INSTRUCTIONS

1-Step Human High-Yield Maxi IVT Kit

88892 2569.0

Number	Description
88892	**1-Step Human High-Yield Maxi IVT Kit,** contains sufficient reagents to perform 2 reactions (2000µL each)

Kit Contents	Cap Color	88892X
HeLa Lysate	Red	4 × 500µL
Accessory Proteins	Green	4 × 100µL
Reaction Mix	Yellow	4 × 200µL
5X Dialysis Buffer	Clear	1 × 23mL
Positive Control DNA: pCFE-GFP (0.5µg/µL, 10µg)	Solid white	20µL
pT7CFE1-NHis-GST-CHA Expression Vector (0.5µg/µL, 10µg)	Clear	20µL

Kit Contents	88892Y
Maxi Dialysis Device	2 each
Nuclease-free Water	2 × 50mL

Note: Completely read the instructions before proceeding with the protocols.

Storage: Upon receipt store 88892X at -80°C and 88892Y at room temperature. 88892X is shipped with dry ice. 88892Y is shipped at ambient temperature.

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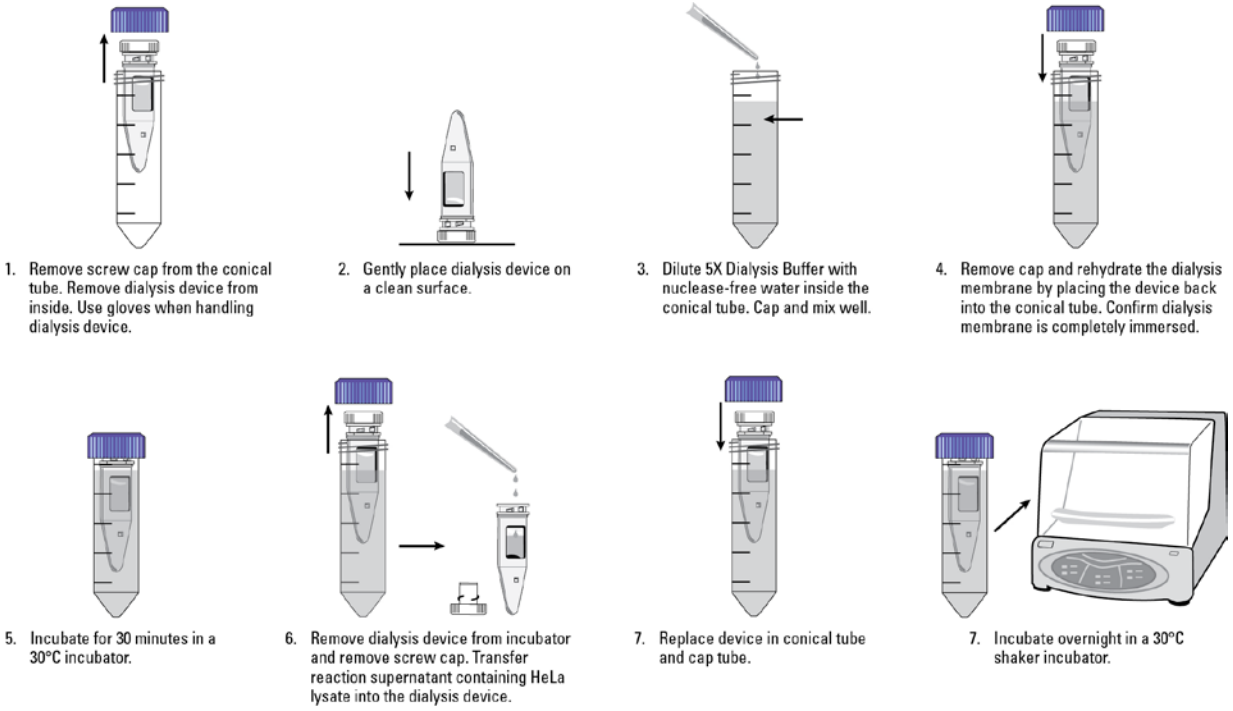
Introduction

The Thermo Scientific™ 1-Step Human High-Yield Maxi IVT Kit is a mammalian *in vitro* translation (IVT) system based on HeLa cell lysates, which contain all of the cellular components required for protein synthesis, including ribosomes, initiation factors, elongation factors and tRNA. When supplemented with the included proprietary Accessory Proteins, Reaction Mix and a DNA template cloned into the Thermo Scientific™ pT7CFE1-NHis-GST-CHA Vector, this system can synthesize protein for up to 16 hours.

The benefits of *in vitro* protein expression over traditional *in vivo* systems include the ability to express toxic proteins, faster protein synthesis and protein labeling with modified amino acids. The optimized kit contains a T7 promoter and an EMCV internal ribosome entry site (IRES) to facilitate high levels of *in vitro* protein expression in a cap-independent fashion. Using a vector containing the EMCV IRES element is critical for obtaining high expression levels in this human *in vitro* protein expression system.

Procedure Summary



1. Remove screw cap from the conical tube. Remove dialysis device from inside. Use gloves when handling dialysis device.
2. Gently place dialysis device on a clean surface.
3. Dilute 5X Dialysis Buffer with nuclease-free water inside the conical tube. Cap and mix well.
4. Remove cap and rehydrate the dialysis membrane by placing the device back into the conical tube. Confirm dialysis membrane is completely immersed.
5. Incubate for 30 minutes in a 30°C incubator.
6. Remove dialysis device from incubator and remove screw cap. Transfer reaction supernatant containing HeLa lysate into the dialysis device.
7. Replace device in conical tube and cap tube.
7. Incubate overnight in a 30°C shaker incubator.

Important Product Information

- **Use the included Thermo Scientific pT7CFE1-NHis-GST-CHA Vector (Product No. 88871) for cloning and expressing the target gene. See the Additional Information Section for additional vector choices, cloning sites and expression-ready clones.**
- Thaw HeLa Lysate on ice, aliquot and quickly store at -80°C. All components of the kit are stable for up to five freeze-thaw cycles as long as the contents are stored at -80°C immediately after use. For faster thawing, gently flick the lysate tubes.
- Undiluted lysate and reactions containing lysate will appear cloudy before and after incubation. Accessory Proteins and Reaction Mix may also appear clear to cloudy upon thawing; mix thoroughly but gently before and after adding each component to the IVT reaction. Undiluted 5X Dialysis Buffer may appear cloudy; mix well before and after dispensing.
- Avoid RNase contamination by wearing gloves; working in a clean, dust-free environment; and using RNase-free tips and microcentrifuge tubes.

Additional Materials Required

- DNA preparation kit (e.g., Thermo Scientific™ GeneJET™ Plasmid Maxi Prep Kit, Product No. K0492)
- Western immunoblot accessories for detecting expressed protein
- FITC filter-containing device to observe the expression of GFP in positive control reactions
- 1.5mL and 15mL RNase-free microcentrifuge tubes for assembling reactions
- RNase-free pipette tips
- Shaker incubator capable of maintaining temperature at 30°C.

Protocol for using the 1-Step Human High-Yield IVT Maxi Kit

A. Protein Expression

1. With the exception of 5X Dialysis Buffer, thaw all other reagents in the kit contents of 88892X and maintain on ice. Thaw 5X Dialysis Buffer at 25-30°C for a maximum of 30 minutes, and after making a 1X mixture, maintain the diluted buffer at 30°C.

   Note: Store any unused 88892X kit components at -80°C.

   Note: The 5X Dialysis Buffer may appear cloudy. Mix or vortex gently. Do not centrifuge before use. Once diluted, the 1X Dialysis Buffer will become clear within minutes.

2. Combine 5X Dialysis Buffer and Nuclease-free Water (volumes per Table 1) in the provided conical tube.

Table 1. Reconstitution of the Dialysis Buffer.

Component	mL
5X Dialysis Buffer	8.0
Nuclease-free Water	32.0
Total	**40.0**

3. Place a dialysis device inside the 50mL tube containing 1X Dialysis Buffer as shown in the Procedure Summary Section.
4. **Optional:** Set up a small reaction to test the integrity of the HeLa Lysate, Accessory Proteins and Reaction Mix. Add 12.5µL of HeLa Lysate, 2.5µL of Accessory Proteins, 5µL of Reaction Mix, 3µL of Nuclease-free Water and 2µL of pCFE-GFP DNA plasmid to a nuclease-free 1.5mL microcentrifuge tube. Incubate at 30°C for 4-5 hours. See Section B, Step 1: Quick visual detection for detecting the expressed GFP protein.
5. Prepare IVT reactions using Table 2. Add the reagents in the order listed into a 15mL RNase/DNase-free tube. Gently mix the reaction after each reagent addition. Incubate HeLa Lysate with Accessory Proteins for 10 minutes at room temperature prior to adding the rest of the components.

Table 2. Components of the IVT reaction.

Component	µL
HeLa Lysate	1000
Accessory Proteins	200
Reaction Mix	400
Cloned DNA(0.5µg/µL)	160
Nuclease Free water	240
Total	**2000**

6. Briefly centrifuge the reaction mix at 10,000 × *g* for 2 minutes. A small pellet will be visible after centrifugation.
7. Transfer the supernatant into the empty dialysis device and screw the cap on the device as described in the Procedure Summary Section.
8. Place the entire dialysis device into the 50mL tube containing Dialysis Buffer and close the screw cap.
9. Incubate the reaction for 6-16 hours at 30°C in a shaker incubator. Shake the entire unit in a shaker incubator using the following guidelines to determine the speed of shaking.

Shaker orbital radius	Recommended RPM
3mm (Eppendorf™ ThermoMixer™)	350
3/4 inch (New Brunswick C24)	250
1 inch (Thermo Scientific™ MaxQ™ 8000)	150

Note: Although protein expression is complete within six hours for most proteins tested, incubating up to 16 hours may increase expression of some proteins. Optimal time to express each protein must be determined empirically. A small white precipitate may be visible, which can be easily removed by centrifugation in the next step.

10. At the end of incubation, collect the contents from the dialysis device equally into two separate 1.5mL microcentrifiuge tubes and centrifuge at 10,000 × *g* for 2 minutes prior to storage.

    Note: Resulting reactions may be stored on ice for same-day use. For long-term storage, transfer the reaction contents from the dialysis device and store separately at -20°C or colder.

11. Proteins expressed using this kit may be purified using the purification guidelines provided in the Product Blog article "Choosing a vector and purification method for *in vitro* protein expression" on our website at: thermoscientific.com/pierce.

B. Determination of Protein Expression Level

Note: The GFP control protein is from the copepod *Pontellina plumata*. This GFP is not reactive to antibodies generated against *Aequorea victoria* GFP (i.e., EGFP or other EGFP mutants). Use polyclonal antibodies to TurboGFP (Product No. PA5-22688).

1. Visualize or quantitate the GFP control protein using one of the following methods:

   Quick visual detection: Place the GFP reaction tubes directly under a microscope or imaging equipment containing a FITC filter (ex/em: 482/502nm); alternatively, spot a small volume (1-2µL) on a piece of plastic wrap or laboratory film and visualize with fluorescent imaging equipment.

   Fluorescent plate reader: Place sample directly into a white or black 96- or 384-well plate. Evaluate signal using a fluorescent plate reader at ex/em: 482/502nm. To quantitate GFP, compare the fluorescence to a recombinant GFP standard curve.

2. Visualize or quantitate non-fluorescent protein expression using one of the following methods:

   Fast Western immunoblot analysis: This is a quick protocol consisting of transfer and detection of proteins separated on SDS-PAGE using ultra-sensitive Thermo Scientific™ SuperSignal™ Substrate. A detailed protocol and reagents required for Western blot detection can be found at thermoscientific.com/pierce; search using "fast western blot."

   SDS-PAGE analysis: Separate proteins by SDS-PAGE and stain using Thermo Scientific™ GelCode™ Blue Stain Reagent (Product No. 24590), Thermo Scientific™ Imperial™ Protein Stain (Product No. 24615) or Thermo Scientific™ PageBlue™ Protein Staining Solution (Product No. 24620) (Figure 2).

C. Purification of IVT-expressed Proteins

Proteins expressed using this kit may be purified using the purification guidelines provided in the Product Blog article "Choosing a vector and purification method for *in vitro* protein expression" on our website at: thermoscientific.com/pierce.

Troubleshooting

Problem	Possible Cause	Solution
GFP not detected by fluorescence in positive control reaction	Incorrect filter set was used	The excitation/emission wavelengths of GFP are 482/502nm
	Lysates have become inactive	Store unused lysate in nuclease-free tubes at -80°C; do not exceed five cycles of freezing and thawing
No expression of target protein	Incorrect vector was used	Use cloning vector pT7CFE1-NHis-GST-CHA provided in the kit to clone and express the gene of interest **Note:** The 1-Step Human IVT Kits are optimized using the pCFE1 vector and its derivatives; for a complete listing, please visit thermoscientific.com/pierce
	HeLa Lysate, Accessory Proteins and Reaction Mix were stored at a suboptimal temperature	Store unused HeLa Lysate, Accessory Proteins and Reaction Mix in nuclease-free tubes at -80°C; do not exceed five cycles of freezing and thawing
	Poor-quality DNA	Ethanol precipitate the DNA to remove trace amounts of inhibitors or salts − see the Additional Information Section for the recommended protocol
	Degradation of mRNA in the translation reaction	Maintain an RNase-free environment by wearing gloves; working in a clean, dust-free environment; and using RNase-free tips and microcentrifuge tubes
	Protein was sensitive to proteases	Add Thermo Scientific™ Halt™ Protease Inhibitor Single-Use Cocktail, EDTA-free (100X) (Product No. 78425) at 0.5X to the reaction mix in Step A, 5, Table 2
Low yield of target proteins	Incorrect incubation temperature	Perform reactions at 30°C
	Incorrect order of reagent addition	Incubate HeLa Lysate with Accessory Proteins for 5-10 minutes before adding remaining components to improve target protein expression
Smaller band size than predicted	Stop codons were in genes of interest	Ensure the cloned genes do not have a stop codon in the open reading frame
Protein appears to be degraded	Proteins were susceptible to proteases	Add Halt Protease Inhibitor Single-Use Cocktail, EDTA-free (100X) (Product No. 78425) at 0.5X to the reaction mix in Step A, 5, Table 2
Larger band size than predicted	Post-translation modifications	HeLa Lysate is capable of protein post-translational modifications, including partial glycosylation and phosphorylation. Validate the presence of glycosylation by digesting a small portion of the sample with Endo H or PNGase (a loss of the higher molecular weight bands indicates proteins were glycosylated)
Low protein yield after purification	Reaction scale was too small	Follow guidelines provided in the Product Blog article "Choosing a vector and purification method for *in vitro* protein expression" on our website at: thermoscientific.com/pierce
		Increase reaction size
Low protein yield after purification	Affinity tag was not accessible	Use different affinity purification for the tagged protein
		Purify protein under denaturing conditions (e.g., 8M urea) using the Thermo Scientific™ HisPur™ Cobalt Purification Kit (Product No. 90090)
		Reduce incubation temperature to 25°C

Additional Information

A. pT7CFE1-NHis-GST-CHA Vector Cloning Sites and Sequence Features

The 1-Step High-Yield IVT Kit has been optimized using the pT7CFE1-NHis-GST-CHA cloning vector, which is designed for high-level protein expression. In addition to multiple purification tags, it contains an HRV 3C cleavage site for tag removal. For a complete listing of pT7CFE1 expression vector derivatives, visit thermoscientific.com/pierce; search using "expression vectors."

Features:

- 10 unique restriction sites are provided in the multiple cloning site for cloning genes of interest (Figure 1)
- 5´ UTR consisting of EMCV internal ribosome entry site (IRES) required for high-level protein expression
- Poly A sequence in the 3´ region promotes mRNA stabilization and protection from nucleases
- T7 terminator ensures synthesis of accurate sized mRNA transcripts

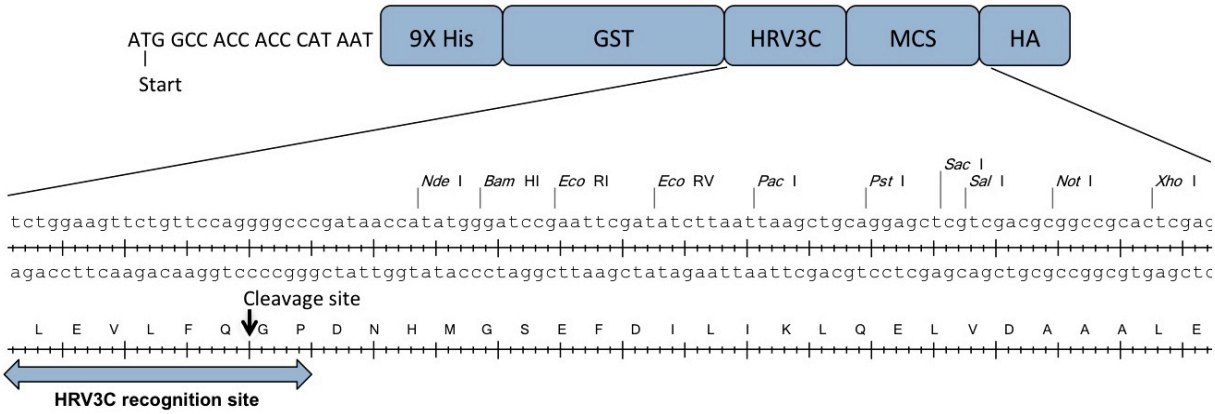


Figure 1. The Thermo Scientific pT7CFE1-NHis-GST-CHA Vector multiple cloning site, with the exception of Msc 1, is common to all of the expression vectors used in the Thermo Scientific 1-Step Human IVT Kits. The translational start site is the ATG found upstream of the His tag region.

B. Vector DNA Clean-up and Concentration Protocol

Prepare DNA using a standard maxi- or mini-prep protocol. To avoid compromising protein expression yield, completely remove contaminating proteins and eliminate the RNase A used in many mini-prep protocols. Perform the following steps to precipitate and, subsequently, concentrate the DNA.

1. Add 1/10 volume of 3M sodium acetate, pH 5.5 and two volumes of ethanol. Thoroughly mix the reaction and incubate at -20°C for 15 minutes.
2. Centrifuge the mixture at 14,000 × *g* for 15 minutes. Remove the supernatant and wash the pellet once with 70% ethanol.
3. Centrifuge at 14,000 × *g* for 5 minutes. Using a fine tip, remove all of the supernatant, including the residual. Air-dry the pellet for 5 minutes at room temperature.
4. Resuspend the pellet in nuclease-free water before measuring the DNA concentration. DNA templates may be stored in a Tris-based buffer. It is not necessary to linearize the plasmid DNA before use.

C. Expression-ready Clones for Use with the 1-Step Human High-Yield IVT Kits

- Custom cloning service; please visit thermoscientific.com/pierce and search for "cloning service."
- The pANT7 vector library from the ASU Biodesign Institute DNASU Plasmid Repository is compatible with our 1-Step High Yield IVT Kit. Visit http://dnasu.asu.edu/DNASU/Home.jsp for information and ordering. Under advanced search options choose "pANT7" for vector selection.
- PCR templates: see Tech Tip #72: PCR protocol for generating optimized templates for Pierce™ Human *In Vitro* Expression Kits on our website.

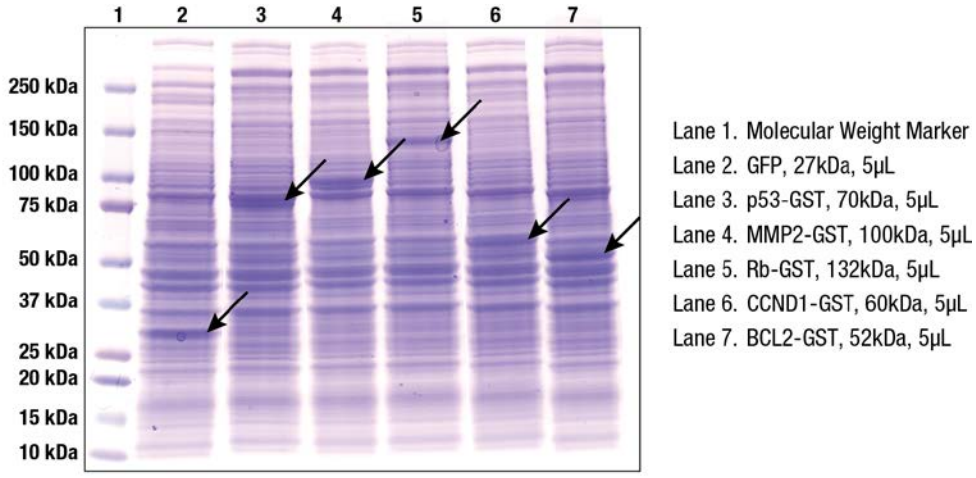


Figure 2. Expression of coomassie-stainable proteins using the Thermo Scientific 1-Step Human High-Yield IVT Kit. Five expression-ready clones (pANT7 vector) obtained from the DNASU Plasmid Repository were used to express the GST-fusion proteins listed in Lanes 3-7. Lane 2 shows expression of the control pCFE-GFP plasmid. Reaction mixtures of 5µL were separated by SDS-PAGE and stained with GelCode Blue Stain Reagent. Arrows indicate the positions of expressed proteins.

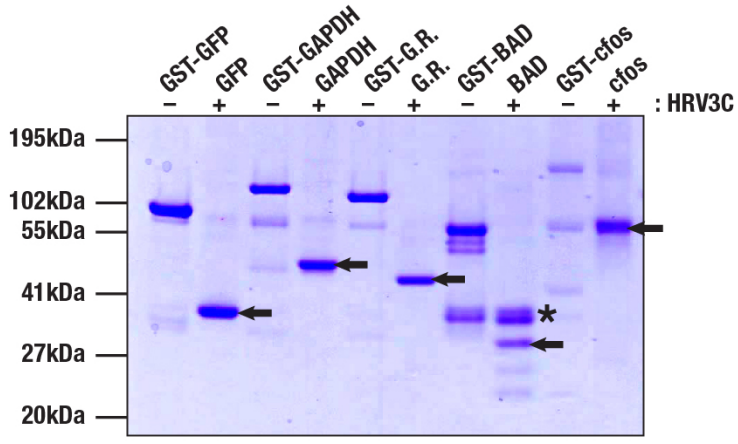


Figure 3. Purification of N-terminal GST fusion proteins with immobilized glutathione. Purification of either GST-fused proteins or untagged protein was performed as described with 10mM glutathione or HRV3C protease, respectively, using instructions provided in the Product Blog article "Choosing a vector and purification method for *in vitro* protein expression" on our website at: thermoscientific.com/pierce. The additional bands denoted with a * found with the purification of Bad are 14-3-3 proteins which co-elute with Bad. Protein identification was verified by mass spectrometry (data not shown).

Related Thermo Scientific Products

88859-71	**pT7CFE1-based Expression Vectors**
88899	**Recombinant GFP Protein**
88881-2	**1-Step Human Coupled IVT Kit – DNA**
88890-1	**1-Step High Yield Mini IVT Kit**
MA121315	**Mouse anti-6x-His Epitope Tag Monoclonal Antibody (HIS.H8)**
26183	**Mouse anti-HA Monoclonal Antibody (2-2.2.14)**
MA4004	**Mouse anti-Glutathione S-transferase Monoclonal Antibody (8-326)**
35035	**Pierce Fast Semi-Dry Transfer Buffer (10X),** 500mL
88217	**Pierce Fast Semi-Dry Blotter**
35050	**Pierce Fast Western Blot Kit, ECL Substrate**
88221	**HisPur Ni-NTA Resin,** see our website for all related products
89964	**HisPur Cobalt Resin,** see our website for all related products
16100	**Pierce Glutathione Agarose,** see our website for all related products
26182	**Pierce Anti-HA Agarose,** see our website for all related products
K0492	**GeneJET Plasmid Maxiprep Kit,** see thermoscientific.com/onebio
PA5-22688	**Anti-TurboGFP Polyclonal Antibody**
88836-7	**Pierce Anti-HA Magnetic Beads**
88821-2	**Pierce Glutathione Magnetic Beads**
88831-2	**HisPur Ni-NTA Magnetic Beads**

General References

Imataka, H., *et al.* (2009). Advantages of human cell-derived, cell-free protein synthesis systems (Japanese). *Seikagaku* **81(4)**:303-7.

Kobayashi, T., *et al.* (2007). An improved cell-free system for picornavirus synthesis. *J Virol Methods* **142(1-2)**:182-8.

Kozak, M. (1983). Comparison of initiation of protein synthesis in prokaryotes, eukaryotes and organelles. *Microbiol Rev* **47(1)**:1-45.

Kozak, M. (2005). Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene* **361**:13-37.

Mikami, S., *et al.* (2006). An efficient mammalian cell-free translation system supplemented with translation factors. *Protein Expr Purif* **46(2)**:348-57.