

**GeneSure™ Probe/Rox qPCR Master Mix (2X)**

Store at -20°C

Cat. No.	Pack Size	
PGK023A-B	200 Rxns	
Component	PGK023A for 200 rxns of 25 µl	PGK023B for 1000 rxns of 25 µl
GeneSure™ Probe/ROX qPCR Master Mix (2X)	2x1.25 ml	10x1.25 ml
Water, nuclease-free	2x1.25 ml	10x1.25 ml

**STORAGE**

Store at -20°C in the dark.

**Description**

Puregene GeneSure™ Probe/ROX qPCR Master Mix (2X) is a ready-to-use solution optimized for quantitative real-time PCR and two-step real-time RT-PCR. The master mix includes GeneSure™ Hot Start Taq DNA polymerase and dNTPs in an optimized PCR buffer. It is supplemented with ROX passive reference dye. Only template and primers need to be added. GeneSure™ Hot Start Taq DNA polymerase in combination with an optimized buffer ensures PCR specificity and sensitivity. dUTP is included in the mix for optional carryover contamination control using uracil DNA glycosylase (UDG). ROX serves as a passive reference dye. The use of GeneSure™ Probe/ROX qPCR Master Mix (2X) in real-time PCR ensures reproducible, sensitive and specific quantification of genomic, plasmid, viral and cDNA templates. The master mix can be used with most real-time thermal cyclers, that are compatible with ROX reference dye; Applied Biosystems: ABI PRISM® 7000, 7300, 7900 HT, StepOne™.

GeneSure™ Hot Start Taq DNA Polymerase is a Taq DNA polymerase which has been chemically modified by the addition of heat-labile blocking groups to amino acid residues. The enzyme is inactive at room temperature, avoiding extension of non-specifically annealed primers or primer dimers and providing higher specificity of DNA amplification. The enzyme provides the convenience of reaction set up at room temperature.

GeneSure™ Probe qPCR Buffer has been specifically optimized for qPCR analysis using sequence-specific probes. It contains both KCl and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to provide high specificity of primer annealing. The buffer composition allows for PCR at a wide range of MgCl<sub>2</sub> concentrations. Therefore, optimization of MgCl<sub>2</sub> concentration in PCR is generally not necessary..

ROX Passive reference dye is included in the master mix to serve as an internal reference for normalization of the fluorescent signal when carrying out reactions using machines that can detect ROX, e.g. Applied Biosystems. ROX allows for correction of well-to-well variation due to pipetting inaccuracies and fluorescence fluctuations. The presence of ROX does not interfere with qPCR using other systems, e.g. iCycler iQ, since it does not participate in PCR and has a different emission spectrum (the excitation/emission maxima are at 580 nm/621 nm, respectively) compared to the fluorescent dyes used for probes.

dUTP is included in the master mix to partially replace dTTP in the accumulated PCR product, allowing for the option to prevent carryover contamination between reactions (1). Uracil-DNA Glycosylase (UDG) pre-treatment of the reaction removes all dU-containing amplicons carried over from previous reactions.

Note. UDG is not included in the GeneSure Probe/ROX qPCR Master Mix and must be purchased separately.

**GUIDELINES TO ASSAY DESIGN**

**Templates**

**DNA.** Genomic DNA up to 100 ng and plasmid DNA up to 10 ng can be used in qPCR with GeneSure Probe/ROX qPCR Master Mix.

**RNA.** Template RNA for RT-qPCR must be free of DNA contamination. We recommend usage of DNase I, RNase-free, to remove trace amounts of DNA from RNA preparations. Always perform an RT-minus control to confirm complete removal of DNA. For two-step RT-qPCR, up to 5 µg of total RNA can be used for cDNA synthesis in the reverse transcription reaction. An aliquot of the first strand cDNA synthesis reaction is then transferred to another tube as a template for qPCR. The volume of the cDNA added (from the RT reaction) should not exceed 10% of the final qPCR volume.

For first strand cDNA synthesis, we recommend using our GeneSure First Strand cDNA synthesis Kit for RT-qPCR

**PRIMERS**

Primer design for qPCR is one of the most important factors to obtain efficient amplification and to avoid the formation of primer dimers. Use primer design software, such as PrimerExpress® or Primer3 (frodo.wi.mit.edu) or follow general recommendations for PCR primer design below:

- GC content: 30-60%.
- Length: 18-30 nucleotides.
- Optimal amplicon length: 70-150 bp.
- Optimal melting temperature (T<sub>m</sub>): 60°C. Differences in T<sub>m</sub> of the two primers should not exceed 2°C.
- Avoid more than two G or C nucleotides in last five nucleotides at 3'-end to lower the risk of nonspecific priming.
- Avoid secondary structures in the amplicon.
- Avoid primer self-complementarities, complementarities between the primers and direct repeats in a primer to prevent hairpin formation and primer dimerization.
- Optimal primer concentration is 0.3 µM for both primers in most cases. The concentration may be optimized between 0.05 and 0.9 µM for individual primers and chosen by the lowest Ct for the amplicon and the highest Ct for primer-dimer formation (if present).



**GeneSure™ Probe/Rox qPCR Master Mix (2X)**

Store at -20°C

**Probes**

For probe design follow general recommendations for primer design described above. Design the probe first then select primers that flank probe region. General guidelines for design of dual-labeled probes, such as TaqMan probes:

- GC content: 30-60%.
- Length: 20-30 bases.
- Melting temperature (T<sub>m</sub>): 68-70°C, 8-10°C higher than the T<sub>m</sub> of the PCR primers.
- Avoid runs of same nucleotides, especially guanine, longer than 3 bases.
- Exclude G at the 5'-end of the probe, which causes quenching.
- Select the template DNA strand containing more C than G bases.
- Avoid secondary structures.
- Avoid dimerization with primers.

**Necessary controls**

- No template control (NTC) is important to assess for reagent contamination or primer dimers. The NTC reaction contains all components except template DNA.
- Reverse Transcriptase Minus (RT-) control is important in all RT-qPCR experiments to assess for RNA sample contamination with DNA. The control RT- reaction contains all components for RT-qPCR except the RT enzyme.

**IMPORTANT NOTES**

- Reaction set-up is at room temperature as the master mix includes hot start Taq DNA polymerase.
- We recommend a reaction volume of 25 µl. Other reaction volumes may be used if recommended for a specific instrument.
- Preparation of a master mix, which includes all reaction components except template DNA, assists in avoiding of pipetting errors and is an essential step in real-time PCR.
- Start PCR cycling with an initial denaturation step of 10 min at 95°C to activate Genesure Hot Start Taq DNA polymerase.
- Minimize exposure of GeneSure Probe qPCR Master Mix (2X) to light during handling to avoid loss of fluorescent signal intensity.
- Readjust the threshold value for analysis of every run.
- When using Bio-Rad iCycler iQ or MyiQ systems collect well factors at the beginning of each experiment using an external well factor plate according to the instrument manufacturer's recommendations. Do not add fluorescein solution to the reaction mix. Well factors are used to compensate for any system or pipetting variations.

**PROTOCOL**

**Primers Reaction set-up**

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Prepare a reaction master mix by adding the following components (except template DNA) for each 25 µl reaction to a tube at room temperature:

GeneSure™ Prob/ROX qPCR Master Mix (2X)*	12.5 µl
Forward Primer	0.3 µM**
Reverse Primer	0.3 µM**
Probe	0.25µM
Template DNA	≤500 ng
Water, nuclease-free to	25 µl
Total volume	25 µl***

\* Provides a final concentration of 4 mM MgCl<sub>2</sub>.

\*\* A final primer concentration of 0.3 µM is optimal in most cases, but may be individually optimized in a range of 0.05 µM to 0.9 µM.

\*\*\* Other reaction volumes can be used if recommended for a specific instrument.

3. Mix the master mix thoroughly and dispense appropriate volumes into PCR tubes or plates.
4. Add template DNA (≤100 ng/reaction) to the individual PCR tubes or wells containing the master mix.

Note. For two-step RT-qPCR, the volume of the cDNA added from the RT reaction should not exceed 10% of the final PCR volume.

5. Gently mix the reactions without creating bubbles (do not vortex). Centrifuge briefly if needed. Bubbles will interfere with fluorescence detection.
6. Program the thermal cycler according to the recommendations below, place the samples in the cycler and start the program.

**Thermal cycling conditions**

Thermal cycling can be performed using a three-step or two-step cycling protocol.

Three-step cycling protocol

Step	Temperature, °C	Time	Number of cycles
Optional: UDG pre-treatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	40
Extension	72	30 s	40

Data acquisition should be performed during the extension step.



**GeneSure™ Probe/Rox qPCR Master Mix (2X)**

Store at -20°C

Two-step cycling protocol

Step cycles	Temperature, °C	Time	Number of cycles
Optional: UDG pre-treatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing/Extension	60	60 s	40

Data acquisition should be performed during the annealing/extension step.

**Optional steps**

- **UDG pre-treatment.** If using carryover decontamination, include a 2 min UDG digestion step at 50°C before the initial denaturation step.
- **Agarose gel electrophoresis of PCR products.** When designing a new assay it is recommended to verify the PCR product specificity by gel electrophoresis, as melting temperatures of a specific product and primer-dimers may overlap depending on the sequence composition.

**TROUBLE SHOOTING**

**Problem**

No amplification curve and no PCR product visible on a gel

**Cause and Solution**

*PCR inhibitors present in the reaction mixture*

.Re-purify your template DNA.  
*Primer design is suboptimal.*

Verify your primer design, use reputable primer design programs or validated pre-designed primers.

*RT-qPCR: inhibition by excess volume of the RT reaction.*  
Volume of RT reaction product added to qPCR reaction should not exceed 10% of the total qPCR reaction volume.

*Pipetting error or missing reagent.* Repeat the PCR reaction; check the concentrations of template and primers; ensure proper storage conditions of all reagents. Make new serial dilutions of template DNA or RNA.

*Degradation of primers.*  
Check PCR primers for possible degradation on polyacrylamide gel.  
*Annealing temperature is not optimal.*

Optimize the annealing temperature in 3°C increments.  
*UDG present in PCR protocol with low annealing temperature.*  
When performing UDG pre-treatment with conventional UDG, the temperature during PCR cycling should always be higher than 55°C. If annealing temperatures must be lower than 55°C, use heat-labile UDG.

**Problem**

No amplification curve but PCR product visible on a gel

**Cause and Solution**

**qPCR instrument settings are incorrect.**  
Check if instrument settings are correct (dye selection, reference dye, filters).

**Inactive fluorescence detection.**  
Fluorescent detection should be activated and set at extension or annealing/extension step of the thermal cycling protocol.

**Instrument problems.**  
Refer to the instrument manual for troubleshooting.

**Problem**

Amplification signal in non-template control

**Cause and Solution**

- DNA contamination of reagents.**
- Follow general guidelines to avoid carry over contamination or include UDG pre-treatment step at the beginning of PCR.
  - Discard reagents and repeat with new reagents.

**RT-qPCR: RNA contaminated with genomic DNA.**  
Design primers on intron/exon boundaries, treat RNA sample with DNaseI, RNA free prior to reverse transcription.

**Problem**

PCR efficiency is >110%

**Cause and Solution**  
**Non-specific products.**

Use gel electrophoresis to identify non specific amplicons. Optimize your primer design to avoid such artifacts or use validated pre-designed primers.

**Problem**

PCR efficiency is <90%

**Cause and Solution**

- PCR inhibitors present in a reaction mixture.**  
Re-purify your template DNA.  
**PCR conditions are suboptimal.**  
Verify the primer concentrations. Verify storage conditions of qPCR master mix.  
**Primer design.**  
Verify your primer design, use primer design programs or validated pre- designed primers. Avoid designing primers in regions with high DNA secondary structure.



**GeneSure™ Probe/Rox qPCR Master Mix (2X)0**

Store at -20°C

**Problem**

Poor standard curve

**Cause and Solution**

**Excessive amount of template.**

Do not exceed maximum recommended amounts of template DNA (500 ng DNA for 25 µl reaction).

**Suboptimal amount of template.**

Increase the amount of template, if possible.

**RT-qPCR: inhibition by excess volume of the RT reaction.**

Volume of RT reaction product added to qPCR reaction should not exceed 10% of the total qPCR reaction volume.

**Problem**

Non-uniform Fluorescence intensity

**Cause and Solution**

**Contamination of the thermal cycler.**

Perform decontamination of your real-time cycler according to the supplier's instructions.

**Poor calibration of the thermal cycler.**

Perform calibration of the real-time cycler according to the supplier's instructions.

**PRODUCT USE LIMITATION**

This product is developed, designed and sold exclusively for research purposes and *in vitro* use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

