AccuScript *PfuUltra* II RT-PCR Kit

INSTRUCTION MANUAL

Catalog #600184

Revision A.01

For In Vitro Use Only

600184-12

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AccuScript *PfuUltra* II RT-PCR Kit

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AccuScript *PfuUltra* II RT-PCR Kit

MATERIALS PROVIDED

Materials provided	Concentration	Quantity[a]
AccuScript High Fidelity RT	—	25 μl
AccuScript RT Reaction Buffer[b]	10×	50 μl
PfuUltra II HS DNA polymerase	—	50 μl
PCR reaction buffer	10×	1 ml
Deoxynucleotide (dNTP) mix	40 mM (10 mM of each dNTP)	100 μl
Oligo(dT) primer (18-mers)	100 ng/μl	3 μg
Random primers (9-mers)	100 ng/μl	3 μg
DTT	100 mM	50 μl
RNase-free water	—	3 × 1.2 ml

[a] Quantities of reagents are sufficient for fifty 10-μl cDNA synthesis reactions.

[b] The 10× AccuScript RT reaction buffer contains 0.5 M Tris-HCl (pH 8.3), 0.75 M KCl, 0.03 M $MgCl_2$.

STORAGE CONDITIONS

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

ADDITIONAL MATERIALS REQUIRED

RNase Block ribonuclease inhibitor (optional, Stratagene Catalog #300151)
Thin-walled PCR tubes (optional)

NOTICE TO PURCHASER

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Revision A.01

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INTRODUCTION

The AccuScript *PfuUltra* II RT-PCR Kit provides a complete system for RT-PCR in applications that demand high fidelity, specificity, and throughput. First, cDNA is synthesized from total or poly(A)$^+$ RNA by Stratagene AccuScript High-Fidelity Reverse Transcriptase* (RT) in a reaction primed with oligo(dT), random primers or gene-specific primers. Then, a portion of the cDNA synthesis reaction is transferred to a new tube and amplified by PCR using *PfuUltra* II Fusion HS DNA Polymerase.** The AccuScript *PfuUltra* II RT-PCR kit can amplify cDNA targets of 0.1–9.6 kb in length synthesized from 10–1000 ng of total RNA or 0.1–10 ng of poly(A)$^+$ RNA.

Reverse transcriptases exhibit significantly higher error rates than other known DNA polymerases, introducing errors at frequencies of one per 1,500 to 30,000 nucleotides during cDNA synthesis.[1] To solve this problem, we developed AccuScript RT, a Moloney murine leukemia virus reverse transcriptase (MMLV-RT) derivative combined with a proofreading 3´-5´ exonuclease.[2] AccuScript RT offers the highest reverse-transcription accuracy while promoting full length cDNA synthesis and superior performance in RT-PCR. AccuScript RT delivers greater than three-fold higher accuracy compared to leading reverse transcriptases, representing a significant advancement in cDNA synthesis accuracy.

PfuUltra II fusion HS DNA polymerase combines fusion polymerase technology with *PfuUltra* DNA polymerase***, hot start antibody, and the ArchaeMaxx polymerase-enhancing factor[3] to achieve extreme accuracy, high specificity, and long target-length capability while dramatically reducing overall PCR extension times.

In applications such as gene cloning, sequencing, and generating error-free clones for protein expression studies, replication fidelity is paramount. The AccuScript *PfuUltra* II RT-PCR kit is ideal for these applications. In addition, the kit provides sensitive and reproducible detection and analysis of RNA molecules. This reduces the number of clones that must be sequenced in order to identify an error-free clone, saving you time and money in downstream sequence verification.

* Patents pending.

** U.S. Patent Nos. 7,045,328, 6,734,293, 6,489,150, 6,444,428, 6,183,997, 5,948,663, 5,866,395, 5,545,552 and patents pending.

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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 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 $OD_{260/280}$ ratio 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 or DEPC-treated water. Although DEPC-treated water may be used for RNA isolation, use the RNase-free water provided, instead of DEPC-treated water, in the cDNA synthesis reaction since DEPC 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.

PfuUltra II fusion HS DNA polymerase has no reverse transcriptase activity under the standard reaction conditions. Therefore, PCR amplification should be entirely dependent on reverse transcription of the RNA template by AccuScript RT. When an RNA template is contaminated with genomic DNA, however, the 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 AccuScript RT is omitted can be performed to ensure that 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

cDNA Synthesis Primers

Oligo$(dT)_{18}$ is recommended for priming polyadenylated RNA and is provided with this kit. Use of oligo(dT) allows the subsequent amplification of products of multiple transcripts from a single first-strand synthesis reaction. Random primers, also provided with this kit, are efficient primers for the detection of multiple short RT-PCR targets. If random primers are used, the first-strand synthesis reaction must be incubated at 25°C for 10 minutes to extend the primers prior to increasing the reaction temperature to 42°C for cDNA synthesis. Gene-specific primers anneal only to defined sequences and are used to synthesize cDNA from particular mRNA transcripts rather than from the entire mRNA population in the sample. Specificity of priming with gene-specific primers may be improved by optimizing annealing and reaction temperatures.

Incubation Temperature and Duration

Denaturation of the RNA template and primer by incubating the reaction at 65°C for 5 minutes is essential.

For RT-PCR applications, AccuScript RT performs optimally at a reaction temperature of 42°C. A 30-minute incubation for the first-strand synthesis reaction is recommended for most targets. For targets <1 kb, a 15-minute incubation is usually sufficient. Rare RNA sequences, long transcripts, or targets at the 5´ end of long transcripts may benefit from a longer incubation (up to 90 minutes).

AccuScript RT Inhibition of PCR

AccuScript RT can inhibit subsequent PCR and must be inactivated by heat treatment at 95°C in the first thermal cycle of PCR. Avoid using more AccuScript RT than the recommended amount. For long RNA targets, it is advisable to increase the incubation time for reverse transcription rather than increasing the amount of AccuScript RT in the reaction.

RNase Inhibitor

RNase Block RNase inhibitor (1 U/10-µl reaction) can be added to the first-strand synthesis reaction following the addition of AccuScript RT. At high concentrations, RNase Block may inhibit the subsequent PCR reaction (do not exceed 10 U/10-µl reaction). If RNase inhibitor is added to the reaction, decrease the volume of water accordingly.

PCR Primer Design

PCR 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.[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 PCR 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.

PCR Amplification

Preparing the Reactions

One microliter of the cDNA synthesis reaction is a sufficient quantity for successful amplification of most targets. For rare cDNA species, optimal results may be achieved using 2–5 μl of cDNA. Because excess salt in the first-strand cDNA synthesis reactions will inhibit the DNA polymerase, use no more than 5 μl of the first-strand cDNA synthesis reaction in the PCR amplification reaction.

After preparing the PCR reactions, transfer them to a preheated thermal cycler (95°C), and immediately start the thermal-cycling program.

Thermal-Cycling Program

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 5°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 step. 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 *PfuUltra* II HS DNA polymerase in RT-PCR is 68°C. The extension time varies with the size of the template. As a starting point, we recommend an extension of time of 30 seconds for targets ≤1 kb and 30 seconds/kb for targets >1 kb. A final extension at 68°C for 5 minutes improves the quality of the final RT-PCR product by extending truncated product to full length.

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.

General Notes

Preparing a Master Mix for Multiple Samples

If running multiple samples, a master mix of components for both reverse transcription and PCR amplification 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.

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.

Mixing and Pipetting Enzymes

Enzymes (including AccuScript RT, the *PfuUltra* II polymerase, and RNase inhibitor) 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.

RT-PCR PROTOCOL

Synthesizing First-Strand cDNA

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

1. Prepare the cDNA synthesis reaction by adding the following components to a microcentrifuge tube *in order*:

   4.9 µl of RNase-free water **(not DEPC-treated water)**
   1.0 µl of 10× AccuScript RT reaction buffer
   0.6 µl of oligo(dT) primer **or** random primers **or** a gene-specific primer (100 ng/µl)
   1.0 µl of dNTP mix (10 mM each dNTP)
   1.0 µl of RNA (*see table below for guidelines on RNA quantity*)

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

2. Incubate the reaction at 65°C for 5 minutes.

3. Cool the reaction at room temperature to allow the primers to anneal to the RNA (approximately 5 minutes).

4. Add 1 µl of 100 mM DTT to the reaction.

5. Add 0.5 µl of AccuScript High-Fidelity RT to the reaction. (The reaction volume is now 10 µl.)

   Notes *As an optional step for protection of RNA during the synthesis reaction, 1 U of RNase Block may also be added to the reaction mixture. If RNase Block is included, decrease the amount of RNase-free water added in step 1 accordingly.*

   To prevent heat inactivation, AccuScript RT and RNase Block (if included) must be added after the reaction has cooled to room temperature following the 65°C incubation.

6. If using random primers, incubate the reaction at 25°C for 10 minutes to extend the primers prior to the 42°C synthesis step. If using oligo(dT) or gene-specific primers, proceed to step 7.

7. Place the tube in a temperature-controlled thermal block at 42°C and incubate the reaction for 30 minutes.

   Note *For targets <1 kb, a 15-minute incubation is usually sufficient. Rare RNA sequences, long transcripts, or targets at the 5′ end of long transcripts may benefit from a longer incubation (up to 90 minutes).*

8. Place the completed first-strand cDNA synthesis reaction on ice for subsequent use in the PCR amplification protocol (see *Amplifying the cDNA Template*). For long-term storage, place the reaction at –20°C.

Amplifying the cDNA Template

Note *Mix each component well then briefly spin in a microcentrifuge before use.*

1. Add the following components *in order* to a sterile thin-walled PCR tube[¤] for each PCR amplification reaction (final volume 50 µl):

   40 µl of RNase-free water
   5 µl of 10× PCR reaction buffer
   1 µl of dNTP mix (10 mM each dNTP)
   1 µl of upstream primer (100 ng/µl)
   1 µl of downstream primer (100 ng/µl)
   1 µl of first-strand cDNA synthesis reaction
   1 µl of *PfuUltra* II HS DNA polymerase

   Note *For most targets, 1 µl of the cDNA synthesis reaction is sufficient for efficient amplification. For rare cDNA species, optimal results may be achieved using 2–5 µl of cDNA.*

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

2. Place the PCR reactions in a thermal cycler, and run the following thermal-cycling program:

Thermal cycler	Cycles	Temperature	Duration
Single or multiple block	1	95°C	1 minute[a]
	40	95°C	30 seconds
		T_m–5°C[b]	30 seconds
		68°C	30 seconds/kb[c]
	1	68°C	5 minutes
RoboCycler temperature cycler	1	95°C	1 minute, 15 seconds
	40	95°C	40 seconds
		T_m–5°C[b]	40 seconds
		68°C	40 seconds/kb[d]
	1	68°C	5 minutes

[a] Longer targets may benefit from a 2-minute initial denaturation time.
[b] Use the annealing temperature appropriate for the specific primer pair used in the reaction.
[c] For targets <1 kb, use a 30-second extension time.
[d] For targets <1 kb, use a 40-second extension time.

Analyzing the PCR Products

Analyze the PCR products by 1.0% (w/v) agarose gel electrophoresis. The products should 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 yield of first-strand cDNA	Verify the integrity of the RNA by denaturing agarose gel electrophoresis to ensure it is not degraded.
	Replace the RNA. Use Stratagene Absolutely RNA or Absolutely mRNA purification kits to isolate intact total RNA or mRNA, respectively.
	Isolate the RNA in the presence of a ribonuclease inhibitor, and ensure that all RT-PCR reagents and labware are free of RNases. Resuspend RNA in DEPC-treated H_20 or elution buffer made with DEPC-treated H_20.
	Reduce the volume of the target RNA or remove RT inhibitors (SDS, EDTA, guanidinium chloride, formamide, Na_2PO_4, or spermidine) during RNA purification with an additional 70% (v/v) ethanol wash following ethanol precipitation.
	In some cases RNase Block ribonuclease inhibitor can inhibit the cDNA synthesis reaction. Reduce or eliminate the RNase Block.
	Increase the length of the 42°C cDNA synthesis reaction to 90 minutes to allow for the synthesis of cDNA from rare or long RNA targets.
	Increase the concentration of the template RNA.
	Add the AccuScript RT after the reactions have cooled to room-temperature following the 65°C denaturation step, and synthesize cDNA at 42°C.
	Confirm that the cDNA synthesis primer is complementary to the target sequence; change the primer type [oligo(dT), gene-specific, or random primers].
	If using random primers, incubate the reaction at 25°C for 10 minutes prior to increasing the temperature to 42°C for cDNA synthesis. This allows better annealing of random primers to RNA.
	In the case of eukaryotic RNA, use oligo(dT) primer.
No or low yield of amplification product	See the discussion under *No or low yield of the first-strand cDNA* for suggestions related to insufficient first strand synthesis.
	Add more cDNA synthesis product to the PCR (up to 5 μl).
	Optimize the annealing temperature, and/or extension time, varying each individually and in increments.
	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 thermocycler is programmed with the correct times and temperatures.
	Increase the number of thermal cycles.
	To maintain adequate quality of dNTPs, keep nucleotides frozen in aliquots, thaw them quickly, and keep them on ice once thawed; avoid multiple freeze–thaw cycles.

(Table continues on the next page)

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Observation	Suggestion
Molecular weight of the amplification product is higher than expected	The RNA preparation may be contaminated with genomic DNA. Verify the presence of contaminating DNA by performing RT-PCR in the absence of AccuScript RT.
	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 to reduce nonspecific amplification.
	Make sure primers are not self-complementary or complementary to each other.
	Try a longer primer.
	To reduce contamination of the reaction with DNA or RNA other than the target, 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.
	Multiple target sequences may exist in the RNA template. In this case, design new primers.

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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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AccuScript *PfuUltra* II RT-PCR Kit

Catalog #600184

QUICK-REFERENCE PROTOCOL

cDNA Synthesis

- Add the following reagents, in order, to a microcentrifuge tube:

Reagent	Volume (for 10-μl reaction)
RNase-free water	4.9 μl
10× AccuScript RT reaction buffer	1.0 μl
Primer	0.6 μl of oligo(dT) primer OR random primer OR gene-specific primer (100 ng/μl)
dNTP mix (10 mM each dNTP)	1.0 μl
Sample RNA	1.0 μl

- Incubate the reaction at 65°C for 5 minutes, then cool to room temperature.

- Add DTT and AccuScript RT as specified:

Reagent	Volume (for 10-μl reaction)
100 mM DTT	1.0 μl
AccuScript high-fidelity RT	0.5 μl

- If using random primers, incubate the reaction at 25°C for 10 minutes to allow primer extension prior to completing the following step.

- Incubate the reaction at 42°C for 30 minutes.

- Place the reaction on ice for subsequent PCR amplification. For long-term storage, place the reaction at –20°C.

PCR Amplification

- Combine the following reagents in order (50 µl final reaction volume):

Reagent	Volume
RNase-free water	40 µl
10× PCR reaction buffer	5 µl
dNTP mix	1 µl
Upstream primer	1 µl (100 ng/µl)
Downstream primer	1 µl (100 ng/µl)
First-strand cDNA synthesis reaction	1 µl
PfuUltra II HS DNA polymerase	1 µl

- Run the following thermal-cycling program:

Thermal cycler	Cycles	Temperature	Duration
Single or multiple block	1	95°C	1 minute
	40	95°C	30 seconds
		T_m–5°C	30 seconds
		68°C	30 seconds/kb
	1	68°C	5 minutes
RoboCycler temperature cycler	1	95°C	1 minute, 15 seconds
	40	95°C	40 seconds
		T_m–5°C	40 seconds
		68°C	40 seconds/kb
	1	68°C	5 minutes