

NZY First-Strand cDNA Synthesis Flexible Pack

Catalogue number	Presentation
MB40001	200 reactions

Description

NZY First-Strand cDNA Synthesis Flexible Pack provides robust transcription of RNA as it includes all the necessary components to synthesize first-strand cDNA from an RNA template. This optimized pack includes the following components: our modified recombinant form of the Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (RNase H minus) that synthesizes the complementary DNA strand using either RNA or single-stranded DNA as template, within an optimal temperature range of 50-55 °C; an optimized Reverse Transcriptases buffer; the NZY Ribonuclease Inhibitor for RNA protection against degradation due to ribonuclease contamination; a dNTPs mix; random hexamers and oligo(dT)₁₈ primers; and finally, an *E. coli* RNase H to specifically degrade the RNA template in cDNA-RNA hybrids after the first-strand cDNA synthesis. This flexible pack offers the possibility to choose the primer to initiate the reaction: you can choose either the primers provided in separate tubes, or a gene-specific primer. The pack is sufficient for up to 200 first-strand cDNA synthesis reactions and it is optimized for RT reactions over a wide range of total RNA concentrations from 10 pg to 5 µg. Downstream applications include real-time PCR, standard PCR and microarrays.

Shipping & Storage Conditions

The product can be shipped from dry ice to blue ice. Upon receipt, store all components of the pack at -30 °C to -15 °C in a constant temperature freezer. Stability can be extended by storing them at -85 °C to -65 °C. These meticulous storage procedures ensure that the NZY First-Strand cDNA Synthesis Flexible Pack delivers consistent and reliable results across its lifespan and usage. All components are formulated to be ready to use. The kit will remain stable till the expiry date if stored as specified.

Components

COMPONENT	MB40001 (200 reactions)	
	TUBES	VOLUME
NZY Reverse Transcriptase (200 U/µL)	1	220 µL
10x Reaction buffer for Reverse Transcriptases	1	750 µL
NZY Ribonuclease Inhibitor (40 U/µL)	1	220 µL
dNTP Mix (10 mM each)	1	200 µL
Random hexamer mix (50 ng/µL)	1	500 µL
Oligo(dT) ₁₈ primer mix (50 µM)	2	100 µL
NZY RNase H (<i>E. coli</i>) (5 U/µL)	1	220 µL
DEPC-treated H ₂ O	3	1 mL

Standard Protocol

Recommendations before starting

- **Nucleic acid manipulation:** High-quality intact RNA, free of residual genomic DNA and RNases, is essential for full-length, high-quality cDNA synthesis, and accurate RNA quantification. To ensure the integrity and purity of RNA, follow these precautions:
 - Maintain aseptic conditions: Always wear gloves, change them if suspected of contamination. We recommend using RNase-free plasticware/reagents, filtered tips and work in an RNase-free area (RNase Cleaner, Cat. No. MB16001, can help removing RNases from surfaces and materials). Designate a dedicated area and equipment solely for RNA work.
 - Store template RNA at -85 °C to -65 °C and avoid subjecting RNA to multiple freeze/thaw cycles. Perform all reaction steps on ice.
 - Assess the RNA purity concerning contaminants by examining the ratio of absorbance at 260 nm and 280 nm (A₂₆₀/A₂₈₀). Ideally, pure RNA should exhibit an A₂₆₀/A₂₈₀ ratio within the range of 1.9-2.1 in a 10 mM Tris-HCl buffer at pH 7.5.
 - DNase I (not provided) may be used to eliminate genomic DNA contamination from the starting total RNA.

- **Reagent usage:** The reaction buffer provided already contains Mg²⁺ at the optimal concentration for NZY Reverse Transcriptase activity. In extremely rare cases, the addition of extra Mg²⁺ for concentration adjustments may be considered. Upon thawing, if any precipitate is observed in the buffer, pulse vortex until the precipitate is completely resuspended.
- **Handling instructions:** Keep all reagents of the kit on ice while setting up the reactions. Minimize the duration of RNA exposure to ice.

Procedure for first-strand cDNA synthesis

1. On ice, in a sterile, nuclease-free microcentrifuge tube, prepare a reaction mixture, combining the following components (not provided)
Note: If setting up more than one reaction, prepare a reaction mixture volume 10% greater than the total required for the number of reactions to be performed.

COMPONENT	1 REACTION VOLUME / AMOUNT
total RNA; or mRNA / poly(A) RNA	10 pg – 5 µg 10 pg – 0.5 µg
Oligo(dT) ₁₂₋₁₈ (50-60 µM); or random hexamer (50-250 ng/µL); or gene-specific primer (2 µM)	1 µL (2.5-3 µM final conc.); (or 2.5-12.5 ng/µL final conc.); (or 0.1 µM final conc.)
dNTP Mix (10 mM each)	1 µL (0.5 mM final conc.)
DEPC-treated water	up to 16 µL

2. For some GC-rich RNAs or nucleic acids with high degree of secondary structure, a denaturation step may be required. If so, centrifuge briefly and incubate the reaction mixture at 65 °C for 5 min. Chill on ice for at least 1 min, briefly centrifuge again and place on ice.
3. On ice, perform the reverse transcription step, by adding the following reaction components to the previous mixture:

COMPONENT	1 REACTION VOLUME
10× Reaction Buffer	2 µL
NZY Ribonuclease Inhibitor	1 µL (40 units)
NZY Reverse Transcriptase	1 µL (200 units)
FINAL VOLUME =	20 µL

4. Mix gently and centrifuge briefly.
5. Incubate at 50 - 55 °C for 10 min.
Notes
 - When using random-hexamer primers, incubate first at 25 °C for 10 min and then proceed with the 50 - 55 °C incubation (step 5).
 - The NZY Reverse Transcriptase is fast enough to allow cDNA synthesis in as little as 10 minutes. However, in certain cases (e.g., for longer or complex templates), increasing the incubation time up to 20 - 30 minutes may enhance cDNA yields.
6. Inactivate the reaction by heating at 85 °C for 5 min, and then chill on ice.
7. **(optional)** Add 1 µL of NZY RNase H and incubate at 37 °C for 20 min.
Note: Addition of NZY RNase H will remove RNA bond to cDNA. This procedure is mainly recommended when util cDNA in downstream applications that either necessitate RNA-free DNA as a template or demand high sensitivity.
8. Store at - 85 °C to -15 °C or proceed to downstream applications.
Notes
 - cDNA can be stored frozen at -30 °C to -15 °C for short-term storage. It is also stable for up to one week when stored at 2 °C to 8 °C. For long-term storage, it is recommended to store at -85 °C to -65 °C. Avoid freeze/thaw cycles of the cDNA.
 - The resulting cDNA can be used for cloning or as a template in PCR or qPCR reactions. Typically, 10% (2 µL) of the first-strand reaction is enough for most PCR applications. Optionally, the cDNA can be diluted in TE buffer.
 - When performing qPCR using the synthesized cDNA as a template, ensure that no more than 1/10 of the final PCR volume is derived from the reverse-transcription product. For example, use up to 5 µL of cDNA obtained in the first-strand synthesis in a 50 µL PCR reaction.

Quality control

Purity

NZY Reverse Transcriptase, NZY Ribonuclease Inhibitor and NZY RNase H are >90% pure as judged by SDS polyacrylamide gel electrophoresis followed by Coomassie Blue staining.

Genomic DNA contamination

The components of the pack comply with internal standards of DNA contamination as evaluated through real-time qPCR.

Nucleases assay

All components of the pack are tested for DNase contamination, using 0.2-0.3 µg of pNZY28 plasmid DNA and 1 µg of RNA, respectively. Following incubation at 37 °C, the nucleic acids are visualized on a GreenSafe-stained agarose gel. There must be no visible nicking or cutting of the nucleic acids.

Functional assay

The components of the pack are tested for performance in a RT-qPCR experiment using a 10-fold serial dilution of total RNA from mouse liver (1 µg to 0.1 ng). The resultant cDNA is then used as template in a quantitative real-time PCR assay using specific primers to amplify mouse housekeeping genes.

Troubleshooting

Troubleshooting is often a systematic, meticulous process where varying one parameter at a time and evaluating impacts can unveil the root cause of issues. These adjusted suggestions, incorporating a blend of specificity and exploratory approaches, aim to enhance the clarity and actionability of your troubleshooting guide. Should any other technical or procedural aspects require attention, your feedback and additional information will always be welcomed.

NO OR INSUFFICIENT AMPLIFICATION PRODUCT IN RT-PCR/RT-qPCR
<ul style="list-style-type: none">• RNA damage or degradation
Analyse RNA on a denaturing gel to verify integrity. Use aseptic conditions while working with RNA to prevent RNase contamination. Replace water, plasticware and even the RNA if necessary.
<ul style="list-style-type: none">• Presence of RT inhibitors
Some inhibitors of RT enzymes include: SDS, EDTA, glycerol, sodium phosphate, spermidine, formamide and guanidine salts. Remove inhibitors by ethanol precipitation of the RNA preparation before use; wash the pellet with 70% (v/v) ethanol. For optimal outcomes, it is advised to start with RNA that has undergone purification using a silica-based method. Check purity of RNA template by determining the A260/A280 ratio.
<ul style="list-style-type: none">• Not enough starting RNA
Increase the concentration of starting RNA by optimizing the RNA extraction or purification process. Employ methods to enhance RNA yield or consider starting with a higher quantity of starting RNA in the reverse transcription reaction. Ensure that the RNA quality is maintained during the extraction process and reevaluate the RNA concentration using a reliable quantification method.
<ul style="list-style-type: none">• Inadequate temperature of reverse transcription
Reverse transcription should be conducted at 50 - 55 °C. However, in some cases, especially for high-complexity RNAs exhibiting a significant degree of secondary structure, it might be beneficial to raise the reaction temperature. The NZY Reverse Transcriptase can withstand temperatures up to 60 °C. However, it is crucial to assess the reaction temperature in the specific application context to avoid compromising enzyme sensitivity and cDNA yield.
<ul style="list-style-type: none">• Short incubation time for reverse transcription
The NZY Reverse Transcriptase is designed for rapid cDNA synthesis, requiring a 10-minute incubation. However, in some cases, such as when working with longer or complex templates featuring significant secondary structures, extending the incubation time up to 20 - 30 minutes can enhance the yield of cDNA.
<ul style="list-style-type: none">• Inadequate amount of Reverse Transcriptase
Using 1 µL of NZY Reverse Transcriptase is a typical starting point for standard first-strand cDNA synthesis. However, in specific cases, such as reverse transcribing long templates, adjusting the enzyme amount may be necessary for optimal results. We highly recommend conducting a titration of the enzyme volume to determine the optimal amount, starting with half or less than 200 units.
<ul style="list-style-type: none">• Incorrect priming
For long templates or when the template is difficult to copy entirely, the use of random hexamers provides versatility by priming throughout the RNA's entire length, resulting in a cDNA pool of varied lengths. On the other hand, oligo(dT) primers are specific for poly(A)+-selected

RNA, which encompasses most eukaryotic mRNAs. Utilizing both types of primers, by employing a mixture of random hexamers and oligo(dT) primers, can improve cDNA yields in certain circumstances.

- **Problems related to the PCR/qPCR setup**

Lack of product or delayed product detection in real-time PCR may arise from issues during cDNA amplification rather than during reverse transcription. To address these challenges, consider the following:

- **Primer Design and Concentration:** Confirm that primer design adheres to best practices. Optimize primer concentrations for efficient amplification.
- **Degradation of Primers/Probe:** Store primers/probe appropriately to prevent degradation. Use fresh, high-quality primers/probes for each experiment.
- **PCR Temperature and Cycling Conditions:** Ensure that PCR temperature profiles and cycling conditions are optimal for the assay. Validate and, if necessary, optimize the annealing and extension temperatures.
- **Insufficient Starting Template:** Increase the concentration of the starting cDNA template if necessary. Reassess the RNA input to ensure adequate cDNA synthesis.
- **Pipetting Errors:** Double-check pipetting accuracy to avoid errors in reagent volumes. Use calibrated pipettes for precision in dispensing reagents.
- **PCR Enzyme/Master Mix:** Verify the integrity and activity of the PCR enzyme/master mix. Consider using a fresh aliquot or a different batch if there are concerns about the quality.
- **Detection step:** Ensure that fluorescence detection occurs during the extension step of the real-time PCR cycling program. Verify that the correct fluorescent channel is being used.

UNEXPECTED BANDS AFTER ELECTROPHORETIC ANALYSIS OF AMPLIFIED PRODUCTS / MULTIPLE PEAKS IN THE MELTING CURVE

- **Non-specific Amplification**

In the reverse transcription step, optimize reverse transcription conditions. Also consider using gene-specific primers or the highly thermostable Supreme NZY Reverse Transcriptase (Cat. No. MB448). For the PCR/real-time PCR step, implement a hot start PCR strategy or use an enzyme designed to minimize nonspecific amplification during the initial stages. Additionally, optimize primer design, and verify primer specificity through bioinformatics tools. Adjust annealing temperatures accordingly.

- **Contaminated Reagents or Equipment**

Use sterile and filtered tips, fresh reagents, and regularly clean pipettes and equipment. Use molecular-grade water and DEPC-treated water.

AMPLIFICATION IN THE NTC

- **Genomic DNA contamination**

Include a control PCR reaction without NZY Reverse Transcriptase (no RT control) to assess the presence of genomic DNA contamination; this control will also help to confirm the specificity of the primers for cDNA amplification. Design primers that span exon-exon junctions to ensure specificity for cDNA amplification. If required, apply DNase I treatment to the RNA samples before reverse transcription to eliminate any residual genomic DNA, by ensuring the DNase I treatment is thorough and follows the recommended protocol. Additionally, the cDNA template can be diluted before being used in the PCR to decrease the chances of genomic DNA contamination, as genomic DNA is present in much higher amounts than cDNA.

For life science research only. Not for use in diagnostic procedures.

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