CellFree Sciences

The natural power of wheat driving science

High Performance Cell-Free Wheat Germ Protein Expression System

INSTRUCTION MANUAL

FLEXIQuant PLUS Expression Kit

This kit provides reagents for $^{13}C/^{15}N$ arginine and lysine stable isotope protein labeling to conduct FLEXIQuant experiments

Product Number(s): CFS-FLEX-MS

Version/date: Version 2.0_eng/March 2019

This Product is for research use only.

Our products are produced under a strike 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 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/

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 transcription and translation experiments at your work place. 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.

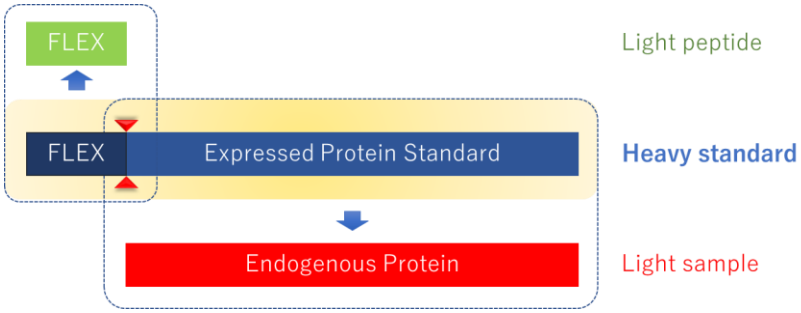
Introduction

Mass spectrometry (MS) has become an important analytic tool to study proteins and their post-translational modifications (PTMs). Quantitative MS is increasingly used to obtain accurate quantitative information on an individual protein or even multiple proteins in proteomic studies. Various approaches have been developed to identify proteins by the mass of representative peptides. During the workflow of such experiments, proteins of interest are commonly first enriched from cell extracts, e.g. by immunoprecipitation with an antibody, before getting digested with a protease, most commonly trypsin, to obtain specific peptides for MS detection. Usually, proteins give rise to multiple peptides in the digestion step. However, only some of those peptides are suitable for specific detection and accurate quantification. Therefore, proteotypic peptides (PTPs) must be selected for each protein, which requires experimental data and validation of each assay. Moreover, there can be distinct forms of the same peptide depending on those PTMs that occurred in the original sample. To accurately quantify peptides in MS experiments, and thus the parental proteins they are derived from, labeled reference peptides of the same sequence are added as internal standards for MS experiments. By the incorporation of stable isotope labeled amino acids e.g. [^{13}C, ^{15}N]-L-Lys and [^{13}C, ^{15}N]-L-Arg into a MS standard, the labeled peptides can be distinguished from the endogenous peptides obtained from the sample by defined mass shifts. Since the amounts of such standards are known, the standard can be used as a reference to quantify the proteotypic peptides in a sample, and by extension also the protein the reference peptide is derived from, by mass spectrometric methods such as selected reaction monitoring (SRM), multiple reaction monitoring (MRM), and/or parallel reaction monitoring (PRM).

The FLEXIQuant (**F**ull-**L**ength **Ex**pressed Stable **I**sotope-Labeled Proteins for **Quant**ification) method developed by the group of Dr. Hanno Steen in the Department of Pathology at Boston Children's Hospital (Boston, MA, USA) allows for in-depth quantification of proteins of interest and their modified peptides by using a heavy, i.e. stable isotope labeled full-length protein standard. To perform SRM, MRM or PRM experiments, it can be advantageous to prepare labeled full-length proteins as MS standards, which can be added directly to cell extracts thereby ensuring identical processing along with the endogenous proteins. In this way, all steps during the entire sample preparation can be monitored including the protein enrichment and digestion steps. A labeled full-length protein will be digested into multiple peptides thus i) allowing for the use of multiple peptides per target protein to increase the reliability of the experiment, ii) easily identifying suitable PTPs, and iii) providing quantitative information about the extent of modification for each observable peptide (see below). The isotope-labeled full-length proteins are prepared in such a way, that they have a unique artificial peptide tag at their N-terminus (aka FLEX-tag-peptide). After sample processing, during trypsinization, the FLEX-tag is liberated from the full-length protein and will be detected in the MS experiment as a single peptide peak. Because this artificial FLEX peptide is within the isotope-labeled full-length protein it is also getting labeled; thus, this peptide can be quantified in the MS experiments by reference to an added unlabeled synthetic FLEX peptide standard. The indirect quantification of the MS standard through the synthetic FLEX peptide is indispensable when preparing only small amounts of labeled protein that are not extensively purified and biochemically quantified.

The FLEXIQuant method uses a wheat germ cell-free protein expression system for preparing the full-length protein standards having the added FLEX-peptide tag. Proteins made in the wheat germ cell-free protein expression system commonly have no PTMs. Thus, all the peptides derived from such full-length proteins should have a defined mass that can be determined from the peptide sequence. PTMs may be identified, and quantified, by reference to the unmodified reference peptide. Because multiple peptides are generally obtained from a full-length protein standard, the method often allows to monitor PTMs at various amino acids along major parts of the protein of interest. Current examples from the Steen lab include Tau in primary human specimens and iPSC-derived samples, TIKI1 in *X. laevis*, and CDC27 and KIFC1 in human cell lines. For further details on the FLEXIQuant method including protocols and application examples, refer to the reference list at the end of the manual.

Figure 1: Principle of FLEXIQuant method



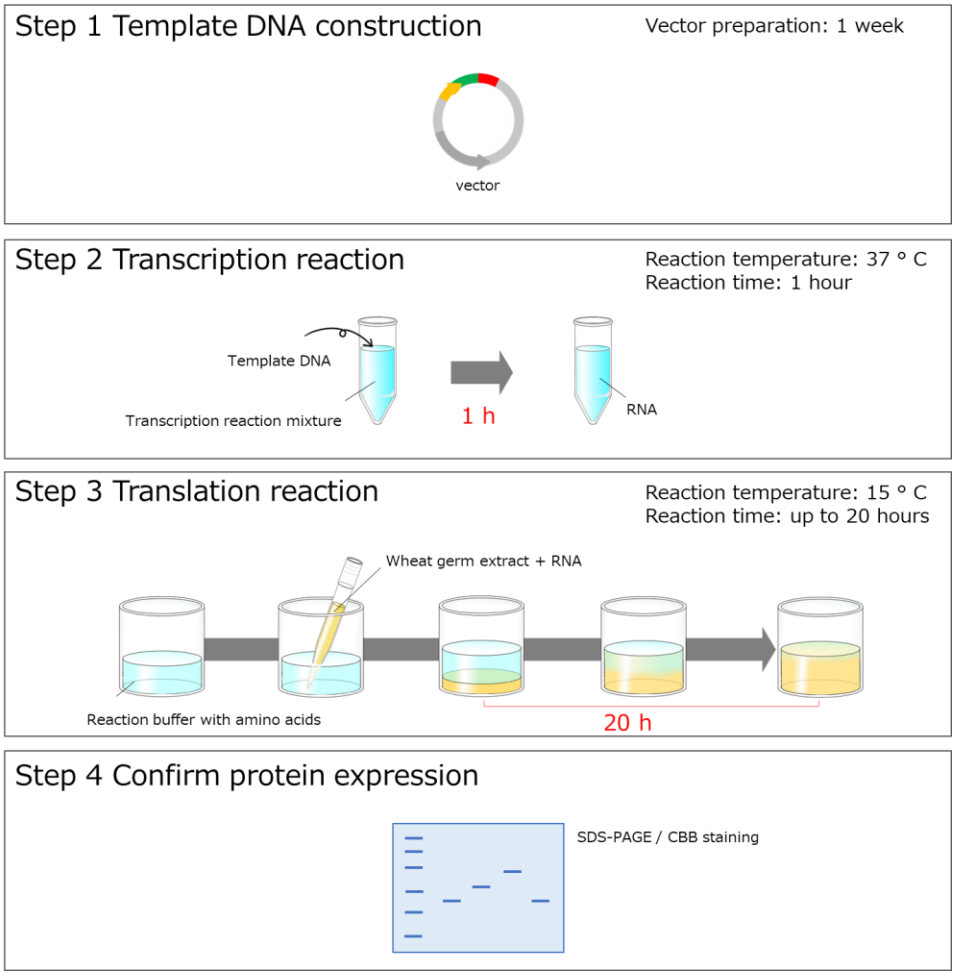
The FLEXIQuant PLUS Expression Kit provides all the necessary reagents to prepare [^{13}C, ^{15}N]-L-Lys and [^{13}C, ^{15}N]-L-Arg labeled full-length proteins for conducting FLEXIQuant experiments. For the FLEXIQuant PLUS Expression Kit, CellFree Sciences developed a reaction format based on WEPRO®9240 with added [^{13}C, ^{15}N]-L-Lys and [^{13}C, ^{15}N]-L-Arg for direct incorporation during protein expression. The reaction conditions allow incorporation efficiencies of about 99% for both labeled amino acids. The protein expression reactions can easily be set up with few pipetting steps by using the ready-to-use, premixed reagents provided for the transcription and translation reactions. The reaction conditions used in this kit have been tested for the expression of many proteins in large-scale proteomics studies, where a 226 µl bilayer expression reaction yields commonly sufficient protein to conduct MS experiments.

The FLEXIQuant PLUS Expression Kit provides all necessary reagents to perform eight protein expression and labeling reactions. In addition, the kit provides an expression vector for wheat germ cell-free protein expression system that allows for the expression of fusion proteins with the FLEX-peptide tag at the N-terminus. An expression vector for a tagged CDC27 protein is provided as a positive control. For more information on the use of our wheat germ cell-free protein expression system for the preparation of labeled proteins and use in mass spectrometry, refer to the references at the end of the manual.

General Information on Wheat Germ Protein Expression System

To prepare proteins in the wheat germ cell-free protein expression system is a straight forward process involving only four steps:

Figure 2: Four steps of a cell-free protein expression experiment



To perform the protein expression experiment, a template DNA is required that has a SP6 RNA polymerase promoter and a suitable enhancer. We advise to clone your cDNA into an expression vector dedicated for use in our wheat germ cell-free protein expression system. For the preparation of FLEX-peptide tagged proteins as needed to conduct FLEXIQuant experiments, this kit provides cloning vector pEU-E01-His-FLEX-MCS-N1. This vector allows for the expression of fusion proteins with the FLEX-tag at the N-terminus of your protein of interest. Moreover, the vector will add a His-tag to the fusion protein to allow for enrichment of the fusion proteins on a nickel matrix. For more details on the pEU-E01-His-FLEX-MCS-N1 vector refer to the vector map in Appendix B. The kit also contains vector pEU-E01-MCS in case you would like to express your protein in the native form without the added His-tag and FLEX-tag.

CellFree Sciences can provide other expression vectors for working with tagged proteins using the DYKDDDDK-, His- or GST-tag; however, those vectors do not contain a FLEX-tag. Refer to our homepage or contact us directly for more information on other expression vectors, that can be obtained from CellFree Sciences (contact information provided at the end of the manual).

Although protein expression reactions for the wheat germ cell-free expression system can be upscaled, commonly the 226 µl reaction format used in this kit provides sufficient protein yields for conducting FLEXIQuant experiments; one 226 µl reaction can for example produce ~23 µg of crude CDC27 protein (i.e. ~1 nmol), the positive control included in the kit. In the following protocol, we provide directions on how to setup a 226 µl bilayer reaction using the premixed reagents provided with the FLEXIQuant PLUS Expression Kit.

Related CFS Products

For performing additional protein labeling reactions, CFS offers the Protein Research Kit for MS (Product number "CFS-PRK-MS"), that provides the same premixed reagents as used in the FLEXIQuant PLUS Expression Kit for conducting 16 times a 226 µl translation reaction. The Protein Research Kit for MS, however, does not contain the pEU-E01-His-FLEX-MCS-N1 vector and extra FLEX-tag peptide. The FLEX-tag peptide can be purchased separately if additional amounts are needed (Catalog number: "EDX-FLEX-PEP"). For even larger reagent requirements, refer to our WEPRO8240MS/WEPRO8240GMS/WEPRO8240HMS Core Kits (Product numbers CFS-C8MS, CFS-C8GMS, CFS-C8HMS).

For conducting more 226 µl bilayer reactions without added labeled amino acids, you can also purchase from CellFree Sciences the Protein Research Kit (S), Product Number CFS-PRK-S24. These kits provide the same WEPRO®9240 premixed reagents to perform 24 small-scale 226 µl bilayer expression reactions for the preparation of unlabeled proteins. 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

The FLEXIQuant PLUS Expression Kit allows for protein synthesis and the incorporation of $^{13}C/^{15}N$ labeled lysine and arginine during the translation reaction (see below more details on the labeled amino acids). In the wheat germ cell-free protein expression system, protein synthesis is carried out by preparing first an RNA from the DNA template in a transcription reaction. The RNA is then used in the following translation reaction for protein synthesis. In the manual, we provide directions for working with a cDNA template cloned into an expression vector.

The manual provides further a protocol for purification of His-tagged proteins using a nickel resin. However, it is not always necessary to purify the labeled protein before use in the FLEXIQuant experiment. Refer to the recommendations on how to perform FLEXIQuant experiments for more information on when a purification step is required.

Protein expression should be confirmed before use in a FLEXIQuant experiment. The manual gives directions on how proteins can be analyzed on SDS-PAGE to see whether a protein of the correct size has been made. We advise to use known amounts of a BSA standard in the SDS-PAGE experiment to estimate the protein yields. Before setting up FLEXIQuant experiments, it is recommended to compare the relative protein amounts obtained from the cell-free protein expression reaction with those in the samples. This can, for example, be done by Western blotting using an antibody against the target protein of interest, which normally detects the heavy exogenous proteins with the same affinity as the light endogenous protein. The FLEXIQuant experiment will give more accurate results when the reference protein and the endogenous protein are within a similar concentration range.

Use of FLEXIQuant PLUS Expression Kit

The FLEXIQuant PLUS Expression Kit provides enough reagents for 8 protein expression reactions on a 226 µl scale to prepare isotope-labeled proteins. In addition, the kit provides expression vectors pEU-E01-MCS, and pEU-E01-His-FLEX-MCS-N1 to prepare an expression template for the protein(s) of interest. An unlabeled FLEX-tag peptide is provided with the kit to assist the conduct of FLEXIQuant experiments.

Materials Provided by the Kit

The FLEXIQuant PLUS Expression Kit comes in two separate boxes 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! Note, the lyophilized reference peptide is provided in a separate box and may be stored at -20°C under dry conditions.

Kit Content

Box holding reference peptide

Item	Quantity	Amount	Content	Vial	Vial Color
FLEX-tag peptide*	8	1 nmol	Dried powder	1.8 ml cryo tube	Yellow

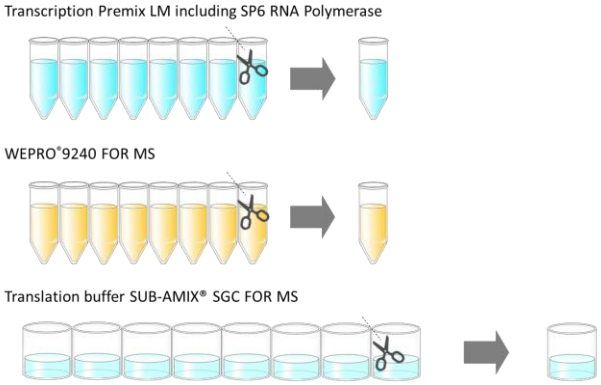
Box holding protein expression reagents and vectors

Item	Quantity	Concentration	Volume	Vial	Vial Color
pEU-E01-MCS	1	1.0 µg/µl	5.0 µl	0.2 ml PCR tube	Red
pEU-E01-His-FLEX-MCS-N1	1	1.0 µg/µl	5.0 µl	0.2 ml PCR tube	Orange
Positive Control pEU-E01-CDC27	1	1.0 µg/µl	5.0 µl	0.2 ml PCR tube	Purple
Transcription Premix LM**	8	1 x	18 µl	Strip of eight 0.2 ml PCR tubes	Blue
WEPRO®9240 for MS**	8		10 µl	Strip of eight 0.2 ml PCR tubes	Clear
SUB-AMIX® SGC for MS**	8	1 x	206 µl	Strip with eight wells	Clear
Aluminum seals	4	-	-	-	-

*The FLEX-tag peptide is provided in separate box. Lyophilized peptides are commonly stable for more than a year when kept under dry conditions preferably at -80°C or otherwise at -20°C. After taking up the lyophilized peptide in a solvent, the solution can be stored at -80°C for possibly up to three months. Ensure to keep the pH of the solution in a 3 to 6 range.

**Use total volume within each vial per one reaction. The premixed reagents for the transcription and translation reactions are provided in strips holding 8 wells. Cut off individual tubes or wells as needed before thawing the reagents. Never thaw reagents that are not needed for the experiment.

Figure 3: Handling of expression reagents



Instructions on Use of Reagents

Item	Description
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-FLEX-MCS-N1	Expression vector for expressing gene of interest with a N terminal His- and FLEX-peptide tag, refer to Appendix B for more details on the vector
pEU-E01-CDC27*	This is a positive control vector for testing protein expression
Transcription Premix LM	Premixed SP6 RNA polymerase and transcription buffer
WEPRO®9240 for MS	WEPRO®9240 wheat germ extract containing $^{13}C/^{15}N$ labeled lysine and arginine as well as other 18 non-labeled amino acids.
SUB-AMIX® SGC for MS	High performance translation buffer containing 18 non-labeled amino acids; lysine and arginine have been excluded from the buffer
Aluminum seal	Seals to cover the wells during translation reaction, cut into appropriate size to cover well
FLEX-tag peptide**	FLEX-tag peptide (TENLYFQGDISR) is provided as 1 nmol of dried peptide per vial. The kit contains 8 vials to enable user to prepare a fresh peptide stock for each experiment. We recommend preparing a 10 □M solution by taking up the dry peptide in 100 µl of 5% (v/v) Formic Acid, 5% (v/v) acetonitrile, and store aliquots at -80°C. For use in the MS experiment, take one aliquot and prepare a serial dilution in the MS loading buffer e.g. with 1, 0.1, and 0.01 □M.

*Vector pEU-E01-CDC27 was described in: "FLEXIQuant: a novel tool for the absolute quantification of proteins, and the simultaneous identification and quantification of potentially modified peptides." Singh S, Springer M, Steen J, Kirschner MW, Steen H.: J Proteome Res. 2009;8:2201-2210.

** Refer to the following reference for more information on how to handle peptides for use in MS experiments: "Recommendations for the Generation, Quantification, Storage, and Handling of Peptides Used for Mass Spectrometry-Based Assays." Hoofnagle A.N. et al., Clin Chem. 2016 Jan;62(1):48-69.

Information on Labeled Amino Acids

Amino Acid	Molecular Weight	Chemical Purity	Chemical Formula
L-ARGININE ($^{13}C6$, 99%; $^{15}N4$, 99%)	220.59	≥99%	H2*N*C(=*NH)*NH(*CH2)3*CH(*NH2)*COOH
L-LYSINE ($^{13}C6$, 99%; $^{15}N2$, 99%)	227.05	≥99%	H2*N(*CH2)4*CH(*NH2)*COOH

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 further phenol extraction of the plasmid DNA. A phenol extraction is not mandatory but can help to remove impurities from standard plasmid DNA preparations.

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 Protein Purification

The following reagents are only needed when purifying the proteins using the His-tag.

Reagents	Description
Nickel resin (*)	Ni Sepharose 6 Fast Flow e.g. from GE Healthcare (17-5268-01). Replace the storage solution with wash buffer B to prepare 50% slurry
Solution A	600 mM imidazole pH 8.0
Wash buffer B	20 mM Na-phosphate pH 7.5, 0.3 M NaCl, 20 mM imidazole
Elution buffer E	20 mM Na-phosphate pH 7.5, 0.3 M NaCl, 500 mM imidazole

*See manufacturer's instructions for details on how to prepare the resin. Details may change depending on provider.

Other Consumables and Instruments Required for Protein Expression

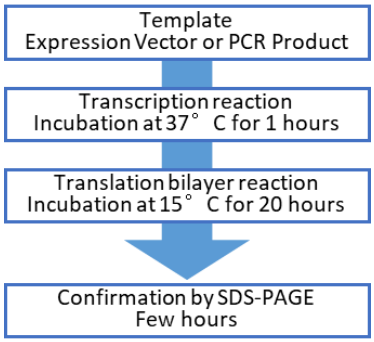
Consumable	Description
Incubator	Temperature range 15 to 37°C
Tabletop centrifuge	Only needed to purify His-tagged proteins
SDS-PAGE	SDS-PAGE*, gel electrophoresis apparatus and power supply

*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 does not mention the time require for template preparation.



Considerations on Obtaining cDNA Templates for Proteins of Interest

Note, 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 the clones and resources they are offering.

Note, that a cDNA clone just represents one possible isoform. However, for most human transcripts 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).

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 on a preparative scale.

In the following we give some brief advice on the use of our expression vectors. This kit contains expression vectors pEU-E01-MCS (for expression of native form; red vial) and pEU-E01-His-FLEX-MCS-N1 (for expression of fusion protein featuring an N-terminal His-tag and FLEX-peptide tag; orange vial). These vectors, 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. 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 multi cloning site (MCS) in the vector map for pEU-E01-MCS (Appendix A) (*1), or in the vector map for pEU-E01-His-FLEX-MCS-N1 (Appendix B). The protein will be translated from the first start codon, an ATG, up to the first stop codon in your cDNA inserted in the MCS. **Note that within vector pEU-E01-His-FLEX-MCS-N1 the EcoRV and XhoI sites cannot be used for cloning. Both restriction sites fall within the FLEX-tag sequence. The BamHI site in vector pEU-E01-His-FLEX-MCS-N1 is at the end of the FLEX-tag sequence. Note that your cDNA must have a stop codon at the 3' end when working with vector pEU-E01-His-FLEX-MCS-N1. This vector does not provide any stop codons to terminate protein synthesis. Note further that your cDNA must have an ATG start codon at the 5' end when working with a vector having no N terminal tag. These vectors do not provide an ATG start codon to initiate protein synthesis. Please make sure that your insert has the same reading frame as the FLEX-tag sequence in the pEU-E01-His-FLEX-MCS-N1 expression vector. Frameshift errors will destroy your experiment!**
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. 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 of 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 C for more information on sequencing primers for our vectors. The vector sequence information for vectors pEU-E01-MCS and pEU-E01-His-FLEX-MCS-N1 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-MGC. 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

For direct use in our expression system, we DO NOT recommend the use of DNA mini-preparation methods by the alkaline elution procedure lacking any further purification step. 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 extended period of time).
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 1.0 µg/µl by adding an appropriate volume of TE buffer (*3).

You need 2 µg purified plasmid DNA per 226 µl 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: 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.

Transcription Reaction Using DNA Template

We recommend using positive control plasmid, pEU-E01-CDC27 (purple vial), in a separate expression experiment to make sure that the experiment has been set up correctly.

Per reaction perform the following steps:

1. Thaw your template DNA before the experiment. You need 2 µg of plasmid DNA.
2. Take one vial with Transcription Premix LM (blue vial) per reaction from storage at -80°C (*1).
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 (*2).
6. After completion of the transcription reaction, leave reaction mixture at room temperature until later use in the translation reaction. Do not cool the reaction mixture, nor store it on ice.
7. Optionally, you can confirm the mRNA quality (*3) after the transcription reaction by agarose gel electrophoresis loading 0.5 µl of the reaction mixture (*4). Refer to a cloning handbook for more information on how to perform RNA gel electrophoresis.

(Notes)

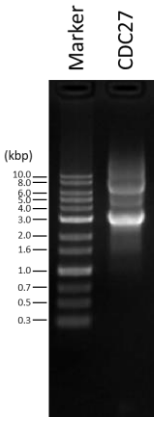
*1: The strip holding the Transcription Premix LM vials can be cut into individual tubes by bending or cutting. Hold the vials firmly so that they do not pop open while separating them.

*2: White precipitate may occur during the 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.

*3: A smear or RNAs smaller than 500 bases indicate degradation of the RNA. Probably this is caused by RNases contaminating the plasmid DNA. In that case, further purification of the plasmid DNA as described in Section 8.4 may resolve the problem. Confirm further that you are working under RNase free conditions.

*4: An example for a good RNA expression product is shown in the Figure 4 below. Note that the size difference between the RNA bands should be in the range of the length of the plasmid sequence. The RNA polymerase tends to fall off at the origin of replication (Ori) of the vector.

Figure 4: Example for RNA transcripts obtained from vector pEU-E01-CDC27 analyzed on a 1% agarose gel (1 µl of reaction mixture loaded; RNA of different length is commonly seen when working with a circular template)



Translation Reaction Using RNA Prepared from DNA Template

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

1. Per reaction take a single vial with WEPRO®9240 for MS (clear vial) and a single well (clear well) containing SUB-AMIX® SGC for MS from storage at -80°C (*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®9240 for MS losses its activity if not kept at -80°C!
2. Thaw reagents on ice. After thawing, briefly spin down each vial with WEPRO®9240 for MS to collect the reagent at the bottom of the vial. Avoid excessive centrifugation of WEPRO®9240 for MS!
3. Resuspend SUB-AMIX® SGC for MS by pipetting gently up and down in the well (*2).
4. Let the reaction mixture from the transcription reaction cool down to room temperature. DO NOT forcibly cool it down on ice or in a refrigerator. Resuspend the transcription mixture by pipetting gently up and down (*3).
5. Prepare "translation mixture" by adding 10 µl of the "transcription mixture" containing the RNA template to the vial containing the WEPRO®9240 for MS as indicated in the table below. Mix gently by pipetting up and down, avoid bubbles.

Reagents	Working Volume	Final Concentration
Transcription mixture (mRNA)	10 µl	0.3 vol.
WEPRO®9240 for MS	10 µl	60 OD
Total	20 µl	-

6. Carefully transfer the translation mixture (20 µl) to the bottom of a single well containing SUB-AMIX® SGC for MS (206 µl) to form bilayer with the translation mixture in the lower layer and SUB-AMIX® SGC for MS in the upper layer. Refer to Figure 5 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.
   DO NOT mix the reagents in the well by pipetting or any other means! It will reduce the yield of the reaction.

Reagent	Working Volume	Final Concentration
SUB-AMIX® SGC	206 µl	1 x
Translation mixture	20 µl	-
Total	226 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)

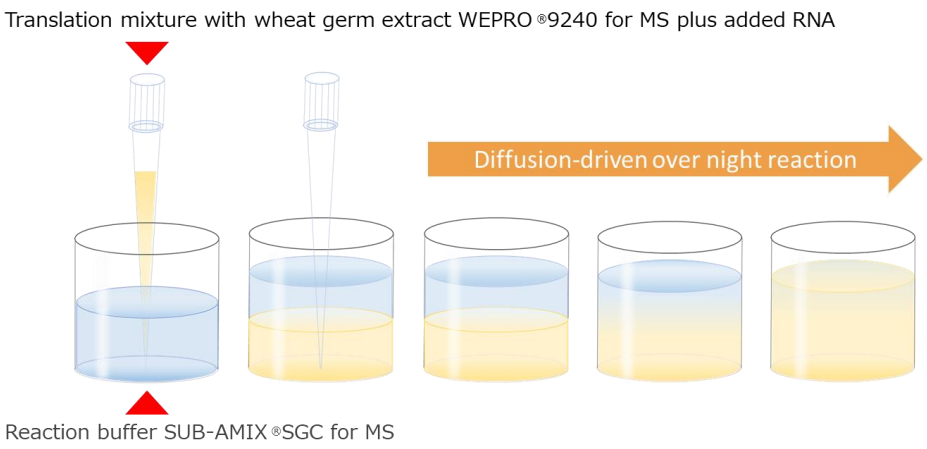
*1: The strips with the WEPRO®9240 for MS vials and SUB-AMIX® SGC for MS wells can be split into individual vials or wells by bending or cutting. Hold the vials with the WEPRO®9240 for MS firmly so that they do not pop open while separating them.

*2: Take particular care to keep the wells with SUB-AMIX® SGC for MS 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®9240 for MS. 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



Purification of His-tagged Fusion Proteins

The pEU-E01-His-FLEX-MCS-N1 vector provided with the kit enables the expression of His-tagged fusion proteins. The His-tag allows for easy protein purification using a batch method. Follow the steps below to enrich His-tagged proteins on a Ni-Sepharose resin. – Note, the kit does not provide reagents for purifying His-tagged proteins. Refer to the table in reagent list for more information on the required reagents.

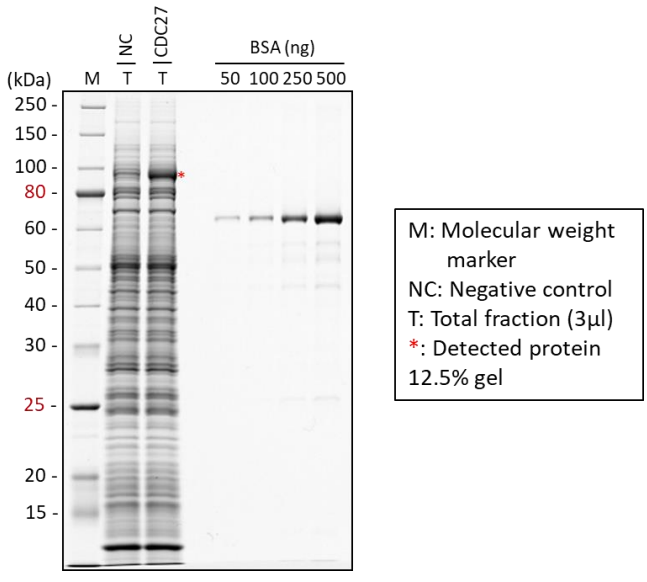
1. Transfer 200 µl of the translation reaction mixture to a 1.5 ml tube. Keep the remaining reaction mixture e.g. for analysis on SDS-PAGE.
2. Add 6.7 µl of 600 mM imidazole pH 8.0 (Solution A) to the tube and mix gently.
3. Add 15 µl of resin (50% slurry, Ni-Sepharose High Performance from GE Healthcare) to the tube.
4. Gently shake the tube at 4°C for 60 min.

5. Centrifuge at 500xg for 5 min to collect resin.
6. Remove supernatant and keep the “flow-through” fraction. Do not discard the flow-through fraction until you have confirmed that all the protein has been recovered from the resin.
7. Add 200 µl of wash buffer (Wash buffer B: 20 mM Na-phosphate pH 7.5, 0.3 M NaCl, 20 mM imidazole) to the tube.
8. Gently shake the tube for 5 min.
9. Centrifuge at 500xg for 5 min to collect resin.
10. Remove supernatant and keep the “wash” fraction.
11. Repeat washing step (steps 7 to 10) 3 times. Do not discard the washing buffer until you have confirmed that all the protein has been recovered from the resin.
12. Add 30 µl of Elution buffer E (20 mM Na-phosphate pH 7.5, 0.3 M NaCl, 500 mM imidazole) to resin.
13. Gently shake the tube for 10 min.
14. Centrifuge at 500xg for 5 min to collect resin.
15. Collect supernatant as “Elution Fraction 1”. Note the supernatant contains the purified protein!
16. Repeat elution steps 12 to 15 for a second time and collect “Elution” Fraction 2”.
17. Keep Elution Fractions 1 and 2 separately, because the protein concentration in Fraction 1 is commonly higher than in Fraction 2.
18. Confirm the purity of the purified proteins by SDS-PAGE.

Confirmation of Protein Expression

We advise to confirm expression of the target protein by SDS-PAGE followed by Coomassie blue staining to identify the expressed protein. SDS-PAGE requires an appropriate acrylamide concentration to distinguish the expressed protein from background proteins originating from wheat germ extract. For a standard mini gel, load 3 µl of translation reaction mixture per well. If the volume is too high or too low to identify the protein, change the volume to obtain a clear result. CDC27, which is used as positive control in this kit, is expressed as a protein of approximately 90 kDa. An example for the expression of the CDC27 protein is shown in Figure 6 below (red asterisk), where we estimated a yield of about 23 µg crude CDC27 protein (~250 pmol) from a 226 µl kit reaction.

Figure 6: SDS-PAGE confirming the expression of the CDC27 protein from vector pEU-E01-CDC27

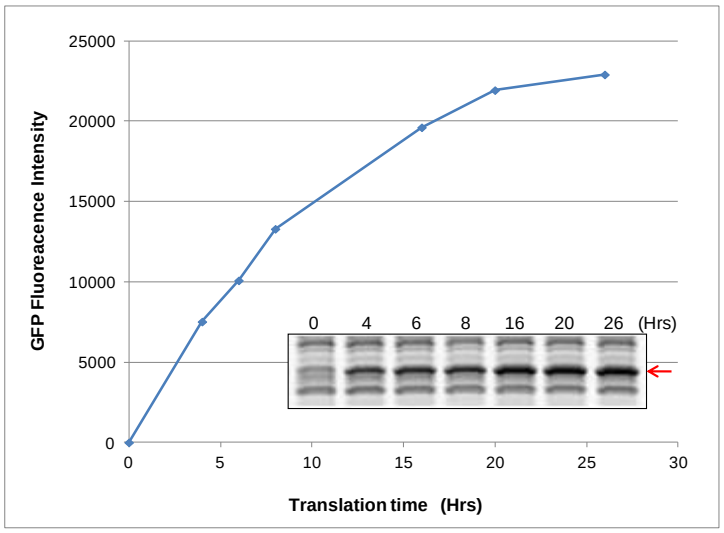


Refer to the following publication for more details on the use of the CDC27 standard: “FLEXIQuant: a novel tool for the absolute quantification of proteins, and the simultaneous identification and quantification of potentially modified peptides.” Singh S, Springer M, Steen J, Kirschner MW, Steen H.: J Proteome Res. 2009;8:2201-2210.

Additional Information on Translation Reaction

The optimal protein yield of the bilayer expression reaction is commonly reached after 24 h. Figure 7 below shows different time points for the expression of a Green Fluorescent Protein (GFP), which was synthesized using a plasmid DNA template. Protein synthesis was confirmed and quantified by measuring the fluorescence intensity in a SDS-PAGE. You may shorten the reaction time of the translation reaction if less protein is needed. Use the data given in the figure as reference for testing different time points.

Figure 7: SDS-PAGE showing a time course for the expression of a GFP protein at 15ºC



Considerations for Use of Labeled Proteins in FLEXIQuant Experiments

A detailed practical guide on how to perform FLEXIQuant experiments has been published by Singh *et al.* in *Methods in Molecular Biology 2012; vol. 893; pages 295-319*. We advise to refer to this protocol and other examples in the literature to learn more about how to use the FLEXIQuant method (see reference list at the end of the manual). The following considerations do not contain a full protocol on how to conduct FLEXIQuant experiments.

Figure 8: Outline of FLEXIQuant experiment

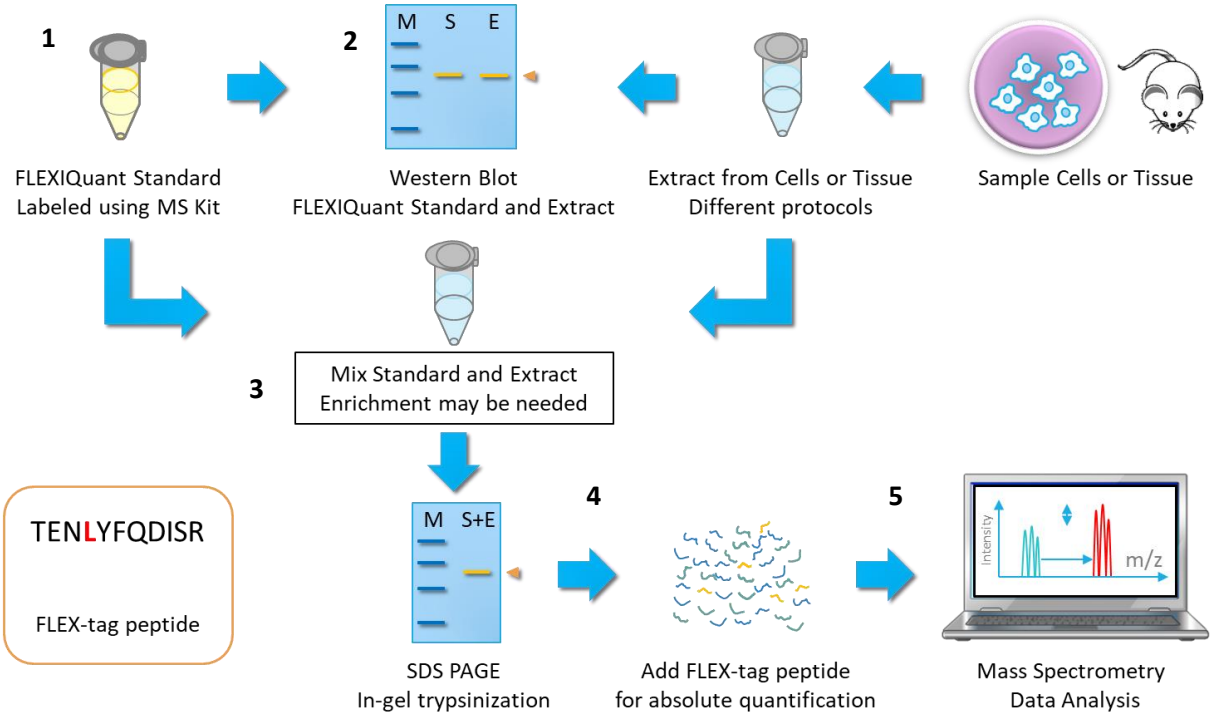


Figure 8 provides an outline of the FLEXIQuant method, where the key steps of the experiment are indicated: 1) At the beginning of the experiment, a FLEXIQuant MS Standard is prepared using the reagents provided with the FLEXIQuant PLUS Expression Kit. 2) It is recommended to compare the protein levels in FLEXIQuant MS Standard with those in the cell or tissue extract used in the analysis. This is most conveniently done by Western blotting using an antibody against the target protein. The FLEXIQuant experiment can be carried out in separate ways depending on the abundance of the protein of interest in the relevant samples. The abundance of the protein of interest dictates as to whether the protein in the sample or the heavy full-length FLEXIQuant MS Standard must be enriched or not; this can be done by e.g. immunoprecipitation of the target protein or using the His-tag on the recombinant FLEXIQuant MS Standard. Doing a Western Blot analysis on the FLEXIQuant MS Standard and the extract at different concentrations should assure that the FLEXIQuant MS Standard is added later in the process at a similar concentration as the protein of interest within the extract. This ensures that the light to heavy peak ratio will be close to 1:1. 3) The endogenous and the exogenous protein samples are combined and processed together to ensure that both samples are handled in the same way. 4) Finally, the sample containing the heavy and the light proteins is digested, and 5) the resulting peptide mixture is analyzed by LC/MS in the presence of the added unlabeled FLEX-tag peptide.

For instance, in the case of analyzing mitotic regulators such as CDC27 (PMID: 19344176) or KifC1 (PMID: 24510915) in whole cell lysates, immunological strategies were used to enrich for the protein of interest prior to the LC/MS experiments. In such a situation, there is no need to purify the heavy full-length recombinant FLEXIQuant MS standard from the translation reaction mixture; instead, the crude reaction mixture, once the *in vitro* transcription/translation reactions are completed, is added to the relevant whole cell lysate prior to immunoprecipitating the endogenous light protein and concomitantly the exogenous heavy FLEXIQuant MS Standard. The most accurate and precise quantitative information is obtained when a mixing ratio of close to 1:1 of the endogenous versus the exogenous protein is achieved. The most straightforward way to balance the appropriate amounts of relevant sample and heavy FLEXIQuant MS Standard-containing solution is to estimate the concentration of the proteins using Western blotting loading some 0.3 to 10 µl of the translation reaction onto an SDS-PAGE mini gel. The relative intensity of the protein bands provides information about the necessary mixing ratio. For example,

if the exogenous protein is 10 times more intense than the endogenous protein, a 1/10 of heavy FLEXIQuant MS standard solution is mixed with the relevant sample.

However, if the protein of interest is present at a sufficiently high concentration so that no enrichment is needed prior to the LC/MS experiments, as in the case of Tau in the sarkosyl insoluble fraction of brain lysates from tauopathy patients (PMID: 26877193), the heavy full-length FLEXIQuant MS Standard must be enriched from the crude translation reaction mixture prior to addition to the relevant sample. Like in the case of the Tau protein, for analyzing proteins that can be directly detected by MS without enrichment from the sample, it is required to enrich the FLEXIQuant MS Standard after completion of the expression reaction. Otherwise, the protein of interest may be too much diluted with wheat proteins from the crude reaction mixture. To this point the His-tag added along with the FLEX-peptide tag can be used to easily and rapidly enrich the fusion protein. Alternatively, preparative methods based on antibodies or protein specific physicochemical properties might also be used, where highly purified proteins are desired.

Note: Commonly the His-tag is sufficient for protein purification and later use in FLEXIQuant experiments. Since LC/MS is used as a read-out, the FLEXIQuant MS standard does not have to be highly purified. Instead enrichment in the 20 to 50% range is perfectly acceptable as i) the protein concentration is determined in a recombinant protein specific manner using the FLEX-tag peptide (derived from the FLEX-peptide tag; see above), and ii) contaminating proteins have minor impact on the qualitative and quantitative FLEXIQuant workflow.

Note: If the endogenous protein must be enriched by e.g. immunoprecipitation (as in the case of CDC27 or KifC1 mentioned above), the protein abundances in the relevant sample and the FLEXIQuant MS Standard-containing samples are estimated prior to the enrichment (as it is assumed that the enrichment yield is the same for the endogenous and the exogenous protein once the solutions are mixed and both isotopologs are simultaneously enriched). In contrast, if the exogenous heavy protein must be isolated/enriched from the wheat germ extract prior to mixing with the relevant sample (as in the case of Tau mentioned above), then the protein abundance estimation must be performed AFTER the purification of the heavy FLEXIQuant MS Standard from translation reaction mixture.

Troubleshooting

The experiments require correct and accurate pipetting during reaction setup. 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 experiment must be done under RNase-free conditions as any loss of the RNA template will prevent protein expression.
- Mark in your protocol each pipetting step you have completed.
- 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 plasmid template will always yield negative results. The same applies if there is a mistake in the expression vector, 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 maps on more information on suitable sequencing primers to confirm the sequence of your expression vector.
- Confirm the DNA and RNA quality if the protein yields are low. Low RNA yields during the transcription reactions 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 recommend to always using fresh Creatine Kinase. Do not freeze/thaw Creatine Kinase as it will rapidly lose activity. Creatine Kinase is required for the energy supply of the translation reactions, and a loss of Creatine Kinase activity will reduce protein yields.
- For the translation reaction, do not mix the two layers during setup of the bilayer translation reaction. Mixing both layers will sharply reduce the protein yields of a 20-hour translation reaction as the reaction will run dead within few hours. A slow mixing of both layers is required to maintain the translation reaction for up to 20 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 a small-scale expression reaction to confirm their integrity. If the results are unclear, you can check the performance of the transcription and translation reactions separately to narrow down the problem. Use a positive control vector to make sure that all reagents work, and the experiment is done correctly. Reconsider the design of your expression template to improve protein yields if all the forgoing steps do not explain low protein yields.
- 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. Follow clearly the recommendations on reagent storage and handling.
- Keep all fractions during protein purification until you have confirmed the recovery of the purified proteins. If you are not able to recover the protein from the resin during the purification experiment, check whether the protein can be found in the flow through or the washing fractions.
- 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.Keep all fractions during protein purification until you have confirmed the recovery of the purified proteins. If you are not able to recover the protein from the resin during the purification experiment, check whether the protein can be found in the flow through or the washing buffer.

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

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Bench Note

Use this Bench Note for setting up your experiments. Mark each step in the protocol after completion.

Setup transcription reaction:

Reagent	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	INCUBATE 1h at 37°C for circular DNA	☐

Setup translation reaction:

Add transcription mixture to wheat germ extract and mix gently by pipetting up and down.

Reagent	Volume	Final Concentration	Checkmark
Transcription mixture (mRNA)	10 µl		☐
WEPRO®9240 for MS	10 µl	60 OD	☐
Total	20 µl		

Place translation reaction mixture below the reaction buffer to form bilayer. DO NOT MIX LAYERS!

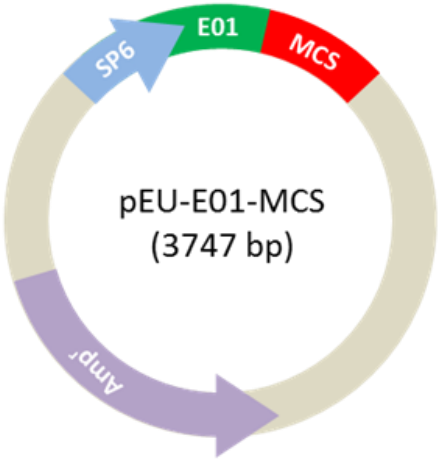
Reagent	Volume	Final Concentration	Checkmark
SUB-AMIX® SGC	206 µl	1x	☐
WEPRO®9240 for MS plus Transcription mixture (mRNA)	20 µl	-	☐
Total	226 µl	INCUBATE 20 h at 15°C	☐

Optional Purification of His-tagged Fusion Proteins:

1. Transfer 200 µl of the translation reaction mixture to a 1.5-ml tube, add Solution A (6.7 µl of 600 mM imidazole pH 8.0) to the tube, and mix gently.
2. Add 15 µl of resin (50% slurry, Ni-Sepharose High Performance from GE Healthcare) to the tube.
3. Gently shake the tube at 4°C for 60 min.
4. Centrifuge at 500xg for 5 min to collect resin, and remove supernatant, and keep the “flow-through” fraction.
5. Add 200 µl of Wash buffer B (20 mM Na-phosphate pH 7.5, 0.3 M NaCl, 20 mM imidazole) to the tube.
6. Gently shake the tube for 5 min.
7. Centrifuge at 500xg for 5 min to collect resin and remove supernatant.
8. Repeat washing steps (steps 7 to 10) 3 times.
9. Add 30 µl of Elution buffer E (20 mM Na-phosphate pH 7.5, 0.3 M NaCl, 500 mM imidazole) to resin.
10. Gently shake the tube for 10 min.
11. Centrifuge at 500xg for 5 min to collect resin and collect supernatant as “Elution Fraction 1”.
12. Repeat elution steps 12 to 15 for 2 times and collect “Elution Fraction 2”.

Appendix A: Vector Map for pEU-E01-MCS

Map:



Multi cloning site:

Vector elements:

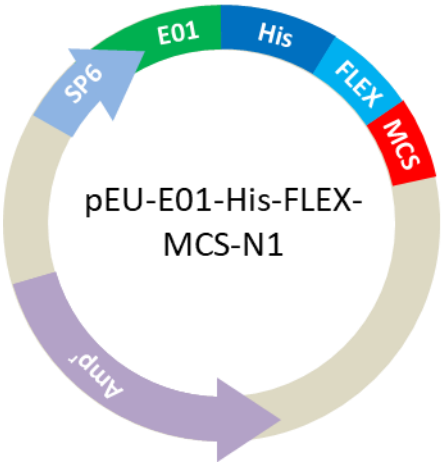
SP6 Promoter:	-17-1
Translation Enhancer E01: 1-73	
Multi cloning site:	74-193
Origin:	1190-1830
Ampicillin resistance gene:	1974-2838

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.

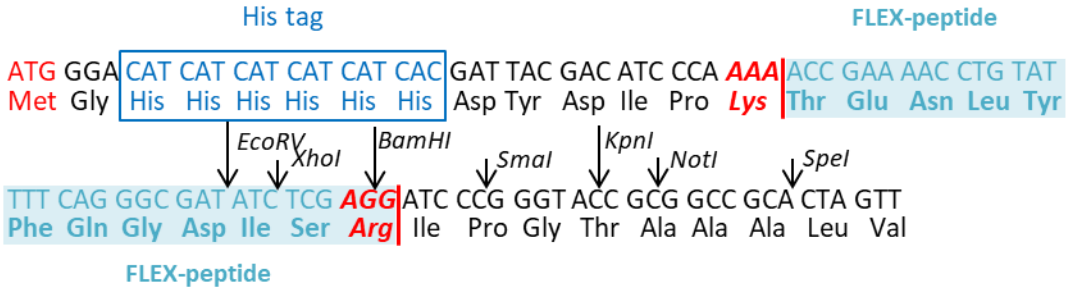
Appendix B: Vector Map for pEU-E01-His-FLEX-MCS-N1

Map:



Multi cloning site:

pEU-E01-His-FLEX-MCS-N1 (3722 bp)



Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine indicated in red.

The vector is derived from vector pEU-E01-His-TEV-MCS-N1, and shares the other vector elements with vector pEU-E01-MCS described in Appendix A.

The TEV site overlaps with the FLEX-tag and can no longer be used for protein digests.

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 C: 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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Others

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