SuperScript™ IV First-Strand Synthesis System

USER GUIDE Pub. No. MAN0013442 Rev. E.0

invitrogen

Contents and storage

Reagents provided are sufficient for 50 or 200 reverse transcription reactions of 20 µL each. **Store at –20°C (non-frost-free)**.

Cat. No.	18091050, 18091150	18091200, 18091300
Component	**50 reactions**	**200 reactions**
SuperScript™ IV Reverse Transcriptase (200 U/µL)	50 µL	4 × 50 µL
5× SSIV Buffer	100 µL	4 × 100 µL
0.1 m DTT	250 µL	4 × 250 µL
Oligo(dT)$_{20}$ primer (50 µM)	50 µL	4 × 50 µL
Random hexamers (50 ng/µL)	250 µL	4 × 250 µL
10 mM dNTP mix	250 µL	4 × 250 µL
Ribonuclease Inhibitor (40 U/µL)	100 µL	4 × 100 µL
E. coli Ribonuclease H (RNase H) (2 U/µL)	50 µL	4 × 50 µL
Total HeLa RNA (10 ng/µL)	20 µL	4 × 20 µL
Sense Control Primer (10 µM)	25 µL	4 × 250 µL
Antisense Control Primer (10 µM)	25 µL	4 × 25 µL
DEPC-treated water	1.2 mL	4 × 1.2 mL

The following reagents are provided only with SuperScript™ IV First-Strand Synthesis System with ezDNase™ Enzyme.

Cat. No.	18091150	18091300
Component	**50 reactions**	**200 reactions**
ezDNase™ Enzyme	50 µL	4 × 50 µL
10× ezDNase™ Buffer	100 µL	4 × 100 µL
Nuclease-free water	1.25 mL	4 × 1.25 mL

Product description

For first strand cDNA synthesis using total RNA or poly(A)+-selected RNA primed with oligo(dT), random primers, or a gene-specific primer.

Required materials

- Template: RNA
- *(Optional)* 2 µM gene-specific primers

Important guidelines

Pre-warm the 5× SSIV Buffer to room temperature before use. Vortex and briefly centrifuge the buffer prior to preparing the reverse transcription reaction mix.

For Research Use Only. Not for use in diagnostic procedures.

Optional materials

For PCR:

Recommended Polymerases:

AmpliTaq™ DNA Polymerase (Cat. No. N808-0160), AmpliTaq™ Gold DNA Polymerase (Cat. No. 4311806), or Platinum™ *Taq* DNA Polymerase (Cat. No. 19066018)

Other Requirements:

- Template: cDNA generated by RT step
- 10 mM dNTP mix (Cat. No. 18427-088)
- Forward and reverse gene-specific primers
- Autoclaved, distilled water
- 0.2-mL or 0.5-mL nuclease-free microcentrifuge tubes
- E-Gel™ General Purpose Gels, 1.2% (Cat. No. G5018-01)
- TrackIt™ 1 Kb Plus DNA Ladder (Cat. No. 10488-085)

For qPCR:

Recommended Mixes:

TaqMan™ Gene Expression Master Mix (Cat. No. 4369016) or EXPRESS qPCR SuperMix, Universal (Cat. No. 11785-200)

Other Requirements:

- Template: cDNA generated by RT step
- 20X Gene-specific fluorescent primers or primer/probe combinations
- Normalization dye for instruments that do not use ROX
- 0.2-mL or 0.5-mL nuclease-free microcentrifuge tubes or MicroAmp™ EnduraPlate™ Optical 96-well or 384-well Plate (Cat. No. 4483354 or 4483285)
- Autoclaved, distilled water
- Real-time instrument

Online resources

Visit our product page for additional information on PCR enzymes and master mixes, RT enzymes and kits, real-time PCR instruments, real-time PCR master mixes, PCR thermal cyclers, and protocols.

For support, visit thermofisher.com/support.

Thermo Fisher Scientific Baltics UAB | V. A. Graiciuno 8 | LT-02241 Vilnius, Lithuania

ThermoFisher SCIENTIFIC

Optional: Remove genomic DNA

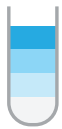
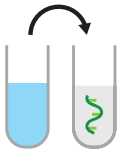
The following example procedure describes the required to remove the genomic DNA (gDNA) from your samples for the RT and No RT Control reactions using the ezDNase™ Enzyme (supplied with Cat. No. 18091150 and 18091300; also available separately at **thermofisher.com**).

The volumes given are for a single **10-µL** gDNA digestion reaction . For multiple reactions, prepare a master mix of components common to all reactions to minimize pipetting error, then dispense appropriate volumes into each reaction.

<table>
<tr><th colspan="2">Step</th><th>Action</th></tr>
<tr><td>1</td><td>  </td><td>Prepare gDNA digestion reaction mix (on ice)</td><td>For each RT reaction and No RT Control reaction, prepare a 10-µL gDNA reaction mix in an RNase-free tube on ice with the following components:
<table>
<tr><th>Component</th><th>Volume</th></tr>
<tr><td>10× ezDNase™ Buffer</td><td>1 µL</td></tr>
<tr><td>ezDNase™ Enzyme</td><td>1 µL</td></tr>
<tr><td>Template RNA (1 pg to 2.5 µg total RNA)</td><td>up to 8 µL</td></tr>
<tr><td>Nuclease-free water</td><td>to 10 µL</td></tr>
</table></td></tr>
<tr><td>2</td><td>  </td><td>Digest gDNA</td><td>Gently mix and incubate at 37°C for 2 minutes.</td></tr>
<tr><td>3</td><td>  </td><td>Inactivate ezDNase™ enzyme</td><td>If the RNA sample is to be used for RT-PCR of fragments ≥3 kb, incubate the sample for 5 minutes at 55°C in the presence of 10 mM DTT to inactivate the enzyme.</td></tr>
<tr><td>4</td><td>  </td><td>Place the reaction on ice</td><td>Chill the tube on ice to bring the sample to room temperature, then briefly centrifuge and place the tube on ice.</td></tr>
</table>

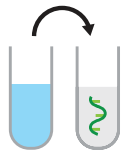
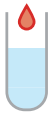
SuperScript™ IV first-strand cDNA synthesis reaction

The following example procedure shows appropriate volumes for a single **20-µL** reverse transcription reaction. For multiple reactions, prepare a master mix of components common to all reactions to minimize pipetting error, then dispense appropriate volumes into each reaction tube prior to adding annealed template RNA and primers.

<table>
<tr><th colspan="2">Step</th><th>Action</th></tr>
<tr><td>1</td><td>  </td><td>Denature template RNA and anneal primers</td><td>a. Add the following components in a PCR reaction tube.
Note: Account for the volumes of all components in steps 1 and 2 to determine the correct amount of water required to reach the final reaction volume.
<table><tr><th>Component</th><th>Volume</th></tr><tr><td>50 µM Oligo d(T)$_{20}$ primer, 50 ng/µL random hexamers, or 2 µM gene-specific reverse primer</td><td>1 µL</td></tr><tr><td>10 mM dNTP mix (10 mM each)</td><td>1 µL</td></tr><tr><td>Template RNA (10 pg–5 µg total RNA or 10 pg–500 ng mRNA)</td><td>up to 11 µL</td></tr><tr><td>DEPC-treated water</td><td>to 13 µL</td></tr></table>b. Mix and briefly centrifuge the components.
c. Heat the RNA-primer mix at 65°C for 5 minutes, and then incubate on ice for at least 1 minute.</td></tr>
<tr><td>2</td><td>  </td><td>Prepare RT reaction mix</td><td>a. Vortex and briefly centrifuge the 5× SSIV Buffer.
b. Add the following components in a reaction tube.
<table><tr><th>Component</th><th>Volume</th></tr><tr><td>5× SSIV Buffer</td><td>4 µL</td></tr><tr><td>100 mM DTT</td><td>1 µL</td></tr><tr><td>Ribonuclease Inhibitor</td><td>1 µL</td></tr><tr><td>SuperScript™ IV Reverse Transcriptase (200 U/µL)</td><td>1 µL</td></tr></table>c. Cap the tube, mix, and then briefly centrifuge the contents.</td></tr>
<tr><td>3</td><td>  </td><td>Combine annealed RNA and RT reaction mix</td><td>Add RT reaction mix to the RNA-primer mix from step 1.</td></tr>
<tr><td>4</td><td>  </td><td>Incubate reactions</td><td>a. For random hexamers, incubate the combined reaction mix at 23°C for 10 minutes, then go to step b.
For oligo d(T)$_{20}$ or gene-specific primer, directly proceed to step b.
b. Incubate the combined reaction mixture at 50–55°C for 10 minutes.
c. Inactivate the reaction by incubating it at 80°C for 10 minutes.</td></tr>
<tr><td>5</td><td>  </td><td>(Optional) Remove RNA</td><td>Note: Amplification of some PCR targets (>1 kb) may require removal of RNA.
To remove RNA, add 1 µL E. coli RNase H, and incubate 37°C for 20 minutes.</td></tr>
<tr><td>6</td><td>  </td><td>PCR amplification</td><td>Use your RT reaction immediately for PCR amplification or store it at –20°C.
Note: As a recommended starting point for PCR, reverse transcription reaction (cDNA) should compose 10% of the total reaction volume</td></tr>
</table>

SuperScript™ IV control reactions - cDNA synthesis reaction

Follow the procedure below to perform the cDNA synthesis step of the SuperScript™ IV RT-PCR control reactions.

<table>
<tr><th colspan="2">Step</th><th>Action</th></tr>
<tr><td>1</td><td>  </td><td>Denature template RNA</td><td>a. Add the following components in each of two PCR reaction tubes.
<table>
<tr><th>Component</th><th>Volume</th></tr>
<tr><td>50 µM Oligo d(T)$_{20}$ primer</td><td>1 µL</td></tr>
<tr><td>10 mM dNTP mix (10 mM each)</td><td>1 µL</td></tr>
<tr><td>10 ng/µL total HeLa RNA (10 ng total)</td><td>1 µL</td></tr>
<tr><td>DEPC-treated water</td><td>10 µL</td></tr>
</table>
b. Mix and briefly centrifuge the components.
c. Heat the RNA-primer mix at 65°C for 5 minutes, and then incubate on ice for at least 1 minute.</td></tr>
<tr><td>2</td><td>  </td><td>Prepare RT reaction mix</td><td>a. Vortex and briefly centrifuge the 5× SSIV Buffer.
b. Prepare two different control reactions. In each reaction tube, add the following:
<table>
<tr><th>Component</th><th>Volume</th></tr>
<tr><td>5× SSIV Buffer</td><td>4 µL</td></tr>
<tr><td>100 mM DTT</td><td>1 µL</td></tr>
<tr><td>Ribonuclease Inhibitor</td><td>1 µL</td></tr>
<tr><td>SuperScript™ IV Reverse Transcriptase (positive control) OR DEPC-treated water (no RT control)</td><td>1 µL</td></tr>
</table>
c. Cap the tube, mix, and then briefly centrifuge the contents.</td></tr>
<tr><td>3</td><td>  </td><td>Combine denatured RNA and RT reaction mix</td><td>Add RT reaction mix to the RNA-primer mix from step 1 (one tube for the positive control reaction, one tube for the no RT control reaction).</td></tr>
<tr><td>4</td><td>  </td><td>Incubate reactions</td><td>a. Incubate the combined reaction mixture at 50°C for 10 minutes.
b. Inactivate the reaction by incubating it at 80°C for 10 minutes.</td></tr>
<tr><td>5</td><td>  </td><td>Remove RNA</td><td>a. Add 1 µL E. coli RNase H and incubate 37°C for 20 minutes.
b. Proceed to PCR amplification (page 5)</td></tr>
</table>

SuperScript™ IV control reactions - PCR amplification

Follow the procedure below to perform the PCR amplification step of the SuperScript™ IV RT-PCR control reactions..

The example procedure below shows the appropriate volumes for a single **50-µL** reaction. For multiple reactions, prepare a master mix of components common to all reactions to minimize pipetting error, then dispense the appropriate volumes into each 0.2-mL or 0.5-mL PCR tube before adding template DNA and primers.

<table>
<tr><th colspan="2">Step</th><th>Action</th></tr>
<tr><td>1</td><td>  </td><td>Assemble PCR amplification mix</td><td>a. Prepare two reactions. In each tube, combine the following:
<table>
<tr><th>Component</th><th>Volume</th></tr>
<tr><td>DEPC-treated water</td><td>37.8 µL</td></tr>
<tr><td>10× High Fidelity PCR Buffer</td><td>5 µL</td></tr>
<tr><td>50 mM $MgSO_4$</td><td>2 µL</td></tr>
<tr><td>10 mM dNTP mix (10 mM each)</td><td>1 µL</td></tr>
<tr><td>Control sense primer (10 µM)
(5′-GCTCGTCGTCGACAACGGCTC-3′)</td><td>1 µL</td></tr>
<tr><td>Control antisense primer (10 µM)
(5′-CAAACATGATCTGGGTCATCTTCTC-3′)</td><td>1 µL</td></tr>
<tr><td>cDNA from positive control reaction (step 5, page 4) or
DEPC-treated water for no RT control</td><td>2 µL</td></tr>
<tr><td>Platinum™ Taq DNA Polymerase High Fidelity (5 U/µL)</td><td>0.2 µL</td></tr>
</table>
b. Mix gently by pipetting up and down and briefly centrifuge the components.</td></tr>
<tr><td>2</td><td>  </td><td>Incubate reactions in a thermal cycler</td><td>a. Place reaction mixture in preheated (94°C) thermal cycler.
b. Perform PCR amplification using the following cycling parameters:
<table>
<tr><th colspan="2">Step</th><th>Temperature</th><th>Time</th></tr>
<tr><td colspan="2">Initial denaturation</td><td>94°C</td><td>2 minutes</td></tr>
<tr><td rowspan="3">35 PCR cycles</td><td>Denature</td><td>94°C</td><td>15 seconds</td></tr>
<tr><td>Anneal</td><td>55°C</td><td>30 seconds</td></tr>
<tr><td>Extend</td><td>68°C</td><td>1 minute</td></tr>
<tr><td colspan="2">Hold</td><td>4°C</td><td>hold</td></tr>
</table></td></tr>
<tr><td>3</td><td>  </td><td>Analyze with gel electrophoresis</td><td>Analyze 10 µL of each reaction using agarose gel electrophoresis and ethidium bromide staining.
A 353-bp band should be visible for the positive control reaction with RT.</td></tr>
</table>

Troubleshooting

Observation	Possible cause	Probable solution
No bands after analysis of amplified products	Procedural error in first-strand cDNA synthesis	Use high quality RNA, such as Cervical Adenocarcinoma (HeLa-S3) Total RNA (Cat. No. AM7852), as a control to verify the efficiency of the first-strand reaction.
	RT inhibitors present in RNA	In samples with inhibitor, addition of less input RNA and/or input cDNA generally improves cDNA synthesis and/or PCR. • Perform reverse transcription reaction varying the amount of SuperScript™ IV Reverse Transcriptase from 10–40 U/µL. • Perform serial dilution of RNA input in the reverse transcription reaction. • Increase reverse transcription time to 50 minutes. • Perform serial dilution of cDNA input into the PCR reaction.
	Low copy and degraded RNA samples	• Troubleshoot using gene-specific primers, random hexamers, or a combination of oligo(dT) and random hexamers. • Increase reverse transcription time to 50 minutes.
	Target RNA contains strong secondary structure	• Increase RT reaction temperature. SuperScript™ IV Reverse Transcriptase has activity up to 65°C. • Increase RT reaction temperature stepwise from 55–65°C. • Use random hexamers or combination of oligo(dT) and random hexamers in RT reaction.
Unexpected bands after analysis of amplified products	Genomic DNA present in RNA sample	General good laboratory practice includes performing RT reaction in the absence of RT enzyme to access the amount of genomic DNA in the RNA sample. • Pretreat RNA with DNase I, Amplification Grade (Cat. No. 18068-015), as described in the DNase I documentation. • Design primers that anneal to sequence in exons on both sides of an intron or at the exon/exon boundary of the mRNA to differentiate between amplified cDNA and potential contaminating genomic DNA.
	Non-specific annealing of primers	• Use hot start PCR enzyme. • Optimize PCR annealing temperature. • Optimize magnesium concentration. • Increase RT reaction temperature from 55–65°C, if using a gene-specific RT primer.
Cloudiness and/or white precipitate in RT reaction	Evaporation of RT reaction	Evaporation of RT reaction may cause cloudiness or a white precipitate in the reaction. This has no effect on the performance of the RT reaction. However, ensure tubes and/or plates are sealed tightly to prevent evaporation. Best RT results also occur when there is no condensation that can be prevented by shorter incubation time or incubation in a thermal cycler using a heated lid. Downstream analysis of RT reactions is more reproducible if there is no variation in evaporation and/or condensation.

Optimization strategies

Primer design

- Design 18- to 30-mers with a similar primer melting temperature (T_m) (55–65°C).
- Avoid internal secondary structures.
- Avoid self-complementary primers and pairs complementary at 3' ends.
- Ensure a balanced distribution of AT and GC-rich domains.
- Design the primer with a 3' GC clamp.
- Primers for GC-rich content templates should have a similar GC content to that of the target sequence and a T_m of at least 70°C, as calculated using the nearest neighbor algorithms.
- Primer design programs are available online.
- Optimal final primer concentrations range from 0.1–0.5 µM each. Start with 0.2 µM.

Template source

cDNA: Use 2 µL of the first-strand reaction for PCR. Increasing the amount of first-strand reaction up to 10 µL in PCR may result in increased product yield for some targets.

Genomic DNA: High quality, purified template is required. Use 1 ng to 1 µg in PCR.

Viral orλ DNA: High quality, purified template is required. Use 1 pg to 1 ng of template in PCR. Use higher DNA concentrations when fewer cycles are required.

Optimization for higher PCR product yields and specificity

[Mg]: Allows polymerase to function.

Vary final concentration from 1.0–4.0 mM.

Annealing temperature: Affects product yield and specificity.

- Start with an annealing temperature that is 5°C lower than your primer T_m.
- Estimate the correct annealing temperature using a T_m calculator.
- Use a gradient thermal cycler, such as the Applied Biosystems™ ProFlex™ PCR System or Veriti™ Thermal Cycler, to determine the optimal annealing temperature for your amplicon.

Cycling parameters: Affects product yield and specificity.

- Vary total PCR cycles from 20–40; 35 cycles is typical.
- Lengthen extension times from 1 min/kb to 1.5 min/kb for difficult templates.

If desired, use touchdown PCR to increase specificity, starting with an annealing temperature higher than the primer T_m in early cycles, which then decreases 1°C every cycle until the desired temperature is reached.

GC-rich or problematic targets: PCR optimization can include adjusting the incubation times and temperature, magnesium, primers, dNTP, *Taq* DNA polymerase, and/or DNA template, and potentially, altering the design of multiple primer sets.

- GC-rich or problematic targets work better with $MgSO_4$ instead of $MgCl_2$.
- Consider using Platinum™ *Taq* PCRx DNA Polymerase.
- Test your target by varying the enhancer solution from 0.5–4X. The co-solvent and amplification buffer offer higher primer specificity, broader [Mg] and annealing temperature optima, as well as improved *Taq* thermostabilization.
- Use 2.5 U of Platinum™ *Taq* for each 50-µL reaction.
- Denature at 95°C for 45 seconds.

RNA sample guidelines

RNA sample preparation guidelines

- To isolate total RNA, we recommend the TRIzol™ Reagent or the PureLink™ 96 Total RNA Purification Kit. Isolation of mRNA is typically not necessary, although incorporating this step may improve the yield of specific cDNAs.
- High-quality, intact RNA is essential for accurate quantification in qRT-PCR.
- DNase I, Amplification Grade, may be used to eliminate genomic DNA contamination from the total RNA.

General handling of RNA

- Use proper microbiological aseptic technique.
- Wear latex gloves while handling reagents, materials, and RNA samples to prevent RNase contamination.
- Use disposable, individually wrapped, sterile plasticware for all procedures.
- Use aerosol-resistant pipette tips.
- Dedicate a separate set of pipettes, buffers, and enzymes for RNA work.
- Use RNase-free microcentrifuge tubes. To decontaminate untreated tubes, soak overnight in a 0.01% (v/v) aqueous solution of diethylpyrocarbonate (DEPC), rinse with sterile distilled water, and autoclave. RNase *Away*™ Reagent, a non-toxic solution available from Invitrogen, can be used to remove RNase contamination from surfaces.

Determining total RNA quality

Total RNA quality can be analyzed using a bioanalyzer such as the Agilent 2100 Bioanalyzer™ with an RNA LabChip™. Alternatively, total RNA can be analyzed by agarose gel electrophoresis. RNA isolated using the PureLink™ kits or TRIzol™ Reagent typically has a 28S-to-18S band ratio of > 1.5. RNA is judged to be intact if discreet 28S and 18S ribosomal RNA bands are observed.

Determining total RNA yield

Total RNA can be quantitated using the Qubit™ RNA HS or the Qubit™ RNA BR Assay Kit, or UV absorbance at 260 nm.

Qubit™ RNA assay kits

The Qubit™ RNA HS (high-sensitivity) and Qubit™ RNA BR (broad-range) Assay Kits provide a rapid, sensitive, and specific method for RNA quantitation with minimal interference from DNA, protein, or other common contaminants that affect UV absorbance readings. The kits provide concentrated assay reagent, dilution buffer, and pre-diluted RNA standards. Simply dilute the reagent using the buffer provided, add your sample (any volume between 1 µL and 20 µL is acceptable), and read the concentration using the Qubit™ 2.0 Fluorometer (or any other suitable instrument).

UV absorbance

1. Dilute an aliquot of the total RNA sample in 10 mM Tris-HCl, pH 7.5. Mix well. Transfer to a cuvette (1-cm path length).

   Note: The RNA must be in a neutral pH buffer to accurately measure the UV absorbance.

2. Determine the OD_{260} of the solution using a spectrophotometer blanked against 10 mM Tris-HCl, pH 7.5.

3. Calculate the amount of total RNA using the following formula:

   Total RNA (µg) = OD_{260} × [40 µg/(1 OD_{260} × 1 mL)] × dilution factor × total sample volume (mL)

   Example: Total RNA was eluted in water in a total volume of 150 µL. A 40-µL aliquot of the eluate was diluted to 500 µL in 10 mM Tris-HCl, pH 7.5. An OD_{260} of 0.188 was obtained.
   The amount of RNA in the sample is:

   Total RNA (µg) = 0.188 × [40 µg/(1 OD_{260} × 1 mL)] × 12.5 × 0.15 = 14.1 µg.

Reverse transcriptase guidelines

About reverse transcription

- Reverse transcriptase binds cDNA and is thus inhibitory to PCR amplification.
- Under the conditions provided (10 U/µL), the 80°C incubation for 10 minutes inactivates the reverse transcriptase and removes the inhibitory effect.
- Under ideal conditions, 10 U/µL produces the highest amount of first-strand cDNA. If reverse transcription is followed by PCR or qPCR, reverse transcriptase amount should be optimized between 1 U/µL and 10 U/µL.
- If the concentration of reverse transcriptase is increased, inactivation of the reverse transcriptase becomes more difficult.
- For synthesis of longer RNA transcripts (>5 kb) that is followed by PCR, optimize the reverse transcription reaction by varying the reverse transcriptase amount between 1 U/µL and 10 U/µL. You can also increase incubation time up to 50 minutes instead of increasing the amount of reverse transcriptase added.
- If increasing the amount of reverse transcriptase is abolutely necessary, heat inactivation followed by phenol/chloroform extraction and ethanol precipitation will remove the PCR inhibitory effect.

RT reaction setup

The protocol in this user guide was created for a single 20-µL reaction. Use the measurements below to prepare a custom RT reaction. Enter your own parameters in the column provided.

Component	20-µL rxn	Custom	Final Conc.
50 µM Oligo d(T)$_{20}$ primer, or 50 ng/µL random hexamers, or 2 µM gene-specific primer	1.0 µL 1.0 µL 1.0 µL	µL	2.5 µM 2.5 ng/µL 0.1 µM
10 mM dNTP mix (10 mM each)	1.0 µL	µL	0.5 mM each
Template RNA*	varies	µL	< 5 µg total RNA or < 500 ng mRNA
DEPC-treated water	to 20 µL	to µL	N/A
5× SSIV Buffer	4.0 µL	µL	1×
100 mM DTT	1.0 µL	µL	5 mM
Ribonuclease Inhibitor (40 U/µL)	1.0 µL	µL	2.0 U/µL
SuperScript™ IV Reverse Transcriptase (200 U/µL)	1.0 µL	µL	10 U/µL

* 10 pg–5 µg total RNA or 10 pg–500 ng mRNA

Primers

The choice of primer for reverse transcription depends on many factors, so you should evaluate all three priming systems.

For short RNAs containing no hairpins, any of the three priming systems usually works equally well. For longer transcripts or sequences containing hairpins, consider these guidelines:

Using random hexamers

- Try using random hexamers first, especially if efficiency of a downstream priming is low, for long reverse transcripts, or for reverse transcripts containing hairpins.
- Use to reverse transcribe all RNA (rRNA, mRNA, and tRNA).

Using downstream gene-specific primers

- Try using downstream gene-specific primers second.
- Use these primers to reverse transcribe only RNA that contains a complementary sequence.
- You must have prior knowledge of the target sequence and must synthesize the primers.

Using oligo d(T)$_{20}$

- Use to increase specificity by reverse transcribing only eukaryotic mRNAs and retroviruses with poly rA tails. (Prokaryotic RNA, rRNA, tRNA, and some eukaryotic mRNA do not have poly rA tails.)
- Use to transcribe entire eukaryotic mRNA messages.

Optimize PCR amplification

- The RNA segment to be transcribed and later amplified can be at least 3 kb long.
- Start with enough copies of the template to ensure that you obtain a signal after 35 cycles — preferably > 10^4 copies but < 1 µg total RNA.
- Sample RNA should be extracted with phenol/chloroform, precipitate with ethanol, and redissolved in RNase-free 1 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl, pH 8.0.
- With high G+C content DNA, melting at 97°C for the first few cycles helps produce a single-stranded template for the PCR amplification.