Cat. # 3281

For Research Use

TaKaRa

Human Cell-Free Protein Expression System

Product Manual

v201807Da

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I. Description

Human Cell-Free Protein Expression System is a cell-free protein synthesis system using human cell extracts. This cell lysate system contains all the required components for protein synthesis (ribosomes, translation initiation/elongation factors and tRNA, etc.). All reactions, from RNA transcription to protein synthesis, are performed in a single tube—by adding 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) to the Cell Lysate and following a simple protocol. The Human Cell-Free Protein Expression System provides the benefits of microliter scale reactions, speed, and cell-free protein synthesis.
The target gene RNA transcribed from the pT7-IRES vector contains an IRES sequence designed to promote protein translation initiation. The translation initiation factor in the Cell Lysate becomes inactivated as protein synthesis progresses. However, it is reactivated by the translation enhancement factor in the reaction mixture, thereby maintaining the translation level. These enhancements of translation efficiency are designed to remedy the drawback of low level protein synthesis common with many mammalian cell-free protein synthesis systems. The composition of the Human Cell-Free Protein Expression System enables synthesis of high-molecular-weight proteins exceeding 150 kDa.
This system is useful in high-throughput protein syntheses with a single-step reaction. It may also be applied to produce toxic proteins that are difficult to synthesize with an expression system using live cells due to the toxic effect on the host cell' s growth and functions.

II. Components (for 10 x 20 μl reactions)

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

*1 Dissolve just prior to use; mix well gently with a micropipette and use immediately. After use, promptly store at -80℃.
Note: Although five cycles of freeze-thaw generally will not lead to any decline in performance, the cell lysate should be stored in aliquots of the required volume.

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

*3 This vector harbors a β-galactosidase gene in pT7-IRES.

III. Materials Required but not Provided

1. Reagents
- Protein gel staining reagents (e.g., CBB staining)
- Destaining reagents

2. Materials
- SDS-PAGE electrophoresis apparatus
- Various tubes
- Thermal cycler or equivalent (for 32℃ incubation)
- Air incubator or equivalent (for 37℃ incubation)

IV. Storage -80℃

V. Protocol

1. Construction of Expression Plasmid

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

It is recommended that the target gene DNA fragment be inserted into the MCS of pT7-IRES vector with the N-terminal initiation codon (ATG) of the target protein in-frame with the ATG of the *Nco* I site at the 5′ -end of the MCS. When inserting the target DNA fragment into a site other than *Nco* I (*Bam*H I-*Xba* I), make sure the ATG initiation codon located in the *Nco* I site is in phase with the reading frame of the target gene.

The example below shows construction of an expression vector using the In-Fusion® method, a simple and convenient directional cloning technology that does not depend on restriction enzyme sites. In this example, the ATG of the *Nco* I site is used as the intiation codon of the target protein.

<Example of expression vector construction using the In-Fusion method>

1. Primer design

   a. N-terminal primer:
   - Design a primer containing the underlined 15-base sequence including the initiation codon **(ATG)**.
   - 5′-ATggCCACAACC**ATg** — N-terminal coding sequence of the ORF —3′

   b. C-terminal primer:
   - Design a primer containing the underlined 18-base sequence including the termination codon **(TCA)**. (Addition of a poly(A) sequence is not required.)
   - 5′-gTTATgCTAgTCTAg**TCA** — C-terminal coding sequence of the ORF —3′

2. DNA insert preparation and In-Fusion cloning
    a. Using primers designed in Step 1, perform PCR amplification with PrimeSTAR® Max DNA Polymerase, PrimeSTAR HS DNA Polymerase, or another high-fidelity PCR enzyme to prepare the insert DNA.
    b. To insert the target DNA, perform an In-Fusion reaction with a vector linearized by *Nco* I and *Xba* I digestion.
    c. Transform into the *E. coli* competent cells provided with the In-Fusion HD Cloning Plus or In-Fusion HD Cloning Plus CE. Select clones harboring the target insert.
    d. Confirm the sequence of the target DNA by sequencing if needed.

Note: For details regarding procedures and primer design for In-Fusion, refer to the appropriate In-Fusion cloning Plus manual.

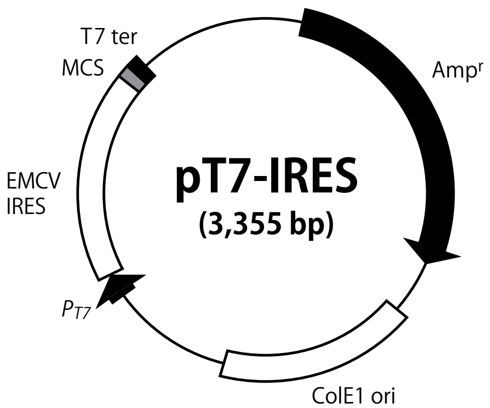

Figure 1. Map of pT7-IRES vector

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

- *Nde* I may not be used as a multicloning site.
- EMCV = Encephalomyocarditis virus

2. Procedure

a. Thaw the components below on ice and dispense into a reaction tube. Mix well by gentle pipetting.

Cell Lysate	9 μl
Mixture-1	6 μl
Mixture-2	1 μl

b. Let stand for 10 minutes at room temperature.

c. Thaw the components below on ice and add to the above reaction tube. After all components have been added, mix the reaction mixture well by gentle pipetting.

Mixture-3	2 μl
Plasmid (0.3 μg/μl)	1 μl
T7 RNA Polymerase	1 μl

- Use the expression plasmid constructed with the target gene (Section V.1).
- As the viscosity of the reaction mixture is very high, make sure to mix well by pipetting 10 - 20 times. An unevenly mixed reaction mixture may result in low expression.

d. Allow to react at 32℃ for 1 - 6 hours.
   - The optimum reaction time differs depending on the protein. Generally, a reaction time of 3 hours is recommended, but reactions may be extended to up to 6 hours.
   - The synthesized protein may be detected with SDS-PAGE or Western blot analysis. Please refer to Sections VII.1 - VII.3.

3. Precautions

- Use all reagents, except plasmid and Mixture-1, immediately after they are thawed on ice and promptly store at -80℃ after use.
- It is recommended that the Cell Lysate be stored in small aliquots.
- The addition of Mixture-3 may generate insoluble matter, which is not expected to compromise performance.

VI. Control Reaction (β-Galactosidase Synthesis)

Perform reaction in accordance with Section V.2. Use 1 μl of Control Vector in Step 2-c and set the reaction time to 3 hours in Step 2-d. After the reaction, detect β-galactosidase by the procedure below.

1. β-galactosidase detection

a. Dispense the following reagents into a microtube and mix well by pipetting.

Control reaction mixture	1 μl
X-Gal solution (25 mg/ml)*	1 μl
Sterile purified water	18 μl

* Prepare by dissolving in N,N-dimethylformamide.

b. Allow reaction to take place in an air incubator at 37℃ for 30 - 60 minutes.

c. Development of blue color as shown in Figure 3 confirms successful protein synthesis.

Color development after 30 min.
Tube 1: Negative control (no color)
Tube 2: + Control Vector (with color)

Figure 3. Color Development (β-galactosidase detection)

2. Time-dependent expression of β-galactosidase and effect of translation enhancement factor (Mixture-2)

Methods :
- 8 reactions were performed using 1 μl of Control Vector per reaction to synthesize β-galactosidase following the protocol (Section V.2).
- At each time point (0, 0.5, 1, 2, 3, 4.5, 6 and 8 hours after start of reaction), 1 reaction tube was checked for β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate.
- To study the effect of translation enhancement factor, a separate experiment was run in the same manner except Mixture-2 was left out.

Results :
- Addition of Mixture-2 markedly increased the amount of protein synthesized.
- β-galactosidase expression increased over time up to approximately 8 hours.

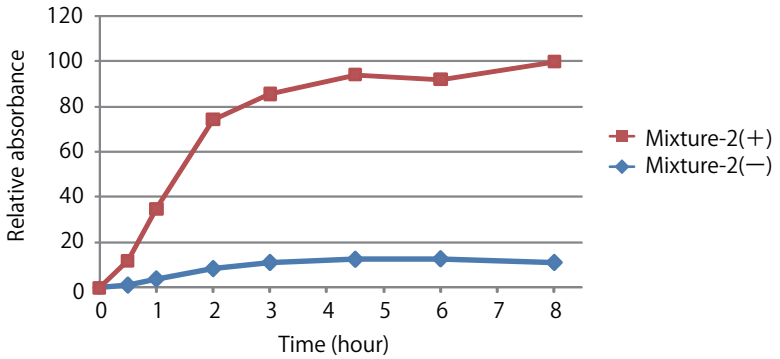


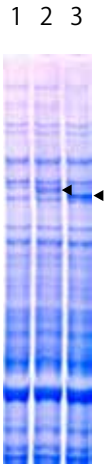
Figure 4. Amount of β-galactosidase synthesized.

VII. Experimental Examples

1. High-molecular-weight protein expression (170 kDa, 200 kDa) using this system

Methods :
- Protein synthesis reactions were performed in accordance with the protocol.
- Target proteins were detected using SDS-PAGE and CBB staining.

Result :
- Proteins of the target sizes (170 kDa, 200 kDa) were successfully detected.



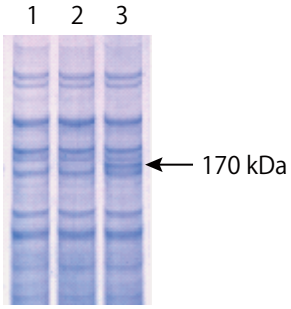
Apply protein synthesis products (equivalent to 2 μl).
Lane 1: Negative Control
Lane 2: Human Dicer (200 kDa)
Lane 3: Human eIF4G (170 kDa)

Figure 5. CBB staining showing bands of appropriate size (170 kDa and 200 kDa).

2. Effect of translation enhancement factor (Mixture-2)

Methods :
- Cell-free protein expression reactions were run with and without translation enhancement factor.
- SDS-PAGE was performed followed by CBB staining to detect the target proteins.

Result :
- The addition of translation enhancement factor improved protein expression.



Apply protein synthesis products (equivalent to 2 μl).
Lane 1: Negative Control
Lane 2: Human eIF4G (170 kDa), − Mixture-2
Lane 3: Human eIF4G (170 kDa), + Mixture-2

Figure 6. Analysis of protein expressed with or without translation enhancement factor.

3. Expression of a tagged protein (Western blot analysis)

Methods :
- Protein synthesis reactions were performed in accordance with the protocol, followed by SDS-PAGE and Western blot analysis to detect the target proteins.
- A tagged protein was detected using an antibody that recognizes the DYKDDDDK-tag sequence.

Result :
- Western blot analysis successfully detected proteins of the target sizes (80 kDa, 200 kDa).

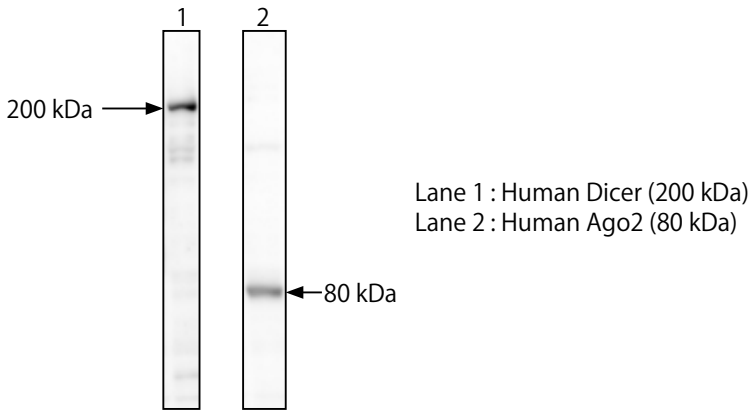


Figure 7. Western blot analysis of DYKDDDDK-tagged proteins.

VIII. Troubleshooting

1. No confirmation of target protein synthesis

Target protein synthesis is largely dependent 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. Procedural issues such as those described below 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 conditions for reagents
  → Please store at an appropriate temperature.

2. Low color development in the control reaction

- 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.
- Unfavorable storage conditions for reagents
  → Please store at recommended temperature.

IX. Related Products

Human Cell-Free Protein Expression Maxi System (Cat. #3285)
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 Plus (Cat. #638909/638910/63638911/638920)
In-Fusion® HD Cloning Plus CE (Cat. #638916/638917/638918/638919)
PrimeSTAR® Max DNA Polymerase (Cat. #R045A/B)
PrimeSTAR® HS DNA Polymerase (Cat. #R010A/B)
PrimeSTAR® HS (Premix) (Cat. #R040A)
DNA Ligation Kit <Mighty Mix> (Cat. #6023)
TaKaRa DNA Ligation Kit LONG (Cat. #6024)
Protein Molecular Weight Marker (Low) (Cat. #3450)
Protein Molecular Weight Marker (High) (Cat. #3451)
Protein Molecular Weight Marker (Broad) (Cat. #3452)
X-Gal (5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside) (Cat. #9031)