IN VITRO PROTEIN EXPRESSION KIT FOR DISULFIDE-CONTAINING PROTEINS

STABLE ISOTOPE LABELING PROTEIN SYNTHESIS KIT

ISOTEC®

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Stable Isotope Labeling Protein Synthesis Kit

Introduction

Thank you for your purchase of the *In Vitro* Protein Expression Kit (*i*PE-SS Kit) for use with disulfide bond containing proteins.

This kit has been developed under license from RIKEN, incorporating its proprietary, advanced cell-free protein synthesizing technology into a kit dedicated to stable isotope labeling. It dramatically reduces isotope dilution compared to conventional products.

This is a protein synthesis system that utilizes *E. coli* cell extract. It allows easy and efficient protein expression by simply adding circular DNA or linear DNA as template DNA, which enables transcription of mRNA with T7 RNA polymerase.

It is important to confirm the expression of target protein by first using the separately sold *i*PE-Quick Kit (**Prod. No. 767824**).

It is important to read this instruction manual thoroughly in order to understand proper handling methods.

Safety Precautions

Review and adhere to all guidelines to avoid potential injury.

- This kit is for experimental and/or research use only. Do not use this kit, the protein, or any other components obtained from this kit for medical care and/or clinical diagnoses of humans and animals and/or add them to beverages or food.
- This kit is intended to be used by experts with experience in general biochemical experiments as well as micropipette operations. Personnel without such experience must not use this product.
- Wear proper safety goggles, gloves, lab coat, and other protective gear when handling the product. If the solution comes into contact with the eyes and/or skin, wash it away using clean running water. If any inflammation occurs, seek medical attention immediately.
- Please note that we assume no liability for any problems that might occur from any use of this product not authorized by this manual.

Storage

- Store at -80 ± 2 °C
- If the kit is stored at temperatures significantly deviating from -80 °C, the protein synthesis performance will be significantly lowered.
- Do not refreeze or store once the kit has been thawed. The synthesis performance will be significantly lowered.

Warning: Do not handle this kit with bare hands. It is stored at -80 °C creating a high risk of frostbite.

Kit Contents

- Internal solution – 685 µL (one 1.5 mL tube)
- External solution – 7.25 mL (one 25 mL tube)
- Natural-Amino acid mixture solution – 1 mL (one 1.5 mL tube)
- Control DNA pUC-BAP* – 50 µL (one 1 mL tube)
- 50 mM Glutathione oxidized form: (GSSG) – 1.2 mL (one 1.5 mL tube)
- 50 mM Glutathione reduced form: (GSH) – 1.2 mL (one 1.5 mL tube)
- Dialysis membrane cup

***Note:** Stable isotope labeled amino acid mixtures are not included in this kit. Please use the separately sold stable isotope labeled amino acids mixtures available from ISOTEC® Stable Isotopes. **Aldrich.com/isotec** (**Prod. No. 767964; 767972; 771031**).

Express alkaline phosphatase (BAP) is derived from *E. Coli*.

Main Components of Solutions Composing the Kit

Internal Solution		
E. coli cell extract	NTPs	Folinic Acid
Creatine Kinase	HEPES-KOH (pH 7.5)	0.05% NaN_3
T7 RNA Polymerase	Polyethylene Glycol	Magnesium Acetate
tRNA	D-Glutamate	Creatine Phosphate

External Solution		
NTPs	Folinic Acid	HEPES-KOH (pH 7.5)
0.05% NaN_3	Polyethylene Glycol	Magnesium Acetate
D-Glutamate	Creatine Phosphate	

Caution: Solutions contain 0.05% sodium azide (NaN_3). Sodium azide is designated as a poisonous substance in the Japanese Poisonous and Deleterious Substances Control Act when reaching 0.1% levels. Handle and discard with care.

Template DNA

This kit does not include template DNA. For protein expression, template DNA for the target protein containing T7 promoter and T7 terminator that enables transcription of mRNA with T7 RNA polymerase is required, as shown in the figure below.

RBS – Ribosome Binding Site **ATG** – Initiation Codon **TAA** – Termination Codon

Prepare template DNA for the target protein before using this kit. Use the following concentrations and amounts as a guideline.

- If circular DNA is used as a template: At least 40 µL (50 µg/mL) is required.
- If linear DNA is used as a template DNA: At least 40 µL (100 µg/mL) is required.
- When using a template DNA containing *lac* operator, the amount synthesized may be increased by adding Isopropyl-β-D(-)-thiogalactopyranoside (IPTG) to achieve a final concentration of 0.5-1.0 mmol/L.

Devices, Reagents, etc. Required for Using the Kit

1. Stable isotope labeled amino acid mixture solution for *i*PE-SS kit. 1 x 1 mL unit containing the labeling pattern of choice is required. (**Prod. No. 767964; 767972; 771031**)
   Note: If no labeling is needed, use Natural-Amino acid mixture solution included in this kit.
2. Template DNA
3. Sterilized distilled water
4. Water bath (30 °C)
5. Magnetic stirrer
6. Dialysis membrane cup (store at room temperature or cool dark place)
7. Incubator (30 °C)
8. Micropipettes
9. Table-top centrifuge
10. Ice bath

Protein Synthesis Operation

Before use, check all packages and containers to ensure that there has not been any damage incurred to the product.

1. Open the aluminum package containing the dialysis cup and remove the dialysis cup. Pour 1 mL of distilled water into the dialysis cup and wait for approximately 30 seconds. Check for absence of leaks and remove the distilled water from the dialysis cup with the micropipette so that the membrane will not break.
2. Open the kit, remove only *i*PE-SS External solution and glutathione solution (50 mM GSH and 50 mM GSSG) from the freezer and thaw it for 15 minutes on a water bath set to 30 °C. Similarly, place the template DNA and amino acids mixture solution of your isotope labeling choice into an ice bath to thaw.

   Note: The thawing time is given as a guideline; adjust the time as required.
3. Mix 50 mM GSH and 50 mM GSSG under one of the conditions shown in the table below to yield a glutathione solution. Proper disulfide bond formation may be promoted by changing the mixing ratio of GSH and GSSG. Optimum conditions differ depending on the type of protein, so perform small scale protein synthesis using microdialysis in advance to optimize the conditions.

Redox conditions	50 mM GSH	50 mM GSSG	Total (µL)
1	1,200	0	1,200
2	960	240	1,200
3	600	600	1,200
4	240	960	1,200
5	0	1,200	1,200

4. Add 1 mL of sterilized water to the amino acid mixture. Make sure the amino acid mixture is in good suspension. Add 750 µL of the aqueous amino acid mixture solution to the external solution container. Mix thoroughly with a micropipette. Add 1 mL of glutathione solution (any one of the redox conditions 1-5 above) and mix with micropipette.

Description	(mL)
*i*PE Kit SS External Solution	7.25
Amino Acid Mixture Solution	0.75
Glutathione Solution (redox condition 1 ~ 5)	1
Sterilized distilled water	1
Total	10

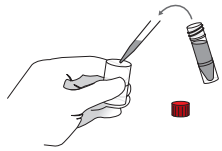
   Notes: Some amino acid mixtures may contain precipitates. This is a normal occurrence as several amino acids have low solubility.
5. Remove *i*PE-SS Internal solution from the freezer and thaw for 3 minutes on a water bath set to 30 °C.

   Note: The thawing time given is a guideline; adjust the time as required.
6. After thawing the Internal solution, immediately spin it down using a table-top centrifuge.

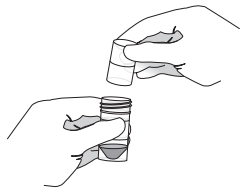
   Warning: The performance may be lowered if the solution is left for an extended period of time.
7. Add 75 µL of aqueous amino acid mixture solution, 100 µL of glutathione solution (any one of the glutathione conditions 1-5 above), 100 µL of sterile distilled water, and 40 µL of template DNA to the Internal solution and stir thoroughly with a micropipette.

Description	(µL)
*i*PE SS External Solution	685
Amino Acid Mixture Solution	75
Glutathione Solution (redox condition 1 ~ 5)	100
Sterilized distilled water	100
Template DNA (50 µg/mL)	40

   When using a template DNA containing lac operator, add IPTG to the internal and external fluids to achieve a final concentration of 0.5 to 1.0 mmol/L.
8. Using a micropipette, gently transfer above solution into the dialysis cup covering the dialysis membrane. This step should be done slowly so that it does not froth or produce any damage to the membrane.



9. Place the dialysis cup into the External solution container cautiously and slowly so that no air bubbles are locked between the External solution surface and the membrane.



10. Place the cap onto the External solution container. Place a stir plate in incubator. Place the External solution container vertically on top of the stir plate inside the incubator at 30 °C.

    Gently stir the solution and let the reaction sit for 4 to 16 hours.

    Note: Adjust the synthesis reaction time according to the type of target protein.
11. Remove the dialysis cup from the External solution container. Collect the reaction solution from the dialysis cup and place it into a 1.5 mL microtube using a micropipette. Place the microtube onto ice to end the reaction.
12. Separate the collected *i*PE Internal solution by centrifugal force at 4 °C and 12,000 rpm for 5 minutes to obtain a solution containing the target soluble protein in the clear upper portion.

    Note: If the solution is left in this state for an extended period of time, the expressed protein may precipitate or decompose. Purify and use immediately with an appropriate method.

Observation of expression by SDS-PAGE

An acetone precipitation is required in order to remove the polyethylene glycol from the reaction mixture before SDS-PAGE. Polyethylene glycol may disturb the protein bands on SDS-PAGE.

Transfer the 3 µL of reaction solution supernatant into a 0.6 mL Eppendorf tube (Total fraction). Centrifuge the reaction solution at 12,000 rpm for five minutes at 4 °C and transfer the 3 µL of supernatant into the 0.6 mL Eppendorf tube (Soluble fraction). Add both 27 µL of distilled water and 60 µL of cold acetone to both the Total fraction and Soluble fraction. Let both tubes stand on ice for five minutes, then centrifuge at 12,000 rpm for five minutes at 4 °C, and discard both supernatants. Dry the pellets at 60 °C for twenty minutes. Add 30 µL of 1 X SDS-PAGE sample buffer into each fraction. After thermal denaturation at 100 °C, apply the solution at 5-10 µL per SDS-PAGE gel well. After electrophoretic separation, stain the gel with Coomassie Brilliant Blue or with a similar stain.

Disposal

The solution of this kit, along with all containers, devices, etc. that may have come into contact with the solution, must be sterilized using an autoclave or similar process, then must be discarded in accordance with local regulations. Refer to the MSDS document for additional details. Protein and other products obtained by this kit must be discarded properly and are the responsibility of the customer.

Caution: *E. coli* bacteria (non-recombinant) may remain within the kit.

Troubleshooting

Protein Purification Method

The target protein expressed in the collected reaction solution can be purified by a method appropriate for the protein. In the case of absorption of a protein in a resin, it is recommended that the collected reaction solution first be diluted to approximately five-fold the original volume with the buffer used for resin equilibration.

If protein does not express, the expressed protein amount is small, or for other issues refer to the following table.

Possible Cause	Recommended Action
Kit performance deterioration	1. Check that the expiration date for use written on the product label is not exceeded. 2. Check that the product was stored at the proper temperature.
Inappropriate template DNA	1. Confirm the protein expression using *i*PE-Quick. 2. Check that the primary structure, sequence, and concentration of the template DNA are correct. If the purity of DNA is low, only a small amount of protein may be expressed. Re-purify the template DNA using commercially available purification kits. 3. For high molecular weight protein genes and large circular DNA, the expression level may be improved by adding a greater quantity.
Problems due to protein property	Some proteins may easily decompose and/or precipitate during the reaction. Collect protein after a short reaction time (approx. 4 hours) and confirm the amount of expression again. The property may be improved by varying the sequence or redesigning the construct.

Warranty

The kit is delivered frozen on dry ice. If there is no dry ice remaining in the delivery box at the time of arrival, or if there is damage to the package and/or solution has leaked out, the quality of the components in this kit may be compromised. Contact us immediately if any of these delivery issues have occurred. The warranty remains in effect until the expiration date marked on the product label.

Disclaimer

We assume no liability for the following even within the warranty period:

- Defects caused by improper storage and/or improper usage.
- Defects unrelated to the performance of this kit.
- Protein expression may decrease due to various factors other than the performance of the kit. For this reason, we do not guarantee the expression of protein.
- Please note that we assume no liability for passive damages due to defects of this kit or damages due to products obtained using this kit.

References

1. 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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4. Seki E. et. al., *Anal. Biochem.*, **377**, 156-161 (2008)
5. Yokoyama J. et. al., *Anal. Biochem.*, **411**, 223-229 (2011)
6. Matsuda T.et. al., *Biochem. & Biophysical Rsch Comm.*, **431**(2), 296-301 (2013)

ISOTEC® Contact Information

We take all possible measures to ensure the correctness of all information contained in this manual. Should you have any questions or comments, notice any omissions, etc., contact us.

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