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

Titan One Tube RT-PCR System

Version: 14
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Cat. No. 11 855 476 001 100 µl
100 reactions

Store the kit at −15 to −25°C.

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1. General Information

1.1. Contents

Vial / bottle	Label	Function / description	Content
1	Titan One Tube RT-PCR System, Enzyme mix	▪ Expand High Fidelity enzyme mix and AMV reverse transcriptase in storage buffer. ▪ Enzyme storage and dilution buffer: 20 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20 (v/v), 0.5% Nonidet P-40 (v/v), 50% glycerol (v/v), pH 7.5 (+25°C).	1 vial, 100 µl
2	Titan One Tube RT-PCR System, RT-PCR buffer, 5x conc.	Optimized RT-PCR buffer with 7.5 mM $MgCl_2$ and DMSO.	1 vial, 1 ml
3	Titan One Tube RT-PCR System, $MgCl_2$ 25 mM Stock Solution	To adjust final Mg^{2}+ concentration.	1 vial, 1 ml
4	Titan One Tube RT-PCR System, Dithiothreitol (DTT) solution	100 mM solution	1 vial, 1 ml

1.2. Storage and Stability

Storage Conditions (Product)

When stored at −15 to −25°C, the kit is stable through the expiry date printed on the label.

Vial / bottle	Label	Storage
1	Enzyme mix	Store at −15 to −25°C.
2	RT-PCR buffer, 5x conc.	
3	$MgCl_2$ 25 mM Stock Solution	
4	Dithiothreitol (DTT) solution	

1.3. Additional Equipment and Reagent 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
- PCR reaction vessels, such as 0.2 ml thin-walled PCR tubes or plates
- Standard benchtop microcentrifuge
- Thermal block cycler

For RT-PCR

- Sequence-specific primers
- Template RNA
- Water, PCR Grade*
- PCR Nucleotide Mix*
- Protector RNase Inhibitor* (40 U/µl)
- Mineral oil (optional)

1.4. Application

The Titan One Tube RT-PCR System uses Reverse Transcriptase AMV for first strand cDNA synthesis, and the Expand High Fidelity enzyme blend consisting of Taq DNA Polymerase and Tgo DNA polymerase which has a proofreading activity for amplification of cDNA by PCR. The proofreading activity, leads to an increased PCR fidelity.
The Titan One Tube RT-PCR System:

- Is designed for fast, sensitive, and reproducible analysis of RNA by high fidelity RT-PCR in a one-step reaction.
- Reduces the error rate in PCR due to the proofreading capability. A threefold higher fidelity is obtained in comparison to Taq DNA polymerase.
- Allows amplification of fragments up to 6 kb due to the use of the Expand PCR System.
- Delivers increased sensitivity due to the high efficiency of all three enzymes.
- Is faster than conventional two-step RT-PCR.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Isolated total or poly(A)+ RNA up to 1 µg or single cells.

Control Reactions

DNA contamination

To exclude artifacts from DNA targets, such as residual genomic DNA contaminations from RNA preparations, and contaminating DNA from previous amplifications, appropriate positive and negative control reactions should be included in the experimental design. Consider setting up the following 3 controls:

1. To control contaminating DNA amplicons from previous PCR, always set up a control reaction without RNA template.
   – No PCR product should be visible.

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2. Set up a control reaction where the RT reaction is not performed.
   – Instead of the Titan Enzyme mix in the kit, use the Expand Enzyme Mix from the Expand High Fidelity or Expand Long Template Systems*, or use 2.5 U Taq DNA Polymerase*.
   – Alternatively, heat the Master Mix 2 for 2 minutes at +94°C to inactivate reverse transcriptase AMV, then follow Step 1 in the RT-PCR protocol and proceed with Step 3 (do not use Step 2).
   i *Consider that even Taq DNA polymerase has a low reverse transcriptase activity.*

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3. Treat your RNA preparation with RNase A, then proceed with the RT-PCR protocol.

Primers

Primer design

Sequence-specific primers must be used for one tube 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. 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.
- The final concentration of primers in the reaction should range from 0.2 to 1 µM.

Mg2+ Concentration

1.5 mM

The optimum magnesium concentration in RT-PCR depends on the final concentration of dNTPs, primers, and template. For most applications, 1.5 mM magnesium will allow amplification of your target RNA using the standard protocol. Depending on the respective target/primer combination, a titration of the magnesium concentration may significantly improve sensitivity and specificity.

General Considerations

The optimal conditions, including incubation times and temperatures, concentration of enzyme, template DNA, $Mg^{2}+$ vary from system to system and must be determined for each individual experimental system. At the very least, titrate the $Mg^{2}+$ concentration and the amount of enzyme mix used per assay to ensure optimal efficiency of DNA synthesis. As a starting point, use the following guidelines:

- Optimal $Mg^{2}+$: In most cases, a $Mg^{2}+$ concentration of 1.5 mM will produce satisfactory results.
- dNTP concentration: For the amplification of RT-PCR products >2 kb, use the sodium salts of dNTPs. Always use equal concentrations of all four dNTPs. The most commonly used concentration is 200 µM. RT-PCR performance can be improved by increasing the concentration to 500 µM dNTPs, each. Since nucleotides strongly bind magnesium ions, the concentration of magnesium will also have to be increased accordingly. A final concentration of 500 µM dNTP typically requires a concentration of 3 mM magnesium chloride.

RNA preparation

Any contamination with RNases from other potential sources, such as glassware, plasticware, and reagent solutions must be avoided.

- Integrity of mRNA is particularly important for the generation of long RT-PCR products. The size of the mRNA can be determined by gel electrophoresis and ethidium bromide staining. The mRNA should appear as a smear between approximately 500 bp and 8 kb. The bulk of the mRNA should be between 1.5 and 2 kb.
- For high quality eukaryotic mRNA preparations, minimize the activity of RNases released during cell lysis by using inhibitors of RNases or methods that disrupt cells and simultaneously inactivate RNases.

RNase inhibitor

For best results, add 5 to 10 U of Protector RNase Inhibitor* to minimize RNase activity. This is particularly important when RT-PCR is started with low amounts of RNA (<10 ng).

Prevention of Carryover Contamination

If dTTP is completely substituted by dUTP, as generally done for carryover prevention using uracil DNA-glycosylase, the yield of PCR products incorporating modified nucleotides can decrease significantly.

Safety Information

For customers in the European Economic Area

Contains SVHC: octyl/nonylphenol ethoxylates. For use in research and under controlled conditions only – acc. to Art. 56.3 and 3.23 REACH Regulation.

2.2. Protocols

Preparation of RT-PCR master mixes

Prepare two RT-PCR master mixes. Master Mix 2 contains enzyme and reaction buffer; Master Mix 1 contains all other reaction components. This circumvents the need for hot start and avoids that the enzyme interacts with primers or template during the reaction setup.

Preparation of master mix 1

⚠ ***Always wear gloves when handling RNA to avoid any contamination with RNases.***

1. Thaw components listed below and place them on ice.

2. Briefly vortex and centrifuge all reagents before setting up the reactions.

3. To a sterile, nuclease-free reaction tube on ice, add the components in the order listed for each 50 µl reaction:

Reagent	Volume [µl]	Final conc.
Water, PCR Grade*	add up to a final volume of 25	–
PCR Grade Nucleotide Mix* (10 mM of each dNTP)	1	200 µM of each dNTP
Forward primer 1	variable	400 nM
Reverse primer 2	variable	400 nM
Template RNA	variable	1 µg – 1 pg total RNA
DTT solution, 100 mM	2.5	5 mM
Protector RNase Inhibitor (40 U/µl)* *Optional: minimize the activity of any RNases present.*	variable	5 – 10 U
Final Volume	**25**	

4. Mix and centrifuge briefly.

Preparation of master mix 2

⚠ ***Always wear gloves when handling RNA to avoid any contamination with RNases.***

1. Thaw components listed below and place them on ice.

2. Briefly vortex and centrifuge all reagents before setting up the reactions.

3. To a sterile, nuclease-free reaction tube on ice, add the components in the order listed for each 50 µl reaction:

Reagent	Volume [µl]	Final conc.
Water, PCR Grade*	add up to a final volume of 25	–
RT-PCR buffer, 5x conc. (Vial 2)	10	1x (1.5 mM $MgCl_2$)
Enzyme mix (Vial 1)	1	–
Final Volume	**25**	

4. Mix and centrifuge briefly.

RT-PCR

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 temperature

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 after addition of the required amount of water at +68°C for 2 minutes or at +94°C for 1 minute.

⚠ ***Do not incubate AMV or RNase inhibitor at this temperature since these components will become inactivated.***

Reverse transcription may be performed between +42 and +60°C. When establishing a new assay, start with +50°C. If the reaction is performed at temperatures >+50°C, only short RNA fragments can be amplified due to partial inactivation of AMV reverse transcriptase. To amplify long fragments >3 kb by RT-PCR, use a temperature range of +45 to +48°C.

PCR temperature

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 elongation temperature should always be +68°C. The denaturation temperature depends on the G + C content of the template. A high G + C content (>60%) of your RNA might require either higher denaturation temperatures or a longer denaturation time.

RT-PCR protocol

⚠ ***Always wear gloves when handling RNA to avoid any contamination with RNases.***

ⓘ *The following thermal profiles are an example. Different thermal cyclers may require different profiles.*

1. For each reaction, combine 25 µl Master Mix 1 and 25 µl Master Mix 2 in a 0.2 ml thin-walled PCR tube on ice.
   – Gently vortex the mixture to produce a homogeneous reaction, then centrifuge briefly to collect the solution at the bottom of the tube.
   – Overlay the reaction carefully with 30 µl mineral oil if required by the thermal cycler.

2. Place your samples in a thermal block cycler equilibrated to +50°C and incubate for 30 minutes.
   ⓘ *Temperatures up to +60°C can be used for the reverse transcription step.*

3. Start cycling using the following thermal profile.

Step	Temperature [°C]	Time	Number of Cycles
Pre-Incubation	94	2 min	1
Denaturation Annealing Elongation	94 45 – 65[1] 68	10 sec 30 sec 45 sec – 4 min[2]	10
Denaturation Annealing Elongation	94 45 – 65[1] 68	10 sec 30 sec 45 sec – 4 min[2] + 5 sec cycle elongation for each successive cycle[3]	25
Final Elongation	68	7 min	1

4. Analyze the samples on a 1 to 2% agarose gel.

(1) Optimal annealing temperature depends on the melting temperature of the primers and on the experimental system.
(2) Elongation time depends on fragment length: 45 seconds for <1 kb, 1 minute for 1.5 kb, 2 minutes for 3 kb, 3 minutes for 4.5 kb, and 4 minutes for 6 kb.
(3) For example, cycle number 11 is 5 seconds longer than cycle 10. Cycle number 12 is 10 seconds longer than cycle 10. Cycle number 13 is 15 seconds longer than cycle 10, etc.

2.3. Parameters

Incorporation of Modified Nucleotides

AMV reverse transcriptase and the Expand High Fidelity enzyme mix both accept modified nucleotides, such as Digoxigenin-11-dUTP*, Biotin-16-dUTP*, or Fluorescein-12-dUTP* at a concentration usually used:

- For probe synthesis, for example, 1:3 to 1:6 DIG-dUTP:dTTP.
- For labeling PCR products to detect products in a ELISA format.

Labeling with DIG-dUTP for ELISA

Follow the protocol described in chapter Preparation of RT-PCR master mixes with one exception. Use instead of the 4 µl in the dNTP mix, use 5 µl of the PCR DIG Labeling Mix* or the PCR ELISA (DIG Labeling)*. Both products use dNTPs with the following final concentration per reaction: 200 µM dATP, dGTP, dCTP each; 190 µM dTTP and 10 µM DIG-11-dUTP.

Maximum Fragment Size

Up to 6 kb.

PCR Cloning

TA or blunt-end cloning.
Resulting fragments are a mixture of fragments with A overhangs at the 3′ end and blunt-ended fragments. Without any treatment of the ends, TA cloning will yield in general, better results than blunt-end cloning.

Sensitivity

10 pg
1,000 copies of RNA targets can be visualized on an agarose gel and ethidium bromide staining, by analyzing 10 µl of the 50 µl reaction after 35 cycles. Sensitivity can easily be improved to the lowest level of RNA targets by using more cycles and/or using labeled nucleotides, such as DIG-dUTP during PCR, and analyzing the reaction in an ELISA format.

Specificity

Specificity of the reaction strongly depends on primer design and DNA contamination. See section, **Control Reactions** for additional information.

Temperature Optimum

+42 to +60°C
Reverse transcription

3. Troubleshooting

Observation	Possible cause	Recommendation
No or very little 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.
	Template RNA degraded.	Prepare fresh RNA template, being careful to prevent RNase activity.
		Check RNA preparation by gel electrophoresis.
	Too much template RNA.	Decrease amount of RNA template. *A too high amount of template RNA may affect/ inhibit performance of RT-PCR.*
	Reaction not optimized.	Increase the concentration of dNTPs in the PCR reaction up to 500 µM maximum. At the same time, raise the final concentration of $MgCl_2$ in the reaction to 3 mM to compensate for the additional dNTPs.
		Increase primer concentration up to 1 µM maximum.
		Synthesize the cDNA at a higher temperature.
	Template secondary structure prevented effective first strand cDNA synthesis.	Optimize DMSO concentration up to 10% maximum.
		Briefly denature the RNA template at +94°C for 1 minute before adding reverse transcriptase. ***Do not incubate reverse transcriptase or RNase inhibitor at this elevated temperature as they will be inactivated.***
	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.
Product is smeared.	Secondary amplification product(s).	Check reagent concentrations and cycling conditions: ▪ Check Mg^2+ concentration. ▪ Optimize temperature of cDNA synthesis step. ▪ Optimize primer concentration. ▪ Decrease number of cycles. ▪ Check and perhaps decrease concentration of template. ▪ Titrate the amount of enzyme(s).
Nonspecific product bands present.	Annealing temperature too low.	Increase annealing temperature during PCR to increase specificity of amplification.
	Contaminating DNA in sample.	Perform a no-RT control, see section, **Control Reactions** for additional information.

4. Additional Information on this Product

4.1. Test Principle

Background information

RT-PCR is the most sensitive technique to determine the mere presence or relative quantity of specific RNA templates, for example, in gene expression studies. Furthermore, RT-PCR allows cloning of rare messages without the necessity to construct cDNA libraries.

- In two-step RT-PCR, reverse transcription of RNA into cDNA is performed prior to amplification of cDNA by PCR in a separate reaction. This reaction setup requires to open the reaction tube after cDNA synthesis and to add the reaction components required for the PCR step of the procedure. Not only is this handling inconvenient, but it also increases the risk of contamination.
- In one-step RT-PCR, cDNA synthesis as well as PCR are performed using an optimized buffer system in one reaction tube in a combined reaction without the requirement to add further reagents between cDNA synthesis and PCR.

Two basic techniques for one-step RT-PCR are described:

- Tth DNA polymerase can be used for both reverse transcription and PCR due to its intrinsic RT-activity. The thermostability of this enzyme can overcome secondary structures in RNAs by performing cDNA synthesis at +60 to +70°C. However, the length of the RT-PCR products is limited to less than 1 kb. Furthermore the polymerase possesses an increased error rate due to the use of manganese ions.
- The combination of AMV reverse transcriptase and Taq DNA polymerase allows amplification of fragments up to 2 kb with decreased error rates compared to the use of Tth DNA polymerase. However, this reaction has to be performed during the cDNA synthesis step at +42°C.

By combining AMV Reverse Transcriptase and the Expand High Fidelity PCR System together with an optimized buffer system in the Titan One Tube RT-PCR System, the limitations of this approach are now overcome allowing an RT reaction temperature of up to +60°C and fragment lengths of up to 6 kb.

Reaction mechanism

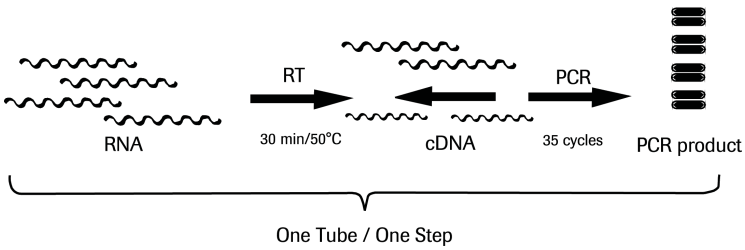

Fig. 1: Reaction mechanism of Titan One Tube RT-PCR System.

4.2. Quality Control

For lot-specific certificates of analysis, see section, **Contact and Support**.

5. Supplementary Information

5.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
❶ ❷ ❸ etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

5.2. Changes to previous version

New information added related to the REACH Annex X.

5.3. Ordering Information

Product	Pack Size	Cat. No.
Reagents, kits		
Uracil-DNA Glycosylase, heat-labile	custom fill	11 780 565 103
Expand High Fidelity PCR System	100 U, 1 x 100 U, 40 reactions in a final volume of 50 µl	11 732 641 001
	500 U, 2 x 250 U, 200 reactions in a final volume of 50 µl	11 732 650 001
	2,500 U, 10 x 250 U, 1,000 reactions in a final volume of 50 µl	11 759 078 001
Expand Long Template PCR System	150 U, 1 x 150 U, 38 reactions in a final volume of 50 µl	11 681 834 001
	720 U, 2 x 360 U, 190 reactions in a final volume of 50 µl	11 681 842 001
	3,600 U, 10 x 360 U, 950 reactions in a final volume of 50 µl	11 759 060 001
Taq DNA Polymerase, 5 U/µl	100 U, 5 U/µl, 80 reactions	11 146 165 001
	500 U, 5 U/µl, 400 reactions	11 146 173 001
	4 x 250 U, 5 U/µl, 800 reactions	11 418 432 001
	10 x 250 U, 5 U/µl, 2,000 reactions	11 596 594 001
	20 x 250 U, 5 U/µl, 4,000 reactions	11 435 094 001
Digoxigenin-11-dUTP, alkali-labile	25 nmol, 25 µl, 1 mM	11 573 152 910
	125 nmol, 125 µl, 1 mM	11 573 179 910
Digoxigenin-11-dUTP, alkali-stable	25 nmol, 25 µl, 1 mM	11 093 088 910
	125 nmol, 125 µl, 1 mM	11 558 706 910
	5 x 125 nmol, 5x 125 µl, 1 mM	11 570 013 910
Biotin-16-dUTP	custom fill	11 093 711 103
Fluorescein-12-dUTP	custom fill	11 375 601 103
Water, PCR Grade	25 ml, 25 x 1 ml	03 315 932 001
	25 ml, 1 x 25 ml	03 315 959 001
	100 ml, 4 x 25 ml	03 315 843 001
PCR Nucleotide Mix	200 µl, 500 reactions of 20 µl final reaction volume	11 581 295 001
	5 x 200 µl, 2,500 reactions of 20 µl final reaction volume.	04 638 956 001
	10 x 200 µl, 5,000 reactions of 20 µl final reaction volume.	11 814 362 001
Protector RNase Inhibitor	2,000 U, 40 U/µl	03 335 399 001
	10,000 U, 5 x 2,000 U	03 335 402 001

5.4. Trademarks

EXPAND is a trademark of Roche.
All other product names and trademarks are the property of their respective owners.

5.5. License Disclaimer

For patent license limitations for individual products please refer to:
List of biochemical reagent products.

5.6. Regulatory Disclaimer

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

5.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

5.8. Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site**.

To call, write, fax, or email us, visit **sigma-aldrich.com**, and select your home country. Country-specific contact information will be displayed.

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