CellFree Sciences

The natural power of wheat driving science

High Performance Cell-Free Wheat Germ Protein Expression System

INSTRUCTION MANUAL

Disulfide Bond PLUS Expression Kit

This kit provides premixed reagents for 8 linked transcription-translation reactions using a modified wheat germ cell-free expression system for the preparation of proteins having disulfide bonds

Product Number(s): CFS-EDX-DB

Version/date: Version 1.0_eng/October 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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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

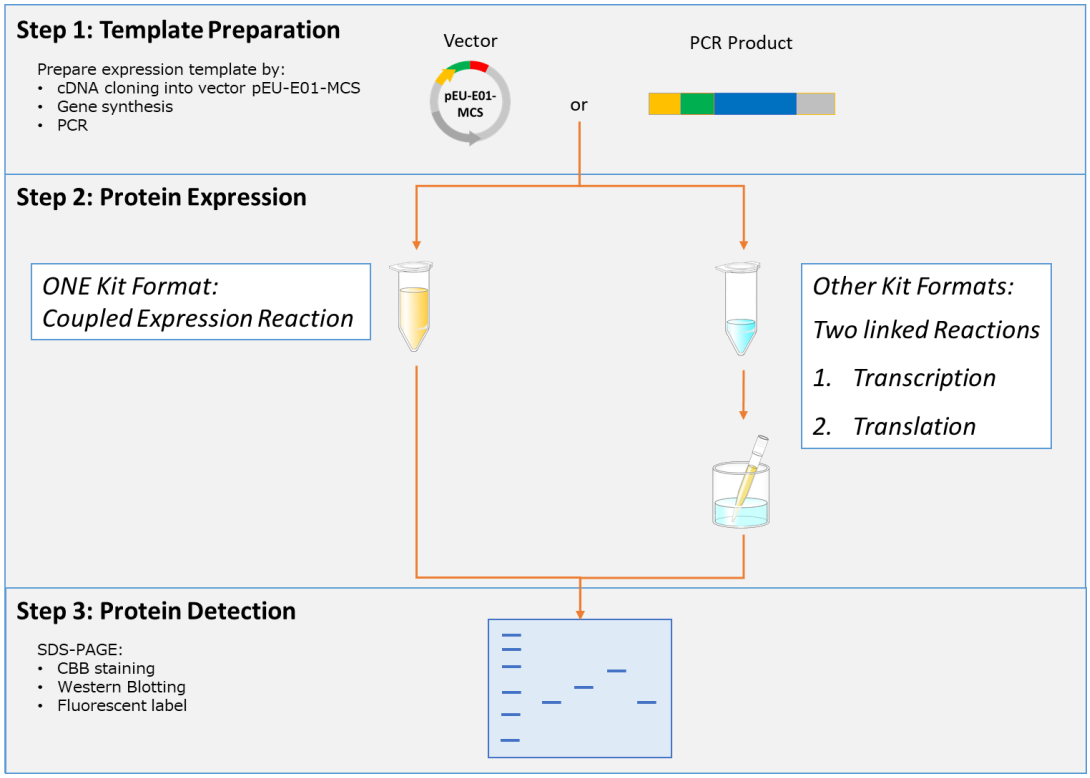
The Disulfide Bond PLUS Expression Kit from CellFree Sciences allows to perform linked transcription and translation reactions to express proteins having disulfide bonds in the wheat germ cell-free protein expression system. Disulfide bonds are among the most important post translational protein modifications and have important effects on protein folding and stability. They are commonly formed under oxidizing conditions within organelles and serve special needs of extracellular proteins. To support the formation of disulfide bonds, this kit provides the human Protein disulfide isomerase (PDI, P07237) and endoplasmic reticulum, oxidoreductin-1α (Ero1α, Q96HE7) that together sustain oxidative protein folding. In addition, we have omitted the 1,4-Dithiothreitol (DTT) from the translation buffer to better stabilize the disulfide bonds in the modified proteins. For easy reaction setup, the kit provides premixed reagents to prepare the transcription and later translation reactions. We developed a new version of our high-performance wheat germ extracts to support small-scale protein expression reactions servicing basic needs in protein research. Here we are using a dedicated version of our wheat germ extract, the WEPRO® for DB PLUS, that gives the user access to the latest version of our high-performance wheat germ cell-free protein expression system. Reagents in the Disulfide Bond PLUS Expression Kit allow to setup the transcription and translation reaction on a 230 µl reaction scale by just a few pipetting steps. Under standard reaction conditions, the expression reaction may yield for the tPA positive control include in the kit some 23 µg in the insoluble fraction and 16 µg. in soluble fraction.

As an entry kit, the Disulfide Bond PLUS Expression Kit provides further 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 Disulfide Bond PLUS 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 Disulfide Bond PLUS 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 experiments and those must be designed by the customer for each target gene. More details on the primer design are provided in this manual.

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 or linked transcription/translation reactions. The Premium ONE Expression Kit was designed to perform 24 reactions on a 55 µl scale as compared to larger 226 µl reactions used in the regular Premium PLUS Expression Kit. Hence the linked reactions in the Premium PLUS Expression Kit have been designed to provide higher yields than possible with the smaller coupled expression reactions. Linked transcription/translation reactions can be further upscaled for large-scale protein production in our expression system and are also the basis to the modified reaction conditions used in the Disulfide Bond 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 cell-free 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 identical to those provided in the Premium PLUS Expression Kit. Our larger WEPRO®7240 Core Kits offer sufficient reagents to perform protein expression reactions on different reaction scales from protein expression tests and 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 linked *in vitro* transcription and translation experiments as further outlined in this protocol working on a 230 µl scale. Because of the special bilayer reaction format, the expression reactions can be maintained for up to 24 hours. The protein yields are most of the time sufficient to view the protein on stained SDS-PAGE after a 24-hour reaction.

In cases where the stained SDS-PAGE does not provide clear results, proteins may also be detected by Western blotting using an antibody against the target protein of interest or an added affinity-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. We advise to use known amounts of a BSA standard in the SDS-PAGE experiment to estimate the protein yields.

The Disulfide Bond PLUS Expression Kit offers special reaction conditions to support correct protein folding. Correct folding can often be confirmed by testing the activity of the protein as further explained in this manual for the positive control (tPA for "tissue plasminogen activator") included into the kit. Protein activity tests must be designed for each protein and no general recommendations can be given to this point.

Use of Disulfide Bond PLUS Expression Kit

The Disulfide Bond PLUS Expression Kit provides enough reagents for 8 linked protein expression reactions on a 230 µl reaction scale. 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-His-tPA (297-562) positive control vector.

Materials Provided by the Kit

The Disulfide Bond PLUS 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!

Kit Content

Item	Quantity	Concentration	Volume	Vial	Vial Color
Transcription Premix LM*	8	—	18 µl	0.2 ml Strip	Blue
WEPRO® for DB PLUS*	8	—	16 µl	0.2 ml Strip	Clear
SUB-AMIX® SGC DTT-free*	8	—	200 µl	Strip with eight wells	Clear
PDI & Ero1α mix	1	75 µM PDI and 18 µM Ero1α	40 µl	0.2 ml PCR 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-His-tPA (297-562)	1	1.0 µg/µl	5.0 µl	0.2 ml PCR tube	Blue
Aluminum seal	2	—	—	—	
Kit manual in print	1	—	—	—	

***Use entire content of each tube for one reaction**

Instructions on Use of Reagents

Item	Description
Transcription Premix LM	Pre-mixed transcription reaction mixture including SP6 RNA polymerase. Avoid unnecessary freeze-thawing of the transcription Premix LM!
WEPRO® for DB PLUS	Pre-mixed wheat germ extract with added creatine kinase for linked protein expression system. Avoid unnecessary freeze-thawing of the wheat germ extract! Store buffer at -80 °C.
SUB-AMIX® SGC DTT-free	Translation reaction buffer for protein expression reactions. Store buffer at -80 °C.
PDI & Ero1α mix	Enzyme mix to support disulfide bond formation. Avoid unnecessary freeze-thawing of the enzyme mix! When used first time, we advise to prepare aliquots of 5 µl each for use in later expression experiments. You need 4 µl of the enzyme mixture per reactions. 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-His-tPA (297-562)	Expression vector for his-tagged tPA domain.

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 an 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.

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 an example for using the *Ex Taq*® DNA Polymerase from Takara Bio Inc. (Catalog # RR001C) to conduct PCR experiments. The enzyme was tested at CellFree Sciences for use in combination with the Disulfide Bond PLUS 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.

Other Consumables and Instruments Required for Protein Expression

Consumable	Description
Incubator	Temperature set to 15 to 25°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
1.5 ml tube	Use DNase, RNase free quality
SDS-PAGE	SDS-PAGE*, gel electrophoresis apparatus and power supply
Optional devises to conduct Western Blotting experiment	Needed when confirming the synthesis of the target protein by Western Blotting

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

Reagents Required for optional tPA Activity Test

The following reagents are only needed when confirming the enzymatic activity of the positive control included in the kit.

Reagents	Description
Reaction buffer	100 mM Tris-HCl (pH 8.0), 0.15% Tween 80
Reaction substrate*	Chromozym® t-PA (Cat. No. 11 093 037 001, Sigma-Aldrich) used at a concentration of 4 mM.
Ultra-pure water	DNase, RNase free: DO NOT use DEPC treated water!

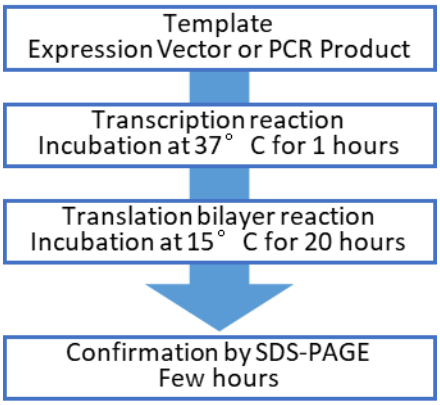
* For more details on this product, refer to the instruction manual provided by the maker.

To read out the results of the activity test, a plate reader is required that can measure absorbance at 405 nm.

Protocols

Time Requirements

Refer to the flowchart below on the estimated time per protein expression reaction step; the transcription reaction may be extended to 4 hours when working with PCR products instead of expression vectors.



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/). UniProt may also provide more information on annotated disulfide bonds.

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 1 µg/µl by adding an appropriate volume of TE buffer (*3).

You need 2 µl purified plasmid DNA per 230 µl expression reaction. In principle, 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: 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

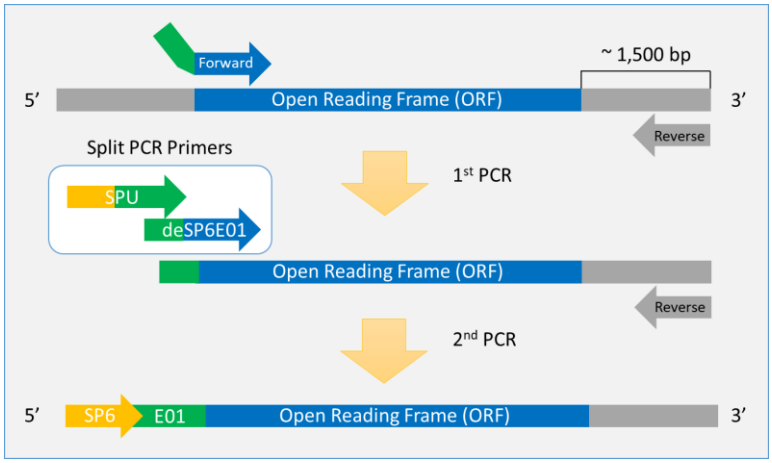
Linear DNA templates can be directly used in *in vitro* transcription/translation reactions. However, they must contain the same regulatory elements as mentioned for the pEU-E01-MCS expression vectors. Hence, during the PCR reactions additional sequences must be introduced adding the SP6 promoter and 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 *Ex Taq*® DNA Polymerase from Takara Bio Inc. (Catalog # RR001C). This is a high-fidelity PCR DNA polymerase 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'- CCACCCACCACCACCAATGNNNNNNNNNNNNNNNNN -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. 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 polymerase. 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.

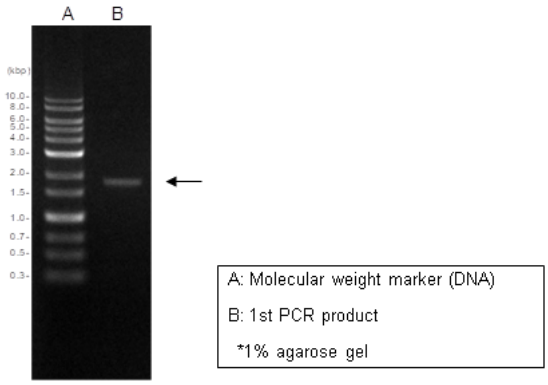
Reagents	Working Volume	Final Concentration
Ultra-pure water	10.35 µl	-
Template plasmid DNA (250 pg/µl)	2 µl	25 pg/µl
10 x *EX Taq*® Buffer	2 µl	1x
2.5 mM dNTP	1.6 µl	0.2 mM
100 nM gene specific primer	2 µl	10 nM
100 nM antisense primer	2 µl	10 nM
***Ex Taq*® DNA Polymerase (5U/µl)**	0.05 µl	0.0125 U/µl
Total	20 µl	

Incubate in thermocycler using the following settings when working with the *Ex Taq*® DNA Polymerase:

Temperature	Incubation time	Cycle Number/Comments
98°C	60 sec	One-time Hot start to activate enzyme
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!
55°C	60 sec	
72°C	1 min/kb	
72°C	1 min/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 template.

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 using the *Ex Taq*® GXL 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.

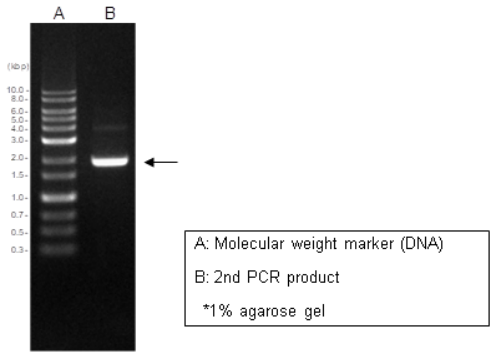
Reagents for *PrimeSTAR GXL DNA Polymerase*	Working Volume	Final Concentration
Ultra-pure water	41.75 µl	-
1st PCR product	10 µl	1/10 vol.
10 x *Ex Taq*® Buffer	10 µl	1x
2.5 mM dNTP	8 µl	0.2 mM
1 µM SPU	10 µl	100 nM
10 nM deSP6E01	10 µl	1 nM
1 µM antisense primer	10 µl	100 nM
***Ex Taq*® DNA Polymerase (5 U/µl)**	0.8 µl	0.0125 U/µl
Total	100 µl	-

Incubate in thermocycler using the following settings when working with the *Ex Taq*® DNA Polymerase:

Temperature	Incubation time	Cycle Number/Comments
98°C	60 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	60 sec	
72°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	
72°C	1 min/kb	
72°C	1 min/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 template.

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



Concentration of PCR Product

We advise to further concentrate the PCR product before use in protein expression experiments. Perform the following step to precipitate the DNA:

1. Transfer the whole content of 2nd PCR reaction mixture into a 1.5-ml tube.
2. Add to the about 100 µl PCR reaction mixture, 2.5 times the volume of the reaction mixture 100% ethanol and one tenth of the volume 3 M Sodium acetate (*1).
3. Mix the solution well and incubate for 10 minutes at -20 ˚C.
4. Centrifuge the mixture at 15,000 rpm for 15 minutes at 4 ˚C.
5. Remove the supernatant and add 300 µl of 70% ethanol to the pellet.
6. Centrifuge the mixture at 15,000 rpm for 5 minutes at 4 ˚C.
7. Remove the supernatant as much as possible and dry it for 15 minutes (*2).
8. Add 10 µl of ultra-pure water to the pellet. Let the tube stand for 10 minutes to loosen the DNA pellet, and then resuspend the pellet gently by pipetting (*3).

You can confirm the PCR product and DNA concentration by running an agarose gel electrophoresis with 0.1 µl of the concentrated PCR product. Two microliters of the concentrated PCR product are required for the transcription reaction.

We recommend extending the transcription reaction time to 4 hours when working with PCR templates to achieve higher protein yields.

(Notes)

*1: Do not dilute the 2nd PCR product before adding the ethanol and sodium acetate.

*2: The DNA pellet should not dry completely before resuspending it in the nuclease-free water.

*3: It may be possible to increase the protein synthesis yields by increasing the concentration of PCR product.

Protein Expression from DNA Template

The cell-free protein expression is performed in two separate reactions, where first an RNA transcript is prepared from the DNA template encoding for the protein of interest. The RNA transcripts are then used in the following translation reaction.

Transcription Reaction Using DNA Template

Per reaction perform the following steps to set up a transcription reaction:

1. Thaw your template DNA before the experiment. You need 2 µg of purified plasmid DNA at a concentration of 100 µg/µl or an equal amount of a PCR product (*1).
2. Take one vial with Transcription Premix LM (blue vial) per reaction from storage at -80°C (*2).
3. Thaw required number of Transcription Premix LM vials on ice. Keep the remaining vials at -80˚C. After thawing, spin the vials briefly to collect the entire volume at the bottom of the vial. Mix the reagent gently before use. Place reagents on ice and keep them cold at all times.
4. Add 2 µl of highly purified plasmid DNA (1.0 µg/µl) to each vial with the Transcription Premix LM as shown in the table below. Then mix gently by pipetting up and down.

Reagents	Working Volume	Final Concentration
Transcription Premix LM	18 µl	1 x
Plasmid (circular DNA, 1.0 µg/µl)	2 µl	100 ng/µl
Total	20 µl	-

5. Incubate at 37°C for 1 hour in an incubator (*3). **We recommend to extend the incubation time to 4 hours when working with PCR templates in the transcription reaction!**

6. Optionally, you can confirm the mRNA quality after the transcription reaction by agarose gel electrophoresis loading 0.5 µl of the reaction mixture. Refer to a cloning handbook for more information on how to perform RNA gel electrophoresis.

(Notes)

*1: Commonly plasmid DNA prepared by a commercial DNA purification kit is suitable for use in protein expression experiments. Do not use plasmid DNA from alkaline lysis without further purification.

*2: The strip holding the Transcription Premix LM vials can be cut into individual tubes by bending or cutting. Hold the vials firmly so that they do not pop open while separating them.

*3: White precipitate may occur during incubation. This is magnesium pyrophosphate, which will not interfere with the following translation experiment. Use the whole reaction mixture including the precipitate in the next step.

Translation Reaction Using RNA Prepared from DNA Template

After completion of the transcription reaction, let the reaction mixture cool down to room temperature. Do not forcibly cool it down on ice or in a refrigerator. Per reaction perform the following steps to set up translation reaction:

1. Per reaction take from storage at -80°C storage a single vial with WEPRO® for DB PLUS (clear vial) and a single well (clear well) containing SUB-AMIX® SGC DTT-free (*1). Do not thaw unneeded vials and wells. Put the remaining vials and wells back into the freezer and store them at -80°C immediately. WEPRO® for DB PLUS loses its activity if not kept at -80°C!
2. Take out the PDI & Ero1α mix and thaw it together with the WEPRO® for DB PLUS and SUB-AMIX® SGC DTT-free on ice. After thawing, briefly spin down each vial with WEPRO® for DB PLUS and PDI & Ero1α mix to collect the reagent at the bottom of the vial. Avoid excessive centrifugation of WEPRO® for DB PLUS!
3. Resuspend SUB-AMIX® SGC DTT-free by pipetting gently up and down in the well (*2).
4. Resuspend the transcription mixture by pipetting gently up and down (*3).
5. Prepare “translation mixture” using 10 µl of the “transcription mixture” containing the RNA template following the instructions in the table below. Mix gently by pipetting up and down, avoid bubbles.

Reagents	Working Volume	Final Concentration
Transcription mixture (mRNA)	10 µl	0.5 vol.
PDI & Ero1α mix	4 µl	0.5 vol.
WEPRO®for DB PLUS	16 µl	120 OD
Total	30 µl	-

6. Carefully transfer the translation mixture (30 µl) to the bottom of a single well containing SUB-AMIX® SGC DTT-free (200 µl) to form bilayer with the translation mixture in the lower layer and SUB-AMIX® SGC DTT-free in the upper layer. Refer to the figure at the end of this section on how to setup a bilayer reaction: Go with the pipette tip to the bottom of the well, and slowly release the translation mixture below the reaction buffer. Because of the yellowish color of the wheat germ extract, you can distinguish the translation mixture from the translation buffer in the well.

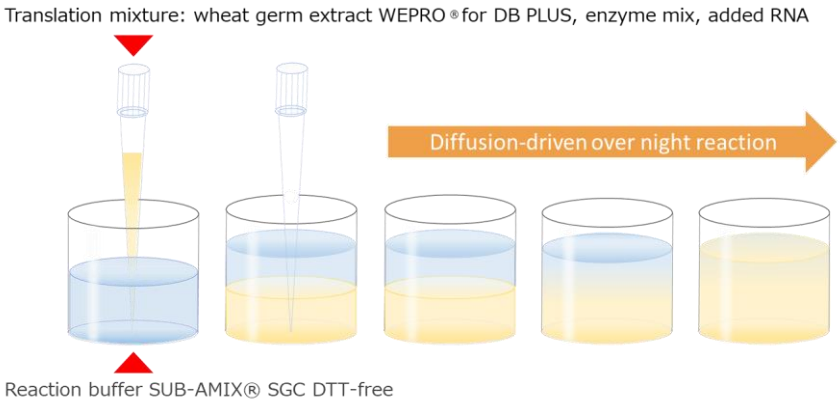
Reagents	Working Volume	Final Concentration
SUB-AMIX® SGC DTT-free	200 µl	1 x
Translation mixture	30 µl	-
Total	230 µl	-

7. Seal the well with an aluminum seal included in the kit to avoid evaporation (*4).
8. Incubate at 15˚C for 20 hours in an incubator. Be careful that the well stably stands on a flat surface.
9. After completion of the translation reaction, mix the bilayer reaction gently by pipetting up and down.

(Notes)

*1: The strips with the WEPRO® for DB PLUS vials and SUB-AMIX® SGC DTT-free wells can be split into individual vials or wells by bending or cutting. Hold the vials with the WEPRO® for DB PLUS firmly so that they do not pop open while separating them.
*2: Take particular care to keep the wells with SUB-AMIX® SGC DTT-free upright at all times. They easily flip over, which would disturb the bilayer.
*3: If you notice a white precipitate after the transcription reaction, resuspend the precipitate by pipetting gently up and down before mixing with WEPRO® for DB PLUS. There is no need to remove the precipitate.
*4: Cut off aluminum seals of an appropriate size to cover the wells. Peel off the brown cover on the back of the seals and glue the seal onto the well. Press down the seal onto the well to make sure it covers the entire surface of the well. The seal can easily be removed after the completion of the reaction by simply pulling it up. Save the remaining seals for later use. Be careful not to disturb the bilayer while placing the seal onto the well.

Figure 5: Illustration on how to setup bilayer reaction

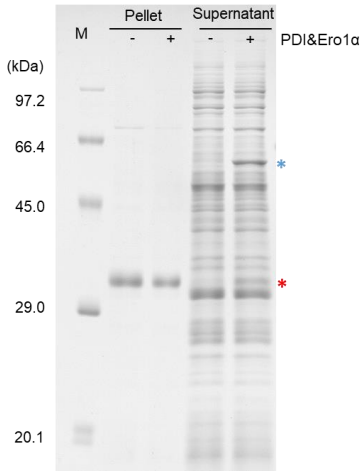


Confirmation of Protein Expression

We recommend confirming protein expression before use in any other experiments. Below we show example data for demonstrating the expression of the tPA positive control included in the Disulfide Bond PLUS Kit. The synthesized tPA protein fragment has a calculated molecular weight of 29,774 Da but runs somewhat higher under standard conditions using SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining (see Figure 6 below). Note, that the added PDI and Ero1α will show on SDS-PAGE as proteins of 56 kDa (PDI) and 52 kDa (Ero1α).

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, 3 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.

*Figure 6: Expression of tPA positive control gene included in the kit. Protein synthesis of the His-tagged tPA protein fragment was confirmed by SDS-PAGE using 2 µl of each sample followed CBB staining and by confirming its activity. The synthesized protein is indicated by **; added PDI/Ero1α is indicated by *. In this experiment, most of the protein was found in the insoluble fraction though solubility could be improved by the addition of PDI and Ero1α during the translation reaction.*



We added to the Disulfide Bond PLUS Expression Kit a template to express a tPA protein fragment. The folding of this protein fragment depends on the formation of disulfide bonds and thus it is a good model to confirm disulfide bond formation using the reagents provide with this kit. Under standard reaction conditions, the enzyme is mostly found in the insoluble fraction and lacks its enzymatic activity. By changing the reaction conditions and working with added PDI & Ero1α, more of the tPA protein fragment can be found in the soluble fraction having a by large greater enzymatic activity. You can confirm the activity of the tPA protein fragment using the following protocol:

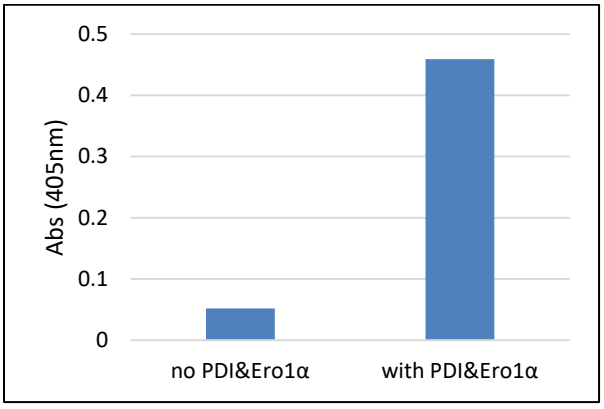
1. After completion of the transcription reaction, centrifuge the reaction mixture containing the tPA protein fragment at 21,000xg for 10 minutes at 4°C. Transfer the supernatant to another tube and place it on ice. You will need 3 µl from the supernatant for doing the activity test.
2. At room temperature set up reaction mixture in a 96-well plate following the directions in the table below:

Reagents	Working Volume	Final Concentration
Ultrapure water	40.7 µl	-
Reaction buffer: 100 mM Tris-HCl (pH 8.0), 0.15% Tween 80	50.0 µl	50 mM Tris-HCl (pH 8.0), 0.075% Tween 80
Reaction substrate: 4 mM Chromozym® t-PA Solution	6.3 µl	0.25 mM
Total	97 µl	-

3. Add 3 µl of the supernatant of the protein expression reaction to the reaction mixture in the 96-well plate and mix gently by pipetting up and down. Avoid the formation of bubbles as they will later interfere with measuring the absorbance.
4. Incubate at room temperature for 30 minutes.
5. After completion of the protease reaction, set the 96-well plate into a plate reader and measure the absorbance at 405 nm. For further details on the measurement, follow the manual that comes with the substrate.

The graph below provides an example comparing the tPA protease activity depending on the presence or absence of PDI and Ero1α during the protein expression reaction.

Figure 7: Protease activity of His-tagged tPA protein fragment.



Troubleshooting

Sometimes the amounts of the expressed proteins are difficult to detect especially when just loading the crude reaction mixtures and using stained SDS-PAGE. Therefore, this kit includes an expression vector for a tPA protein fragment that can be used as a control on how the system works. Refer to the Figure 6 above for reference on the expected signal after a 20h reaction time; you should be able to see the tPA protein on SDS-PAGE after an overnight reaction. The positive control can be used to confirm that the expression reactions are handled correctly and that all reagents work properly. When loading crude reaction mixtures on SDS-PAGE, the wheat germ proteins in the extract may make it difficult to assign the right band for the overexpressed protein. Therefore, we advise to run a negative control experiment without added template to view the protein present in the wheat germ extract. As an alternative, also Western Blotting can be used to better detect the expressed protein as the wheat germ proteins in the extract should not be detected by the antibody. Follow the advice given below if you have problems to see expression of the control or your proteins of interest.

Please consider the size of your target protein: The added PDI and Ero1α will be visible on SDS-PAGE as proteins of some 56 kDa and 52 kDa respectively. If your protein has a similar size, it may be hard to detect on a stained gel, and you may want to confirm protein expression for example by doing a Western Blotting experiment with a suitable antibody.

While we provide a protocol for testing the protease activity of the tPA protein fragment to confirm the formation of disulfide bonds for this specific protein, there are several other methods described in the literature to study disulfide bond formation. Please inform yourself on what could be the most suitable approach to confirm disulfide bond formation for your protein of interest.

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 expression reactions are dependent on accurate use of the DNA template and setting the reaction temperature preferably to 15°C. However, the wheat germ extract can be used at temperatures of up to 25°C to test other expression conditions. At even higher temperatures, the wheat germ extract will lose its activity leading to low protein yields.

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 *Ex Taq* DNA Polymerase from Takara Bio Inc. (Catalog # RR001C) for the protocol given in this manual, but other high fidelity polymerases having a proof-read function may work equally well when preparing PCR templates for *in vitro* protein expression. However, we cannot exclude that other PCR conditions will give lower template concentrations 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 a 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 Disulfide Bond PLUS Expression Kit contain the SP6 RNA Polymerase and other RNA polymerase promoters are not supported.

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

- 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. We advise in the manual on doing an additional phenol/chloroform extraction if the OD260/280 ratio is not correct or poor protein yields had been obtained.
- 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 1 µg/µl.
- Make sure to run the transcription and translation reactions at indicated reaction temperature. The wheat germ extract will lose activity above 25°C. If possible, we recommend using a thermocycler as they are more accurate on keeping the set temperature, but the cups provided with this kit can be incubated in an incubator as well.
- 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.
- 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.

- 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. Similarly, we recommend avoiding unnecessary freeze/thawing of the PDI/Ero1α enzyme mixture. Prepare aliquots at first use if not all the mixture is used at once.
- 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. Mark each step in the protocol after completion.

Transcription Reaction Using DNA Template

Per reaction perform the following steps to set up transcription reaction:

1. Thaw your template DNA before the experiment. You need 2 µg of purified plasmid DNA at a concentration of 1 µg/µl.
2. Take one vial with Transcription Premix LM per reaction from storage at -80°C.
3. Thaw required number of Transcription Premix LM vials on ice. Keep the remaining vials at -80˚C. After thawing, spin the vials briefly to collect the entire volume at the bottom of the vial. Mix the reagent gently before use. Place reagents on ice and keep them cold at all times.
4. Add 2 µl of highly purified plasmid DNA (1.0 µg/µl) to each vial with the Transcription Premix LM as shown in the table below. Then mix gently by pipetting up and down.

Reagents	Working Volume	Final Concentration	Checkmark
Transcription Premix LM	18 µl	1 x	☐
Plasmid (circular DNA, 1.0 µg/µl)	2 µl	100 ng/µl	☐
Total	20 µl		

5. Incubate at 37°C for 1 hour when working with expression vector, incubate for 4 hours when using PCR product.

Translation Reaction Using RNA Prepared from DNA Template

After completion of the transcription reaction, let the reaction mixture cool down to room temperature. Do not forcibly cool it down on ice or in a refrigerator.

Per reaction perform the following steps to set up translation reaction:

10. Per reaction take from storage at -80°C storage a single vial with WEPRO® for DB PLUS, a single well containing SUB-AMIX® SGC DTT-free, and PDI & Ero1α mix. Do not thaw unneeded vials and wells.
11. Thaw reagents on ice. After thawing, briefly spin down each vial with WEPRO® for DB PLUS and PDI & Ero1α mix to collect the reagent at the bottom of the vial. Avoid excessive centrifugation of WEPRO® for DB PLUS!
12. Resuspend SUB-AMIX® SGC DTT-free by pipetting gently up and down in the well.
13. Resuspend the transcription mixture by pipetting gently up and down.
14. Prepare “translation mixture” by adding 10 µl of the “transcription mixture” containing the RNA template to the vial containing the WEPRO® for DB PLUS as indicated in the table below. Mix gently by pipetting up and down, avoid bubbles.

Reagents	Working Volume	Final Concentration	Checkmark
Transcription mixture (mRNA)	10 µl	0.5 vol.	☐
PDI & Ero1α mix	4 µl		
WEPRO® for DB PLUS	16 µl	120 OD	☐
Total	30 µl	-	

15. Carefully transfer the translation mixture (30 µl) to the bottom of a single well containing SUB-AMIX® SGC DTT-free (200 µl) to form bilayer with the translation mixture in the lower layer and SUB-AMIX® SGC DTT-free in the upper layer. Refer to the figure in the manual on how to setup a bilayer reaction: Go with the pipette tip to the bottom of the well, and slowly release the translation mixture below the reaction buffer. Because of the yellowish color of the wheat germ extract, you can distinguish the translation mixture from the translation buffer in the well.

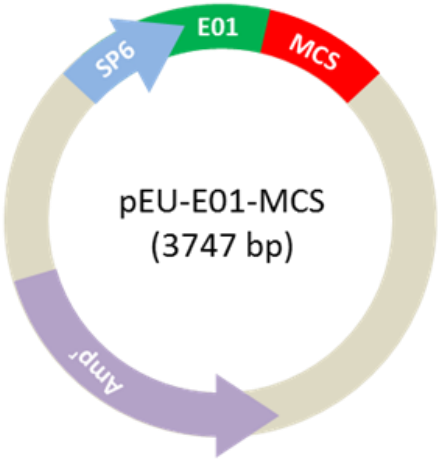
Reagent	Working Volume	Final Concentration	Checkmark
SUB-AMIX® SGC DTT-free	200 µl	1 x	☐
Translation mixture	30 µl	-	☐
Total	230 µl	-	

16. Seal the well with an aluminum seal included in the kit to avoid evaporation.
17. Incubate at 15˚C for 20 hours in an incubator. Be careful that the well stably stands on a flat surface.
18. After completion of the translation reaction, mix the bilayer reaction gently by pipetting up and down.

Refer to the full manuals provided with our reagent kits and our homepage for more advice on how to use our wheat germ cell-free protein expression system. Please contact our support team if you have further questions or needs.

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.
Chromozym® is a registered trademark of Sigma-Aldrich Co. LLC.
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.

Contact

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