thermoscientific USER GUIDE

RevertAid Master Mix, with DNase I

Catalog Number M16315, M16325

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support.**

Contents and storage

Cat. No.	Contents		Amount	Storage
M16315 (50 rxn)	1 box of RevertAid Master Mix (Cat. No. M1631), containing:	20X Enzyme mix (MuLV and RNase inhibitor)	50 µL	-25 °C to -15 °C
		2X RT Buffer Mix (includes dNTPs, random primers, and oligo dT)	500 µL	
	1 box of DNase I (Cat. No. EN0529), containing:	DNase I, RNase-free (1 U/µL)	200 µL	
		10X Reaction Buffer with $MgCl_2$ for DNase I	1.25 mL	
		50 mM EDTA	1 mL	
M16325 (100 rxn)	2 boxes of RevertAid Master Mix (Cat. No. M1631), each containing:	20X Enzyme mix (MuLV and RNase inhibitor)	50 µL	
		2X RT Buffer Mix (includes dNTPs, random primers, and oligo dT)	500 µL	
	1 box of DNase I (Cat. No. EN0529), containing:	DNase I, RNase-free (1 U/µL)	200 µL	
		10X Reaction Buffer with $MgCl_2$ for DNase I	1.25 mL	
		50 mM EDTA	1 mL	

Description

Thermo Scientific RevertAid Master Mix with DNase I is a complete system for efficient synthesis of first strand cDNA from mRNA or total RNA templates. The kit contains recombinant endonuclease (DNase I) to remove contaminating genomic DNA from RNA preps.

For reverse transcription the kit uses RevertAid Reverse Transcriptase (RT), which has lower RNase H activity compared to AMV reverse transcriptase. RevertAid Master Mix provides all cDNA synthesis reaction components in a convenient two-tube master mix. It is optimized for highly efficient cDNA synthesis for two-step quantitative RT-PCR (RT-qPCR) applications. The two-tube formulation reduces reaction time and requires fewer pipetting steps than with the RevertAid RT Reverse Transcription Kit, while maintaining high performance.

Procedural guidelines

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 laboratory environments. General recommendations to avoid RNase contamination:

- Use certified nuclease-free labware or DEPC-treat all tubes and pipette tips to be used in cDNA synthesis.
- Wear gloves when handling RNA and 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).
- Keep the kit components tightly sealed when not in use. Keep all tubes tightly closed during the reverse transcription reaction.

Template RNA
The kit is compatible with total cellular RNA isolated by standard methods. Purified RNA must be free of salts, metal ions, ethanol, and phenol to avoid inhibiting the cDNA synthesis reaction.
For RT-qPCR applications, template RNA must be free of DNA contamination. Prior to cDNA synthesis, treat RNA samples with DNase I, RNase-free to remove residual DNA.
Always perform a No RT Control reaction, which includes all components for reverse transcription except the RevertAid RT.

RNA sample quality
Assess RNA integrity prior to cDNA synthesis. Total eukaryotic RNA can be analyzed by agarose gel electrophoresis followed by ethidium bromide staining.
The RNA is considered intact, if both 18S and 28S rRNA appear as sharp bands after electrophoresis of total RNA. The 28S rRNA band should be approximately twice as intense as the 18S rRNA. Smearing of rRNA bands is an indication of degraded mRNA, and a new sample of total RNA should be prepared.
Alternatively, total RNA can be analyzed using a bioanalyzer (e.g., Agilent 2100) to obtain an RNA integrity number, which provides quantitative information about the general state of the RNA sample (2). A reference gene/target gene 3'-5' integrity assay (3) can also be used to determine the integrity of the RNA sample.

Primers
RevertAid Master Mix contains oligo (dT) and random primers to prime synthesis of first strand cDNA. This primer mixture ensures high sensitivity in low copy number transcript detection assays.

Protocol

Removal of genomic DNA from RNA preparations:
1. Add to an RNase-free tube:

Component	Volume
RNA	1 µg
10X Reaction Buffer with $MgCl_2$	1 µL
DNase I, RNase-free (1 U/µL)	2 µL
Water, nuclease-free	to 10 µL

2. Incubate at 37 °C for 5 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.

First Strand cDNA Synthesis:
Allow kit components to thaw, then mix and briefly centrifuge. Store the tubes on ice.
1. Add the following reagents into a sterile, RNase free tube or plate on ice in the following order:

Component	Volume
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
2X RT Buffer Mix	10 µL
20X RT Enzyme Mix	1.0 µL
Water, nuclease-free	to 20 µL
Total volume	**20 µL**

2. Seal the plates or tubes with the appropriate seal or caps.
3. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
4. Place the plate or tubes on ice until you are ready to start the reverse transcription reaction.
5. Incubate the reaction for 42 °C for 30 minutes.

Note: For using lower than 1000 ng of high-quality RNA input, incubation time could be shortened to 15 minutes. For using higher than 1000 ng of challenging RNA input, incubation for 60 minutes is recommended.
6. Stop the reaction by heating to 95 °C for 5 minutes and hold at 4 °C. For convenience, the incubation may be performed in a thermal cycler.
The cDNA is ready for use in PCR applications or for storage at -20 °C for less than one week. For longer storage, -70 °C is recommended.

qPCR
Use the product of the cDNA synthesis reaction directly in qPCR. Normally, 2 µL of the RT product is used as template for subsequent qPCR in a 25 µL total volume.

Control reactions
Use the following negative control reactions to verify the results of the first strand cDNA synthesis.
- **No RT control** to assess genomic DNA contamination of the RNA sample.
- **No template control (NTC)** to assess for reagent contamination. The NTC reaction includes all reagents necessary for the reverse transcription reaction except the RNA template.

Prepare control reactions according to the following instructions.
Note: Template RNA amount and reverse transcription incubation conditions should reflect the conditions used for experimental samples.
1. Add the following reagents into a sterile, RNase free tube or plate on ice in the following order:

Component	No RT control	No template control
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	—
2X RT Buffer Mix	10 µL	10 µL
20X RT Enzyme Mix	—	1 µL
Water, nuclease-free	to 20 µL	to 20 µL
Total volume	**20 µL**	**20 µL**

2. Seal the plates or tubes with the appropriate seal or caps.
3. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
4. Place the plate or tubes on ice until you are ready to start the reverse transcription reaction.
5. Incubate the reaction for 42 °C for 30 minutes.
Note: For using lower than 1000 ng of high-quality RNA input, incubation time could be shortened to 15 minutes. For using higher than 1000 ng of challenging RNA input, incubation for 60 minutes is recommended.
6. Stop the reaction by heating to 95 °C for 5 minutes and hold at 4 °C. For convenience, the incubation may be performed in a thermal cycler.

Troubleshooting

No qPCR product generated or product appears late in qPCR
Poor integrity of RNA template RNA purity and integrity is essential for synthesis and quantification of cDNA. • Always assess the integrity of RNA prior to cDNA synthesis. • Use freshly prepared RNA. • Multiple freeze/thaw cycles of the RNA sample and synthesized cDNA is not recommended. • Follow general recommendations to avoid RNase contamination and discard low quality RNA. **Low template purity (inhibitors in RNA sample)** 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). Remove trace contaminants by re-precipitating the RNA with ethanol and wash the pellet with 75 % ethanol. **Insufficient template quantity** Increase the amount of RNA template in the first strand reaction to the recommended level. Following DNase I treatment, terminate the reaction by heat inactivation in the presence of EDTA (to bind magnesium ions), RNA hydrolyzes during heating in the absence of a chelating agent (1). **GC-rich template** If the RNA template is GC-rich or is known to contain secondary structures, the temperature of the reverse transcription reaction can be increased up to 45 °C. **Excess amount of cDNA in qPCR** Decrease amount of cDNA synthesis reaction in qPCR. The volume of the cDNA synthesis reaction mixture should not exceed 10 % of the final PCR reaction mixture.
RT-qPCR product in No RT Control
RNA template is contaminated with DNA. The presence of a PCR product in the negative control (No RT Control) reaction indicates that the reaction is contaminated with DNA. Follow the protocol carefully and make sure that DNase I treatment step prior reverse transcription is not omitted. To avoid DNA contamination perform DNase I digestion prior reverse transcription (see protocol on p. 2).

References

1. Wiame, I., et al., Irreversible heat inactivation of DNaseI without RNA degradation, BioTechniques, 29, 252-256, 2000.
2. Fleige, S., Pfaffl, M.W., RNA integrity and the effect on the real-time qRT-PCR performance, Mol. Aspects Med., 27, 126-139, 2006.
3. Nolan, T., et al., Quantification of mRNA using real-time RT-PCR, Nat. Protoc., 1, 1559-1582, 2006.

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