AccuScript High Fidelity 1st Strand cDNA Synthesis Kit

INSTRUCTION MANUAL

Catalog #200820

Revision C.0

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AccuScript High Fidelity 1st Strand cDNA Synthesis Kit

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MATERIALS PROVIDED

Materials provided	Concentration	Quantity[a]
AccuScript High Fidelity RT	—	50 reactions
AccuScript RT buffer[b]	10×	100 µl
Oligo(dT) primer (18-mers)	0.5 µg/µl	25 µg
Random primers (9-mers)	0.1 µg/µl	15 µg
100 mM dNTP Mix	25 mM each dNTP	40 µl
100 mM DTT	100 mM	100 µl
RNase-free water	—	1.2 ml
RNase Block	40 U/µl	1000 U

[a] The AccuScript high fidelity 1st strand cDNA synthesis kit provides enough reagents for 50 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 Reagents: –20°C

ADDITIONAL MATERIALS REQUIRED

PfuUltra DNA polymerase & reaction buffer (optional)
Thin-walled PCR tubes (optional)

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INTRODUCTION

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. Additionally, the AccuScript high fidelity 1st strand cDNA synthesis kit applies this superior performance to the amplification of specific complementary DNA (cDNA) fragments from limited amounts of RNA using a reverse transcriptase-mediated polymerase chain reaction (RT-PCR) which results in high yield, full length cDNA. The first-strand synthesis protocol generates a heterogeneous population of cDNA molecules from all available poly(A)+ mRNA or total RNA, while subsequent amplification with sequence-specific primers yields a homogeneous population of the specific cDNA of interest, eliminating the need for amplification and screening of a cDNA library. Resulting populations of cDNA can be used for microarray, conventional and real time PCR amplification.

The AccuScript high fidelity 1st strand cDNA synthesis kit delivers robust first strand cDNA, superior RT-PCR yields from low RNA input amounts and exceptional full-length cDNA capability—making it the perfect choice for any application requiring premium first strand cDNA.

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 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 Stratagene RNase Block Ribonuclease Inhibitor, is recommended when isolating RNA from samples high in RNase activity.

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 60-minute incubation for the first-strand synthesis reaction is recommended for most targets. A shorter incubation time (15–30 minutes) may be sufficient for some targets and 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 is inactivated by incubation at 70°C after the cDNA synthesis reaction is complete. 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

We recommend adding RNase Block RNase inhibitor (20 U per 20-µl reaction) to the first-strand synthesis reaction, as specified in the following protocol. The use of RNase inhibitor at higher concentrations may reduce product yield. If RNase inhibitor is omitted from the reaction, increase the volume of water accordingly.

THE REVERSE TRANSCRIPTASE-MEDIATED POLYMERASE CHAIN REACTION PROTOCOL

Note *Wear gloves at all times during the first-strand cDNA synthesis and PCR amplification procedures and while handling materials and equipment to prevent contamination by ribonucleases (RNases).*

Synthesis of First-Strand cDNA Using Reverse Transcriptase

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*:

   RNase-free water to total volume 16.5 µl
   2.0 µl of AccuScript RT Buffer (10×)
   1.0 µl of a gene-specific primer (0.1 µg /µl) OR oligo(dT) primer (0.5 µg/µl) OR 3 µl of random primers (0.1 µg/µl)
   0.8 µl of dNTP mix (25 mM each dNTP)
   X 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	200–5000 ng
mRNA (all targets)	0.1–100 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 the following components to the reaction, in order, for a final reaction volume of 20 µl:

   2 µl of 100 mM DTT
   1 µl of AccuScript RT
   0.5 µl of RNase Block ribonuclease inhibitor (40 U/ µl)

   Note *To prevent heat inactivation, AccuScript RT and RNase Block must be added after the reactions have cooled to room temperature following the 65°C incubation.*

5. 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 6.

6. Place the tube in a temperature-controlled thermal block at 42°C and incubate the reaction for 60 minutes.

7. Terminate cDNA synthesis by incubating the reaction at 70ºC for 15 minutes.

8. Place the completed first-strand cDNA synthesis reaction on ice for use in downstream applications. If performing RT-PCR, proceed to the PCR amplification protocol (see *Amplification of First-Strand cDNA Using the Polymerase Chain Reaction*). For long-term storage, place the reaction at –20°C.

Amplification of First-Strand cDNA using the Polymerase Chain Reaction

Notes *cDNA synthesis reaction products should be stored at –20°C until needed.*

Mix and spin each component in a microcentrifuge before use.

A protocol for amplification using Stratagene high-fidelity PfuUltra *DNA polymerase is detailed below. This protocol could be adapted to other PCR enzymes. Amplification with a high-fidelity PCR enzyme is strongly recommended, however, in order to realize the benefits of high-fidelity reverse transcription provided by AccuScript RT.*

Amplification using *PfuUltra* DNA Polymerase

1. Add the following components *in order* to sterile thin-walled PCR tubes[¤] for each PCR amplification reaction:

   37.6 µl of RNase-free water
   5 µl of 10× *PfuUltra* PCR buffer
   2 µl of 25 mM $MgSO_4$[§]
   0.4 µl of dNTP mix (25 mM each dNTP)
   1 µl of upstream primer (0.1 µg/µl)
   1 µl of downstream primer (0.1 µg/µl)
   2 µl of experimental first-strand cDNA reaction
   1 µl of *PfuUltra* DNA polymerase (2.5 U/µl)

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

[§] The *PfuUltra* reaction buffer (supplied with the enzyme) contains $MgSO_4$ at a final concentration of 2 mM. For amplification of cDNA using this enzyme, the reaction mixture should be supplemented with $MgSO_4$ to a final concentration of 3 mM.

[¤] Thin-walled 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.

3. Place the amplification reactions in a thermal cycler, and run the thermal-cycling program below.

PCR Cycling Program for *PfuUltra* DNA Polymerase in RT-PCR

Thermal cycler	Cycles	Temperature	Duration
Single or multiple block	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
RoboCycler temperature cycler	1	95°C	1 minute
	40	95°C	1 minute
		T_m–5°C[a]	1 minute
		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.

4. Analyze the PCR products by loading 10 µl of each PCR amplification reaction (taken from below the mineral oil layer) into separate lanes of a 0.8% (w/v) agarose gel.

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.
	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 the oligo(dT) primer.

REFERENCES

1. Roberts, J. D., Bebenek, K. and Kunkel, T. A. (1988) *Science* 242:1171-1173.
2. Arezi, B. and Hogrefe, H. H. (2007) *Anal Biochem* 360(1):84-91.

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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Catalog #200820

QUICK-REFERENCE PROTOCOL

cDNA Synthesis

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

Reagent	Volume (for 20 µl final reaction volume)
RNase-free water	To final volume of 16.5 µl
10 × AccuScript RT buffer	2.0 µl
Primer	1 µl gene-specific primer (0.1 µg/µl) OR 1 µl oligo(dT) primer (0.5 µg/µl) OR 3 µl random primers (0.1 µg/µl)
dNTP mix (25 mM each)	0.8 µl
Sample RNA	X µl

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

- Add the following components, in order:

  2 µl of 100 mM DTT
  1 µl of AccuScript RT
  0.5 µl of RNase Block

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

- Incubate the reaction at 42°C for 60 minutes.

- Terminate cDNA synthesis by incubating the reaction at 70°C for 15 minutes. Place the reaction on ice for use in downstream applications or at –20°C for long-term storage.