User Manual

Titanium®
One-Step RT-PCR Kit
User Manual

Clontech

United States/Canada
800.662.2566
Asia Pacific
+1.650.919.7300
Europe
+33.(0)1.3904.6880
Japan
+81.(0)77.543.6116

Clontech Laboratories, Inc.
A Takara Bio Company
1290 Terra Bella Ave.
Mountain View, CA 94043
Technical Support (US)
E-mail: tech@clontech.com
www.clontech.com

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Contact Us For Assistance	
Customer Service/Ordering:	**Technical Support:**
Telephone: 800.662.2566 (toll-free)	Telephone: 800.662.2566 (toll-free)
Fax: 800.424.1350 (toll-free)	Fax: 650.424.1064
Web: www.clontech.com	Web: www.clontech.com
E-mail: orders@clontech.com	E-mail: tech@clontech.com

I. Introduction & Protocol Overview

The **Titanium One-Step RT-PCR Kit** offers a unique system for performing RT-PCR in a single step, in a single tube. Four attributes—a highly purified Moloney murine leukemia virus reverse transcriptase (MMLV-RT), a Thermostabilizing Reagent, GC-Melt™, and Titanium *Taq* DNA Polymerase—set the Titanium One-Step RT-PCR Kit apart from other similar RT-PCR kits. This novel technique allows you to detect and estimate the amounts of specific mRNAs with high sensitivity and unparalleled convenience.

RT-PCR is a highly sensitive and versatile technique that is widely used for measuring gene expression in tissues and cultured cells (Powell *et al.*, 1987; Wilkinson, 1998). Traditionally, RT-PCR is performed in two reaction steps. In the initial reaction, first-strand cDNA is reverse-transcribed from total or poly A^+ RNA using a reverse transcriptase. Then, in a separate reaction, the cDNA is amplified by PCR using a thermostable DNA polymerase. Such two-step procedures require multiple handling steps, including the serial dilution of cDNA templates, and either multiple tubes or the sequential addition of enzymes and other reagents to a single tube. In contrast, the Titanium One-Step RT-PCR Kit allows cDNA synthesis and PCR to be performed in a single optimized buffer, with a single enzyme mix. No additional reagents are required after the reaction is initiated. This method reduces the possibility of cross-contamination and provides a very convenient technique for detecting gene expression (Murakawa *et al.*, 1988; Aatsinki *et al.*, 1994; Sellner and Turbett, 1998).

One-Step RT-PCR is recommended for general use in analyzing gene expression in tissue samples or cell culture systems. This method is particularly useful for applications in which the expression of a small number of genes must be analyzed in many different total RNA samples. In contrast, for applications in which many genes must be analyzed in few RNA samples, two-step RT-PCR is usually a better approach. For these cases, we recommend our Advantage® RT-for-PCR Kit (Cat Nos. 639505, 639506).

I. Introduction & Protocol Overview continued

Titanium One-Step RT-PCR offers the following additional benefits:

- **Wide dynamic range** — Reliably amplifies cDNAs from both low-abundance and high-abundance mRNAs, so low-level gene expression is not missed.
- **High sensitivity** — Amplifies target transcripts from as little as 10 pg of total RNA or 50 copies of synthetic RNA.
- **Robust RT-PCR** — Successfully amplifies high-abundance transcripts from very crude total RNA preparations.
- **Flexible amplicon design** — Our MMLV-RT is highly purified. This results in less template degradation, and leads to the production of longer cDNAs, allowing you to target primers near the 5' end of transcripts.
- **Reduces RNA secondary structure** — Inclusion of our Thermostabilizing Reagent in the reaction allows reverse transcription to be performed at 50°C, minimizing problems caused by RNA folding (Carninci *et al.*, 1998).
- **Advanced PCR enzyme system** — Titanium *Taq* DNA Polymerase contains a 5'-exonulcease-deficient, thermostable DNA polymerase, and TaqStart™ Antibody for automatic hot-start PCR. In addition, because Titanium *Taq* works best at the high Mg^{2+} concentrations favored by reverse transcriptases, it is far superior for RT-PCR applications than other *Taq*-based systems.
- **Reads through GC-rich templates** — This powerful PCR feature is made possible by the addition of our GC-Melt reagent, which destabilizes base-pairing in GC-rich regions.

II. List of Components

Store Control Mouse Liver Total RNA at –70°C.

Store all other components at –20°C.

Sufficient reagents are supplied for 30 (Cat. No. 639503) or 100 (Cat. No. 639504) RT-PCR reactions.

30 rxns	100 rxns	
• 30 µl	100 µl	**50X Titanium *Taq* RT Enzyme Mix** (includes MMLV-RT, Titanium *Taq* DNA Polymerase, and TaqStart Antibody)
• 150 µl	500 µl	**10X One-Step Buffer** (400 mM Tricine, 200 mM KCl, 30 mM $MgCl_2$, 37.5 µg/ml BSA)
• 30 µl	100 µl	**50X dNTP Mix** (10 mM each of dATP, dCTP, dGTP, and dTTP; final rxn concentration: 0.2 mM each)
• 750 µl	2.8 ml	**Thermostabilizing Reagent** Thaw Thermostabilizing Reagent at room temperature before use and vortex to resuspend crystals. If residual precipitate fails to dissolve, spin and use supernatant.
• 300 µl	1 ml	**GC-Melt**
• 30 µl	100 µl	**Oligo(dT) Primer** (20 µM; dT[18])
• 15 µl	50 µl	**Recombinant RNase Inhibitor** (40 units/µl; cloned from human placenta)
• 30 µg	100 µg	**Control Mouse Liver Total RNA** (1 µg/µl)
• 30 µl	100 µl	**Control Mouse β-Actin Primer Mix** (45 µM each) 5' Primer: 5'–GTGGGCCGCCCTAGGCACCAG–3' 3' Primer: 5'–CTCTTTGATGTCACGCACGATTTC–3'
• 1.5 ml	3 ml	**RNase-Free H_2O**

III. Additional Materials Required

The following reagents are required but not supplied.

- [optional] **Mineral oil** (We recommend Sigma Cat. #M-3516.)
- **PCR reaction tubes**
- **Dedicated pipettors**
- **PCR pipette tips** suitable to the above pipettors and preferably equipped with hydrophobic filters.
- **DNA size markers**
- **5X Stop/loading buffer** (Sambrook & Russell [2001] provides several recipes.)

IV. General Considerations

A. RNA Preparation

The use of non-degraded, pure total RNA is critical for synthesizing high-quality cDNA for PCR. RNA should have an A_{260}/A_{280} ratio of 1.7 or higher and should be evaluated on a denaturing formaldehyde/agarose gel to verify integrity prior to cDNA synthesis. The sensitivity of Titanium One-Step RT-PCR decreases as RNA purity decreases. For detecting medium- and low-abundance transcripts, an A_{260}/A_{280} ratio $\geq$ 1.8 is recommended.

Total RNA can be prepared by any standard method. See Sambrook, *et al*. (1989) as a reference. For optimal results use 1 µg–1 ng of RNA per RT-PCR reaction.

To avoid contamination and degradation of RNA, follow these precautions:

- Wear gloves to avoid RNase contamination from hands.
- Wipe all pipettes with 70% ethanol or isopropanol before RNA work.
- Use sterile pipette tips.

Store pure RNA at –70°C or as an ethanol precipitate at –20°C.

B. PCR Primer Design

Primer design is the single largest variable in PCR applications and the single most important factor in determining the success or failure of PCR reactions. Always check and recheck your primer design before constructing or ordering primers.

1. Location of primers
   Our robust RT ensures the synthesis of long cDNAs, enabling you to target primers to any location in the transcript. However, for best results, we recommend that primers anneal within 4 kb of the transcript's 3' end and that they amplify a fragment that is less than 1.5 kb in length. If necessary, primers can be targeted to the transcript's 5' end and can amplify fragments longer than 1.5 kb. Additionally, to distinguish between cDNA and genomic DNA amplification, target primers to different exons that are separated by one or more introns. Such primers will produce larger fragments when amplified from genomic DNA. Alternatively, if intron-separated primer sites cannot be used, a negative control reaction can be performed as described in Section IV.D.
2. Length and G-C content
   Primers should have a T_m of around 70°C and, whenever possible, primers should be at least 22 nucleotides (nt) long (25–30-mers are preferred) and should have a G-C content of 45–60%. Furthermore, the 3'-terminal ends of each primer should not be complementary to each other and should have a low G-C content.
3. Concentration
   Prepare a mix of your 5' and 3' primers that is 45 µM in concentration for each primer.

IV. General Considerations continued

C. Good PCR practices

1. Use dedicated pipettors in a dedicated work space
   Due to the tremendous amplification power of PCR, minute amounts of contaminating DNA can produce nonspecific amplification; in some instances, contaminants can cause DNA bands even in the absence of added template DNA. We recommend that you use small aliquots of starting material to avoid contaminating your stocks. Set up your PCR reactions in a dedicated lab area or non-circulating containment hood and use dedicated pipettors, PCR pipette tips with hydrophobic filters, and dedicated solutions. We also recommend performing post-PCR analysis in a separate area with a separate set of pipettors.
2. Pipetting
   Because of the small volumes used in PCR experiments and the potential for tube-to-tube variation, careful pipetting technique is extremely important. Always be sure that no extra solution is on the outside of the pipette tip before transfer. When adding solution to a tube, immerse the tip into the reaction mixture and deliver the contents from the pipette tip into the mixture, then rinse the tip by pipetting up and down several times.
3. Use a Master Mix
   Assembling a Master Mix, which contains the appropriate volumes of all the reagents required for multiple RT-PCR reactions, eliminates the need for repeated pipetting of individual reaction components into each reaction tube. We highly recommend using a Master Mix for setting up multiple reactions because it greatly reduces tube-to-tube variation. The Master Mix should be thoroughly mixed before use (i.e., vortexed without bubbling).

D. Control Reactions

You should perform a positive control reaction using the enclosed Control Mouse Liver Total RNA and Control Mouse β-Actin Primer Mix. This reaction amplifies a 540-bp fragment of the mouse β-actin cDNA.

An optional negative control can be performed to help distinguish between fragments amplified from cDNA and those derived from genomic DNA contaminants in your RNA sample. In this control, use a PCR enzyme mix without RT (such as Titanium *Taq* DNA Polymerase; Cat. No. 639208) instead of the Titanium *Taq* RT Enzyme Mix included in the One-Step kit. This reaction will require a separate PCR Master Mix in Section V.A. Alternatively, a standard One-Step reaction can be preheated at 94°C for 5 min to inactivate reverse transcriptase before using the thermal cycler procedure in Section V.B. For the latter option, preheat the thermal cycler to 94°C before adding the tube(s) to ensure that minimal cDNA synthesis occurs prior to inactivation.

V. One-Step RT-PCR Procedure

A. Preparing an RT-PCR Master Mix

1. Quickly thaw each tube by warming in your hands; place on ice.

   Note: Thaw Thermostabilizing Reagent at room temperature before use and vortex to resuspend crystals. If residual precipitate fails to dissolve, spin and use supernatant.

2. Spin each tube briefly in a microcentrifuge and return to ice.
3. Prepare a Master Mix as described below. Prepare sufficient Master Mix for all of your reactions plus one additional reaction to ensure adequate volume. You should plan to perform one positive control reaction in addition to your experimental reactions. An optional negative control (described in Section IV.D) can be performed as well.

5 µl	10X One-Step Buffer
1 µl	50X dNTP Mix
0.5 µl	Recombinant RNase Inhibitor (40 units/µl)
25 µl	Thermostabilizing Reagent
10 µl	GC-Melt
1 µl	Oligo(dT) Primer
1 µl	50X Titanium *Taq* RT Enzyme Mix
43.5 µl	**Total volume**

B. Setting Up the Reactions

Set up reactions as shown in Table I.

TABLE I. ONE-STEP RT-PCR REACTIONS

Reagent	Positive control	Experimental reaction(s)
Control Mouse Liver Total RNA	1 µl	–
Control Mouse β-Actin Primer Mix	1 µl	–
Experimental RNA sample (1 ng–1 µg)	–	1–5.5 µl
Experimental PCR primer mix (45 µM each)	–	1 µl
Master Mix	43.5 µl	43.5 µl
RNase-Free H_2O	4.5 µl	to 50 µl
Final volume	**50 µl**	**50 µl**

V. One-Step RT-PCR Procedure continued

C. Running the Reactions

Commence thermal cycling using the following program. This protocol works with most hot-lid and non hot-lid thermal cyclers. However, the optimal cycling parameters may vary with different templates, primers, experimental protocols, tubes, and thermal cyclers.

- 50°C for 1 hr
- 94°C for 5 min
- 25–40 cycles[a]:
  - 94°C 30 sec
  - 65°C 30 sec
  - 68°C 1 min[b]
- 68°C for 2 min

[a] Optimal number of cycles depends on transcript abundance and template complexity and must be determined empirically.

[b] For experimental reactions, use 1–1.5 min of extension time per kb.

VI. Troubleshooting Guide

A. Low Yield or No Products

- Your RNA may be degraded. RNA should be evaluated by running a denaturing formaldehyde/agarose gel to verify integrity prior to cDNA synthesis.
- There may be impurities in your RNA sample that interfere with the RT-PCR reaction. Ensure that your sample has an A_{260}/A_{280} ratio of 1.7 or higher. Reprecipitate or re-isolate RNA from the source if necessary.
- Poor primer design. Be sure to follow the guidelines in Section IV.B when designing primers.
- PCR parameters may need to be optimized for your transcript of interest.
- Your target gene may be very weakly expressed in the analyzed sample.

B. Multiple Amplification Products

- Your sample may contain alternatively spliced forms of the target transcript.
- The gene may be a member of a multigene family, in which case your "gene-specific" primers may simultaneously amplify several highly homologous cDNAs.
- Poor primer design. Be sure to follow the guidelines in Section IV.B when designing primers.
- Your RNA sample may be contaminated by another RNA or DNA sample.
- PCR parameters must be optimized for analyzing your target gene.

VII. References

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