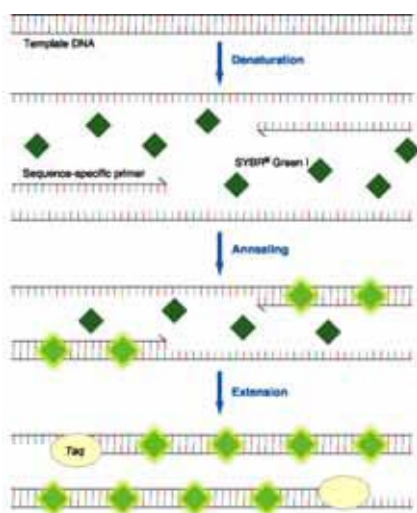


# Optimisation of Absolute™ QPCR SYBR® Green Mix for Quantitative PCR



The Quantitative Polymerase Chain Reaction (QPCR) is one of the most powerful and sensitive gene analysis techniques. Its versatility has made it one of the fastest growing tools in molecular biology. The simplest method for monitoring fluorescence in quantitative PCR is to use a dye that emits light when intercalated into double stranded DNA<sup>1</sup>. The intensity of the fluorescent signal is directly proportional to the amount of dsDNA present in the reaction. As these dyes do not distinguish between the different dsDNA species, it is essential that the formation of non-specific dsDNA (including primer dimers) be kept to a minimum.

The first intercalating dye to be used in real-time QPCR was Ethidium Bromide (EtBr)<sup>2</sup>. EtBr will fluoresce 40 times brighter when bound to dsDNA than when free in the PCR mix. In the late 1990's Molecular Probes released the first of the new generation of dyes, SYBR® Green I, which is less mutagenic than EtBr and also increases in fluorescence 200-fold upon binding to dsDNA<sup>3</sup>.



## Importance of SYBR® Green I

The success of a QPCR SYBR® Green assay depends on many factors, but the concentration of SYBR® Green I is critical. If the levels of SYBR® Green I are too low, a significant increase in fluorescence will not be generated, meaning low copy number templates will be masked by the background fluorescence. At high concentrations (< 1 in 10,000–20,000 dilution<sup>4</sup>) the PCR will be inhibited (figure 1).

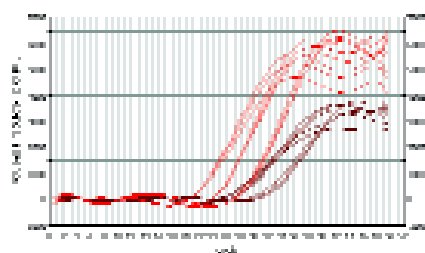


Figure 1. Amplification of GAPDH from human genomic DNA 10-fold serial dilution. MgCl<sub>2</sub> and dNTP concentration were kept constant. Red lines represent SYBR® Green I at a 1 in 50,000 dilution and the brown lines show SYBR® Green I at a 1 in 20,000 dilution.

## MgCl<sub>2</sub> concentration

Increasing the MgCl<sub>2</sub> concentration relieves SYBR® Green inhibition and can also improve specificity. However, the levels cannot be increased too dramatically as this itself can eventually inhibit PCR, resulting in a decrease in the end point reading and an increase in cycle threshold (Ct) value.

ABgene® Absolute™ QPCR SYBR® Green Mixes include a balanced level of MgCl<sub>2</sub>, optimised to work over a broad spectrum of templates. An additional vial of MgCl<sub>2</sub> is included in our SYBR® Green kits to allow user optimisation for complex templates such as plant DNA.

## Influence of dNTPs

There is a close correlation between MgCl<sub>2</sub> and dNTP concentration. dNTPs are MgCl<sub>2</sub> chelators and if too concentrated will leave no free MgCl<sub>2</sub> available to facilitate the Taq polymerase in the reaction.

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An additional consideration is the choice between the use of dUTP or dTTP. dUTP can be used instead of dTTP in conjunction with Uracil-DNA-Glycosylase (UNG) to prevent carry-over contamination. However, dUTP is not as efficient a substrate as dTTP<sup>5</sup> and this substitution will result in a reduction in PCR efficiency, leading to higher Ct values. The data below (figure 2) clearly shows that using dTTP instead of dUTP in the QPCR mixes maximises sensitivity.

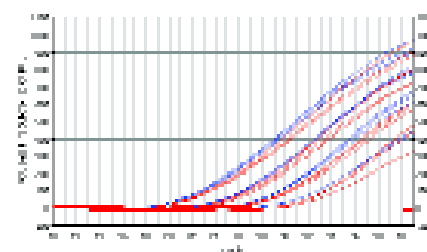


Figure 2. Amplification of /-tubulin from human genomic DNA. A 10-fold dilution series from 250ng–0.25ng DNA. dTTP represented in blue and dUTP represented in red.

## Importance of hot-start polymerases in QPCR

As SYBR® Green I reports all dsDNA generated in the PCR reaction, it is essential that the QPCR mix and primers used offer not only good sensitivity but also the highest specificity possible. For this reason the Absolute™ QPCR SYBR® Green Mixes contain ABgene®'s Thermo-Start® DNA Polymerase, a chemically modified (patented) polymerase that is inactive until 'switched on' by high temperature incubation. In-house tests were performed to assay the effect of varying concentrations of Thermo-Start® on Ct value.

The data (figure 3) shows that increasing Thermo-Start® concentration has no positive effect on Ct value and can introduce inconsistency due to decreased specificity. The concentration of Thermo-Start® in our QPCR mixes helps generate reproducible results each time.

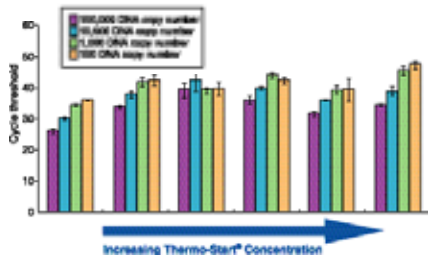


Figure 3. The effect of varying Thermo-Start® concentrations in Absolute™ SYBR® Green QPCR Mix.

### Effect of enhancing additives

A variety of additives have been reported to enhance PCR performance (e.g. sucrose and polyethylene glycol). In QPCR, however, it was found that most had no positive effect on end point or Ct values (data not shown).

One low molecular weight compound was identified that significantly enhanced SYBR® Green QPCR, even when using templates containing GC rich regions (figure 4). This enhancer is included in all of our Absolute™ QPCR SYBR® Green Mixes.

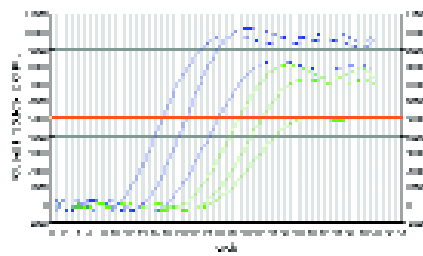


Figure 4. Effect of enhancer on 18s rRNA SYBR® Green QPCR. A 10-fold dilution series (2,500pg–25pg) of *Saccharomyces cerevisiae* template. Amplified using Absolute™ QPCR SYBR® Green Mix (blue), Absolute™ QPCR SYBR® Green Mix without enhancer (green).

### Absolute™ QPCR SYBR® Green Mix

Absolute™ QPCR SYBR® Green Mixes contain the optimal balance of MgCl<sub>2</sub>, SYBR® Green I and dNTPs to minimise the time needed for

experiment-specific optimisation. The mixes are therefore ideally suited to rapid screening and target identification assays. They offer high sensitivity (figure 5) and specificity and also have a broad working range.

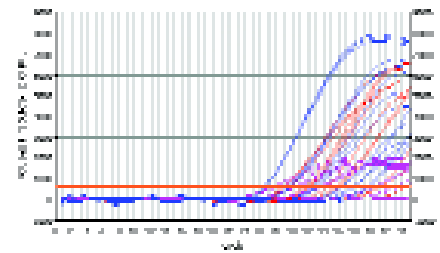


Figure 5. Amplification of GAPDH. Human Genomic DNA was diluted in a 10-fold dilution series (2,000pg–20pg per reaction) and amplified using Absolute™ SYBR® Green Fluorescein Mix (blue), Supplier A's SYBR® Green mix (red) and Supplier B's SYBR® Green mix (pink). The data shows that ABgene® mix is 2–3 cycles more sensitive than the competitor mixes.

### References

1. Wittwer CT, Hermann MG, Moss AA and Rosmussen RP (1997). Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* 22: 130–138
2. Higuchi R, Fockler C, Dollinger G and Watson R (1993). Kinetic PCR analysis real-time monitoring of DNA amplification reactions. *Biotechnology (NY)* 11(9): 1026–30.
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4. Nath K, Sarosy JW, Hahn J and Di Como CJ (2000). Effects of ethidium bromide and SYBR® Green I on different polymerase chain reaction systems. *J Biochem Biophys Methods*. Jan 3;42(1–2):15–29
5. Longo *et al* (1990). Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 93: 125–128

Cat. No.	Description	Quantity
<b>SYBR® Green Mixes for ABI PRISM®:</b>		
AB-1163	Absolute™ QPCR SYBR® Green ROX Mix	200 x 50µl rxns
AB-1162/b	Absolute™ QPCR SYBR® Green ROX Mix	800 x 50µl rxns
<b>SYBR® Green Mixes for Bio-Rad iCycler™:</b>		
AB-1220	Absolute™ QPCR SYBR® Green Fluorescein Mix	200 x 50µl rxns
AB-1219/b	Absolute™ QPCR SYBR® Green Fluorescein Mix	800 x 50µl rxns
<b>SYBR® Green Mixes for RotorGene™ and MJ Opticon™:</b>		
AB-1159	Absolute™ QPCR SYBR® Green Mix	200 x 50µl rxns
AB-1158/b	Absolute™ QPCR SYBR® Green Mix	800 x 50µl rxns
<b>SYBR® Green Mixes for Stratagene Mx4000™:</b>		
AB-1167	Absolute™ QPCR SYBR® Green Mix plus ROX vial	200 x 50µl rxns
AB-1166/b	Absolute™ QPCR SYBR® Green Mix plus ROX vial	800 x 50µl rxns



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Optimised for QPCR

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