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PRODUCT INFORMATION

Thermo Scientific
RevertAid First Strand cDNA Synthesis Kit
#K1621, #K1622

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#K1621
Lot __

CERTIFICATE OF ANALYSIS

RT-PCR using 100 fg of control GAPDH RNA and control primers generated a prominent 496 bp product on 1% agarose gel after ethidium bromide staining.

Quality authorized by:  Jurgita Zilinskiene

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COMPONENTS OF THE KIT

RevertAid First Strand cDNA Synthesis Kit	**20 rxns** #K1621	**100 rxns** #K1622
RevertAid M-MuLV Reverse Transcriptase (200 u*/µl)	25 µl	120 µl
RiboLock RNase Inhibitor (20 u**/µl)	25 µl	120 µl
5X Reaction Buffer 250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT	150 µl	500 µl
10mM dNTP Mix	50 µl	250 µl
Oligo$(dT)_{18}$ Primer 100 µM, 0.5 µg/µl (15 A_{260} u/ml)	25 µl	120 µl
Random Hexamer Primer 100 µM, 0.2 µg/µl (6 A_{260} u/ml)	25 µl	120 µl
Forward GAPDH Primer, 10 µM 5' – CAAGGTCATCCATGACAACTTTG – 3'	20 µl	20 µl
Reverse GAPDH Primer, 10 µM 5' – GTCCACCACCCTGTTGCTGTAG - 3'	20 µl	20 µl
Control GAPDH RNA 1.3 kb 3'-poly(A) tailed RNA transcript, 0.05 µg/µl	20 µl	20 µl
Water, nuclease-free	2x1.25 ml	2x1.25 ml

* One unit of RevertAid M-MuLV RT incorporates 1 nmol of dTMP into a polynucleotide fraction (adsorbed on DE-81) in 10 min at 37°C.
** One unit of RiboLock RNase Inhibitor inhibits the activity of 5 ng RNase A by 50%.

STORAGE

All components of the kit should be stored at -20°C. Keep control RNA at -70°C for longer storage.

DESCRIPTION

The Thermo Scientific RevertAid First Strand cDNA Synthesis Kit is a complete system for efficient synthesis of first strand cDNA from mRNA or total RNA templates. The kit uses RevertAid™ M-MuLV Reverse Transcriptase, which has lower RNase H activity compared to AMV reverse transcriptase. The enzyme maintains activity at 42-50°C and is suitable for synthesis of cDNA up to 13 kb.
The recombinant Thermo Scientific RiboLock RNase Inhibitor, supplied with the kit, effectively protects RNA from degradation at temperatures up to 55°C.
The kit is supplied with both oligo$(dT)_{18}$ and random hexamer primers. Random hexamer primers bind non-specifically to the RNA template and are used to synthesize cDNA from all RNAs in the total RNA population. The oligo$(dT)_{18}$ primer selectively anneals to the 3'-end of poly(A) RNA, synthesizing cDNA only from poly(A) tailed mRNA. Gene-specific primer may also be used with the kit to prime synthesis from a specified sequence.
First strand cDNA synthesized with this system can be directly used as a template in PCR or real-time PCR. It is also ideal for second strand cDNA synthesis or linear RNA amplification. Radioactively and non-radioactively labeled nucleotides can be incorporated into first strand cDNA for use as a probe in hybridization experiments, including microarrays.

IMPORTANT NOTES

Avoiding ribonuclease contamination

RNA purity and integrity is essential for synthesis of full-length cDNA. RNA can be degraded by RNase A, which is a highly stable contaminant found in any laboratory environment. All components of the kit have been rigorously tested to ensure that they are RNase free. To prevent contamination both the laboratory environment and all prepared solutions must be free of RNases.

General recommendations to avoid RNase contamination:

- DEPC-treat all tubes and pipette tips to be used in cDNA synthesis or use certified nuclease-free labware.
- Wear gloves when handling RNA and all reagents, as skin is a common source of RNases. Change gloves frequently.
- Use RNase-free reagents, including high quality water (e.g., Water, nuclease-free, #R0581).
- Use an RNase inhibitor, such as RiboLock™ RNase Inhibitor (provided with the kit) to protect RNA from the activity of RNases.
- Keep all kit components tightly sealed when not in use. Keep all tubes tightly closed during the reverse transcription reaction.

Template RNA

Total cellular RNA isolated by standard methods is suitable for use with the kit. Purified RNA must be free of salts, metal ions, ethanol and phenol to avoid inhibiting the cDNA synthesis reaction. Trace contaminants can be removed by ethanol precipitation of the RNA followed by two washes of the pellet with cold 75% ethanol.

For RT-PCR applications, template RNA must be free of DNA contamination. Prior to cDNA synthesis, RNA can be treated with DNase I, RNase-free (#EN0521) to remove trace amounts of DNA. Always perform a control (RT-minus) reaction which includes all components for RT-PCR except for the reverse transcriptase enzyme.

Removal of genomic DNA from RNA preparations

1. Add to an RNase-free tube:

RNA	1 µg
10X Reaction Buffer with $MgCl_2$	1 µl
DNase I, RNase-free (#EN0521)*	1 µl (1 u)
Water, nuclease-free	to 10 µl

2. Incubate at 37°C for 30 min.
3. Add 1 µl 50 mM EDTA and incubate at 65°C for 10 min. RNA hydrolyzes during heating with divalent cations in the absence of a chelating agent (1). Alternatively, use phenol/chloroform extraction.
4. Use the prepared RNA as a template for reverse transcriptase.

* Do not use more than 1 u of DNase I, RNase-free per 1 µg of RNA.

RNA sample quality

Assess RNA integrity prior to cDNA synthesis. The most common method is denaturing agarose gel electrophoresis followed by ethidium bromide staining. If both 18S and 28S rRNA appear as sharp bands after electrophoresis of total eukaryotic RNA, the RNA is considered to be intact. The 28S rRNA band should be approximately twice as intense as the 18S rRNA. Any smearing of rRNA bands is an indication of degraded mRNA. If this occurs, a new sample of total RNA should be prepared.

To evaluate the suitability of purified RNA (human, mouse or rat) for RT-PCR applications a control RT-PCR can be performed using template RNA and the control GAPDH primers provided in the kit. The GAPDH-specific control PCR primers are designed to be complementary to human, mouse and rat GAPDH genes and generate a 496 bp RT-PCR product.

RNA quantity

- Use 0.1 ng - 5 µg of total RNA or 1 ng - 500 ng of poly(A) mRNA to generate first strand cDNA as the initial step of a two-step RT-PCR protocol.
- Use 1 µg of isolated mRNA to generate first strand cDNA for second-strand synthesis and subsequent cloning reactions.

Primers

Synthesis of first strand cDNA can be primed with either oligo$(dT)_{18}$ primer, random primers or gene-specific primers.

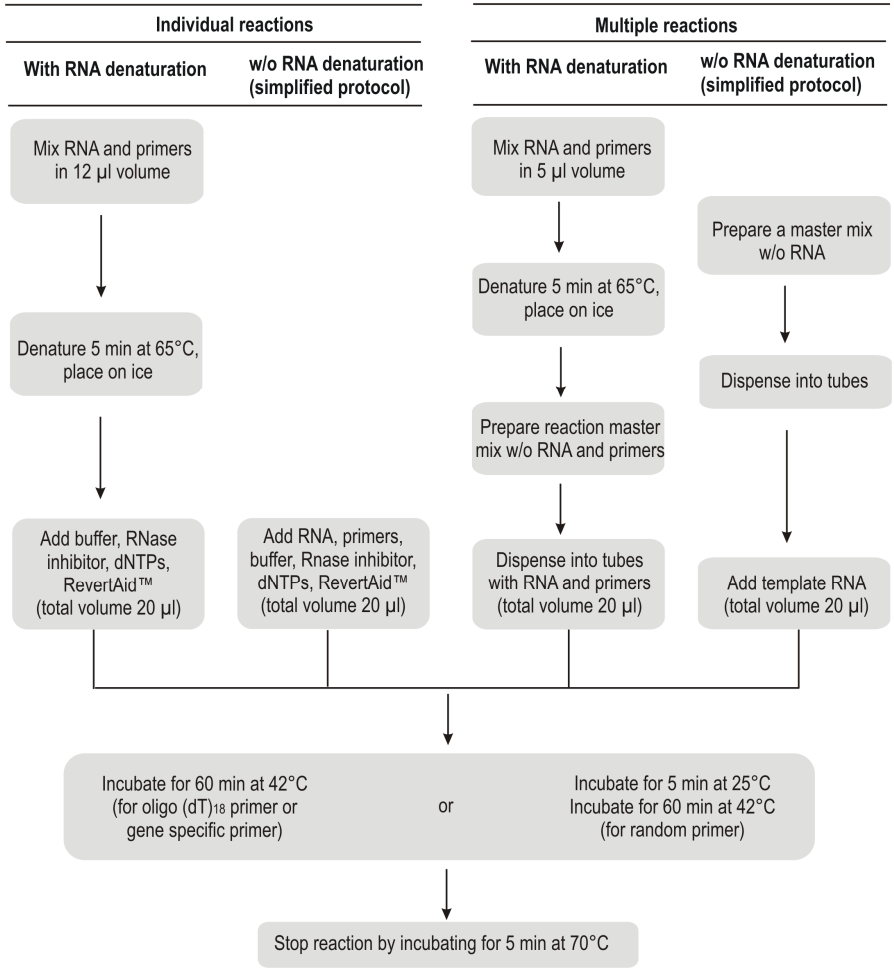
Oligo$(dT)_{18}$ primes cDNA synthesis from the poly(A) tail present at the 3'-end of eukaryotic mRNA. Random primers initiate cDNA synthesis from the total RNA population (rRNA and mRNA). Therefore, using random primers for first strand synthesis results in a greater complexity of the generated cDNA compared with the oligo$(dT)_{18}$ primer. As a consequence, the sensitivity and specificity of subsequent PCR reactions may be reduced. However, there are several applications where it is beneficial to use random primers, such as cDNA synthesis using mRNAs without a poly(A) tail, or cDNA synthesis using poly(A)-enriched RNA samples. Gene-specific primers are used to synthesize specific cDNA from a pool of total RNA or mRNA and must be obtained by the user.

First Strand cDNA synthesis procedure

The first strand cDNA reaction can be performed as an individual reaction or as a series of parallel reactions with different RNA templates. Therefore, the reaction mixture can be prepared by combining reagents individually or a master mix containing all of the components except template RNA can be prepared. Depending on the structure of the RNA template, separate steps for RNA denaturation and primer annealing may improve RT-PCR results.

The table below describes the main steps of the first strand cDNA synthesis protocol. A detailed protocol is provided on p.6. The protocol describes the set-up of an individual reaction that includes an RNA denaturation step. Other reaction layouts use same reagent amounts per 20 µl reaction, but require different order of reagent assembly (*see* table below).

Table 1. Overview of cDNA synthesis procedures in individual and multiple reactions.



PROTOCOLS

Please read the Notes section of the manual (p.3-5) before starting.

RT-PCR

I. First Strand cDNA Synthesis

After thawing, mix and briefly centrifuge the components of the kit. Store on ice.

1. Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order:

Template RNA	total RNA *or* poly(A) mRNA *or* specific RNA	0.1 ng - 5 µg 10 pg - 0.5 µg 0.01 pg - 0.5 µg
Primer	oligo $(dT)_{18}$ primer *or* random hexamer primer *or* gene-specific primer	1 µl 1 µl 15-20 pmol
Water, nuclease-free		to 12 µl
	Total volume	12 µl

2. *Optional.* If the RNA template is GC-rich or contains secondary structures, mix gently, centrifuge briefly and incubate at 65°C for 5 min. Chill on ice, spin down and place the vial back on ice.
3. Add the following components in the indicated order:

5X Reaction Buffer	4 µl
RiboLock RNase Inhibitor (20 u/µl)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuLV Reverse Transcriptase (200 u/µl)	1 µl
Total volume	20 µl

4. Mix gently and centrifuge.
5. For oligo$(dT)_{18}$ or gene-specific primed cDNA synthesis, incubate for 60 min at 42°C. For random hexamer primed synthesis, incubate for 5 min at 25°C followed by 60 min at 42°C.
   Note. For GC-rich RNA templates the reaction temperature can be increased up to 45°C.
6. Terminate the reaction by heating at 70°C for 5 min.

The reverse transcription reaction product can be directly used in PCR applications or stored at -20°C for less than one week. For longer storage, -70°C is recommended.

II. PCR Amplification of First Strand cDNA

The product of the first strand cDNA synthesis can be used directly in PCR or qPCR. The volume of first strand cDNA synthesis reaction mixture should not comprise more than 1/10 of the total PCR reaction volume. Normally, 2 µl of the first strand cDNA synthesis reaction mixture is used as template for subsequent PCR in 50 µl total volume. *Taq* DNA polymerase, PCR Master Mix (2X) or Thermo Scientific PyroStart Fast PCR Master Mix (2X) can be used to amplify fragments less than 3 kb. Thermo Scientific DreamTaq DNA polymerase is suitable for amplification of longer fragments up to 6 kb. The Long PCR Enzyme Mix and High Fidelity PCR Enzyme Mix are recommended to generate amplicons up to 20 kb.

cDNA Synthesis for Cloning

I. First strand cDNA synthesis

Mix and briefly centrifuge all components after thawing, keep on ice.

1. Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order:

Template RNA	poly(A) mRNA *or* specific RNA	1 µg 0.5-1 µg
Primer	oligo $(dT)_{18}$ primer *or* random hexamer primer *or* gene-specific primer	1 µl 1 µl 100 pmol
Water, nuclease-free		to 12 µl
	Total volume	12 µl

2. *Optional.* If the RNA template is GC-rich or contains secondary structures, mix gently, centrifuge briefly and incubate at 65°C for 5 min. Chill on ice, spin down and place the vial back on ice.
3. Add the following components in the indicated order:

5X Reaction Buffer	4 µl
RiboLock RNase Inhibitor (20 u/µl)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuLV Reverse Transcriptase (200 u/µl)	1 µl
Total volume	20 µl

4. Mix gently and centrifuge.
   For oligo$(dT)_{18}$ or gene-specific primed cDNA synthesis, incubate for 60 min at 42°C.
   For random hexamer primed synthesis, incubate for 5 min at 25°C followed by 60 min at 42°C.
   Note. For GC-rich RNA templates the reaction temperature can be increased up to 45°C.
5. Terminate the reaction by heating at 70°C for 5 min.

The reverse transcription can be used immediately in second strand cDNA synthesis reactions or stored at -20°C for less than a week. For longer storage, -70°C is recommended.

II. Second strand cDNA synthesis

1. Add the following reagents to 20 µl of the first strand cDNA synthesis reaction mixture on ice:

10X Reaction Buffer for DNA Polymerase I	8 µl
RNase H, *E.coli* (#EN0201)	1 u
DNA Polymerase I, *E.coli* (#EP0041)	30 u
Water, nuclease-free	to 100 µl
Total volume	100 µl

2. Gently vortex and briefly centrifuge.
3. Incubate at 15°C for 2 hours. Do not let the temperature to rise above 15°C.
4. Add 12.5 u of T4 DNA Polymerase (#EP0061) and incubate at 15°C for 5 min.
5. Terminate the reaction by adding 5 µl of 0.5 M EDTA, pH 8.0 (#R1021).
6. Purify blunt-end cDNA by phenol/chloroform extraction and use for further cloning-related procedures e.g., adapter ligation, phosphorylation, size fractionation, ligation and transformation.

CONTROL REACTIONS

Positive and negative control reactions should be used to verify the results of the first strand cDNA synthesis steps.

- **Reverse transcriptase minus (RT-) negative control** is important in RT-PCR or qRT-PCR reactions to assess for genomic DNA contamination of the RNA sample. The control RT- reaction contains every reagent for the reverse transcription reaction except for the RT enzyme.
- **No template negative control (NTC)** is important to assess for reagent contamination. The NTC reaction contains every reagent for the reverse transcription reaction except for RNA template.
- **Positive control** RNA template and gene-specific primers are supplied with the kit. The human GAPDH control RNA (1.3 kb) was produced by *in vitro* transcription. The GAPDH-specific control PCR primers are designed to be complementary to human, mouse and rat GAPDH genes and generate 496 bp RT-PCR product. The protocol for the positive control RT-PCR is provided below.

I. Positive control first strand cDNA synthesis reaction

Mix and briefly centrifuge all components after thawing, keep on ice.

1. Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order:

Control GAPDH RNA (50 ng/µl)	2 µl
Oligo $(dT)_{18}$ Primer *or* Random Hexamer Primer *or* Reverse GAPDH Primer	1 µl
5X Reaction Buffer	4 µl
RiboLock RNase Inhibitor (20 u/µl)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuLV Reverse Transcriptase (200 u/µl)	1 µl
Water, nuclease-free	9 µl
Total volume	20 µl

2. Mix gently and centrifuge.
3. For oligo$(dT)_{18}$ or gene-specific primed cDNA synthesis, incubate for 60 min at 42°C.
   For random hexamer primed synthesis, incubate for 5 min at 25°C followed by 60 min at 42°C.
4. Terminate the reaction by heating at 70°C for 5 min.
5. Briefly centrifuge and proceed with control PCR amplification on p.9.

II. Control PCR amplification

1. Dilute the cDNA generated with the control first strand cDNA reaction (p. 8) 1:1000 in Water, nuclease-free.
2. Gently vortex and briefly centrifuge all PCR reagents after thawing.
3. Place a thin-walled PCR tube on ice and add the following reagents:

cDNA from control RT reaction (1:1000 dilution)	2 µl
10X PCR buffer	5 µl
10 mM dNTP Mix	1 µl (0.2 mM each)
25 mM $MgCl_2$	3 µl
Forward GAPDH Primer	1.5 µl
Reverse GAPDH Primer	1.5 µl
Taq DNA polymerase (5 u/µl)	0.5 µl
Water, nuclease-free	35.5 µl
Total volume	50 µl

4. Perform PCR in a thermal cycler with a heated lid or overlay with 25 µl of mineral oil.

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 s	35
Annealing	58	30 s	
Extension	72	45 s	

5. Load 5-10 µl of the RT-PCR product on 1% agarose gel. A distinct 496 bp PCR product should be visible after ethidium bromide staining.

TROUBLESHOOTING

Problem	Cause and Solution
Low yield or no RT-PCR product	**Degraded RNA template.** RNA purity and integrity is essential for synthesis of full-length cDNA. Always assess the integrity of RNA prior to cDNA synthesis. Sharp 18S and 28S RNA bands should be visible after denaturing agarose gel electrophoresis of total eukaryotic RNA. Follow general recommendations to avoid RNase contamination (p. 3). **Low template purity.** Trace amounts of agents used in RNA purification protocols may remain in solution and inhibit first strand synthesis, e.g., SDS, EDTA, guanidine salts, phosphate, pyrophosphate, polyamines, spermidine. To remove trace contaminants, re-precipitate the RNA with ethanol and wash the pellet with 75% ethanol. **Insufficient template quantity.** Increase the amount of template to the recommended level. Following DNase I treatment, terminate the reaction by heat inactivation in the presence of EDTA (to bind magnesium ions), *see* protocol on p. 3. RNA hydrolyzes during heating in the absence of a chelating agent (1). **Incorrect primer choice.** Use the correct primer for the RNA template. Use the random hexamer primer instead of the oligo$(dT)_{18}$ primer with bacterial RNA or RNA without a poly(A) tail. Ensure sequence-specific primers are complementary to 3'-end of the template RNA. **GC rich template.** If the RNA template is GC rich or is known to contain secondary structures, increase the temperature of the reverse transcription reaction up to 45°C.
RT-PCR product longer than expected	**RNA template is contaminated with DNA.** Amplification of genomic DNA containing introns. Perform DNase I digestion prior reverse transcription (*see* protocol on p. 3). To avoid amplification of genomic DNA, design PCR primers on exon-intron boundaries.
RT-PCR product in negative control	**RNA template is contaminated with DNA.** PCR product in the negative control (RT-) indicates the reaction is contaminated with DNA. Perform DNase I digestion prior reverse transcription (*see* protocol on p. 3).

Reference

1. Wiame, I., et al., Irreversible heat inactivation of DNaseI without RNA degradation, BioTechniques, 29, 252-256, 2000.

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and *in vitro* use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

Please refer to www.thermoscientific.com/fermentas for Material Safety Data Sheet of the product.