

## Frequently asked questions

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### USB® VeriQuest™ qPCR Master Mixes

#### USB VeriQuest Probe qPCR Master Mix

##### **What is the VeriQuest Probe qPCR Master Mix?**

VeriQuest Probe qPCR Master Mix is a ready-to-use mix for real-time quantitative PCR (qPCR) on all PCR instruments that use ROX™ as a passive reference dye. The master mix is formulated for use with fluorescent probes such as TaqMan® MGB probes. The master mix is supplied at a 2X concentration with all components except probes, primers, and template, and should be used at a 1X concentration in the reaction.

##### **What does the VeriQuest Probe qPCR Master Mix contain?**

The master mix contains a chemically-modified Taq DNA polymerase, MgCl<sub>2</sub>, ultrapure nucleotides with an optimized dUTP:dTTP ratio, *E. coli* Uracil-DNA Glycosylase (UDG), and ROX Passive Reference Dye in a proprietary reaction buffer. The proprietary reaction buffer with optimum MgCl<sub>2</sub> concentration is specially designed for robust probe hybridization and efficient cleavage of TaqMan probes.

##### **How does the VeriQuest Probe Master Mix work?**

The VeriQuest Probe qPCR Master Mix uses a chemically-modified Taq DNA Polymerase (VeriQuest Taq DNA Polymerase for hot-start PCR<sup>(1,2)</sup>). This hot start Taq polymerase has no polymerase activity prior to the initial heat activation step which allows reaction assembly at room temperature as well as higher specificity and sensitivity. The initial incubation step of 95°C for 10 minutes before PCR cycling removes the blocking chemical moiety resulting in activation of the polymerase.

The master mix is formulated for use with fluorescent probes such as TaqMan probes. TaqMan probes consist of a fluorophore covalently attached to the 5'-end of an oligonucleotide probe and a quencher at the 3'-end. As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals. The Taq DNA Polymerase used in the master mix has the 5' to 3' exonuclease activity necessary for efficient removal of the 5'-fluorophore from the 3'-quencher when encountering the TaqMan probe anneals to its target. Since fluorescent probes are designed to hybridize to the target of interest, detection specificity is greatly increased relative to nonspecific dsDNA binding dyes such as SYBR® Green I.

Our product has been validated with several different fluorophores (e.g. FAM™, HEX, VIC, Cy3, and Cy5) and quenchers (e.g. , Black Hole Quencher® (BHQ®) and TAMRA™) as well as with minor-groove binder containing probes.

##### **What is Uracil-DNA Glycosylase used for in the mix?**

The *E. coli* Uracil DNA Glycosylase (UDG or UNG) and dUTP in VeriQuest Probe qPCR Master Mix offer an option for carry-over contamination prevention from previous PCR amplifications, which is especially important for high-throughput applications<sup>(3)</sup>. The dUTP in the master mix ensures that amplicons contain uracil which can be destroyed prior to subsequent amplification reactions by the enzymatic activity of the UDG in conjunction with the initial heating step. The UDG eliminates at least 10<sup>5</sup> copies of dUTP-containing templates. After the initial denaturation step, the UDG is inactivated, and only the desired target sequences are amplified.

When the reaction products must be analyzed by gel electrophoresis, remove the plate from the cycler immediately after completion of the reaction and place on ice prior to loading the gel. The reason is that the UDG in the mix can regain low-level activity over time at temperatures less than 60°C.

##### **What is the purpose of the ROX Passive Reference Dye?**

ROX Passive Reference Dye is used to normalize fluorescent signal intensities when reactions are performed on ABI and Stratagene real time PCR instruments. This signal normalization is necessary to correct for well-to-well variations that may occur due to pipetting errors or instrument optical limitations. The fluorescence of this inert dye does not change during the reaction. Instead it provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Software-based normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the passive reference to obtain a ratio defined as the Rn (normalized reporter) for a given well. The Rn value less the baseline fluorescence equals the ΔRn value, which reliably indicates the magnitude of the signal generated in each well.

**On which real-time PCR instruments can I use this master mix? Do I have to adjust the ROX concentration? Do I need to order a “low” or “high” ROX mix specific for certain instruments?**

The VeriQuest Probe qPCR Master Mix includes ROX which is optimized for detection on all real-time PCR instruments that use ROX as a Passive Reference Dye (e.g. Applied Biosystems 7300, 7500, 7900, StepOne™, and StepOnePlus™; Stratagene Mx3000P®, and Mx3005P™; etc.). Unlike most other products, you do not need to adjust the ROX concentration or choose a “low” or “high” ROX mix.

**Can I use this master mix in fast cycling mode protocols?**

No, VeriQuest Probe qPCR Master Mix is formulated for standard mode cycling protocols (e.g. 1 cycle of 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on ABI real-time PCR instruments). Be sure to select standard mode and follow the instrument manufacturer’s protocol for instrument setup.

**What stability data is available for VeriQuest Probe qPCR Master Mix?**

VeriQuest Probe qPCR Master Mix is stable for up to 1 year at -20°C and up to 3 months at 4°C. The master mix withstands up to 10 freeze-thaw cycles without a loss in performance. In addition, preassembled PCR reactions with VeriQuest Probe qPCR Master Mix are stable for up to 72 hours at room temperature. This extended benchtop stability provides flexibility to process samples using high-throughput liquid handling systems that may require assembled reactions to sit at room temperature until they are ready to be run in the real-time PCR instrument.

**Can I use VeriQuest Probe qPCR Master Mix to validate my microarray results?**

Yes, VeriQuest Probe qPCR Master Mix has been validated for quantification of abundant and limited targets from genomic DNA and cDNA samples. Our validation study showed detection from 6.6 pg of human genomic DNA, which corresponds to 2 copies of a single-copy gene. We also validated that VeriQuest Probe qPCR Master Mix can be used to discriminate a 1.33 to 10-fold dilution series of cDNAs with 99.9% confidence.

**Can I use VeriQuest Probe qPCR Master Mix for duplex PCR detection?**

Yes, VeriQuest Probe qPCR Master Mix can be used for single target and duplex PCR detection with similar PCR efficiency. We also validated that the VeriQuest Probe qPCR Master Mix can be used with various commonly used reporter dyes such as FAM, HEX, VIC, Cy3, Cy5, etc.

**Can I use VeriQuest Probe qPCR Master Mix for SNP genotyping?**

Yes, VeriQuest Probe qPCR Master Mix has been validated for genotyping assays.

**What do you suggest when amplifying AT-rich, GC-rich and/or difficult templates?**

VeriQuest Probe qPCR Master Mix has been validated for amplification of target amplicons with 30-71% GC content with a PCR efficiency of 92% or higher over three orders of magnitude. Higher %GC content target may be amplified but due to the presence of secondary structure, the PCR reaction may result in mispriming and polymerase stalling. Try increasing the annealing/extension temperature while keeping it lower than the primer  $T_m$  (e.g. 65°C) to improve specificity.

**What control reactions are necessary for probe-based qPCR assays?**

A No Template Control (NTC) in which the template is omitted should be performed at least in triplicate to assess the presence of contaminating nucleic acids that may come from pipette tips, plates, primers, probes, water or mixes. When carrying out RT-qPCR assays, a no reverse-transcriptase control (no RT) should be performed to test for genomic DNA contamination in the RNA sample. Positive controls may be necessary for quantification analysis (i.e. performing a standard curve from a cloned PCR fragment or a synthetic oligo or amplifying a housekeeping gene).

**How does VeriQuest Probe qPCR Master Mix compare to other probe qPCR master mix products?**

VeriQuest Probe qPCR Master Mix has been validated to have the same or better performance, compared to ABI TaqMan Gene Expression Master Mix and ABI TaqMan Universal Master Mix II.

## USB VeriQuest SYBR Green qPCR Master Mix

**What is the VeriQuest SYBR Green qPCR Master Mix?**

VeriQuest SYBR Green qPCR Master Mix is a ready-to-use mix for real-time quantitative PCR (qPCR) on all the PCR instruments that use ROX as a Passive Reference Dye. It is formulated for SYBR Green I detection of dsDNA products. The master mix is supplied at a 2X concentration with all components except primers, and templates and should be used at a 1X concentration in the reaction.

**What does the VeriQuest SYBR Green qPCR Master Mix contain?**

The master mix contains a chemically-modified Taq DNA polymerase,  $MgCl_2$ , ultrapure nucleotides with an optimized dUTP:dTTP ratio, *E. coli* Uracil-DNA Glycosylase (UDG), SYBR Green I, and ROX Passive Reference Dye in a proprietary reaction buffer. The proprietary reaction buffer and the hot-start polymerase enhance SYBR Green-based qPCR reactions by reducing primer-dimer formation which increases specificity and sensitivity.

### **How does the VeriQuest SYBR Green Master Mix work?**

VeriQuest SYBR Green qPCR Master Mix uses a chemically-modified Taq DNA Polymerase (VeriQuest Taq DNA Polymerase for hot-start PCR<sup>(1,2)</sup>). The hot start Taq polymerase has no polymerase activity prior to the initial heat activation step which allows reaction assembly at room temperature as well as higher specificity and sensitivity. The initial incubation step of 95°C for 10 minutes before PCR cycling removes the blocking chemical moiety resulting in activation of the polymerase.

The VeriQuest SYBR Green qPCR Master Mix (2X) uses SYBR Green I dye to detect any double-stranded DNA that is generated during the amplification process. SYBR Green I dye binding to dsDNA results in an increase in fluorescence intensity proportional to the amount of PCR product. Unlike the Taqman probe detection where one fluorophore is generated from one amplified product, multiple SYBR Green I dye molecules bind to a single amplified molecule. Therefore, if the PCR efficiencies are the same, amplification of a longer target will generate more signal than a shorter one by SYBR Green detection.

### **What is Uracil-DNA Glycosylase used for in the mix?**

The *E. coli* Uracil DNA Glycosylase (UDG or UNG) and dUTP in VeriQuest SYBR Green qPCR Master Mix offer an option for carry-over contamination prevention from previous PCR amplifications, which is especially important for high-throughput applications<sup>(3)</sup>. The dUTP in the master mix ensures that amplicons contain uracil which can be destroyed prior to subsequent amplification reactions by the enzymatic activity of the UDG in conjunction with the initial heating step. The UDG eliminates at least 10<sup>5</sup> copies of dUTP-containing templates. After the initial denaturation step, the UDG is inactivated, and only the desired target sequences are amplified.

When the reaction products must be analyzed by gel electrophoresis, remove the plate from the cycler immediately after completion of the reaction and place on ice prior to loading the gel. The reason is that the UDG in the mix can regain low-level activity over time at temperatures less than 60°C.

### **What is the purpose of the ROX Passive Reference Dye?**

ROX Passive Reference Dye is used to normalize fluorescent signal intensities when reactions are performed on ABI and Stratagene real time PCR instruments. This signal normalization is necessary to correct for well-to-well variations that may occur due to pipetting errors or instrument optical limitations. The fluorescence of this inert dye does not change during the reaction. Instead it provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Software-based normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the passive reference to obtain a ratio defined as the Rn (normalized reporter) for a given well. The Rn value less the baseline fluorescence equals the  $\Delta Rn$  value, which reliably indicates the magnitude of the signal generated in each well.

### **On which real-time PCR instruments can I use this master mix? Do I have to adjust the ROX concentration? Do I need to order a "low" or "high" ROX mix specific for certain instruments?**

The VeriQuest SYBR Green qPCR Master Mix includes ROX which is optimized for detection on all real-time PCR instruments that use ROX as a Passive Reference Dye (e.g. Applied Biosystems 7300, 7500, 7900, StepOne, and StepOnePlus; Stratagene Mx3000P, and Mx3005P; etc.). Unlike most other products, you do not need to adjust the ROX concentration or choose a "low" or "high" ROX mix.

### **Can I use this master mix in fast cycling mode protocols?**

No, VeriQuest SYBR Green qPCR Master Mix is formulated for standard mode cycling protocols (e.g. 1 cycle of 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute on ABI real-time PCR instruments). Be sure to select standard mode and follow the instrument manufacturer's protocol for instrument setup.

### **What stability data is available for VeriQuest SYBR Green qPCR Master Mix?**

VeriQuest SYBR Green qPCR Master Mix is stable for up to 1 year at -20°C and up to 3 months at 4°C. The master mix withstands up to 10 freeze-thaw cycles without a loss in performance. In addition, pre-assembled PCR reactions with VeriQuest SYBR Green qPCR Master Mix are stable for up to 72 hours at room temperature. This extended benchtop stability provides flexibility to process samples using high-throughput liquid handling systems that may require assembled reactions to sit at room temperature until they are ready to be run in the real-time PCR instrument.

### **Can I use VeriQuest SYBR Green qPCR Master Mix to validate my microarray results?**

Yes, VeriQuest SYBR Green qPCR Master Mix has been validated for quantification of abundant and limited targets from genomic DNA and cDNA samples. Our validation study showed detection from 13.2 pg of human genomic DNA, which corresponds to 4 copies of a single-copy gene. We also validated that VeriQuest SYBR Green qPCR Master Mix can be used to discriminate a 1.33 to 10-fold dilution series of cDNAs with 99.9% confidence.

### **What do you suggest when amplifying AT-rich, GC-rich and/or difficult templates?**

VeriQuest SYBR Green qPCR Master Mix also has been validated for amplification of target amplicons with 30-71% GC content with a PCR efficiency of 92% or higher over four orders of magnitude. Higher %GC content target may be amplified but due to the presence of secondary structure, the PCR reaction may result in mispriming and polymerase stalling. Try increasing the annealing/extension temperature while keeping it lower than the primer T<sub>m</sub> (e.g. 65°C) to improve specificity.

### **What control reactions are necessary for SYBR Green-based qPCR assays?**

A No Template Control (NTC) in which the template is omitted should be performed at least in triplicate to assess the presence of contaminating nucleic acids that may come from pipette tips, plates, primers, probes, water or mixes. When carrying out RT-qPCR assays, a no reverse-transcriptase control (no RT) should be performed to test for genomic DNA contamination in the RNA sample. The addition of a Melt-Curve program and analysis is strongly recommended to distinguish specific products from non-specific ones such as primer-dimers. Formation of specific PCR products may be confirmed by gel electrophoresis. Positive controls may be necessary for quantification analysis (*i.e.* carrying out a standard curve from a cloned PCR fragment in a plasmid or a synthetic oligo in solution or amplifying a housekeeping gene).

### **How does VeriQuest SYBR Green qPCR Master Mix compare to other SYBR Green qPCR master mix products?**

VeriQuest SYBR Green qPCR Master Mix has been validated to have similar performance, and in some cases better, compared to ABI Power SYBR Green PCR Master Mix and BioRad iTaq™ SYBR Green Supermix with ROX.

## Primers and Probes

### **How can I design primers and/or probes suitable for qPCR?**

There are many computer programs available to select appropriate primers and probes for qPCR (*e.g.* Primer3) as well as several public databases (*e.g.* NCBI Probe Database, Quantitative PCR Primer Database, PrimerBank and RTPrimerDB). As a general guide when designing TaqMan probes for gene expression analysis: (1) use a probe length of 20-30 bases; (2) avoid repeat stretches of identical nucleotides, especially Gs; (3) a G residue should not be present at the 5'-probe terminus, as it may quench the adjacent fluorophore; (4) primer melting temperature ( $T_m$ ) should be around 60°C and probe  $T_m$  about 8-10°C higher; (5) probe G+C content should be in the 30-80% range, with more Cs than Gs; (6) primers spanning exon-exon junctions are preferable to avoid detection of false-positive signal due to the amplification of contaminating genomic DNA. Other resources for PCR primers, oligo databases and design tools can be found at [http://www.hsls.pitt.edu/guides/genetics/obrc/dna/pcr\\_oligos](http://www.hsls.pitt.edu/guides/genetics/obrc/dna/pcr_oligos). For best results, primers should be designed with an amplicon size ranging between 70 and 200 bp. For PCR efficiencies close to 100%, the amplicon length should be less than 150 bp.

### **How can I validate my primers and/or probes for my qPCR assay?**

A general strategy for new qPCR primer selection includes the validation of both the primers and probe for specificity. Initial primer validation may be performed with conventional end-point PCR and analyzed on a gel (*i.e.* no primer-dimer formation and no mispriming that would result in non-specific product formation). Alternatively, SYBR Green melt curve analysis is a powerful tool to identify primer-dimer formation and non-specific product amplification. SYBR Green detection also offers a flexible and inexpensive option to evaluate the primer quality and optimize the qPCR reaction conditions (*e.g.* annealing temperature) for TaqMan probe detection. Then, further optimization can be performed by running a standard curve to determine PCR efficiency and sensitivity (see also "How do I evaluate the PCR efficiency of my reaction?").

### **How can I determine the presence of primer-dimers in my qPCR reaction?**

When using SYBR Green dye, run and analyze the melting/dissociation curve following the amplification step. The presence of primer-dimers will appear in the NTC and most likely in the experimental wells as a peak with a lower  $T_m$  than the  $T_m$  of the specific amplicon. Gel electrophoresis is also a quick and easy way to monitor primer-dimer formation as bands shorter than 75 bp. Formation of primer-dimers can be eliminated by reducing the amount of primers in the reaction without affecting the amplification efficiency. Be sure that the primers have been stored properly to avoid their degradation. Alternatively, primer design optimization may be necessary (see above for information). Web-based software, such as AutoDimer, can also help to screen selected PCR primers for potential primer-dimer formation.

### **What are the recommended storage conditions for primers and probes?**

We recommend resuspending the primers and labeled probes in TE Buffer, pH 7-8, rather than water and storing them at -20°C for long-term storage. The dye-labeled probes may be stored at -20°C in an amber-colored tube to avoid light exposure. A working stock of primers may be stored at 4°C for up to one month.

## Templates

### **When should you use one-step versus two-step RT-qPCR?**

Two-step RT-qPCR is best suited for detecting multiple transcripts from a single RNA sample and allows the use of different priming strategies (*e.g.* oligo(dT), random primers, mixture of oligo(dT) and random primers (see USB First Strand cDNA Synthesis Kit for Real-Time PCR, PN 75780) ) during the reverse-transcription step. One-step RT-qPCR is best suited when processing multiple samples and minimizes potential carryover contamination. It generally delivers greater sensitivity since gene-specific primers are used for the reverse transcription step.

### **What type of template can I use?**

VeriQuest qPCR Master Mixes have been tested and validated with a wide range of template sources such as cDNA prepared from reverse transcription of RNA, genomic DNA and plasmid DNA.

### How much template should be used? What is the linear detection range of the mixes?

This depends on the expression level and/or copy number of your target. Optimal template input quantities for cDNA is the amount of cDNA corresponding to 1 pg to 500 ng of total RNA. One pg is the amount of total RNA from 1/10<sup>th</sup> of a typical human cell. If template is cDNA from a first-strand cDNA synthesis reaction that has not been purified or diluted, do not exceed 10% of the final PCR reaction volume (*i.e.* 5 µl of a RT reaction into a 50 µl PCR reaction). For genomic DNA, single-copy targets have been detected from 6.6 pg of human gDNA which is the amount from about 1 typical human cell. For genomic DNA, do not exceed 100 ng.

The linear detection range of VeriQuest Probe qPCR Master Mix is up to 9 orders of magnitude and the linear detection range of VeriQuest SYBR Green qPCR Master Mix is up to 7 orders of magnitude.

## Instrument Set-up and Data Analysis

### How can I program my ABI real-time instrument for use with the VeriQuest qPCR Master Mixes?

While we recommend following the instrument manufacturer's instructions for setting-up an experiment (*e.g.* plate document), we have validated VeriQuest qPCR Master Mixes with the standard mode cycling protocols on ABI instruments (1 cycle of 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute).

### What quantification method should I use to determine the levels of my target mRNA by RT-qPCR?

Determining the appropriate quantification method for your particular qPCR assay will mostly depend on the experimental goals. Absolute quantification allows determination of copy number of the target of interest using a standard curve of known concentrations. In comparative or relative quantification  $\Delta\Delta C_t$  method, changes in gene expression is measured in a given sample relative to a reference sample (*a.k.a.* calibrator) normalized to an endogenous or exogenous control (*e.g.* housekeeping gene) that does not change under the experimental conditions. In this case, it is important to show that amplification efficiencies of the target and the control genes are similar.

### How do I evaluate the PCR efficiency of my reaction?

By performing a standard curve using a dilution series of the target (minimum 3 logs of template concentration or 4 data points if using orders of magnitude), PCR efficiency can be determined using the following equation:  $E = 10^{(-1/\text{slope})} - 1$ . Generally, an efficiency of between 90-110% is considered acceptable. This corresponds to a slope of the curve between -3.58 and -3.1 from the plot of the  $C_t$  vs. log-template amounts. Since high PCR efficiency enables greater sensitivity and more accurate quantification, an efficiency nearing 100% (*i.e.* doubling of the amplicon every cycle) is desired. Factors that affect the PCR efficiency include length of the amplicon, primer design, presence of inhibitors, secondary structure, etc.

### Why should I determine the efficiency of the qPCR reaction?

First, it is important to determine whether factors such as PCR inhibitors are not present and that the PCR reaction is optimal. Also, when comparing the relative expression of 2 genes (*e.g.* target and reference), PCR efficiency should be similar for both messages especially when using the  $\Delta\Delta C_t$  comparative quantification method.

### How do I analyze my results on the ABI instruments?

This step is instrument and software-dependent, so we recommend carefully following the instrument manufacturer's instructions. Some general guidelines to analyze the amplification data include: (1) reviewing the amplification plot for each well of the plate; (2) setting the baseline and threshold value (automatically first followed by manual adjustment if necessary) to calculate the  $C_t$  threshold cycles for amplification traces; (3) analyzing and extracting the data (*e.g.* relative or absolute quantification).

## References

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2. Birch, D. E. *et al.*, (1996) *Nature* **381**, 445-446.
3. Longo, M. *et al.*, (1990) *Gene* **93**, 125-128.
4. Bustin, S. *et al.*, (2009) *Clinical Chemistry* **55**(4), 611-622.

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