MB172_IFU_EN_V2401

NZY M-MuLV First-Strand cDNA Synthesis Kit

Catalogue number	Presentation
MB17201	50 reactions
MB17202	250 reactions

Features

- Cost-effective kit using NZY M-MuLV Reverse Transcriptase
- Provides high yields of full-length cDNA products for use in qPCR/PCR assays
- Formulated to increase sensitivity in RT-qPCR
- Primer type: oligo(dT)$_{18}$ and random hexamers
- Starting material: 10 pg to 5 µg of total RNA
- Optimal reaction temperature: 37 - 42 °C

Description

NZY M-MuLV First-Strand cDNA Synthesis Kit is a system that includes all the necessary components to synthesize first-strand cDNA, except the template RNA. The resulting single-stranded cDNA is suitable for use in real-time quantitative PCR (qPCR). NZY M-MuLV First-Strand cDNA Synthesis Kit is formulated to provide high yields of full-length cDNA products and to increase sensitivity in downstream applications. Starting material can range from 10 pg up to 5 µg of total RNA. The kit includes a combination of random hexamers and oligo(dT)$_{18}$ primers to increase priming efficiency and yield. The primers are included in the NZYRT 2× Master Mix, which also contains dNTPs, Mg^{2+} and an optimized RT buffer. NZYM-MuLV RT Enzyme Mix includes both NZY M-MuLV Reverse Transcriptase (RNase H minus) and a powerful NZY Ribonuclease Inhibitor to protect RNA against degradation due to ribonuclease contamination. RNase H (from *E. coli*) is provided in a separate tube to specifically degrade the RNA template in cDNA-RNA hybrids after the first-strand cDNA synthesis. This procedure may improve the sensitivity of subsequent real-time PCR reactions since PCR primers will bind more easily to the cDNA.

Shipping & Storage Conditions

This product is shipped in dry ice. Upon receipt, store all components of the kit 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 M-MuLV First-Strand cDNA Synthesis Kit, separate oligos, 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

	MB17201 (50 reactions)		MB17202 (250 reactions)	
COMPONENT	**TUBES**	**VOLUME**	**TUBES**	**VOLUME**
NZYM-MuLV RT Enzyme Mix (1)	1	100 µL	5	100 µL
NZYRT 2× Master Mix (2)	1	500 µL	5	500 µL
NZY RNase H (*E. coli*)	1	50 µL	5	50 µL
DEPC-treated Water	1	1 mL	2	1 mL

(1) Includes NZY M-MuLV Reverse Transcriptase and NZY Ribonuclease Inhibitor

(2) Includes oligo(dT)$_{18}$, random hexamers, Mg^{2+} and dNTPs

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 kit components already include a Ribonuclease inhibitor and dNTPs. Do not add extra RNase inhibitor and dNTPs. Note that this kit does not include a control RNA. We highly recommend using the provided DEPC-treated water; however, other sterile, nuclease-free water may also be used.

▪ Handling instructions: Keep all reagents of the kit on ice while setting up the reactions. Minimize the duration of RNA exposure to ice.

Procedure

1. Thaw kit components and RNA (not provided) on ice. Gently mix each reagent by flicking the tubes before pulse-spinning them prior to opening.
2. On ice, add the following reaction components to a sterile, nuclease-free microcentrifuge tube.
   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.*
   Note: *It is highly recommended to include a negative control without RNA.*

COMPONENT	1 REACTION VOLUME
NZYRT 2× Master Mix	10 µL
NZYM-MuLV RT Enzyme Mix	2 µL
RNA (up to 5 µg)	X µL
DEPC-treated Water	up to 20 µL

3. Mix gently and incubate at 25 °C for 10 min.
4. Incubate at 37 - 42 °C for 50 min.
5. Inactivate the reaction by heating at 70 °C for 15 min (or at 85 °C for 5 min), and then chill on ice.
6. **(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.*
7. Use the cDNA product directly in PCR or qPCR diluted in TE buffer or undiluted; or store at -85 °C to -15 °C until required.
   Notes
   - *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.*
   - *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.*

Quality control

Purity

NZY M-MuLV Reverse Transcriptase and NZY Ribonuclease Inhibitor present in the NZYM-MuLV RT Enzyme Mix are >90% pure as judged by SDS polyacrylamide gel electrophoresis followed by Coomassie Blue staining.

Genomic DNA contamination

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

Nucleases assay

To test for DNase contamination, 0.2-0.3 µg of pNZY28 plasmid DNA are incubated with the kit component in test for 14-16 h at 37 °C. To test for RNase contamination, 1 µg of RNA is incubated with the kit component in test for 1 h at 37 °C. Following incubation, 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

NZY M-MuLV First-Strand cDNA Synthesis Kit is 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
• **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.
• **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.
• **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.
• **Inadequate temperature of reverse transcription**
The optimal reaction temperature for NZY M-MuLV Reverse Transcriptase activity is 37 °C. However, for high-complexity RNAs with considerable secondary structure, raising the temperature to 42 °C may be advantageous. In cases of very complex template structures, the NZY First-Strand cDNA Synthesis Kit (Cat. No. MB124), featuring the thermostable NZY Reverse Transcriptase, may be preferable.
• **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 NZYM-MuLV RT Enzyme Mix (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.