CellFree Sciences

The natural power of wheat driving science

High Performance Cell-Free Wheat Germ Protein Expression System

INSTRUCTION MANUAL

Premium ONE Expression Kit

This kit provides reagents for 24 coupled transcription-translation reactions using the wheat germ cell-free expression system

Product Number(s): CFS-EDX-ONE

Version/date: Version 2.0_eng/June 2020

This Product has a shelf life of 1 year being safely stored at -80°C.

CFS products are for research use only.

Our products are produced under a strict quality management system offering high-quality reagents including wheat germ extracts from wheat obtained by natural farming in Japan.

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Web: https://www.cfsciences.com/eg/ Follow us on Twitter: @CFSciences

Table of Contents

Important Information

Shipment and Storage

Our products are shipped on dry ice. Wheat germ extracts are temperature sensitive and must always be kept frozen. Store kit at -80°C right upon arrival and only thaw reagents when needed. Avoid repeated freeze/thawing cycles. Prepare aliquots of the wheat germ extract on first use if you want to keep on making more expression experiments later; refer to the protocol below on how much extract is needed per reaction. Do not freeze/thaw the wheat germ extract more than three times.

Safety

This kit can be used in a regular molecular biology laboratory. We strongly advise to work under RNase-free conditions. Refer to a laboratory handbook for more information on how to work under RNase-free conditions.

Read the protocol carefully before starting the experiment.

Do not drink or eat in the laboratory, and always wear gloves and a lab coat while working in the lab.

Wash hands before and after doing an experiment. If you get any reagent(s) in your eyes or on your skin, wash eyes or skin immediately with water. Although this kit does not contain any hazardous reagents, do not take any risk.

Inform yourself about the necessary precautions for performing SDS-PAGE experiments using high voltage, and toxic chemicals in case you wish to prepare your own gels.

Safety Data Sheets (SDS) for our products can be downloaded from our homepage at:

https://www.cfsciences.com/eg/

Look up your product to find the matching SDS.

Contact CellFree Sciences for further support and advice if you have any questions on the experiments described herein and materials provided with this product. Contact information is given at the end of this manual.

For your convenience

CellFree Sciences is providing short versions of our protocols (“Bench Notes”). Use these Bench Notes to setup your experiments at your workplace. They only contain the basic information needed for setting up the experiments. Use the checkmarks in the Bench Notes to assure that all pipetting steps have been completed correctly.

Introduction

The Premium ONE Expression Kit from CellFree Sciences allows to perform coupled transcription and translation reactions to express proteins in the wheat germ cell-free protein expression system. By combining the transcription and translation reactions to be performed at the same time in the same reaction mixture, protein expression experiments can be setup with little effort and conducted with shorter overall reaction times. These conditions are optimal for doing small-scale protein expression experiments, where one 55 µl reaction may yield some 1 µg of protein for a 24 h reaction. This is sufficient to detect most proteins on SDS-PAGE or by more sensitive detection methods like Western Blotting or working with a label. The kit contains a small amount of FluoroTect™ GreenLys (sold separately by Promega: Catalog # L5001) that is incorporated during *in vitro* translation reactions and thus allows to prepare a fluorescently labeled protein for easy and sensitive detection during expression tests on new templates. The label provided with this kit may be used to test up to four templates in protein expression experiments. Refer to reference 4 in the reference list for more information on how to use the FluoroTect™ GreenLys label.

Commonly one 55 µl reaction of the Premium ONE Expression Kit provides sufficient protein yields to conduct some biochemical experiments including studies on enzymatic activity, protein modifications, or binding experiments studying the interaction with antibodies, other proteins, DNA or RNA. Protein requirements will vary depending on the assay and the detection methods used to analyze the proteins. Customers can adopt the reaction time and setup to meet with their protein needs as yield will vary for different proteins.

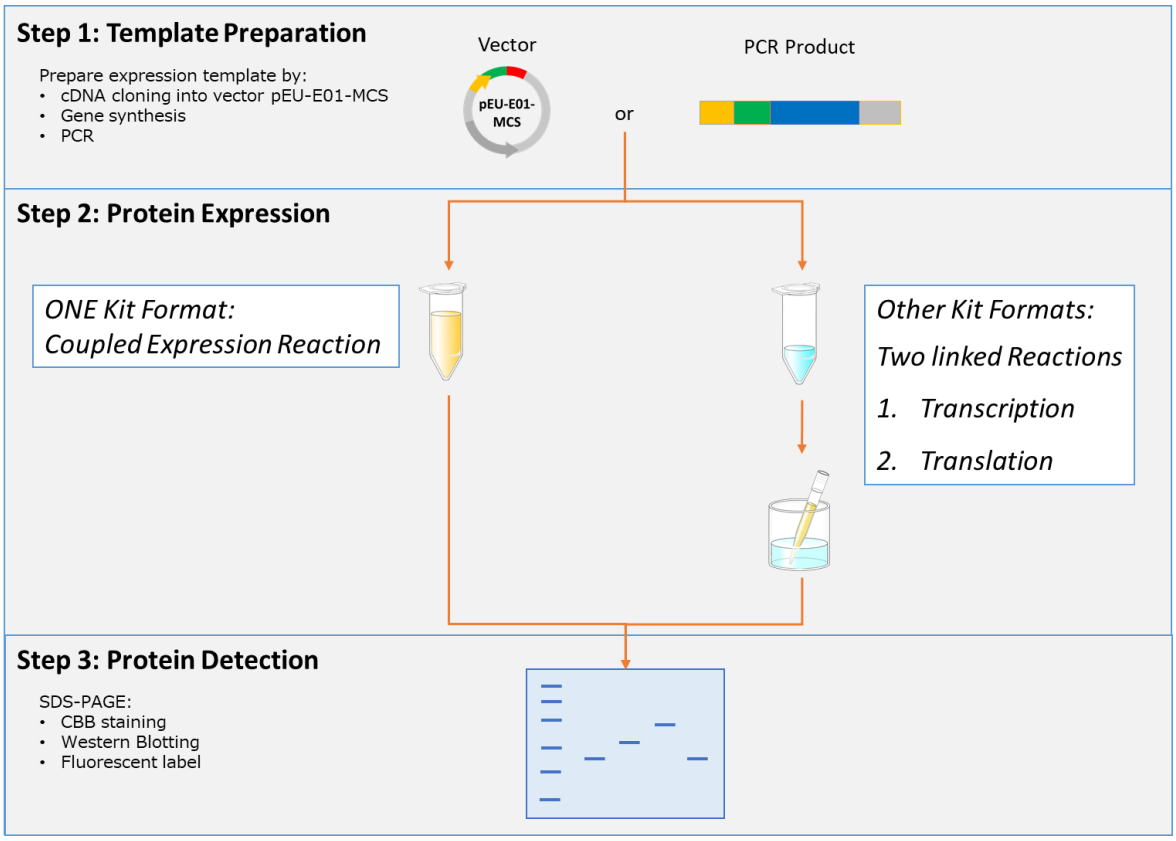
The Premium ONE Expression Kit provides means to support template preparation for protein expression experiments. All templates used in the wheat germ cell-free protein expression system require an SP6 RNA polymerase promoter and a dedicated translation initiation site (E01 translational enhancer) that supports Cap-independent initiation of translation. Being an open *in vitro* translation system, proteins can be expressed from linear DNA templates as well as using an expression vector encoding the Open Reading Frame (ORF) for the protein of interest. Commonly, it is preferable to work with an expression vector to achieve higher protein yields and to obtain reproducible results. To this point, the Premium ONE Expression Kit provides the pEU-E01-MCS expression vector that had been optimized for use in the wheat germ cell-free system; refer to our homepage for more information on other available expression vectors for our system. However, for rapid testing of different templates it may be desirable to work directly with PCR products in the protein expression experiments. The Premium ONE Expression Kit provides primers covering the regulatory elements needed to drive the transcription and translation reactions for use in PCR experiments. In addition, gene-specific primers are required to perform PCR and those must be designed by the customer for each target gene.

While we give one standard reaction size in this manual for doing coupled transcription/translation reactions, the reagents provided with the kit can be used on different reaction scales. Please refer to the table at the end of the protocol on how to use the reagents for different reaction sizes. The conditions in the table have been tested to confirm the scalability of the system. For large-scale protein expression experiments, however, we recommend using our linked reaction systems to perform separate transcription and translation reactions. Separating the two reactions allows for optimal reaction conditions for each step and provides more flexibility to further modify protein expression reactions for meeting with certain protein requirements. Please refer to our homepage for more information on our protein expression kits to perform linked protein expression reactions.

General Information on Wheat Germ Protein Expression System

To prepare proteins in the wheat germ cell-free protein expression system is a straightforward process with the easy setup of the coupled transcription/translation reactions. Working with coupled reactions requires two separate reagents that must form a bilayer for effective protein expression. The Premium ONE Expression Kit was designed to perform 24 reactions on a 55 µl scale as compared to larger 226 µl reactions commonly used in our entry kits for linked transcription and translation reactions like the Premium PLUS Expression Kit.

Figure 1: Comparison of coupled and linked cell-free protein expression reactions using different CFS kit formats



CellFree Sciences offers several reagent kits for conducting linked protein expression reactions in the wheat germ system. For conducting simplified bilayer reactions, you can purchase the Protein Research Kit S16, Product Number CFS-PRK-S16. This kit provides WEPRO®9240 premixed reagents to perform 16 small-scale 226 µl bilayer expression reactions. Our larger WEPRO®7240 Core Kits offer sufficient reagents to perform protein expression reactions on different reaction scales from protein expression tests to scaling up protein expression to a milligram scale. Protein Research Kits and Core Kits are provided in different versions to better enable working with GST- and His-tagged proteins, synthesis of membrane proteins, or to prepare labeled proteins. CellFree Sciences offers dedicated protein expression kits to prepare isotope-labeled protein standards for protein MS (FLEXIQuant Standards) and samples for protein NMR experiments. While requiring extra time, linked protein expression experiments give higher protein yields from the same template as compared to working with a coupled transcription/translation system that compromises on the reaction conditions for each reaction. For more information on other CFS products, refer to our homepage or contact us directly using the contact information at the end of the manual.

Protocol Overview

This manual provides advice on how to prepare expression templates by cloning DNA fragments into expression vector pEU-E01-MCS, or as an alternative approach to prepare templates by a two-step PCR process. Both templates can be used to setup the coupled *in vitro* transcription/translation experiments as further outlined in this protocol working on a 55 µl scale. The expression reactions can be maintained for up to 24 hours, where some 2-hour reactions may be sufficient to detect the expressed protein by Western Blotting. To view the protein on stained SDS-PAGE, the translation reactions may be maintained for some 12 to 24 hours.

Additional directions are provided to for doing labeling experiments with the FluoroTect™ GreenLys including further advice on how these proteins can be analyzed on SDS-PAGE to see whether a protein of the correct size was made. We advise to use known amounts of a BSA standard in the SDS-PAGE experiment to estimate the protein yields. Proteins may also be detected by Western blotting using an antibody against the target protein of interest or an added tag. For conducting Western Blot experiments, refer to the recommendations for the antibodies used in the experiment. Antibodies may require different dilutions or washing conditions and signal detection can be performed by several methods.

Use of Premium ONE Expression Kit

The Premium ONE Expression Kit provides enough reagents for 24 coupled protein expression reactions on a 55 µl reaction scale; the provided FluoroTect™ GreenLys lasts for 4 labeling reactions. In addition, the kit provides expression vector pEU-E01-MCS, and primers SPU and deSP6E01 to prepare an expression template for the protein(s) of interest. To confirm the correct use of the reagents and reaction conditions, we further include the pEU-E01-DYKDDDDK-DHFR positive control vector.

Materials Provided by the Kit

The Premium ONE Expression Kit comes in one box and is shipped on dry ice. Upon arrival, store the kit immediately at -80°C. Do not thaw reagents at any time until starting the actual experiment. Avoid unnecessary freeze/thawing cycles. The wheat germ extract will rapidly lose activity when kept above -80°C! Prepare aliquots of the wheat germ extract when used for the first time. Refer to the table at the end of the protocol on reagent needs for different reaction sizes.

Kit Content

Item	Quantity	Concentration	Volume	Vial	Vial Color
WEPRO® TTmix	1	—	70 µl	0.5 ml Tube	Clear
SUB-AMIX® TT	1	—	1.3 ml	2 ml Tube	Clear
SPU Primer	1	1 µM	100 µl	0.2 ml PCR tube	Orange
deSP6E01 Primer	1	10 nM	100 µl	0.2 ml PCR tube	Purple
pEU-E01-MCS	1	1.0 µg/µl	5.0 µl	0.2 ml PCR tube	Red
pEU-E01-DYKDDDDK-DHFR (*)	1	15 ng/µl	10 µl	0.2 ml PCR tube	Clear
FluoroTect™ GreenLys tRNA (**)	1	—	4 µl	0.5 ml PCR tube	Black

*DYKDDDDK is the sequence for the Flag™-tag; ** Sourced and manufactured by Promega Corp. Keep reagent in the dark.

Instructions on Use of Reagents

Item	Description
WEPRO® TTmix	Pre-mixed wheat germ extract for coupled protein expression system. Avoid unnecessary freeze-thawing of the wheat germ extract! When melting the first time, prepare aliquots depending on your needs, e.g. 10 µl per aliquot. Freeze aliquots in liquid nitrogen and store them at -80 °C. Per 55 µl reaction some 2.5 µl of extract are required. Avoid freeze-thawing of the wheat germ extract for more than 3 times.
SUB-AMIX® TT	Translation reaction buffer for coupled protein expression system. When using SUB-AMIX® TT in multiple reactions, prepare small aliquots and freeze them in liquid nitrogen. Store buffer at -80 °C.
SPU Primer	2nd PCR sense primer
deSP6E01 Primer	2nd PCR sense primer
pEU-E01-MCS	Standard expression vector for use in wheat germ cell-free protein expression system, refer to Appendix A for more details on the vector.
pEU-E01-DYKDDDDK-DHFR	Expression vector for DYKDDDDK-tagged DHFR protein.
FluoroTect™ GreenLys tRNA	Fluorescent-labeled lysine-loaded tRNA: the labeled lysine is randomly incorporated during protein synthesis to obtain a labeled protein. **Always keep reagent in the dark!**

Materials to Be Prepared by User

Reagents for optional Plasmid DNA Purification

Plasmid DNA should be prepared by a commercial DNA purification kit. The following reagents are only needed for optional phenol extraction of the plasmid DNA. A phenol extraction is not mandatory but can help to remove impurities from standard plasmid DNA preparations when a vector gives low protein yields.

Reagents	Description
Phenol/Chloroform	phenol:chloroform:isoamyl alcohol (25:24:1 v/v), pH 7.9
Chloroform	> 99%
Ethanol	100%
Ethanol	70 %
Sodium acetate	3 M, pH 5.2
TE buffer	10 mM Tris, 1 mM EDTA, pH 8.0. Sterilized. It is highly recommended to use nuclease-free water when preparing TE buffer. - DO NOT use DEPC treated water!

Reagents Required for optional PCR Experiment

The following reagents are only needed when preparing the expression template by PCR. Two options are given for the PCR reagents described in this manual.

Reagents	Description
Gene specific forward primer (1st PCR sense primer)	Gene specific forward primer must be designed by customer following instructions within this manual.
Gene specific reverse primer (used in 1st PCR and 2nd PCR)	Gene specific reverse primer must be designed by customer following instructions within this manual.
PCR reagents*	This kit does not include the DNA polymerase, reaction buffer, dNTP mix required to do PCR reactions. The manual gives examples for using PrimeSTAR® GXL DNA Polymerase from Takara Bio Inc. and Platinum™ SuperFi™ DNA Polymerase from Thermo Fisher to conduct PCR experiments. Both enzymes have been tested at CellFree Sciences for use in combination with the Premium ONE Expression Kit.
Ultra-pure water	DNase, RNase free: DO NOT use DEPC treated water!

*See manufacturer's instructions for details on how to use PCR reagents.

Reagent Required for Protein Labeling using FluoroTect™ GreenLys

The following reagent is only needed when FluoroTect™ GreenLys for protein labeling.

Reagents	Description
RNase A (10 µg/µl) stock solution	RNase A is needed to reduce background on SDS-PAGE when confirming protein synthesis using fluorescently labeled tRNA in expression reaction.

Other Consumables and Instruments Required for Protein Expression

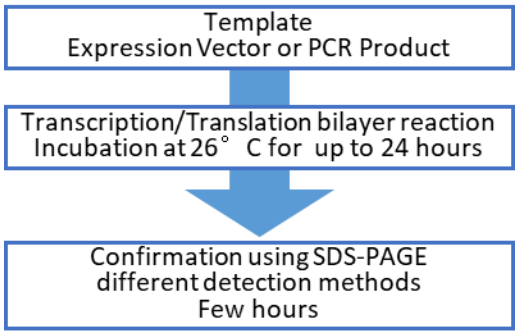
Consumable	Description
Incubator	Temperature set to 26°C, the reaction can be performed in a thermocycler
Thermocycler	Needed when using PCR for template preparation
0.2 ml PCR tube	Use DNase, RNase free quality
SDS-PAGE	SDS-PAGE*, gel electrophoresis apparatus and power supply
Devises to conduct Western Blotting experiment	Needed when confirming the synthesis of the target protein by Western Blotting
Fluorescent gel scanner	Needed when detecting of target protein after fluorescent lysine incorporation. Please use equipment which can measure at excitation wavelength 502 nm and detection wavelength 510 nm

*Using a commercially available SDS-PAGE can give better results and avoids the risk of working with toxic chemicals.

Protocols

Time Requirements

Refer to the flowchart below on the estimated time per protein expression reaction step. The flowchart covers only time required for template preparation by two-step PCR.



Considerations on Obtaining cDNA Templates for Proteins of Interest

Gather information on your protein of interest before preparing your expression template. Expression templates can be easily made by standard cloning methods or gene synthesis, which may be further used to do some codon optimization for expression in a wheat system. However, codon optimization is not required for using our expression system. Otherwise, for many protein coding genes cDNA clones are available in the public domain. There are large cDNA collections from which cDNA clones encoding for your protein of interest may be available. These clones are commonly distributed through clone distributors or public depositories. Searching such cDNA collections can be an easy, and convenient way to find cDNAs clones other than requesting published materials from other researchers. In general, for inquires on certain genes you can make a search at “Gene” on NCBI homepage at:

https://www.ncbi.nlm.nih.gov/gene/

Gene holds information on reference sequences from RefSeq, maps, pathways, variations, phenotypes, and links to genome-, phenotype-, and locus-specific resources worldwide. While the sequence information may be useful for using gene synthesis services, the links to worldwide resources are most useful to look for matching cDNA clones.

At the very end of the page, you will find “Additional links”, where you must get the list under “Gene LinkOut” (you may have to click on the + sign to see the complete list). NCBI allows suppliers to put there their links to products and services on the specified gene shown in the output page.

Click on the links for getting more information on each product to see whether this is what you want to obtain. For making a protein, you require information on available cDNAs for cloning into an expression vector. There had been several large cDNA cloning projects to provide ORF clones for most human protein coding genes. Look under “Research Materials” to see those resources, where you commonly will find various providers distributing academic clone collections like the ones offered by the “NITE Biological Resource Center” (a Japanese National Project), or the international ORFeome Collaboration clones.

As an alternative to searching Gene at NCBI, you may consider to directly searching the databases of different clone providers and public depositories. They commonly provide comprehensive information on their clones and the resources they are offering.

Note, that a cDNA clone just represents one possible isoform. However, for most transcripts from higher organisms there are multiple splice variants that commonly encode for different proteins. As an alternative to searching matching cDNA clones, gene synthesis services offer a convenient way to get access to ready to use expression vectors. Working with a gene synthesis provider allows you to fully avoid any cloning experiments. However, you should be careful about selecting the correct sequence information and correct insertion into the expression vector (see below). Gene synthesis may also be used to design templates for fusion proteins.

For more information on your protein of interest, refer to the UniProtKB database (https://www.uniprot.org/). Basic physical and chemical parameters for a protein can be calculated by the tool ProtParam tool (https://web.expasy.org/protparam/).

Remarks on Expression Vector Construction

We recommend preparing an expression vector for your protein(s) of interest prior to doing the protein expression experiments. While PCR products can be used in a cell-free protein expression system, plasmid DNA templates commonly provide better yields and give more reproducible results. Plasmid DNA templates are essential for continuous protein production and to up-scale protein production to a preparative scale.

In the following we give some brief advice on the use of our expression vectors. This kit contains expression vector pEU-E01-MCS (this vector does not encode any tag; refer to Appendix A for more details; red vial). The pEU-E01-MCS vector, and the positive control vector included in the kit, contain a SP6 promoter, an E01 translational enhancer, and an ampicillin resistance gene. If you obtained a standard cDNA clone, it may be necessary to isolate the coding region (Open Reading Frame or “ORF”) for later cloning into any of our expression vectors. Noncoding regions flacking the ORF must be removed when preparing an expression vector. Additional considerations apply when expressing protein fragments to assure proper use of the starting methionine and stop codon. Refer to a cloning handbook for more information on how to conduct vector design and DNA cloning experiments.

1. Insert the coding region for your protein of interest into the multiple cloning site (MCS) of the vector using one or two restriction enzyme sites properly selected according to the information on the MCS in the vector map for vector pEU-E01-MCS (Appendix A) (*1). The protein will be translated from the first start codon, an ATG, up to the first in frame stop codon in your cDNA.
2. After the ligation step, transform a suitable *E. coli* strain (e.g. JM109) with the vector DNA containing the cDNA-inserted into the expression vector. Grow transformed bacteria on Lysogeny Broth (LB) medium plus added ampicillin at a concentration of 100 µg/mL; the same ampicillin concentration can be used to growing bacteria transformed with the positive control vector included into the kit. Our vectors are high-copy

vectors and should commonly give good yields for DNA preparations in line with the yield ranges expected for a plasmid DNA purification kit.

3. Once you have selected an expression vector having the correct insert with the correct orientation and reading frame, we advise to prepare glycerol stocks from the transformed bacteria, and to store bacteria for future use. It is also possible to store DNA aliquots of the expression vector.

We recommend confirming correct insertion of the cDNA into the expression vector by at least end-sequencing of the insert and both cloning sites. In case the cDNA insert was prepared by PCR, sequencing of the entire insert is recommended to exclude inserts with PCR errors. Refer to Appendix B for more information on sequencing primers for our vectors. The vector sequence information for vector pEU-E01-MCS and all our other vectors can be downloaded from our homepage at http://www.cfsciences.com/eg/vector.html.

(Notes)

*1: To efficiently express the target protein, it is recommended to select a restriction enzyme site as close as possible to the E01 translational enhancer when cloning into vector pEU-E01-MCS. For cloning cDNAs into this vector, do not add a Kozak consensus sequence. The E01 translational enhancer is enough to induce translation.

Preparation of Plasmid DNA Template

We do not recommend the use of DNA mini-preparation methods based on alkaline elution procedures lacking any further purification step. Those may not work when directly using the vector in our expression system. Instead prepare plasmid DNA from *E. coli* cultures using a commercially available DNA purification kit. We recommend a QIAGEN Plasmid Midi Kit (Catalog No. 12143) or QIAGEN Plasmid Maxi Kit (Catalog No. 12163), which have commonly worked well in combination with our expression system. Comparable products from another provider may as well provide suitable results.

A highly purified plasmid DNA is essential for successful transcription and subsequent translation reactions. The protein synthesis may not proceed well, if the plasmid DNA does not have a proper A260/A280 ratio (proteins remained in the DNA preparation). Remaining proteins in the DNA preparation may lead to a low quality of RNA transcripts, or poor RNA yields. Therefore, we recommend in such cases a further purification of the plasmid DNA by phenol/chloroform extraction that can remove proteins and some other contaminations:

1. Add an equal volume of phenol/chloroform to the plasmid DNA solution and mix well (*1).
2. Centrifuge the mixture at 15,000 rpm for 5 min at room temperature.
3. Carefully transfer the upper aqueous phase to a new tube. Do not take the intersection.
4. Add an equal volume of chloroform into the tube with the aqueous phase and mix well.
5. Centrifuge this mixture at 15,000 rpm for 5 min at room temperature.
6. Carefully transfer the upper aqueous phase to another new tube. Do not take the intersection.
7. To this aqueous solution, add 2.5 times the volume 100% ethanol, and 3M sodium acetate (pH 5.2) at a 1/10 of the volume of the aqueous phase to precipitate the DNA.
8. Mix solutions and store at -20°C for 10 min.
9. Centrifuge at 15,000 rpm for 20 min at 4°C.
10. Remove the supernatant. Do not disturb the DNA pellet.
11. Add 800 µl of 70% ethanol to wash the DNA pellet in the tube.
12. Centrifuge the tube at 15,000 rpm for 10 min at 4°C.
13. Remove the supernatant. Do not disturb the DNA pellet.
14. Dry the DNA pellet for 10 to 20 min (do not dry pellet for longer period).
15. Add an appropriate volume of TE buffer to resuspend the DNA pellet.

16. Determine the concentration of the DNA with a spectrophotometer at wavelengths of 260 nm and 280 nm. Absorbance at 260 nm represents the concentration of DNA. The ratio of absorbance at 260 nm and at 280 nm indicates the purity of the DNA (*2).
17. Adjust the DNA concentration to 15 ng/µl by adding an appropriate volume of TE buffer (*3,4).

You need 2.5 µl purified plasmid DNA per 55 µl coupled transcription-translation reaction.

Plasmid DNA can be stored for a long time at -20°C. We advise to keep aliquots of the vector DNA for later use.

(Notes)

*1: Phenol and chloroform are hazardous chemicals and should only be handled with appropriate care and precautions. Note that phenol and chloroform must be discarded as special chemical waste.

*2: Purity of plasmid DNA should have an A260/A280 ratio between 1.70 and 1.85. Ratios outside this range indicate that the plasmid DNA is still not suitable for conduction the expression experiment. In that case, repeat the phenol extraction from the beginning.

*3: Make sure that the DNA concentration is accurately set to 15 ng/µl. Lower concentrations, e.g. 10 ng/µl, or higher concentrations, e.g. 20 ng/µl, will reduce protein yields from the coupled transcription/translation reactions.

*4: Plasmid DNA quality can be further confirmed by agarose gel electrophoresis loading some 0.1 to 0.2 µg of DNA on a standard or small agarose gel.

Preparation of DNA Template by PCR

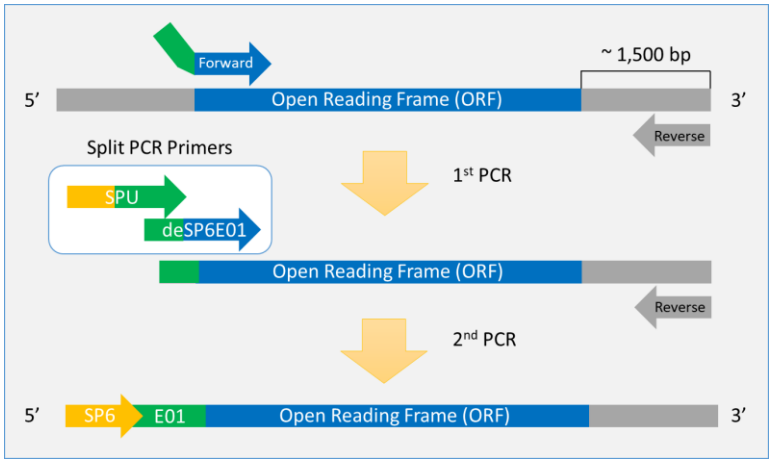
Linear DNA templates can be directly used in *in vitro* transcription/translation reactions. However, they must contain the same regulatory elements as mentioned for the pEU-E01-MCS expression vectors. Hence, during the PCR reactions additional sequences must be introduced adding the SP6 promoter and E01 translation enhancer elements at the 5' end of the open reading frame. The open reading frame must have its own stop codon at its 3' end. Working with the SP6 RNA polymerase in the transcription reaction, no terminator sequence has to be added to the 3' end of the template. In our PCR protocol, the 3' end of the ORF is further extended by some 1,500 bp to better stabilize the linear DNA against exonucleases. While the additional sequences at the 3' end can be directly taken from the vector used as the PCR template, we are using two primer extension reactions to add the SP6 promoter and E01 enhancer to the 5' end. Only during the second PCR step, the SP6 promoter and E01 translation enhancer sequences are added using two separate primers. Since we are avoiding primers containing both elements together, the method is also called "Split Primer PCR". Separating the SP6 promoter and E01 enhancer avoids protein expression from miss-primed PCR products. The PCR protocol given in this manual assumes that customer wants to amplify the full-length ORF of the target gene. Please contact us for more information on other PCR protocols to prepare templates for protein fragments or working with the His-tag.

Customers must design their own gene specific primers to amplify their genes of interest for template preparation. Follow the directions given in the protocol below on how to prepare those primers. We advise to check primer sequences before ordering oligonucleotide synthesis, e.g. using a free online tool like Primer3 (http://primer3.ut.ee/) or Primer3PLUS (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

We noticed in the past that target genes inserted into vectors of the pET-24 or pET-28 series may not yield any PCR product with the primers given in this protocol. We further advise to make sure that your PCR templates do not have any SP6 promoter sequence; having an SP6 promoter sequence in the template may cause mis-priming during the second PCR step. If you are uncertain about your PCR template, we recommend to rather subcloning the inserts into vector pEU-E01-MCS. Expression vectors based on our pEU-E01 vectors can be directly used in our expression system and no further PCR step is required.

For the protocol given in this manual, we tested the PrimeSTAR® GXL DNA Polymerase from Takara Bio Inc. (Catalog # R050B) and Platinum™ SuperFi™ DNA Polymerase from Thermo Fisher (Catalog # 12351010), which use different reaction conditions. Both are high-fidelity PCR DNA polymerases having low error rates to avoid any sequence errors during amplification. Even if you wish to use another DNA Polymerase, we strongly recommend using only high-fidelity PCR DNA polymerases to prepare expression templates. Using different PCR conditions may reduce protein yields.

Figure 2: Preparation of linear templates by Split Primer PCR method



Primers Design for 1st PCR

Forward Primer	Description
Forward Primer	Forward gene specific primer placed at 5' end of target ORF
Length (bp)	About 35 bases
Sequence	5'- CCACCCACCACCACCAATGNNNNNNNNNNNNNNNN -3'
Description	Green: Part of E01 translation enhancer sequence (Overlapping sequence with primer "deSP6E01" used for 2nd PCR) Red: Start codon for protein translation Blue: Gene specific sequence of interest (use about 20 bases)

Reverse Primer	Description
Reverse Primer	Reverse primer (1st PCR and 2nd PCR)
Length (bp)	About 20 bases
Sequence	Based on vector sequence into which the target gene was inserted
Description	Design with a length of about 20 bases in the downstream region about 1.5 kb or more downstream from the 3' end of the target gene. **This primer is also used for 2nd PCR step.**

Setup of 1st PCR Reaction

In the following we provide the conditions to setup and run the 1st PCR reaction either with the PrimeSTAR® GXL DNA Polymerase or the Platinum™ SuperFi™ DNA Polymerase. Note, that the setup for both enzymes is different. Reaction setup may vary if you are using a different PCR enzyme and reaction mixture. Take special precautions when pipetting the exceedingly small volumes for the DNA polymerases. Refer to the instructions of the providers for more information on the PCR reagents. PCR reactions are setup in 0.2 ml PCR tubes following the directions given in the tables below.

Working with PrimeSTAR® GXL DNA Polymerase from Takara Bio Inc.

Reagents for *PrimeSTAR GXL DNA Polymerase*	Working Volume	Final Concentration
Ultra-pure water	16 µl	-
Template plasmid DNA (250 pg/µl)	4 µl	25 pg/µl
5x PrimeSTAR GXL Buffer	8 µl	1x
2.5 mM dNTP	3.2 µl	0.2 mM
100 nM gene specific primer	4 µl	10 nM
100 nM antisense primer	4 µl	10 nM
PrimeSTAR GXL DNA Polymerase	0.8 µl	0.025 U/µl
Total	40 µl	

Incubate in thermocycler using the following settings when working with the PrimeSTAR GXL DNA Polymerase:

Temperature	Incubation time	Cycle Number/Comments
98°C	30 sec	One-time Hot start to activate enzyme
98°C	10 sec	Perform 30 to 35 cycles: Adjust annealing temperature and extension time in accordance with your primer design and length of your PCR product!
55°C	15 sec	
68°C	1 min/kb	
68°C	1 min/kb	One-time to complete last extension reaction
20°C	Hold	Hold until sample is further processed

Working with Platinum™ SuperFi™ DNA Polymerase from Thermo Fisher

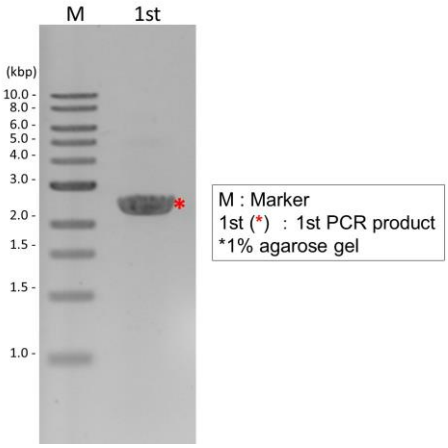
Reagents for *Platinum™ SuperFi™ DNA Polymerase*	Working Volume	Final Concentration
Ultra-pure water	10.8 µl	-
Template plasmid DNA (250 pg/µl)	4 µl	25 pg/µl
5x SuperFi™ Buffer	8 µl	1x
5x SuperFi™ GC Enhancer	8 µl	1x
10 mM dNTP mix	0.8 µl	0.2 mM
100 nM gene specific primer	4 µl	10 nM
100 nM antisense primer	4 µl	10 nM
Platinum™ SuperFi™ DNA Polymerase	0.4 µl	0.02 U/µl
Total	40 µl	

Incubate in thermocycler using the following settings when working with the Platinum™ SuperFi™ DNA Polymerase:

Temperature	Incubation time	Cycle Number/Comments
98°C	30 sec	One-time Hot start to activate enzyme
98°C	10 sec	Perform 30 to 35 cycles: Adjust annealing temperature and extension time in accordance with your primer design and length of your PCR product!
55°C	10 sec	
72°C	15–30 sec/kb	
72°C	15–30 sec/kb	One-time to complete last extension reaction
20°C	Hold	Hold until sample is further processed

To confirm the successful amplification of the target sequence, load 2 µl of the PCR reaction mixture onto an agarose gel. The PCR product should be visible as a single band of the expected size. If no PCR product can be found, you may proceed still with the 2nd PCR in the hope to further amplify the template. Figure 3 below given an example amplifying the insert of DHFR positive control provided with the Premium ONE Expression Kit.

Figure 3: Agarose gel electrophoresis showing amplified DNA from 1st PCR reaction



Primers Design for 2nd PCR

Forward Primer	Description
First Primer	SPU provided with the kit
Length (bp)	21 bases
Sequence	5'- GCGTAGCATTTAGGTGACACT -3'
Description	Yellow: 5' end of SP6 promoter sequence
Forward Primer	**Description**
Second Primer	deSP6E01 provided with the kit
Length (bp)	100 bases
Sequence	5'-GGTGACACTATAGAACTCACCTATCTCCCCAACACCTAATAACATTCAATCACTCT TTCCACTAACCACCTATCTACATCACCACCCACCACCACCAATG-3'
Description	Yellow: 3' end of SP6 promoter sequence Green: E01 translation promoting sequence (Underline: Overlapping sequence with the 1st PCR product) Red: Start codon

Reverse Primer	Description
Reverse Primer	Reverse primer
Length (bp)	About 20 bases
Sequence	Based on vector sequence into which the target gene was inserted
Description	Same as used in 1st PCR reaction

Setup of 2nd PCR Reaction

In the following we provide the conditions to setup and run the 2nd PCR reaction again either using the PrimeSTAR® GXL DNA Polymerase or the Platinum™ SuperFi™ DNA Polymerase. Note, that the setup for both enzymes is again different. PCR reactions are setup in 0.2 ml PCR tubes following the directions given in the tables below.

Working with PrimeSTAR® GXL DNA Polymerase from Takara Bio Inc.

Reagents for *PrimeSTAR GXL DNA Polymerase*	Working Volume	Final Concentration
Ultra-pure water	12 µl	-
1st PCR product	4 µl	1/10 vol.
5x PrimeSTAR GXL Buffer	8 µl	1x
2.5 mM dNTP	3.2 µl	0.2 mM
1 µM SPU	4 µl	100 nM
10 nM deSP6E01	4 µl	1 nM
1 µM antisense primer	4 µl	100 nM
PrimeSTAR GXL DNA Polymerase	0.8 µl	0.025 U/µl
Total	40 µl	-

Incubate in thermocycler using the following settings when working with the PrimeSTAR GXL DNA Polymerase:

Temperature	Incubation time	Cycle Number/Comments
98°C	30 sec	One-time Hot start to activate enzyme
98°C	10 sec	Perform 5 cycles: Adjust annealing temperature and extension time in accordance with your primer design and length of your PCR product!
55°C	30 sec	
68°C	1 min/kb	
98°C	10 sec	Perform 35 cycles: Adjust annealing temperature and extension time in accordance with your primer design and length of your PCR product!
60°C	40 sec	
68°C	1 min/kb	
68°C	1 min/kb	One-time to complete last extension reaction
20°C	Hold	Hold until sample is further processed

Working with Platinum™ SuperFi™ DNA Polymerase from Thermo Fisher

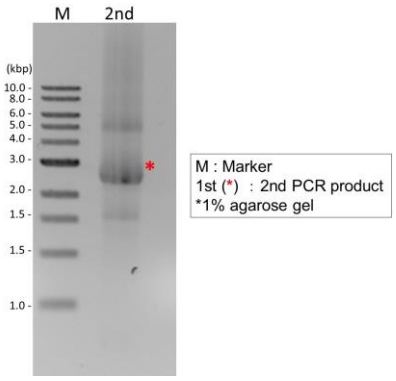
Reagents for *Platinum™ SuperFi™ DNA Polymerase*	Working Volume	Final Concentration
Ultra-pure water	6.8 µl	-
1st PCR product	4 µl	1/10 vol.
5x SuperFi™ Buffer	8 µl	1x
5x SuperFi™ GC Enhancer	8 µl	1x
10 mM dNTP mix	0.8 µl	0.2 mM
1 µM SPU	4 µl	100 nM
10 nM deSP6E01	4 µl	1 nM
1 µM antisense primer	4 µl	100 nM
Platinum™ SuperFi™ DNA Polymerase	0.4 µl	0.02 U/µl
Total	40 µl	-

Incubate in thermocycler using the following settings when working with the Platinum™ SuperFi™ DNA Polymerase:

Temperature	Incubation time	Cycle Number/Comments
98°C	30 sec	One-time Hot start to activate enzyme
98°C	10 sec	Perform 5 cycles: Adjust annealing temperature and extension time in accordance with your primer design and length of your PCR product!
55°C	10 sec	
72°C	15–30 sec/kb	
98°C	10 sec	Perform 35 cycles: Adjust annealing temperature and extension time in accordance with your primer design and length of your PCR product!
60°C	40 sec	
72°C	15–30 sec/kb	
72°C	15–30 sec/kb	One-time to complete last extension reaction
20°C	Hold	Hold until sample is further processed

To confirm the successful amplification of the target sequence, load 2 µl of the PCR reaction mixture onto an agarose gel. The PCR product should be visible as a single band of the expected size. We advise to always confirm the formation of the correct PCR product before starting the protein expression experiment. Figure 4 below given an example amplifying the insert of DHFR positive control provided with the Premium ONE Expression Kit.

Figure 4: Agarose gel electrophoresis showing amplified DNA from 2nd PCR reaction



Protein Expression Reaction on Bilayer Format

Per 55 µl reaction perform the following steps to set up coupled *in vitro* transcription and translation reaction. The volumes given below do not include any added label; refer to the additional information given in Notes 2 and 3 for using the FluoroTect™ GreenLys tRNA label provided with the kit.

While setting up the reactions, always keep the reagents on ice. Be careful **NOT** to mix the two layers when forming the bilayer. Do **NOT** vortex or further mix the reaction mixture after the bilayer has been formed. The reagents are designed for bilayer experiments, and you will lose the translation activity of the reaction when mixing both layers!

1. Per reaction prepare two 0.2 ml PCR tubes with proper label: "**M**" for "Template Mixture" and "**R**" for "Reaction". Set a thermocycler at 26°C for later incubation of the reaction mixture (*1).
2. Thaw the WEPRO® TTmix (clear tube) and SUB-AMIX® TT (clear tube) on ice. After thawing, gently mix both reagents by pipetting up and down, then slightly spin the tubes to collect the entire volume at the bottom of the vial. Avoid excessive centrifugation as this may damage the reagents. Do not thaw unneeded tubes from the kit. After use, immediately freeze reagents in liquid nitrogen and put vials with the remaining reagents back into the freezer for storing them at -80°C. The WEPRO® TTmix and SUB-AMIX® TT lose its activity if not kept at -80°C!
3. Add 50 µl of SUB-AMIX® TT to the bottom of the 0.2 ml PCR tube marked as "**R**". Avoid any air bubbles.
4. In other 0.2 ml PCR tube marked as "**M**", mix 2.5 µl of WEPRO® TTmix and 2.5 µl of a 15 ng/µl plasmid DNA template (*2). Avoid air bubbles when mixing the extract with the DNA template. The same volume is used regardless of whether you are working with an expression vector or a linear DNA template made by PCR. Do not store the **Template Mixture** but use it immediately to setup the bilayer reaction.
5. Setup bilayer reaction by carefully placing the **Template Mixture** in tube **"M"** at the bottom of PCR tube marked "**R"** containing already 50 µl of the SUB-AMIX® TT reaction buffer. Because of the higher density of the wheat germ extract, the **Template Mixture** with the extract and DNA template will form a separate layer below the reaction buffer. Avoid air bubbles when pipetting the Template Mixture below the reaction buffer. Refer to *Figure 5* for more details on how to set up the bilayer.

6. Do not mix the template mixture and reaction buffer by any means: Destroying the bilayer will reduce protein yields!
7. Incubate expression reaction at 26°C for at least 2 hours; the reaction can be maintained for up to 24 hours for improving protein yields. Refer to the example data below for guidance on suitable incubation times for your experimental needs. Commonly, expressed proteins can be detected by Western Blotting after some 2 to 4 hours. To observe expressed proteins on SDS-PAGE most of the time longer expression reactions are required.
8. After completion of the protein expression reaction, the reaction mixture should be gently mixed by pipetted up and down before use in subsequent experiments (*3).

When setting up the expression system, we recommend performing a negative control experiment without added DNA template and a positive control experiment using the control vector provided with the kit. Use 2.5 µl of the pEU-E01-DYKDDDDK-DHFR vector provided with the kit as a template. The positive control has already the correct DNA concentration.

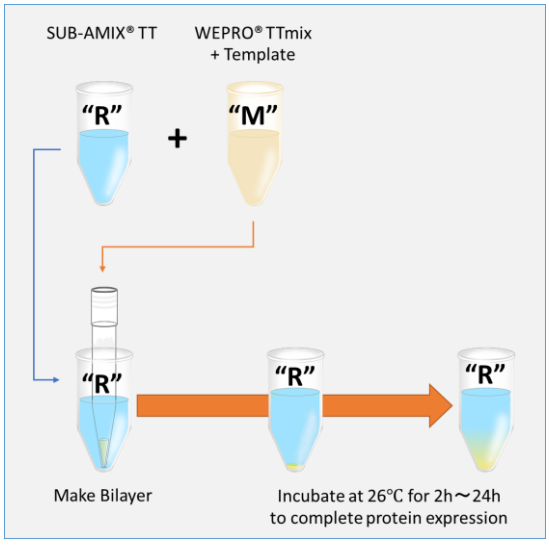
(Notes)

*1: We highly recommend running the incubations in a thermocycler as those keep the temperatures more accurately than for example other heating blocks or a water bath. The protein yields will drop well running the reactions at higher or lower temperatures.

*2: When working with the FluoroTect™ GreenLys tRNA label provided with the kit, mix 2.5 µl of WEPRO® TTmix, 2.5 µl of DNA template and 0.5 µl of FluoroTect™ GreenLys tRNA. The labeling reagent must be added to the **Template Mixture**. Keep the FluoroTect™ GreenLys tRNA in the dark at all time as the label is light sensitive.

*3: The FluoroTect™ GreenLys tRNA must be destroyed before detecting the labeled protein. Otherwise, multiple bands are detected in the gel at a molecular weight of about 30,000 Da or below. Therefore, take out 5 µl from the reaction mixture and transfer it to another tube. Add 1 µl of RNase A (10 µg/µl) and incubate at 26° C for 30 minutes to digest any remaining RNA. Keep reaction mixture in the dark not to destroy the label! After completion of the digestion step, add 6 µl of 2x SDS sample buffer and heat at 70° C for 3 min. Load 10 µl of sample onto an SDS-PAGE and run the gel in the dark. The labeled protein can be detected on a fluorescent gel scanner set at an excitation wavelength of 502 nm and detection wavelength of 510 nm.

Figure 5: Illustration on how to setup bilayer reaction



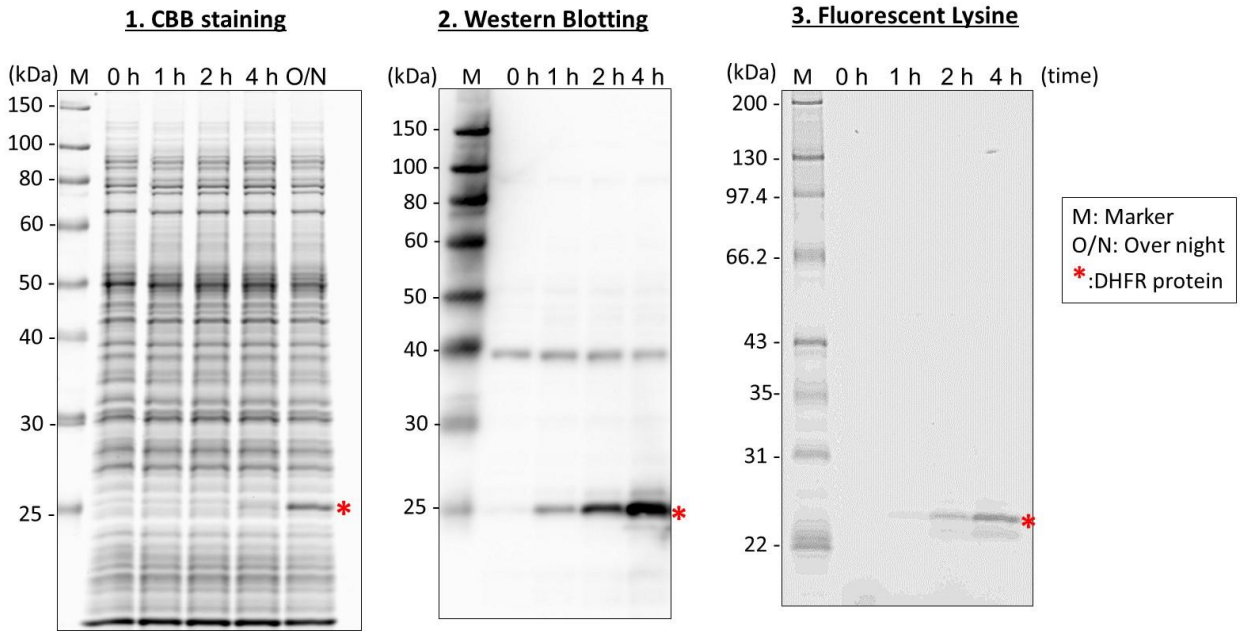
Confirmation of Protein Expression

We recommend confirming protein expression before use in any other experiments. Below we show example data for demonstrating the expression of the positive control included in the Premium ONE Kit. The synthesized DYKDDDDK-DHFR protein is a 19 kDa protein that may show a different molecular weight under standard conditions using SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining, Western Blotting using an anti-DYKDDDDK antibody, and fluorescent lysine incorporation. In the figure below, the protein was detected at different time points to guide the user on how long to maintain the protein expression reactions.

When performing an analysis by SDS-PAGE, use a polyacrylamide gel with a high resolution and appropriate concentration to clearly distinguish the background proteins derived from the wheat embryo extract from the overexpressed protein of interest. Commonly, 5 µl of the reaction mixture are sufficient to detect the overexpressed protein. However, please increase or decrease the amount of sample according to the result seen on the gel and repeat the SDS-PAGE analysis. As shown our example, the DYKDDDDK-DHFR protein is synthesized as a protein of about 25 kDa. Protein yields may further be analyzed on SDS-PAGE by reference to a BSA standard.

Please inquire for antibodies suitable for detecting your target protein and follow the directions given by the provider on how to perform Western Blotting experiments. The positive control provided with the kit has an added DYKDDDDK tag, that can be detected by an anti-DYKDDDDK tag antibody.

Figure 6: Expression of positive control gene included in the kit. Protein synthesis of the DYKDDDDK-DHFR protein was confirmed by SDS-PAGE followed CBB staining (1), Western Blotting using anti-DYKDDDDK antibody (2), and fluorescent lysine incorporation (3). Timepoints are indicated in the figures. Note, proteins contained in the wheat germ extract are not detected during Western Blotting or working with an incorporated label. A fluorescently labeled marker had been used for reference under (3). The DYKDDDDK-DHFR positive control protein has a theoretical MW of about 19 kDa, but bands may appear at different molecular weight positions depending on the running conditions and marker used.



Using different Reaction Sizes

The reagents provided with the Premium ONE Expression Kit can be used to run the transcription/translation reactions on different reaction scales. Please refer to the table below if you want to use other reaction sizes than the 55 µl standard reaction given in the protocol above. It can be preferable to run multiple reaction on different

well-plate formats. Contact us on purchase on larger amounts of the WEPRO® TTmix wheat germ extract and SUB-AMIX® TT reaction buffer. The Template Mixture in the table below is always prepared from equal volumes of the wheat germ extract and the DNA template. Note the ratio of surface of the well versus the height of the reaction mixture. The height should not exceed some 0.8 mm as it will reduce the protein yields.

Format	Volume Template Mixture	Volume Reaction Mixture	Total reaction size
0.2 ml PCR Tube	5 µl	50 µl	55 µl
96-well plate	30 µl	300 µl	330 µl
24-well plate	150 µl	1500 µl	1650 µl
6-well plate	500 µl	5000 µl	5500 µl

Troubleshooting

The experiments require correct and accurate pipetting during reaction setup. Avoid air bubbles as they will reduce protein yields. Any mistake in the volumes added to the reactions, mixing the reagents, or forgetting any of the reagents will lead to wrong results. Therefore, carefully check the label for each reagent prior to starting the pipetting step. The good protein yields of the coupled transcription/translation reactions are dependent on accurate use of the DNA template and setting the reaction temperature to 26°C.

We cannot provide with this manual detailed advice on how to do recombinant DNA experiments or how to design and perform PCR reactions. Refer to laboratory textbook on how to conduct cloning experiments and PCR reactions and how to solve common problems on failed PCR reactions or the amplification of artifacts. Further, make sure to follow the instructions of the provider, from whom you obtained your PCR reagents. We only tested the PrimeSTAR® GXL DNA Polymerase from Takara Bio Inc. (Catalog # R050B) and Platinum™ SuperFi™ DNA Polymerase from Thermo Fisher (Catalog # 12351010) for the protocol given in this manual, but other high fidelity polymerases having a proof-read function may work equally when to prepare PCR templates for *in vitro* protein expression. However, we cannot exclude that other PCR conditions will give lower template concentration that will later also reduce the protein yields.

Working with an expression vector commonly gives better protein yields and more reproducible results. For routinely making the same protein, we advise to prepare an expression vector using one of the vectors provided by CellFree Sciences for use in our expression system. There are other expression vectors on the market and in public depositories for use in wheat germ cell-free protein expression system. We have not confirmed how expression vectors from other providers work in combination with our expression system. When using expression vectors from other sources, always make sure that the vector has an SP6 RNA Polymerase promoter. The reagents for the Premium ONE Expression Kit contain the SP6 RNA Polymerase and other RNA polymerase promoters are not supports.

If you cannot obtain the desired protein, please confirm the following:

- Confirm your DNA template especially when working with linear DNA prepared by PCR. Expression vectors do not have to be linearized for use in the protein expression reactions. Make sure that the vector was used at a concentration of 15 ng/µl.
- Do not store the Template Mixture with the wheat germ extract and the DNA template. The Template Mixture should be prepared right before setting up the bilayer reaction.
- Make sure to run the transcription/translation reactions at exactly 26°C. If possible, we recommend using a thermocycler as they are more accurate on keeping the set temperature.
- Make sure not to miss any reagents: Mark in your protocol each pipetting step you have completed.
- The experiment must be done under RNase-free conditions as any loss of the RNA template will prevent protein expression.

- Change the pipetting tip after each pipetting step. Do not use the same pipetting tip to pipette different reagents or reaction mixtures. Always change the pipetting tip after use.
- Leaving out the DNA template will always yield negative results. The same applies if there is a mistake in the expression vector or PCR product, e.g. leaving out the starting ATG, forgetting a stop codon, or having a frame shift error.
- Confirm that your expression vector is correct and has a start and stop codon in line with the reading frame for the protein. Refer to our vector map primer information in Appendix A and B on more information on suitable sequencing primers to confirm the sequence of your expression vector.
- Confirm the DNA quality and concentration if the protein yields are low. Low RNA yields during the transcription reaction will also reduce the protein yields. Perform a phenol/chloroform extraction on the template DNA if RNA yields are low and ensure working under RNase-free conditions. An OD260/280 ratio of ~1.8 for your plasmid DNA preparations is commonly considered as pure enough for use in protein expression experiments. Lower ratios may indicate remaining proteins and/or other contaminations absorbing near 280 nm. Note that the actual values for the OD260/280 ratio can vary from vector to vector as the actual OD values depend also on the nucleotide composition of your DNA vector. It is important to confirm the OD260/280 ratio for your vector DNA before use in protein expression experiments because low DNA purity prevents RNA and protein expression.
- For the protein expression reactions, do not mix the two layers during setup of the bilayer translation reaction. Mixing both layers will sharply reduce the protein yields. Let both layers slowly mix by diffusion without any further manipulation. The translation reaction can be maintained for up to 24 hours.
- When working with the fluorescent lysine the reaction mixture must be shielded from light during incubation and all later steps. Covering the tubes with aluminum foil for protection and run electrophoresis in the dark. The fluorescent label is light sensitive, and the signal will be lost after longer exposure to light.
- It is possible to work with linear templates in cell-free protein expression experiments, which can be easily prepared by PCR methods. Working with PCR products, however, can reduce protein yields. PCR products are used to quickly find the best expression construct or to test expressing different protein fragments. If the yields obtained with PCR products are too low, it may be worthwhile to prepare an expression vector already for doing the test expression experiments. Always use expression vectors for large-scale protein production to have stable and reproducible conditions.
- If the protein is not expressed, check reaction conditions, reagents, and DNA template in an expression reaction with the positive control vector provided with the kit to confirm their integrity. Reconsider the design of your expression template to improve protein yields if all the forgoing steps do not explain low yields for your target protein while the positive control works.
- Make sure that the wheat germ extract was always keep frozen before use. Avoid repeated freeze/thawing; it will inactivate the extract.
- Store wheat germ extract at -80°C; storage at higher temperature will lead to low activity or even total loss of activity. Clearly follow the recommendations on reagent storage and handling given in this manual.
- Some proteins may have special requirements and do not express well under standard conditions. Gather information on your target protein before the expression experiments to see whether additional considerations are needed. Contact us for more information on how to modify cell-free protein expression experiments.

Contact the technical support of CellFree Sciences for further help using the contact information on the last page of the manual.

References

Wheat Germ Cell-Free Expression System:

1: Sawasaki T, Ogasawara T, Morishita R, Endo Y.: A cell-free protein synthesis system for high-throughput proteomics. Proc Natl Acad Sci U S A. 2002 Nov 12;99(23):14652-7. Epub 2002 Oct 30. PMID: 12409616

2: Takai K, Sawasaki T, Endo Y.: Practical cell-free protein synthesis system using purified wheat embryos. Nat Protoc. 2010 Feb;5(2):227-38. doi: 10.1038/nprot.2009.207. Epub 2010 Jan 21. PMID: 20134421

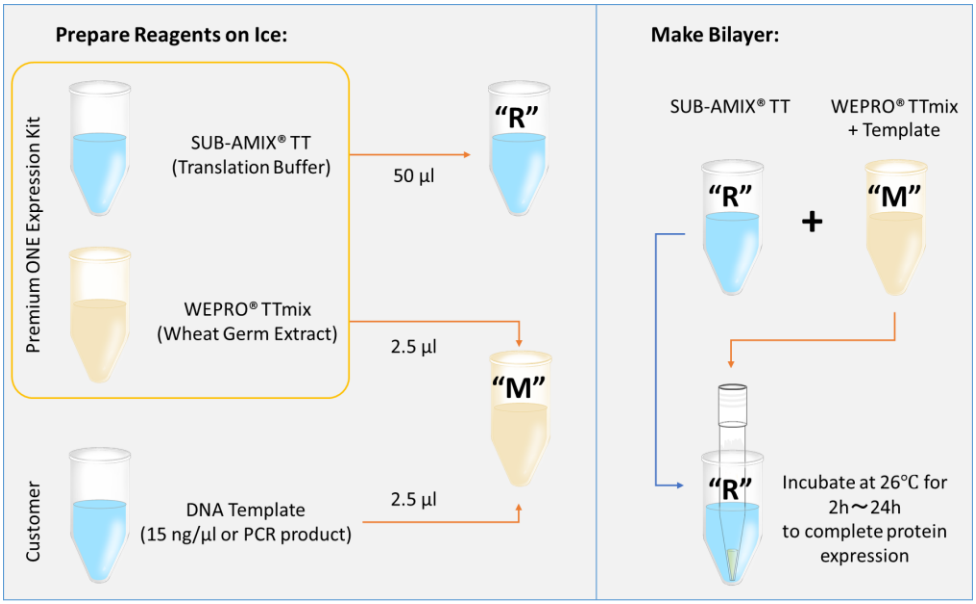
3: Harbers M.: Wheat germ systems for cell-free protein expression. FEBS Lett. 2014 Aug 25;588(17):2762-73. doi: 10.1016/j.febslet.2014.05.061. Epub 2014 Jun 12. PMID: 24931374

4: Novikova IV et al.: Protein Structural Biology Using Cell-Free Platform From Wheat Germ. Adv Struct Chem Imaging

. 2018;4(1):13. doi: 10.1186/s40679-018-0062-9. Epub 2018 Nov 10. PMID: 30524935

Bench Note

Use this Bench Note for setting up your protein expression experiments using the Premium ONE Expression Kit. Volumes are given for making reactions on 55 µl scale. Mark each step in the protocol after completion.



Prepare reagents on ice and set thermocycler:

Reagent/Instrument	Action	Checkmark
Thermocycler	Set at 26°C to incubate the reaction mixture	☐
DNA Template	Expression vector with 15 ng/µl or PCR product	☐
SUB-AMIX® TT	Thaw on ice, softly mix with pipette, slightly spin to collect translation buffer	☐
WEPRO® TTmix	Thaw on ice, softly mix with pipette, slightly spin to collect wheat germ extract	☐
PCR Tubes	Label one 0.2 ml PCR tube with "M" like "Template Mixture" Label one 0.2 ml PCR tube with "R" like "Reaction"	☐

Prepare translation buffer in tube "R":

Reagents	Volume	Final Concentration	Checkmark
SUB-AMIX® TT	50 µl	-	☐

Prepare wheat germ extract and template in tube "M":

Reagents	Volume	Final Concentration	Checkmark
WEPRO® TTmix	2.5 µl	-	☐
DNA Template	2.5 µl	7.5 ng/µl	☐
OPTIONAL: FluoroTect™ GreenLys tRNA*	*0.5 µl*		☐

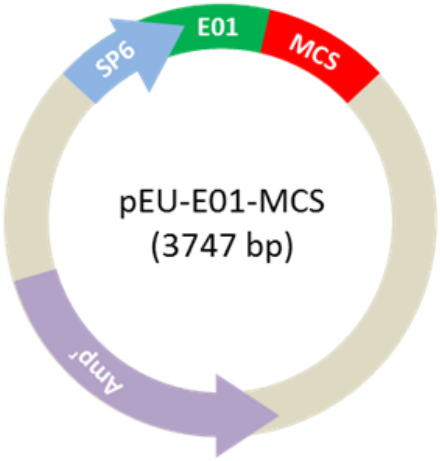
Set up bilayer translation reaction:

1. As shown in the figure, take up entire Template Mixture in tube **"M"** and place it below the Translation Buffer at the bottom of tube **"R"**. **Do not mix both layers!**
2. Incubate expression reaction in thermocycler set to 26°C for at least 2 hours; the reaction can be maintained for up to 24 hours for improving protein yields.
3. After completion of the expression reaction, mix the reaction mixture to work with a homogenous sample.

*: Treat sample with RNase when working with the FluoroTect™ GreenLys tRNA.

Appendix A: Vector Map for pEU-E01-MCS

Map:



Multi cloning site:

Vector elements:

SP6 Promoter:	-17-1
Translation Enhancer E01:	1-73
Multi cloning site:	74-193
Origin:	1190-1830
Ampicillin resistance gene:	1974-2838

Position 1 is located at the final G of the SP6 Promoter: ATTTAGGTGACACTATA**G**

CellFree Sciences can provide the vector sequence as a text file. For downloading vector maps and sequences visit our homepage at: http://www.cfsciences.com/eg/vector.html.

Appendix B: Sequencing Primers for Vectors of pEU-E01-MCS Family

Standard M13 sequencing primers are available from different providers. Alternatively, customized sequencing primers can be prepared by DNA synthesis. All sequencing primers should be purified by gel electrophoresis or HPLC.

pUC/M13 Sequencing Primers

The pUC/M13 Primers are designed for sequencing inserts cloned into the M13 vectors and pUC plasmids. These primers can also be used for sequencing other *lac*Z-containing plasmids such as the pGEM®-Z and pGEM®-Zf Vectors.

Forward (17mer): 5´-d(GTTTTCCCAGTCACGAC)-3´

Reverse (17mer): 5´-d(CAGGAAACAGCTATGAC)-3´

Reverse (22mer): 5´-d(TCACACAGGAAACAGCTATGAC)-3´

Forward (24mer): 5´-d(CGCCAGGGTTTTCCCAGTCACGAC)-3´

For 5' end sequence: SP6 Primer

5'-ATTTAGGTGACACTATAGAA-3'

For 3' end sequence

5'-CCTGCGCTGGGAAGATAAAC-3'

Customer Information

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pGEM® is a registered trademark of Promega Corporation.
Company names and product names mentioned herein are the trademarks to the indicated owner as stated above.

Others

All product specifications and information in the manual may be changed without prior notice.

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