1st edition: 2024.3
Cat. No. A29-0059

Protein synthesis kit
Musaibo kun SI
Operating instructions

The product does not contain stable isotope-labeled amino acids. Please utilize the stable isotope-labeled amino acids mixture, which is available for separate purchase, in order to prepare stable isotope-labeled proteins. Prior to usage, we kindly request that you thoroughly peruse this manual to acquire a comprehensive understanding of the appropriate handling techniques.

- In order to enhance the quality, the specifications, designs, text, etc. included in this manual are subject to change without prior notice.
- We make every effort to ensure the accuracy of all information provided in this manual. If you have any inquiries, comments, or notice any omissions, please contact us at the following email address.

Customer Support E-mail: Isotope.TNS@tn-sanso.co.jp

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1. Introduction

Thank you for your purchase of the Protein Synthesis Kit “Musaibo kun SI”. This kit has been developed under license from RIKEN, incorporating their exclusive and advanced cell-free protein synthesis technology into a convenient kit.

This protein synthesis system employs E. coli cell extract, enabling straightforward and efficient protein expression through the addition of circular or linear DNA as template DNA. This allows for the transcription of mRNA using T7 RNA polymerase.

To ensure proper handling, we kindly request that you carefully read and familiarize yourself with the contents of this manual.

2. Safety Precautions

This product is intended for research purposes only. It is strictly prohibited to utilize this kit, the protein, or any other components obtained from this kit for medical care and/or clinical diagnoses of humans and animals, or to incorporate them into beverages or food items.

This kit is designed for use by experienced individuals with expertise in general biochemical experiments and micropipette operations. Individuals lacking such experience must refrain from using this product.

When handling the product, it is imperative to wear appropriate safety goggles, gloves, lab coat, and other protective equipment. In the event of contact with the solution on the eyes and/or skin, promptly rinse with clean running water. If any inflammation occurs, seek immediate medical attention.

Please be aware that we assume no responsibility for any issues that may arise from unauthorized use of this product, not in accordance with this manual.

The caution box below highlights areas that require special attention to ensure safe usage. Please carefully observe the points indicated in this caution.

Caution	Risk of minor injury or physical damage may occur due to improper handling.

3. Kit Contents

● Musaibo kun SI Internal solution	775 µL	1
● Musaibo kun SI External solution with a stir bar	8.25 mL	1
● Musaibo kun Unlabeled-amino acid mixture	1 mL	1
● Control DNA (pUC-CAT)*, 50 ng/µL *Expresses chloramphenicol acetyl transferase (CAT).	50 µL	1
● Dialysis device (MWCO: 15,000)		1

4. Storage

Store at -80°C ±2°C

Deviations from the recommended storage temperature of -80°C significantly reduce protein synthesis performance.

During transportation or temporary storage, utilize a container such as Styrofoam with dry ice.

Once thawed, the reaction mixture premix should be immediately placed on ice, divided into the necessary quantity, and rapidly frozen using liquid nitrogen or powdered dry ice. Store at -80°C. Other solutions should be aliquoted and stored at -30°C or below. Please ensure to use the divided solution completely during subsequent use, as repeated freeze-thaw cycles diminish protein expression efficiency.

For transportation or temporary storage, it is recommended to use an insulated container with dry ice, such as Styrofoam.

5. Main Components

Musaibo kun SI Internal solution

E. coli cell extract	Folinic acid	NTP
Magnesium acetate	Creatine phosphate	HEPES
T7 RNA polymerase	Polyethylene glycol	tRNA
Sodium azide	Creatine kinase	DTT
Ammonium acetate	D-glutamate	cAMP

Musaibo kun SI External solution

Magnesium acetate	Creatine phosphate	NTP
Potassium acetate	HEPES	Sodium azide
Polyethylene glycol	Folinic acid	DTT
D-glutamate	Ammonium acetate	cAMP

Note: Contains 0.05% sodium azide (NaN_3), which is classified as a designated poisonous substance under the Deleterious Substances and Deleterious Substances Control Law in Japan. However, since the content is 0.1% or less, the aforementioned regulation does not apply. Nevertheless, please exercise caution when handling and disposing of this substance.

6. Disposal

Caution	The kit may contain *E. coli* bacteria (non-genetically modified). The solution, as well as any containers, devices, etc. that come into contact with the solution, must be sterilized using an autoclave or similar method, and then disposed of in compliance with local regulations. Please refer to “5. Main Components” and the attached MSDS for further information.

The customer is responsible for the proper disposal of proteins and other products obtained using this kit.

7. Warranty

Warranty period

Please ensure to use this kit before the expiration date indicated on the product label. The kit will be delivered frozen on dry ice. If there is no dry ice remaining in the delivery box upon arrival or if there is any damage to the package and/or leakage of the solution, it is possible that the quality may have been compromised. In such cases, please contact us and we will provide a free replacement of the product.

Disclaimer

We do not assume any liability for the following circumstances, even within the warranty period:

- Defects resulting from improper storage and/or improper usage.
- Defects unrelated to the performance of this kit.
- The protein synthesis activity may decrease due to various factors unrelated to the performance of this kit. Therefore, we cannot guarantee the expression of proteins. Please be aware that we do not accept responsibility

for any indirect damages resulting from defects in this kit or damages arising from the use of products obtained using this kit or similar methods.

8. Template DNA

In order to perform protein expression using this kit, it is necessary to prepare a template DNA that contains the essential components for mRNA transcription by T7 RNA polymerase and translation. These components consist of the T7 promoter, the T7 terminator, the ribosome binding site (RBS), and the target gene, as depicted in the diagram below. It is crucial to ensure that all the required elements are present in the template DNA to achieve successful protein expression.

Note: Protein yield is greatly dependent on the amino acid and/or DNA sequence, particularly in the vicinity of the N-terminus. Addition of high-yield N-terminal tags (Yabuki, 2007) is recommended.

RBS: Ribosome binding site **ATG**: Initiation codon **TAA**: Termination codon

9. Protein Synthesis

Caution	Do not handle this kit with bare hands, as there is a risk of frostbite due to the storage temperature of -80°C. Please ensure to wear appropriate safety goggles, gloves, lab coat, and other protective gear when handling this kit. In the event that the solution of the kit comes into contact with the eyes and/or skin, it may cause inflammation.

Devices, Reagents, etc. Prepared by User

Before using the kit, please prepare the following items:

1) Stable isotope-labeled amino acid mixture solution for Musaibo kun (Please refer to Chapter 12 for more information).
2) Template DNA
   - A minimum of 50 µL of circular DNA (at a concentration of 50 ng/µL or higher) or linear DNA (at a concentration of 100 ng/µL or higher) is required. It is recommended to optimize the concentration of template DNA within the range of 1–10 ng/µL in the final reaction solution (Internal solution).
   - If using plasmid DNA prepared with a commercially available plasmid purification kit, please perform additional purification through phenol/chloroform extraction followed by ethanol precipitation to eliminate any residual nucleases.
   - When using template DNA containing the lac operator, protein yield may be increased by adding IPTG at a final concentration of 1 mM.

3) Sterilized water
4) Acetone
5) Micropipette
6) Desktop centrifuge
7) Water bath (30°C)
8) Air incubator (30°C)
9) Magnetic stirrer
10) Ice bath
11) Sample tube

Protein Synthesis Reaction

Prepare the External solution

1. Add 750 µL of the suspended amino acid mixture solution and 1 mL of sterilized water to the External solution. Stir the mixture thoroughly and let it sit at room temperature.

   Note: The Musaibo Kun amino acid mixture may form a suspension due to the presence of undissolved amino acids.

   A stir bar is included within the External solution container.

Reagents	Volume (mL)	Final conc.
External solution	8.25	82.5% v/v
Unlabeled or SI-labeled amino acid mixture	0.75	1.5 mM ea.
Sterilized water	1.00	
Total	10.00	

Prepare the Internal solution

1. Mix the Internal solution gently with a pipette until it is uniformly mixed, ensuring that no bubbles are formed.
2. Add 75 µL of the amino acid mixture, 100 µL of sterilized water, and 50 µL of template DNA to the Internal solution. Mix thoroughly with a micropipette, being cautious not to introduce bubbles.

Reagents		Volume (µL)	Final conc.
Internal solution		775	77.5% v/v
Unlabeled or SI-labeled amino acid mixture		75	1.5 mM ea.
Template DNA	[Circular: 50 (20–200) ng/µL] [Linear: 100 (20–200) ng/µL]	50	2.5 (1–10) ng/µL 5.0 (2–20) ng/µL
Sterilized water		100	
Total		1000	

3. Gently transfer the Internal solution into the dialysis cup using a micropipette. Please exercise caution to avoid foaming.

Preparation

1. Please ensure that there is no leakage from the dialysis cup:
   - Remove the dialysis cup from the container lid.
   - Fill the dialysis cup with 1 mL of distilled water and verify that there is no water leakage from the bottom dialysis membrane.
   - Carefully remove the distilled water from the dialysis cup using a micropipette, taking care not to damage the dialysis membrane.

   Note: Be sure to check before thawing the kit.

   The dialysis cup is initially temporally fixed under the container lid.

   If any leakage is detected in the dialysis cup, we will provide a free replacement.

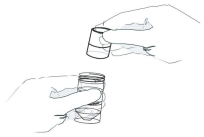
2. Thaw the External solution in a 30°C water bath for approximately 15 minutes, then let it reach room temperature after thawing.
3. Thaw the template DNA (control DNA or user's template DNA), then place it on ice.
4. Thaw the Internal solution in a 30°C water bath for about 1 minute, and immediately place it on ice after thawing.

   Note: Prior to thawing, ensure that the lid is securely fastened and the container is undamaged.

   The indicated thawing time is a general guideline, so please adjust as needed.

   After thawing, promptly place the solution on ice and use it immediately to prevent deterioration.

5. Spin down the thawed Internal solution and template DNA in a desktop centrifuge, and place them on ice.
4. Float the dialysis cup on the External solution and position the dialysis membrane to prevent the entrapment of air bubbles beneath it.



5. Close the lid of the External solution container and vertically place it inside the air incubator set at 30°C. Start stirring the External solution by gently rotating the stirrer bar at 100–200 rpm.

6. Incubate for 6 hours to synthesize protein. Keep gentle stirring during incubation.

   Note: Adjust the synthesis reaction time according to target protein specification.

   Shorter reaction times (1–4 hours) may be appropriate for the synthesis of labile proteins.

   Longer reactions (up to 16 hours) may increase the yield for certain stable proteins.

7. Remove the dialysis cup from the External solution container and collect the Internal solution using a micropipette.
8. Transfer the collected Internal solution into a 1.5 mL sample tube and place it on ice for 5 minutes to precipitate inorganic phosphate salts.

   Note: Unstable proteins may precipitate during this step. In such cases, omit cooling and/or dilute with an appropriate buffer and proceed immediately to the next step.

9. If the total fraction, including the insoluble fraction, is needed, use it as is (total fraction). If the soluble fraction is required, centrifuge the Internal solution at 15,000 x g for 1 minute at 4°C, and then use the supernatant (soluble fraction).

   Note: Confirming protein expression by SDS-PAGE is recommended. Certain proteins may precipitate.

   If the internal solution is left on ice for an extended period after the completion of the reaction, the expressed protein may precipitate or degrade. Use immediately or purify using an appropriate method.

   If the protein is to be purified using a His-tag affinity resin, it is recommended to dilute the collected internal solution 5 to 10 times with an equilibration buffer prior to applying it onto the affinity resin. Failure to do so may result in a decrease in the capture yield of the target protein due to the presence of a moderate concentration of amines in the Internal solution, which can interfere with the binding of the His-tag.

Prepare SDS-PAGE samples using acetone precipitation

The Musaibo kun SI kit reaction solution contains polyethylene glycol (PEG), which can often interfere with protein separation on SDS-PAGE. To remove PEG prior to SDS-PAGE analysis, follow the acetone precipitation procedure outlined below:

1. Mix 3 µL of the suspended Internal solution with 27 µL of ice-cold water (total fraction).
2. Centrifuge the remaining Internal solution at 15,000 x g for 1 minute at 4°C. Mix 3 µL of the supernatant with 27 µL of ice-cold water (supernatant fraction).
3. Add 60 µL of ice-cold acetone to each prepared fraction. Chill them on ice for 10 minutes.
4. Centrifuge at 15,000 x g for 20 minutes at 4°C and carefully discard the supernatant. Air-dry the remaining pellet for 20 minutes at 60°C.
5. Dissolve the pellet in 60 µL of 1 x SDS-PAGE sample buffer and denature it for 5 minutes at 95°C. If the pellet remains undissolved, repeat the process of suspending and denaturing the sample until the pellet is dissolved.
6. Apply 10 µL of the denatured sample (equivalent to 0.5 µL of the Internal solution) per lane on a gel and perform SDS-PAGE.

10. Troubleshooting

If the protein is not expressed or the expression yield is low, please refer to the FAQ on our website. For any further inquiries, please feel free to contact us.

Possible causes	Solutions
Reduced kit performance	– Please ensure that the product is used before the indicated expiration date on the product label. – Please verify that the product has been stored at the appropriate temperature. – If the product has been stored correctly within the expiration date, please reach out to us for further assistance.
Template DNA quality	– When utilizing plasmid DNA prepared using a commercially available plasmid purification kit, perform additional purification through phenol/chloroform extraction followed by ethanol precipitation to eliminate any residual nucleases. – Please evaluate the DNA concentration through agarose gel electrophoresis as Abs_{260} measurements can occasionally lead to overestimation. – Please verify the suitability of the template DNA sequence. (Please refer to Chapter 8. Template DNA) – Protein yield is greatly dependent on the amino acid and/or DNA sequence, particularly in the vicinity of the N-terminus. Consider adding or modifying the N-terminal tag.
Problems related to the nature of the protein	Certain unstable proteins may degrade or precipitate during the protein synthesis reaction. It is recommended to optimize the reaction conditions. – Lowering the reaction temperature to 25°C may decrease degradation or enhance solubility, although it may result in reduced yield. – For kits with a standard reaction time of 6 hours or longer, reducing the reaction time to 4 hours or less may improve solubility albeit at the expense of reduced yield. – Consider reaction condition optimization (addition of co-factors that is specific to the target protein etc.) using smaller-scaled reactions. – Inserting a tag sequence that enhances solubility may alter the solubility of the entire protein. – Based on the protein specifications, attempt optimizing the construct through deletion, extension, or mutation.

11. References

- Kigawa T. *et al.*, Cell-free Protein Synthesis Methods and Protocols (Spirin, A. S. & Swartz, J. R., eds.), 83-97, 2007
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- Yabuki T. *et al.*, J. Struct. Funct. Genomics, 8 (4), 173-191, 2007
- Seki E. *et al.*, Anal. Biochem., 377, 156-161, 2008
- Yokoyama J. *et al.*, Anal. Biochem., 411, 223-229, 2011
- Matsuda T. *et al.*, J. Bioorganic & Medicinal Chem., 20, 6579-6582, 2021

12. Stable Isotope-Labeled Amino Acid

Stable isotope-labeled amino acids for Musaibo kun are available for separate purchase. Please utilize them in combination with the kit.

Product name	Catalogue number
Amino acids mixture solution-UL-d 1 ml	A107-0144
Amino acids mixture solution-UL-^{15}N 1 ml	A39-0072
Amino acids mixture solution -UL-^{13}C,^{15}N 1ml	A40-0073
Amino acids mixture solution -UL-^{15}N,D 1 ml	A41-0074
Amino acids mixture solution -UL-^{13}C,^{15}N,D 1 ml	A42-0075
Amino acids mixture solution -Lys, Arg-^{13}C,^{15}N 1 ml	A91-0128
Amino acids mixture solution -Lys, Leu-^{13}C,^{15}N 1 ml	A92-0129
Amino acids mixture solution -SeMet 1 ml	A108-0145

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