

Atlas[®] Arrays RT-PCR 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

Catalog No. 634564
PT3270-1 (PR1X380)
Published 30 October 2001

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

Table of Contents

I. Introduction	3
II. Required Materials	4
III. RT-PCR Protocol	5
A. General Considerations	5
B. RNA Preparation	5
C. Considerations for cDNA Priming	5
D. cDNA Synthesis	6
E. PCR	7
F. Troubleshooting	10
IV. Related Products	11

Notice to Purchaser

Clontech products are to be used for research purposes only. They may not be used for any other purpose, including, but not limited to, use in drugs, *in vitro* diagnostic purposes, therapeutics, or in humans. Clontech products may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without written approval of Clontech Laboratories, Inc.

Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,079,352 and 6,127,155. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim (such as method claims in U.S. Patents Nos. 5,210,015, 5,487,972, 5,994,056 and 6,171,785) and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby conveyed by the purchase of this product expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

No license is conveyed with the purchase of this product under U.S. Patents Nos. 5,210,015, 5,487,972, 5,804,375, 5,994,056 and 6,171,785, and corresponding patent claims outside the U.S., relating to the 5' nuclease and dsDNA-binding dye processes. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Clontech, the Clontech logo and all other trademarks are the property of Clontech Laboratories, Inc. Clontech is a Takara Bio Company. ©2006

I. Introduction

Clontech's Atlas cDNA Expression Arrays include hundreds of cDNA fragments spotted on positively charged nylon membranes. Negative and positive controls are also included. The Atlas RT-PCR Primer Sequences CD-ROM contains the forward and reverse primer sequences for all cDNA fragments included on human, mouse, and rat nylon Atlas Arrays. This User Manual provides an RT-PCR protocol and related information for use of these primer sequences.

Each Atlas Array experiment yields a gene expression profile that provides data on hundreds of genes. Atlas Arrays provide semi-quantitative, comparative expression data for genes whose expression differs by as little as two-fold between two samples. Based on our experience, RT-PCR is the most efficient, high-throughput method for corroborating differences in gene expression levels detected by Atlas Arrays

Clontech's Advantage RT-for-PCR Kit (Cat. No. 639505) is a complete kit for efficient first-strand cDNA synthesis from any RNA in just one hour. This kit contains an improved, longer oligo (dT) primer that provides more effective cDNA priming. The RT-for-PCR Kit also includes random hexamer primers, control PCR primers, and control RNA.

The protocol in this User Manual, adapted from the Advantage RT-for-PCR Kit, is included as a guideline, and is optimized for compatibility with Atlas primers. Several positive control amplimers are also available from Clontech, including G3PDH (glyceraldehyde-3-phosphate-dehydrogenase) and β -actin (see Section IV).

II. Required Materials

For your reference, the components provided with Clontech's Advantage RT-for-PCR kit are listed here.

Note: Store all components *except* RNA at -20°C . Store RNA at -70°C .

- **DTT** (100 mM)

Provided with Advantage® RT-for-PCR Kit:

- **5X Reaction Buffer:**
 - 250 mM Tris-HCl, pH 8.3
 - 375 mM KCl
 - 15 mM MgCl_2
- **Oligo (dT) primer** (20 μM)
- **Random hexamer primer** (20 μM)
- **MMLV Reverse Transcriptase** (200 units/ μl)
(MMLV: Moloney-Murine Leukemia Virus, recombinant)
- **Recombinant RNase inhibitor** (40 units/ μl)
- **dNTP mix, 10 mM each of dATP, dGTP, dCTP, and dTTP**
- **Control RNA** (human placenta total RNA) (1 $\mu\text{g}/\mu\text{l}$)
- **Premixed human G3PDH amplimers** (10 μM each primer)
- **Milli-Q-grade deionized or DEPC-treated water**

III. RT-PCR Protocol

A. General Considerations

- Sterile, disposable plasticware is essentially free of RNases and can be used for the preparation and storage of RNA without pretreatment. However, general laboratory glassware and plasticware may be contaminated with RNases and should be treated appropriately. We recommend that you avoid using multiple-use glassware. However, if you do use glassware, remove RNases by baking at 300°C for 4 hr (or 180°C for 8 hr). To remove RNases from polypropylene plasticware, rinse with chloroform. Do not use autoclaved glass or plasticware without first washing it with SDS and ethanol.
- We strongly recommend that you prepare all solutions with Milli-Q-grade-deionized water. You may also use DEPC-treated water.

B. RNA Preparation

- The use of undegraded and pure RNA is critical for synthesizing high-quality cDNA for PCR. RNA should have a A_{260}/A_{280} ratio of 1.8 or higher. Its integrity should be verified prior to cDNA synthesis by running it on a denaturing formaldehyde/agarose gel. The ratio of 28S/18S rRNA should be at least 2 to 1.
- Successful RT-PCR requires high-quality RNA free of DNA contaminants. We highly recommend the use of our Atlas Pure Total RNA Labeling System (Cat. No. 634562) for the isolation of RNA. If you do not use this kit, use a DNase I treatment step to ensure high-quality RNA. The procedure for this step is included in the Atlas cDNA Expression Array protocol.
- Store RNA at -70°C as a water solution, or as an ethanol precipitate at -20°C or -70°C .

C. Considerations for cDNA Priming

- In the most commonly used method for this procedure, the entire population of mRNA molecules is converted into cDNA by priming with oligo(dT) or random hexamers. Two gene-specific PCR primers are then added for amplification. Several different genes can be amplified from the same pool of cDNA. This procedure facilitates side-by-side comparison of control and experimental gene expression levels.
- In our experiments, we typically start with oligo(dT) priming, which we have found to be more sensitive, and on average, to yield fewer nonspecific PCR products than random priming. There may be situations, however, when random priming of cDNA may be beneficial. For instance, the reverse transcriptase may fail to fully transcribe an mRNA template if the 5' primer is located greater than about 2–3 kb from the poly-A⁺ tail or if secondary structures exist that impede the processivity of the reverse transcriptase.

III. RT-PCR Protocol *continued*

D. cDNA Synthesis

1. Quickly thaw any frozen reagents 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. In a sterile 0.5-ml microcentrifuge tube, add your RNA preparation to a volume of H₂O that will give a total volume of 11.5 µl. Use 0.2–1 µg of total RNA.
4. Add 1.0 µl of either random hexamer primer or oligo(dT) primer.
5. Heat the RNA at 70°C for 2 min. *Then quench rapidly on ice before proceeding to the next step.*
6. Add the components listed in the table below according to the volumes given.

TABLE I: cDNA SYNTHESIS

Reagent	Volume
5X Reaction Buffer	4.0 µl
DTT 100 mM	1.0 µl
dNTP mix (10 mM each)	1.0 µl
Recombinant RNase inhibitor	0.5 µl
MMLV reverse transcriptase	1.0 µl
Total Volume	7.5 µl

Note: We recommend preparing a master reagent mix when more than one RNA sample will be used for RT-PCR. This procedure 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 sufficient master mix for all tubes.

7. Mix the contents of the tube by pipeting 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; then spin down the contents of the tube.
10. Dilute the reaction to a final volume of 100 µl by adding 80 µl of H₂O. Vortex and spin again. The dilution will allow more accurate pipeting of the cDNA.

III. RT-PCR Protocol *continued*

11. The cDNA is now ready for immediate use or storage. Store at -20°C or below, and avoid multiple freeze/thaw cycles. After thawing samples stored at -20°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 + H ₂ O	11.5 μl	0.2–1 μg total RNA
Random hexamer primer or oligo (dT) primer	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
DTT (100 mM)	1.0 μl	5 mM
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	\geq 200 units/ μg RNA
Total Volume	20 μl	

E. PCR

Note: A control RT-PCR reaction with at least two housekeeping genes should be performed prior to amplification of your gene of interest. This protocol allows you to estimate the relative abundance level of an amplified fragment between two experimental samples.

1. Place the following components into a 0.5-ml PCR reaction tube:

Deionized H ₂ O	37 μl
10X PCR buffer	5 μl
dNTP mix (10 mM each)	1 μl
Atlas primer pair (20 μM each)	1 μl
cDNA (from Step D.10)	5 μl
TITANIUM™ <i>Taq</i> or other DNA polymerase	1 μl
Total	50 μl

III. RT-PCR Protocol *continued*

2. Thermal cycling parameters (optimized using the Perkin-Elmer DNA Thermal Cycler 480):
 - 18 cycles
 - 94°C 30 sec
 - 68°C 2 min
 - 5 min final extension at 68°C.
3. Remove 5 µl from each reaction and place it in a clean tube. Put the rest of the reaction back into the thermal cycler for five more cycles.
4. Repeat step 3 three times (i.e., remove 5 µl after 28, 33, and 38 cycles).
5. Examine the 5-µl samples (i.e., the aliquots that were removed from each reaction after 18, 23, 28, 33, and 38 cycles) on a 2.0% agarose/EtBr gel.

When a PCR reaction reaches a saturation plateau, the intensity of PCR product bands cannot be accurately compared. Thus, for best results, you should compare the intensities of amplified cDNA fragments at the beginning of the exponential phase of amplification, when visible products first appear.

A control RT-PCR reaction with at least two housekeeping genes should be performed in parallel with your gene(s) of interest. Alternatively, the control reaction can be performed prior to amplification of your gene of interest. The amplification of the housekeeping genes should be identical in both samples, confirming that the same concentration of cDNA was used in each sample (see Figure 1 below). Normalizing your RT-PCR signals with two housekeeping genes will help prevent using a housekeeping gene that is differentially expressed in your samples.

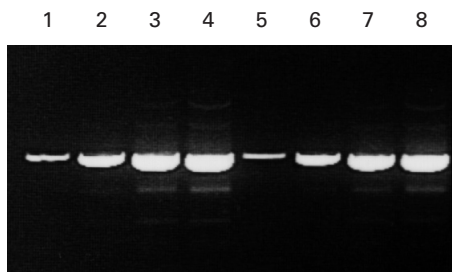


Figure 1. RT-PCR amplification of control mRNA. PCR was performed on cDNA sample 1 (Lanes 1–4) and cDNA sample 2 (Lanes 5–8) with G3PDH 5' and 3' primers. Lanes 1 & 5: 18 cycles; Lanes 2 & 6: 23 cycles; Lanes 3 & 7: 28 cycles; Lanes 4 & 8: 33 cycles. Band size: approximately 600 bases.

III. RT-PCR Protocol *continued*

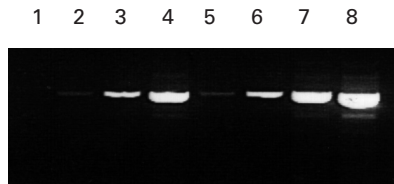


Figure 2. Comparison of expression levels of mRNA by RT-PCR. PCR was performed on cDNA sample 1 (Lanes 1–4) and cDNA sample 2 (Lanes 5–8) with two gene-specific PCR primers. Lanes 1 & 5: 18 cycles; Lanes 2 & 6: 23 cycles; Lanes 3 & 7: 28 cycles; Lanes 4 & 8: 33 cycles. Band size: approximately 600 bases.

Figure 1 shows an experiment, performed on a housekeeping gene, in which there is no difference in the abundance of the housekeeping mRNA. Note: if PCR products from both housekeeping genes are not the same, *i.e.* they do not appear simultaneously, dilute the sample whose concentration of cDNA is greater. Dilute the sample in order to equalize the concentrations of cDNA in the two samples, and then repeat your PCR analysis.

Figure 2 shows an example of amplification of a gene of interest from two cDNA samples. The quantity of PCR cycles required for visualization of PCR products reflects the abundance of the gene. For more abundant RNAs, you may see a band after 18–23 cycles. However, for less abundant RNAs, you should see a product at 28, 33, or 38 cycles.

The difference in the number of cycles required for equal intensity of amplified PCR products reflects the difference in sequence abundance between your two cDNA samples. Five cycles corresponds roughly to an approximate 10-fold difference in cDNA concentration. In Figure 2, PCR of sample 1 produces a band after 23 cycles, while PCR of sample 2 produces a band of comparable intensity after 18 cycles. Based on this result, we can estimate that the gene of interest is 10-fold more abundant in sample 2 than in sample 1.

III. RT-PCR Protocol *continued*

F. Troubleshooting

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

We typically use 5–10 μl of the diluted cDNA for each 50- μl PCR reaction. The efficiency of the cDNA synthesis can be estimated by using Clontech's positive control amplimers (G3PDH or β -actin; see the following page for a detailed product listing). The absence of a band when using positive control 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.

IV. Related Products

For the latest and most complete listing of all Clontech products, please visit www.clontech.com

- | | |
|---|------------------|
| • Advantage® RT-for PCR Kit | 639505
639506 |
| • Atlas® Pure Total RNA Labeling System | 634562 |
| • TITANIUM™ <i>Taq</i> DNA Polymerase | 639208
639209 |
| • Advantage® 2 PCR Kit | 639207
639206 |
| • Advantage® 2 Polymerase Mix | 639201
639202 |
| • TaqStart® Antibody | 639250
639251 |
| • Multiple Tissue Northern Blots (MTN® Blots) | many |

Clontech offers the following RT-PCR Control Amplimer Sets, available in 100 or 25 reaction sizes :

- | | |
|---|------------------|
| • Human G3PDH Control Amplimer Set | 639005
639006 |
| • Mouse G3PDH Control Amplimer Set | 639009
639010 |
| • Rat G3PDH Control Amplimer Set | 639013
639014 |
| • Human β -Actin Control Amplimer Set | 639001
639002 |
| • Mouse β -Actin Control Amplimer Set | 639007
639008 |
| • Rat β -Actin Control Amplimer Set | 639011
639012 |