PRODUCT INSERT

GeneSure™ SYBR Green Fluorescein qPCR Master Mix (2X)

Store at -20°C

Cat. No.		Pack Size
PGK024A-B		200 Rxns

Component	PGK024A for 200 rxns of 25 µl	PGK024B for 1000 rxns of 25 µl
GeneSure™ SYBR Green Fluorescein 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™ SYBR Green Fluorescein qPCR Master Mix (2X) is a ready-to-use solution optimized for quantitative real-time PCR and two-step real-time RT-PCR on Bio-Rad iCycler iQ®, MyiQ™ or iQ5™ machines. The master mix includes Hot Start Taq DNA polymerase and dNTPs in an optimized PCR buffer. It contains SYBR® Green I dye and is supplemented with fluorescein passive reference dye. Only template and primers need to be added. Hot Start Taq DNA polymerase in combination with an optimized buffer ensures PCR specificity and sensitivity. The SYBR Green I intercalating dye allows for DNA detection and analysis without using sequence-specific probes. dUTP is included in the mix for optional carryover contamination control using uracil-DNA glycosylase (UDG). The use of SYBR Green Fluorescein qPCR 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 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.

SYBR Green qPCR Buffer has been specifically optimized for qPCR analysis using SYBR Green I. It contains both KCl and $(NH_4)_2SO_4$ to provide high specificity of primer annealing. The buffer composition allows for PCR at a wide range of $MgCl_2$ concentrations. Therefore, optimization of $MgCl_2$ concentration in PCR is generally not necessary.

Fluorescein is included into the master mix to serve as an internal reference for normalization of the SYBR Green I fluorescent signal between different wells to compensate for any instrument or pipetting variation. The fluorescein allows for the generation of Dynamic Well Factors on Bio-Rad iCycler iQ, iQ5 and MyiQ machines, but does not affect qPCR efficiency.
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 Maxima SYBR Green/Fluorescein 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 SYBR Green Fluorescein 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.

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 (Tm): 60°C. Differences in Tm 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).

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 genomic DNA. The control RT- reaction contains all components for RT-qPCR except the RT enzyme.

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IMPORTANT NOTES

- Reaction set-up is at room temperature as the master mix includes Hot Start Taq DNA polymerase. A reaction volume of 25 µl is recommended. 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, helps to avoid 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 Hot Start Taq DNA polymerase.
- Minimize exposure of GeneSure™ SYBR Green Fluorescein qPCR Master Mix (2X) to light during handling to avoid loss of fluorescent signal intensity.
- Readjust the threshold value for analysis of every run.

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™ SYBR Green/ Fluorescein qPCR Master Mix (2X)*	12.5 µl
Forward Primer	0.3 µM**
Reverse Primer	0.3 µM**
Template DNA	≤100 ng
Water, nuclease-free to	25 µl
Total volume	25 µl***

* Provides a final concentration of 2.5 mM $MgCl_2$.
** 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.

Two-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/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.
- **Melting curve analysis** may be performed to verify the specificity and identity of the PCR product. Primer-dimers may occur during PCR if the primer design is not optimal. The dimers are distinguished from the specific product by a lower melting point.

- **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.

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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
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.
Primer-dimers.
Use melting curve analysis to identify primer-dimers by the lower melting temperature compared to amplicon. If presence of dimers is confirmed:
- Redesign your primers according to recommendations or use validated pre-designed primers.
- Optimize annealing temperature by increasing in 3°C increments.

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
PCR efficiency is >110%

Cause and Solution
Non-specific products.
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.

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.

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-univorm Fluorescense 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.

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