

## PRODUCT INSERT

PUREGENE

GeneSure Probe PCR Master Mix (2X),  
ROX Solution provided

Store at -20°C

Cat. No.  
PGK026Pack Size  
200rxn & 1000rxn

Store at -20°C

Component	PGK026-A for 200 rxns of 25µL	PGK026-B for 1000 rxns of 25 µL
GeneSure Probe qPCR Master Mix (2X), no ROX	2x1.25 ml	10x1.25 ml
ROX Solution, 50 µM	50 µL	250 µL
Water, nuclease free	2x1.25 ml	10x1.25 ml

## DESCRIPTION

GeneSure Probe PCR Master Mix (2X) is an universal ready to use solution for quantitative realtime PCR and two step realtime RT-PCR on most realtime PCR machines. The master mix includes Hot Start Taq DNA polymerase and dNTPs in an optimized PCR buffer. ROX Solution is provided separately for use with machines that require ROX. 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). The use of GeneSure Probe PCR Master Mix in real time PCR ensures reproducible, sensitive and specific quantification of genomic, plasmid, viral and cDNA templates.

Hot Start TAQ DNA Polymerase is a Taq DNA polymerase which has been chemically modified by the addition of heatlabile 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.

Probe PCR Buffer 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.

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). UracilDNA Glycosylase (UDG) pretreatment of the reaction removes all dUcontaining amplicons carried over from previous reactions.

Note. UDG is not included in the GeneSure Probe qPCR Master Mix, no ROX and must be purchased separately.

ROX Solution. ROX passive reference dye is supplied in a separate tube at 50 µM concentration. It can be added to a whole 2X master mix tube or to individual reaction mixture. ROX serves as an internal reference for normalization of fluorescent signal when using machines which can detect ROX, e.g. from Applied Biosystems. ROX allows for correction of wellto well variation due to pipetting inaccuracies and fluorescence fluctuations. ROX does not participate in PCR and has a different emission spectrum (the excitation/emission GeneSure are at 580 nm/621 nm, respectively) compared to dyes used for probes. (Refer to a Table 1 to determine the recommended amounts of ROX concentrations for a specific instrument you use).

Table 1. Recommended amounts of ROX for a specific instrument.

Instrument	Amount of ROX per 25 µl reaction	Amount of ROX per 1.25 ml of 2X master mix	Final ROX concentration
Applied Biosystems: 7300, 7900HT, StepOne™, StepOnePlus™, ABI PRISM™7000 and 7700	0.15 µl	15 µl	300 nM
Applied Biosystems: 7500 Stratagene: Mx3000P™, Mx3005P™, Mx4000®	0.15 µl 10X diluted*	15 µl 10X diluted*	30 nM
Bio-Rad: iCycler® iQ, iQ5 and MyiQ™, Opticon®, CFX 96, CFX 384 Roche: LightCycler® 480, LightCycler® 2.0 Corbett: Rotor-Gene™ 3000, 6000 Eppendorf: MasterCycler™ ep realplex Cepheid: Smart Cycler®	Not required	Not required	Not required

\* add 2 µl of ROX Solution to 18 µl of Water, nuclease-free, mix and use 0.15 µl for 25 µl qPCR reaction.

## Guidelines to Assay Design

## Templates

**DNA.** Genomic DNA up to 500 ng and plasmid DNA up to 10 ng can be used in qPCR with GeneSure Probe qPCR Master Mix, no ROX.

**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 RTminus control to confirm complete removal of DNA (see below). 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, #PGK162

## 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 primerdimer formation (if present).

## 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 duallabeled 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 RTqPCR experiments to assess for RNA sample contamination with genomic DNA. The control RT reaction contains all components for RTqPCR except the RT enzyme.



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### IMPORTANT NOTES

- Reaction setup is at room temperature as the mix includes Hot Start Taq DNA polymerase.
- We recommend a reaction volume of 25 µl.
- Preparation of a master mix, which includes all reaction components except template DNA, helps to avoid pipetting errors and is an essential step in realtime PCR.
- Start PCR cycling with an initial denaturation step of 10 min at 95°C to activate Hot Start Taq DNA polymerase.
- Minimize exposure of GeneSure Probe PCR Master Mix (2X), no ROX and ROX Solution to light during handling to avoid loss of fluorescent signal intensity.
- Readjust the threshold value for analysis of every run.

### Protocol

#### Reaction setup

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 Probe qPCR Master Mix (2X), no ROX*	12.5µl
Forward Primer	0.3 µM**
Reverse Primer	0.3 µM**
Probe	0.2 µM
ROX Solution	30 nM/ 300 nM***or not required
Template DNA	≤ 500 ng
Water, nucleasefree	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.

\*\*\*see table 1 on p.3 for final concentration of ROX optimal for your instrument.

3. Mix the master mix thoroughly and dispense appropriate volumes into PCR tubes or plates.
  4. Add template DNA (≤ 500 ng/reaction) to the individual PCR tubes or wells containing the master mix.
- Note.** For twostep RTqPCR, 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 twostep cycling protocol.

#### Three step cycling protocol

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

Data acquisition should be performed during the annealing step.

#### Twostep cycling protocol

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

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

• **UDG pretreatment.** 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 primerdimers may overlap depending on the sequence composition.

### TROUBLESHOOTING

Problem	Possible cause and solution
No amplification curve and no PCR product visible on a gel	<p><b>PCR inhibitors present in the reaction mixture.</b> Repurify your template DNA.</p> <p><b>Primer design is suboptimal.</b> Verify your primer design, use reputable primer design programs or validated predesigned primers.</p> <p><b>RT-PCR: inhibition by excess volume of the RT reaction.</b> Volume of RT reaction product added to qPCR reaction should not exceed 10% of the total qPCR reaction volume.</p> <p>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.</p>



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Problem	Possible cause and solution
No amplification curve but PCR product visible on a gel	<p><b>qPCR instrument settings are incorrect.</b> Check if instrument settings are correct (dye selection, reference dye, filters). Inactive fluorescence detection. Fluorescent detection should be activated and set at annealing or annealing/extension step of the thermal cycling protocol.</p> <p><b>Instrument problems.</b> Refer to the instrument manual for troubleshooting. Degradation of probe. Check the probe for possible degradation on polyacrylamide gel.</p>
Amplification signal in non template control	<p><b>DNA contamination of reagents.</b> ● Follow general guidelines to avoid carry over contamination or include UDG pretreatment step at the beginning of PCR. ● Discard reagents and repeat with new reagents.</p> <p><b>RT-qPCR: RNA contaminated with genomic DNA.</b> Design primers on intron/exon boundaries, treat RNA sample with DNaseI, RNA free prior to reverse transcription.</p>
PCR efficiency is >110%	<p><b>Non specific products.</b> Use melting curve analysis and gel electrophoresis to identify non specific amplicons. Optimize your primer design to avoid such artifacts or use validated pre designed primers.</p>
PCR efficiency is <90%	<p><b>PCR inhibitors present in a reaction mixture.</b> Repurify your template DNA. <b>PCR conditions are suboptimal.</b> Verify the primer/probe concentrations. Verify storage conditions of qPCR master mix. <b>Primer design.</b> Verify your primer design, use primer design programs or validated pre designed primers. Avoid designing primers in regions with high DNA secondary structure.</p>
Poor standard curve	<p><b>Excessive amount of template.</b> Do not exceed maximum recommended amounts of template DNA (500 ng DNA for 25 µl reaction). <b>Suboptimal amount of template.</b> Increase the amount of template, if possible. <b>RT-qPCR: inhibition by excess volume of the RT reaction.</b> Volume of RT reaction product added to qPCR reaction should not exceed 10% of the total qPCR reaction volume.</p>
Nonuniform fluorescence intensity	<p><b>Contamination of the thermal cycler.</b> Perform decontamination of your realtime cycler according to the supplier's instructions. <b>Poor calibration of the thermal cycler.</b> Perform calibration of the real time cycler according to the supplier's instructions.</p>

## REFERENCE

1. Longo, M.C., et al., Use of uracil DNA glycosylase to control carryover contamination in polymerase chain reactions, Gene, 93, 125128, 1990.

## 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.



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