Platinum® Quantitative RT-PCR ThermoScript™ One-Step System

Cat. No. 11731-015 **Size: 100 reactions**
Cat. No. 11731-023 **Size: 500 reactions**

Store at –20°C

Description

The Platinum® Quantitative RT-PCR ThermoScript™ One-Step System is a one-step real-time quantitative RT-PCR (qRT-PCR) system that is designed for the convenient, sensitive, and reproducible detection and quantification of RNA molecules. This system combines ThermoScript™ Plus Reverse Transcriptase (RT) and Platinum® *Taq* DNA Polymerase in a single enzyme mix. Both cDNA synthesis and PCR are performed in a single tube using gene-specific primers and either total RNA or mRNA. Reagents are provided for 100 or 500 amplification reactions of 50 µl each.

The system enables highly sensitive detection from as few as 10 copies of RNA template, with a broad dynamic range that supports accurate quantification of high-copy mRNA at up to 1 µg of total RNA.

- **ThermoScript™ Plus RT** is an engineered mutant of avian retroviral reverse transcriptase that has increased thermostability compared to other RTs. Maximum activity for ThermoScript™ Plus RT occurs between 45°C and 50°C; however, it supports cDNA synthesis up to 70°C.
- **Platinum® *Taq* DNA polymerase** is recombinant *Taq* DNA polymerase complexed with a proprietary antibody that blocks polymerase activity at ambient temperatures (1, 2). Activity is restored after the denaturation step in PCR cycling, providing an automatic "hot start" in PCR for increased sensitivity, specificity, and yield.
- **2X ThermoScript™ Reaction Mix** consists of a proprietary buffer system optimized for reverse transcription and PCR amplification, 6 mM $MgSO_4$ optimized for universal use, 0.4 mM of each dNTP, and stabilizers. This convenient 2X format allows further addition of template and primer at any desired concentration.

Reaction conditions for the system have been optimized to support both end-point RT-PCR and qRT-PCR. In qRT-PCR, the mix has been specifically formulated to provide optimal performance using fluorogenic probe-based detection technologies, such as TaqMan® probes (3–9). For one-step qRT-PCR using LUX™ Primers, we recommend the SuperScript® III Platinum® One-Step Quantitative RT-PCR System. For one-step qRT-PCR using SYBR® Green I dye, we recommend the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR System. See **Additional Products**, below.

Component	100-Rxn Kit	500-Rxn Kit
ThermoScript™ Plus/Platinum® *Taq* Mix	100 µl	500 µl
2X ThermoScript™ Reaction Mix	2 × 1.25 ml	12.5 ml
50 mM Magnesium Sulfate	1 ml	2 × 1 ml
ROX Reference Dye	100 µl	500 µl

Storage

Store components at –20ºC. Stability can be extended by storage at -80ºC. **ROX Reference Dye must be stored in the dark.**

Additional Products

The following products are also available from Invitrogen.

Product	Amount	Catalog no.
SuperScript® III Platinum® One-Step Quantitative RT-PCR System	100 rxns	11732-020
	500 rxns	11732-088
SuperScript® III Platinum® SYBR® Green One-Step qPCR System	100 rxns	11736-051
	500 rxns	11736-059
PureLink™ Micro-to-Midi™ Total RNA Purification System	50 rxns	12183-018
TRIzol® Reagent	100 ml	15596-026
	200 ml	15596-018
RNaseOUT™ Recombinant Ribonuclease Inhibitor	5000 units	10777-019
DNase I, Amplification Grade	100 units	18068-015

Recommendations and Guidelines for Quantitative RT-PCR

Template

Starting material can range from 1 pg to 1 µg of purified total RNA. If you are using purified mRNA, the amount of template may be reduced to as low as 0.5 pg.

RNA should be free of RNase contamination and aseptic conditions should be maintained. RNA may be treated with DNase I (Catalog no. 18068-015) to remove any contaminating genomic DNA.

Optional: An RNase inhibitor such as RNaseOUT™ (Cat. no. 10777-019) may be added to the reaction after the 2X Reaction Mix to safeguard against degradation of target RNA due to ribonuclease contamination.

Isolating Total RNA

To isolate total RNA, we recommend the PureLink™ Micro-to-Midi™ Total RNA Purification System (Cat. no. 12183-018), TRIzol® Reagent (Cat. nos. 15596-026 and 15596-018), or the PureLink™ 96 Total RNA Purification Kit for high-throughput applications (Cat. no. 12173-011). Isolation of mRNA from total RNA is typically not necessary, although incorporating this step may improve the yield of specific cDNAs.

Magnesium Concentration

The 2X ThermoScript™ Reaction Mix includes magnesium at a final concentration of 3 mM. This works well for most targets; however, the optimal concentration may range from 3 to 6 mM. If necessary, use the separate tube of 50-mM magnesium sulfate to increase the magnesium concentration. Use the following table to determine the amount of $MgSO_4$ to add to achieve the specified concentration (in a 50-µl PCR with 25 µl of 2X Reaction Mix):

For a Final $MgSO_4$ Conc. of	Add 50-mM $MgSO_4$ (per 50-µl Rxn)
4.0 mM	1 µl
5.0 mM	2 µl
6.0 mM	3 µl

Decrease the amount of water in the reaction accordingly.

ROX Reference Dye

ROX Reference Dye can be included in the reaction to normalize the fluorescent reporter signal, for instruments that are compatible with that option. ROX is supplied at a 25 µM concentration, and is composed of a glycine conjugate of 5-carboxy-X-rhodamine, succinimidyl ester in 20 mM Tris-HCl (pH 8.4), 0.1 mM EDTA, and 0.01% Tween® 20. Use the following table to determine the amount of ROX to use with a particular instrument:

Instrument	Amount of ROX per 50-µl reaction	Final ROX Concentration
ABI 7000, 7300 7700, 7900HT	1.0 µl	500 nM
ABI 7500; Stratagene Mx3000™, Mx3005P™, and Mx4000™	0.1 µl*	50 nM

*To accurately pipet 0.1 µl per reaction, we recommend that you dilute ROX 1:10 immediately before use and use 1 µl of the dilution.

Note that some TaqMan® probes utilize a TAMRA quencher, which can be used as a reference dye for normalization of data. This technique is only valid for an RT-PCR containing a single TaqMan® probe, and should not be used in multiplex applications.

Instrument Guidelines

This kit can be used with a variety of real-time instruments, including but not limited to the ABI PRISM® 7000, 7700, and 7900HT; the ABI 7300 and 7500 Real-Time PCR Systems; the ABI GeneAmp® 5700; the Bio-Rad iCycler™; the Stratagene Mx3000P®, Mx3005P™, and Mx4000®; the Corbett Research Rotor-Gene™; the MJ Research DNA Engine Opticon™, Opticon® 2, and Chromo 4™ Real-Time Detector; and the Cepheid Smart Cycler®. For instrument-specific guidelines, visit www.invitrogen.com/qpcr. Optimal cycling conditions will vary with different instruments.

Primers

Gene-specific primers (GSPs) are required. A final concentration of 200 nM per primer is effective for most reactions. Doubling the amount of reverse primer (to 400 nM) may improve the performance of certain reactions. Optimal results may require a primer titration between 100 and 500 nM.

Design primers that anneal to exons on both sides of an intron or within the exon/exon boundary of the mRNA to allow differentiation between amplification of cDNA and potential contaminating genomic DNA.

For multiplex applications, limit the amount of primer for the reference gene (*e.g.*, β-actin or GAPDH) to avoid competition between amplification of the reference gene and sample gene. In general, the final concentration of the reference gene primer should be between 25 and 100 nM. However, a primer titration is recommended for optimal results.

Dual-Labeled Probes

- The optimal concentration of probe may vary between 50 and 800 nM. A recommended starting concentration is 100 nM.
- The probe sequence should be free of secondary structure and should not hybridize to itself or to primer 3′ ends.
- For multiplex RT-PCR applications, the concentration of each probe may need to be adjusted independently to obtain optimal fluorescent signals. The amount of probe for the reference gene, such as β-actin or GAPDH, should be limited as described above for primers.

Reaction Setup and Conditions

- Keep all components, reaction mixes and samples on ice. After reaction assembly, transfer them to a thermal cycler pre-heated to the desired cDNA synthesis temperature to start the reaction.
- Efficient cDNA synthesis can be accomplished in a 15–30 min incubation at 50–70°C. The optimal temperature will vary for different primers and templates. 50°C is a good general starting point. For problematic templates, or to increase the specificity of cDNA priming, increase the cDNA synthesis temperature to 60°C. Use 70°C as a final option, because RNA template integrity in the presence of Mg^{2+} is short-lived at elevated temperatures.
- ThermoScript™ Plus RT is inactivated, the RNA/cDNA hybrid is denatured, and Platinum® *Taq* DNA polymerase is activated during the 5-minute incubation at 95°C.
- The annealing temperature should be 5–10°C below the melting temperature of the primers used.
- The extension time will vary with the size of the amplification product (approximately 1 minute per 1 kb).

One-Step qRT-PCR Protocol

Follow the protocol below for one-step qRT-PCR using TaqMan® Probes on ABI real-time instruments. Note the lower amount of ROX Reference Dye required for the ABI 7500 systems (see page 2). This generic protocol may also be used for other real-time instruments.

For instrument-specific guidelines, visit www.invitrogen.com/qpcr. A standard 50-µl reaction size is provided; component volumes can be scaled as desired (*e.g.*, scaled down to a 20-µl reaction volume for 384-well plates).

1. Program your real-time instrument to perform cDNA synthesis immediately followed by PCR amplification, as shown below. Optimal temperatures and incubation times may vary for different target sequences.

   cDNA Synthesis: 50–70°C for 15–30 minutes (use 50°C for 30 minutes as a general starting point)
   PCR: 95°C for 5 minutes (1 cycle)
   35–45 cycles of:
   95°C for 15 seconds
   60–65°C for 60 seconds

2. Set up reactions **on ice**. Volumes for a single 50-µl reaction are listed below. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well on ice, and then add the unique reaction components (*e.g.*, template). **Note:** Preparation of a master mix is essential in qRT-PCR to reduce pipetting errors.

Component	Single rxn
ThermoScript™ Plus/ Platinum® *Taq* Enzyme Mix	1 µl *
2X ThermoScript™ Reaction Mix	25 µl
Forward primer, 10 µM	1 µl
Reverse primer, 10 µM	1 µl
Fluorogenic probe, 10 µM	0.5 µl
ROX Reference Dye (optional)	1 µl/0.1 µl**
RNaseOUT™, 40 U/µl (optional)	1 µl
Template (1 pg to 1 µg total RNA)	≤ 10 µl
DEPC-treated water	to 50 µl

   *To test for genomic DNA contamination of the RNA template, prepare a control reaction containing 2 units of Platinum® *Taq* DNA polymerase instead of the ThermoScript™ Plus/Platinum® *Taq* Mix.

   **See the table on page 2 for the amount/concentration of ROX to use for your specific instrument.

3. Cap or seal the reaction tube/PCR plate, and gently mix. Make sure that all components are at the bottom of the tube/plate; centrifuge briefly if needed.

4. Place reactions in a preheated real-time instrument programmed as described above. Collect data and analyze results.

One-Step End-Point RT-PCR Protocol

Follow the protocol below for one-step end-point RT-PCR. The cycling conditions were established using a DNA Thermal Cycler 9600 or 2400 (Perkin-Elmer) and may need to be altered for other thermal cyclers. Optimal temperatures and cycling conditions will also depend on primer and target sequences. A standard 50-µl reaction size is provided; component volumes can be scaled as desired (*e.g.*, scaled down to a 20-µl reaction volume for 384-well plates).

Cycling Program

cDNA synthesis: 50–70°C, 15–30 minutes[1]
PCR: 95°C, 5 minutes (1 cycle)
35–40 cycles:
95°C, 15 seconds[2]
55-60°C, 30 seconds
68–72°C, 1 minute/kb

[1]Use 60°C for 30 minutes at as a general starting point.
[2]If using Perkin-Elmer Model 480, use 30 seconds denaturation instead of 15 seconds.

Reaction Mix

Component	Per 50-µl Rxn
ThermoScript™ Plus/ Platinum® *Taq* Enzyme Mix[3]	1 µl
2X ThermoScript™ Reaction Mix	25 µl
Template RNA (10 pg–1 µg)	x µl
Forward primer, 10 µM	1 µl
Reverse primer, 10 µM	1 µl
RNaseOUT™, 40 U/µl (optional)	1 µl
DEPC-treated water	to 50 µl

[3]To test for genomic DNA contamination of the RNA template, prepare a control reaction containing 2 units of Platinum® *Taq* DNA polymerase instead of the ThermoScript™ Plus/Platinum® *Taq* Mix.

1. Program the thermal cycler to perform cDNA synthesis immediately followed by PCR amplification, as specified above.
2. Set up reactions **on ice**. Add the reaction mix to a microcentrifuge tube or each well of a PCR plate placed on ice. When assembling multiple reactions, prepare a master mix of all components except template.
3. Gently mix and make sure that all the components are at the bottom of the amplification tube. Centrifuge briefly if needed. Depending on the thermal cycler used, overlay with silicone oil, if necessary.
4. Place reactions in a preheated thermal cycler programmed as described above. Collect data and analyze results.

Quality Control

The Certificate of Analysis (CofA) provides detailed quality control information for each product. The CofA is available on our website at www.invitrogen.com/cofa, and is searchable by product lot number, which is printed on each box.

Troubleshooting Guide

Problem	Possible Cause	Probable Solution
No amplification product Relative fluorescent signal ≤ background or no template control	cDNA synthesis temperature too high, low priming efficiency	Lower incubation temperature.
	RT or cDNA primer blocked by secondary structure	Raise incubation temperature. Redesign primer(s).
	RNA has been damaged or degraded	Replace RNA if necessary.
	RNase contamination	Maintain aseptic conditions; add RNase inhibitor.
	Fluorescent probe not functional	Validate probe design and presence of fluorophore and quencher: Treat TaqMan® probe with DNase, and check for increase in fluorescence. Redesign and/or resynthesize probe if necessary.
Poor sensitivity	Not enough starting template RNA	Increase the concentration of template RNA; use 10 ng to 1 µg of total RNA.
Product detected at higher than expected cycle number	RNA has been damaged or degraded	Replace RNA if necessary.
	RNase contamination	Maintain aseptic conditions; add RNase inhibitor.
	RT inhibitors are present in RNA	Remove inhibitors in the RNA preparation by an additional 70% ethanol wash. Inhibitors of RT include SDS, EDTA, guanidium salts, formamide, sodium phosphate and spermidine (10, 11).
	Inefficient cDNA synthesis	Adjust cDNA synthesis temperature and/or primer design.
	Inefficient PCR amplification	Optimize PCR conditions: Adjust annealing temperature as necessary. Increase magnesium concentration. Redesign primers.
Higher than expected signal	Too much sample added to reactions	Decrease the concentration of template RNA.
Product detected at lower-than-expected cycle number, and/or positive signal from no-template controls	Template or PCR carry-over contamination	Isolate source of contamination and replace reagent(s). Use separate dedicated pipettors for reaction assembly and post-PCR analysis. Assemble reactions (except for target addition) in a DNA-free area. Use aerosol-resistant pipet tips or positive displacement pipettors.
Unexpected bands after electrophoresis	RNA contamination with genomic DNA	Pre-treat RNA with DNase I.
	Oligo dT or random primers used for 1st strand synthesis	Use gene-specific primers.
	Low specificity in PCR	Optimize PCR conditions as described above.

References

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