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ThermoScript™ RT-PCR System

Catalog nos. (25 reactions):
11146-024
11146-057 (w/ Platinum® *Taq* DNA polymerase)

Catalog nos. (100 reactions):
11146-016
11146-032 (w/ Platinum® *Taq* DNA polymerase)
11146-040 (w/ Platinum® *Taq* DNA polymerase High Fidelity)

Store at –20°C (stability can be extended by storing at -70°C)

Doc. part no. 11146.pps **Pub. no.** MAN0000941 **Rev.** 3.0

Product description

The ThermoScript™ RT-PCR System is designed for the sensitive and reproducible detection and analysis of RNA molecules in a two-step process. ThermoScript™ RT, an avian reverse transcriptase with reduced RNase H activity, is engineered to have higher thermal stability, produce higher yields of cDNA, and produce more full-length cDNA transcripts than AMV RT. cDNA synthesis is performed in the first step using either total RNA or poly(A)$^+$-selected RNA primed with oligo(dT), random primers or a gene-specific primer, at 50-65°C. In the second step, PCR is performed in a separate tube using primers specific for the gene of interest. RNA targets from 100 bp to >12 kb can be detected with this system, using 10 pg to 5 µg of total RNA. PCR is performed with Platinum® *Taq* DNA Polymerase or Platinum® *Taq* DNA Polymerase High Fidelity. Platinum® *Taq* DNA Polymerase High Fidelity is suitable for templates from 100 bp to >12 kb. Platinum® *Taq* DNA polymerase (1) provides automatic hot-start conditions for increased specificity up to 3 kb.

Reagents are provided for 25 or 100 cDNA synthesis reactions of 20 µL each and 25 or 100 amplification reactions of 50 µL each.

Component	25 rxn kit	100 rxn kit
ThermoScript™ RT (15 U/µL)	25 µL	100 µL
5X cDNA Synthesis Buffer*	500 µL	500 µL
0.1 M DTT	250 µL	250 µL
10 mM dNTP Mix	100 µL	2 × 250 µL
RNaseOUT™ (40 U/µL)	25 µL	100 µL
Oligo $(dT)_{20}$ (50 µM)	25 µL	100 µL
Random Hexamers (50 ng/µL)	50 µL	250 µL
DEPC-Treated Water	1.25 mL	1.25 mL
E. coli RNase H (2 U/µL)	50 µL	2 × 50 µL

*250 mM Tris acetate (pH 8.4), 375 mM potassium acetate, 40 mM magnesium acetate, stabilizer

Catalog numbers 11146-057 (25 rxns) and 11146-032 (100 rxns) include the following, in addition to the components to the left:

Component	25 rxn kit	100 rxn kit
Platinum® *Taq* DNA polymerase	24 µL	60 µL
10X PCR buffer Minus Mg	1.25 mL	2 × 1.25 mL
50 mM $MgCl_2$	1 mL	1 mL
KB Extender	1.3 mL	1.3 mL

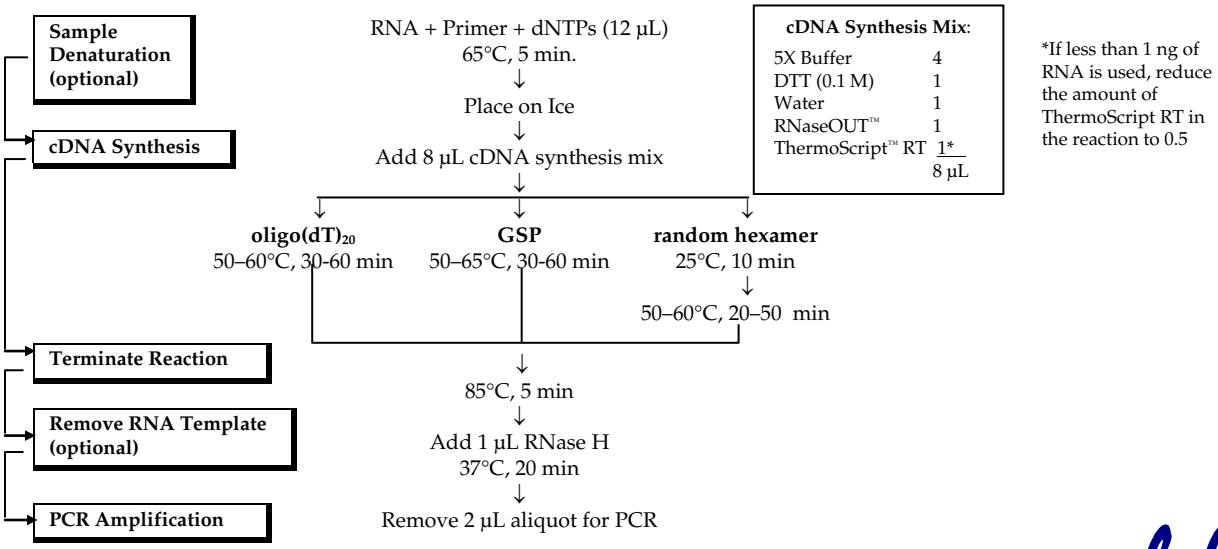
Catalog number 11146-040 (100 rxns) includes the following, in addition to the components to the left:

Component	100 rxn kit
Platinum® *Taq* DNA Polymerase High Fidelity	100 units
10X High Fidelity PCR Buffer	1.0 mL
50 mM $MgSO_4$	1.0 mL

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.

Summary of Procedure



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Important Guidelines and Parameters

RNA

- High quality intact RNA is essential for successful full-length cDNA synthesis and successful long RT-PCR.
- RNA should be devoid of any RNase contamination and aseptic conditions should be maintained.
- Recommended methods of total RNA isolation include the Micro-to-Midi Total RNA Purification System (Catalog no. 12183-018) and TRIzol® Reagent (Catalog no. 15596-026) (2, 3). Oligo(dT)-selection for poly(A)+ RNA is typically not necessary, although incorporating this step may improve the yield of specific cDNAs.

cDNA Synthesis Primers

- Oligo(dT)$_{20}$ (50 pmoles/reaction) is recommended for priming poly(A)+ RNA.
- Random hexamers (50-250 ng/reaction) are efficient primers for the detection of multiple short RT-PCR targets. Use of more than 50–100 ng primer/µg of RNA can increase the yield of short products but may inhibit detection of long targets (>3kb) or rare transcripts. If random hexamers are used, the first-strand reaction must be incubated at 25°C for 10 min to extend the primers prior to synthesis.
- Gene-specific primers (GSP) should be used at 10–20 pmol/reaction. Specificity of priming may be improved by optimizing annealing/reaction temperature.
- Treatment of cDNA with RNase H to remove the complementary RNA prior to PCR is optional. RNase H digestion will improve the RT-PCR signal of many targets and is required for the efficient and consistent amplification of long RT-PCR templates.

cDNA Synthesis Reaction

- Optional: Denature the RNA template and primer by incubating at 65°C for 5 min. Most targets can be reverse transcribed without this step; however, it can remove secondary structure that may impede cDNA synthesis.
- We recommend incubation at 50–55°C for most RT-PCR targets. However, incubation at 50–60°C for oligo(dT) and 50–65°C for gene-specific primers can reduce secondary structure and improve specificity.
- Most targets can be amplified after only a 30-min incubation for the first-strand reaction. Rare RNAs, long transcripts, or targets at the 5′ end of long transcripts benefit from longer incubation times (50–60 min).

PCR Primers

- A final concentration of 0.2– 0.4 µM per primer is generally optimal. A primer titration is recommended for best results.
- Design primers that anneal to sequence in exons on both sides of an intron or exon/exon boundary of the mRNA to allow differentiation between amplification of cDNA and potential contaminating genomic DNA.
- Primers should not be self-complementary or complementary to each other at the 3′ ends.

PCR Reactions

- Most targets will be efficiently amplified using 2 µL or less of the cDNA synthesis reaction.
- The optimum magnesium concentration varies from 1.5 to 3 mM. In general, the Mg amounts specified in the following protocols are optimal for most primer sets, but titration is recommended for best results. Each µL of the cDNA synthesis reaction adds 0.16 mM to the final magnesium concentration in a 50-µL PCR reaction.
- Assemble the PCR reactions on ice, transfer them to a pre-heated thermal cycler (85–95°C) and immediately start the PCR amplification program.
- The annealing temperature should be 10°C below the melting temperature of the primers used.
- The optimum extension time for Platinum® Taq DNA Polymerase High Fidelity varies with the size of the amplicon (approximately 1 min per 1 kb of amplicon).

cDNA Synthesis

1. In a 0.2- or 0.5-mL tube, combine primer (50 µM Oligo(dT)$_{20}$, 50 ng/µL random primer or 10 µM gene-specific primer), RNA, and dNTP mix and adjust volume to 12 µL with DEPC-treated water.

Component	Amount
Primer	1 µL
RNA (10 pg to 5 µg)	X µL
10 mM dNTP Mix	2 µL
DEPC-treated water	to 12 µL

2. Denature RNA and primer by incubating at 65°C for 5 min and then place on ice (optional).
3. Vortex the 5X cDNA Synthesis Buffer for 5 s just prior to use.
4. Prepare a master reaction mix on ice and vortex gently.

Component	Single Reaction	10 Reactions
5x cDNA Synthesis Buffer	4 µL	40 µL
0.1 M DTT	1 µL	10 µL
RNaseOUT™ (40 U/µL)	1 µL	10 µL
DEPC-treated water	1 µL	10 µL
ThermoScript™ RT (15 units/µL)	1 µL*	10 µL*

***NOTE**: If less than 1 ng of template RNA is used, reduce the amount of ThermoScript™ RT in the reaction to 0.5 µL/reaction (5 µL/10 reactions). Increase the amount of DEPC-treated water in the master reaction mix to 1.5 µL/reaction (15 µL/10 reactions).

5. Pipet 8 µL of master reaction mix into each reaction tube on ice.
6. Transfer to a thermal cycler preheated to the appropriate cDNA synthesis temperature and incubate as follows:
   - Oligo(dT)$_{20}$ primed: 30–60 min at 50°C (or 50–60°C)
   - Gene-specific primed: 30–60 min at 50°C (or 50–65°C)
   - Random-hexamer primed: 25°C for 10 min, followed by 20–50 min at 50°C (or 50–65°C)
7. Terminate the reaction by incubating at 85°C for 5 min.
8. Add 1 µL of RNase H and incubate at 37°C for 20 min (optional).
9. cDNA synthesis reactions can be stored at –20°C or used for PCR immediately.

PCR with Platinum® *Taq* DNA Polymerase

Use only 10% of the cDNA synthesis reaction (2 µL) for PCR. Use of 50 mM $MgCl_2$ and 2 µL of cDNA will result in a final magnesium concentration of 1.82 mM, which is adequate for most primers and targets. However, titration of magnesium concentration is recommended for best results.

KB Extender: Enhances the extension of cDNA targets longer than 4 kb, with the greatest benefits for targets longer than 5 kb. For these targets, add 1 µL of KB Extender per 50-µL reaction.

Single reactions: To avoid pipetting volumes <1 µL, for single reactions dilute 1 µL Platinum® *Taq* DNA Polymerase in 4 µL of nuclease-free water (a 1:5 dilution) and use 1 µL of dilution.

1. Add the following to a 0.2- or 0.5-mL thin-walled PCR tube:

Component	Volume per reaction	10 Reactions
10X PCR Buffer Minus Mg	5 µL	50 µL
50 mM $MgCl_2$	1.5 µL	15 µL
10 mM dNTP mix	1 µL	10 µL
Primer Mix (10 µM each)	1 µL	10 µL
Optional: KB Extender, for targets >4–5 kb (see note above)	1 µL	10 µL
Platinum® *Taq* DNA Polymerase *(for single reactions, dilute 1:5 and use 1 µL as noted above)	0.2 µL*	2 µL
cDNA (from synthesis reaction)	2 µL	20 µL
Nuclease-free water	38.1 µL	381 µL
Final volume	50 µL	500 µL

2. Cap the tubes, mix, and centrifuge briefly to collect the contents.
3. Incubate tubes in a thermal cycler at 94°C for 30 seconds to 2 minutes to completely denature the template and activate the enzyme.
3. Perform 20 to 40 cycles of PCR amplification as follows:

Step	Temp	Time
Denature	94°C	30 seconds
Anneal	55°C	30 seconds
Extend	68–72°C	1 minute per kb
Hold	4°C	Indefinitely

4. Samples can be stored at –20°C until use. Analyze the products by agarose gel electrophoresis.

PCR with Platinum® *Taq* DNA Polymerase High Fidelity

Use only 10% of the cDNA synthesis reaction (2 µL) for PCR. Use of 2 µL of 50 mM $MgSO_4$ and 2 µL of cDNA (0.32 mM magnesium in a 50-µL PCR) results in a final concentration of 2.32 mM magnesium, which is effective for most primer sets. However, titration of the magnesium concentration with the provided 50 mM $MgSO_4$ is recommended for best results.

Single reactions: To avoid pipetting volumes <1 µL, for single reactions dilute 1 µL Platinum® *Taq* DNA Polymerase in 4 µL of nuclease-free water (a 1:5 dilution) and use 1 µL of dilution.

1. Add the following to a 0.2- or 0.5-mL thin-walled PCR tube:

Component	Volume per reaction	10 Reactions
10X High Fidelity PCR Buffer	5 µL	50 µL
50 mM $MgSO_4$	2 µL	20 µL
10 mM dNTP Mix	1 µL	10 µL
Primer Mix (10 µM each)	1 µL	10 µL
Platinum® *Taq* High Fidelity *(for single reactions, dilute 1:5 and use 1 µL as noted above)	0.2 µL*	2 µL
cDNA (from cDNA synthesis reaction)	2 µL	20 µL
DEPC-treated water	37.8 µL	378 µL
Final volume	50 µL	500 µL

2. Mix gently and overlay with silicone oil or mineral oil if the thermal cycler lacks a heated lid.
3. Incubate at 94°C for 2 min, then perform 20 to 40 cycles of PCR with optimized conditions for your sample (1 min/kb extension time at 68°C).
4. Analyze 10 µL of the amplified sample by agarose gel electrophoresis.

Control Reactions

An RT-PCR Primer and Control Set is available separately for monitoring the performance of the system (Cat. Number 10929-016).

1. Use 1 ng of the Control RNA in the cDNA Synthesis Reaction.

2. Perform the PCR using the appropriate polymerase, as described above.

Troubleshooting Guide

Problem	Possible cause	Possible solution
No RT-PCR product	No cDNA synthesis (temperature too high)	For the cDNA synthesis step, incubate at 45–50°C.
	Incomplete synthesis of target cDNA (secondary structure of RNA blocks synthesis)	For the cDNA synthesis step, incubate at 50–70°C. For long mRNAs, increase cDNA synthesis incubation time (up to 50 min)
	RNase contamination	Maintain aseptic conditions; add RNaseOUT™ (RNase inhibitor).
	Concentration of template RNA in reaction is too low	Increase the concentration of template RNA; use 1–5 µg of total RNA or reduce the volume of ThermoScript™ RT used in the reaction.
	RNA has been damaged or degraded	Replace RNA.
	RT inhibitors are present in RNA	Remove inhibitors in the RNA preparation by an additional 70% ethanol wash after ethanol precipitation. Note: Inhibitors of RT include SDS, EDTA, guanidinium chloride, formamide, sodium phosphate and spermidine (4).
	Cycle number is too low	Increase cycle number.
Low yield/low specificity in PCR	Reaction conditions not optimal	Optimize magnesium concentration. Optimize the primer concentration
		Optimize the annealing temperature and extension time.
		Increase temperature of RT reaction to 50-60°C.
Unexpected bands after electrophoresis	RNA contamination with genomic DNA	Pre-treat RNA with DNase I. Redesign PCR primers to anneal to sequence in exons on both sides of an intron in the target gene.

References

1. Westfall, B., Sitaraman, K., Solus, J., Hughes, J., and Rashtchian, A. (1997) *Focus® 19*, 46.
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3. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) *Biochemistry 18*, 5294.
4. Gerard, G.F (1994). *Focus® 16*, 102.

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