FOR *in vitro* USE ONLY

Reconstituted *in vitro* translation kit

PURESYSTEM™ advance

Instruction Manual

advance8 #Pad008

advance96 #Pad096

advanceS-S8 #PaS008

advanceS-S96 #PaS096

Postgenome Co., Ltd.

9-1 Azanaka Kiriura, Nishiitsushiro, Ichinomiya, Aichi,JAPAN

Tel: +81-586-59-4385 Fax: +81-586-58-2339

IMPORTANT NOTICE to Purchaser

- This kit cannot be used in human subjects, in clinical trials or for diagnostic purposes involving human subjects.
- Contents of this kit are subject to change without notice.

Safety Instructions

Please note that Sol. A and Sol. B contains <1% DTT (Dithiothreitol). No other hazardous substances are included in reportable quantities. The synthesized protein may have strong toxicity or show pathogenic properties. To insure your safety, please take all the necessary precautions such as using the safety cabinet or protecting your eyes and skin by use of eyewear and gloves at all times. If your skin comes in direct contact with any of the reagents, or if you have swallowed any reagents by mistake, please obtain appropriate medical assistance. Please also insure to inactivate all unused reagents by autoclave or dry-heat sterilization before disposal. If your institution or country holds its own laws or regulations concerning experimental procedures, material transfer, export and disposal of the materials, please follow the instructions as stated.

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1. Quick Protocol for Protein Synthesis Reaction

1. Thaw Sol. A and Sol. B on ice.
2. Assemble in new reaction tube(s) as follows;

Nuclease-free water	µl
Sol. A	25 µl
Sol. B	10 µl
Template DNA	µl*[1]
Total	50 µl

*1; 1 pmol PCR product or 0.5 µg plasmid DNA

3. Mix gently and centrifuge briefly.
4. Incubate the reaction(s) at 37°C for 1 hour.
5. Stop the reaction by placing the tube(s) on ice.
6. Analyze the synthesized protein(s).

2. Kit Components

Please check that your kit contains the reagents listed below.

2-1. PURESYSTEM™ advance 8 (Prod.#Pad008)

Each kit contains sufficient reagents for 8 x 50 µl reactions.

Label (Tube Color)	Amount
Sol. A (Purple)	8x 25 µl
Sol. B (Green)	8x 10 µl
Temp. DHFR (Blue)	1 x 5 µl
Univ. Primer (Clear)	1x 50 µl

2-2. PURESYSTEM™ advance 96 (Prod.#Pad096)

Each kit contains sufficient reagents for 96x 50 µl reactions.

Label (Tube Color)	Amount
Sol. A (Purple)	96 x 25µl
Sol. B (Green)	96 x 10 µl
Temp. DHFR (Blue)	2x 5µl
Univ. Primer (Clear)	2 x 500 µl

- Sol. A and Sol. B must be thawed just before use and multiple freeze-thaw cycle must be avoided.
- Temp. DHFR is plasmid DNA containing dihydrofolate reductase (DHFR) gene (0.2 µg/µl) and can be used as positive control. It is added directly to the synthesis reaction.
- Univ. Primer is a forward primer for second-step PCR to add the T7 RNA polymerase recognition sequence to the template DNA and its concentration is 2 µM.

3. Storage Condition

Store all the components in the kit at -80°C. Avoid multiple freeze-thaw cycles.

4. Description

4-1. Background of PURESYSTEM Technology

PURESYSTEM is a novel reconstituted *in vitro* translation system which consists of about 30 purified enzymes necessary for transcription, translation and energy recycling.* In **PURESYSTEM**, all the factors for transcription and translation are tagged with a hexahistidine; 3 initiation factors (IF1, IF2, IF3), 3 elongation factors (EF-G, EF-Tu, EF-Ts), 3 release factors (RF1, RF2, RF3), ribosome recycling factor, 20 aminoacyl-tRNA synthetases, methionyl-tRNA formyltransferase and T7 RNA polymerase. The reagents also contains *E. coli* 70S ribosome, amino acids, NTPs, *E. coli* tRNA and energy recycling system, so the target protein can be synthesized just by addition of the template DNA to the reaction. **PURESYSTEM advance** is a kit base on the **PURESYSTEM** technology.

* Shimizu Y., *et al.*, (2001) *Nature Biotechnology*, vol.19, p.751-755.

4-2. PURESYSTEM advance Kit

PURESYSTEM advance is a kit based on **PURESYTEM** technology.

Milligram Yield and Hassle-Free

Using **PURESYSTEM advance**, milligram scale protein is in your hand within an hour and even purified protein is also in your hand in next two hours. Since the experimental protocol is nearly same as our **PURESYSTEM originator**, users who are familiar with originator can start using an **advance** kit without new training.

Low cost

The efficiency of protein synthesis of **PURESYSTEM advance** is more than twice as much as that of PURESYSTEM originator. In almost all case, the cost of synthesis per protein amount is lower than that of originator.

High Purity

Because **PURESYSTEM** is a reconstituted system in which all the components are identified, the reagents in **PURESYSTEM advnce** are essentially free from unidentified components such as protease, RNase and kinase.

No Tags Required

Because all the proteinous factors are His-tagged, the synthesized protein can be reverse-purified by using metal affinity resin (Figure 1). Addition of tags such as His, FLAG, and GST often inactivates protein of interest. You do not have to worry about choice of the tags and location of tags (e.g., N-terminal and C-terminal).

Time Saving

All you need is to follow the 3 steps as shown in Figure 1. It takes only 10 minutes for your handling and 3 hours for incubation and centrifugation.

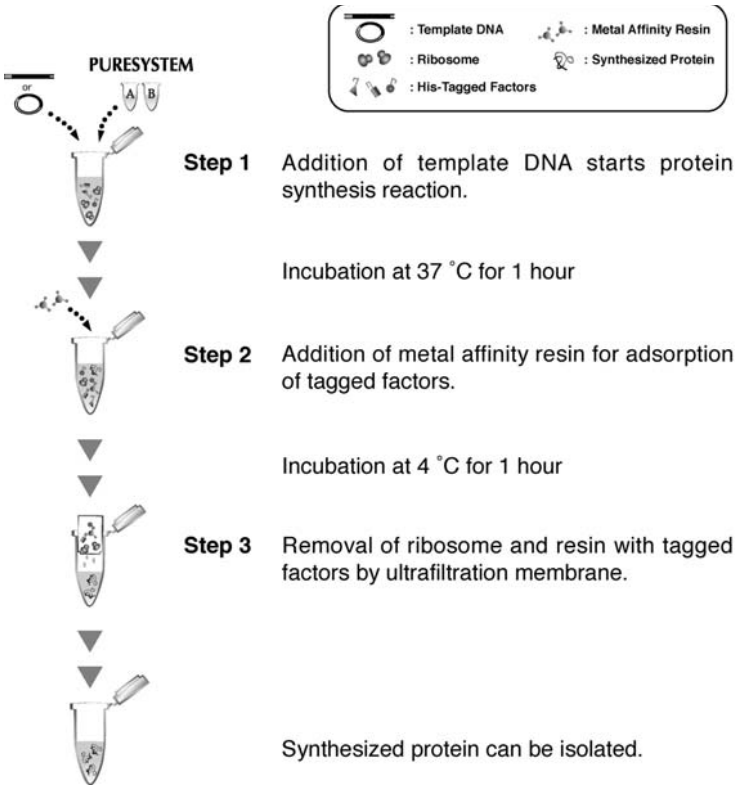

Figure 1.

Schematic diagram of protein synthesis and purification by PURESYSTEM

5. Procedures

5-1. Preparation of Template DNA

5-1-1. Considerations for Template DNA Construction

Both PCR product and plasmid DNA can be used as the template DNA for protein synthesis reaction in **PURESYSTEM**. The template DNA needs to contain the following;

- start codon (ATG)
- stop codon (TAG, TGA or TAA)
- T7 promoter sequence at upstream from the coding sequence
- Ribosomal binding site (RBS; SD sequence) at approximately 10 bp upstream from start codon
- Additional sequence of 6 bp or more downstream from stop codon (for PCR product)
- Terminator sequence at downstream from stop codon (for plasmid DNA)

We recommend PCR product as the template DNA due to the following reasons;

- PCR product can be used without further purification.
- No need for time-consuming construction of plasmid DNA suitable for protein synthesis.
- Multiple templates can be prepared at a time.
- The coding sequence can be easily modified.
  eg) deletion, point mutation, division into several domains and addition of tag sequence.

5-1-2. Generation of the Template DNA by 2-step PCR

The template DNA can be generated by 2-step PCR (Figure 2). In the first (1^{st}) PCR, the adapter sequence is added at 5'-terminus of the gene of your interest, and all the regulatory sequences are added in the second (2^{nd}) PCR. It is possible to amplify the template DNA by 1-step PCR using forward primer containing 5'-terminal region of the coding sequence and all regulatory sequences.

1. Design the forward and reverse primers as shown in Table 1.

2. Perform 1st PCR from DNA containing the gene of interest using these primers and high-fidelity polymerase.

3. Analyze the 1st PCR product by agarose gel electrophoresis. When detected as a single band, the 1st PCR reaction mix can be used in the second step without further purification. If any additional bands are detected, please optimize the PCR conditions or excise the band of the product from agarose gel.

4. Perform 2nd PCR from the 1st PCR product using the universal primer included in this kit and reverse primer used at the 1st PCR.

5. Analyze the 2nd PCR product by agarose gel electrophoresis. The 2nd PCR product will be approximately 70 bp longer than the1st PCR product, and this can be used for protein synthesis reaction without further purification.

Table 1. Primer List

primer	sequence
Forward primer	5'-AAGGAGATATACCA-ATG-N_{14-20}-3'
Reverse primer	5'-TATTCA-TTA-N_{14-20}-3'
Universal primer (Univ. Primer)	5'-GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCC CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCA-3'

5-1-3. Considerations for plasmid DNA

Plasmid DNA can also be used as the template DNA for **PURESYSTEM**. The plasmid DNA will need to fulfill the following elements and structural features;

- Any commercially available expression vectors can be used which contains T7 promoter sequence, RBS (approximately 10 bp upstream from the initiation codon) and terminator sequence.
- Some commercially available plasmid purification kits are not suitable for preparation of template DNA for **PURESYSTEM**.
- The plasmid DNA needs to be dissolved in RNase-free water or buffer without EDTA, in order to avoid inhibition of protein synthesis reaction.
- RNA needs to be removed. When RNase is used for the removal of RNA, complete inactivation of RNase is necessary by Phenol treatment or by similar inactivation

treatments.

• Quick purification method described in this manual cannot be used when the target gene is introduced to expression vector with a histidine-tag sequence.

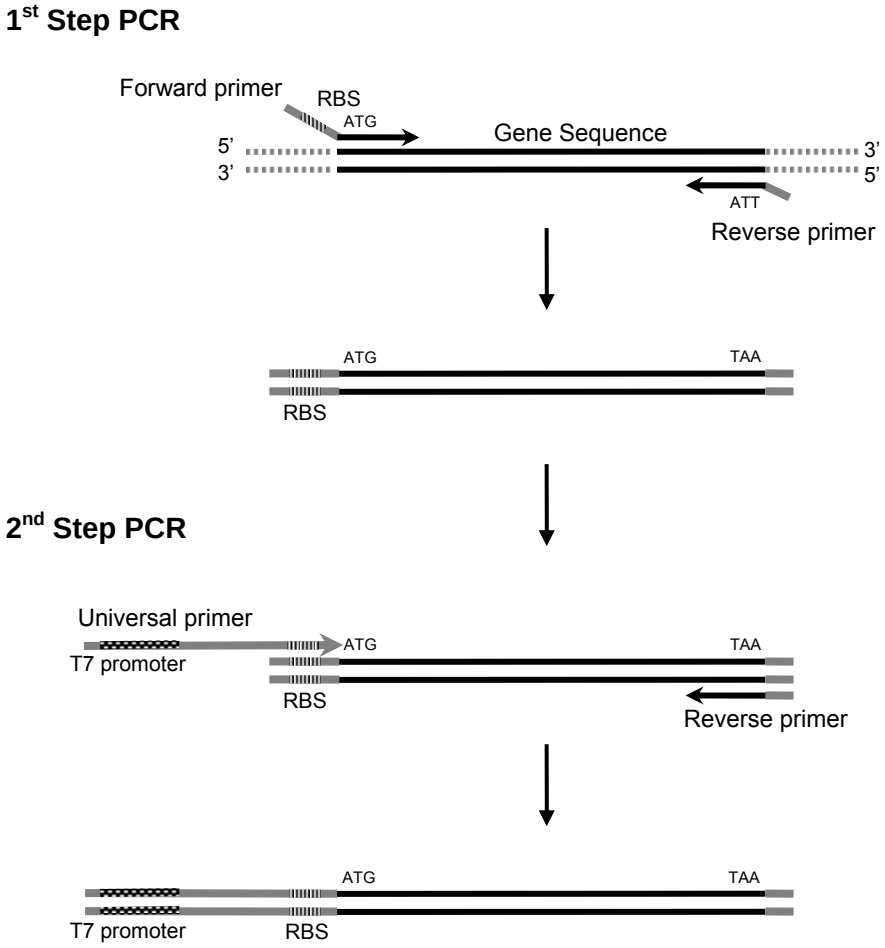

Figure 2. Generation of template DNA by 2-step PCR

5-2. Protein Synthesis Reaction

5-2-1. Precautions concerning reagents and experimental procedures

If you are unfamiliar with an *in vitro* translation, we recommend you to check the experimental procedures by using positive control (DHFR) included in the kit.
In order to avoid nuclease contamination, please use Nuclease-free tubes and tips, and wear gloves and mask.

5-2-2. Procedures

1. Thaw the necessary number of Sol. A and Sol. B on ice.
   Please avoid multiple freeze-thaw cycles, because some enzymes in the reagents may be inactivated.
2. Assemble in new reaction tube(s) as follows;

Nuclease-free water	µl
Sol. A	25 µl
Sol. B	10 µl
Template DNA	µl*[1]
Total	50 µl

*1; 1 pmol PCR product or 0.5 µg plasmid DNA

When labeled amino acid is incorporated into synthesized protein, add the labeled amino acid just before protein synthesis. For example, add 1 µl of 0.4 MBq/µl [^{35}S]methionine before addition of template DNA.

3. Mix gently and centrifuge briefly.
4. Incubate the reaction(s) at 37°C for 1 hour.
5. Stop the reaction by placing the tube(s) on ice.
   If purificaion step is unnecessary, the reaction mixture may be directly used for your experiment. Some proteins may require couple of hours for correct folding. Place the tube in an incubator and measure the activity if protein at different incubation time in order find an optimum incubation time.

5-3. Purification of Synthesized Protein - Quick Purification Protocol

If your proteins are characterized and you are familiar with the purification methods for your proteins, please proceed with the methods of your choice.
If the purification methods of the synthesized protein are unknown and the molecular weight is less than 100 kDa, the protein may be purified by the quick purification method as follows.

5-3-1. Additional Equipments necessary for Quick Purification

Ni-NTA Agarose (QIAGEN)
Nanosep 100K (Pall)
Micro Bio-Spin Chromatography Column (BIO-RAD)
Vortex Mixer

5-3-2. Procedure 1 (for 50 µl scale reaction)

1. Add equal volume of H_2O to the reaction mixture.
2. Apply the diluted reaction mixture to Nanosep 100K.
3. Centrifuge at 1,500x*g* at 4°C for 30 minutes.
4. Transfer the permeate to a new tube (preferably 2 ml round-bottom tube).
5. Add 0.1 vol. of Ni-NTA Agarose.
6. Vortex for 1 hour at 4°C vigorously.
7. Apply the mixture to the Micro Bio-Spin Chromatography Column.
8. Centrifuge at 1,500x*g* at 4°C for 1 minute.
9. Recover the permeate.

5-3-3. Procedure 2 (for 50 µl scale reaction)

This procedure is recommended when the synthesized protein could be purified according to the Procedure 1.

1. Add equal volume of H_2O to the reaction.
2. Transfer the diluted reaction mixture to a new tube (preferably 2 ml round-bottom tube).
3. Add 0.1 vol. of Ni-NTA Agarose.

4. Vortex for 1 hour at 4°C vigorously.
5. Apply the mixture to Nanosep 100K.
6. Centrifuge at 1,500x*g* at 4°C for 30 minutes.
7. Recover the permeate fraction.

5-3-4. Procedure 3 (for ml scale reaction)

1. Divide the reaction mixture into 200 µl-aliquot and apply each aliquot to Nanosep 100K.
2. Centrifuge at 1,500x*g* at 4°C for 30 minutes.
3. Pool the permeate in a new 2 ml round-bottom tube.
4. Add 0.1 vol. of Ni-NTA Agarose.
5. Vortex for 1 hour at 4°C vigorously.
6. Apply the mixture to the Micro Bio-Spin Chromatography Column.
7. Centrifuge at 1,500x*g* at 4°C for 3 minutes.
8. Recover the permeate.

5-4. Analysis of Synthesized protein

Synthesized proteins can be detected and analyzed by methods such as SDS-PAGE followed by gel staining or western blotting. In this section, a general method for SDS-PAGE and gel staining is shown.
If protein synthesis reaction is carried out in the presence of radiolabeled amino acids, the product can be analyzed by autoradioraphy.

1. Mix 0.5-2 µl of samples with SDS-PAGE sample buffer.
2. Load the samples to SDS-PAGE gel and carry out electrophoresis.
3. Stain the gel by SyproOrange.

Depending on the staining method tRNA may interfere with the detection of the proteinsof which molecular mass is 20-30 kDa. The interference may be reduced by treatment with RNase after synthesis reaction. For example, add 3.75 µg of RNaseA in 50 µl of reaction and incubate at 37°C for 15 min. We recommend SYPRO Orange as staining since the interence is minimal.

6. Synthesis and Quick Purification of DHFR (Positive Control)

1. Thaw Sol. A, Sol. B and Temp. DHFR on ice.
2. Assemble in a new reaction tube as follows;

Nuclease-free water	12.5 µl	
Sol. A	25 µl	
Sol. B	10 µl	
Temp. DHFR	2.5 µl	(0.5 µg)
Total	50 µl	

3. Mix gently and centrifuge briefly.
4. Incubate the reaction at 37°C for 1 hour.
5. Stop the reaction by placing the tube on ice.
6. Add 50 µl of H_2O to the reaction.
7. Apply the diluted reaction mixture to Nanosep 100K.
8. Centrifuge at 1,500x*g* at 4°C for 30 minutes.
9. Transfer the permeate to a new 2 ml round-bottom tube.
10. Add 25 µl of Ni-NTA Agarose.
11. Vortex for 1 hour at 4°C vigorously.
12. Apply the mixture to the Micro Bio-Spin Chromatography Column.
13. Centrifuge at 1,500x*g* at 4°C for 1 minute.
14. Recover the permeate.
15. Mix 10 µl of each sample (equiv. 5 µl of the synthesis reaction) with SDS-PAGE sample buffer.
16. Incubate the samples at 95°C for 5 minutes.
17. Load to 12.5 % SDS-PAGE gel.
18. Carry out electrophoresis.
19. Stain the gel by SYPRO Orange.

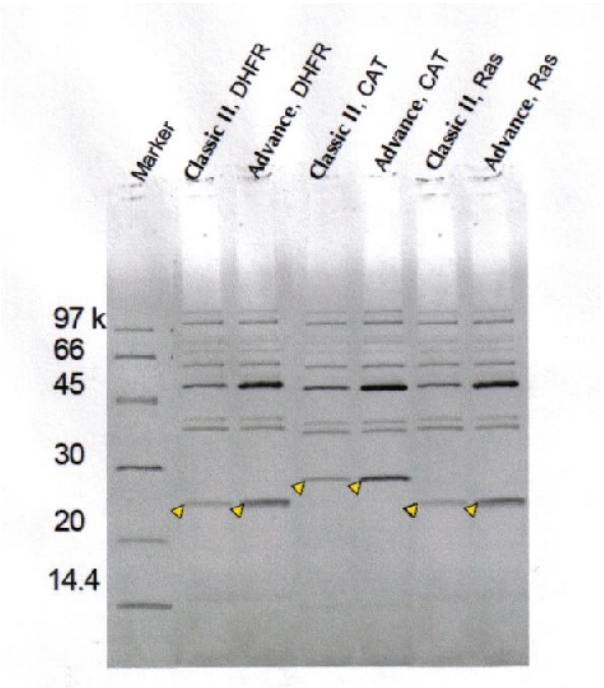

Figure 3

Comparison with efficiency of PURESYSTEM™ advance and originator

DHFR, chlorramphenicol acetyl transferase (CAT) and Ras weresynthesized by using **PURESYSTEM originator** or **advance**. After ultrafiltration,0.5 ul of reaction mixture was separated by SDS-PAGE and stained by SYPRO Orange. The arrows indicate the synthesized proteins. The amount of protein synthesized by **PURESYSTEM advance** is more than twice as much as that by originator.

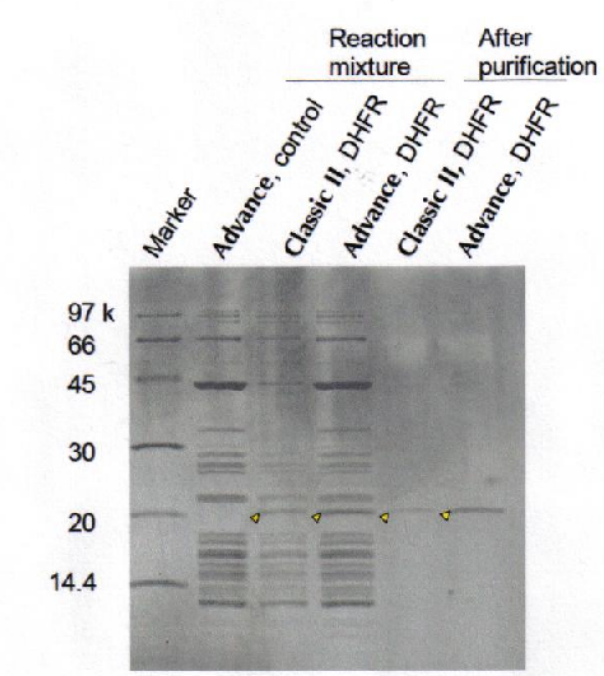

Figure 4

Protein synthesis and purification of PURESYSTEM™ advance and originator

DHFR was synthesized by using **PURESYSTEM originator** or **advance**. The synthesized proteins was purified by ultrafiltration and Ni-chelating chromatography. The arrows indicate the synthesized protein. The purity ofthe final product is more than 90% both in **PURESYSTEM advance** and originator.

7. Troubleshooting

7-1. Preparation of Template DNA

No product at the 1st step PCR

Primer is not optimal.

→Redesign the primer.

The reaction condition is not optimal.

→Check reaction conditions such as annealing temperature, extension time and number of cycles.

→Perform the PCR reaction in the presence of additives such as DMSO.

No product at the second-step PCR

The primer is not optimal.

→Redesign the primer.

→Prepare the template DNA by 1 step PCR using the long primer as below.

5'- GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCC

CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCA- ATG- N_{14-20}-3'

The reaction condition is not optimal.

→Check reaction conditions such as annealing temperature, extension time and number of cycles.

→Perform the PCR reaction in the presence of additives such as DMSO.

7-2. Protein Synthesis by PURESYSTEM

No control is synthesized.

Some components in the kit are inactivated.

→Store the reagents in the kit at -80°C and avoid multiple freeze-thaw cycles.

The reaction is contaminated with nuclease.

→Wear gloves and mask and use nuclease-free tubes and tips.

The control is synthesized, but the target is not.

The sequence of template DNA is incorrect.

→Check the sequence of the template DNA.

Purity and/or concentration of the template DNA is not optimal.

→Check purity and concentration of the template DNA.

The reaction is contaminated with nuclease.

→Wear gloves and mask and use nuclease-free tubes and tips.

The target protein is synthesized, but the yield is very low.

The concentration of the template DNA is not optimal.

→The concentration of the template DNA may need optimization. In some case, 1 pmol DNA per 50 µl reaction is not optimum. Try a lower or higher amount of template DNA (e.g., 0.1-10 pmol/50 µl).

Transcript may have formed a rigid secondary structure.

→Introduce the silent mutations in the sequence. See precautions.

Products (transcripts and/or polypeptides) may inhibit protein synthesis reaction.

→Synthesize control protein (DHFR) and protein of interest in the same reaction mixture and check the yield of DHFR. If the yield of DHFR is lower than 10 micrograms per milliliter, consider using additive such as inhibitors and excessive substrates of target protein.

The target protein is synthesized, but it is insoluble or not active.

Protein folding problem.

→Protein solubility is improved when protein synthesis reaction is performed at lower temperature than 37°C.

→Addtion of chaperones such as Hsp 70 and 90 in a reaction mixture may improve the solubility of the synthesized protein. We provide the DnaK and GroE supplement for this purpose. See the related product on page 19.

→Disulfide bonds formation may be needed. Try **PURESYSTEM S-S**.

→Synthesize the protein of interest together with cofactor(s). Before proceeding the experiment, make sure that the added cofactor does not inhibit protein synthesis by using positive control (i.e., DHFR).

8. Technical Service

For information about **PURESYSTEM** technology and kits (including **custom** kit)

info@post-genome.com

9. Related Products

PURESYSTEM™ S-S

PURESYSTEM S-S is the *in vitro* transcription/translation system which allows the synthesized protein to form correct disulfide bond(s). The high purity and flexibility of **PURESYSTEM** enables the redox conditions of reaction mixture to be reproducible.