INSTRUCTIONS

1-Step Human Coupled IVT Kit – DNA

88881 88882 2317.2

Number	Description
88881	**1-Step Human Coupled IVT Kit – DNA,** contains sufficient reagents to perform 8 reactions (25µL each)
88882	**1-Step Human Coupled IVT Kit – DNA,** contains sufficient reagents to perform 40 reactions (25µL each)

Kit Contents	Cap Color	88881	88882
HeLa Lysate	Red	110µL	500µL
Accessory Proteins	Green	25µL	100µL
Reaction Mix	Yellow	40µL	200µL
Nuclease-free Water	Solid blue	1.5mL	1.5mL
Positive Control DNA: pCFE-GFP (0.5µg/µL, 10µg)	Solid white	20µL	20µL
pT7CFE1-CHis Expression Vector (0.5µg/µL, 10µg)	Clear	20µL	20µL

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

Storage: Upon receipt store at -80°C. Kits are shipped with dry ice.

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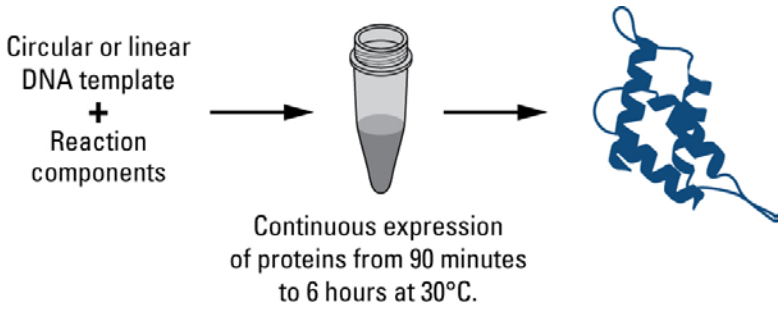
Introduction

The Thermo Scientific 1-Step Human Coupled IVT Kit – DNA is a mammalian *in vitro* translation (IVT) system based on HeLa cell lysates. The kit contains 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-based Vector, this system can synthesize protein for up to 6 hours. The kit includes a pT7CFE-CHis vector for ease of use in downstream applications.

The benefits of *in vitro* expression of proteins over traditional *in vivo* systems include expression of toxic or insoluble 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



Important Product Information

- **Use the included Thermo Scientific pT7CFE1-CHis Vector (Product No. 88860) for cloning and expressing the target gene. See the Additional Information Section for additional vector choices, cloning sites and sequence features.**
- Thaw HeLa Lysate on ice, aliquot and quickly store at -80°C. HeLa Lysate may be freeze-thawed up to five times without loss of activity. 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 gently before adding to the IVT reaction.
- 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., Qiagen Miniprep or Maxiprep Kit)
- Western immunoblot accessories for detecting expressed protein
- FITC filter-containing device to observe the expression of GFP in positive control reactions
- RNAse-free microcentrifuge tubes (0.5 or 1.5mL)
- RNAse-free pipette tips
- Incubator capable of maintaining temperature at 30°C

Protocol for using the 1-Step Human Coupled IVT Kit – DNA

A. Protein Expression

Note: Thaw reagents immediately before use and keep on ice. Assemble the IVT reaction at room temperature. Store any unused lysate or kit components immediately at -80°C.

1. Thaw the HeLa Lysate, Accessory Proteins, Reaction Mix and plasmid DNA on ice. To hasten the thawing process, warm vials in gloved hands. Once thawed half way, place back on ice.
2. Use Table 1 to prepare reactions at room temperature. Add the reagents **in the order listed** into a 1.5mL nuclease-free tube. Gently mix the reaction after each reagent addition. Scale-up of the reaction may be done using the component ratios shown in Table 1.

Table 1. Components for the IVT reaction.

Component	No DNA Control (µL)	GFP Control (µL)	Target Protein (µL)
HeLa Lysate	12.5	12.5	12.5
Accessory Proteins	2.5	2.5	2.5
Reaction Mix	5	5	5
pCFE-GFP DNA (0.5µg/µL)	–	2	-
Cloned DNA (0.5µg/µL)	–	-	2
Nuclease-free Water	5	3	3
Total	**25**	**25**	**25**

3. Incubate the reaction for 90 minutes to 6 hours at 30°C.

   Note: For same-day use, maintain reactions on ice for downstream applications and analysis. For long-term storage, maintain reactions at -20°C or colder.

   Note: Reactions may be centrifuged at 10,000 × *g* for 5 minutes; however, before performing this step it is important to determine if the expressed protein is in the soluble supernatant fraction or the pellet portion by performing a Western blot analysis of the total reaction as described in Section B: Determination of Protein Expression Level.

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 a polyclonal antibody to TurboGFP (e.g., 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: 482/512nm); 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: 482/512nm. 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 *www.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), Imperial Protein Stain (Product No. 24615) or PageBlue Protein Staining Solution (Product No. 24620).

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 482/512nm
	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-CHis 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 *www.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 EDTA-free Aprotinin or Leupeptin protease inhibitor at the recommended concentration to the lysate
Low yield of target proteins	Incorrect incubation temperature	Perform translation reactions at 30°C
	Incorrect order of reagent addition	Incubate 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 EDTA-free Aprotinin or Leupeptin protease inhibitor at the recommended concentration to the lysate
Larger band size than predicted	Post-translation modifications	HeLa Lysate is capable of protein PTMs, 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	Increase reaction size
	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)

Additional Information

A. pT7CFE-CHis Vector Cloning Sites and Sequence Features

The 1-Step Human Coupled IVT Kit – DNA has been optimized using the pT7CFE1-CHis cloning vector, which is designed for high-level protein expression. For a complete listing of pT7CFE1 expression vector derivatives, visit *www.thermoscientific.com/pierce;* search using "expression vectors."

Features:

- 14 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

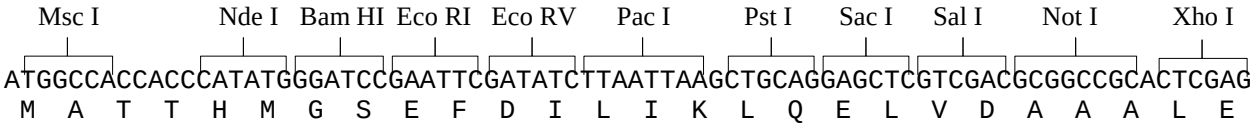


Figure 1. pT7CFE1-CHis Multiple Cloning Site common to all of the expression vectors used in the Thermo Scientific 1-Step Human IVT Kits. For the expression vector pT7CFE1-CHis, the preferred 5´ cloning site is Nde I, which produces maximal expression using the EMCV IRES. The translational start site is the ATG partially found in the Msc I site. Sequences cloned into Nde I will have methionine followed by alanine, threonine, threonine and histidine at the N-terminus of the protein. Sequences cloned into Msc I will have methionine followed by alanine at the N-terminus of the protein.

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 at 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 Coupled IVT Kit

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

Related Thermo Scientific Products

88859-69	**pT7CFE1-based Expression Vectors**
88899	**Recombinant GFP Protein**
88883-4	**1-Step Human IVT Kit – mRNA**
88887	**1-Step Human High-Yield Midi IVT Kit**
88889	**1-Step Human High-Yield Maxi IVT Kit**
88330-1	**1-Step Heavy Protein IVT Kit**
69570	**Slide-A-Lyzer MINI Dialysis Device, 10K MWCO, 0.1mL**
88401	**Slide-A-Lyzer MINI Dialysis Device, 10K MWCO, 0.5mL**
88404	**Slide-A-Lyzer MINI Dialysis Device, 10K MWCO, 2mL**
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
PA5-22688	**TurboGFP Antibody**

General References

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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.

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