Easy-A One-Tube RT-PCR System

INSTRUCTION MANUAL

Catalog #600182
Revision B.01

For in Vitro Use Only
600182-12

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Easy-A One-Tube RT-PCR System

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Easy-A One-Tube RT-PCR System

MATERIALS PROVIDED

Materials provided	Quantity[a]
Reverse Transcriptase (RT)	50 µl
Easy-A High-Fidelity PCR cloning enzyme (5 U/µl)	125 U
10× RT-PCR buffer	1 ml
40 mM Deoxynucleotide (dNTP) mix (10 mM of each dNTP)	50 µl
RNase-free water	3 × 1.2 ml

[a] Sufficient reagents are provided for 50 RT-PCR reactions.

STORAGE CONDITIONS

All Materials: Store at –20°C upon receipt.

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Revision B.01

INTRODUCTION

The Easy-A One-Tube RT-PCR System* combines cDNA synthesis, high-fidelity PCR amplification, and the generation of 3´-A overhangs. Both poly(A)$^+$ and total RNA samples can be used as starting material in this convenient and easy-to-use one-step format. The kit combines 1st-strand cDNA synthesis reagents with the Stratagene Easy-A high-fidelity PCR cloning enzyme, which is 6-fold more accurate than *Taq* DNA polymerase, for PCR. In addition to the exonuclease activity that improves PCR fidelity, the Easy-A enzyme possesses terminal transferase activity, which adds 3´-A overhangs, generating amplicons that can be easily cloned into any TA/UA cloning vector including the vectors in the Stratagene StrataClone PCR Cloning Kit.

The Easy-A one-tube RT-PCR system can readily detect RNA targets of 0.1–6 kb in length using 10–200 ng of total RNA or 0.1–10 ng of poly(A)$^+$ RNA.

The reagents for both 1st-strand cDNA synthesis and PCR amplification are combined in one tube. Complementary DNA synthesis and PCR take place successively in a specially optimized RT-PCR buffer during an uninterrupted thermal-cycling program. Because the reaction tube is not handled between cDNA synthesis and PCR amplification, the Easy-A one-tube RT-PCR system requires less hands-on time and involves a lower risk of sample contamination than two-tube systems.

The Easy-A one-tube RT-PCR system is well-suited for detecting and quantifying gene expression and for generating products with high fidelity for cloning and sequencing. The one-step format is useful for running multiple reactions simultaneously for high-throughput screening of RNA samples.

* U.S. Patent Nos. 7,045,328, 6,734,293, 6,489,150, 6,444,428, 6,183,997, and patents pending

PREPROTOCOL CONSIDERATIONS

RNA Isolation

High-quality intact RNA is essential for successful synthesis of full-length cDNA and yield of long RT-PCR products. Total and poly(A)$^+$ RNA can be rapidly isolated and purified using the Stratagene Absolutely RNA purification kits. Oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary, although including this step may improve the yield of specific cDNA templates. RNA samples with an $OD_{260/280}$ of 1.8–2.0 are optimal.

Take precautions to minimize the potential for contamination by ribonucleases (RNases). RNA isolation should be performed under RNase-free conditions. Wear gloves and use sterile tubes, pipet tips, and RNase-free water (not DEPC-treated water, because it can inhibit PCR). Use of an RNase inhibitor, such as Stratagene RNase Block Ribonuclease Inhibitor, is recommended when isolating RNA from samples high in RNase activity.

Easy-A high-fidelity PCR cloning enzyme has negligible reverse transcriptase activity under the standard reaction conditions. Therefore, PCR amplification should be dependent on reverse transcription of the RNA template by the reverse transcriptase (RT). When an RNA template is contaminated with genomic DNA, however, the RT-PCR product may be the result of amplification of trace amounts of genomic DNA rather than the cDNA template. A negative control reaction in which RT is omitted can be performed to ensure that the RT-PCR product is the result of amplification of the cDNA template.

Contaminating DNA can be removed from the RNA preparation using an RNase-free DNase. Alternatively, PCR primers can be designed to distinguish between amplification of target cDNA and possible contaminating genomic DNA (see *PCR Primer Design*).

cDNA Synthesis Reaction

Primers

The gene-specific downstream primer is used to prime cDNA synthesis. Specificity of priming may be improved by optimizing the reaction temperature and including a 65°C 5-minute incubation for annealing prior to the addition of the reverse transcriptase. The purity and quality of the primers can impact the yield of the RT-PCR reaction, particularly for low-abundance targets. In such cases, try increasing the concentration of template RNA in the reaction.

Incubation Temperature and Duration

The reverse transcriptase (RT) included with the kit is an engineered version of the Moloney Murine Leukemia Virus (MMLV) RT. For most targets, we recommend a 30-minute, 45°C incubation for the 1st-strand synthesis reaction. For targets >4 kb, lowering the 1st-strand synthesis incubation temperature to 42°C may improve yield.

RNase Inhibitor

RNase inhibitor (1 U/reaction) can be added to RT-PCR reaction mixtures. The use of RNase inhibitor in higher concentrations may reduce product yield. If RNase inhibitor is added to the reaction, decrease the volume of water accordingly.

RT-PCR Reaction

Inactivating Reverse Transcriptase

The RT enzyme must be inactivated prior to PCR to obtain high yields of amplification product. Heat treatment at 95°C for 1 minute in the first thermal cycle of PCR inactivates RT and also denatures the RNA–cDNA hybrid.

Thermal-Cycling Program

Complementary DNA synthesis and PCR take place during an uninterrupted thermal-cycling program. A 45°C incubation for cDNA synthesis is immediately followed by the thermal cycles for PCR amplification.

A typical amplification cycle consists of a denaturation step (95°C), a template–primer annealing step (42–60°C), and an extension step (68°C). PCR primer sequence is a major consideration in determining the annealing temperature of the thermal-cycling program. The annealing temperature should be 10°C below the T_m of the primers. For a primer with a high T_m, it may be advantageous to increase the suggested temperatures of the annealing and extension steps. A higher temperature minimizes nonspecific primer annealing, thus increasing the amount of specific product. For a primer with a low T_m, it may be necessary to decrease the annealing temperature to allow the primer to anneal to the template.

The optimal extension temperature for Easy-A high-fidelity PCR cloning enzyme in RT-PCR is 68°C. The extension time varies with the size of the template. A reasonable starting point is 2 minutes for targets <1 kb and 2 minutes/kb for targets >1 kb. A 10-minute final extension at 68°C improves the quality of the final product by extending truncated product to full length.

Number of Thermal Cycles

Forty cycles of amplification is sufficient to detect most RNA targets. If the target RNA is rare or if only a small amount of starting material is available, it may be necessary to increase the number of cycles.

PCR Primer Design

Primer pairs that exhibit similar melting temperatures and are completely complementary to the template are recommended. Depending on the primer design and the desired specificity of the amplification reaction, melting temperatures between 55° and 80°C generally yield the best results.[1] The following formula[2] is commonly used for estimating the melting temperature (T_m) of the primers:

$$T_m(°C) \cong 2(N_A + N_T) + 4(N_G + N_C)$$

where N equals the number of primer adenine (A), thymidine (T), guanidine (G), or cytosine (C) bases. Several other articles present additional equations for estimating the melting temperature of the primers.[3, 4] Care must be taken when using degenerate primers. Degenerate primers should be designed with the least degeneracy at the 3´ end. Optimization of degenerate primer concentration is necessary. Finally, primers should not be self-complementary or complementary to each other at their 3´ ends.

To differentiate between amplification of target cDNA and possible contaminating genomic DNA, the primers can be designed to anneal to sequences in exons on opposite sides of an intron. An amplification product derived from genomic DNA will be much larger than the product of the RT-PCR reaction. This size difference makes it possible to differentiate the two products by gel electrophoresis. Alternatively, PCR primers can be designed to anneal to the exon–exon boundary of the mRNA. With these primers, amplification of genomic DNA will be highly inefficient.

General Notes

Preventing Cross-Contamination

Take precautions to minimize the potential for carryover of nucleic acids (RNA and DNA) from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips.

Preparing a Master Mix for Multiple Samples

If running multiple samples, a master mix of components for RT-PCR can be prepared by combining the desired multiple of each component. Individual samples can then be prepared by aliquoting the master mix into individual tubes using a fresh pipet tip for each addition. Using a master mix facilitates accurate dispensing of reagents, minimizes loss of reagents during pipetting, and makes repeated dispensing of each reagent unnecessary, all of which help minimize sample-to-sample variation.

Mixing and Pipetting Enzymes

Enzymes (including RT and Easy-A PCR cloning enzyme) should be mixed by gentle vortexing without generating bubbles. Pipet the enzymes carefully and slowly; otherwise, the viscosity of the 50% glycerol in the buffer can lead to pipetting errors.

SINGLE-TUBE RT-PCR PROTOCOL

Note *Mix and spin each component in a microcentrifuge before use.*

1. Prepare the reaction by adding the following components *in order* to a separate sterile thin-wall PCR tube[‖] or sterile 0.5-ml microcentrifuge tube:

   39.5 µl of RNase-free water **(not DEPC-treated water)**
   5.0 µl of 10× RT-PCR buffer
   1.0 µl of upstream primer (100 ng)
   1.0 µl of downstream primer (100 ng)
   1.0 µl of dNTP mix (40 mM)
   1.0 µl of RNA (*The quantity of RNA depends on the RNA purity, primer quality, message abundance, and size of the target. See table below for guidelines.*)

RNA	Quantity
Total RNA, target <2 kb	10–200 ng
Total RNA, target >2 kb	200–500 ng
mRNA (all targets)	0.1–10 ng

3. Add 1.0 µl of reverse transcriptase (RT).

4. Add 0.5 µl of Easy-A High-Fidelity PCR cloning enzyme.

5. Vortex the reaction gently without creating bubbles.

6. If the thermal cycler does not have a hot top assembly, overlay the reaction with one or two drops (20–40 µl) of nuclease-free mineral oil to prevent evaporation and condensation during thermal cycling.

[‖] Thin-wall tubes are highly recommended for use with Stratagene thermal cyclers. These tubes maintain ideal contact with the blocks, permit efficient heat transfer, and maximize thermal-cycling performance.

7. Place the reaction in a thermal cycler. Run the following thermal-cycling program to synthesize 1st-strand cDNA from the RNA template and then to amplify the cDNA via PCR.

Thermal cycler	Cycles	Temperature	Duration	
			<1 kb target	>1 kb target
Single or multiple block	1	45°C	15 minutes	30 minutes
	1	95°C	1 minute	1 minute
	40	95°C	30 seconds	30 seconds
		60°C[a]	30 seconds	30 seconds
		68°C	2 minutes	2 minutes/kb
	1	68°C	5 minutes	10 minutes
RoboCycler temperature cycler	1	45°C	15 minutes	30 minutes
	1	95°C	1 minute	1 minute
	40	95°C	1 minute	1 minute
		60°C[a]	1 minute	1 minute
		68°C	2 minutes	2 minutes/kb
	1	68°C	5 minutes	10 minutes

[a] Use the annealing temperature appropriate for the specific primer pair used in the reaction.

Analyzing the RT-PCR Products

Analyze the RT-PCR products by 1.0% (w/v) agarose gel electrophoresis. The products will be readily visible by UV transillumination of the ethidium bromide-stained agarose gel.

Store the reaction products at –20°C until needed. The RT-PCR products may be purified using the Stratagene StrataPrep PCR Purification Kit.

TROUBLESHOOTING

Observation	Suggestion
No or low product yield	Verify the integrity of the RNA by denaturing agarose gel electrophoresis.
	Replace the RNA. Use Stratagene RNA isolation kits to isolate intact RNA or mRNA.
	Isolate the RNA in the presence of a ribonuclease inhibitor and ensure that all RT-PCR reagents and labware are free of RNases.
	Reduce the volume of the target RNA or remove RT inhibitors (SDS, EDTA, guanidinium chloride, formamide, Na_2PO_4, or spermidine) with an additional 70% (v/v) ethanol wash following ethanol precipitation.
	If RNase inhibitor is inhibiting the reaction, reduce the quantity of or eliminate the RNase inhibitor.
	For low-abundance targets, increase the quantity of template RNA added to the reaction and/or increase the number of thermal cycles.
	For targets >4 kb, incubate the cDNA synthesis reaction at 42°C instead of 45°C.
	For RNA targets prone to secondary structure formation, increase the temperature of the cDNA synthesis reaction up to 48°C.
	Increase the concentration of the template RNA.
	Optimize the primer concentration, annealing temperature, and/or extension time, varying each individually and in increments.
	Increase the quantity of Easy-A high-fidelity PCR cloning enzyme and/or the extension time to compensate for insufficient primer extension.
	Make sure primers are not self-complementary or complementary to each other
	Verify that the primers are designed to be complementary to the appropriate strands.
	Try a longer primer.
	Verify that the times and temperatures are correct and that the programs for cDNA synthesis and PCR amplification are correctly linked.
	Increase the number of thermal cycles.
	Ensure that the dNTPs have not degraded. Keep the deoxynucleotide mix frozen in aliquots, thaw them quickly, and keep them on ice once thawed; avoid multiple freeze–thaw cycles.
Molecular weight of the amplification product is higher than expected	In some cases, the RNA preparation may be contaminated with genomic DNA. Verify the presence of contaminating DNA by performing RT-PCR in the absence of reverse transcriptase. The RNA may be treated with RNase-free DNase prior to RT-PCR to remove contaminating genomic DNA.
	Treat the RNA preparation with RNase-free DNase I.
	Redesign the PCR primers to anneal to sequences in the exon–exon boundary of the target gene (see *PCR Primer Design*).
Multiple nonspecific amplification products	Increase the annealing temperature.
	Incubate the mixture at 65°C for 5 minutes for annealing (before adding RT).
	Make sure primers are not self-complementary or complementary to each other.
	Try a longer primer.
	Use positive displacement pipets or aerosol-resistant pipet tips to reduce cross-contamination during pipetting; use separate work areas and pipettors for pre- and post-amplification steps; wear gloves and change them often.
	It is possible that multiple target sequences exist in the RNA template. In this case, design new primers.

REFERENCES

1. Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (1990). *PCR Protocols: A Guide to Methods and Applications*. Academic Press, New York.
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3. Rychlik, W., Spencer, W. J. and Rhoads, R. E. (1990) *Nucleic Acids Res* 18(21):6409-12.
4. Wu, D. Y., Ugozzoli, L., Pal, B. K., Qian, J. and Wallace, R. B. (1991) *DNA Cell Biol* 10(3):233-8.

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

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Easy-A One-Tube RT-PCR System

Catalog #600182

QUICK-REFERENCE PROTOCOL

- Combine the following components *in order*:

Component	Volume
RNase-free water	39.5 µl
10× RT-PCR buffer	5.0 µl
Upstream primer (100 ng)	1.0 µl
Downstream primer (100 ng)	1.0 µl
dNTP mix (40 mM)	1.0 µl
Sample RNA	1.0 µl

- Add 1.0 µl of reverse transcriptase (RT).
- Add 0.5 µl of Easy-A High-Fidelity PCR cloning enzyme and vortex gently.
- Run the appropriate thermal-cycling program below:

Thermal cycler	Cycles	Temperature	Duration	
			<1 kb target	>1 kb target
Single or multiple block	1	45°C	15 minutes	30 minutes
	1	95°C	1 minute	1 minute
	40	95°C	30 seconds	30 seconds
		60°C[a]	30 seconds	30 seconds
		68°C	2 minutes	2 minutes/kb
	1	68°C	5 minutes	10 minutes
RoboCycler temperature cycler	1	45°C	15 minutes	30 minutes
	1	95°C	1 minute	1 minute
	40	95°C	1 minute	1 minute
		60°C[a]	1 minute	1 minute
		68°C	2 minutes	2 minutes/kb
	1	68°C	5 minutes	10 minutes

[a] Use the annealing temperature appropriate for the specific primer pair used in the reaction.