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Maxima™ H Minus Double-Stranded cDNA Synthesis Kit

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

Product description

The Maxima™ H Minus Double-Stranded cDNA Synthesis Kit is a complete system for efficient synthesis of double-stranded cDNA from total RNA or mRNA. First- and second-strand cDNA synthesis reactions are performed in the same tube without the need for intermediate organic extraction or ethanol precipitation steps. This convenient one-tube format speeds up the synthesis procedure and maximizes cDNA recovery. The kit contains premixed components to reduce the number of pipetting steps necessary to complete the procedure.

First Strand cDNA Synthesis Reaction

The First Strand Enzyme Mix is a blend of Maxima™ H Minus Reverse Transcriptase and Thermo Scientific™ RiboLock™ RNase Inhibitor. Maxima™ H Minus Reverse Transcriptase (RT) is an advanced enzyme derived through *in vitro* evolution of M-MuLV RT. The enzyme features the highest thermostability among M-MuLV RT derivatives and lacks RNase H activity. Recombinant RiboLock™ RNase Inhibitor effectively protects RNA from degradation by RNases A, B and C at temperatures up to 55 °C.
The Maxima™ H Minus Double-Stranded cDNA Synthesis Kit is capable of synthesizing high yields of cDNA up to 11 kb in length from a wide range of starting RNA amounts (0.5 µg to 20 µg total RNA, 50 ng to 5 µg mRNA) and at elevated temperatures (50-60 °C). Due to the rapid synthesis rate of the Maxima™ H Minus RT, the first strand synthesis reaction can be completed in only 30 min. The optimal temperature for reverse transcription is 50 °C but for transcription of GC-rich RNA the reaction temperature can be increased to 60 °C.
The kit is supplied with both oligo(dT)$_{18}$ and random hexamer primers. The oligo(dT)$_{18}$ primer selectively anneals to the 3'-end of poly(A)$^+$ RNA, synthesizing cDNA only from poly(A)$^+$ tailed mRNA. Random hexamer primers bind non-specifically and are used to synthesize cDNA from total RNA. Gene-specific primers may also be used with the kit to prime synthesis from a specified sequence.

Second Strand cDNA Synthesis Reaction

Second strand cDNA is generated using the first strand cDNA as a template. The Maxima™ H Minus Double-Stranded cDNA Synthesis Kit uses nick translational replacement of the mRNA to synthesize the second strand cDNA. First described by Okayama and Berg (1), and later popularized by Gubler and Hoffman (2), second strand cDNA synthesis is catalyzed by *E. coli* DNA polymerase I in combination with *E. coli* RNase H and *E. coli* DNA ligase. *E.coli* RNase H inserts nicks into the RNA, providing 3' OH-primers for DNA polymerase I.
The 5'-3' exonuclease activity of *E. coli* DNA polymerase I removes the RNA strand in the direction of synthesis, while its polymerase activity replaces the RNA with deoxyribonucleotides. *E. coli* DNA ligase links the gaps to complete the ds cDNA strand.
The second strand reaction is performed at 16 °C to prevent spurious synthesis by DNA polymerase I due to its tendency to strand-displace (rather than nick translate) at higher temperatures.

Contents and storage

Contents	Amount		Storage
	Cat. No. K2562 50 rxns	Cat. No. K2563 200 rxns	
First Strand Enzyme Mix	100 µL	4 x 100 µL	-25 °C to -15 °C
4X First Strand Reaction Mix	500 µL	4 x 500 µL	
Second Strand Enzyme Mix	250 µL	4 x 250 µL	
5X Second Strand Reaction Mix	1 mL	4 x 1 mL	
0.5 M EDTA, pH 8.0	1 mL	4 x 1 mL	
RNase I, 10 U/µL	500 µL	4 x 500 µL	
Control RNA, 0.5 µg/µL	100 µL	4 x 100 µL	
Oligo(dT)$_{18}$ Primer, 100 µM, 0.5 µg/µL	125 µL	4 x 125 µL	
Random Hexamer Primer, 100 µM, 0.2 µg/µL	125 µL	4 x 125 µL	
Water, nuclease-free	3 x 1.25 mL	4 x 3 x 1.25 mL	

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 kit components 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:

- 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 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).
- Ensure that the kit components are tightly sealed when not in use. Keep all tubes tightly closed during the reverse transcription reaction.

Template RNA

Synthesis of high quality ds cDNA begins with the preparation of high quality RNA. Purified RNA must be free of salts, metal ions, ethanol and phenol to avoid inhibiting the cDNA synthesis reaction. It is important to optimize the isolation of total RNA or mRNA from eukaryotic tissues or from cell cultures to prevent introduction of RNases into the preparation.
We recommend using the Thermo Scientific™ GeneJET™ RNA Purification Kit (#K0731, #K0732) or GeneJET™ Whole Blood RNA Purification Mini Kit (#K0761) for isolation of total RNA.

RNA sample quality

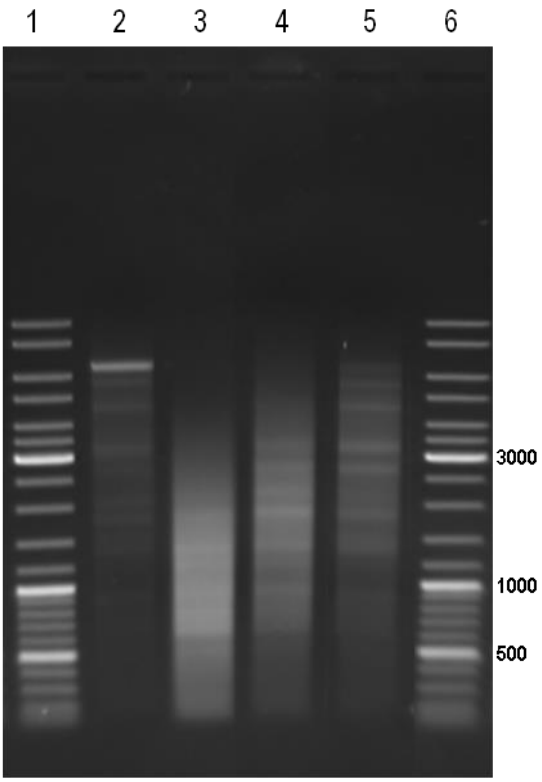
The quality of the template RNA greatly influences the yield and size distribution of the synthesized cDNA. The integrity of the template RNA should be assessed prior to cDNA synthesis. Total eukaryotic RNA can be analyzed by agarose gel electrophoresis followed by ethidium bromide staining. The RNA is considered to be intact if after electrophoresis both 18S and 28S rRNA appear as sharp bands. The 28S rRNA band (~5 kb) should be approximately twice as intense as the 18S rRNA (~2 kb). Any smearing of the rRNA bands is an indication of mRNA degradation and a new sample of total RNA should be prepared. Alternatively, total RNA integrity can be estimated using microfluidic chip-based analyzers, which provide quantitative and qualitative information about the general state of the RNA sample in the form of RIN (RNA integrity number) or RQI (RNA quality indicator), depending on the manufacturer (3). A reference gene/target gene 3':5' integrity assay (4) can also be used to determine the integrity of the RNA sample.
mRNA preparations should appear as a smear from ~7 kb to 200 bp when mRNA integrity is assayed by agarose gel electrophoresis. The gel should reveal a dense smear centered in the 1–3 kb range. If the mRNA distribution is not centered in this range, a new sample of mRNA should be prepared or the RNA isolation procedure may require troubleshooting.

Primers

Synthesis of first strand cDNA can be primed with either the oligo(dT)$_{18}$ primer, random hexamer 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 the first strand synthesis results in a greater complexity of the generated cDNA compared to the oligo(dT)$_{18}$ primer. As a consequence, the sensitivity and specificity of ds cDNA synthesis reactions may be reduced. However, there are several applications where it is beneficial to use random primers, such as cDNA synthesis of mRNAs which lack 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.
Note. When using random primers, the length of the cDNA obtained will depend on the amount of primer added. As a general rule, the more primer added, the shorter the cDNA obtained. Therefore it may be necessary to optimize the primer concentration to obtain the best result. Fig. 1 illustrates this point by comparing the size range of ds cDNA prepared with oligo(dT)$_{18}$ and three different random hexamer primer to mRNA ratios. Lower ratios produce longer cDNAs.

Recommended RNA quantity for first strand synthesis

When using Oligo(dT)$_{18}$ Primers: Use 1 µg - 20 µg of total RNA or 50 ng - 5 µg of poly(A)$^+$ mRNA.
When using Random Hexamer Primers: Use 0.5 µg - 5 µg of total RNA or 50 ng - 5 µg of poly(A)$^+$ mRNA.



Lane 1: Thermo Scientific™ GeneRuler™ DNA Ladder Mix
Lane 2: 100 pmol Oligo(dT)$_{18}$ Primer
Lane 3: 500 pmol Random Hexamer
Lane 4: 100 pmol Random Hexamer
Lane 5: 10 pmol Random Hexamer
Lane 6: GeneRuler™ DNA Ladder Mix
1 % TAE agarose

Fig. 1. cDNA size dependence on primer choice and concentration

First and second strand cDNA synthesis reactions were performed as described in the first and second strand synthesis protocols outlined in this manual using a 6.4 kb polyadenylated synthetic RNA template (0.5 µg/reaction) with the indicated primers. The reaction products were purified using the GeneJET™ PCR Purification Kit (#K0701) and analyzed by agarose gel electrophoresis and ethidium bromide staining.

Control RNA

GAPDH poly(A)$^+$ mRNA is provided for use as control template in the first- and second-strand synthesis reactions.

Protocol

Please read the **Important Notes** section of the manual (p.2-3) before starting.

I. First Strand cDNA Synthesis

Thaw reaction components, mix, briefly centrifuge and place on ice. Use an appropriate combination of total RNA or poly(A)$^+$ mRNA with oligo(dT)$_{18}$ or random hexamer primers.

1. Add the following reagents into a sterile, RNase-free tube on ice:

Primer		**Template RNA**	
		total RNA ***Or***	**poly(A)$^+$ mRNA**
oligo(dT)$_{18}$ ***Or*** random hexamer	1 µL (100 pmol) ***Or*** variable	1 µg - 20 µg	50 ng - 5 µg
		0.5 µg - 5 µg	50 ng - 5 µg
Water, nuclease-free		to 14 µL	

2. Mix gently, centrifuge briefly and incubate at 65 °C for 5 min. Chill on ice, briefly centrifuge again and place on ice.
3. Add the following components in the indicated order:

Component	Amount
4X First Strand Reaction Mix	5 µL
First Strand Enzyme Mix	1 µL

Mix gently and centrifuge.

4. Incubate:
   - if an oligo(dT)$_{18}$ primer or gene-specific primer is used, incubate for 30 min at 50 °C.
   - if a random hexamer primer is used, incubate for 10 min at 25 °C followed by 30 min at 50 °C.
5. Terminate the reaction by heating at 85 °C for 5 minutes.
6. Place the tube on ice.
7. Continue immediately with the second strand synthesis reaction as described below.

II. Second Strand cDNA Synthesis

Thaw reaction components, mix, briefly centrifuge and place on ice.

1. For one 100 µL reaction, pipette the following components directly into the first strand reaction tube on ice in the indicated order:

Component	Amount
First Strand cDNA Synthesis Reaction Mixture	20 µL
Water, nuclease-free	55 µL
5X Second Strand Reaction Mix	20 µL
Second Strand Enzyme Mix	5 µL
Total volume	**100 µL**

2. Mix gently and centrifuge briefly.
3. Incubate at 16 °C for 60 min.
4. Stop the reaction by adding 6 µL 0.5 M EDTA, pH 8.0 and mixing gently.
5. Keep the reaction on ice (4 °C) until you are ready to continue with residual RNA removal or ds cDNA purification procedures.

III. Removal of RNA

If total RNA was used as a starting material, we recommend the following steps to remove residual RNA from the ds cDNA preparation:

1. Add 10 µL (100 U RNase I to the second strand synthesis reaction tube.
2. Incubate for 5 minutes at room temperature.
3. Proceed with cDNA purification or store the reaction mixture at -20 °C.

IV. Purification of ds cDNA

Purify blunt-end double-stranded cDNA using the GeneJET™ PCR Purification Kit (#K0701, #K0702) or by phenol/chloroform extraction; use in downstream applications immediately or freeze at -20 °C.

V. Analysis of cDNA products

The quantity and size distribution of the synthesized ds cDNA can be estimated spectrophotometrically by measuring the absorbance at 260 nm (e.g. using NanoDrop™ ND-1000) and by gel analysis.

Note. Prior to spectrophotometrical analysis of cDNA, unincorporated dNTPs must be removed by purification via GeneJET™ PCR Purification Kit, gel filtration column or by ethanol precipitation.

VI. Downstream applications

Cloning of ds cDNA

Following purification, the blunt-end double-stranded cDNA can be used in the following cloning applications:

- direct ligation with pJET1.2/blunt cloning vector (Thermo Scientific™ CloneJET™ PCR Cloning Kit #K1231, #K1232) following the Blunt-End Cloning Protocol outlined in the CloneJET™ PCR Cloning Kit manual.
- ligation with any other blunt-end vector. If necessary, T4 PNK (#EK0031, #EK0032) can be used to phosphorylate the 5' ends of the ds cDNA.
- ligation with specially designed adaptors, enabling cloning into sticky-end vectors. The choice of the adaptors depends on the vector used.

Restriction Digestion of ds cDNA

Following purification, blunt-end ds cDNA can be used in restriction enzyme digestion applications. FastDigest™ Restriction Enzymes can be used to rapidly digest ds cDNA in just 5-15 minutes.

Control Reaction

The performance of the kit components can be assayed with the Control RNA (1.3 kb poly(A)$^+$ mRNA) and the Oligo(dT)$_{18}$ Primer.

I. First Strand cDNA Synthesis

Thaw reaction components, mix, briefly centrifuge and place on ice.

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

Component	Amount
Oligo(dT)$_{18}$ Primer	1 µL (100 pmol)
Control RNA	4 µL (2 µg)
Water, nuclease-free	9 µL

III. Mix gently, centrifuge briefly and incubate at 65 °C for 5 min. Chill on ice, briefly centrifuge again and place on ice.

IV. Add the following components in the indicated order:

Component	Amount
4X First Strand Reaction Mix	5 µL
First Strand Enzyme Mix	1 µL
Total volume	**20 µL**

V. Mix gently, centrifuge briefly and incubate for 30 min at 50 °C.

VI. Terminate the reaction by heating at 85 °C for 5 minutes, then place the tube on ice.

VII. Remove 10 µL of the first strand cDNA synthesis reaction mixture into a new tube for analysis and keep on ice until purification of the first strand reaction products.

VIII. Use the remaining 10 µL of first strand synthesis reaction mixture for the second strand synthesis reaction as described below.

II. Second Strand cDNA Synthesis

Thaw all reaction components, mix, briefly centrifuge and place on ice.

1. Pipette the following components directly into the first strand reaction tube on ice in the indicated order:

Component	Amount
First Strand cDNA Synthesis Reaction Mixture	10 µL
Water, nuclease-free	27.5 µL
5X Second Strand Reaction Mix	10 µL
Second Strand Enzyme Mix	2.5 µL
Total volume	**50 µL**

2. Mix gently and centrifuge briefly.
3. Incubate at 16 °C for 60 min.
4. Stop reaction by adding 3 µL 0.5 M EDTA, pH 8.0.
5. Purify the first and second strand synthesis products (separately) using the GeneJET™ PCR Purification Kit (#K0701, #K0702) or by phenol/chloroform extraction.

If the cDNA is purified using the GeneJET™ PCR Purification Kit (#K0701, #K0702), the elution volume should be reduced to increase the cDNA concentration. We recommend adding 20 µL of Elution Buffer to the center of the purification column membrane for cDNA elution. If cDNAs are purified by phenol/chloroform extraction, dissolve the pellet in ≤20 µL water following the final precipitation step.

III. Analysis of Control Reaction Products

Use 10 µL of purified ds cDNA from the control reaction in a digestion reaction with the HinP1I (Hin6I) restriction enzyme. To digest ds cDNA in just 5 minutes, use the FastDigest™ HinP1(Hin6I) restriction enzyme (#FD0484).

Load 5 µL of first and second strand synthesis products and 10 µL of the HinP1I (Hin6I) digested ds cDNA on a 1% agarose gel. Following electrophoresis the first strand reaction products (RNA/cDNA hybrids) will migrate slightly longer than the second strand reaction products. To estimate the actual size of the first strand cDNA reaction product, the first strand cDNA should be analyzed by alkaline agarose gel electrophoresis. HinP1I (Hin6I) digested ds cDNA should migrate as 615, 363, 227 and 143 bp DNA fragments.

Troubleshooting

Problem	Cause and Solution
Low (or no) yield of first strand cDNA product	**Low quality RNA template.** RNA purity and integrity is essential for synthesis and quantification of cDNA. Always assess the integrity of RNA prior to cDNA synthesis (*see* p. 2-3). 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 (page 2) and discard low quality RNA. **Low template purity (e.g. inhibitors in RNA sample).** Trace amounts of reagents 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, or re-purify the RNA with the GeneJET™ RNA Purification Kit (#K0731, #K0732) following the RNA Cleanup Protocol outlined in the kit manual. **Insufficient template quantity.** Increase the amount of RNA template in the first strand reaction to the recommended level. **GC-rich template.** If RNA template is GC-rich or is known to contain secondary structures, the temperature of the reverse transcription reaction can be increased up to 60 °C. If cDNA synthesis is performed at temperatures higher than 50 °C, use 2 µL of First Strand Enzyme Mix in the reaction.
Low yield of long cDNA products	**Suboptimal priming.** Use Oligo(dT)$_{18}$ primer or a gene specific primer. If random primers are used, reduce the amount of random primers to 20 pmol per 20 µL of the first strand synthesis reaction mixture.
Low yield of second strand cDNA products	**Incomplete RT inactivation.** Always perform the RT inactivation step after first strand cDNA synthesis (see First Strand cDNA Synthesis protocol (p 3-4). **Improper preparation of the second strand reaction mixture.** Dilute the first strand reaction as outlined in the Second Strand cDNA Synthesis protocol on page 4. Failure to properly dilute the first strand reaction changes the pH of the second strand reaction and influences the activity of the 3'-5' and 5'-3' exonuclease activities of DNA polymerase I. **Incorrect reaction temperature.** The second strand reaction must be incubated at 16 °C to prevent spurious synthesis by DNA polymerase I. At higher temperatures DNA polymerase I tends to strand-displace rather than nick translate.

References

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5. Ausubel, F.M. et al. Current Protocols in Molecular Biology. *John Wiley and Sons*, Section 5.5, 1987.

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