Advantage® RT-for-PCR Kit Protocol

PT1107-2

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I. Introduction

The **Advantage RT-for-PCR Kit** (Cat. Nos. 639505 & 639506) 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—but it does not contain the reagents needed for PCR. This abbreviated protocol (PT1107-2) is provided for your convenience, but is not intended for first-time users. For additional details, see the Advantage RT-for-PCR Kit User Manual (PT1107-1).

II. General Considerations

A. RNA Handling

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

- Wear gloves to avoid RNase contamination from hands.
- Use 70% ethanol or isopropanol to wipe all pipetting devices clean before use for RNA work and make sure to use sterile pipette tips.

B. RNA Preparation & Storage

For optimal cDNA synthesis, prepare and store RNA according to the following criteria:

- RNA should have an $A_{260/A280}$ ratio of 1.7 or higher, and should be evaluated by running a denaturing formaldehyde/agarose gel to verify integrity prior to cDNA synthesis.
- RNA should be stored at –70°C or below, or as an ethanol precipitate at –20°C.

NOTE: Sambrook, J. and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY is a good reference for RNA preps.

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C. Primers for cDNA Synthesis

The following four types of primer strategies are used to synthesize first-strand cDNA for generating cDNA libraries (1–3), or for cDNA cloning and qRT-PCR (4). See PT1107-1 for more information.

1. **Oligo(dT) primers** anneal to the polyA$^+$ tail (3' end) of mRNA, ensuring that only mRNA is reverse transcribed. However, the RT enzyme is not always able to completely synthesize longer transcripts.
2. **Random hexamer primers** randomly anneal to any RNA template, and typically generate a mix of relatively short cDNA transcripts representing all of the RNA in the sample. These primers are used to reverse transcribe total RNA or the 5' region of long mRNA transcripts.
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, and achieve higher cDNA yields.
4. **Gene-specific primers** (~18 bp long, intron-spanning) are used to target the mRNA of a specific gene.

III. Protocols

A. cDNA Synthesis Protocol

1. Quickly thaw each tube in the Advantage RT-for-PCR Kit and place it 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)—or 0.5 ml each of both primers. Both random hexamer and oligo(dT)$_{18}$ primers are provided in the kit. For more information on primer selection, see Section III.C of the User Manual (PT1107-1).
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 Table 1 as indicated.

Table 1. 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: Prepare a master mix 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 to make extra master mix so that there will be enough for all of your reactions.

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

11. The cDNA is now ready for immediate use or storage. 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 2 provides the final composition of the reaction mixture.

Table 2. Reaction Mixture Composition

Reagent	Volume	Final Concentration/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	

B. PCR Protocol for Determining the Efficiency of cDNA Synthesis

The efficiency of cDNA synthesis can be estimated using the Control RNA and PCR primers 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 Amplimers in the following PCR protocol:

1. Control PCR Reaction Mixture

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 Amplimers	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.

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