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Roche

Transcriptor One-Step RT-PCR Kit

Hot start one step RT-PCR Kit for end-point analysis up to 6.5 kb

Cat. No. 04 655 877 001 50 reactions including 10 control reactions
Cat. No. 04 655 885 001 150 reactions without control reactions

Version 07
Content version: June 2011

Store the kit at −15 to −25°C.
Store vial 4 (Control RNA) at − 60°C or below.

1. What this product does

Number of Tests

The kit is designed for 50 or 150 reactions (depending on pack size):

- a) Cat. No. 04 655 877 001 contains 50 reactions including 10 control reactions.
- b) Cat. No. 04 655 885 001 contains 150 reactions without control reactions.

Kit contents

Vial	Label	Content
1	Transcriptor Enzyme Mix	a) 1 vial / 50 µl b) 3 vials / 50 µl each contains Transcriptor Reverse Transcriptase, Expand System and Protector RNase Inhibitor
2	RT-PCR Reactions Buffer, 5× conc.	a) 1 vial / 500 µl b) 3 vials / 500 µl each including Tris, $MgCl_2$, sodium salts of dNTPs (1.5 mM each) and additives for hot start PCR
3	Water, PCR Grade	a) 2 vial / 1 ml each b) 6 vials / 1 ml each
4	Control RNA	a) 50 µl, 200 copies/µl HAV RNA, *in vitro* transcript
5	Control Primer Mix	a) 20 µl, HAV RNA upstream and downstream primer to amplify a 246 bp-fragment, each primer 10 µM

Storage and Stability

Store the kit at −15 to − 25°C.

⚠ Store the supplied control RNA (vial 4) separately at −60°C or below.

If properly stored, all kit components are stable until the expiration date printed on the label.

The kit is shipped on dry ice.

⚠ Avoid repeated freezing and thawing.

Application

The Transcriptor One-Step RT-PCR Kit is designed for fast, sensitive, and specific end-point RT-PCR analysis using gene-specific primers. It combines the high sensitivity and yield of Transcriptor Reverse Transcriptase with the fidelity and yield of the Expand System. It adds high specificity and overall improved performance like reduced primer-dimer formation by the innovative hot-start buffer. It allows reaction temperatures up to 60°C and achieves full-length transcripts up to 6.5 kb.

The kit may be applied to:

- Qualitative, semi quantitative or quantitative analysis of RNA transcription levels even in single cells in combination with gel-based detection methods or ELISA-based detection methods.
- Cloning of RNA up to 6.5 kb.
- Mutation analysis at RNA level in combination with sequencing or other mutation.
- Scanning techniques.

Additional Equipment and Reagents Required

- Standard laboratory equipment
  - nuclease-free, aerosol-resistant pipette tips
  - pipettes with disposable, positive-displacement tips
  - sterile reaction tubes for preparing PCR mixes and dilutions
  - standard bench top microcentrifuge
- For the RT-PCR reaction
  - standard block cycler instrument
  - PCR primers
  - template RNA
  - PCR reaction vessels (thin-walled PCR tubes or plates are recommended)

Assay Time

1 to 2 h for RT-PCR of a 246 bp fragment (*e.g.*, control reaction supplied with the kit)

2. How to use this product

2.1 Before you begin

Precautions

Take special precautions when working with RNA:

- Always wear gloves when working with RNA. After putting on gloves, do not touch surfaces and equipment to avoid reintroduction of RNases to decontaminated material.
- Designate a special area for RNA work only.
- Treat surfaces of benches and glassware with commercially available RNase inactivating agents. Clean benches with 100% ethanol.
- Use commercially available sterile and RNase-free disposable plasticware only.
- Purchase reagents that are free of RNases. Reserve separate reagents for RNA work only. Use DEPC - treated H_2O for all solutions.
- Keep all reagents on ice.
- Extract RNA as quickly as possible after obtaining samples. For best results, use either fresh samples or samples that have been quickly frozen in liquid nitrogen and stored at – 60°C or below.

Sample material

Isolated total RNA from 1 fg up to 1 µg, poly $(A)^+$ RNA, *in vitro* transcribed RNA, or lysates from cell culture.

RNA preparation

For high quality eukaryotic RNA preparations it is necessary to minimize the activity of RNases released during cell lysis by using inhibitors of RNases or methods that disrupt cells and simultaneously inactivate RNases. A good overview of appropriate methods is given in ref. 1 and 2.

This kit can be used in combination with RNA purified with *e.g.,* Roche's High Pure Kits, Roche's MagNA Pure Kits and with lysates of cells.

Avoid contamination with RNases from other potential sources like glass ware, plastic ware, and reagent solutions. Ref. 1 lists several measures to avoid problems with RNase contamination.

The enzyme mix (vial 1) contains the Protector RNase Inhibitor, which inhibits a wide spectrum of RNases and is active up to 60°C. Protector RNase Inhibitor protects the RNA during cDNA synthesis, where other RNase inhibitors fail. Integrity of mRNA is particularly important when longer fragments are analyzed.

The size of the mRNA can be determined by gel electrophoresis and ethidium bromide staining. The mRNA should appear as a smear between approx. 500 bp and 8 kb. The bulk of the mRNA should be between 1.5 and 2 kb.

Primer design

Sequence-specific primers have to be used for one-step RT-PCR.

To differentiate between amplification of cDNA and amplification of contaminating genomic DNA, primers can be designed that anneal to sequences in exons on both sides of an intron. With this approach PCR products from genomic DNA will be much longer compared to the intronless mRNA derived products. Alternatively, a primer designed on an exon/exon boundary of the mRNA should not amplify genomic DNA.

DNA contamination

Include appropriate positive and negative control reactions to exclude artifacts from DNA targets like residual genomic DNA contaminations from RNA preparations or contaminating DNA from previous amplifications.

2.2 Setup of the RT-PCR reaction

Step	Action
❶	Thaw the components listed below and place them on ice.
❷	Vortex briefly and centrifuge all reagents before setting up the reactions.
❸	Set up the reaction components in a nuclease-free microcentrifuge tube placed on ice:

Component	Volume	Final conc.
Water, PCR Grade (vial 3)	× µl	
5× Reaction Buffer (vial 2)	10 µl	1×
Fwd primer	× µl	0.4 µM
Rev primer	× µl	0.4 µM
Transcriptor Enzyme Mix (vial 1)	1 µl	
Template RNA	× µl	100 ng (down to 1 fg)
Total	**50 µl**	

❹	Mix the reagents and centrifuge briefly to collect the sample at the bottom of the tube.

2.3 RT-PCR Protocol

RT-PCR is performed in a standard block cycler instrument. Depending on the instrument used, the recommended program may require optimization. The cycle number is dependent on the abundance of the respective mRNA. For rare mRNA messages 40 cycles or a second nested PCR could be necessary.

Reverse transcription: Reverse transcription may be performed between 45°C and 60°C. When establishing a new assay, it is recommended to start with 50°C for 30 min. If a higher stringency in primer annealing is required or if difficult targets (*e.g.*, GC-rich templates or templates with a high degree of secondary structures) are reverse transcribed the temperature can be raised up to 60°C. For higher reaction temperatures primers with appropriate melting temperatures have to be used.

PCR: During the initial denaturation step Transcriptor Reverse Transcriptase is inactivated and the RNA/cDNA hybrid is denatured. The recommended denaturation temperature is 94°C. A high GC content (>60%) of the template might require either a higher denaturation temperature or a longer denaturation time.

The annealing temperature in PCR depends on the melting temperature of the respective primer pair. Use an appropriate computer program to calculate the optimal temperature for your primers. The recommended annealing temperature is the melting temperature of the primers or 2°C below.

The elongation temperature is always 68°C. The elongation time depends on the template length: 60 seconds per kb. After 10 cycles it is recommended to extend the elongation time by 5 seconds per kb for each successive cycle. This may result in higher yield and higher sensitivity.

Standard RT-PCR protocol

Step	Action			
Sample loading	Overlay the reaction with 30 µl mineral oil, if required by the type of block cycler used.			
Reverse transcription	50°C for 10 - 30 min			
Initial denaturation	94°C for 7 min			
Standard PCR Profile	**Step**	**Temp.**	**Time (s)**	**Cycles**
	Denaturation	94°C	10	10
	Annealing	× [1] °C	30	
	Elongation	68°C	× [2]	
	Denaturation	94°C	10	25
	Annealing	× [1] °C	30	
	Elongation	68°C	×[2] + ×[3]	
	Final Elong.	68°C	7 min	
Analyze samples	1 - 3% agarose gel.			

Short RT-PCR protocol[4]

In most cases the short RT-PCR protocol is sufficient. Due to the shortened reaction time, yield could be reduced. Therefore use the standard protocol if highest sensitivity is necessary.

Step	Action			
Sample loading	Overlay the reaction with 30 µl mineral oil, if required by the type of block cycler used.			
Reverse transcription	50°C for 5 min			
Initial denaturation	94°C for 5 min			
Short PCR Profile	**Step**	**Temp.**	**Time (s)**	**Cycles**
	Denaturation	94°C	10	35
	Annealing	× [1] °C	30	
	Elongation	68°C	× [2]	
	Final Elong.	68°C	5 min	
Analyze samples	1 - 3% agarose gel.			

[1] Melting temperatures of primers or up to 2°C below.

[2] Elongation time: 60 s per kb.

[3] Cycle elongation of 5 s per kb for each successive cycle. Cycle elongation may result in higher yield and specificity.

[4] Evaluated by using a fast cycling instrument (*e.g.*, 2720 Thermal Cycler, Applied Biosystems).

2.4 Setup of the control reaction

Step	Action
❶	Thaw the components listed below and place them on ice.
❷	Vortex briefly and centrifuge all reagents before setting up the reactions.
❸	Set up the control reaction in a nuclease-free microcentrifuge tube placed on ice:

Component	Volume	Final conc.
Water, PCR Grade (vial 3)	32 µl	
5 × Reaction Buffer (vial 2)	10 µl	1×
Control Primer Mix (vial 5)	2 µl	0.4 µM
Transcriptor Enzyme Mix (vial 1)	1 µl	
Control RNA (vial 4)	5 µl	1000 copies
Total	**50 µl**	

❹	Mix the reagents and centrifuge briefly to collect the sample at the bottom of the tube.

RT-PCR performed on a dilution series of HAV RNA, *in vitro* transcript (provided with Cat. No. 04 655 877 001)

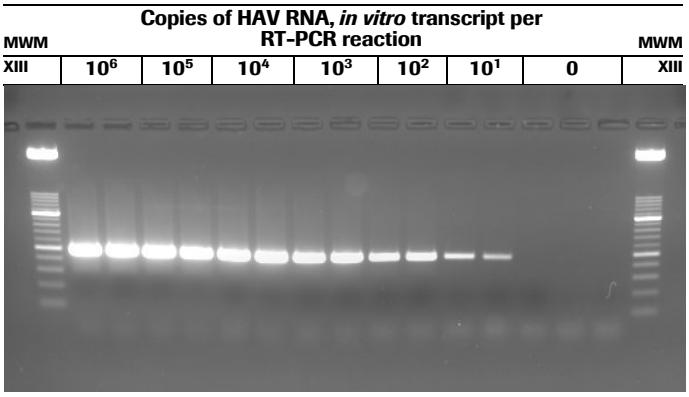

Fig. 1: RT-PCR was performed on a dilution series of the HAV RNA, *in vitro* transcript provided with Cat. No. 04 655 877 001. The setup of the control reaction and the subsequent RT-PCR with primers for a 246 bp HAV fragment was performed according to the standard RT-PCR protocol (reverse transcription at 50°C for 30 min, PCR annealing at 55°C).

3. Typical Results

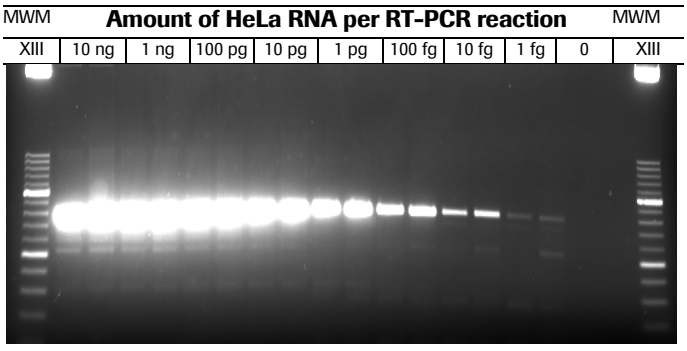

Fig. 2: High sensitivity for difficult templates. RT-PCR was performed on a dilution series of the HeLa RNA. A 389 bp fragment was amplified with primers specific for human 28S ribosomal RNA according to the standard RT-PCR protocol (reverse transcription at 50°C for 30 min). The GC content of the amplified fragment is 64%. With 1 fg of RNA a clearly visible band is obtained after agarose gel electrophoresis and ethidium bromide staining.

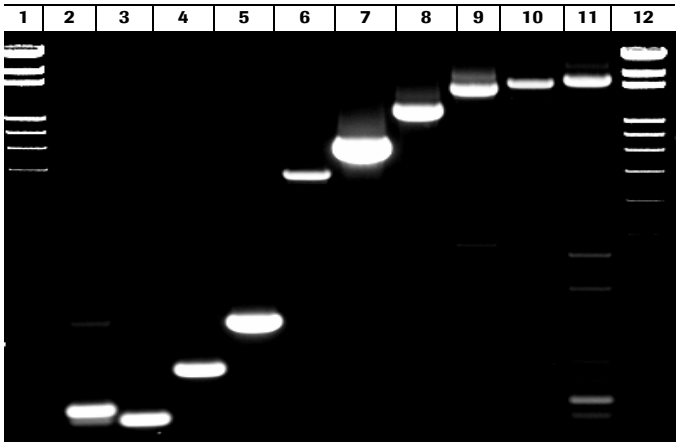

Fig. 3: Long fragments from a variety of templates. The Transcriptor One-Step RT-PCR Kit generates fragments up to 6.5 kb. RT-PCR on various fragments from different sources was performed according to the standard RT-PCR protocol. Aliquots of 15 µl of each RT-PCR reaction were analyzed on an agarose gel.

Lanes	Content
1 and 12	Molecular Weight Marker IV
2	246 bp HAV (10^6 copies of HAV)
3	139 bp Apo E (10 ng of human liver RNA)
4	387 bp ß-Actin (10 ng of K562 RNA)
5	587 bp ß-Actin (10 ng of K562 RNA)
6	1.8 kb Dystrophin (10 ng human skeletal muscle RNA)
7	2.5 kb Apo B (100 ng human liver RNA)
8	3.6 kb Dystrophin (100 ng human skeletal muscle RNA)
9	5.3 kb hTSF (100 ng HeLa RNA)
10	6.0 kb Dystrophin (100 ng human skeletal muscle RNA)
11	6.3 kb mfas (100 ng mouse liver RNA)

4. Troubleshooting

Problem	Possible cause	Solution
No PCR product or very little amount of PCR product	Insufficient amount of template RNA	▪ Increase amount of RNA template in cDNA reaction. ▪ Use poly(A)$^+$ mRNA rather than total RNA as template. ▪ Raise enzyme amount to 1.5 to 2-fold.
	Template RNA degraded	▪ Prepare fresh RNA template, being careful to prevent RNase activity. ▪ Check RNA preparation by gel electrophoresis.
	Too much template RNA	A too high amount of template RNA may affect/inhibit performance of RT-PCR: Decrease amount of RNA template.
	RT-PCR Inhibitors are present in the RNA	Make sure that the RNA is free of RT-PCR inhibitors, *e.g.*, by using Roche's High Pure Kits or Roche's MagNA Pure Kits for RNA purification and isolation.
	Reaction not optimized	▪ Increase primer concentration (up to 1 µM maximum). ▪ Synthesize the cDNA at a higher temperature.
	Template secondary structure prevented effective first strand cDNA synthesis	Raise temperature for reverse transcription reaction up to 60°C.
	Template secondary structure inhibits effective formation of full-length products	If GC content of RNA is high (>60%), increase denaturation temperature or denaturation time in PCR cycles.

Problem	Possible cause	Solution
	Incubation temperature too high	For higher reverse transcription reaction temperatures, primers with appropriate melting temperatures have to be used. The annealing temperature in PCR depends on the melting temperature of the respective primer pair. Use an appropriate computer program to calculate the optimal temperature for the primers used. The recommended annealing temperature is the melting temperature of the primers or 2°C below.
Background smear	Secondary amplification product(s)	Check reagent concentrations and cycling conditions. ▪ Optimize temperature of cDNA synthesis step. ▪ Optimize primer concentration. ▪ Decrease number of cycles. ▪ Check and perhaps decrease concentration of template.
Non-specific product bands	Annealing temperature too low	Increase annealing temperature during PCR to increase specificity of amplification.
	Primer formed dimers	Design primers without complementary sequences at the 3' ends.
	Contaminating DNA in sample	▪ Perform a control without reverse transcription step. ▪ Design primers that anneal to sequence in exons on both sides of an intron or at the exon / exon boundary of the mRNA to differentiate between amplified cDNA and potential contaminating DNA.

5. Additional Information on this Product

5.1 How this Product Works

The Transcriptor One-Step RT-PCR Enzyme Mix contains a blend of active components: Transcriptor Reverse Transcriptase, Protector RNase Inhibitor, and a DNA polymerase enzyme blend, consisting of Taq DNA Polymerase and a proofreading polymerase. Transcriptor Reverse Transcriptase enables for sensitive and robust reverse transcription. Protector RNase Inhibitor is fully active at elevated temperatures to provide maximum template protection during reverse transcription. For the PCR step an optimized enzyme blend is included to assure high fidelity and minimize the probability of introduction of mutations.

An optimized RT-PCR buffer, including dNTPs and additives, ensures ease of use and improved performance of a hot-start system: Proprietary hot-start component binds and sequesters primers at lower temperatures to prevent the primers from unspecific binding. This new formulation is effective during reverse transcription as well as during PCR. It results in increased specificity and sensitivity. The buffer is also suited for one-step RT-PCR of difficult templates with high secondary structure and high-GC content, without an increase in the reaction temperature.

Quality control

Each lot of the Transcriptor One-Step RT-PCR Kit is function tested in RT-PCR. RT-PCR is performed with 1000 copies of HAV RNA, *in vitro* transcript (also provided with Cat. No. 04 655 877 001). The setup of the control reaction and the subsequent RT-PCR with primers for a 246 bp HAV fragment is performed according to the standard RT-PCR protocol (reverse transcription at 50°C for 30 min) provided in this package insert. With 1000 copies of the target RNA a clearly visible band is obtained after agarose gel electrophoresis and ethidium bromide staining.

In addition RT-PCR is performed on a dilution series of human liver total RNA (10 ng, 1 ng and 0.1 ng). RT-PCR with specific primers for a 2.5 kb fragment of ApoB is performed according to the standard RT-PCR protocol (reverse transcription at 50°C for 30 min) provided in this package insert. With 1 ng of RNA a clearly visible band is obtained after agarose gel electrophoresis and ethidium bromide staining.

References

1 Sambrook, J. et al (1989) Molecular Cloning. A Laboratory Manual. Second Edition (Cold Spring Harbour Laboratory Part 7. and 8.)

2 Rolfs, A. et al. (1992) PCR: Clinical Diagnostics and Research, Springer Verlag, 99-111.

6. Supplementary Information

6.1 Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, www.roche-applied-science.com, and our Special Interest Sites including:

- Amplification - Innovative tools for PCR: http://www.roche-applied-science.com/pcr
- Nucleic Acid Isolation and Purification: http://www.roche-applied-science.com/napure
- Automated Sample Preparation (MagNA Lyser Instrument, MagNA Pure Compact System, and MagNA Pure LC System): http://www.magnapure.com
- Transcriptor High Fidelity: http://www.transcriptor-highfidelity.com

Product	Pack size	Cat. No.
Water, PCR Grade	25 ml (25 vials of 1 ml) 25 ml (1 vial of 25 ml) 100 ml (4 vials of 25 ml)	03 315 932 001 03 315 959 001 03 315 843 001
Protector RNase Inhibitor	2000 U 10 000 U	03 335 399 001 03 335 402 001
Expand Long Range dNTPack	175 U (50 react.)	04 829 034 001
Expand High Fidelity PCR System dNTPack	100 U	04 738 250 001
High Pure RNA Isolation Kit	50 purifications	11 828 665 001
High Pure RNA Tissue Kit	50 purifications	12 033 674 001
High Pure RNA Paraffin Kit	100 purifications	03 270 289 001
mRNA Isolation Kit	> 70 µg mRNA	11 741 985 001
mRNA Isolation Kit for Blood/ Bone Marrow	30 - 100 purifications	11 934 333 001
High Pure Viral RNA Kit	100 purifications	11 858 882 001
Transcription High Fidelity cDNA Synthesis Kit	50 reactions 100 reactions 200 reactions	05 081 955 001 05 091 284 001 05 081 963 001
Transcriptor First Strand cDNA Synthesis Kit	50 reactions 100 reactions 200 reaction	04 379 012 001 04 896 866 001 04 897 030 001
Transcriptor Reverse Transcriptase	250 U (25 reactions) 500 U (50 reactions) 2,000 U (200 reactions)	03 531 317 001 03 531 295 001 03 531 287 001

6.2 Changes to previous version

Disclaimer of License updated.

6.3 Trademarks

6.4 Regulatory Disclaimer

For life science research only. Not for use in diagnostic procedures.

6.5 Disclaimer of License

NOTICE: This product may be subject to certain use restrictions. Before using this product please refer to the Online Technical Support page (http://technical-support.roche.com) and search under the product number or the product name, whether this product is subject to a license disclaimer containing use restrictions..

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