YOUR ONE-STOP-SHOP
REAL-TIME PCR SUPPLIER

EUROGENTEC

EQT GROUP

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Introduction

Real-Time PCR is a method that has been introduced relatively recently. The technology combines DNA amplification with detection of the products in a single tube. The homogeneous format is highly beneficial as it removes the significant contamination risk caused by opening tubes for post-PCR manipulation. It is also less time consuming than gel based analysis and can supply a quantitative result.

Current detection methods are based on changes in fluorescence proportional to the increase in product, whether specific or non-specific. Fluorescence is monitored during each PCR cycle to provide an amplification plot, allowing the user to follow the reaction (Figure 1.).

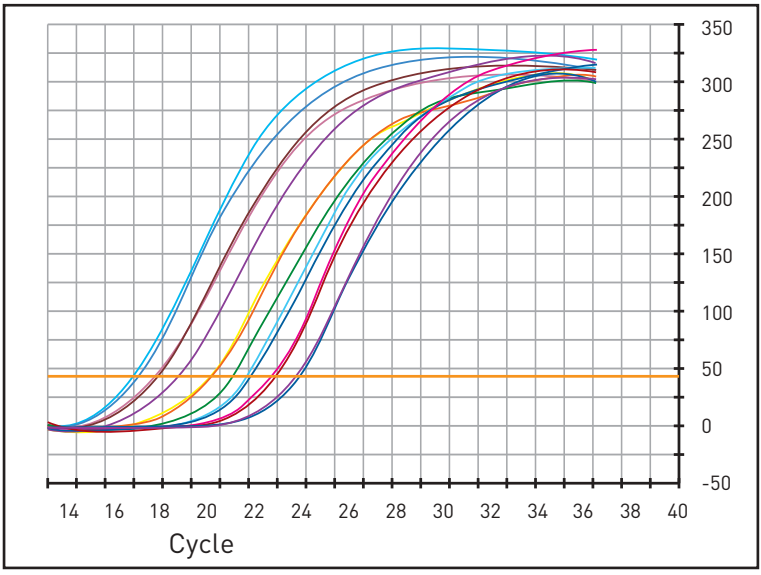

Figure 1. Amplification plot of 2 x serial diluted cDNA derived from primary fibroblasts using the 18S rRNA Control Kit Yakima Yellow®.

There are now a number of Real-Time PCR thermocyclers designed to carry out Real-Time reactions and analyze the results. With the introduction of easy-to-use reagent kits Real-Time PCR has become an attractive option for applications such as expression profiling (quantitative PCR) and allelic discrimination. This document aims at providing a comprehensive, simple guide to Real-Time PCR, covering the different technologies, applications, designs and protocols. There are a number of decisions to be made once the initial decision to use Real-Time PCR has been made for a particular application. First, there is the choice of the instrument and the technology. Many Real-Time thermocyclers are capable of running most of the current technologies but care should be taken when matching the machine with technology.

Instrumentation

There are currently several Real-Time PCR thermocyclers on the market. They can be classified in two categories in function of the needs of the user: high-throughput instruments and flexible instruments.
The high-throughput instruments, which have 96 or 384-well plate formats, are dedicated for laboratories running large batches of samples and few different parameters.
The flexible instruments are more dedicated to experiments with smaller batches of samples but are faster and present more flexibility, allowing the user to run different parameters each time.

High-throughput systems

GeneAmp® SDS 5700

The GeneAmp® Sequence Detection System 5700 is a system for Real-Time PCR, which includes a GeneAmp® 9600 thermal cycler combined with precision optics. It is a 96-well plate format instrument, where excitation is done via a halogen lamp. It has been optimized to detected dyes such as SYBR® green I and FAM. The experiment is completed within 2 hours. The machine was one of the first out on the market, but has been discontinued in the mean time and has been replaced by the cost-effective multichannel ABI Prism® 7000.
Fore more detailed information: http://home.appliedbiosystems.com

ABI Prism® SDS 7000

The ABI Prism® SDS 7000 is a 96-well plate format instrument; all the wells are illuminated by a tungsten – halogen lamp. There are 4 optical filters, which are optimized for the use of FAM or SYBR® green I, VIC or Yakima Yellow®, TAMRA and ROX dyes that enable multiplexing. A complete experiment can be run in 2 hours, a dissociation curve can be displayed and the results are displayed during the run of the experiment.
Fore more detailed information: http://home.appliedbiosystems.com

ABI Prism® SDS 7700

The ABI Prism® SDS 7700 is a 96 well-plate format system with multiplexing capabilities. Excitation of the fluorescent dyes is done through a laser. Fluorophores with an emission between 500 and 660 nm can be detected on these platforms. The standard set-up is such that the common dyes like FAM, TET, JOE, Yakima Yellow® or VIC, ROX, TAMRA and SYBR® green I can be used. A typical experiment takes about 2 hours.
The ABI Prism® SDS 7700 has been discontinued and is replaced by the ABI Prism® SDS 7900 HT.
Fore more detailed information: http://home.appliedbiosystems.com

ABI Prism® SDS 7900 HT

The ABI Prism® SDS 7900 HT is available as either a 96 or a 384-well plate format system for high-throughput applications. The ABI Prism® 7900 HT is sold with a robotic plate loading system. Excitation of the fluorescent dyes is done through a laser. Fluorophores with an emission between 500 and 660 nm can be detected on these platforms. The standard set-up is such that the common dyes like FAM, TET, JOE, Yakima Yellow® or VIC, ROX, TAMRA and SYBR® green I can be used. A typical experiment takes about 2 hours.
Fore more detailed information: http://home.appliedbiosystems.com

ABI 7300 and 7500 Real-Time PCR Systems

The ABI 7300 and 7500 Real-Time PCR System machines have been recently introduced to the market. The software of these third-generation Real-Time thermocyclers has been improved. Both platforms are 96-well plate formats and use a tungsten halogen lamp for excitation. Both machines can run an experiment with samples of 25-100 µl in 2 hours. The ABI 7300 has been optimized for the use of FAM or SYBR® green I, VIC or Yakima Yellow® or JOE, TAMRA and ROX. The ABI 7500 provides 5 colour detection and supports a broader range of fluorophores like FAM or SYBR® green I, VIC or Yakima Yellow® or JOE, NED or TAMRA or Cy® 3, ROX or Texas Red® and Cy® 5. The ABI 7500 allows a better sensitivity with longer wavelength dyes.
Fore more detailed information: http://home.appliedbiosystems.com

iCycler iQ®

The iCycler iQ® is a 96-well PCR machine, which is a standard PCR machine with an additional optical unit. The unit uses a CCD camera to measure emission from each well simultaneously and can detect 4 different dyes at once being FAM or SYBR® green I, VIC or Yakima Yellow® or HEX or TET or Cy® 3, TAMRA, ROX or Texas Red® and Cy® 5. A halogen lightsource illuminates the samples. Experiments can be run in 2 hours.
Fore more detailed information: http://www.bio-rad.com

Mx3000p® Multiplex quantitative PCR system

The Mx3000p® Multiplex quantitative PCR systems is a low cost personal thermocycler. This machine can also detect four fluorophores within the same reaction using four different channels (customized filters). It is a 96-well plate format, which is illuminated by a halogen lamp, which excites in the range of 350-750 nm. The detection is done by a scanning photomulitplier tube (PMT). The light is red through selected filters, which permit detecting the following dyes: FAM or SYBR® green I, TET, HEX or JOE or VIC or Yakima Yellow®, TAMRA or Cy® 3, Texas Red® or ROX and Cy® 5 or Alexa Fluor® 350.
The extended excitation range offers good multiplexing possibilities in which 4 targets can be detected at the same time. Experiments can be run in 2 hours.
Fore more detailed information: http://www.stratagene.com/

Mx4000®

The Mx4000® Multiplex Quantitative PCR system uses a halogen lamp for excitation of dyes with excitation wavelengths between 350-750 nm. It can detect 4 fluorophores (350 nm-830 nm) in one reaction. Different excitation and emission filter sets are available for the detection of FAM or SYBR® green I, TET, HEX or JOE or VIC or Yakima Yellow®, TAMRA or Cy® 3, Texas Red® or ROX and Cy® 5. These filter sets can be customized for the end user. This machine is a 96-well format.
Fore more detailed information: http://www.stratagene.com

DNA Engine Opticon®

The DNA Engine Opticon® is a Real-Time system based on a PTC 200 thermal cycler. It combines a 96-well DNA engine and an optical system, the Opticon fluorescence detector. An array of 96 blue LEDs illuminates the cycler wells one at a time, and a photomultiplier tube detects fluorescence.
The DNA Engine Opticon® excites fluorescent dyes with absorption spectra in the 450 to 495 nm range. The system is optimized for dyes with emission spectra in the 515 to 545 nm range as SYBR® green I and FAM, which only enables singleplex reactions. An experiment can be run in 2 hours.
Fore more detailed information: http://www.mjr.com

DNA Engine Opticon® 2

DNA Engine Opticon® 2 is a 96-well plate thermocycler, which has a multicolour capacity. The 96-wells are illuminated by an array of 96 LEDs for excitation in a range of 470-505 nm and for sensitive detection. The fluorescence detection is done in two different channels; in channel 1 the wavelength range detected is 523-543 and in channel 2 it is 540-700 nm. This permits detecting in channel 1 SYBR® green I or FAM and in channel 2 TET, HEX, VIC / Yakima Yellow® or TAMRA, which enable to do duplex detection. The machine allows either quantitative or allelic discrimination; it is also a flexible machine as it is possible using 12 different temperatures to optimize reactions in a single run.
Fore more detailed information: http://www.mjr.com

Chromo 4 Real-Time detector

The Chromo 4 is a 96-well plate machine that can be adapted to any DNA Engine thermal cycler; it is fully swappable with all user-changeable sample blocks available. The instrument has a four-colour capability, which allows multiplexing. It also has swappable detection modules (with LEDs) that can be ordered with customized filter sets. The 96-wells are independently excited and detected to minimize cross talk. It is a four-channel detection instrument; in channel 1 wavelengths between 515 and 530 nm are detected, in channel 2 between 560 and 580 nm, in channel 3 between 610 and 650 nm and in channel 4 between 675 and 730 nm. Different reaction volumes can be used (10-100 µl), with a recommended reaction volume of 20 µl.
Fore more detailed information: http://www.mjr.com

Quantica® Real-Time nucleic acid detection system

The Quantica® is a 96-well plate machine, which has recently been launched on the market. The halogen white light source and PMT detector gives an excitation range between 470 and 650 nm and a detection range between 500 and 710 nm. It is possible to work either with a single or a dual channel instrument, which enables multiplexing with the possibility of using different chemistries. It is a fast instrument with ramp rates up to 3 °C / second.
For further information: http://www.techne.com

InSite Real-Time Nucleic Acid Detection/Analysis System

The InSite is a 96-well plate format using reaction volumes between 10 and 50 µl. The machine can be used with every standard chemistry and dye. The ECP technology (Patented Electrically Conducting Polymer) that the machine is using permits to heat the vessel directly; each vessel has an infrared sensor to ensure independent thermal control. The machine emits light with a wavelength between 520 and 720 nm using a multichannel laser collector. It is ultra rapid cycling, which enables to run 30 cycles in 20 minutes.
For further information: http://www.biogene.com

Flexible instruments

LightCycler®

Another popular machine is the LightCycler®. This is very similar to the Idaho Rapid Cycler, from which it was developed. Rather than using plastic tubes and plates this machine uses glass capillaries. The LightCycler® is extremely fast, with a ramp rate of 20 °C per second, but is only capable of doing 32 samples in one run. Dye excitation is from a single LED at 470 nm but it is only capable of detecting at 530 nm (FAM or SYBR® green I), 640 nm and 710 nm. There are very few dyes that are excited at 470 nm and emit at 640 or particularly 710 nm. This causes a problem for multiplexing probes. The machine was originally set up for the Roche technology, hybridization probes, which use FRET (Fluorescence resonance energy transfer-Ju *et al.* 1995) to transfer energy from FAM to the Roche dyes LC red 640 and LC red 705. This machine is suitable for fast cycling using a FAM Double-Dye Oligonucleotide probe or SYBR® green I. There is a novel Scorpions® and Molecular Beacons design that also allows detection of ROX in channel 2 (640 nm).
Fore more detailed information: http://biochem.roche.com

LightCycler® 2.0 Instrument

The new LightCycler® 2.0 Instrument has been specially designed to enable multiplex assays. The 6 detection channels provide detection of the following wavelength: 530, 560, 610, 640, 670, and 710 nm. This enables to detect dyes such as SYBR® green I / FAM / Fluorescein, HEX / VIC or Yakima Yellow®, LC Red 610, LC red 640, LC Red 670 and LC red 705. A new software is available with the new machine, which permits to design 4 primers and probes at the same time. It is a 32-glass capillary system but it is now also possible to run either 20 or 100 µl reactions.
Fore more detailed information: http://biochem.roche.com

Smartcycler®

The Smartcycler® system is a highly versatile and efficient thermal cycler with Real-Time optical detection, specifically tailored to the rapidly evolving needs of today's molecular biology laboratory.
The core of the smart cycler system is a I-Core® (Intelligent Cooling / Heating Optical reaction) module, incorporating state-of-the-art microfluidic and microelectronic design. Each Smartcycler® processing block contains sixteen independently programmable I-Core® modules, each of which performs four-colour Real-Time fluorometric detection. Samples are amplified and measured in the Smartcycler® proprietary sealable reaction tubes, which are designed to optimize rapid thermal transfer and optical sensitivity. Six blocks can be linked together to give a high throughput machine.
The Smartcycler® software enables single or multiple operators to define and simultaneously carry out any number of separate experiments, each with a unique set of cycling protocols, threshold criteria, and data analysis.
In addition, thermal and optical data from each and all sites can be monitored in Real-Time and graphs of temperature, growth curves, and melt curves can be charted as the data are collected. This will increase considerably the throughput of the system, and will be ideal for optimization of different cycling conditions. It is an open system being able to run all existing chemistries as SYBR® green I, Double-Dye oligonucleotides, Molecular Beacons, Scorpions® primers and FRET technology. The machine enables the use of 16 tubes at the same times, and four colours can be detected – FAM / TET / TAMRA / ROX. Experiments can be run in 0.5 hour.
Fore more detailed information: http://www.cepheid.com

Smartcycler® II system

The Smartcycler® II is built with 16 modular I-Core®, which all maintain a specific temperature. The new machine has four optical channels, which enable the use of different dyes compared to the previous one. The instrument allows the use of different techniques such as intercalating dyes like SYBR® green I, Molecular Beacons, Double-Dye Oligonucleotide probes, Amplifluor® and Scorpions® primers. The machine displays the graphs in real time and enables the use of two different reaction volumes (25 µl and 100 µl).
The new software enables more operators to define and carry out in the mean time different separate experiments. Moreover, thermal growth and meltcurve can be performed during data collection. It allows the detection in four different channels in the same time of wavelength in between 510 nm and 750 nm. The dyes detected are FAM or SYBR® green I in channel 1, Cy® 3 or TET in channel 2, ROX or Texas Red® in channel 3 and Cy® 5 in channel 4, which enable multiplex assays.
The machine uses a precise heating and cooling and has a fast thermal ramp rate, which permits a run of 0.5 hour. It is also possible to run different protocols simultaneously in each of the 16 tubes.
Fore more detailed information: http://www.cepheid.com

RotorGene 2000

The RotorGene 2000 combines a Real-Time PCR machine with a centrifuge. The samples are held in a rotor, which spins at high speed and eliminates all sample-to-sample variation in temperature. The rotor takes 32 samples. There are 2 excitation sources, a LED emitting at 470 nm and one emitting at 530 nm. It has 2 detection channels at 510 and 550 nm and there is no spectral overlap between the channels. This machine can detect SYBR® green I, FAM, TET, JOE and VIC or Yakima Yellow®.
Fore more detailed information: http://www.corbettresearch.com

RotorGene 3000 Four Channel Multiplexing System

The RotorGene 3000 multi filter system can be used for the detection of either 32 or 72 samples (two rotors are available) and can detect all available Real-Time chemistries including SYBR® green I, Double-Dye Oligonucleotide probes, FRET probes and Molecular Beacons...
The fluorescence of up to 4 different probes can be detected in a single tube; in channel 1 the detection of fluorescence can be done at 510 nm, in channel 2 at 555 nm, in channel 3 at 610 nm and in channel 4 at 660 nm. This permits the detection of the following fluorophores, FAM, TET, JOE, VIC or Yakima Yellow®, Max, ROX, Cy® 3, Cy® 5, Texas Red®.
The instrument uses a heat cooler, which permits to run an experiment within 1 hour.
Fore more detailed information: http://www.corbettresearch.com

Quantitative and qualitative PCR technologies

Introduction

To understand how technologies work it is first necessary to understand PCR (figure 2). In brief, this technology was first developed in 1983 to amplify fragments of DNA. It uses the ability of a thermo-stable DNA polymerase enzyme (*Taq* polymerase) to extend short single-stranded synthetic oligonucleotides (primers) during repeated cycles of heat denaturation, primer annealing and primer extension. The primers are designed to bind the DNA fragment to be amplified. The *Taq* polymerase uses the target DNA added to the reaction as a template for primer extension. At each cycle, more DNA is synthesized, providing additional template. The reaction proceeds in an exponential manner, doubling the amount of target each cycle, until one of the reagents becomes limiting and the reaction reaches a plateau.

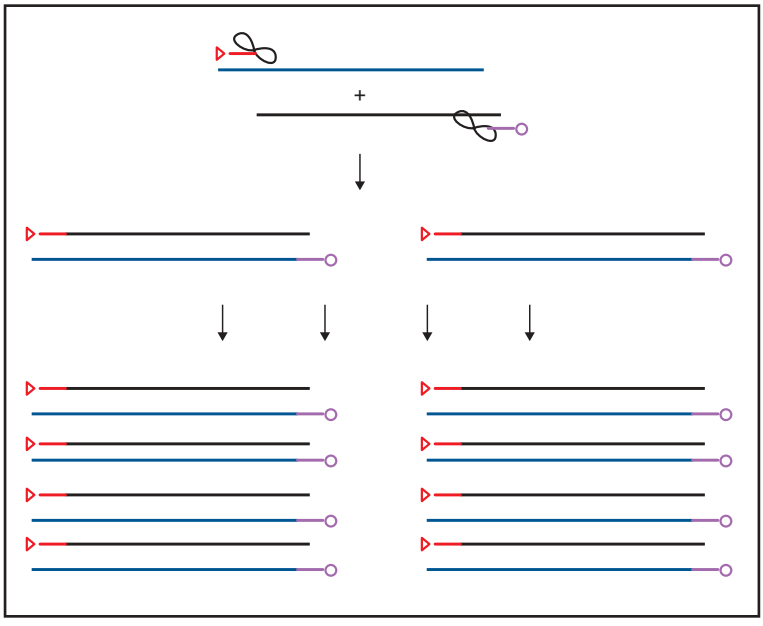

Figure 2. The Polymerase Chain Reaction. (K. Mullis and F. Fabona, 1987). The red and pink lines represent the primers that the polymerase extends from. The blue and black lines represent the single-stranded DNA template produced from denaturation of the double-stranded DNA template.

There are 2 types of DNA detection chemistries that are used in Real-Time PCR. Specific sequence detection distinguishes between the sequence of interest and primer dimers or non-specific amplification. It can also be used to detect different alleles. Non-specific detection detects all double-stranded DNA produced during the reaction.

Non-specific detection systems

The standard method for non-specific detection is a double-stranded DNA intercalating dye that fluoresces once bound to the DNA. The most commonly used dye is SYBR® green I (http://www.probes.com/). This dye is excited at 497 nm and emits at 520 nm. The different products detected by SYBR® green I can be observed by doing a melt curve at the end of the reaction. The reaction is heated slowly from 40 °C to 95 °C whilst continuously monitoring the fluorescence. The point at which the double-stranded DNA melts is observed as a drop in fluorescence as the SYBR® green I dissociates. Different length of products and products of different sequences will melt at different temperatures and will be observed as distinct peaks when plotting the first negative derivative of fluorescence vs temperature (many machines will do this calculation in the analysis software). If the PCR reaction is fully optimized it is possible to produce a melting peak profile that contains only a single peak that represents the specific product expected from the primer pair. If this can be obtained, SYBR® green I can be useful for quantification. SYBR® green I can often be useful for optimizing a PCR reaction and checking that the primers are working well before moving on to use one of the specific methods. One of the advantages of using such a dye is that several target genes can be analyzed simultaneously without the need to synthesize expensive double labeled probes. However, with SYBR® green I assays it is of the utmost importance to do a very good primer design to make sure that only specific products are obtained and primer dimers are avoided. Only in this way an accurate quantitation can be done. An alternative to SYBR® green I is SYBR® Gold (http://www.probes.com/). The spectrum of this dye is slightly shifted compared with SYBR® green I, with the excitation peak at 495 nm and the emission peak at 537 nm. As most machines are set up for SYBR® green I the SYBR® Gold emission peak will be missed by the machines unless otherwise calibrated. The advantage of using SYBR® Gold is that it is far more stable than SYBR® green I, which degrades very quickly.

Specific detection systems

There are a number of specific detection systems that use probes. These probes can be labeled with a wide range of dyes such as those mentioned in the instrumentation section as well as others. Their different excitation and emission spectra allow the dyes to be distinguished from one another. Alternative dyes that improve signal and make probe synthesis more efficient have been developed (www.epochbio.com). Below are listed some of the most popular methods currently used.

TaqMan® probes

TaqMan® probes also called Double-Dye Oligonucleotide or dual labeled probes, are the most widely used type of probes and are often the method of choice for scientists who have just started using Real-Time PCR. They were developed by Roche (Basel, Switzerland) and ABI (Foster City, USA) from an assay that originally used a radiolabeled probe (Holland *et al.* 1991) and consist of a single-stranded probe sequence that is complementary to one of the strands of the amplicon. A fluorophore is attached to one end of the probe and a quencher to the other end. The fluorophore is excited by the machine and passes its energy, via FRET, to the quencher. Traditionally the FRET pair has been FAM as the fluorophore and TAMRA as the quencher. TAMRA is excited by the energy from the FAM and fluoresces. As this fluorescence is detected at a different wavelength to FAM the background level of FAM is low. The probe binds to the amplicon during each annealing step of the PCR. When the *Taq* polymerase extends from the primer bound to the amplicon it displaces the 5' end of the probe, which is then degraded by the 5'-3' exonuclease activity of the *Taq* polymerase. Cleavage continues until the remaining probe melts off the amplicon. This process releases the fluorophore and quencher into solution, spatially separating them compared to when they were held together by the probe. This leads to an irreversible increase in fluorescence from the FAM and a decrease in the TAMRA. Other quenchers can also be used, especially dark quenchers that give higher signal to noise ratios. For recommendation of dye combination see p 28-29.

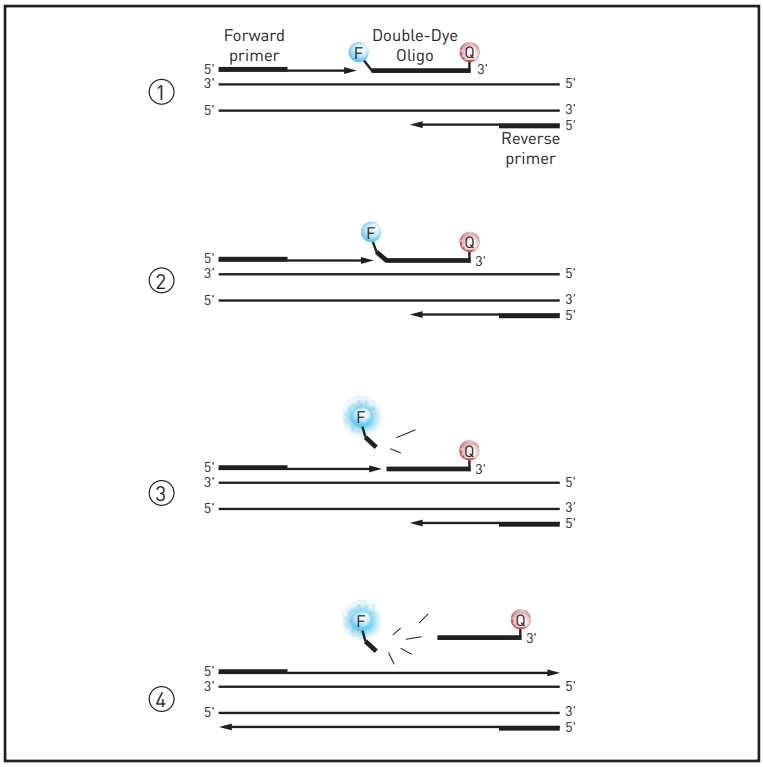

Figure 3: TaqMan® probes mode of action

TaqMan® probes can be used for both quantification and mutation detection and most designs appear to work well. They are convenient either for allelic discrimination or expression profiling and are usually easy to design, easy to use in a standard protocol and need minimal optimization. For mutation detection the probe is designed to hybridize over the mutation and can be made specific enough to detect single base differences. To obtain robust allelic data it is vital that a probe is used for each different mutation otherwise negative results with one probe, caused by failed PCR reactions, can be scored as an absence of a particular allele. Ideally these probes would be multiplexed to make the assays faster and cheaper.

TAMRA, whilst being used as a quencher, can also be used as a fluorophore. Using a different quencher would free up TAMRA to improve multiplexing capability. There are now several 'Dark quenchers' available. These absorb the energy emitted by the fluorophore but release it as heat rather than fluorescence. Examples of these are Deep Dark Quencher I or Deep Dark Quencher II (Eurogentec), Dabcyl (Nasarabadi *et al.* 1999) and Eclipse® Dark Quencher. A dark quencher gives a higher signal to noise ratio, thus more sensitive assay; that is why we do recommend to use dark quenchers when doing multiplexing assay (see p 28). Multiplexing is often a difficult question to address and the choice of the correct fluorophore dyes is therefore crucial. Eurogentec is for example a licensed provider of a fluorescent dye developed by Epoch Biosciences (Bothell, USA). This fluorophore-phosphoramidite called Yakima Yellow® can conveniently be incorporated under normal fluorescent probes synthesis conditions. Use of the Yakima Yellow® dye significantly increases both the number of independent reporters possible in an assay, and thus increases the wavelength range available for use. For best results, the combination of FAM (target) and Yakima Yellow® (endogenous control) is recommended. As so, Yakima Yellow® offers the most affordable alternative to the use of VIC dye (absorption and emission of maximum wavelengths are respectively of 528 nm – 546 nm for VIC and 525-548 nm for Yakima Yellow®).

LNA Double-Dye Oligonucleotide probes

LNA (Locked Nucleic Acid) has been developed by Exiqon® (Vedbaek, Denmark). By changing the conformation of the helix and increasing the stability of the duplex, the integration of LNA bases into Double-Dye Oligonucleotides probes opens great perspectives to improve techniques requiring high affinity probes as specific as possible, like SNP detection, expression profiling and *in situ* hybridization.

LNA is a bicyclic RNA analogue, in which the ribose moiety in the sugar-phosphate backbone is structurally constrained by a methylene bridge between the 2'-oxygen and the 4'-carbon atoms (Obika *et al.* 1997, Koskhin *et al.* 1998, Singh *et al.* 1998) (figure 4).

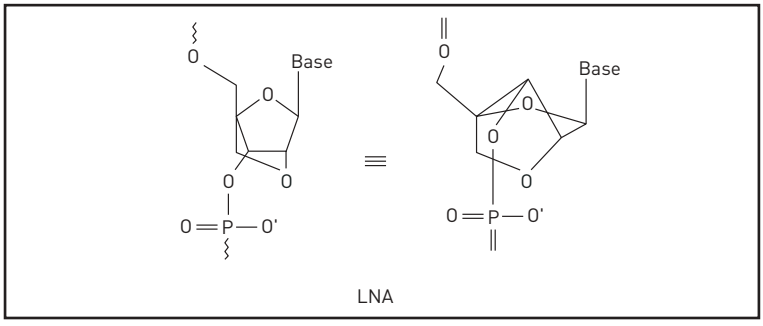

Figure 4: LNA bases

The integration of LNA bases into probes changes the conformation of the duplex when the annealing with DNA bases occurs. The integration of LNA moieties on every third position changes the structure of the double helix from the B to A type (figure 5).

This conformation allows a much better stacking and therefore a higher stability. By increasing the stability of the duplex, the integration of LNA monomers into the oligonucleotides sequence allows to increase consequently the Tm of the duplex, thus resulting to reduce the size of the probe, which increases the specificity of the probe.

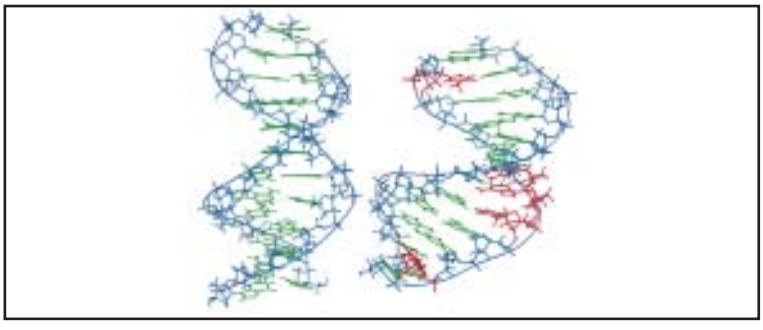

Figure 5: B helix and A helix containing LNA bases

A project has evaluated the performance of LNA and MGB® probes in 5' nuclease PCR assays and the results have shown that there were very slight differences between both technologies and that these results could be considered as similar either if MGB® or LNA were used. Both assays were showing the same sensitivity and specificity (Lelertre *et al.*, 2003).

Molecular Beacon probes

Molecular Beacons (Tyagi and Kramer, 1996) differ from TaqMan® probes in both their structure and mode of action. They consist of a hairpin loop structure where the loop is a single-stranded probe that is complementary to the amplicon. The stem is approximately 6 bases long, should mainly consist of C's and G's and holds the probe in the hairpin configuration. A fluorophore is attached to one end of the stem and a quencher (usually Deep Dark Quencher I or Dabcyl) to the other. The stem holds the two in close proximity and quenching occurs by collisional quenching.

When the amplicon is produced during the PCR, the probe is able to bind to a specific sequence providing the design such that the probe-target duplex is thermodynamically more stable than the hairpin structure at the fluorescent acquisition temperature. Once the probe binds to its target the hairpin is opened and the fluorophore and quencher are separated.

The increase in fluorescence that occurs is reversible, unlike TaqMan® probes, as the probe will dissociate at high temperatures and close back into the hairpin structure. Eventually, as temperature is increased, the stem will melt giving a linear probe that is high in fluorescence. This gives a melt curve that is very useful for observing the dynamics of the reaction and the best temperature for fluorescent acquisition.

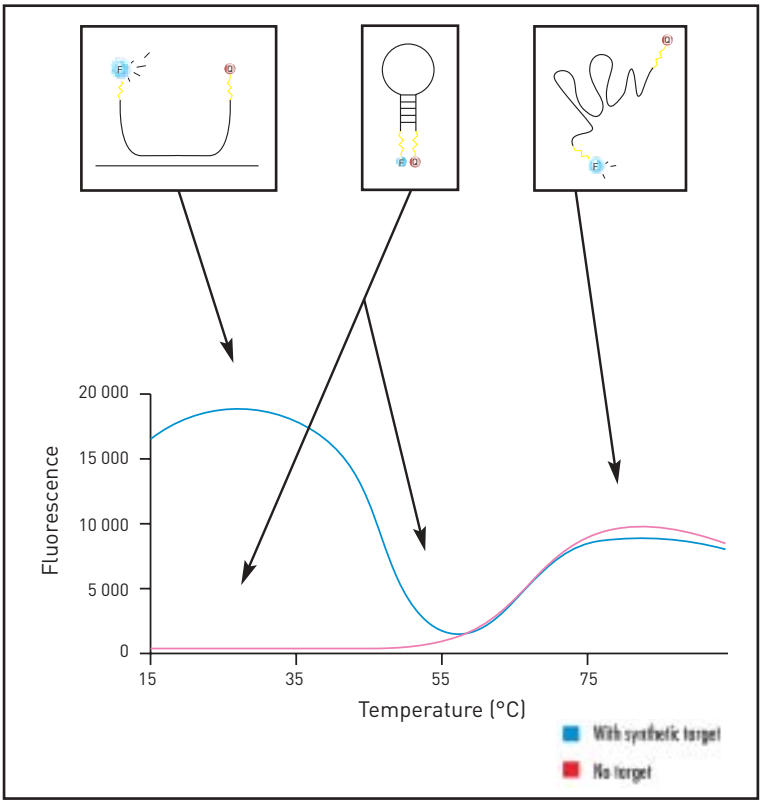

Figure 6: Melt curve of a Molecular Beacon with and without a synthetic complement as a target. Annotations show the configuration of the beacon and target at different temperatures.

The stem structure adds specificity to this type of probe as the hybrid formed between the probe and target has to be stronger than the intramolecular stem association. Mismatched targets will form a probe-target duplex but this will dissociate at a lower temperature than a perfectly matched duplex. The associated differences in the melt curves will show this and a mutation detection assay can be developed to monitor fluorescence at a temperature where the probe binds to the perfectly matched target but has dissociated from a mismatched target. Therefore Molecular Beacons are convenient either for allelic discrimination or gene expression; they give easily a discrimination and are convenient for multiplex assays. One problem with molecular beacons is that although good designs can give nice results, the signal is often very poor, as no physical separation of fluorophore from quencher occurs.

An easy way to improve signal strength is to use the **wavelength-shifting Molecular Beacons** (Tyagi *et al.*, 2000). These Molecular Beacons are nucleic acid probes that contain a harvester fluorophore that absorbs strongly into the wavelength range of monochromatic light source, an emitter fluorophore of the desired emission colour, and a non-fluorescent quencher. On the absence of complementary nucleic acid targets, the probes are non-fluoresent. In the presence of targets, they fluoresce, not in the emission range of the harvester fluorophore that absorb the light, but rather in the emission range of the emitter fluorophore. This shift in emission spectrum is due to the transfer of the absorbed energy from the harvest fluorophore to the emitter fluorophore by FRET, and it only takes place in probes that are bound to targets. Wavelength shifting Molecular Beacons are substantially brighter than conventional Molecular Beacons that contain a fluorophore that cannot efficiently absorb energy from the available monochromatic light source.

To detect the various RNA classes in living cells, several approaches have been developed. One of these is based on the use of **2'-O-Methyl RNA Molecular Beacon probes** for the detection of small nuclear RNAs. 2'-O-methyl RNA probes are considered to perform better than DNA oligonucleotides because they are not only nuclease resistant, but also possess a higher affinity, increased specificity, faster hybridization kinetics, and a superior ability to bind to structured targets compared to DNA oligonucleotides. Recently Molecular Beacons were introduced for RNA detection in living cells. The rationale for using Molecular Beacons to detect RNAs in living cells was to improve signal to noise ratios by eliminating fluorescence signals derived from non-hybridized probe sequences. It appears that linear 2'-O-Methyl RNA probes are usually more suitable for specific detection of these RNAs, representing different classes of RNA, in the nuclei of living cells. Molecular Beacons result in images with improved signal to noise ratios, thereby leading to better detection sensitivity.

Scorpions® primers

Scorpions® primers allow quantitative Real-Time PCR and genotyping. They are also suitable for end-point analysis of specific DNA targets.
Scorpions® differ (Whitcombe *et al.* 1999; Thelwell *et al.* 2000) from the specific detection methods discussed so far in that their mechanism of probing is intramolecular.
They are PCR primers with a "stem-loop" tail containing a fluorophore and a quencher. The "stem-loop" tail is separated from the PCR primer sequence by a "PCR blocker", a chemical modification that prevents the *Taq* polymerase from copying the stem loop sequence of the Scorpions® primer. Such read-through would lead to non-specific opening of the loop, causing a non-specific fluorescence signal.
The hairpin loop is linked to the 5' end of a primer via a PCR stopper. After extension of the primer during PCR amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched and an increase in signal is observed (Figure 7.).
Unimolecular probing is kinetically favourable and highly efficient. Covalent attachment of the probe to the target amplicon ensures that each probe has a target in the near vicinity. Enzymatic cleavage is not required, thereby reducing the time needed for signaling compared to TaqMan® probes, which must bind and be cleaved before an increase in fluorescence is observed.

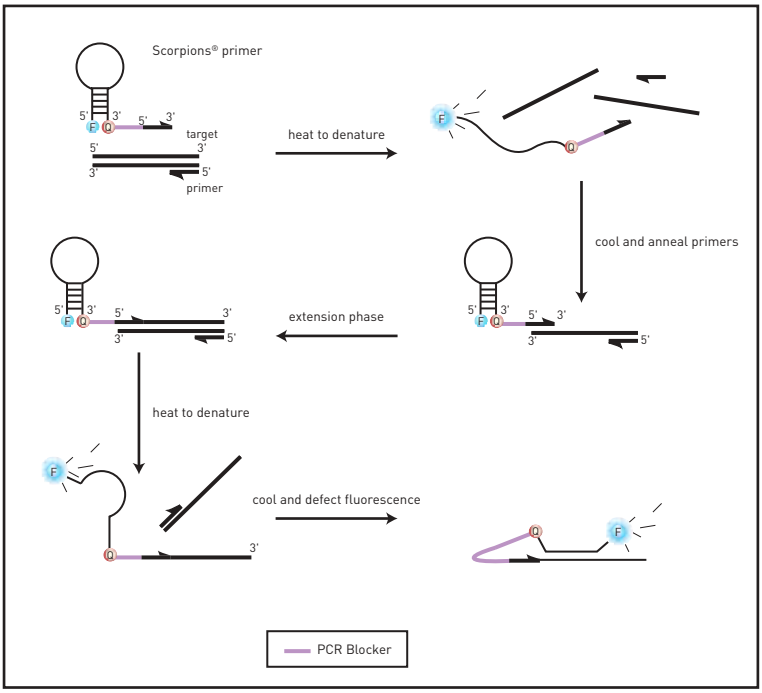

Figure 7: Scorpions® unimolecular mode of action. Progression through a PCR reaction.

Scorpions® primers have successfully been used for mutation detection and quantification, having the specificity and melt curve analysis of the Molecular Beacons with additional speed and efficiency.
The main advantages of Scorpions® primers are their lower background compared to TaqMan® probes, their fast reaction mechanism, their improved allelic discrimination capabilities and their extended multiplex possibilities.

There are three types of Scorpions® primers.
Standard Scorpions®, which consist of a bi-labeled probe with a fluorescent dye at the 5' end and an internal non-fluorescent quencher. If Scorpions® are being used on a LightCycler® system for detection channel 2 (620 nm), a specific design, called **FRET Scorpions®**, is required. As the LightCycler® will only excite at 470 nm (FAM absorption wavelength), it is necessary to incorporate a FAM within the stem. A ROX is placed at the 5' end of the Scorpions® primer. During the PCR, the FAM dye is excited and passes its energy onto the ROX. This is quenched in the closed form of the Scorpions® but once the probe binds to the amplicon, the increase in ROX fluorescence can be observed. The FRET between ROX and FAM is extremely efficient, and the FAM signal observed in channel 1 remains constant so the FAM Scorpions® can be multiplexed with the FRET Scorpions® without interference from the ROX.

A **Duplex Scorpions®** has also been developed to give much better signal intensity than the normal Scorpions® format. In a Standard Scorpions® the quencher and fluorophore remain within the same strand of DNA and some quenching can occur even in the open form. In the Duplex Scorpions® the quencher is on a different oligonucleotide and separation between the quencher and fluorophore is greatly increased, decreasing the quenching when the probe is bound to the target.

Hybridization probes

Roche (Basel, Switzerland) has developed hybridization probes (Caplin *et al.* 1999) for use with their LightCycler®. Two probes are designed to bind adjacent to one another on the amplicon. One has a 3' label of FAM, whilst the other has a 5' LC dye, LC red 640 or 705.

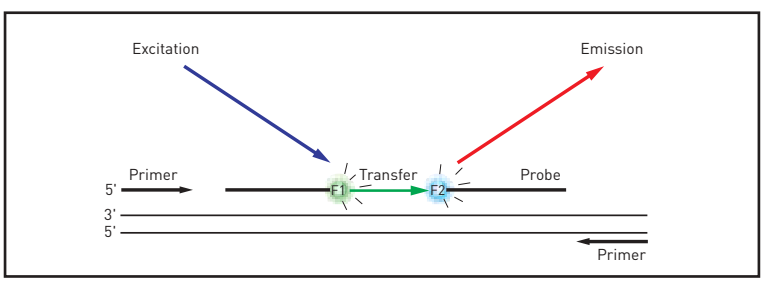

Figure 8: FRET probes principle

During the PCR reaction the 2 probes bind to the specific amplicon sequence and the capillary system excites the FAM. This passes its energy on to the LC dye through FRET and the increase in fluorescence, proportional to the increase in amplicon, can be observed in channels 2 or 3 depending on the LC dye used. This technology can be used for quantitative PCR or mutation detection. For mutation detection one probe is positioned over the polymorphic site. Melt curve analysis after the PCR indicates which alleles are present since the mismatch causes the probe to dissociate at a different temperature to the fully complementary amplicon. One probe dissociating from the amplicon causes a decrease in fluorescence, as FRET can no longer occur. It should be noted that FRET is not efficient between the LC dyes and FAM, particularly the LC red 705 dye, due to the small overlap between the spectra.

TaqMan® MGB® probes

TaqMan® MGB® probes have been developed by Epoch Biosciences (Bothell, USA) and Applied Biosystems (Foster City, USA) ; they bind to the minor groove of the DNA helix with strong specificity and affinity. When TaqMan® MGB® probe is complemented with DNA, it forms a very stable duplex with DNA. The probe carries the MGB® moiety at the 3' end, and permits to work with higher Tm, thus shorter probes, which are more specific.

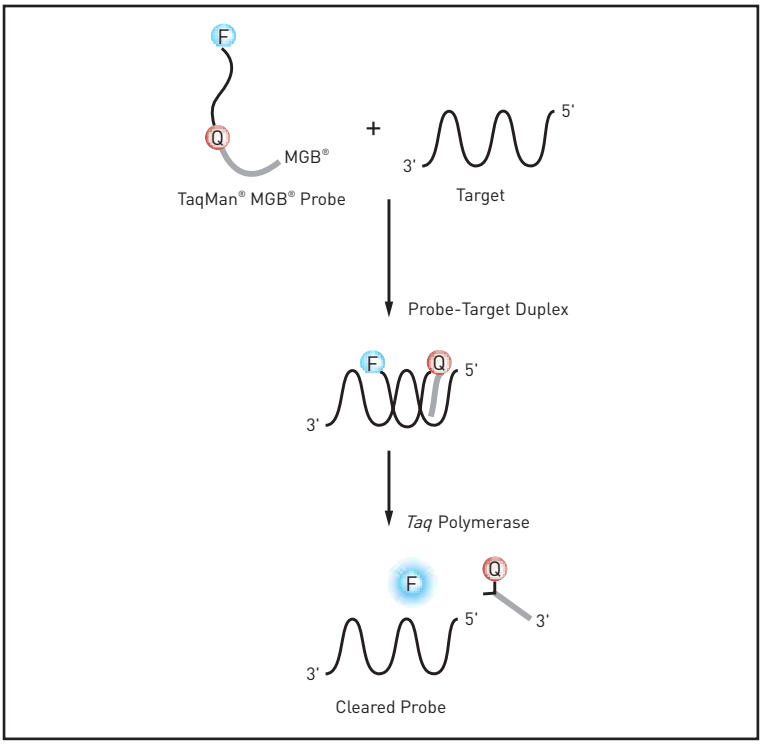

Figure 9: TaqMan® MGB® probe principle

The probe performs particularly very well with A / T rich regions and is very successful for SNPs detection (Walburger *et al.*, 2001). It is also a good alternative when it has been hard to design conventional probes, which should be located in the splice junction. Smaller probes can be designed with Tm as 65-67 °C, which permits a better discrimination (the probe is more specific for single mismatch).
During the primer extension step, the hybridized probe is cleaved by the 5' exonuclease activity of *Taq* polymerase and an increase in fluorescence is seen. Fluorescence of the cleaved probe during PCR is monitored in real time by the thermocycler.

MGB Eclipse® probes

MGB Eclipse® probes also known as QuantiProbes, have originally been developed by Epoch Biosciences (Bothell, USA). MGB Eclipse® probes carry a minor groove binder moiety that allows the use of short probes for very high specificity. These are short linear probes that have a minor groove binder and a quencher on the 5' end and a fluorophore on the 3'end. This is the opposite orientation to TaqMan® MGB® probes and it is thought that the minor groove binder prevents the exonuclease activity of the *Taq* polymerase from cleaving the probe. Quenching occurs when the random coiling of the probe in the free form brings the quencher and the fluorophore close to another. The probe is straightened out when bound to its target and quenching is decreased, leading to an increase in fluorescent signal. The minor groove binder works together with the quencher to improve quenching, reducing the background level of fluorescence.
As with TaqMan® MGB® probes, short probes can be used and allelic discrimination can be performed. They also frequently contain superbases (which increase the stability) that stabilize the probe hybridization (even for regions where hybridization is hard, such as AT-rich region).

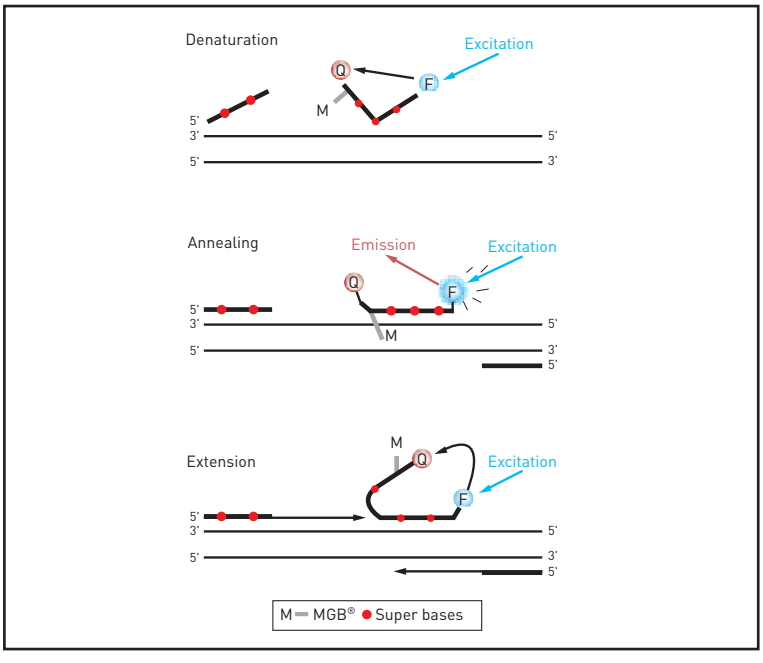

Figure 10: MGB Eclipse® probe principle

As we said before, the MGB® bound to the 5' end of the MGB® Eclipse® probe that binds to the minor groove of the DNA helix, stabilizing the duplex formed, results in an increased Tm (15 to 30 °C) and allows design of shorter probes.
The quencher used for the probe is NFQ (Non Fluorescent Quencher) also known as Eclipse® Dark Quencher, which enables to quench the fluorophore over a broad spectrum, allowing a greater choice for fluorescent labeling.
These probes are suitable for viral detection, gene expression and allelic discrimination.
The disadvantage of MGB® Eclipse® probes is the low signal to noise ratio.

Other technologies

The technologies that have been discussed above are the most widely used today but other technologies have been published or are available on the market.

Resonsense probes

Resonsense probes are single labeled probes that bind to a specific template. A DNA intercalator, such as SYBR® Gold, which can pass energy to the dye on the probe via FRET, is also added to the reaction. Once the probe has bound to its target it gives a double-stranded template for the SYBR® Gold to bind to. Energy is passed from the SYBR® Gold (excited by the machine) to the dye on the probe leading to an increase in fluorescence from the dye. It should be noted that this technology cannot be used on all machines.

Light-up probes

Light-up probes (Wolfs *et al.*, 2001 – Svanvik *et al.*, 2000) are another alternative, which use the ability of some dyes to bind to the target and increase in fluorescence once the probe has bound.
These probes are PNA probes (Peptide Nucleic Acids) rather than DNA probes. PNA has properties that make the hybridization faster and stronger than when using DNA probes.

HyBeacon® probes

HyBeacon® probes have been developed by LGC (Laboratory of Government Chemist: http://www.lgc.co.uk) and consist of a specific linear probe with a dye label. Assays use the change in fluorescence observed when the probe binds to its target, compared to when it is in its single-stranded form, for sequence detection. The probes are more suitable for allelic discrimination than for expression profiling.

LUX primers

LUX ("Light Upon eXtension") primers are single-labeled fluorogenic primers with a hairpin structure (20-30 bases in length). Their principle depends on a single fluorophore, which is located close to the 3'end in a specific sequence context that provides self-quenching. The particularity of the probe is that there is no quencher. The DNA sequence and the secondary structures with the labeled primer provide fluorescence quenching. Therefore, the design of such a probe or primer has to be done on the effect of the primary and secondary structure of the oligonucleotide. When the primer is incorporated into double-stranded PCR product, the fluorophore is no longer quenched and the fluorescence signal increased.

The advantages of the technology are that no probe is needed and a single-labeled oligo is simpler to synthesize. Moreover, a design software is available and the technology can be applied on every Real-Time thermocycler. But on the other hand, the technology is only available with FAM and JOE labels. The results give a low signal to noise ratio and thus a lower sensitivity.

Yin-yang probes

Yin-yang probes are specific to the gene of interest. They are double-stranded probes, which are composed of two complementary oligonucleotides of different lengths. The longer positive strand is labeled with a fluorophore and the shorter negative strand is labeled with a quencher. The probe set is low-fluorescent due to the close proximity of the fluorophore and the quencher. In the presence of target the negative strand is displaced by the target and an increase in fluorescence can be detected. The presence of a complementary strand enhances the specificity of the probe. Single nucleotide discrimination is easily detected with such a probe. The probes, which are easy to synthesize, simple to design, and highly sensitive can be used for quantitation or allelic discrimination.

For more information: (Li *et al.*, 2002).

Amplifluor® (non specific probe system)

The alternative to using a SYBR® dye for non-specific detection is the Amplifluor®, Universal Amplification and Detection System. This system has 2 steps carried out in the same PCR reaction. One of the specific primers has a universal sequence (Z sequence) added to the 5' end. During amplification the Z sequence is incorporated into the amplicon. A second primer (UniPrimer®) that is complementary to the Z sequence is then used for amplification. The UniPrimer® has a hairpin structure attached to its 5' end. The hairpin is similar in structure to a Molecular Beacon, as described below, but the single-stranded loop sequence is not specific to the amplicon sequence and does not bind to the amplicon. The hairpin is incorporated into the PCR product when the UniPrimer® extends. When the extension product becomes a template for the opposite primer the *Taq* polymerase extends through the hairpin loop causing the loop to open. This separates the fluorophore and quencher that were previously held in close proximity by the hairpin. A corresponding increase in fluorescence is then observed.

For more information: Myakishev *et al.*, 2001

Technical Notes

Table 1: Technology compatibility table with Real-Time thermocycler

	SYBR® green I	Double - Dye Oligos	Molecular Beacons	Scorpions® primers	FRET probes	LNA ddo	TaqMan® MGB®	MGB Eclipse®
GeneAmp® 5700	X	X	X	X		X	X	X
ABI Prism® 7000	X	X	X	X		X	X	X
ABI Prism® 7700	X	X	X	X		X	X	X
ABI Prism® 7900	X	X	X	X		X	X	X
ABI 7300	X	X	X	X		X	X	X
ABI 7500	X	X	X	X		X	X	X
iCycler iQ®	X	X	X	X	X	X	X	X
Mx4000®	X	X	X	X		X	X	X
Mx3000p®	X	X	X	X		X	X	X
RotorGene 2000	X	X	X	X	X	X	X	X
RotorGene 3000	X	X	X	X	X	X	X	X
DNA Engine Opticon® 1	X	X	X	X		X	X	X
DNA Engine Opticon® 2	X	X	X	X		X	X	X
Chromo 4	X	X	X	X		X	X	X
Smartcycler®	X	X	X	X	X	X	X	X
Smartcycler® II	X	X	X	X	X	X	X	X
LightCycler®	X	X *	X *	X	X	X *	X *	X *
LightCycler 2.0	X	X	X	X	X	X	X	X
Quantica®	X	X	X	X		X	X	X
InSite	X	X	X	X		X	X	X

* singleplex

Fluorophore and quencher

Many of the commonly employed techniques for the detection of nucleic acid sequences in a homogenous manner use fluorescence as the signaling technology. Typically a single-stranded probe is labeled with a fluorophore and a quencher molecule. Changes in quenching of the fluorophore caused by hybridization of the probe to its target nucleic acid lead to signal generation.
Thus, Real-Time PCR relies not only on the choice of one technology but also in the choice of a fluorophore and a quencher, whether the assay would be singleplex or multiplex. The choice of a fluorophore and its combination with a quencher will give different results in terms of sensitivity of the assay.

A fluorophore is a molecule that emits light of a certain wavelength after having absorbed light of a specific but different wavelength first. The emission wavelength is always higher than the absorption wavelength.

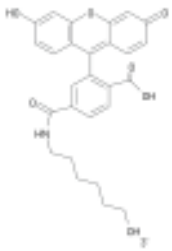

Figure 11: FAM structure

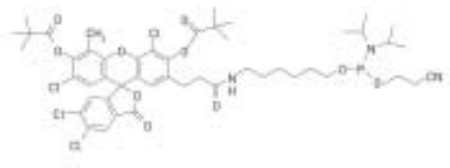

Figure 12: Yakima Yellow® structure

A quencher is a molecule that accepts energy from a fluorophore in the form of light and dissipates this energy either in the form light or heat.

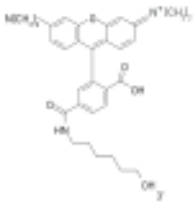

Figure 13: TAMRA structure

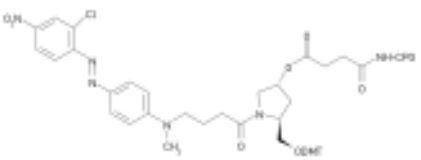

Figure 14: Eclipse® Dark Quencher structure

Different ways of quenching

Quenchers are molecules that can accept energy from a fluorophore and dissipate it by two mechanisms, called proximal and FRET quenching. A fluorophore absorbs light energy and is promoted to an excited state. In the absence of a quencher the fluorophore falls back to the ground state and the excess of energy is released as fluorescence.

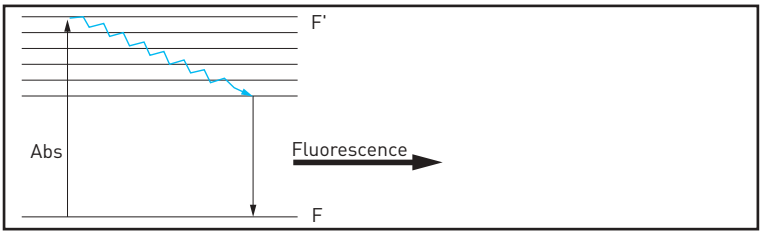

Figure 15: Fluorescence principle

Proximal quenching

When the fluorophore is in close proximity of a quencher molecule, the energy is transferred from the fluorophore to the quencher, which then dissipates the energy as heat (no fluorescence is observed). It is also known as collisional quenching.Examples of quenchers for such a mechanism are Deep Dark Quencher I, Dabcyl or Dabsyl.

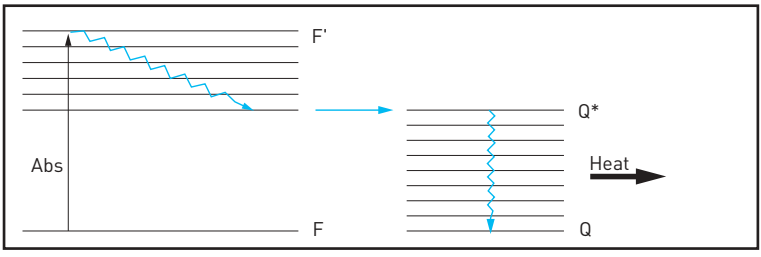

Figure 16: Proximal quenching principle

FRET quenching

The fluorophore transfers its energy to the quencher (which may be another fluorophore); the energy is released from the quencher as radiative (i.e. fluorescence) of a higher wavelength. The efficiency of this process is dependent on the distance between fluorophore and quencher, more precisely on the Förster distance $1/r^6$ (where "r„ is the fluorophore – quencher distance).

An example of quencher for such a mechanism is TAMRA.

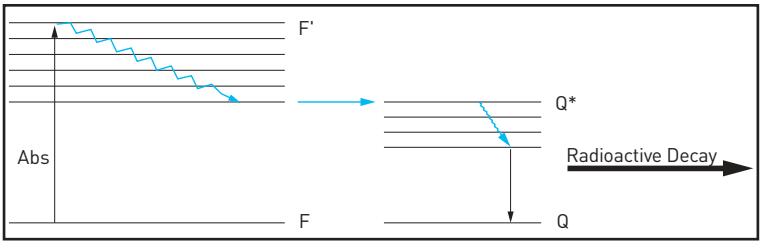

Figure 17: FRET quenching principle

Optimal fluorophore quencher combination

The quencher has to fit the fluorophore

One fluorophore can be combined with different quenchers, but the absorption spectrum of the quencher needs to have good overlap with the emission spectrum of the fluorophore to achieve optimal quenching. For example, Deep Dark Quencher II absorbs over a large range of the visible spectrum and therefore efficiently quenches most of the commonly used fluorophores, especially those emitting at higher wavelengths.
Deep Dark Quencher I and Eclipse® Dark Quencher quench the lower wavelength dyes such as FAM but they are not good at quenching those that emit at high wavelength such as Cy® dyes.

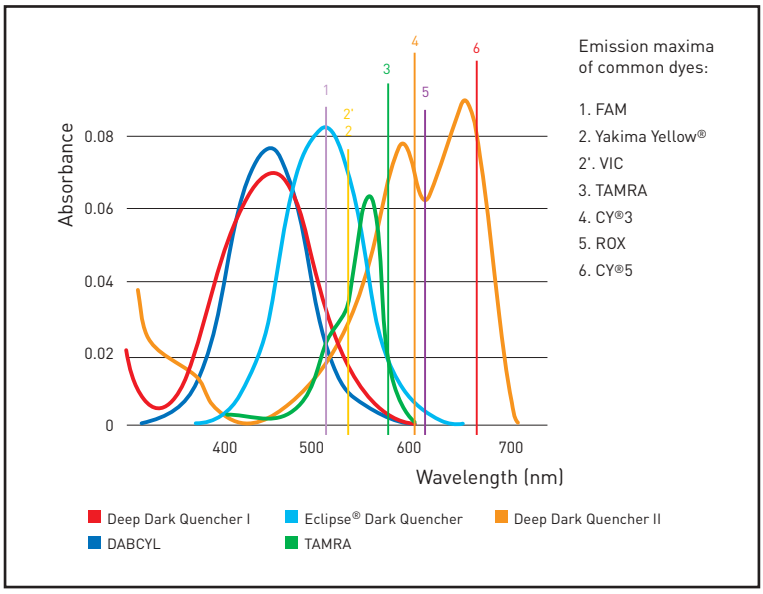

Figure 18: UV-Vis absorbance spectra of oligonucleotides labeled with 3'-Deep Dark Quencher I, 3'-Deep Dark Quencher II, 3'-Eclipse® Dark Quencher and 3' TAMRA

Quenchers have a quenching capacity throughout their absorption spectrum, but the performance is best close to the absorption maximum.

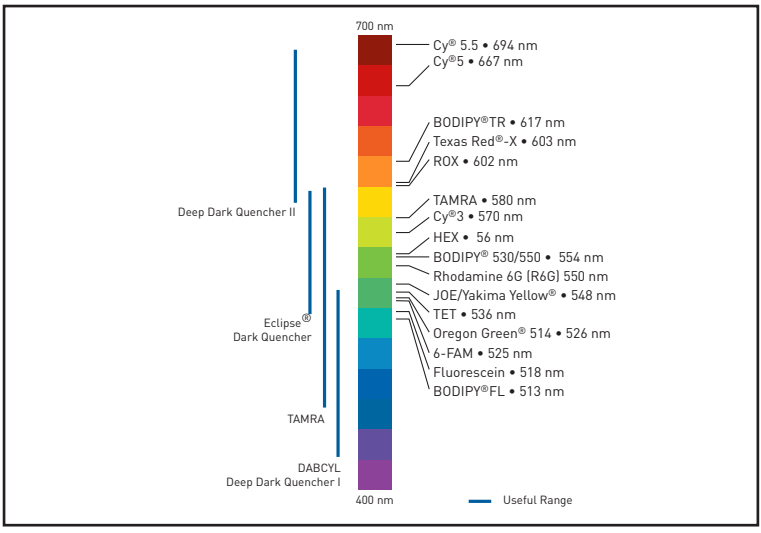

Figure 19: Absorbance range of Dark Quenchers and emission maxima for Reporter Dyes

Efficient quenching of fluorophores

If the fluorescence is too high because of incomplete quenching the relative fluorescence signal (signal to noise ratio) will decrease.
Therefore the fluorophore-quencher combination, which gives the highest signal to noise ratio should be selected.

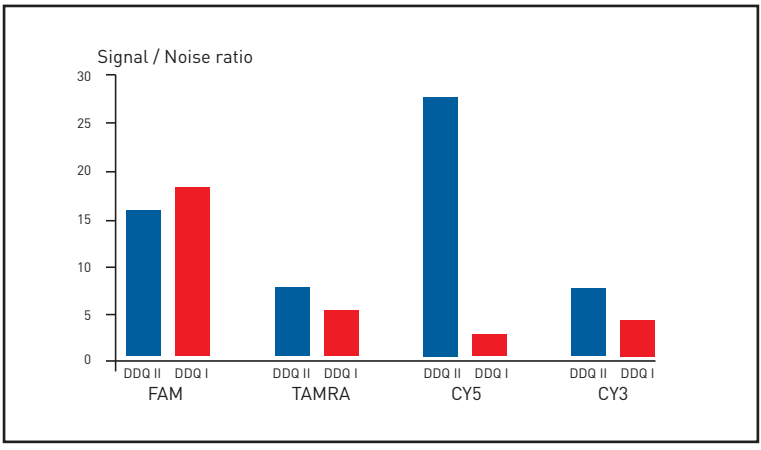

Figure 20: Comparative analysis of quenching efficiency of 3'-Deep Dark Quencher I and 3'-Deep Dark Quencher II on 4 different dyes (represent a broad range of emission maxima).

Why using dark quenchers?

Dark quenchers are completely non-fluorescent and extremely low background quenchers. Using dark quenchers significantly improves signal to noise ratios (higher ΔRn, a lower background), and thus give a higher sensitivity in multiplex PCR.

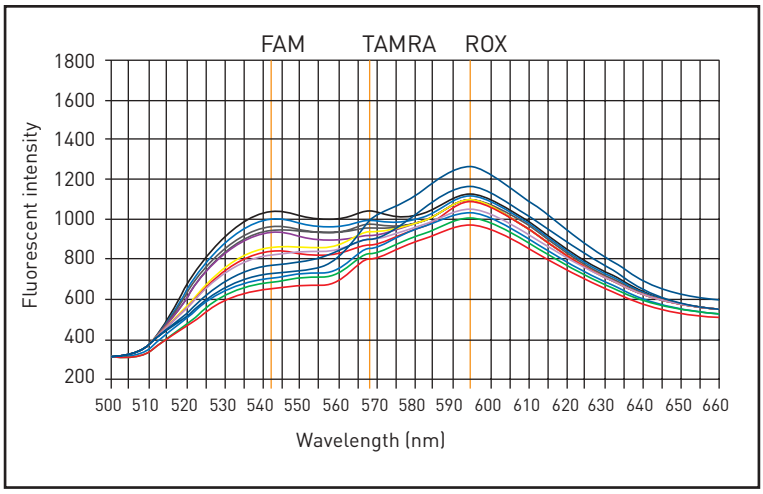

Figure 21: Raw spectrum of FAM-TAMRA probe in ROX containing MasterMix

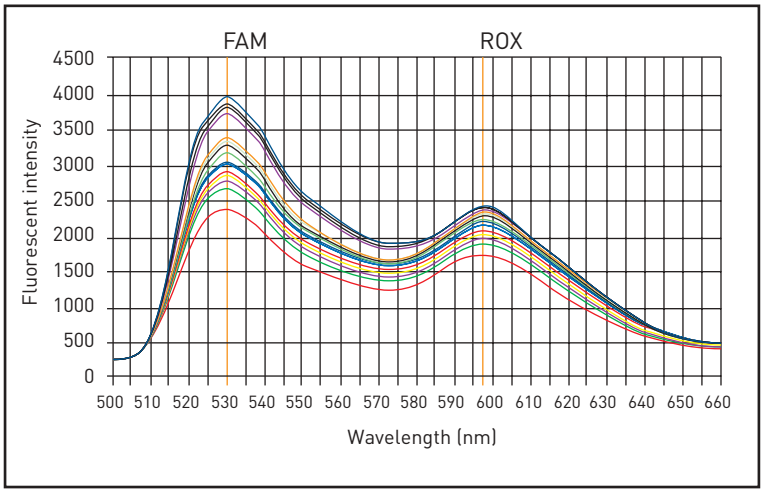

Figure 22: Raw spectrum of FAM-DDQI probe in ROX containing MasterMix

Eclipse® Dark Quencher superior to TAMRA

When using a dye quencher pair like FAM-TAMRA, a number of drawbacks can be met. These include intrinsic fluorescence of the quencher and poor spectral overlap between the fluorescent dye and quencher molecule, both leading to a poor signal to noise ratio. Dark quenchers like Eclipse® Dark Quencher eliminate high background signals. Thus, Eclipse® Dark Quencher is superior to TAMRA and it is the quencher of choice, as it does not fluoresce. The combination of FAM, TET or Yakima Yellow® with Eclipse® Dark Quencher leads to improved Ct values and signal to noise ratio. Therefore, we recommend to use such a quencher for multiplex assays.

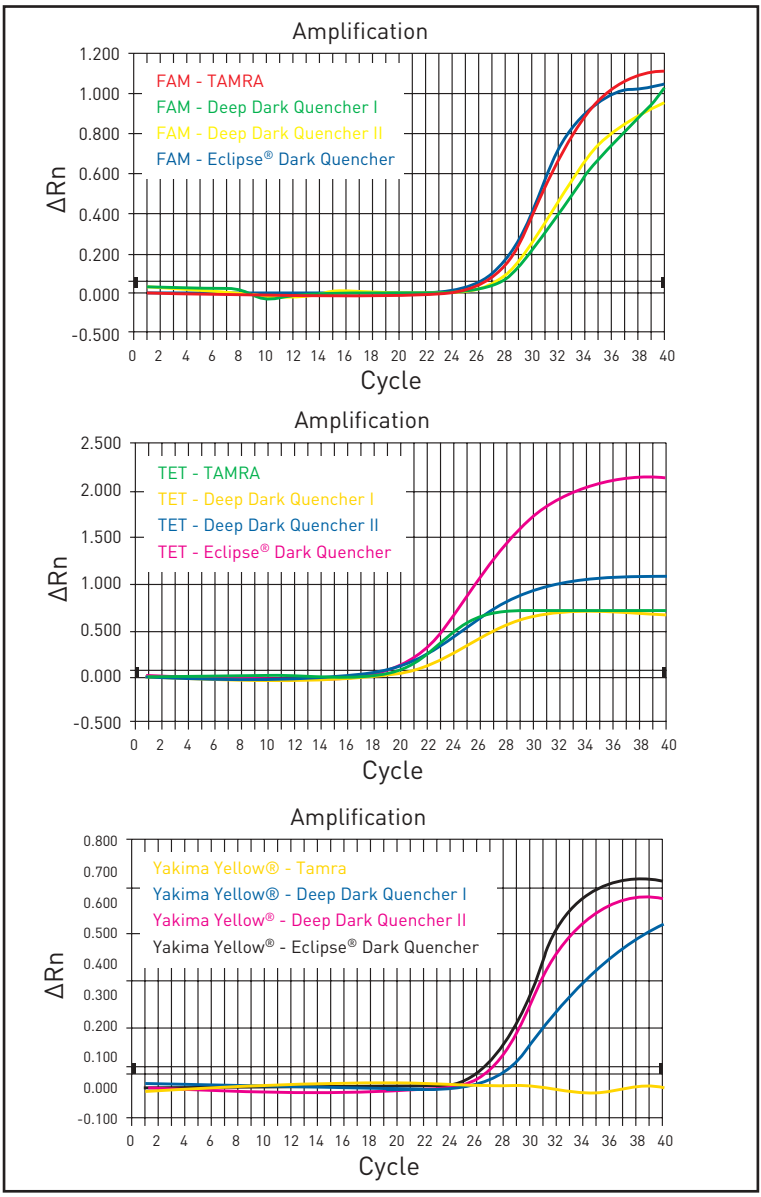

Figure 23: Effects of different quenchers (TAMRA - Deep Dark Quencher I - Deep Dark Quencher II - Eclipse® Dark Quencher) on FAM, TET and Yakima Yellow®

Optimal combination for multiplexing using the Double-Dye Oligonucleotides technology

Double-Dye Oligonucleotides in single-colour detection
For single-colour detection we recommend to use the combination FAM TAMRA or FAM- Eclipse® Dark Quencher. This combination is the most standard one; it can be detected on every Real-Time PCR and is easy to synthesize.

Double-Dye Oligonucleotides in multicolour detection
For multiplex Real-Time PCR, it is very important to select dyes that give a good spectral separation to avoid overlap of the signal.
For the first colour we recommend the use of FAM-Eclipse® Dark Quencher. This is the choice to make for duplex or multiplex PCR as Eclipse® Dark Quencher gives a lower background compared to TAMRA.
For the second colour, we recommend the use of Yakima Yellow® - Eclipse® Dark Quencher, which is the best choice to be combined to FAM-Eclispe® Dark Quencher as Yakima Yellow® gives a good spectral separation from FAM. Furthermore, it is a good cost effect alternative to VIC and it can be detected in the same channel.
The choice of the third colour is dependent on the thermocycler (cf the dye compatibility table with Real-Time thermocycler p.29). We recommend to use if possible either Cy® 3-DDQII or Texas Red®-DDQII. These two dyes have both a good spectral separation from FAM and Yakima Yellow® and give both efficient synthesis with high yields. Moreover, as Deep Dark Quencher II is also a dark quencher it does not give any background. The choice of the fourth dye is also dependent on the Real-Time thermocycler. We usually recommend the use of Cy® 5-DDQII as Cy® 5 has a good spectral separation from FAM, Yakima Yellow®, Cy® 3 and Texas Red®. Furthermore, this last one has also an efficient synthesis and gives high yields.

Dye compatibility with Real-Time thermocyclers

Almost every detection system type works on almost every Real-Time thermocycler. The detection channels determine which fluorophores have to be used, and the combination of excitation and detection channels determine which probe systems can be used. As previously seen, for singleplex we recommend the use of FAM, as a second Yakima Yellow® dye, which is a good alternative to VIC and which can be detected by almost every thermocycler. The third and fourth dyes are usually dependent on the thermocycler. The level of cross-talk between the channels determines which are the best fluorophores to combine for duplex or multiplex Real-Time PCR.

Table 2: Dye compatibility table with Real-Time thermocycler

Thermocycler	Ch 1	Ch2	CH3	Ch4	Ch5	Ch6	Ch7
GeneAmp® 5700	FAM						
ABI Prism® 7000	FAM	VIC/YY/JOE/TET	HEX	ROX/TAMRA			
ABI Prism® 7700	FAM	VIC/YY/JOE/TET	HEX	ROX/TAMRA			
ABI Prism® 7900	FAM	VIC/YY/JOE/TET	HEX	ROX/TAMRA			
ABI 7300	FAM	VIC/YY/JOE	TAMRA/ROX		Cy® 5		
ABI 7500	FAM	VIC/YY/JOE	NED/TAMRA Cy® 3	ROX/TR	Cy® 5		
iCycler iQ®	FAM	VIC/HEX/TET/Cy®3/YY	Cy® 3/TAMRA	ROX/TR	Cy® 5		
Mx4000®	FAM	TET/YY	HEX/JOE/VIC/YY	TAMRA	Cy® 3	TR/ROX	Cy® 5
Mx3000p®	FAM	TET/YY	HEX/JOE/VIC/YY	TAMRA	Cy® 3	TR/ROX	Cy® 5/Alexa®350
RotorGene 2000	FAM	TET/JOE/VIC/YY	ROX/TAMRA/Cy® 3/TR	Cy® 5			
RotorGene 3000	FAM	TET/JOE/VIC/YY	MAX/ROX/Cy® 3/TR	Cy® 5			
DNA Engine Opticon® 1	FAM						
DNA Engine Optiicon® 2	FAM	TET/HEX/VIC/YY/TAMRA					
Smartcycler® I	FAM	TET/JOE/VIC/YY	TAMRA/Cy® 3/Alexa®	ROX/TR			
Smartcycler® II	FAM	TET/Cy® 3/YY	ROX/TR	Cy® 5			
LightCycler®	FAM	LCRed 640 / ROX	LCRed 705/Cy® 5				
LightCycler® 2.0	FAM	HEX/VIC/YY	LCRed 610	LCRed 640	LCRed 670	LCRed 705	

With a ROX passive reference everything is relative

On both ABI and Stratagene Real-Time thermocyclers the use of a ROX passive reference is recommended. The ROX passive reference is used to correct for pipetting inaccuracies. It is a dye, which does not participate in the 5' exonuclease PCR. The ROX passive reference provides an internal fluorescent reference to which the reporter fluorescence can be normalized.
The Rn (normalized reporter signal) is calculated by dividing the reporter signal by the ROX passive reference signal. This signal is only corrected for pipetting inaccuracies. When correction for variations in background is required, the ΔRn is used. The ΔRn is calculated by taking the Rn and subtracting the normalized baseline (Bn). The normalized baseline is the baseline signal divided by the ROX passive reference signal.

Rn= (reporter signal / ROX passive reference signal)

Bn= (baseline signal / ROX passive reference)

ΔRn = Rn-Bn

ΔRn (reporter signal / ROX passive reference signal) – (baseline signal / ROX passive reference signal)

As can be deducted from the formulas above, the ROX passive reference influences both the level of the Rn and the Bn. In most software an automatic threshold is used to determined a Ct value. The automatic threshold is calculated as the average baseline plus x standard deviations of that baseline.

automatic threshold = average baseline + x STD baseline

The noise of the baseline is the value, which determines the standard deviation of the baseline. The ROX passive reference influences this baseline level and therefore also the noise level. When the level of ROX passive reference increases, the noise will decrease, but also the ΔRn will decrease. It is therefore important to determine the right level of ROX that does not compromise the ΔRn.

The Ct value is defined as the cycle in which a significant increase in reporter signal above the threshold can be detected, i.e. the cycle in which the growth curve crosses the threshold.
The Ct is therefore dependent on the threshold level and therefore indirectly also on the baseline level and the ROX passive reference level.

Only a minor variation in the ROX passive reference will already cause a change in the Rn, Bn, ΔRn, threshold and Ct value. Therefore we recommend to use MasterMixes that already contain a ROX passive reference to avoid these variations, which might be interpreted as real differences, but are in reality just experimental artifacts.

Dyes and quenchers chemistry

There are a number of different dyes and quenchers available for use in qualitative and quantitative PCR applications, the most common ones are listed in the table below.

Table 3: Dye and quencher chemistry table

Dyes	Absorption (nm)	Emission (nm)
Fluorescein	495	518
FAM	494	525
TET	521	536
JOE	520	548
Yakima Yellow® (alternative to VIC)	526	548
R6G	524	550
HEX	535	556
Cy® 3	552	570
TAMRA	565	580
Cy® 3.5	581	596
ROX	575	602
Texas Red®	583	603
Cy® 5	643	667
Cy® 5.5	683	707

Quenchers		
Deep Dark Quencher I	410	None
Deep Dark Quencher II	630	None
Eclipse® Dark Quencher	522	None
Dabcyl	453	None
Dabsyl	466	None
TAMRA	565	580

There are different methods by which a dye can be incorporated into a probe. The method used depends on the structures of the dye and of the length of the probes.

• **Manual coupling**

Manual coupling can be done via amine or thiol groups using activated dyes such as Texas Red®, TAMRA, JOE, Rhodamine (R6G) or ROX, BODIPY® and other dyes (Alexa®, Marina Blue®...). These labels can be linked internally to any dT-residue, dR-residue or to either terminus. The fluorescent molecule is linked to the oligonucleotide via a spacer (a C-6 spacer generally) to reduce the steric hindrance.
After synthesis, but before labeling, these oligonucleotides are purified, and then they are labeled and purified by HPLC or PAGE.

• Automatic coupling

Automatic coupling uses the appropriate phosphoramidite to which the dye is already coupled like Cy® dyes, FAM, Fluorescein, HEX, TET and Yakima Yellow®. These labels are coupled during the synthesis at the 5' terminus. For internal labeling, this process is also used to introduce Fluorescein-dT and TAMRA-dT as a replacement for a dT-residue.
After synthesis, these oligos are purified either by HPLC or PAGE.

The easiest probes to synthesize and to purify are Double-Dye Oligonucleotide probes, as they are not too long. Moreover, the probes where you can use phosphoramidites (automatic coupling method) are usually the easiest ones to synthesize and purify, as there is only one set up for synthesis and one purification step. They also give the highest yields. 3' TAMRA probes as well as probes quenched with Dabcyl, Eclipse® Dark Quencher, Deep Dark Quencher I and Deep Dark Quencher II, have also very efficient synthesis, as the coupling method with CPG is very easy to achieve. Eurogentec is very experienced in the synthesis of dye labeled oligos.
The probes, which require post-labeling procedures (manual coupling method) are more laborious to synthesize and will give in general lower yield: they need a second purification step and the dyes are usually supplied as NHS esters.

Applications

Quantitative PCR

Quantitative PCR can be used for a variety of applications such as monitoring gene expression levels and viral load determination (Bustin 2000 - Bustin 2002 - Freeman *et al.* 1999- Halford 1999 - Heid *et al.* 1996). Monitoring gene expression is one of the most frequently used applications for Real-Time quantitative PCR. In this way, effects of different treatments on the level of mRNA transcription can be assessed. To be able to determine the level of mRNA expression, the RNA must be transcribed in cDNA, which can then be amplified by PCR. cDNA differs from genomic DNA in that it does not contain any introns. Reverse transcription can be carried out in a separate reaction (Two step RT qPCR) or in one and the same reaction (One step RT qPCR). The RT step requires the use of primers. It is possible to use the same specific primers as required for the PCR step. In a One step RT qPCR this is always the case. Note that the RT step is a linear process rather than exponential and it is the reverse primer that is used to produce the cDNA strand of the mRNA. In a two-step reaction there is a choice of assay specific primers, random hexamers, random nonamers or oligo d(T). If the target is rRNA, oligo d(T) should be avoided as this primes at the poly-A tail of the mRNA. The merits of the different primers for RT are a very discussed subject, with some claiming that using specific primers will introduce a bias and others feeling that using oligo d(T), priming at the end of the transcript, will reduce the number of full length cDNA's produced. It has also been suggested that a mixture of oligo d(T) and random hexamers should be used. Once the cDNA has been synthesized a standard Real-Time PCR reaction can be run and quantification carried out by the machine software.

Quantitative PCR software uses the exponential phase of PCR for quantification. PCR is initially an exponential process but eventually reaches a plateau phase as one of the reagents becomes limited. Reactions can plateau at different levels even if they have the same starting concentration of target. During the exponential phase the amount of target is assumed to be doubling every cycle and no bias is expected due to limiting reagents. Analyses use the Ct, the point (cycle number) at which the signal is detected above the background and the amplification is in exponential phase. The more abundant template samples reach this point the earliest. Differences in Ct then have to be related to some other quantitative values to make them meaningful. There are two types of quantification, relative quantification and absolute quantification.

Relative quantitation

Relative quantification is the most widely used technique.
An endogenous control is amplified from the cDNA sample as well as the gene of interest (GOI).

What is a control?

An endogenous control gene is used to standardize the amount of sample RNA or DNA added to the reaction. It should be a stabile expressed gene through the experiment that is used to normalize the results of a variable target gene and to correct for sample-to-sample variations.

What is a good control?

A good control should have a constant level of expression between individuals, among different tissues of an organism, at all stages of development, and should not be affected by the experiment treatment. The control gene should also be expressed at similar level as the gene of interest and the range of linear amplification should be known (Bustin, 2000). If a control gene is up and down regulated by the experimental intervention, this will lead to an incorrect normalization and thus to misinterpretation of the results. Therefore, proper validation of presumed stability of expression of the control genes should be done before studying the target gene.

Normalization of Real-Time PCR

When analyzing and comparing results of Real-Time PCR assays many researchers are confronted with several uncontrolled variables, which can lead to misinterpretation of the results. Those uncontrolled variables can be the amount of starting material, enzymatic efficiencies, and differences between tissues, individuals or experimental conditions.
To be able to make a good comparison, normalization can be used as a way to correct for these variables.
There are several ways to normalize, which all have some advantages and disadvantages.
The most commonly known and used ways of normalization are normalization to the original number of cells, normalization to the total RNA mass, normalization to one or more housekeeping genes and normalization to an internal or external calibrator.
The various methods of normalization can then also be combined with different calculation methods, like the absolute standard curve method, the relative standard curve method, the ΔΔCt method and the geometrical averaging method. (See http://www.wzw.tum.de/gene-quantification/ for further information).

Normalization to number of cells can actually only be done for cell culture and blood samples. In solid tissue and tumours the amount of cells can not be counted, only estimated and therefore leads to inaccuracies (Bustin, 2000).

Normalizing to the RNA mass quantity will lead to inaccuracies as the total RNA mass (rRNA, tRNA and mRNA) might contain imbalances between rRNA and mRNA (Solinas *et al.*, 2001). The determination of the RNA amount by photospectrometry is another factor causing inaccuracy as the A260 quantitation is strongly influenced by the impurity of the sample, caused by contaminating DNA, free nucleotides and proteins (Bustin, 2000 – Bustin, 2002). As very few is known about total RNA content per cell in different tissues, a variation between individuals and between normal or highly proliferating tissues, like tumours will cause unwanted inaccuracies (Bustin, 2000 – Bustin, 2002). In general 18S and 28S rRNA are used for this method.
Normalization to one housekeeping gene excludes some of the above-mentioned drawbacks, but is also not the perfect method. The advantage is that the variation due to different amounts of RNA can be excluded. However, one must assure oneself that the housekeeping gene is expressed constantly at the level throughout the experiment and between samples. A housekeeping gene like GAPDH is for example strongly upregulated in patients with diabetes type II. It is therefore recommendable to first validate the housekeeping gene or to go for more than one housekeeping gene. Vandesompele *et al.* (2002) have described a method to normalize using more than one housekeeping gene.

Normalization by an internal or external calibrator can also be done. By spiking DNA into the sample (external calibration) a correction for inter-reaction variablity can be made, but not for intrareaction variability. The opposite is true for internal calibrators.
Most of the calculation methods used for normalization relate to the use of housekeeping genes.
The absolute standard curve method allows deducting the initial amount of mRNA from the Ct values, assuming that there is no variability in the RT step.
The relative standard curve makes a comparison between Ct values of target and calibrator and allows to express this in an index. The ratio of specific gene signal to endogenous control signal in a sample can be calculated in each sample. One sample has to be nominated as the calibrator, e.g. the untreated sample. Each normalized value can then be divided by the calibrator to obtain relative values for each sample. When using this method, DNA can be used as standard if the RT efficiency is identical for all the samples (Giulietti *et al.* 2001).
The ΔΔCt method is an easy-to-use method as no standard curve is needed. But it requires some assumptions that need verification. The PCR efficiency of target and calibrator should be close to 100 % and not vary more than 5 %. The method consists of calculation of the relative expression levels, compared with a control. The amount of target is normalized to endogenous housekeeping genes and relative to the calibrator.
The following equations are used for this calculation
ΔCt= endogenous Ct-Gene of interest Ct
ΔΔCt= ΔCt of sample-ΔCt of calibrator
Amount of target normalized to a control and relative to a calibrator = $2^{(\Delta\Delta Ct)}$

The outcome of the method is similar to one of the relative standard curve method, but not identical.
The normalization by geometrical averaging of multiple internal control genes (Vandesompele *et al.*, 2002) is a robust and innovative approach for accurate normalization. It relies on the use of several housekeeping genes to even out variations in the expression of these genes.
Only the most stabile expressed housekeeping genes are taken into the calculation. The calculation can be performed using the freely available software geNorm (http://medgen31.ugent.be/jvdesomp/genorm/). The major drawback of this method is the need for many primer pairs and the complicated way to process the data.

Normalization using singleplex or multiplex assay

The endogenous control can be used as a singleplex reaction where the gene of interest and control are amplified in separate reactions. It can also be used in a multiplex reaction, which prevents pipetting errors between the control reaction and gene of interest reaction that can occur when using singleplex reactions. In that case, normalizing in the same well will account for the variability in individual PCR assay variability like inhibitors. Moreover, multiplex assays have the advantage of being cost effective in terms of reagents required. Multiplexing Real-Time PCR reactions is possible as most Real-Time thermocycler can detect more than one dye in each sample. It is important to consider, which dyes are the best for multiplexing on the particular machine being used and also any requirements for spectral calibration. This is required because dyes exhibit some spectral overlap, making it necessary to calibrate the machine to enable it to distinguish between the dyes. Spectral calibration kits can be bought for the majority of machines where this needs to be considered. Problems arise when the endogenous control and gene of interest compete for PCR reagents. A highly abundant endogenous control can out-compete a less abundant template leading to a bias in the results. Limiting the primer concentration for the most abundant template can avoid this competition. However, this takes a considerable amount of development. Limiting primer concentrations will not alter the Ct compared to a fully optimized reaction but will lead to a much earlier plateau phase. This solution is not ideal as the signals obtained can be less reliable. Furthermore, even when working with limiting primer concentrations the detection of rarer target genes may not be possible or may be detectable at much reduced efficiency. Optimizing can also mean adding more units of *Taq* polymerase per reaction and therefore increasing the costs again.
To summarize, when targeting two genes with equal expression levels with PCR reactions of similar amplification efficiency, multiplex Real-Time PCR can be performed without any doubt.
When analyzing two genes with different expression levels we recommend to separate amplification of the control kit and the target gene. The difference between a single or a multiplex detection can be crucial when detecting close to the limits of the method.

How to use a control gene?

When doing singleplex PCR, the target gene and the control are in different reactions, which means that only one dye is used. We recommend to use the following combination: FAM-TAMRA or FAM-Eclipse® Dark Quencher. In that case, every reaction should be run at maximum efficiency. There will be no competition due to the presence of several amplicons. Instead of FAM it is also possible to use SYBR® green I.

When running a duplex PCR the target genes and control genes are in the same reaction, which means that two dyes should be used to enable separate detection. We recommend to use the following combinations: FAM - Eclipse® Dark Quencher for the target gene and Yakima Yellow® - Eclipse® Dark Quencher for the control gene.

When running a multiplex PCR, several targets and controls are in the same reaction, which means that multicolour detection is used. In that case we recommend to use the following combinations if possible. The combinations used in that case are dependent on the Real-Time thermocycler possibilities (table 2 p.29). FAM - Eclipse® Dark Quencher / Texas Red® - Eclipse® Dark Quencher/Cy® 5 - Eclipse® Dark Quencher for the target and Yakima Yellow®-Eclipse® Dark Quencher for the control.
In this case, well-to-well variations are avoided, but it should be clear that strong competition could alter the assay, as several amplicons are present within the same reaction.

Commonly used control kits

The table below describes the expression level of commonly used control genes. The target gene should ideally be combined with a control gene of about the same level of expression, which does not alter throughout the experiment. However, it should be taken into account that the expression level of these genes can vary per tissue (figure 24).

Table 4: Relative expression level and presence (+) or absence (-) of pseudogenes in the genome concerning the most commonly used control genes.

Symbol	Accession number	Name	Pseudogene	Relative expression level
-	M10098	18S rRNA		Very high
-	M11167	28S rRNA		Very high
ACTB	NM_00101	Beta actin	+	High
GAPD	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	+	High
UBC	M26880	Ubiquitin C	-	High
B2M	NM_004048	Beta-2-microglobulin	-	High
YWHAZ	NM_003406	Tyrosine 3-monooxygenase activation protein, Zeta polypeptide (Phospholipase A2)	+	Medium
RPL13A	NM_012423	Ribosomal protein L13a	+	Medium
SDHA	NM_004168	Succinate dehydrogenase complex, subunit A	+	Medium
HPRT1	NM_0000194	Hypoxanthine phosphoribosyl-transferase I	+	Medium
TBP	NM_003194	TATA box binding protein	-	Low
HMBS	NM_000190	Hydroxymethyl-bilane synthase	-	Low

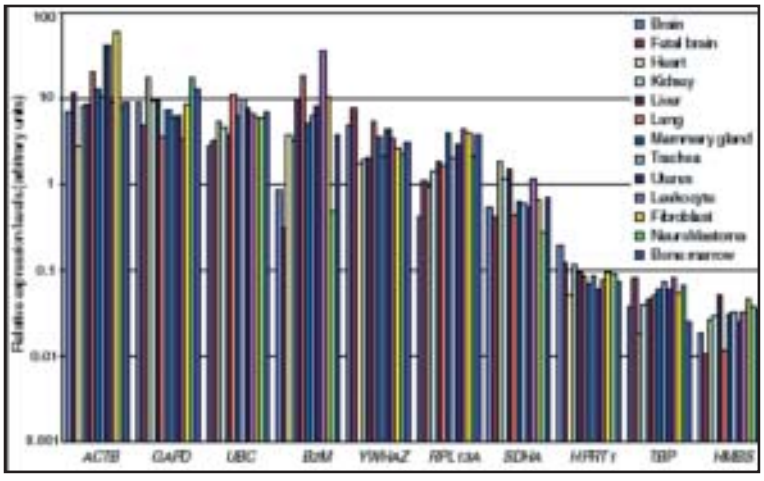

Figure 24: Logarithmic histogram of the expression level of no internal control genes determined in 13 different human tissues, normalized to the geometric mean of 6 control genes (Vandesompele et al., 2002).

Absolute quantitation

Absolute quantitation requires a standard curve of known copy numbers. It can be constructed using several methods. The amplicon being studied can be cloned, or a synthetic oligonucleotide can be used. Either DNA or RNA standards can be used. For RNA standards the cloned amplicon is transcribed to RNA or a single-stranded sense oligonucleotide can be made. There are several criteria for absolute standards. The standard must be amplified using the same primers as the gene of interest and must amplify with the same efficiency. The standards must also be quantified accurately. This can be carried out by reading the absorbance at A260, although this does not distinguish between DNA and RNA, or by using a fluorescent ribonucleic acid stain such as RiboGreen. Once a copy number value has been obtained for a sample it must be normalized to something. Often number of cells or total RNA is used. This requires accurate measurement of input RNA, again either by reading the absorbance or using a nucleic acid stain. Alternatively, an exogenous control can be used for normalization. An exogenous control gene is used to standardize the PCR reaction itself. It is an external DNA, which is spiked into the PCR reaction, and which would always come up at the same Ct value. The problem when using in vitro transcribed RNA as standard is that the construction of cDNA plasmid has to be done and has to be reverse transcribed. Moreover, it is not very stable for long term storage. The advantages of using cDNA plasmid standards is that cDNA plasmids are easy to construct, they can be prepared in large amount and can be stored for a very long time without any difficulties.

Qualitative PCR or Allelic Discrimination

In quantitative Real-Time PCR the goal is to detect the presence or absence of a certain sequence. It can be for virus sub-typing and bacterial species identification. It can also be used for allelic discrimination between wildtype and mutant, between different SNPs (Single Nucleotide Polymorphisms) or between different splicing forms. When using SYBR® green I, alleles can be distinguished by the difference in amplicon melting peaks caused by the slight differences in sequence. Specific detection methods are more often used for these applications and probes can be used to detect single base mutations or small deletions. They can also be used to detect different splice sites. The design and optimization of the assays for allelic discrimination is vital and is discussed further in later sections. An alternative to placing the probe over a polymorphism is to place the 3' end of one of the primers over the mutation. A mismatch in this region of the primer should prevent amplification and the probe can be used to simply detect whether there is specific amplification or not. It is often the case that mismatched primers will amplify the template to some degree but a difference in Ct will be observed.

How to set up a good assay?

PCR efficiency

In order to get accurate quantitation and reproducible results, Real-Time PCR reactions and especially assays performed in One step RT qPCR SYBR® green I format should have an efficiency close to 100 %.

The PCR efficiency is influenced by the following actors:
- length of the amplicon
- GC content of the amplicon
- secondary structures in primers and / or probe and / or amplicon
- concentration of reaction components

With all Eurogentec kits PCR efficiencies of 100 % can be obtained if the primers are well designed. Good primers can be found when following the design guidelines as mentioned on p.42.
If the slope of this standard curve is –3.32 the PCR is 100 % efficient.
To ensure that only one product has been amplified a meltcurve can be made.
If the PCR efficiency is drastically lower than 100 % we recommend to:
- check the primer design
- optimize the primer concentration
- optimize the temperature reaction and times
- go for a 3-step instead of a 2-step protocol

How to determine the PCR efficiency?

The slope of the standard curve can be used to determine the exponential amplification and efficiency of the PCR reaction:

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

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

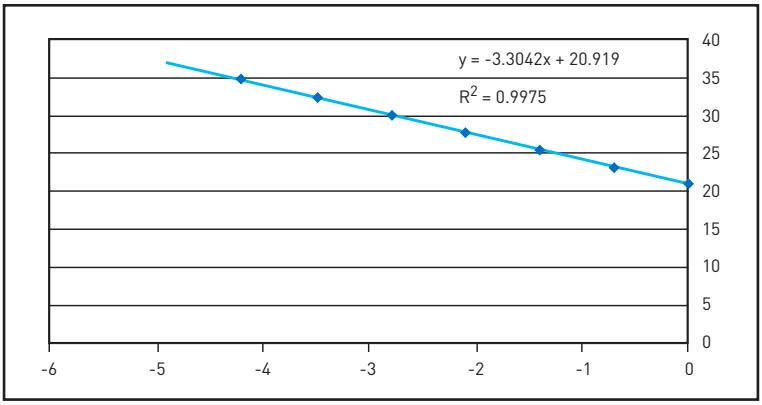

FIG.26: Standard curve made with a 10x dilution series using the qPCR MasterMix for SYBR® green I

A 100 % efficiency corresponds to a slope of –3.32.
With 100 % efficiency, a 2x dilution gives a ΔCt of 1 between every dilution (every cycle the amount of amplification is doubled).
With a 100 % efficiency, a 10x dilution gives a ΔCt of 3.2 values between each dilution (every 3.2 cycles the amount of amplification is 10 fold higher).
The R^2 is a parameter, which tells you how well the datapoints lie on line. If the $R^2 < 0.95$ there is an indication that either the reactions have not been pipeted accurately or that their is no linear relation between the Ct and the 10log of the DNA concentration. The latter can be caused by inhibitory factors that are diluted out.

Quality of starting material

Using poor quality RNA can limit the efficiency of the reverse transcriptase reaction and limit the yield of the RT reaction. Therefore RNA extraction should always be performed carefully. We recommend the use of commercially available kits to obtain good quality RNA.
Diluted solutions of RNA should be aliquoted to avoid several freeze / thaw cycles.
RNA samples should be stored between –20 °C and –70 °C in RNase free water (DEPC treated water).

Standard curve

By preparing a standard curve for every gene, which needs to be analyzed, a good idea can be obtained about the performance and efficiency of the PCR. The standard curve should cover the complete range of expected expression levels (in general 6 logs of magnitude).
The standard curve gives much information on the assay (PCR efficiency, pipeting errors, and sensitivity).

Multiplex negative controls

- Use a no Reverse Transcriptase control to exclude genomic DNA contamination (reaction with all the components except the reverse transcriptase).
- Use a no target control to exclude any contamination from reagents or previous PCR (reaction with all the components except the template).
- In some cases, it could be necessary to have a positive control containing a known concentration of template to test presence or absence of the target gene to check for inhibitory factors.

Primer and probe design guidelines

Well-designed primers and probes are a prerequisite for successful RT qPCR. By using well-designed primers and probes PCR efficiencies of 100 % can be obtained when using Eurogentec RT qPCR or qPCR kits.

If the following primer design guidelines are taken into account you will achieve high PCR efficiencies, specific PCR products, non co-amplification of gDNA and therefore the most sensitive results.
We do recommend in general to use a design software (for example Oligo® Primer Analysis Software) to check for all following criteria.

Design guidelines for SYBR® green I assays

As SYBR® green I binds to any dsDNA it is important to avoid primer-dimers and / or non-specific products in SYBR® green I assays. This can only be avoided by carefully selecting primers that only bind to the selected target. By selecting amplicons between 100 and 150 bp a high level of fluorescence can be obtained without compromising the PCR efficiency.

Primers

- length
  - 18-30 bases
- GC content
  - 30-80 % (ideally 40-60 %)
- Tm
  - 63-67 °C (ideally 64 °C)(Tm=2(number A+T)+4(number G+C)), so that Tannealing is 58-62 °C (ideally 59 °C)
  - ΔTm 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 with Oligo® Primer Analysis Software)

- avoid complementarity between the primers to avoid primer dimers, especially at 2 or more bases at the 3' ends of the primers (check with Oligo® Primer Analysis Software)
- design intron spanning or intron flanking primers to prevent or identify amplification of contaminating genomic DNA. For intron spanning primers the first half of the oligo must hybridize to the 3' end of one exon and to the 5'end of the other exon. In this way only cDNA will be amplified and gDNA not. For intron flanking primers the forward primer must hybridize to one exon and the reverse primer to the other exon. Amplicons from cDNA, without intron, will be smaller than the amplicons from gDNA, which will contain the intron. The bigger amplicon will be amplified less efficiently. The difference in size of these amplicons can be determined via meltcurve analysis. If genes of only one exon are studied contamination with gDNA can only be avoided by DNase treatment of the RNA with RNase free DNase (Vandesompele, 2002).
- check if primers are unique and specific (check with BLAST: www.ncbi.nlm.nih.gov/BLAST/).

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 Double-Dye Oligonucleotide probe assays

In probe assays, primer dimers and non-specific products will not be detected, however, they will influence the PCR dynamics and efficiency. Therefore, also in probe assays they should be avoided as much as possible. For probe assays the amplicons should be kept as short as possible, with the 5' end of the probe as close as possible to the 3' end of the forward primer in case the probe is on the same strand, and as close as possible to the 3' end of the reverse primer in case the probe is on the opposite strand. In this way the 5' nuclease reaction will be optimal.

Experience has learned that it is easier to first design the probe and then the primers than the other way around.

By selecting quenchers that fit the fluorophores used with the Double-Dye Oligos you will be able to obtain high signal. Eclipse® Dark Quencher gives better signal to noise ratios than TAMRA. In general, we recommend to take fluorophores that can be coupled efficiently to the oligo during the synthesis. Examples of fluorophores which can be coupled efficiently are FAM, HEX, TET, Yakima Yellow®, Texas Red®, Cy® 3 and Cy® 5. ROX is an example of label, which is hard to couple efficiently.

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 (normally coupled to a T)
- 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 the probe as close as possible to the primers without overlapping them
- avoid mismatch between probe and target
- avoid complementarity with in the probe
- 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

Primers

- length
  - 18-30 bases
- GC content
  - 30-80 % (ideally 40-60 %)
- Tm
  - 63-67 °C (ideally 64 °C)(Tm=2(number A+T)+4(number G+C), so that Tannealing is 58-62 °C (ideally 59 °C)
  - ΔTm 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 with Oligo® Primer Analysis Software)
- avoid complementarity between the primers to avoid primer dimers, especially at 2 or more bases at the 3' ends of the primers (check with Oligo® Primer Analysis Software)
- design intron spanning or intron flanking primers to prevent or identify amplification of contaminating genomic DNA for intron spanning primers the first half of the oligo must hybridize to the 3' end of one exon and to the 5'end of the other exon. In this way only cDNA will be amplified and gDNA not. For intron flanking primers the forward primer must hybridize to one exon and the reverse primer to the other exon. Amplicons from cDNA, without intron, will be smaller than the amplicons from gDNA, which will contain the intron. The bigger amplicon will be amplified less efficiently. The difference in size of these amplicons can be determined via meltcurve analysis. If genes of only one exon are studied contamination with gDNA can only be avoided by DNase treatment of the RNA with RNase free DNase.
- check if primers are unique and specific (check with BLAST: www.ncbi.nlm.nih.gov/BLAST/)

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/).

Design guidelines for Scorpions® primers assays

When designing Scorpions® primers the folding of the primer and probe is the crucial feature. The probe sequence should be close to the 3' end of the primer intended to be attached to the probe. The probe should be complementary to the extension of this primer, making probing intramolecular. In order to be in the correct orientation the probe should be written so that the 5' end is complementary to the 3' end of the target (the probe should be the reverse complement of the target). The probe sequences should be about 17-27 bases. The probe target should be 11 bases or less from the 3' end of the Scorpions®. Although a greater distance works, the further away the probe target the lower the probing efficiency, and the advantage of using intramolecular probe is lost. A stem sequence of 6-7 bases, mostly C's and G's, avoiding repetitive motifs (as CGCGCG), should be added to the end of the probe so that the two regions of stem can bind to one another and hold the probe in a loop configuration. The 5' end stem sequence should begin with a C, as a G may quench the dye.
The following web site: http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi should be used to fold Scorpions® (probe, stem and primer) at 0.004 M $MgCl_2$ and 0.01 M Na^+. The folding temperature should be the temperature at which the fluorescence will be monitored. This parameter often needs optimizing and a good starting temperature for the folding is 60 °C. Fold both the Scorpions® sequence alone and the Scorpions® with amplicon. The design should be such that a stem loop is produced for Scorpions® with amplicon. This may mean altering the stem sequence and / or length of the probe sequence or adjusting the temperature. The ΔG value should be negative and ideally the stem loop should have a Tm of 5-10 °C higher than the probe bound to the target. The ΔG value is more important. When designing a probe for mutation detection the same principles apply. A design should be found where the probe is bound to the perfectly matched target but has dissociated from the target with a mismatch. Once the sequence has been designed the fluorophore is placed on the 5' end and Deep Dark Quencher I, and a PCR stopper are placed between the primer and the hairpin.

Design guidelines for Molecular Beacons probe assays

Design of Molecular Beacons hairpin loops is fairly similar to Scorpions® primers design except that intramolecular folding does not need to be taken into account. The hairpin structure needs to be checked in the DNA folding programme. The Tm of the Beacon probe sequence should be 7-10 °C higher than the annealing temperature of the primers. In contrast to the probes discussed above, Molecular Beacons should be designed in the centre of the amplicon with greater than 6 bases between the Beacon and the 3' end of the primers.

Protocols

RNA extraction or DNA extraction

For the extraction of RNA or DNA from the samples, the use of an appropriate commercially available kit is recommended. There are many different kits available, which will contain all the required reagents for the full extraction / purification procedure.
These kits will also outline general guidelines, such as the storage conditions and shelf life of the extracted RNA or DNA. However these guidelines may vary between kits due to the different composition of buffers. It is also recommended that the buffers supplied with each kit are used according to the protocol.

The final product should be cleaned and free from any residual buffers that may inhibit the PCR such as EDTA or solvent containing buffers. This is usually not a problem when using spin column kits instead of manual extraction techniques.

Reverse Transcription reaction

If RNA is used, we recommend for the reverse transcription step the use of the Eurogentec Reverse Transcriptiase Core Kit as the formulation of this is adapted to the formulation of the Eurogentec qPCR Core Kits and qPCR MasterMixes.

Two step RT qPCR set-up

The Eurogentec kits are supplied with random nonamers and oligo d(T) VN primers, but the kit can also be used with sequence specific primers. Random nonamers will bind to and transcribe through out the RNA in the reaction leading to the creation of random cDNAs. Oligo d(T) VN primers bind to the poly-A region of the RNA and transcribe from these points. The use of sequence specific primers for RT reaction means that only the sequence of interest is transcribed from the RNA in to the cDNA. Full details of the content of this reaction mix are supplied in the technical data sheet supplied with the kit.

The RT reaction should be set up in a clean environment to avoid contamination; cleaning solutions are available to avoid any RNAse contamination (RNASE away). We also recommend to work with RNAse free plastics. The tubes containing the reaction should be maintained on ice during the set up of the reaction. This will avoid early reaction generated before the incubation.

The RT reactions are incubated at 48 °C for 30 minutes with a final hold of 5 minutes at 95 °C to inactivate the RT enzyme. If sequence specific primers or oligod(T) VN primers are used and initial 10 minutes prehybridization at 25 °C should be performed prior to the 30 minutes incubation. The cDNA generated should be stored between –70 °C to 80 °C.

The use of a separate RT step is recommended when the reaction is performed with a limiting amount of starting material.

One step RT qPCR set-up

In a One step RT qPCR, both the RT reaction and the PCR reaction are performed in a single tube. With a One step RT qPCR only sequence specific primers can be used. These primers are the same as those to be used in your PCR.

The reaction is incubated at 48 °C for 30 minutes, and then the PCR is performed after the RT. The 95 °C hold required for the activation of the hotstart enzyme will also cause deactivation of the RT enzyme.
The precautions described for the set-up of two-step RT qPCR reaction should also be followed for the set-up of a One step RT qPCR reaction.

qPCR reaction

When setting up a qPCR reaction, it is always recommended to prepare a reaction mix, containing the primers, probes and all the reagents required for the reaction. This minimizes differences across the plate and allows for more accurate pipeting (as the volume required per sample is usually very small).

The reaction mix should ideally be prepared in a separate room, different from the room where the DNA samples have been prepared, to avoid any contamination.
The DNA samples should always be added to the side of each well before the reaction mix and then rinsed down while adding the reaction mix. As the reaction mix is heavier than the DNA, the DNA will be mixed within the reaction mix.

The plate can be shaken on a plate shaker and spun before placing the plate on the machine. This is not an essential step but will ensure that the reactions are toughly mixed and collected at the bottom of the reaction tube. It is useful to also check the wells for bubbles, as bubbles at the bottom of the well can produce unusual plots on the results, when using machines that read from the top.

The no template controls should be placed on the plate in such a position that cross contamination is avoided during the set up; thus, they should be placed if possible away from the highest DNA concentrations, at the top or bottom of the rows to avoid going over these wells when pipeting.

When sealing the PCR plates, it is important to ensure that it has been correctly done. If optical films are used, fingerprints and marks should be avoided on the top of the film.
The Real-Time thermocycler used should be programmed according to the manufacturers instruction manual.
The ideal thermal cycling conditions can vary from assay to assay. The technical data sheet supplied with the Eurogentec qPCR kits should be used as a good starting point, but optimization of these conditions may be required for certain assays to achieve optimal results.

Standard cycling conditions for Double-Dye Oligonucleotides are as follows:

50 °C for 2 minutes if UNG is used	
95 °C for 10 minutes	
40 cycles of	
	95 °C for 15 seconds
	60 °C for 1 minute

This is a two-step PCR where the extension and annealing step are a combined 60 °C step. This is possible since the amplicons are designed to be very short and can be copied without requiring a 72 °C extension. This protocol should be used initially when testing a new assay.

Double-Dye Oligonucleotides probes, which are designed using Primer Express®, will work optimally under these conditions. However, Molecular Beacons and Scorpions® primers may need an additional step for monitoring the fluorescence as the optimal temperature for fluorescent signal or allelic discrimination may be different to the annealing / extension step. Melt curves are useful for determining this temperature. The annealing temperature may require optimization for any of the technologies.

In the following cases we recommend to perform a 3-step protocol to obtain better results: when performing SYBR® green I assays, if results show late Ct values, if only primer dimers are detected or if the results give a less steep growth curve and if in this special case it is not possible to re-do the design.
The protocol will then be as follows:

40 cycles	denaturation	95 °C for 15 seconds
	annealing	60 °C for 30 seconds
	extension	72 °C for 30 seconds

Extension time can be increased with 10-second steps.
It is also possible to add a data collection step after the extention step. To do so, ensure that the detection temperature is at least 3 °C lower than the Tm of the amplicon.

In addition to the cycling parameters there are two temperature steps at the beginning of the reaction that have specific functions.
The 50 °C step is activating the UNG (Uracil-N-Glycosilase). UNG is used as a contamination control system. In that case, dUTP is used instead of dTTP and UNG will weaken glucosidics bonds in DNA with U's instead of T's. A heating step will then deactivate the UNG and break the glycosidic bonds fragmenting the DNA so it cannot be amplified. UNG is useful if any PCR products from a previous amplification can contaminate the reaction.
The 95 °C step for 10 minutes is used to activate the hotstart *Taq* polymerase. A hotstart enzyme is inactive at low temperatures (room temperature). This prevents from formation of non-specific amplification and reduces primer dimer formation. Heating at 95 °C during 10 minutes activates the enzyme and the amplification can begin once the primers are annealed.

Results Analysis

All instruments are supplied with some analysis software. Instructions related to the use of this software will be contained within the manual.

Some analysis packages will allow more interaction and manipulation of the data than others, but most will allow baseline and threshold / fit point adjustment.

Literature available from Eurogentec

Available on the Eurogentec web page:
- Frequently Asked Questions
- Troubleshooting Guide
- Technical Datasheets
- Material Safety Datasheets
- (Certificates of analysis)

Eurogentec products

Kits and consumables

Our MasterMixes are developed to work with standard temperature profiles, to be completely adapted to any assay. However, it is recommended to generate a primer optimization matrix and a primer and probe ratio matrix, as this is a crucial step to obtain the lowest signal to noise ratio, the earliest Ct value and to save maximum reagents.

Primer optimization matrix

Optimize according to the table 5, performs qPCR's selecting the one with the lowest Ct value.
By doing this type of optimization it is also possible to compensate for differences up to 2 °C in melt temperature of the primers.

Table 5. Primer-probe ratio matrix

	Forward		
Reverse	50 nM	300 nM	900 nM
50 nM	50 / 50	300 / 50	900 / 50
300 nM	50 / 300	300 / 300	900 / 300
900 nM	50 / 900	300 / 900	900 / 900

Primer-probe ratio matrix

Select optimal primer concentration as described above and test with all probe concentrations like in table 6. Select the concentration, which gives the lowest value.

Table 6. Primer-probe ratio matrix

	Probe		
	50 nM	125 nM	250 nM
Opt. Primers	50 / opt	125 / opt	250 / opt

qPCR kit compatibility with Real-Time thermocycler

			ABI GeneAmp® SDS 5700 ABI Prism® SDS 7000 / 7700 / 7900HT 7500 / 7300	iCycler iQ®	Mx4000® Mx3000p®	DNA Engine Opticon® DNA Engine Opticon® 2 Chromo 4	RotorGene 2000 RotorGene 3000	SmartCycler® I SmartCycler® II	Capillary thermocycler
With ROX passive reference	Reference	# RXN							
qPCR MasterMix plus w/o UNG	RT-QP2X-03WOU+	300	X		X				
qPCR SYBR® MasterMix Plus	RT-SN2X-03WOU+	300	X		X				
qPCR MasterMix	RT-QP2X-03	300	X		X				
qPCR MasterMix for SYBR® green I	RT-SN2X-03T	300	X		X				
qPCR MasterMix Plux 7.5 ml	RT-QP2X-03-075+	300	X		X				
qPCR SYBR® MasterMix Plus I 7.5 ml	RT-SN2X-03+	300	X		X				
qPCR MasterMix Plus 15 ml	RT-QP2X-03-15+	600	X		X				
qPCR SYBR® MasterMix Plus 15 ml	RT-SN2X-06+	600	X		X				
qPCR MasterMix Plus 50 ml	RT-QP2X-03-50+	2000	X		X				
qPCR SYBR® MasterMix Plus 50 ml	RT-SN2X-20+	2000	X		X				
qPCR Core kit	RT-QP73-05	500	X		X				
qPCR Core kit for SYBR® green I	RT-SN10-05	500	X		X				
Without ROX passive reference									
qPCR MasterMix No ROX	RT-QP2X-03NR	300		X	X	X	X		
qPCR SYBR® MasterMixPlus	RT-SN2X-03+NR	300		X	X	X	X		
qPCR Core kit No ROX	RT-QP73-05NR	500		X	X	X	X		
qPCR SYBR® Core kit	RT-SN10-05NR	500		X	X	X	X		
Fluorescein Additive	RT-FLUO-ADD	1 ml		X					
Smart kit	RT-QP2X-CE	500						X	
Smart kit for SYBR® green I	RT-SN2X-CE	500						X	
Lithos qPCR MasterMix 2 mM	RT-SN73-35LC2	350							X
Lithos qPCR MasterMix 3 mM	RT-SN73-35LC3	350							X
Lithos qPCR MasterMix 4 mM	RT-SN73-35LC4	350							X
Lithos qPCR MasterMix HotStart 2 mM	RT-SNHS-35LC2	350							X
Lithos qPCR MasterMix HotStart 3 mM	RT-SNHS-35LC3	350							X
Lithos qPCR MasterMix HotStart 4 mM	RT-SNHS-35LC3	350							X

RT qPCR kit compatibility with Real-Time thermocycler

	Reference	# RXN	ABI GeneAmp® SDS 5700 ABI Prism® SDS 7000 / 7700 / 7900HT 7300 / 7500	iCycler iQ®	Mx4000® Mx3000p®	DNA Engine Opticon® DNA Engine Opticon® 2 Chromo 4	RotorGene 2000 RotorGene 3000
With ROX passive reference							
One step RTqPCR MasterMix	RT-QPRT-032X	300	X		X		
One step RTqPCR SYBR® MasterMix	RT-SNRT-032X	300	X		X		
Two step RTqPCR MasterMix	RT-QP2X-03RT	300	X		X		
Two step RTqPCR SYBR® MasterMix	RT-SN2X-03RT	300	X		X		
Two step RTqPCR Core kit	RT-QP73-05RT	500	X		X		
Two step RTqPCR SYBR® Core kit	RT-SN73-05RT	500	X		X		
Without ROX passive reference							
One step RTqPCR MasterMix No ROX	RT-QPRT-032XNR	300		X	X	X	X
One step RTqPCR SYBR® MasterMix	Please inquire	300		X	X	X	X
Two step RTqPCR MasterMix kit No ROX	RT-QP2X-03NRRT	300		X	X	X	X
Two step RTqPCR SYBR® MasterMix	RT-SN2X-03NRRT	300		X	X	X	X
Two step RTqPCR Core kit No ROX	RT-QP73-05NRRT	500		X	X	X	X
Two step RTqPCR SYBR® Core kit	RT-SN73-05NRRT	500		X	X	X	X
Fluorescein additive	RT-FLUO-ADD	1 mL		X			

Control kits	Reference	# RXN	ABI GeneAmp® SDS 5700 ABI Prism® SDS 7000 / 7700 / 7900HT 7300 / 7500	iCycler iQ®	Mx4000® Mx3000p®	DNA Engine Opticon® DNA Engine Opticon® 2 Chromo 4	RotorGene 2000 RotorGene 3000	SmartCycler® I SmartCycler® II
18S rRNA FAM-TAMRA	RT-CKFT-18S	250	X	X	X	X	X	X
18S rRNA Eclipse®-Yakima Yellow®	RT-CKYD-18S	250	X	X	X	X	X	X
23S rRNA	RT-CKFT-23S	250	X	X	X	X	X	X
28S rRNA	RT-CKYD-28S	250	X	X	X	X	X	X
ß-actin	RT-CKYD-ACTB	250	X	X	X	X	X	X
GAPDH	RT-CKYD-GAPD	250	X	X	X	X	X	X
ß -2-microglobulin	RT-CKYD-B2M	250	X	X	X	X	X	X
Phospholipase A2	RT-CKYD-YWHAZ	250	X	X	X	X	X	X
Hydroxymethyl-bilane synthase	RT-CKYD-HBMS	250	X	X	X	X	X	X
Hypoxanthine phosphoribosyltransferase	RT-CKYD-HPRT1	250	X	X	X	X	X	X
Ribosomal protein L13a	RT-CKYD-RPL13A	250	X	X	X	X	X	X
Succinate deshydrogenase complex	RT-CKYD-SDHA	250	X	X	X	X	X	X
TATA box binding protein	RT-CKYD-TBP	250	X	X	X	X	X	X
Ubiquitin C	RT-CKYD-UBC	250	X	X	X	X	X	X
Universal RT kits								
Reverse Transcriptase Core kit	RT-RTCK-03	300	X	X	X	X	X	
Reverse Transcriptase Core kit	RT-RTCK-05	500	X	X	X	X	X	
Carryover prevention								
UNG (Uracyl-N-Glycosylase)	RT-0610-03	500 U	X	X	X	X	X	X
UNG (Uracyl-N-Glycosylase)	RT-0610-15	1500 U	X	X	X	X	X	X
96-well Plates								
96-well Plates natural	RT-PL96-01N	4 x 5 plates + caps	X (except ABI Prism® 7900 HT)	X	X	X		
96-well Plates black	RT-PL96-01B	4 x 5 plates + caps	X (except ABI Prism® 7900 HT)	X	X	X		

Double-Dye Oligonucleotide probes

5'FAM + 3'TAMRA	40 nmol	OL-0361-01004/TA
5'FAM + 3'TAMRA or Eclipse® Dark Quencher or DDQI	0.2 µmol	OL-0361-0102/TA/DQ
5'FAM + 3'TAMRA or Eclipse® Dark Quencher or DDQI	1.0 µmol	OL-0361-0110/TA/DQ
5'HEX + 3'TAMRA or Eclipse® Dark Quencher or DDQI	0.2 µmol	OL-0361-0102/TA/DQ
5'HEX + 3'TAMRA or DABCYL or Eclipse® DQ or DDQI	1.0 µmol	OL-0361-0110/TA/DQ
5'TET + 3'TAMRA or Eclipse® Dark Quencher or DDQI	0.2 µmol	OL-0361-0102/TA/DQ
5'TET + 3'TAMRA or Eclipse® Dark Quencher or DDQI	1.0 µmol	OL-0361-0110/TA/DQ
5'TAMRA + 3'DDQII or DDQI	0.2 µmol	OL-0361-0502EQ/MR
5'TAMRA + 3'DDQII or DDQI	1.0 µmol	OL-0361-0510EQ/MR
5'Texas Red® + 3'DDQII or DDQI	0.2 µmol	OL-0361-0902EQ/MR
5'Texas Red® + 3'DDQII or DDQI	1.0 µmol	OL-0361-0910EQ/MR
5'Cy® dyes + 3' DDQII	0.2 µmol	OL-0361-1102EQ
5'Cy® dyes + 3' DDQII	1.0 µmol	OL-0361-1110EQ
5' Yakima Yellow® + 3' Eclipse® Dark Quencher or DDQI	0.2 µmol	OL-0361-1202DQ/MR
5'YakimaYellow® + 3' Eclipse® Dark Quencher or DDQI	1.0 µmol	OL-0361-1210DQ/MR

If other labels required, please inquire

Molecular Beacons

5'FAM + 3' DABCYL	0.2 µmol	OL-0371-0102DA
5'FAM + 3' DABCYL	1.0 µmol	OL-0371-0110DA
5'TET + 3' DABCYL	0.2 µmol	OL-0371-0302DA
5'TET + 3' DABCYL	1.0 µmol	OL-0371-0310DA
5'Texas Red® + 3'DABCYL	0.2 µmol	OL-0371-0902DA
5'Texas Red® + 3'DABCYL	1.0 µmol	OL-0371-0910DA
5'Cy® dyes + 3'DABCYL	0.2 µmol	OL-0371-1102DA
5'Cy® dyes + 3'DABCYL	1.0 µmol	OL-0371-1110DA

if other labels required, please inquire

Scorpions® primers

Scorpions® primers ROX/FAM FRET	0.2 µmol	OL-0361-5801
Scorpions® primers	0.2 µmol	OL-0361-S901
Duplex Scorpions® primers	0.2 µmol	OL-0361-S1001

Scorpions® primers are available with FAM / HEX / TET / JOE and ROX labeling and are quenched with Deep Dark Quencher I.

FRET probes

3'FAM	0.2 µmol	OL-0390-LCFAM02
5'ROX	0.2 µmol	OL-0390-LRROX02
5'Cy®5	0.2 µmol	OL-0390-LCCY02

Dye compatibility table with Real-Time thermocyler (p.29)

Product citations

Oligonucleotides and Real-Time qPCR probes

ASSAF S., HAZARD D., PITEL F., MORISSON M., ALIZADEH M., GONDRET F., DIOT C., VIGNAL A., DOUAIRE M., and LAGARRIGUE S., "Cloning of cDNA Encoding the Nuclear Form of Chicken Sterol Response Element Binding Protein-2 (SREBP-2), Chromosomal Localisation, and Tissue Expression of Chicken SREBP-1 and –2 Genes", Science, Vol.82, 54-61, 2003

DALPKE A.H., SCHÄFER M.K.H, FREY M., ZIMMERMANN S., TEBBE J., WEIHE E. and HEEG K., "Immunostimulatory CpG-DNA Activates Murine Microglia", The Journal of Immunology, Vol.168, 4854-4863, 2002

DE KERCHOVE A., EXAERDE D., CARTAUD J.C., RAVEL-CHAPUIS A., SEROZ T., PASTEAU F., ANGUS L.M., JAMSIN B.J., CHANGEUX J. and SCHAEFFER L., "Expression of mutants Ets protein at the neuromuscular synapse causes alterations in morphology and gene expression", EMBO reports, Vol.3 (11), 2002

FAURE M., GLOMOT F., PAPET I., "Branched-Chain Amino Acid Aminotransferase Activity Decreases during Development in skeletal Muscles of sheep", JH. Nutr, Vol.131, 1528-1534, 2001

HERNANDEZ M., PLA M., ESTEVE T., PRAT S., PUIDOMENECH P. and FERRANDO A., "A specific real-time quantitative PCR detection system for event MON810 in Maize YieldGard® based on the 3'-transgene integration sequence", Transgenic Research, Vol.12, 179-189, 2003

ILVANDERBROUCKE I., VANDESOMPELE J., DEPAEPE A. and MESSIAEN L., "Quantification of splice variants using Real-Time PCR", Nucleic Acids Research, Vol.29 (13), 2001

MASON M., PROVERO P., VAIRA A.M. and PAOLO P., ACCOTTO, "Estimating the number of integrations in transformed plants by quantitative real-time PCR",.BMC Biotechnology, Vol.2 (20), 2002

OVERBERGH L., GIULIETTI A., VALCKS D., DECALLONNE B., BOUILLON R. and MATHIEU C. J., "The use of real-time transcriptase PCR for the quantification of Cytokine Gene Expression", Bimol.Tech, Vol.14, 33-43, 2003

ULVIK A., UELAND P.M., "Single nucleotides Polymorphism (SNP) genotyping in unprocessed whole blood and serum by Real Time PCR: Application to SNPs affecting homocistein and Folate Matabolism", Clinical Chemistry, Vol.47 (11), 2050-2053, 2001

VERSTREPEN W.A., KUHN S., KOCKX M.M., VAN DE VYVERE M.E. and MERTENS A.H., "Rapid detection of anterovirus RNA in cerebrospinal fluid specimens with a novel single tube real time reverse transcription PCR assay", Journal of Clinical Microbiology, Vol.39 (11), 4093-4096, 2001

VOGT M.H.J, VAN DEN MUIJSENBERG J.W., GOULMY E., SPIERINGS E., KLUCK P., KESTER M.G., VAN SOEST R.A., DRIJFHOUT J.W., WILLEMZE R. and FALKENBURG BLOOD J.H.F., "The DBY gene codes for an HLA-DQ5-rstricted human male-specific minor histocompatibility antigen involved in graft-versus-host disease", Blood, Vol.99 (8), 3027-3032, 2002

VON DER HARDT V., SCHOOF E., KANDLER M.A., DÖTSH J. and RASCHER W., "Aerolized perfluorocarbon Supress Early Pulmonary Inflammatory Response in a surfactant-Depleted Piglet Model", Pediatric Research, Vol.51, (2), 2002

qPCR and RTqPCR kits

ABOU-BACAR A., PFAFF A.W., GEORGES S., LETSHER-BRU V., FILISETTI D., VILLARD O., ANTONI E., KLEIN J.P., CONDOLFI E., "Role of NK cells and gamma interferon in transplacental passage of toxoplasma gondii in mouse model of primary infection", Infection and Immunity, Vol.72 (3), 1397-1401, 2004

GORBERT V., GOTTAR M., MATSKEVICH A., RUTSCHMANN S., ROYET J., BELVIN M., HOFFMAN J., FERANDON D., Dual Activation of Drosophila Toll pathway by two pattern recognition receptors, Science, Vol.302 (5653), 2126-30, 2003

GAO L., CUETO M.A., ASSELBERGS F., ATADJA P., "Cloning & and functional characterization of HDAC11, a novel member of the human histone Deacetylase Family", The journal of Biological Chemistry, Vol.277 (28), 25748-25755, 2002

GOBERT V., GOTTAR M., MASKEVICH A.A., RUTSCHMANN S., ROYET J., BELVIN M., HOFFMAN J.A., FERRANDON D., HAYETTE M.P., VAIRA D., SUSIN F., BOLAND P., CHRISTIANAENS G., MELIN P. and DEMOL P., "Detection of Aspergillus Species DNA by PCR in Bronchoalveolar lavage Fluid", Journal of Clinical Biology, Vol.39 (6), 2338-2340, 2001

HEIBREDER M., FRÖHLICH F., JÖHREN O., DENDORFER A., QADRI F., DOMINIAK P., "Sendai virus targets inflammatory responses, as well as the interferon induced antiviral state, in multifaceted manner", Journal of Viorology, Vol.77, 7903-7913, 2003

HEMMINGS-MIESZCZAK M., DORN G., NATT F.J., HAL J. and WISHART W., "Independent combinatorial effect of antisense oligonucleotides and RNAi-mediated specific inhibition of the recombinant Rat P2X3 receptor", Nucleic Acids Research, Vol.31 (8), 2117-2126, 2003

LARITZ EN I., ZANZURI M., HONORE E., DUPRAT F., EHRENGRUBER M., LAZDUNSKI M., PATEL A., "K+ dependent cerebelar Granule Neuron Apoptosis", The Journal of Biological Chemistry, Vol.278 (34), 32068-32076; 2003

LE CANN P., RANARIJAONA S., MONOEHO S., LEGYADER S., FERRE V., "Quantification of human astroviruses in sewage using real-time RT-PCR", Res. Microbiol., Vol.155 (1), 11-15, 2003

LI-WEBER M., WEIGAND M.A., GIAISI M., SÜSS D., TREIBER M.K, BAUMANN S., RITSOU E., BREITKREUTZ R. and KRAMMER P.H., "Vitamin E inhibits CD95 ligand expression and protects T cells from activation-induces cell death", Clin. Invest, Vol.110, 681-690, 2002

MARTI D., GROSSNIKLAUS-BÜRGIN C., WYDER S., WYLER T. and LANZREIN B., "Ovary development and polydnavirus morphogenesis in the parasitic wasp Chelonus inanitus. I. Ovary morphogenesis, amplification of viral DNA and ecdysteroid titres", Journal of General Virology, Vol.84, 1141-1150, 2003

MASTERNAK K., REITH W., "Promoter-specific functions of CIITA and the MHC class II enf-hanceosome in transcriptional activation", The EMBO Journal, Vol.21 (6), 1379-1388, 2002

WANG S., NEALE Y., FISHER D., ZEREMSKI M., COI R., ZHU J., BAGS F.A., HAMPTON G., COHEN D., "Histone deacetylase 1 represses the small GTPase RhoB expression in human non small lung carcinoma cell line", Norvatis research corporation, Oncogene, Vol.22, (13), 6204-62, 2003

YANG Z., TSCHOPP O., HEMMINGS-MIESCAK M., FENG J., BRODBECK D., PERENTES E., and HEMMINGS B.A., "Protein Kinase B_/Akt1 regulates placental Development and fetal Growth", The Journal of Biological Chemistry, Vol.278 (34), 32124-32131, 2003

References

BUSTIN S., "Absolute quantification of mRNA using Real-Time reverse transcriptin polymerase chain reaction assays", Journal of Molecular Endocrinology, Vol.25, p.169-193, 2000

BUSTIN S., "Quantification of mRNA using Real-Time reverse transcription PCR (RT-PCR); trends and problems", Journal of Molecular Endocrinology, 29, 23-39, 2002

CAPLIN B.E, RASMUSSEN R.P., BERNAR P.S. and WITTWER C.T, "LigthCycler® Hybridisation probes – the most direct way to monitor PCR amplification and mutation detection"; Biochemica; Vol.1, 5-8, 1999

FREEMAN W.M., WALKER S.J., VRANA K.E., "Quantitative Rt-PCR:pitfalls and potential", Biotechniques, Vol.112 (22), 124-125, 1999

GIULIETTI A., OVERBERGH L., VACKX D., DECALLONNE B., BOUILLON R. and MATHIEU C., "An overview of Real-Time Quantitative PCR: Application to quantify cytokine gene expression", Methods, Vol.25, 386-401, 2001

HALFORD W.P., "The essential prerequisites for quantitative RT-PCR: pitfalls and potetial", Nature Biotechnology, Vol.17 (19), 835, 1999

HEID C.A, STEVENS J., LIVAK K.J., WILLIAMS P.M., "Real-Time quantitative PCR", Genome Research, Vol.6 (10), 986-94, 1996

HOLLAND P., ABRAMSON R.D., WATSON R. and GELLAND D.H., "Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of thermos aquatus.", Proc. Natl, Acad, Sci. USA, Vol.88, 7276-7280, 1991

JU J., RUAN C., FULLER C.W., GLAZER A.A. and MATHIES R.A., ''Fluorescence energy transfer dye-labeled primers for DNA sequencing and Analysis'', Proc. Natl. Acad. Sci. USA., Vol.92, 4347-4351, 1995

KUTYAVIN I., AFONINA A., MILLS A., GORN V.V., LUJHTANOV E.A., BELOUSOV E., SINGER M., WALBURGER D.K., LOKHOV S.G., GALL A.A., DEMPCY R., REED M., MEYER R., HEDPETH J., "3' minor groove DNA probes increase sequence specificity at PCR extension temperature", Nucleic Acids Research, Vol.28 (2), 2000

LETERTRE C., PERELLE S., DILASSE F., ARAR K., FACH P., "Evaluation of the performance of LNA and MGB probes in 5' nuclease assays", Molecular and cellular probes, Vol.17 (6), 307-11, 2003

LI Q., LUAN G., GUO Q., LIANG J., "A new class of homogenous nucleic acid probes based on specific displacement hybridization", Nucleic Acid Research, Vol.30 (2), 2002

MYAKISHEV M., KHRIPIN Y., MU S., DEAN M., ''High-Throughput SNP Genotyping by Allele-Specific PCR with Universal Energy-Transfer-Labeled primers'', Genome Research, Vol.1, 163-169, 2001

MULLIS K. and FOBONA F., In methods in Enzymology, Vol.155, 335, 1987

NASARABADI S., MILANOVICH F., RICHARDS J., and BELGRADER P., "Simultaneous detection of Taqman probes containing FAM and TAMRA reporter fluorophores", Biotechniques, Vol.27 (6), 1116-1117, 1999

SOLINAS A., BROWN L.J., MXKEEN C., MELLOR J.M., NICOL J., THELWELL N., BROWN T., "Duplex Scorpions primers in SNP analysis and FRET applications", Nucleic Acids Res, Vol.29, E96, 2001

SUZUKI T., HIGGINS P.J., CRAWFORD D.R., "Control selection for RNA quantitation", Biotechniques, Vol.29, 332-7, 2000

SVANIK N., STAHLBERG A., SEHISTEDT U., SJOBACH R., and KUBISTA M., "Detection of PCR product in Real-Time using Ligthup probes", Analytical Biochemistry, Vol.287, 179-182, 2000

TYAGI S., AND KRAMER F.R., ''Molecular Beacons probes that fluoresce upon hybridization'', Nature Biotechnology, Vol.14, 303-308, 1996

TYAGI S., MARRAS S.A.E. and KRAMER F.R., ''Wavelength-Shifting Molecular Beacons'', Nature Biotechnology, Vol.18, 1191-1196, 2000

VANDESOMPELE J., DE PETER K., PATTYN F., POPPE B., VAN ROT N., DE PAEPE A. and SPELEMAN F., "Accurate normalization of real time quantitative RT-PCR data by geometric averaging of multiple internal control genes", Genome Biology, Vol.3 (7), 2002

WALBURGER DK., AFONIA IA., LUYDRO R., ''An improved Real-Time PCR, method for simultaneous detection of C282Yand H63D mutations in the HFE gene associated with hereditary hemochromatosis'', Mutation Research, Vol.432, 69-78, 2001

WHITCOMBE D., THEAKER J., GUY S.P., BROWN T., and LITTLE S., "Detection of PCR product usinf self-probing nM amplicons and fluorescence", Nature and Biotechnology, Vol.17 804-807, 1999

WOLFFS P., KNUTSSON R., SJOBACK R., RADSTOM P., "PNA- based light-up probes for Real-Time detection of sequence specific PCR products", Biotechniques, Vol. 766, 769-71, 2001

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Notes

European offices

BELGIUM
Eurogentec Headquarters
Tel.: +32 4 372 74 00
Fax: +32 4 372 75 00
info@eurogentec.com

FRANCE
Eurogentec France s.a.s.u.
Tel.: +33 2 41 73 33 73
Fax: +33 2 41 73 10 26
info@eurogentec.com

GERMANY
Eurogentec Deutschland GmbH
Tel.: +49 221 258 94 55
Fax: +49 221 258 94 54
info@eurogentec.com

SWITZERLAND
Eurogentec Succursale de Genève
Français :
Tel.: +32 4 372 74 00
Fax: +32 4 372 75 00
Deutsch :
Tel.: +49 221 258 94 55
Fax: +49 221 258 94 54
info@eurogentec.com

THE NETHERLANDS
Eurogentec Nederland b.v.
Tel.: +31 43 363 40 37
Fax: +31 43 363 77 65
info@eurogentec.com

UNITED KINGDOM
Eurogentec Ltd.
Tel.: +44 1794 511 411
Fax: +44 1794 522 417
info.uk@eurogentec.com

US offices

USA
Eurogentec North America, Inc.
Tel.: +1 215 966 6135
Fax: +1 215 966 6001
info.usa@eurogentec.com

Eurogentec North America, Inc.
Production site
Tel.: +1 858 793 2661
Fax: +1 858 793 2666
info.usa@eurogentec.com

Asian offices

JAPAN
Nippon EGT
Tel.: +81 76 411 02 77
Fax: +81 76 452 03 99
n-egt@nifty.com

SINGAPORE
RB EGT
Tel.: +65 6273 1066
Fax: +65 6273 4914

Ref.: q&qPCR booklet-0304-V1

EUROGENTEC
EGT GROUP

LIEGE SCIENCE PARK • 4102 SERAING • BELGIUM • Tel.: +32 4 372 74 00 • Fax: +32 4 372 75 00
info@eurogentec.com • order@eurogentec.com • **www.eurogentec.com**