User Manual

Advantage® RT-for-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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I. Introduction

The Advantage RT-for-PCR Kit allows efficient and rapid first-strand cDNA synthesis from either total or polyA$^+$ RNA. Starting with nanogram quantities of any RNA, this kit provides sufficient quantities of first-strand cDNA for PCR; however, this kit does not contain the reagents needed for PCR.

II. List of Components

Store all components except the RNA at –20°C.

Store RNA at –70°C.

Each Advantage RT-for-PCR Kit contains the following reagents for 25 or 100 cDNA synthesis reactions.

639505 (25 rxns)	639506 (100 rxns)	
• **100 µl**	**400 µl**	**5X Reaction Buffer** 250 mM Tris-HCl, pH 8.3 375 mM KCl 15 mM $MgCl_2$
• **25 µl**	**100 µl**	**Oligo $(dT)_{18}$ Primer, 20 µM**
• **25 µl**	**100 µl**	**Random Hexamer Primer, 20 µM**
• **30 µl**	**120 µl**	**MMLV Reverse Transcriptase, 200 units/µl** (MMLV: Moloney Murine Leukemia Virus, recombinant)
• **15 µl**	**60 µl**	**Recombinant RNase Inhibitor, 40 units/µl**
• **25 µl**	**100 µl**	**dNTP Mix, 10 mM each**
• **15 µl**	**60 µl**	**Control RNA (mouse liver total RNA), 1 µg/µl**
• **50 µl**	**200 µl**	**Premixed Mouse G3PDH Amplimers, 10 µM each primer**
• **1.0 ml**	**4.0 ml**	**DEPC-treated Water** [treated with diethyl pyrocarbonate, (1:1000) and autoclaved]

III. Advantage RT-for-PCR Kit Protocol

PLEASE READ ENTIRE PROCEDURE BEFORE STARTING.

A. General Considerations

To avoid contamination and degradation of RNA, use the following precautions:

1. Wear gloves to avoid RNase contamination from hands.
2. Use 70% ethanol or isopropanol to wipe all pipettes clean before use for RNA work. Use sterile pipette tips.

B. RNA Preparation

The use of pure, high-quality RNA is critical for synthesizing high-quality cDNA for PCR. RNA should have a A_{260}/A_{280} ratio of 1.7 or higher and should be evaluated by running a denaturing formaldehyde/agarose gel to verify integrity prior to cDNA synthesis. Molecular Cloning: A Laboratory Manual, by Sambrook and Russell (2001) is a good reference for RNA preparation.

RNA should be stored at –70°C or below, or as an ethanol precipitate at –20°C.

C. Primer Selection

In general, four types of primer strategies are used in RT reactions. Typically, first-strand cDNA synthesis is primed with either oligo(dT) primers, random hexamer primers, a combination of the two, or gene specific primers (GSP). When selecting primers, consider the following:

1. Oligo(dT) primers anneal to the polyA$^+$ tail of mRNA, ensuring that only mRNA is reverse transcribed. Since these primers only anneal to the 3' end of the mRNA, all of the transcripts begin at approximately the same place. One drawback to using Oligo(dT) primers is that the RT enzyme is not always able to completely synthesize longer transcripts, so these transcripts may not be fully represented in the cDNA. These primers are most often used to make cDNA libraries.
2. Random hexamer primers randomly anneal to any RNA template and typically generate a mix of relatively short cDNA transcripts that represent all of the RNA in the sample. Random hexamer primers are typically used to reverse transcribe total RNA, or the 5' region of long mRNA transcripts. These primers are useful for generating cDNA libraries, including those of single-stranded RNA viruses.
3. Oligo(dT) primers and Random hexamer primers are often combined in one reaction to synthesize a cDNA pool that is more representative of the RNA in a sample. Because each type of primer anneals to a different subset of the template—oligo(dT) primers

III. Advantage RT-for-PCR Kit Protocol continued

only bind to the polyA$^+$ tail of mRNA, while random hexamer primers bind randomly over all of the RNA—cDNA synthesis is more evenly distributed over the RNA template and higher yields can be achieved.

4. Gene-specific primers are used when you want to target the RNA of a specific gene. Gene-specific primers should be approximately 18 bases long, and should span an intron in order to specifically target fully processed mRNA. These primers are used for cDNA cloning and for qRT-PCR.

D. cDNA Synthesis

1. Quickly thaw each tube in the Advantage RT-for-PCR Kit and place on ice. Carry out all dilutions and additions on ice.
2. Spin each tube briefly in a tabletop microcentrifuge and return to ice.
3. Place 0.2–1 µg of your total RNA prep in a sterile 0.5 ml microcentrifuge tube and bring the volume up to 12.5 µl with DEPC-treated H_2O (clear tube).
4. Add 1.0 µl of either the random hexamer primer (yellow tube) or the oligo(dT)$_{18}$ primer (orange tube). Both random hexamer and oligo(dT) primers are provided in the kit. For additional information, please refer to Section III.C.
5. Heat the RNA at 70°C for 2 min, then cool rapidly on ice before proceeding to the next step.
6. Add the components listed in the table below as indicated.

Table I: cDNA Synthesis

Reagent	Volume
5X Reaction Buffer	4.0 µl
dNTP Mix (10 mM each)	1.0 µl
Recombinant RNase Inhibitor	0.5 µl
MMLV Reverse Transcriptase	1.0 µl
Total Volume	6.5 µl

Note: We recommend that a master reagent mix be prepared when more than one RNA sample will be used for RT-PCR. This will help to ensure tube to tube consistency in the cDNA synthesis reaction. Be sure, however, to make extra master mix so that there will be enough for all of your reactions.

7. Mix the contents of the tube by pipeting up and down.
8. Incubate the reaction at 42°C for 1 hr.

III. Advantage RT-for-PCR Kit Protocol continued

9. Heat at 94°C for 5 min to stop the cDNA synthesis reaction and to destroy any DNase activity; then spin down the contents of the tube.
10. Dilute the reaction to a final volume of 100 µl by adding 80 µl of DEPC-treated H_2O. Vortex and spin again. The dilution will allow more accurate pipeting of the cDNA.
11. The cDNA is now ready for immediate use or storage. If storing the cDNA, store at –70°C or below. Avoid multiple freeze/thaw cycles. After thawing samples stored at –70°C, vortex and spin briefly before use.

Table II provides the final composition of the reaction mixture:

Table II: Reaction Mixture

Reagent	Volume	Final Conc./Amount
RNA + DEPC-treated H_2O	12.5 µl	0.2–1 µg total RNA
Primer—random hexamer or oligo$(dT)_{18}$	1.0 µl	20 pmol
5X Reaction Buffer	4.0 µl	50 mM Tris-HCl, pH 8.3 75 mM KCl 3 mM $MgCl_2$
dNTP mix (10 mM each)	1.0 µl	0.5 mM each
RNase Inhibitor	0.5 µl	1 unit/µl
MMLV Reverse Transcriptase	1.0 µl	≥ 200 units/µl RNA
Total Volume	20 µl	

E. PCR

We typically use 5–10 µl of diluted cDNA for each 50 µl PCR reaction. The efficiency of the cDNA synthesis can be estimated by using the Premixed Mouse G3PDH Amplimers to amplify G3PDH from cDNA generated with the Control RNA. The absence of a band when using these amplimers suggests that a component of the cDNA synthesis reaction or PCR reaction was omitted. Poor amplification results (as indicated by faint gel bands) suggest that more cDNA should be used for gene amplification. (Do not exceed 20 µl of the diluted cDNA). Poor amplification of experimental samples may indicate that the quality of the RNA is poor.

III. Advantage RT-for-PCR Kit Protocol continued

PCR primers should be 23–30 nucleotides in length, have a GC-content of ~50% , and contain no internal secondary structures. In addition, the 3' ends of the primers should not contain complementary sequences.

F. Controls

Control RNA and PCR primers are included in this kit. Use 1 µl of Control RNA (mouse liver total RNA, 1 µg/µl) for cDNA synthesis (Section D). Then use the premixed G3PDH primers in the following PCR protocol:

1. Place the following components into a tube:

Sterile H_2O	36 µl
10X PCR buffer	5 µl
dNTP mix (10 mM each)	1 µl
Premixed G3PDH primers	2 µl
cDNA, (1:100)	5 µl
TITANIUM™ *Taq* or another DNA polymerase	1 µl
Total	50 µl

2. Thermal cycling parameters:

   Use the following guidelines when setting up your initial experiments with the Advantage system. These are general guidelines—the optimal parameters may vary with different thermal cyclers and will depend on your particular primers, template, and other experimental variables.

   - 30 cycles

     94°C 45 sec
     60°C 45 sec-
     72°C 2 min

   - 7 min final extension at 72°C.

   Under some conditions, 30 cycles may be needed. Upon gel electrophoresis (2% agarose in 0.5X TBE) a single band of 983 bp should be visible.

   Note: If you are using a different enzyme and/or thermal cycler, PCR conditions may need to be modified.

V. References

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