Cat. # 3285

For Research Use

TaKaRa

Human Cell-Free Protein Expression Maxi System

Product Manual

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Table of Contents

I. Description

The Human Cell-Free Protein Expression Maxi System further enhances the protein-producing performance of the Human Cell-Free Protein Expression System (Cat. #3281), which has a much greater protein synthesis capacity than conventional mammalian protein *in vitro* synthesis systems. Through use of a dialysis technique to continually replenish ATP and amino acids throughout the translation reaction, the Human Cell-Free Protein Expression Maxi System can produce a large amount of the target protein.

The Human Cell-Free Protein Expression System utilizes human cell extracts. The Cell Lysate in this system contains all factors required for *in vitro* protein synthesis (ribosomes, translation initiation/elongation factor, tRNA, etc.). Using a simple protocol, both the RNA transcription reaction and the protein synthesis reaction take place in a single tube. Reactions are started by mixing plasmid (a target gene cloned into a pT7-IRES vector), T7 RNA polymerase, Mixture-3 (amino acids and ATP, etc.), and Mixture-2 (translation enhancement factor) into the Cell Lysate.

During the reaction, the Maxi System allows continual supply of amino acids and ATP, etc. through a dialysis membrane. Ordinarily, these factors are depleted as the protein synthesis progresses. Supply of these components allows protein synthesis to occur at high efficiency over a long period of time, thereby greatly increasing protein yield.

Use the Maxi System for large-scale synthesis of a target protein after initial screening/expression validation using the Human Cell-Free Protein Expression System. The Maxi system in combination with a tagged protein expression vector (sold separately*) can be used to perform large-scale synthesis of His-tagged or c-Myc-tagged protein. The expression of tagged proteins can also be verified using Western blot analysis.

Note: Tagged protein expression vectors (available separately):
pT7-IRES His-N DNA（Cat. #3290）
pT7-IRES His-C DNA（Cat. #3291）
pT7-IRES Myc-N DNA（Cat. #3292）

II. Components (for 5 reactions)

(1)	Human Cell-Free Protein Expression System (Cat. #3281) ＊1	2 Kits
(2)	Supplement Buffer (Human Cell-Free Protein Expression) (Cat. #3286)	15 ml
(3)	Dialysis Columns & Accessories (Cat. #3287) ＊2	5 Sets

Note: Items (2) and (3) are not available for purchase individually.

＊1： Components of the Human Cell-Free Protein Expression System (Cat. #3281):

1.	Cell Lysate ＊3	100 μl
2.	Mixture-1	60 μl
3.	Mixture-2 ＊4	10 μl
4.	Mixture-3 ＊4	20 μl
5.	T7 RNA Polymerase (200 U/μl)	10 μl
6.	pT7-IRES Vector (0.5 μg/μl)	20 μl
7.	Control Vector ＊5 (0.3 μg/μl)	5 μl

＊2: This product is manufactured by G-Biosciences and includes the following components.

- Tube-O-DIALYZER™, Micro 15K MWCO (5 tubes)
  Note: Each tube is filled with a preservative solution. Each package also contains a small amount of preservative solution to maintain moisture.
- Micro Dialysis Cup (5 cups)
- Float for Micro, for Dialysis Cup (5 floats)

Tube-O-DIALYZER™, Micro 15K MWCO

Micro Dialysis Cup

Float for Micro, for Dialysis Cup

＊3: Thaw Cell Lysate just prior to use. Mix gently and thoroughly with a micropipette and use immediately. After use, promptly store at -80℃.
Note： Although up to five cycles of freeze-thaw generally does not lead to any decline in performance, we recommend storing the Cell Lysate in aliquots of the required amount.

＊4: Mixture-2 and Mixture-3 contain proteins. To avoid deactivating proteins, do not stir excessively or vortex. Mixture-2 contains an HN-tagged protein.

＊5: This vector includes a β-galactosidase gene.

III. Materials Required but not Provided

- Microtubes
- Incubator (capable of 32℃ temperature setting)
- Magnetic stirrer
- Mini stir bar with tapered ends (e.g., $\varphi 4 \times 10$ mm)

  Note: If a bar is used that does not have recommended dimensions, the Micro Dialysis Cup may be ground down and form abrasive powder. There is no effect on the protein expression, even if the Supplement Buffer becomes turbid due to this powder.
- SDS-PAGE electrophoresis system
- Coomassie brilliant blue (CBB) stain and destaining solution

IV. Storage

- Dialysis Columns & Accessories (Cat. #3287): 4℃
  (The Micro Dialysis Cup and Float for Micro, for Dialysis Cup may be stored at room temperature.)

  Note: After removing the required number of tubes of Tube-O-DIALYZER™, Micro 15K MWCO from the package, reseal the package to prevent the remaining dialysis membranes from drying out.
- Store all other components at -80℃.

V. Precautions

1. Use all reagents except the expression plasmid and Mixture-1 immediately after they are thawed on ice. After use, promptly store at -80℃.
2. Store the Cell Lysate in aliquots of the required amount.
3. Addition of Mixture-3 to the reaction may generate insoluble precipitate. This does not affect performance of the reaction.
4. In addition to this manual, refer to the User Manual for the Human Cell-Free Protein Expression System (Cat. #3281).

VI. Construction of Expression Plasmids

Construct an expression plasmid by inserting a DNA fragment encoding the target gene into the multiple cloning site (MCS) of pT7-IRES vector. DNA fragments may be generated by PCR, by restriction enzyme digestion to transfer an ORF cloned into another plasmid, or by artificial gene synthesis. Addition of a poly(A) sequence to the inserted sequence is not required.

Takara recommends that the target gene DNA fragment be inserted into the MCS of pT7-IRES vector such that the 5′ (N-terminal) ATG initiation codon of the target protein is in frame with the ATG of the *Nco* I site at the 5′ end of the MCS. When inserting the target gene DNA fragment into a site other than *Nco* I (i.e., any restriction site sequence in the MCS from *Bam*H I to *Xba* I), make sure the ATG initiation codon located in the upstream *Nco* I site is in frame with the reading frame of the target gene.

The following example describes construction of an expression vector using the In-Fusion® method, a simple and convenient directional cloning technology that does not limit cloning strategy to particular restriction enzyme sites. In this example, the 5′ region encoding the N-terminal end of the target protein is inserted using the ATG of the *Nco* I site.

Example of expression vector construction using the In-Fusion method (cloning into the *Nco* I/*Xba* I site)

1. Primer design
   - N-terminal primer:
     Design a primer with an 5′ -end that includes the following underlined 15-base sequence, with the initiation codon **(ATG)**.
     5′ -ATGGCCACAACC**ATG** —target coding sequence—3′
   - C-terminal primer:
     Design a primer with a 5′ -end that includes the following underlined 15-base sequence. Include a stop codon or downstream sequence. (No poly(A) is required.)
     5′ -GTTATGCTAGTCTAG**TCA (stop codon)**—target coding sequence—3′

2. Insert DNA preparation and In-Fusion*1 cloning

Using primers designed as described above, prepare DNA encoding the target protein by performing PCR amplifications with PrimeSTAR Max DNA Polymerase or another high-fidelity PCR enzyme. Insert the target DNA by performing an In-Fusion reaction with a pT7-IRES vector linearized by *Nco* I and *Xba* I digestion. Transform into *E. coli* cells suitable for high-efficiency transformation such as *E. coli* HST08 Premium Competent Cells*2 and select clones harboring the target insert. Confirm the sequence of the target DNA by sequencing if needed. For details regarding procedures and primer design for In-Fusion, refer to the instruction manual of the In-Fusion cloning kit or system.

* 1: This kit can be used in combination with the In-Fusion HD Cloning System (Cat. #639645/ 639646/639692/ 639647) or In-Fusion HD Cloning System with CE (Cat. #639636/ 639637/639693/ 639638), each supplied with competent cells. Alternatively, use the In-Fusion HD Cloning Kit (Cat. #639648/639649/639650) or In-Fusion HD Cloning Kit w/ Cloning Enhancer (Cat. #639633/639634/639635), not supplied with competent cells, in combination with Stellar™ Comptent Cells (Cat. #636763/636766).

Availabilty of In-Fusion systems, kits, and Stellar Competent cells varies by geographic location; check for availability in your area.

* 2: *E. coli* HST08 Premium Competent Cells (Cat. #9128) are available in Japan.

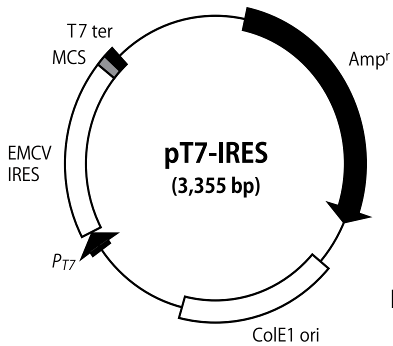


Figure 1. Map of pT7-IRES vector

```
                                 BamH I
               EMCV IRES      Nco I            EcoR I   (Nde I)*   Bgl II    Nhe I
5'–TAATATGGCCACAACC ATG GGA TCC GAA TTC CAT ATG AGA TCT GCT AGC
3'–ATTATACCGGTGTTGG TAC CCT AGG CTT AAG GTA TAC TCT AGA CGA TCG
                    Met Gly Ser Glu Phe His Met Arg Ser Ala Ser

 Sal I
 Hinc II  EcoR V  Xho I   Xba I         T7 terminator
GTC GAC GAT ATC CTC GAG TCT AGA CTAGCATAACCCCTTGGGGCCTCTAAA–3'
CAG CTG CTA TAG GAG CTC AGA TCT GATCGTATTGGGGAACCCCGGAGATTT–5'
Val Asp Asp Ile Leu Glu Ser Arg
```

Figure 2. Sequence of the multicloning site (MCS) on pT7-IRES vector

*: *Nde* I may not be used as a multicloning site

Note: Various tagged fusion expression vectors are available (sold separately). Refer to the appropriate data sheet to see the MCS for each vector.

pT7-IRES His-N DNA (Cat. #3290)
pT7-IRES His-C DNA (Cat. #3291)
pT7-IRES Myc-N DNA (Cat. #3292)

VII. Protocol

VII-1. Preparation of using Tube-O-DIALYZER™ and Supplement Buffer

1. Remove the Tube-O-DIALYZER from its packaging. Remove the cap with the dialysis membrane. Use a pipette, a microfuge, or other suitable apparatus to completely remove the preservative solution from both the internal and external sides of the tube. Protect the tube from dust prior to the dialysis reaction.
2. Gently clean the cap with dialysis membrane by dipping it into a sterile 50 ml tube or other suitable container containing an appropriate amount of sterile water. Keep the cap soaked in sterile water until use to prevent the dialysis membrane from drying out.
3. Remove Supplement Buffer from storage at -80°C and allow it to thaw at room temperature.

VII-2. Protein synthesis reaction

1. Thaw the following components of the Human Cell-Free Protein Expression System on ice and dispense into a microfuge tube. Mix well by gentle pipetting.

Cell Lysate	36 μl
Mixture-1	24 μl
Mixture-2	4 μl

2. Let stand for 10 minutes at room temperature. In the meantime, add 3 ml of the thawed Supplement Buffer into a Micro Dialysis Cup along with a mini stir bar. Protect the buffer from dust before use.
3. Thaw the reagents listed below on ice and add to the reaction tube from Step 2. After all components have been added, mix the reaction mixture well by gentle pipetting.

   Note: Since the viscosity of the reaction mixture is very high, mix well by pipetting between 10 and 20 times. An unevenly mixed reaction mixture may result in low yield.

Mixture-3	8 μl
Expression plasmid*1 (0.3 μg/μl)	4 μl
T7 RNA Polymerase	4 μl

   * 1: Use plasmid with the target sequence prepared according to Section VI. Construction of Expression Plasmids.

4. Transfer the reaction mixture (80 μl) from Step 3 into the prepared Tube-O-DIALYZER tube. Take the cap with dialysis membrane out of the sterile water and remove water carefully from both sides of the membrane. Take care to avoid damaging the membrane. Replace the cap on the tube.
5. Place the tube upside down to allow the reaction mixture in the tube to come in contact with the dialysis membrane. If any portion of the reaction mixture remains at the top of the inverted tube, shake gently to bring it down so that the entire reaction mixture is at the bottom of the tube and in contact with the dialysis membrane.

6. Set the inverted dialysis tube in the Float and place them into the Micro Dialysis Cup prepared in Step 2 such that the dialysis membrane is in contact with Supplement Buffer in the bottom of the Cup. Make sure no air bubbles are present between the dialysis membrane and Supplement Buffer. (See Figure 3)

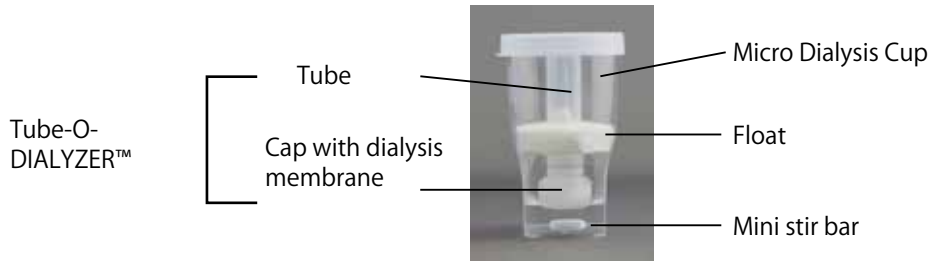


Figure 3. Tube-O-DIALYZER and Micro Dialysis Cup assembled for *in vitro* translation

7. Stir gently with the magnetic stirrer in an incubator＊2 during the *in vitro* translation (IVT) reaction. Incubate at 32℃ for 12 - 24 hours.＊3

   Note: When initiating stirring, make sure once again that no air bubbles are present between the dialysis membrane and the Supplement Buffer. If air bubbles have formed, tilt the Micro Dialysis Cup to free the bubbles.

   ＊2: We recommend using a magnetic stirrer in order to achieve adequate mixing.

   ＊3: The optimum reaction time may differ depending on the target protein. In general, a 12- to 18-hour reaction is recommended.

8. After the reaction is complete, briefly centrifuge to collect the reaction mixture at the bottom of the dialysis tube. Remove the cap with dialysis membrane and transfer the reaction mixture to a new tube for storage.
9. Store the reaction mixture at -80℃ until analysis.

The synthesized protein may be analyzed by SDS-PAGE, Western blot, or another suitable method. See Section VIII. Experimental Examples. This system, in combination with a tagged fusion expression vector (available separately), can be used to attach a tag (i.e., His, c-Myc) to the target protein. Tagged proteins may be analyzed by Western blot or purified by affinity chromatography.

VIII. Experimental Examples

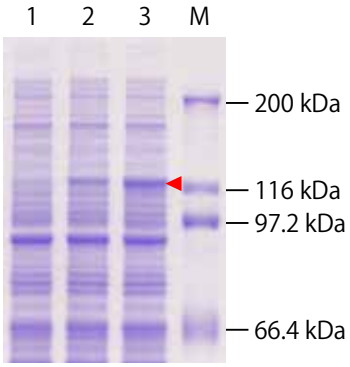
VIII-1. Large-scale expression of β-galactosidase (116 kDa) with Maxi System

[Method]

β -Galactosidase (116 kDa) was synthesized according to the protocol described in this manual. An 18-hour reaction was performed at 32℃. For comparison, the protein was also synthesized by the batch method with Human Cell-Free Protein Expression System (Cat. #3281) as described in the User Manual during a 3-hour reaction at 32℃. Analyses were subsequently performed using SDS-PAGE and an Agilent 2100 Bioanalyzer to detect the target protein.

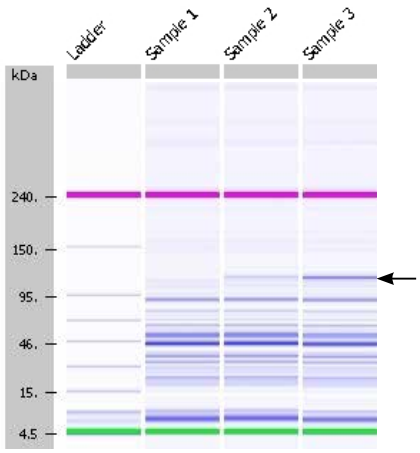
[Result]

A protein of the expected size (116 kDa) was detected. The results showed that the Human Cell-Free Protein Expression Maxi System provided approximately 4-fold higher yield compared with the batch method when the same volume of sample was analyzed.



Protein synthesis reaction
volume: equivalent to 0.4 μl of the reaction
Lane 1：Negative Control
Lane 2：Synthesis by the batch method
Lane 3：Synthesis by the Human Cell-Free Protein Expression Maxi System (dialysis)
Lane M：Protein Molecular Weight Marker (Broad)

Figure 4. Result of SDS-PAGE analysis (CBB stain)



Sample No.	Reaction condition	Concentration [mg/ml]
2	batch method	2.70
3	dialysis	11.22

Protein synthesis reaction
volume: equivalent to 0.25 μl of the reaction
Sample 1：Negative Control
Sample 2：Synthesis by the batch method
Sample 3：Synthesis by the Human Cell-Free Protein Expression Maxi System
Ladder：Protein 230 Ladder

Figure 5. Result of analysis with Agilent 2100 Bioanalyzer

VIII-2. Large-scale expression of Human Ago2 (approx. 80 kDa) with Maxi System

[Method]

Human Ago2 protein (approx. 80 kDa) was synthesized according to the protocol described in this User Manual. An 18-hour reaction at 32℃ was performed. For comparison, the protein was also synthesized by the batch method with the Human Cell-Free Protein Expression System (Cat. #3281) as described in the Cat. #3281 User Manual during a 3-hour reaction at 32℃. Analyses were subsequently performed by SDS-PAGE and Western blot (colorimetric) using Anti-Human Ago2, Monoclonal (Clone 1B1) (Cat. #M211) to detect the target protein.

[Result]

A protein of the expected size (approx. 80 kDa) was detected. The results showed that the Human Cell-Free Protein Expression Maxi System provided higher protein yield compared with the batch method.

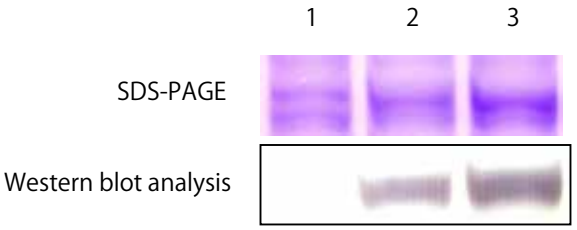


Figure 6. Results of SDS-PAGE (CBB stain) and Western blot detections

Protein synthesis reactions volume: equivalent to 2 μl (SDS-PAGE)

Lane 1： Negative Control

Lane 2： Synthesis by the batch method

Lane 3： Synthesis by the Human Cell-Free Protein Expression Maxi System (dialysis)

IX. Troubleshooting

1. Low yield of protein

   The yield of target protein largely depends on factors such as the origin and sequence of the target gene as well as the stability of both the RNA transcript and the protein generated in the reaction mixture. The following factors may also contribute to a low level of protein synthesis:

   - Unevenly mixed reaction mixtures

     With high-viscosity solutions such as the Cell Lysate, be sure to gently pipette reaction mixtures during preparation to achieve thorough mixing.

   - High nuclease concentration in the prepared plasmid

     Extract with phenol/chloroform followed by alcohol precipitation. Remove all residual phenol by adequate washing with alcohol.

   - Low plasmid purity

     Extract with phenol/chloroform followed by alcohol precipitation. Remove all residual phenol by adequate washing with alcohol.

   - Low plasmid concentration

     Concentrate by alcohol precipitation.

   - Unfavorable storage condition for reagents

     Store reagents at the recommended temperatures.

2. Use of the dialysis technique does not lead to increased yield of target protein synthesis

   The yield of target protein synthesis by the dialysis technique largely depends on factors such as the stability of the target protein in the reaction mixture. The following factors may also contribute to a low level of protein synthesis:

   - Part of the reaction mixture was trapped at the top of the inverted tube during the dialysis reaction and did not come into contact with the membrane, resulting in inadequate dialysis.

     If any portion of the reaction mixture remained on the top of the inverted tube, shake gently to bring it down so that the entire reaction mixture is at the bottom of the tube and in contact with the dialysis membrane.

   - Air bubbles were present between Supplement Buffer (solution in the bottom of the Micro Dialysis Cup) and the dialysis membrane, resulting in inadequate dialysis.

     Make sure to get rid of any bubbles present when the Supplement Buffer comes into contact with the dialysis membrane. Check for air bubbles periodically after starting the reaction. In particular, bubbles may appear once stirring begins. Remove any air bubbles that form by tilting the Micro Dialysis Cup to free the bubbles.

   - The Supplement Buffer was unevenly mixed.

     If the mini stir bar stops rotating, inadequate mixing will occur. Check periodically after starting the reaction to make sure the stirrer is operating properly.

＊ For additional troubleshooting, see the instruction manual for the Human Cell-Free Protein Expression System (Cat. #3281).

X. Related Products

Human Cell-Free Protein Expression System（Cat. #3281）
pT7-IRES His-N DNA（Cat. #3290）
pT7-IRES His-C DNA（Cat. #3291）
pT7-IRES Myc-N DNA（Cat. #3292）
In-Fusion® HD Cloning System (Cat. #639645/ 639646/639692/ 639647)＊
In-Fusion® HD Cloning System CE (Cat. #639636/ 639637/639693/ 639638)＊
In-Fusion® HD Cloning Kit (Cat. #639648/639649/639650)＊
In-Fusion® HD Cloning Kit w/ Cloning Enhancer (Cat. #639633/ 639634/ 639635)＊
Stellar™ Comptent Cells (Cat. #636763/ 636766)＊
E. coli HST08 Premium Competent Cells (Cat. #9128)＊
DNA Ligation Kit <Mighty Mix>（Cat. #6023）
TaKaRa DNA Ligation Kit LONG（Cat. #6024）
PrimeSTAR® Max DNA Polymerase（Cat. #R045A）
Protein Molecular Weight Marker (Low)（Cat. #3450）
Protein Molecular Weight Marker (High)（Cat. #3451）
Protein Molecular Weight Marker (Broad)（Cat. #3452）

<For His-tag fusion protein purification>
TALON® Metal Affinity Resin（Cat. #635501 etc.)
His60 Ni Superflow™ Resin（Cat. #635659 etc.)

＊： Not available in all geographic locations. Check for availability in your region.