

RobusT™ II RT-PCR Kit

Instruction manual

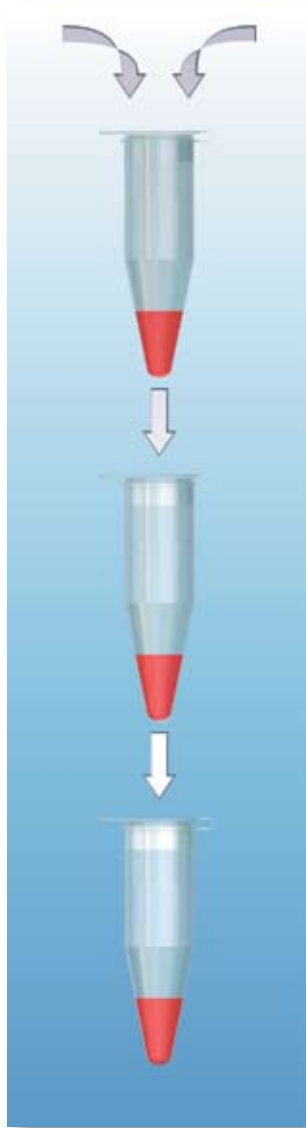
F- 590S Sufficient for 20 reactions

F- 590L Sufficient for 100 reactions

Kit Description	3
Applications	3
Advantages	3
Kit Components.....	4
Quality control	4
Storage stability.....	4
Important Parameters to Consider Prior to Setting up Your Reaction	4
RNA preparation	4
Primers.....	5
Magnesium concentration.....	5
dNTP concentration	5
Use of RNase inhibitor.....	5
General Notes	6
Preventing cross-contamination	6
Master mix for multiple samples.....	6
Pipetting enzymes	6
Application Protocol.....	7
Control reactions.....	8
Positive control reaction	8
Negative control reaction	8
Notes about Cycling Conditions	9
Reverse transcription step.....	9
RT inactivation and denaturation.....	9
Primer annealing.....	9
Primer extension	9
Number of amplification cycles	9
Troubleshooting.....	10
Appendix I: Specification of Kit Components.....	12
Appendix II: Determination of concentration and purity of RNA. Storage of RNA.	13
Appendix III: Related products.....	14
Appendix IV: References.....	14
Appendix V: Warranty.....	14
Appendix VI: Trademark and Licensing Information	15

One-Tube RobusT™ RT-PCR protocol

RNA
Template



RT-PCR Mix

- Reverse transcriptase
- DyNAzyme™ EXT DNA Polymerase
- Optimized Buffer
- dNTPs
- Primers

First strand cDNA
synthesis at 37-50°C

High-fidelity PCR
amplification of
the cDNA with
DyNAzyme™ EXT
DNA Polymerase

Completed RT-PCR
product ready for further
analysis.

Kit Description

Robus™ II RT-PCR Kit is designed for convenient, sensitive and reproducible amplification of a specific target RNA up to 6 kb in length. The system uses Moloney Murine Leukemia Virus Reverse Transcriptase RNase H⁻ (M-MuLV RT RNase H⁻) for the first strand cDNA synthesis and DyNAzyme™ EXT DNA Polymerase for the second strand cDNA synthesis and DNA amplification. cDNA synthesis and high-fidelity PCR amplification of cDNA are performed successively in a single tube during a continuous thermal-cycling program.

Compared to other RT-PCR protocols (two-step PCR, uncoupled RT-PCR, discontinuous one-tube RT-PCR), continuous RT-PCR (one-step PCR) is the most efficient system. The risk of contamination is minimized by combining cDNA synthesis and PCR, and eliminating component addition following cDNA synthesis¹. The one-tube one-buffer system minimizes hands-on time required. Either total RNA, messenger RNA or viral RNA can be used as a template for the first strand synthesis. One-step RT-PCR format is useful for running multiple reactions simultaneously for high-throughput screening of RNA samples.

Applications

- RNA amplification.
- Detection of gene expression.
- Quantification of gene expression.
- Generation of DNA products with high fidelity for cloning and sequencing.

Advantages

- Robust system with high fidelity.
- One-tube system allows quick, sensitive and reproducible analysis of RNA with minimal risk of sample contamination.
- Minimal hands-on time required.
- High sensitivity compared to competing systems.
- Capable of amplifying long targets, up to 6 kb.
- The absence of RNase H activity enhances the synthesis of long cDNAs².
- Amplifies products from a wide variety of RNA sources.
- Saves time by eliminating the need to construct and screen cDNA libraries.

Kit Components

The kit contains sufficient materials for 100 (F-590L) or 20 (F-590S) reactions including 20 control reactions.

Table 1. Kit components.

RobusT™ II RT-PCR Kit	F-590L	F-590S
10x RobusT Reaction Buffer	1.5 ml	1.5 ml
50 mM MgCl ₂	1.5 ml	1.5 ml
dNTP mix (10 mM each)	100 µl	20 µl
M-MuLV Reverse Transcriptase, RNase H ⁻ 5 U/µl	200 µl	40 µl
DyNAzyme EXT DNA Polymerase 1 U/µl	200 µl	40 µl
Upstream Contol Primer 10 pmol/µl	20 µl	20 µl
Downstream Contol Primer 10 pmol/µl	20 µl	20 µl
Control RNA with Carrier	20 µl	20 µl

For detailed information, see Appendix I: Specification of Kit Components.

Quality control

The product is tested functionally using 1 pg of MS2 RNA. Template RNA can be reverse transcribed and amplified into a clear, discrete 1011 bp DNA product.

Storage stability

RobusT II RT-PCR Kit is shipped in gel ice. Store all kit components at -20 °C. For long term storage, the control RNA may be stored at -70 °C. The RobusT II RT-PCR Kit is stable for one year from the date of packaging when stored and handled properly.

Important Parameters to Consider Prior to Setting up Your Reaction

RNA preparation

Successful full length cDNA synthesis is dependent on the integrity and purity of the template RNA. RNA preparation should be free of any DNA or RNase contamination. RNA isolation should be performed under RNase-free conditions. Furthermore, any contamination with RNases from other potential sources like glassware, plasticware and reagent solutions has to be avoided.

The minimum amount of RNA that can be amplified with RT-PCR depends both on the template and the primers that are used. By using total RNA template in the range of 1 pg-1 µg per reaction or poly(A)⁺ template in the range of 1 pg-100 ng, excellent amplification results can be obtained.

Primers

Sequence specific primers should be used for RT-PCR. Primers may be designed to anneal to sequences in two exons on opposite sides of an intron to differentiate between amplification of cDNA and contaminating genomic DNA. The amplification product from genomic DNA will be larger than the mRNA derived product that lacks the intron sequence. Alternatively, primers can be designed to anneal to the exon-exon boundary of the mRNA. With such primers, amplification of genomic DNA will be highly inefficient. When designing primers, avoid complementary sequences at the 3' end of the primer pairs to prevent primer-dimer formation. Avoid internal complementary sequences to minimize internal hairpins. Using 10 pmol of each primer (0.2 µM final concentration for each) in a reaction is recommended.

Magnesium concentration

The optimal magnesium concentration is dependent on the final concentration of dNTPs, primers and template. Although 1.5 mM MgCl₂ concentration is suitable for most applications, titration of the magnesium concentration for each experimental target/primer combination can significantly improve the sensitivity and specificity. To determine the optimal magnesium concentration for a specific application, prepare a reaction series containing 0.5-3.0 mM MgCl₂ in 0.5 mM increments.

dNTP concentration

200 µM dNTP concentration is optimal for most RT-PCR reactions.

Use of RNase inhibitor

The use of RNase inhibitor is optional. We recommend, however, to use always RNase inhibitor (5-20 U in a 50 µl reaction; see manufacturer's recommendation) to minimize the activity of any RNases present. This is particularly important when RT-PCR is started with low amounts of template RNA (less than 10 ng).

General Notes

Preventing cross-contamination

Care should be taken to minimize the carryover of nucleic acids from one experiment to another. The use of aerosol-resistant pipet tips is recommended. If possible, separate work areas and pipettes for pre- and post-amplification steps are recommended.

Master mix for multiple samples

Master mix of components for RT-PCR can be prepared if running multiple samples. Using a master mix facilitates accurate dispensing of reagents, minimizes loss of reagents during pipetting, minimizes the hands-on time required and makes repeated dispensing of each reagent unnecessary, all of which help to minimize sample-to-sample variation.

Pipetting enzymes

The viscosity of the 50 % glycerol in the enzyme storage buffer can lead to pipetting errors. Enzymes should be pipetted carefully and slowly and mixed with gently pipetting up and down avoiding bubble formation.

Application Protocol

Keep all components, reaction mixes and samples on ice. Add the following reaction components to a nuclease free PCR tube placed on ice:

Table 2. Reaction setup.

RT-PCR mix components	Volume
10x RobusT Reaction Buffer	5 µl
50 mM MgCl ₂	1.5 µl
dNTP mix (10 mM each)	1 µl
RNase inhibitor*	x µl
template RNA	10 pg - 1 µg
downstream primer	10 pmol
upstream primer	10 pmol
M-MuLV RT RNase H ⁻ 5 U/µl	2 µl
DyNAzyme EXT DNA Polymerase 1 U/µl	2 µl
RNase free H ₂ O	add to 50 µl

* Recommended, see: Important Parameters to Consider Prior to Setting Up Your Reaction.

Gently mix the components and spin briefly. Depending on the thermocycler used, overlay with mineral oil if required. A typical temperature profile is given below. Cycling conditions have to be optimized for each amplicon according to the instructions on page 9, Notes about Cycling Conditions.

Table 3. Cycling instructions.

Cycle step	Temp.	Time	Number of cycles
cDNA synthesis	37-50 °C	15-60 min	1
Inactivation of M-MuLV Reverse Transcriptase and denaturation of the cDNA-RNA hybrid	94 °C	2 min	1
PCR Amplification	94 °C 45-72 °C * 72 °C	30 s 30 s 1-1.5 min/kb	25-40 **
Final Extension	72 °C	5-10 min	

* The annealing temperature is dependent on the melting temperature of the primers used.

** The number of cycles is dependent on the abundance of the target RNA.

Control reactions

To monitor your RT-PCR reactions and to facilitate optimization and possible troubleshooting, perform both positive and negative control reactions.

Positive control reaction

For positive control reaction, use the Control RNA and Upstream and Downstream Control Primers supplied with the kit. Control RNA is MS2 viral RNA including a carrier RNA. With the positive control primers, a 1011 bp sequence in the viral RNA is amplified.

Setting up the positive control reaction

Table 4. Pipetting instructions.

Component	Volume
10x Reaction Buffer	5 µl
50 mM MgCl ₂	1.5 µl
Upstream Control Primer 10 pmol / µl	1 µl
Downstream Control Primer 10 pmol / µl	1 µl
Control RNA	1 µl
RNase inhibitor	x µl
dNTP mix (10 mM each)	1 µl
M-MuLV RT RNase H ⁻ 5 U/µl	2 µl
DyNAzyme EXT DNA Polymerase 1 U/µl	2 µl
RNase free H ₂ O	add to 50 µl

Table 5. Cycling protocol.

Cycle step	Temp.	Time	Number of cycles
cDNA synthesis	48 °C	30 min	1
Inactivation of M-MuLV RT and denaturation of the cDNA-RNA hybrid	94 °C	2 min	1
PCR amplification	94 °C 60 °C 72 °C	30 s 30 s 1 min	25
Final extension	72 °C	7 min	1

Negative control reaction

For a negative control, set up a reaction identical with your application except omit M-MuLV RT. This negative control is performed to exclude products amplified from a potential DNA contamination. No PCR product should be visible in a negative control reaction.

Note: Due to weak reverse transcriptase activity of DyNAzyme EXT DNA Polymerase, short target sequences can be reverse transcribed even in the absence of M-MuLV.

Notes about Cycling Conditions

Reverse transcription step

First strand cDNA synthesis can be efficiently accomplished in a 15-60 minute incubation at 37-50 °C. A 30 minute incubation at 40 °C works well in most applications and is recommended as a general starting point. A template denaturation step prior to initiation of the reverse transcription is not required. If desired, a denaturation step may be incorporated by incubating primers and template RNA at 68 °C for 2 min or at 94 °C for 1 min. Do not incubate M-MuLV RT or RNase inhibitor at this temperature, since these enzymes will be inactivated.

RT inactivation and denaturation

M-MuLV RT RNase H⁻ is inactivated after the first strand cDNA synthesis step. Inactivation and denaturation of the cDNA-RNA hybrid is accomplished by incubating the reaction for 2 minutes at 94 °C. This step is directly followed by the second strand synthesis and PCR amplification.

Primer annealing

We suggest the primers to be annealed for 1 minute or less at the highest temperature that will permit annealing of the primer to the template. Annealing temperatures are usually set about 5 °C below the T_m of primers. It is best that both primers have a similar T_m. T_m is recommended to be calculated with the nearest-neighbor method. Instructions for T_m calculation and a link to a calculator using the nearest-neighbor method can be found on Finnzymes' website www.finnzymes.com.

Primer extension

A constant extension time of 1-1.5 minutes per one kb at 72 °C is recommended. However, if the target sequence is long (4-6 kb), increasing the extension time up to 2 min/kb may give better results. A final 5-10 minute extension step at 72 °C improves the quality of the final product by allowing extension of truncated products to full-length.

Number of amplification cycles

The number of amplification cycles is dependent on the abundance of the respective RNA. For most RNA samples 30 cycles is adequate, but if the target RNA is rare or if only a small amount of starting material is available, it may be necessary to increase the number of cycles to 35-40.

Troubleshooting

No amplification product or low product yield	
Possible causes	Comments and suggestions
Error in cycler setup	<ul style="list-style-type: none"> • Check that instrument settings correspond with the experiment.
Missing components (e.g. primers or template) or pipetting error	<ul style="list-style-type: none"> • Check the assembly of the reaction. • Check the concentrations and storage conditions of the reagents.
RNA is degraded or poor quality	<ul style="list-style-type: none"> • Verify the integrity of RNA by gel electrophoresis. • Replace the RNA if necessary. • Isolate the RNA in the presence of a RNase inhibitor and ensure that reagents, tips and tubes are RNase-free.
Inhibitors are present in RNA	<ul style="list-style-type: none"> • Reduce the volume of the target RNA. • Remove inhibitors in the RNA preparation by ethanol, precipitation and 70% ethanol wash. <p>Note: Inhibitors of RT include SDS, EDTA, guanidium salts, formamide, sodium phosphate and spermidine.</p> <ul style="list-style-type: none"> • Increase the amount of DyNAzyme EXT DNA Polymerase in reaction. Pipet 2.5-3 μl instead of the usual 2 μl.
Incorrect temperature in cDNA synthesis reaction	<ul style="list-style-type: none"> • The recommended temperature in cDNA synthesis step is 40 °C. It can be optimized between 37-50 °C if necessary.
RNase contamination	<ul style="list-style-type: none"> • Maintain aseptic conditions, always include RNase inhibitor in reaction.
Not enough starting template RNA	<ul style="list-style-type: none"> • Increase the amount of template RNA.
RNA target is rare or long	<ul style="list-style-type: none"> • Increase the length of RT step to 60 minutes. • Increase the number of cycles.
Incomplete synthesis of target cDNA because of the secondary structure of the RNA template	<ul style="list-style-type: none"> • Increase the temperature of RT step to 50 °C. • Add oligo(dT) to reaction together with gene specific primers (10 pmol of each primer per 50 μl reaction). Gene specific primer may be unable to anneal to the RNA template because of secondary structures (e.g. loops) or M-MuLV RT RNase H⁻ may not be efficient at extending from this primer on the template. Adding oligo(dT) allows efficient priming and enables the enzyme to read through these structures. • Add 2-10 % DMSO to reaction³. <p>Note: The annealing temperature must be lowered when DMSO is used because DMSO decreases the melting point of the primers. E.g. 10 % DMSO decreases the melting temperature 5.5-6.0 °C.</p>
Reaction conditions are suboptimal	<ul style="list-style-type: none"> • Optimize annealing temperature and/or extension time, varying each individually. • Optimize magnesium chloride concentration. • Increase the number of thermal cycles. • Set extension time for at least 1.5 min per kb of target length. • If smear appears, increase the dNTP concentration in 40 μM increments. • Increase temperature of RT step to 50 °C. • Redesign the primers.

Target sequence genuinely not present in target RNA	<ul style="list-style-type: none"> • Redesign experiment or try other sources of target RNA.
Low specificity	
Possible causes	Comments and suggestions
Reaction conditions not optimal	<ul style="list-style-type: none"> • Increase the annealing temperature. • Optimize magnesium chloride concentration. • Optimize extension time. • Decrease the amount of DyNAzyme EXT DNA Polymerase in reaction. Pipet 1-1.5 U instead of the usual 2 U per reaction.
Primer design not optimal	<ul style="list-style-type: none"> • Make sure primers are not self-complementary or complementary to each other. • Verify that the primers are designed to be complementary to the appropriate strands. • Try a longer primer.
Contamination by another target RNA/DNA	<ul style="list-style-type: none"> • Use aerosol resistant tips to reduce cross-contamination during pipetting. • Use separate work areas and pipettes for pre- and post-amplification. • Always wear gloves and change them often. • Aliquot all reagents used and use one aliquot for one experiment only.
Unexpected bands after electrophoresis	
Possible causes	Comments and suggestions
RNA preparation is contaminated with genomic DNA	<ul style="list-style-type: none"> • Verify the presence of contaminating DNA by performing RT-PCR in the absence of M-MuLV RT, RNase H⁻. • Treat the RNA preparation with RNase-free-DNase I. • Redesign the primers to anneal to sequences in the exon-exon boundary of the target gene (see Parameters to consider: Primers on page 5)
Multiple target sequences genuinely exist in target RNA	<ul style="list-style-type: none"> • Design new primers.

Appendix I: Specification of Kit Components

M-MuLV Reverse Transcriptase, RNase H⁻

Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase RNase H⁻ (minus) is an RNA-directed DNA polymerase used for synthesizing a complementary DNA strand initiating from a primer using either single stranded RNA or single stranded DNA template. The absence of RNase H activity enhances the synthesis of long cDNAs².

Source: M-MuLV Reverse Transcriptase, RNase H⁻ is purified from a strain of *E. coli* that carries the Reverse Transcriptase gene from M-MuLV modified by site-directed mutagenesis to eliminate the RNase H activity.

Concentration: 5 U/μl

Storage Buffer: 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 % Nonidet P 40, 50 % glycerol (v/v).

DyNAzyme EXT DNA Polymerase

In the RobusT II RT-PCR System second strand cDNA synthesis and high-fidelity PCR amplification of cDNA are performed by DyNAzyme EXT DNA Polymerase, which is a versatile and easy-to-use enzyme with powerful advantages for all PCR applications. DyNAzyme EXT DNA Polymerase is an optimal mixture of DyNAzyme II DNA Polymerase and a proofreading enzyme. DyNAzyme EXT DNA Polymerase contains sufficient amounts of proofreading activity to remove misincorporated nucleotides which would otherwise either be extended and thus produce mutations or block further chain extension.

Concentration: 1 U/μl

Storage Buffer: 20 mM Tris-HCl (pH 7.4 at 25 °C), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, stabilizers, 200 μg/ml BSA, 50 % glycerol (v/v).

10x RobusT Reaction Buffer

RobusT II RT-PCR Kit is supplied with a 10x RobusT Reaction Buffer, which is specifically optimized for RobusT II RT-PCR reactions.

50 mM MgCl₂ Solution

dNTP Mix

Concentration: 10 mM of each

The dNTP Mix is a premixed ready-to-use solution consisting of the following compounds: dATP, dGTP, dCTP and dTTP. The deoxynucleosidetriphosphates are dissolved in water, pH 7.0.

Upstream Control Primer

20 μl 10 pmol/μl in ddH₂O
5'-GGCCCGCAGGTGGTTGGAGT-3'
Tm 74.3 °C

Downstream Control Primer

20 μl 10 pmol/μl in ddH₂O
5'-GGAGTTTGCTGCGATTGCTGAGG-3'
Tm 72.4 °C

Control RNA with Carrier

20 μ l 10 ng MS2 RNA/ μ l
 30 ng carrier RNA/ μ l
 in ddH₂O

Appendix II: Determination of concentration and purity of RNA. Storage of RNA.

Concentration of RNA

The concentration of RNA may be determined spectrophotometrically at 260nm, where 1 absorbance unit (A_{260}) = 40 μ g of single stranded RNA/ml.

Purity of RNA

The ratio of absorbances at 260 and 280nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. Pure RNA should exhibit an A_{260}/A_{280} ratio of 1.9-2.1 in 10 mM Tris-HCl, pH 7.5.

The A_{260}/A_{280} ratio is influenced by pH and since water is not buffered, it is recommended to measure the absorbance of RNA sample in 10 mM Tris-HCl, pH 7.5.

Storage of RNA

Purified RNA may be stored at -20 °C or -70 °C in water. For long term storage, ethanol precipitation of the RNA is recommended; add Sodium Acetate, pH 4.0, to 0.25 M, and 2.5 volumes of 100 % ethanol. Store at -70 °C.

Appendix III: Related products

M-MuLV Reverse Transcriptase RNase H⁻

F-572S	10 000 U	200 U/μl
F-572L	50 000 U	200 U/μl

DyNAzyme™ EXT DNA Polymerase

with Optimized DyNAzyme™ EXT Buffer and Buffer Pack for Mg²⁺ Optimization

F-505S	200 U	1 U/μl
F-505L	1000 U	1 U/μl

Appendix IV: References

References:

- (1). Mallet, F. *et al.* (1995) *Biotechniques* 18, 678.
- (2). Sambrook, J. *et al.* (1989) *Molecular Cloning; A Laboratory Manual*, second edition, pp. 5.52-5.55, 8.11-8.17 Cold Spring Harbor Laboratory, Cold Spring Harbor.
- (3) Sidhu, M. *et al.* (1996) *Biotechniques* 21, 44.

Appendix V: Warranty

Finnzymes Oy warrants that its products will meet the specifications stated on the technical data section of the data sheets, and Finnzymes Oy agrees to replace the products free of charge if the products do not conform to the specifications. Notice for replacement must be given within 60 days of receipt. In consideration of the above commitments by Finnzymes Oy, the buyer agrees to and accepts the following conditions:

1. That this warranty is in lieu of all other warranties, express or implied;
2. That ALL WARRANTIES OF MERCHANT ABILITY OR OF FITNESS FOR A PARTICULAR PURPOSE ARE HEREBY EXCLUDED AND WAIVED;
3. That the buyer's sole remedy shall be to obtain replacement of the product free of charge from Finnzymes Oy; and
4. That this remedy is in lieu of all other remedies or claims for damages, consequential or otherwise, which the buyer may have against Finnzymes Oy.

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Finnzymes Oy recommends that the buyer and other persons using the products follow the N.I.H. guidelines published in the Federal Register, Volume 41, No. 131, July 7, 1976, and any amendments thereto. Finnzymes Oy disclaims any and all responsibility for any injury or damage which may be caused by the failure of the buyer or any other person to follow said guidelines.

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Appendix VI: Trademark and Licensing Information

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