Polaris® Lyo RT-LAMP Master Mix 4x

Catalogue number	Presentation
MD06971	for 660 µL (100 rxns of 25 µL)
MD06972	for 25 x 660 µL (2.5k rxns of 25 µL)

Introducing the Polaris® brand

NZYtech, with its established expertise in enzyme development and IVD kit production, proudly introduces Polaris® - a groundbreaking series of newly developed diagnostic enzymes, master mixes and reagents. Polaris® brand products set the standard in purity, extreme stability, diagnostic performance, reliability, and regulatory compliance. These attributes are housed in functional packaging tailored for stringent laboratory applications. Polaris® stands at the forefront of innovation, designed to meet the complex demands of molecular diagnostics with a steadfast focus on quality and scientific integrity. At its core, Polaris® adheres to stringent international quality standards, including ISO 9001 and ISO 13485, ensuring its enzymes and reagents are perfectly suited for a wide array of IVD applications. These products surpass the stringent European IVDR requirements, demonstrating a commitment to quality management and excellence in every aspect of their development and production. Utilizing cutting-edge manufacturing protocols, precise control measures, and rigorous validation, Polaris® becomes the new benchmark for diagnostic testing. NZYtech's state-of-the-art facilities are optimized to produce these high-precision diagnostics tools, ensuring unmatched accuracy and performance. Our team is always ready to offer comprehensive support to our customers and partners, assisting with IVDR compliance and ensuring smooth integration, upon request. NZYtech is committed to advancing the field of molecular diagnostics, thereby expanding access to clinical results, enabling rapid diagnostics, and fostering research advancement.

Features

- Lyophilized mixture, with all essential components for RT-LAMP testing, except template and primers.
- Superior specificity at 65-70°C, enhancing diagnostic accuracy and reducing NTC amplifications.
- Provided freeze-dried, offering superior stability for extended storage and simplified shipping.
- Incorporates a powerful Bst DNA polymerase with strong strand displacement capabilities for efficient and reliable isothermal amplification.
- Extensively validated to deliver consistent performance across various conditions and biological matrices.

Description

Polaris® Lyo RT-LAMP Master Mix 4x is a state-of-the-art solution crafted for efficient, precise, and economical reverse transcription loop-mediated isothermal amplification (RT-LAMP) testing. This Master Mix integrates the latest generation of strand-displacing DNA polymerases, specifically optimized for LAMP assays within diagnostic settings, as well as new and highly efficient reverse transcriptases. It includes a finely adjusted reaction buffer and additives, meticulously selected to maximize enzymatic performance. Additionally, this Master Mix is supplied already freeze-dried, facilitating storage and transport. The use of the supplied Reconstitution buffer for Polaris® Lyo RT-LAMP Master Mix 4x allows for immediate use of the Master Mix. Engineered for enhanced performance, our Master Mix enables robust RT-LAMP amplification at elevated temperatures (65 to 70 °C, optimally at 68-69 °C), improving assay specificity and minimizing undesired amplification. The ability to function at higher temperatures not only sharpens assay specificity but also strengthens resistance to common amplification inhibitors found in a wide range of samples. Offered in a concentrated 4x format, it allows for immediate use upon reconstitution for molecular research and diagnostics in remote areas where refrigerated shipping is problematic. Polaris® Lyo RT-LAMP Master Mix 4x, through rigorous validation and its flexible design, stands out as a versatile and reliable tool for diagnostics. It is crafted to fulfil a variety of diagnostic needs, providing consistent and reliable outcomes across different biological matrices and testing conditions.

Shipping & Storage

This product is shipped at room temperature. Upon receipt, preferably (obligatory after reconstitution) store all components at -85 °C to -15 °C in a constant temperature freezer. Avoid direct sunlight exposure. Immediately after use, swiftly return the components to a temperature between -85 °C and -15 °C to minimize exposure to room temperature. This product is stable through a minimum of 10 freeze-thaw cycles. Adhering to these meticulous storage procedures ensures that Polaris® Lyo RT-LAMP Master Mix 4x will remain stable until the expiry date and deliver reliable and consistent performance in all applications.

Components

This product provides the following set of reagents in sufficient amounts to perform 100 or 2500 *in vitro* RT-LAMP reactions of 25 µL each.

COMPONENT		MD06971		MD06972	
		TUBES	VOLUME	TUBES/ BOTTLES	VOLUME
Lyo RT-LAMP MMix 4x	Polaris® Lyo RT-LAMP Master Mix 4x	1	For 660 µL	25	For 660 µL
RBuffer for Lyo RT-LAMP MMix	Reconstitution buffer for Polaris® Lyo RT-LAMP Master Mix 4x	1	700 µL	1	18 mL
$MgSO_4$ 100 mM	Polaris® $MgSO_4$ 100 mM	1	160 µL	1	4 mL

Reagents, Materials and Equipment Required but Not Provided

To utilize Polaris® Lyo RT-LAMP Master Mix 4x in RT-LAMP protocols, additional reagents are necessary, though not provided with this product. For optimal efficiency, we recommend the use of complementary reagents from NZYtech's innovative Polaris® series. Among the following recommended reagents, particularly the Polaris® Speedy LAMP Fluorescent dye 25x might not be required if not conducting a fluorescence-based evaluation of the amplification; however, this dye also contains several reaction accelerators that might be necessary in certain circumstances, even when fluorescence analysis is not performed (please refer to the technical note bellow – Signal Detection).

REAGENTS NOT PROVIDED	TEST COMPONENT	CAT. NO.
Polaris® Speedy LAMP Fluorescent dye 25x	**Speedy LAMP dye 25x***	MD0756
DEPC-treated water	**DEPC-treated water**	MB43701

* Only required if the RT-LAMP reaction is to be monitored in real-time through fluorescence in a specialized thermocycler.

Other Essential Materials and Equipment required but not provided are:

- Real-time PCR Instrument (in case RT-LAMP reaction is to be run in a thermocycler): Ensure the instrument is capable of detecting the FAM™/SYBR fluorescent dyes (emission wavelengths of approximately 520 nm).
- RNase & DNase-free PCR Plasticware: including PCR tubes, strips, caps, 96-well plates and adhesive films.
- Pipettors and Filter Tips: ensure that they are RNase & DNase-free.
- Disposable Gloves: to prevent contamination and maintain sample integrity.
- Vortex and Centrifuge: essential for mixing and reaction preparation.

Ensure that all reagents and equipment used comply with the appropriate standards for molecular diagnostic use. Follow all relevant guidelines and manufacturer recommendations for handling and use.

Standard Protocol

Recommendations before starting.

- Handling instructions:
  - To help prevent any carry-over DNA/RNA contamination, you should assign independent areas for reaction set-up and RT-LAMP amplification. It is essential that any tubes containing amplified product should not be opened in the RT-LAMP set-up area. Use sterile filtered tips.
  - All pipetting actions and experimental plate preparations must be diligently performed on benchtop coolers or ice to safeguard the integrity of the reagents and to mitigate the risk of generating RT-LAMP artifacts, which could compromise the sensitivity and/or specificity of detection.
  - Upon reaction setup, swiftly progress to initiating the RT-LAMP protocol; any delay or prolonged incubation of reaction mixes at room temperature may inadvertently foster the emergence of artifacts.
- Reagent usage:
  - It is strongly recommended to thoroughly review the usage instructions of all involved reagents before assay execution.
  - Reconstitute the lyophilized Master Mix by following the instructions below before any work is performed with these reagents.
  - Ensure homogeneity of the reagents prior to use. To achieve this, gently flick the tubes provided to homogenise the contents, then centrifuge for a few seconds to collect the contents at the bottom of the tube. Maintain tubes on ice.

- To avoid cross-contamination, we strongly recommend pipetting the template and particularly the Positive Controls last, only after all the other components have been used and remaining material properly stored.
- Always use sterile molecular grade, nuclease free water.

▪ Controls: To verify the absence of contamination, prepare a negative control reaction without a template (No-template control or negative control). Additionally, include a positive control to serve as a reference for ensuring the correct functioning of the RT-LAMP reaction and detection system. The positive control should exhibit the expected amplification and/or fluorescence signal, confirming the assay's ability to accurately detect the target sequence.

Procedure for RT-LAMP testing

This standard protocol provides a foundational guideline for conducting RT-LAMP reactions. While it serves as a reliable starting point, some parameters may require adjustments based on specific needs, such as reaction temperature or the initial quantity of template and, as such, this protocol can be adapted accordingly. The effectiveness of this suggested protocol is contingent on the proper storage and condition of all supplied components.

1. Thaw all components on ice. Before any tube is opened, quickly vortex the tubes RBuffer for Lyo RT-LAMP MMix and $MgSO_4$ 100 mM (do not vortex the lyophilized Master Mix before reconstitution) before pulse-spinning their contents.
Note: *Avoid using the same vortex and centrifuge used for the template with the other reaction components, particularly once they have been used.*

2. Reconstitute the lyophilized RT-LAMP Master Mix 4x by adding 660 µL of RBuffer for Lyo RT-LAMP MMix to the Lyo RT-LAMP MMix 4x tube. Eject the reconstitution buffer slowly, against the inner wall of the tube, making sure that all lyophilized material is dissolved into it. Briefly pulse vortex and pulse spin the Master Mix tube. Leave the Master Mix on ice for 10 min to ensure proper reconstitution. The Master Mix is now ready to be used.

3. In the clean reaction setup area, prepare a RT-LAMP reaction mixture according to the table below that specifies the volumes for 1 and *n* reactions, as required for your experiments, into a sterile tube (not provided).
Note: *Include sufficient reactions for the No-Template and positive controls, if required. We strongly recommend performing replicates of all reactions.*

COMPONENT	1 REACTION VOLUME (µL)	*n* REACTIONS VOLUME (µL)
Reconstituted Lyo RT-LAMP MMix 4x	6.25	*n* x 6.25
Primer Mix 10x (not provided)	2.5	*n* x 2.5
Polaris® $MgSO_4$ 100 mM (*)	1.5	*n* x 1.5
Polaris® Speedy LAMP Fluorescent dye 25x (not provided)	1	*n* x 1
Ultra-pure, molecular-grade water (not provided) (**)	8.75	*n* x 8.75
TOTAL	**20**	***n* x 20**

(*) The Lyo RT-LAMP Master Mix 4x includes $MgSO_4$ for a final (1x) concentration of 2 mM. The addition of this extra volume of $MgSO_4$ results in a final magnesium concentration of 8 mM in the reaction. The final magnesium content can be adjusted to meet the requirements of specific reactions.
(**) If necessary, the water volume can be reduced to allow for a maximum template usage of 12.5 µL. In this instance, the volume is tailored for using 5 µL of template, although this amount may vary.

4. Pipette 20 µL of the RT-LAMP reaction mixture into individual wells, according to your RT-LAMP experimental plate/strip/tube configuration.

5. For the No-Template Control, add 5 µL of ultra-pure, molecular-grade water, instead of the RNA template, into the designated well(s). The final volume in each well should be 25 µL. Cover wells with appropriate caps.

6. For the remaining reactions, add up to 5 µL of your template into the respective wells (add molecular-grade water to fill up to 5 µL if needed). The final volume should be 25 µL.

7. Cover and seal the plate/strip/tube with appropriate caps or optical adhesive film before proceeding with the RT-LAMP detection steps.

8. Place the reaction plate/strip/tube within the real-time instrument and run the general RT-LAMP protocol defined below. These conditions might be adapted to suit your specific needs, within sensible limits.

NUMBER OF CYCLES	TEMPERATURE	TIME	STAGE
40	69 °C (65-70 °C)	30 seconds	Amplification
1	95 °C	3 minutes	Enzyme inactivation
1	65 → 99 °C	10 second intervals	Melting curve *

***** *While not mandatory, the production of a Melting curve profile is invaluable when evaluating reaction efficiency and specificity.*

9. To assess the specificity of the RT-LAMP amplification reaction when performing fluorescence-based real-time RT-LAMP, it is highly recommended to include a Melt Curve step: 65 → 99 °C at 10 second intervals.

10. Store at - 85 °C to -15 °C or directly proceed to downstream applications.

Technical Notes

Sample material

Achieving optimal results in RT-LAMP molecular testing requires meticulous attention to various factors, including the protocol for sample collection from biological specimens and the methods of sample transport, storage, and processing. Upon collection, samples should be promptly tested and must be transported and stored at low temperatures, complying with local biosafety regulations. Ensure the suitability of RNA samples in terms of purity, concentration, and nucleic acid integrity. The optimal amount of starting material may vary depending on its quality and complexity. In general, we recommend using 1 ng to 500 ng of mammal mRNA templates (>100 target copies/reaction).

Primers

A typical RT-LAMP assay incorporates a set of four primers designed to specifically recognize distinct regions within the target sequence. These primers are categorized into two pairs: two outer primers and two inner primers, commonly referred to as FIP (Forward Inner Primer), BIP (Backward Inner Primer) for the inner pair, and F3 (Forward Outer Primer) and B3 (Backward Outer Primer) for the outer pair. To enhance the efficiency and reduce the reaction time of the isothermal amplification, an additional pair of loop primers, LoopF (Forward Loop Primer) and LoopB (Backward Loop Primer), can be incorporated. For the preparation of a RT-LAMP Primer Mix, both sets comprising either 4 or 6 primers (including Loop primers) can be utilized. A recommended 10x RT-LAMP Primer Mix should include the following concentrations: 16 µM each for FIP and BIP, 2 µM each for F3 and B3, and 4-8 µM each for LoopF and LoopB, diluted in TE Buffer or water. Please follow the general guidelines for RT-LAMP Primer Design:

- Primer Length: Primers should be between 15 and 25 nucleotides in length.
- Amplicon Characteristics: Aim for an amplicon length of less than 300 base pairs, with the distance between the FIP and BIP primers ranging from 120 to 160 base pairs.
- GC Content: Maintain a GC content within the range of 45-60%. It is critical to avoid regions prone to forming secondary structures or containing single or dinucleotide repeats.
- Melting Temperature (Tm): Ensure that the melting temperatures of primer pairs are closely matched, with less than a 5°C difference, to facilitate uniform annealing across all primers.

Leveraging available online software for primer design is highly recommended, especially when targeting novel genes. These software tools are equipped with algorithms that consider the intricacies of primer design, including specificity, melting temperature (Tm), GC content, and potential for secondary structures or primer-dimer formation. For novel targets, it is advisable to design and evaluate multiple sets of primers to ensure optimal performance in RT-LAMP assays. Using online software for primer design streamlines the process, making it more efficient and less prone to error. These tools can quickly analyze genetic sequences to identify optimal primer binding sites while minimizing the risk of non-specific amplification. Moreover, these software tools can assist in predicting potential issues such as primer-dimer formation or secondary structures, which are critical for the success of RT-LAMP assays.

Testing multiple primer sets with control kits, such as those offered by NZYtech, NZY LAMP Positive Control Kit (Cat. No. MB48001), or NZY RT-LAMP Positive Control Kit (Cat. No. MB48101), provides a systematic approach to selecting the best primer set, ultimately ensuring the success of molecular diagnostics applications. In addition, introducing a melting curve analysis is valuable for confirming the specificity and purity of the products generated during the RT-LAMP reaction. A melt curve helps to distinguish between specific amplification products and nonspecific products or primer dimers – specific products typically exhibit a sharp and distinct melting peak, corresponding to the expected Tm of the target sequence, while nonspecific products or primer dimers may produce different melting temperatures or broad peaks, indicating less specificity.

$MgSO_4$

Mg^{2+} concentration optimization can be performed by testing a range between 2 – 16 mM of $MgSO_4$ final concentration. It is essential to note that the Polaris® Lyo RT-LAMP Master Mix 4x already incorporates $MgSO_4$ in its formulation for a final (1x) concentration of 2 mM.

Signal Detection

The optional Polaris® Speedy LAMP Fluorescent Dye 25x (not included) is a DNA intercalating dye. As the amount of DNA increases during the amplification process, the dye intercalates into the DNA strands. This results in a fluorescent signal, which can be easily detected and quantified in real time. The intensity of the fluorescence is proportional to the amount of DNA amplified, providing a direct measure of the reaction's progress. Furthermore, this dye also contains reaction accelerators, which significantly reduce the time-to-result of a typical RT-LAMP reaction. The combination of these characteristics makes the use of the Polaris® Speedy LAMP Fluorescent Dye 25x in a RT-LAMP assay a powerful tool for the rapid, specific, and sensitive detection of nucleic acids. Polaris® Lyo RT-LAMP Master Mix 4x leverages these principles to deliver reliable and efficient diagnostic results, making it an invaluable asset in molecular biology and diagnostic laboratories.

Negative Control

Including a negative control in RT-LAMP assays is crucial to validate positive results and ensure the reliability of the assay. Due to the nature of RT-LAMP reactions, which involve multiple primers, there is a significant risk of non-specific amplification, especially when the initial template amounts are low. A negative control (i.e. a reaction without template, or No-Template Control), accompanied by melting curve analysis, is essential for identifying any non-specific amplification and serves as an internal validation of the assay's accuracy. To perform a negative control, simply replace the RNA template in the reaction mix with molecular-grade ultra-pure water. Since Polaris® Lyo RT-LAMP Master Mix 4x includes an engineered Bst enzyme that operates at higher temperatures, the elevated amplification temperature minimizes the risk of non-specific amplification typically observed in Non-Template Controls. In any case, melting curve analysis enables the differentiation of non-specific templates when compared to the target product.

Positive control

Including a positive control in each experiment is crucial to verify the assay's functionality. The absence of a signal in the positive control indicates a potential issue with the experimental setup, requiring further investigation and correction. If needed, NZYtech provides a RT-LAMP Positive Control kit (MB481), purposedly designed for validating RT-LAMP reagents, set-ups, and reactions.

Data

Typical amplification plots obtained with Polaris® Lyo RT-LAMP Master Mix 4x (blue) with human genomic template compared with the with Polaris® Lyophilized RT-LAMP Master Mix 4x (MD0681, red) are illustrated in Figure 1.

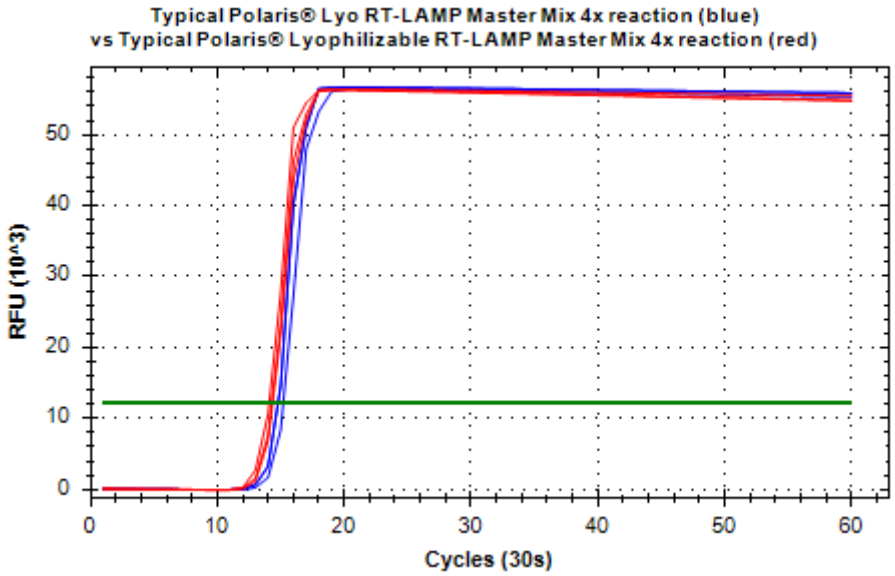

Figure 1. Typical RT-LAMP Amplification Plot. The graph represents the amplification curves of a typical RT-LAMP assay using Polaris® Lyo RT-LAMP Master Mix 4x (blue traces) comparatively against Polaris® Lyophilizable RT-LAMP Master Mix 4x (red traces). It displays the real-time progression of DNA synthesis as measured by fluorescence intensity on the y-axis against the elapsed reaction time on the x-axis.

Quality control assays

Purity

All enzymes present in Polaris® Lyo RT-LAMP Master Mix 4x are > 98% pure as judged by SDS polyacrylamide gel electrophoresis followed by Coomassie Blue staining.

Genomic DNA contamination

Polaris® Lyo RT-LAMP Master Mix 4x components are tested to verify that ≤ 0.75 copies of bacterial gDNA and no human DNA are present. This is evaluated through qPCR detection.

Nucleases assay

No nuclease contamination is detected in Polaris® Lyo RT-LAMP Master Mix 4x given that there is no visible nicking or cutting of the nucleic acids. To test for DNase contamination, 0.2-0.3 µg of pNZY28 plasmid DNA are incubated with each of the Polaris® Lyo RT-LAMP Master Mix 4x components for 14-16 h at 37 °C. To test for RNase contamination, 1 µg of RNA is incubated with these components for 1 h at 37°C. Following incubation, the nucleic acids are visualized on a GreenSafe-stained agarose gel.

Functional assay

The Polaris® Lyo RT-LAMP Master Mix 4x is tested for performance in Reverse Transcription Loop-mediated isothermal amplification (RT-LAMP) reactions using viral samples.

Troubleshooting

Troubleshooting RT-LAMP assays requires a methodical approach, where altering one variable at a time and evaluating its impact can reveal the root cause of any issues encountered. The following recommendations are aimed at addressing common problems that may arise during RT-

LAMP amplification using Polaris® Lyo RT-LAMP Master Mix 4x. These adjusted suggestions, incorporating a blend of specific and exploratory approaches, aim to enhance the clarity and applicability of your troubleshooting guide. Should any other technical or procedural aspects require attention, your feedback and additional information will always be welcomed.

NO AMPLIFICATION DETECTED
• **Inappropriate storage conditions.**
Store Polaris® Lyo RT-LAMP Master Mix 4x away from light, preferably within the product box in a freezer (-85 °C to -15°C). Limit exposure to ambient laboratory lighting and avoid direct sunlight.
• **Excessive amount of sample in the reaction**
You may use up to 50% of the sample/template in a RT-LAMP reaction. In the case of sample-caused reaction inhibition, this value should optimally be reduced to 4 to 10% per reaction, which corresponds to 1 to 2.5 µL of lysate per 25 µL reaction.
• **Suboptimal RT-LAMP conditions**
Explore varying the reaction temperature, adjusting reaction time, or increasing the amplification protocol over 20 min to enhance amplification. Ensure that these alterations do not result in non-specific product amplification.
• **Contamination with DNases**
Ensure that all labware, including pipettes, tubes, and containers, is clean and free from residual DNase/RNase contamination. Use DNase/RNase-free, autoclaved, or sterile equipment whenever possible. Use DNase/RNase-free water (we highly recommend using NZYtech's ultra-pure, DEPC-water – MB43701). Change gloves frequently. If need to store extract samples for an extended period, always freezing them between -85 °C and -65 °C; this will help prevent DNase/RNase activity.
LOW AMPLIFICATION YIELD
• **Ineffective reaction temperature**
Optimize the RT-LAMP protocol by tweaking the reaction temperature and time, while observing the impact on amplicon yield.
• **Presence of inhibitors**
While robust to most inhibitors, Polaris® Lyo RT-LAMP Master Mix 4x is nevertheless susceptible to inhibition. Most common inhibitors are well tolerated in normal quantities, but larger amounts may reduce reaction efficiency and amplicon yield. Take care not to use overly crude samples in large amounts or less-than-ideal reaction components (for example, molecular-grade, DEPC-treated water is always recommended).
AMPLICON WITH INCORRECT SIZE OR UNEXPECTED AMPLICON NUMBERS
• **Primer mis-design or mis-binding**
Verify the design of the primers and validate their ability to selectively amplify the desired fragment from 1-10 ng of purified RNA. Poor RT-LAMP primer design is responsible for most non-specific amplifications.
• **Cross-contamination**
Create separate work areas for sample processing and RT-LAMP setup. Use separate, dedicated pipettes, and disposable tips for each setup. Always follow good laboratory practices to avoid contamination. Include negative controls (no RNA template) in RT-LAMP reactions to monitor for contamination in reagents or labware.

By systematically addressing these troubleshooting aspects, users can enhance the performance and reliability of RT-LAMP assays conducted with Polaris® Lyo RT-LAMP Master Mix 4x. Remember that troubleshooting is a progressive process, and careful attention to detail can lead to resolutions. If persistent issues occur, please consult with NZYtech's technical support for further assistance.

NZYtech genes & enzymes

Suitable for veterinary, agriculture, water, and pharmaceutical testing procedures. Do not use in human diagnostic (IVD) procedures.