dye Oligos can be detected with VIC settings. As Yakima Yellow™ is normally combined with Eclipse® Dark Quencher, the background correction for TAMRA should be turned off (in plate set up window).

Should anything be changed in the software when analyzing Double-Dye Oligos with quenchers other than TAMRA?

When analysing fluorescent signals from Double-Dye Oligos labeled dark quenchers the background correction for TAMRA should be turned off (in plate set up window).

Does Eurogentec do 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@eurogentec.com

4. No template control is positive

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.

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.

How can negative control be positive when detecting *E. Coli* sequences?

- 1- If you are detecting bacterial sequences other than *E. Coli*, the primers will have to exclude any part of the DNA that is shared between *E. Coli* and the bacteria used (in other words, the primers have to be designed only in the part which is unique for the bacteria used).
- 2- If detecting *E. Coli* sequences, There are some traces of bacterial DNA in the *Taq* polymerase and in the UNG (as it is produced in *E. Coli*). You will have to determine the minimum level and subtract it from the positive signal. Everything above this signal should be considered as positive.