AccuScript High Fidelity RT-PCR System

Instruction Manual

Catalog #600180

Revision F0

Laboratory Reagent.

600180-12

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AccuScript High Fidelity RT-PCR System

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MATERIALS PROVIDED

Materials provided	Concentration	Quantity[a]
AccuScript High Fidelity RT	—	50 reactions
AccuScript RT Buffer[b]	10×	100 µl
PfuUltra HF DNA polymerase	2.5 U/µl	125 U
PCR 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	100 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 buffer contains 0.5 M Tris-HCl (pH 8.3), 0.75 M KCl, 0.03 M $MgCl_2$.

STORAGE CONDITIONS

All Materials: –20°C

ADDITIONAL MATERIALS REQUIRED

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

Revision F0

INTRODUCTION

The AccuScript high fidelity RT-PCR system features the highest-fidelity reverse transcriptase available today. The AccuScript high fidelity RT-PCR system can amplify cDNA 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 reverse transcriptase, a Moloney murine leukemia virus reverse transcriptase (MMLV-RT) derivative combined with a proofreading 3'-5' exonuclease.[2] AccuScript reverse transcriptase delivers the highest reverse-transcription accuracy while promoting full length cDNA synthesis and superior performance in RT-PCR. AccuScript reverse transcriptase delivers greater than three-fold higher accuracy compared to leading reverse transcriptases, representing a significant advancement in cDNA synthesis accuracy.

When performing RT-PCR, first, cDNA is synthesized from total or poly(A)$^+$ RNA by AccuScript RT in a reaction primed with oligo(dT) or random primers or gene-specific primers. Second, a portion of the cDNA synthesis reaction is transferred to a new tube and then amplified by PCR using *PfuUltra* high fidelity (HF) DNA polymerase, which delivers the highest accuracy of any DNA polymerase on the market today. *PfuUltra* HF DNA polymerase is a special formulation containing a genetically engineered mutant of *Pfu* DNA polymerase and the ArchaeMaxx polymerase-enhancing factor.[3] The accuracy of *PfuUltra* HF DNA polymerase is 18 times that of *Taq* DNA polymerase and three to six times that of DNA polymerase blends.

In applications such as gene cloning, sequencing, and ensuring that error-free clones are used in protein expression studies, replication fidelity is paramount. The AccuScript high fidelity RT-PCR system is ideal for these applications. In addition, the AccuScript high fidelity RT-PCR system 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.

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 Agilent's 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 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, as the water component in the cDNA synthesis reaction since DEPC can inhibit PCR.) Use of an RNase inhibitor, such as Agilent RNase Block Ribonuclease Inhibitor, is recommended when isolating RNA from samples high in RNase activity.

PfuUltra HF 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*).

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 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.

Mixing and Pipetting Enzymes

Enzymes (including AccuScript RT, *PfuUltra* HF DNA 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.

cDNA Synthesis Primers

Oligo$(dT)_{18}$ is recommended for priming polyadenylated RNA and is provided with this kit. Use of Oligo$(dT)_{18}$ allows the subsequent amplification of products of multiple transcripts from a single first-strand synthesis reaction. Random 9-mers, also provided with this kit, are efficient primers for the detection of multiple short RT-PCR targets. If random 9-mers 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.

cDNA Synthesis Reaction

Incubation Temperature and Duration

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

AccuScript RT is effective between 37 and 42°C. A 30-minute incubation for the first-strand synthesis reaction is sufficient for most targets in RT-PCR applications. Rare RNA sequences, long transcripts, or targets at the 5′ end of long transcripts benefit from a longer incubation at 42°C (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

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] The following formula[5] 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.[6, 7] 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 efficient amplification of most targets. Excess salt in the first-strand cDNA synthesis reactions will inhibit the DNA polymerase; therefore use up to (but not more than) 5 µl of the first-strand cDNA synthesis reaction in any subsequent 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* HF DNA polymerase in RT-PCR is 68°C. The extension time varies with the size of the template. A reasonable starting point is 3 minute/kb. A 10-minute final extension at 68°C improves the quality of the final RT-PCR product by extending truncated products 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.

The thermal cycling program provided in the *RT-PCR Protocol* section is specialized for RT-PCR using *PfuUltra* HF DNA polymerase. This thermal cycling program generally yields superior RT-PCR results compared to the cycling program provided with *PfuUltra* HF DNA polymerase when purchased separately, which is optimized for general PCR applications.

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*:

   4.4 µl of RNase-free water **(not DEPC-treated water)**
   1.0 µl of 10× AccuScript RT 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. The quantity of RNA depends on the RNA purity, message abundance, and size of the target:

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 1.0 µl of AccuScript 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.

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

Notes *Mix and spin each component 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 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 reaction
   1 µl of *PfuUltra* HF DNA polymerase (2.5 U/µl)

   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.*

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

Cycles	Temperature	Duration
1	95°C	1 minute
40	95°C	30 seconds
	T_m–5°C[a]	30 seconds
	68°C	3 minutes/kb[b]
1	68°C	10 minutes

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

[b] For targets <1 kb, use a 3-minute 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 a StrataPrep PCR purification kit (Agilent catalog #400771).

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 Agilent 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.
	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.
	In some cases RNase Block ribonuclease inhibitor can inhibit the cDNA 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].
	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.
	Increase the quantity of *PfuUltra* HF DNA polymerase and/or the extension time.
	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.
	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 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

Material Safety Data Sheets (MSDSs) are provided online at *www.agilent.com*. MSDS documents are not included with product shipments.

AccuScript High Fidelity RT-PCR System

Catalog #600180

QUICK-REFERENCE PROTOCOL

cDNA Synthesis

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

Reagent	Volume (for 10-µl reaction)
RNase-free water	4.4 µl
10× AccuScript RT 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 RT	1.0 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.

PCR Amplification

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

Reagent	Volume
RNase-free water	40 µl
10× PCR buffer	5 µl
dNTP mix	1 µl
Upstream primer	1 µl
Downstream primer	1 µl
First-strand cDNA synthesis product	1 µl
PfuUltra HF DNA polymerase	1 µl

- Run the following thermal-cycling program:

Cycles	Temperature	Duration
1	95°C	1 minute
40	95°C	30 seconds
	T_m–5°C	30 seconds
	68°C	3 minutes/kb
1	68°C	10 minutes