Reconstituted cell-free protein synthesis kit

PURE*frex*® 2.0

PF201-0.25-EX

PF201-0.25-5-EX

Reconstituted cell-free protein synthesis kit

PURE*frex*® 2.1

PF213-0.25-EX

PF213-0.25-5-EX

Instruction Manual

October 2020

Table of Contents

Kit Information

PURE*frex*® *2.0 / 2.1*

Product information

Store the unopened kit at -80ºC.

Product no.	Product name	Volume	Expiration date
PF201-0.25-EX	PURE*frex*® *2.0*	For 250 µL reactions	See the attached document
PF201-0.25-5-EX	PURE*frex*® *2.0*	For 5x 250 µL reactions	See the attached document
PF213-0.25-EX	PURE*frex*® *2.1*	For 250 µL reactions	See the attached document
PF213-0.25-5-EX	PURE*frex*® *2.1*	For 5x 250 µL reactions	See the attached document

Product contents

PURE*frex*® *2.0*

Reagent name	Volume	Description	Storage temperature
Solution I	125 µL	Amino acids, NTPs, tRNAs, enzyme substrates, etc.	≤ -20ºC
Solution II	12.5 µL	Proteins (dissolved in 30% glycerol solution)	≤ -20ºC*
Solution III	25 µL	20 µM Ribosomes	-80ºC*
DHFR DNA	10 µL	Template DNA containing gene encoding *E. coli* dihydrofolate reductase (DHFR) for positive control of protein synthesis (20 ng/µL)	≤ -20ºC

PURE*frex*® *2.1*

Reagent name	Volume	Description	Storage temperature
Solution I	125 µL	Amino acids, NTPs, tRNAs, enzyme substrates, etc.	≤ -20ºC
Solution II	12.5 µL	Proteins (dissolved in 30% glycerol solution)	≤ -20ºC*
Solution III	25 µL	20 µM Ribosomes	-80ºC*
Cysteine	20 µL	10 mM Cysteine	≤ -20ºC
DTT	20 µL	40 mM Dithiothreitol	≤ -20ºC
GSH	20 µL	80 mM Reduced glutathione	≤ -20ºC
DHFR DNA	10 µL	Template DNA containing gene encoding *E. coli* dihydrofolate reductase (DHFR) for positive control of protein synthesis (20 ng/µL)	≤ -20ºC

*For storage at -80ºC: If storing the remaining Solution II and III at -80ºC after use, store after quickly freezing with liquid nitrogen or dry ice/ethanol. Divide into aliquots as necessary to avoid repeated freeze-thaw cycles whenever possible.

Caution about the use of the kit

PURE*frex*® is a kit for research purposes. Administration to human and other animals and use in the clinic, for diagnostic purposes, and other uses are prohibited. Do not use in foods or for domestic purposes. It contains no compound regulated under the relevant laws of Japan (Act on Confirmation of Release Amounts of Specific Chemical Substances in the Environment and Promotion of Improvements to the Management Thereof, Industrial Safety and Health Act, and Poisonous and Deleterious Substances Control Act).

About PURE*frex*®

What is PURE*frex*®?

PURE*frex*® is a product based on the PURE system technology that is a reconstituted cell-free protein synthesis system developed by a group led by Professor Takuya Ueda of the University of Tokyo (1). The kit mainly consists of 3 reagents. Solution I contains amino acids, NTPs, other small-molecule compounds and tRNAs. Solution II contains RNA polymerase, translation factors and other proteins. Finally, Solution III contains purified ribosomes. The purified components are mixed at a concentration to achieve the maximum protein synthesis activity. For the protein synthesis, DNA (or mRNA) encoding the protein of interest is added to the mixture of Solutions I, II, and III and then the reaction mixture is incubated. The preparation methods of the proteins, ribosomes, and tRNAs constituting the reagents of PURE*frex*® are improved over that of the original PURE system (1). The improved preparation method reduce contaminant substances unrelated to the protein synthesis. Lipopolysaccharides from *E. coli*, for example, are reduced to around 0.1 EU per microliter of the reaction mixture. Amounts of RNase, beta-galactosidase and other contaminating proteins are also reduced. All translation factors and other proteins contained in the reagents of PURE*frex*® do not contain any tag sequences. This enables the use of any tag sequence for purification and detection of the synthesized protein.

About the PURE System

The PURE system is a reconstituted cell-free protein synthesis system developed by a group led by Professor Takuya Ueda of the University of Tokyo (1, 2). The system contains 3 initiation factors (IF1, IF2, IF3), 3 elongation factors (EF-Tu, EF-Ts, EF-G), 3 release factors (RF1, RF2, RF3), ribosome recycling factor, 20 aminoacyl-tRNA synthetases, and methionyl-tRNA formyltransferase, that involved in translation in *E. coli*, as well as T7 RNA polymerase and 4 enzyme proteins necessary for transcription and energy regeneration, respectively. These proteins are individually purified and then mixed with amino acids, NTPs, other small-molecule compounds, tRNAs and purified ribosomes. In the PURE system purified factors are only used rather than the cell extracts used in other cell-free protein synthesis systems. This means it is almost free of proteins unrelated to the protein synthesis and allows its composition to be freely adjusted, which are the superior characteristics of the PURE system.

References:

1. Shimizu Y. *et al*. (2001) *Nat. Biotechnol*., vol. 19, p. 751.
2. Shimizu Y. *et al*. (2005) *Methods*, vol. 36, p. 299.

Experiment workflow

General procedures for using PURE*frex*® to synthesize functional proteins are shown in Figure 1.

Start by preparing the template DNA suitable to the protein synthesis with PURE*frex*® and examine whether the protein of interest is synthesized. Redesign the nucleotide sequence of the template DNA if too little protein is synthesized. Add supplement such as molecular chaperones or changing the synthesis conditions if the solubility or activity of the synthesized protein is low or absent. The kit contains enough reagents for examining whether the target protein can be synthesized.

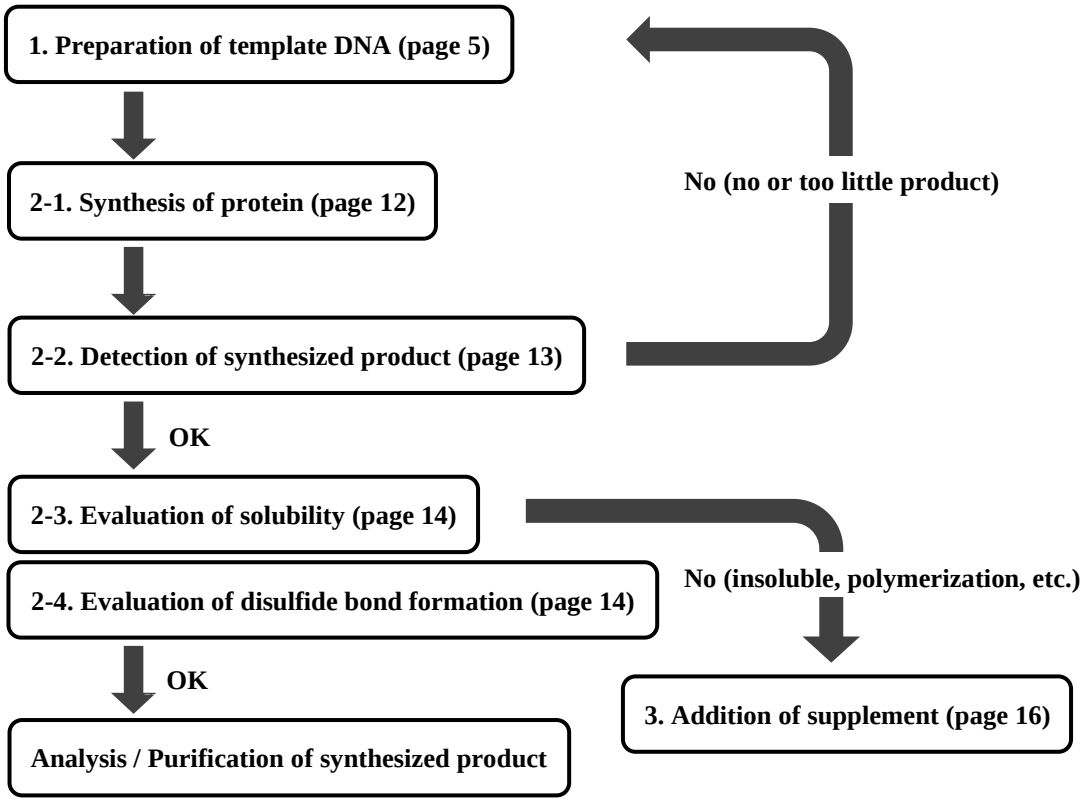

Figure 1. Workflow of protein synthesis with PURE*frex*®

Click on text with a page number to jump to that page in this manual.

1. Preparation of the template DNA

Transcription and translation take place sequentially in the same reaction mixture after template DNA is added to the mixture by combining Solutions I, II, and III of the kit. Preparing the template DNA suited to the protein synthesis with PURE*frex*® is essential. Consider the following information when preparing the template DNA encoding the protein of interest to be synthesized with PURE*frex*®.

About the template DNA for PURE*frex*®

Both circular and linear DNA (including PCR product and circular DNA digested with a restriction enzyme) can be used as the template DNA for PURE*frex*® in case it contains at least the sequence described in the following section. Alternatively, mRNA can be also used to synthesize protein with PURE*frex*®.

About the upstream region of the gene encoding the protein of interest

The template DNA must contain T7 promoter sequence and ribosome binding site (SD sequence) upstream of the gene encoding the protein of interest (Figure 2).

Figure 2. Construct of the template DNA

About stop codon of the gene encoding the protein of interest

All of 3 stop codons (TAA, ochre; TAG, amber; TGA, opal) can be used with PURE*frex*®, because it contains 2 release factors (translation termination factors) recognize all 3 stop codons.

About the downstream region of the gene encoding the protein of interest

When using circular DNA, T7 terminator sequence to terminate transcription must be placed downstream of the gene encoding the protein of interest. When using linear DNA, add at least 10 nucleotides downstream from stop codon. See “Examples of primers used for 2-step PCR” (page 7) for an example of nucleotide sequence to add. T7 terminator sequence is not necessary when linear DNA is used.

Tips for dissolving the template DNA

EDTA contained in TE buffer may reduce the efficiency of protein synthesis because it chelates magnesium ion necessary for transcription and translation reaction. We recommend dissolving the template DNA in EDTA-free buffer or Milli-Q water. Since contaminating RNase digests RNA (transcription products and tRNAs) in the reaction mixture, we recommend using only nuclease-free water, reagents, and instruments and wearing gloves and a mask.

In case using PCR product as the template DNA

PCR product purity

If bands other than that of the desired product are detected in electrophoresis after PCR, modify the PCR condition to reduce byproducts. The purity of the PCR product affect the efficiency of protein synthesis because proteins can be also synthesized from PCR-byproducts.

If byproducts are unable to eliminate by changing the PCR conditions, excise the target band from the gel and purify the product. Excise the band from the gel without using ultraviolet light, which can damage DNA (i.e., impair transcription). Blue light may be used, but exposure time should be kept to a minimum.

How much PCR product to add

The concentration of the template DNA added to the reaction mixture of PURE*frex*® is defined as the number of DNA molecules (molar concentration) and it is added at a final concentration around 2 nM. About 2 nM is approximately 0.5 to 3 ng per 1 kbp for 1 µL of the reaction mixture.

Although unpurified PCR reaction can be directly used with PURE*frex*®, do not add more than 10% (v/v) to the reaction mixture of PURE*frex*®. The efficiency of both transcription and translation reaction may decrease when the salt concentration is altered by carryover from the PCR mixture. If the concentration of the PCR product is low, prepare DNA solution with a sufficient concentration using a DNA purification kit instead of adding more unpurified PCR mixture to the reaction mixture of PURE*frex*®.

Overview of preparing the template DNA by 2-step PCR

Two-step PCR for preparing the template DNA is summarized in Figure 3. Use a high fidelity PCR enzyme (e.g., KOD (Toyobo), PrimeSTAR (TakaraBio), Pfu (Promega)) to prepare the template DNA. At the first step, use FOR primer and REV primer to amplify fragments containing a sequence from the ribosome binding site (RBS (SD sequence)) upstream of the gene encoding the protein of interest to a stop codon and at least 10 nucleotides. Adjust the PCR condition so that even minimal byproducts are produced at 1st PCR. At the second step, use T7PRO-SD primer and REV primer to amplify PCR fragments containing T7 promoter sequence.

FOR, REV, and T7PRO-SD primers are not included in the kit and should be purchased separately.

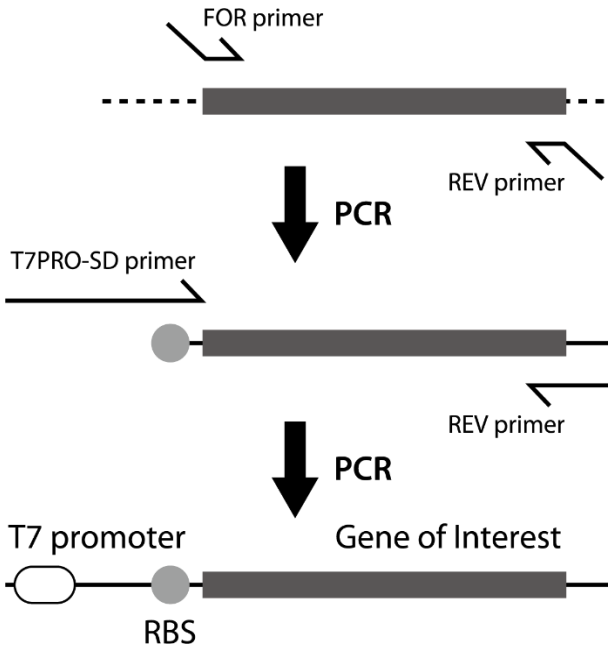

Figure 3. Overview of 2-step PCR

Examples of primers used for 2-step PCR

Primer sequences used for 2-step PCR are shown in Table 1. Also refer to the following site:

https://www.genefrontier.com/solutions/dhfr_dna/

Primer	Sequence
FOR primer	5′-AAGGAGATATACCA-ATG-N(10-20)-3′ RBS
REV primer	5′-GGATTAGTTATTCA-TTA-N(10-20)-3′ Any sequence of at least 10 nt
T7PRO-SD primer	5′-GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCT T7 promoter CTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCA-3′ RBS

Table 1. Primer sequences

In case using plasmid DNA as the template DNA

Usable vectors

Vectors that contain T7 promoter, SD sequence, and T7 terminator can be used such as pET vectors (Merck) and pQE vectors (Qiagen). The presence of the lac operator sequence may decrease the protein yield. We recommend using vectors without the lac operator sequence (e.g., pET17).

Plasmid DNA preparation

When preparing plasmid DNA, make sure that the activity of RNase used for purification is not contaminated in the final purified product. Using a filter-based purification kit such as QIAprep Spin Miniprep Kit (Qiagen) or Wizard Plus SV Minipreps DNA Purification System (Promega), for example, results in contamination of the final purified DNA solution by the RNase A contained in Lysis buffer. If adding this solution without further treatment to the PURE*frex*® reaction mixture as template DNA, transcription products and other RNA in the reaction mixture are digested, inhibiting protein synthesis. Therefore, treat with phenol/chloroform to denature RNase and then re-purify by ethanol precipitation to prepare a DNA solution free of RNase activity. Adding RNase inhibitor to the PURE*frex*® reaction mixture is also effective.

Plasmid DNA purified with Plasmid Mini Kit (Qiagen) has minimal contamination with RNase because the plasmid DNA eluted from resin is precipitated by adding isopropanol. Plasmid DNA purified with this kit are verified to use without any treatment.

How much plasmid DNA to add

The concentration of the template DNA added to the reaction mixture of PURE*frex*® is defined as the number of DNA molecules (molar concentration) and it is added at a final concentration around 2 nM. About 2 nM is approximately 0.5 to 3 ng per 1 kbp for 1 µL of the reaction mixture. A 6 kbp plasmid DNA, for example, is be added at (0.5-3)×6 = 3-18 ng/µL, regardless of the length of the ORF.

In case using RNA as a template

In case using mRNA to synthesize the protein of interest, make sure that the mRNA contains SD sequence upstream of start codon. Add mRNA usually at a concentration of 0.1 to 1 µM to the reaction mixture. Since the optimal concentration depend on the sequence and purity of the mRNA, we recommend examining the optimal concentration in reference to this concentration range.

Tips about the template DNA sequence

Certain nucleotide or amino acid sequence may reduce the yield of the protein of interest. In such cases, optimizing the sequence may improve the yield. Ribosomes, tRNAs, and translation factors in PURE*frex*® are derived from *E. coli*. Therefore, in principle, it is recommended to design the gene encoding the protein of interest with codons optimized for translation in *E. coli*. Consider the following points including the used codons when designing the template DNA sequence for PURE*frex*®.

Codon usage

Nucleotide sequences optimized for *E. coli* by available optimization tool may use single codon frequently used in *E. coli*. Only CTG codon, for example, may be used for leucine. The yield of the synthesized protein may reduce when using such DNA as a template DNA. If the codon usage is excessively imbalanced in the optimized sequence, reassign properly according to the codon usage in the *E. coli* genome.

The codon usage in the *E. coli* genome (W3110) is referred to the following web site:
https://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=316407

AT content just after the start codon

Select codons to maximize AT content in the region immediately after the start codon (usually up to 6th codon) (Figure 4 on page 10). In this region, use AT-rich codons over codons frequently used in *E. coli*.

Secondary structure of mRNA around the start codon

If mRNA can form a rigid secondary structure around the start codon, from SD sequence to the N-terminal of the ORF (about 6-10 codons), and SD sequence is hidden, binding of mRNA to ribosome is impaired and then the yield may be decreased. Substitute N-terminal codons with synonymous codons if a rigid secondary structure to form in this region is predicted.

Example of secondary structure prediction is illustrated in Figure 5 (page 10). In this example, RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) is used to analyze DHFR DNA included as the positive control in the kit. Since the region around the start codon does not form rigid secondary structure, the yield is not reduced.

Amino acid sequence at N-terminus

A low yield may occur if the second and third amino acids right after the first methionine are proline or glycine. Avoid using proline and glycine in this region if possible.

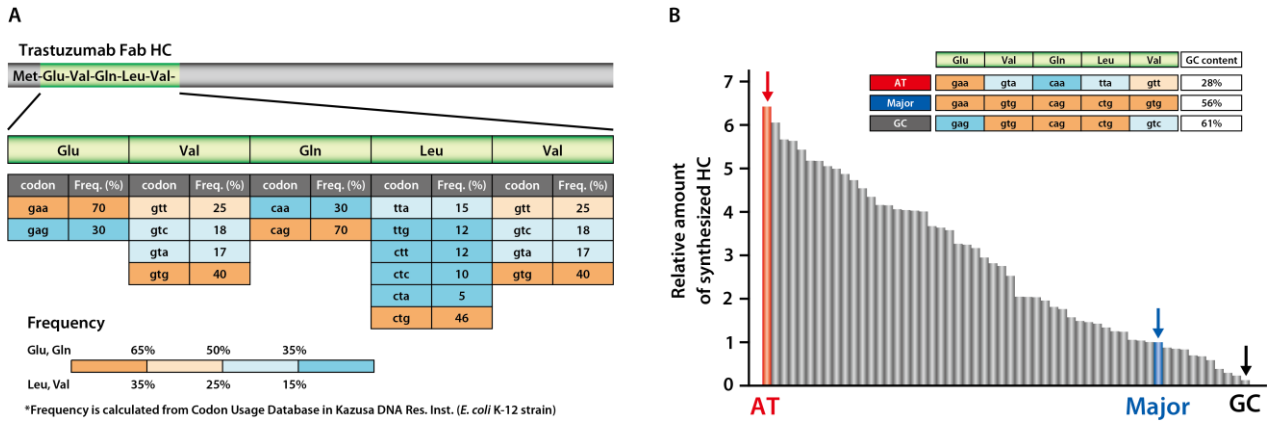

Figure 4. Effects of codons near N-terminal region on protein yield

A. Codons of N-terminal region of heavy chain (HC) of trastuzumab (Herceptin)

B. Comparison of yields from template DNA with different N-terminal codons

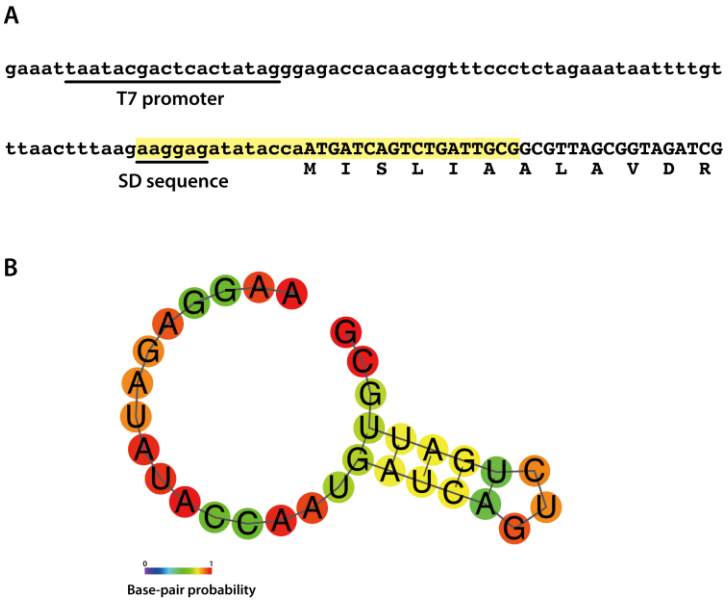

Figure 5. Secondary structure prediction of the translation initiation region

A. Nucleotide sequence of 5'-terminal region of DHFR DNA. The region analyzed is highlighted in yellow.

B. Secondary structure prediction by RNAfold

Sequence that cause frameshift

Nucleotide sequences that are likely to cause frameshift during elongation, such as X/XXY/YYZ, should be replaced with other codons. A nucleotide sequence of “A/AAA/AAA” for 2 consecutive lysine residues, for example, should be replaced with “A/AAG/AAA” to avoid frameshift.

Reference: Sharma V. *et al*. (2014) *Nucleic Acids Res*., vol.42, p.7210.

Sequences containing consecutive proline residues

Proteins with consecutive proline residues may be produced at a low yield. A translation factor called EF-P is known to be involved in elongation at consecutive proline residues in *E. coli*, but the kit (PURE*frex*® *2.0/2.1*) contains no EF-P and may therefore produce a lower-than-normal yield. EF-P is available as a supplement (#PFS052-0.5-EX).

Examples of effect of EF-P on the increase of the yield are shown in the following poster.

https://www.genefrontier.com/files/sites/2/p21_MBSJ2018.pdf

2. Synthesis of Protein using PURE*frex*® *2.0*

Template DNA is added to the reaction mixture prepared by combining Solutions I, II, and III of the kit and then he reaction mixture is usually incubated for 2 to 6 hours at 37ºC.

Contamination of RNase digests the transcription products, tRNA, and other RNA, inhibiting synthesis or substantially reducing the yield. Be sure to use RNase-free tubes and tips and work with gloves.

For the first protein synthesis, we recommend concurrently performing the reaction without template DNA (negative control) and the reaction with DHFR DNA included in the kit (positive control).

2-1 Synthesis of protein

What is needed

PURE*frex*® *2.0* (GeneFrontier)

Template DNA

(containing gene encoding the protein of interest, and sequences necessary for transcription and translation)

Nuclease-free water

37ºC heat block or water bath

Protocol

1. **Warm Solution I at a temperature between room temperature and 37ºC for approximately 1 minute to completely thaw (so that any turbidity is removed) and then place on ice.**
   - If the solution again becomes turbid after being placed on ice, keep at room temperature and use as soon as possible, storing the remaining reagent at a temperature less than -20ºC.
2. **Thaw Solutions II and III on ice.**
3. **Vortex or tap the thawed Solutions I, II, and III to achieve uniform and then centrifuge to collect the contents at the bottom of the tubes.**
4. **Prepare the following reaction mixture to synthesize protein in 20 µL:**

Nuclease-free water	7-X µL	
Solution I	10 µL	
Solution II	1 µL	
Solution III	2 µL	
Template DNA	X µL	(0.5 to 3 ng/µL per 1 kbp)
Total	20 µL	

- Adjust the amount of template DNA added according to the type and concentration of the DNA used.

See “1. Preparation of the template DNA ” (page 5) for more information.

- The volume of reaction mixture can be freely changed. Synthesis is possible with as little as several microliters.

5. **Synthesize the protein of interest by allowing the tube to incubate for 2 to 6 hours on a 37ºC heat block or water bath.**
   - Gas-phase chambers (e.g., incubators) take time to warm the reaction mixture and produce a lower yield.
   - Adjust the temperature and incubation time of synthesis to best suit the protein synthesized.
6. **Detect the synthesized protein with SDS-PAGE.**

2-2 Detection of the synthesized product

Apply the reaction mixture to SDS-PAGE to examine if the protein of interest is synthesized. Compare the lane of the reaction mixture containing the template DNA to the lane of the reaction mixture without template DNA and see if the band is present near the expected molecular weight of the synthesized protein. The maximum yield obtained by the kit is almost the yield of DHFR synthesized from DHFR DNA included in the kit. Redesign the template DNA sequence if the yield of the protein of interest is substantially lower than that of DHFR. Refer to “Tips about the template DNA sequence ” (page 9) when designing the template DNA sequence.

The following method can be used for the detection of the synthesized product with SDS-PAGE.

- Stain the gel with fluorescent dye instead of CBB and then detect the protein bands by fluorescent imager.
- Detect the product by western blotting using an antibody against the protein of interest. Alternatively, synthesize the target protein fused with tag sequence and detect with an antibody against the tag.
- Synthesize the protein in the presence of [^{35}S]methionine and detect radioactive bands in the SDS-PAGE gel.
- Synthesize the protein in the presence of FluoroTect™ Green$_{Lys}$ (Promega) and detect fluorescent bands in the SDS-PAGE gel.

An example of stain with Coomassie Brilliant Blue (CBB) following SDS-PAGE to detect the product is shown as below. Change the reaction mixture volume and gel concentration for SDS-PAGE according to the molecular weight and yield of the protein of interest, the gel staining method, and other factors.

What is needed (in case of staining with CBB)

PURE*frex*® reaction mixture after reaction

3x Sample Buffer for SDS-PAGE (see Appendix for composition on page 33)

Water

95ºC heat block or water bath

SDS-PAGE gel

Molecular weight marker

CBB stain

Protocol

1. **Add <u>equal volume</u> of water to the reaction mixture after protein synthesis.**
   - Salt concentration of the reaction mixture is relatively high. Add 3x Sample Buffer after diluting with water or buffer because addition of 3x Sample Buffer to the undiluted reaction mixture and heating may cause a white precipitate to form.
2. **Add <u>equal volume</u> of 3x Sample Buffer to the diluted reaction mixture.**
3. **Heat for 5 minutes at 95ºC.**
4. **Apply 3 µL of the sample per lane and molecular weight marker to the SDS-PAGE gel and run electrophoresis.**
   - This is equivalent to 1 µL of reaction mixture per lane.
   - A 10-20% gradient gel is recommended for analyses over a wide range of molecular weight.
5. **After electrophoresis, stain the gel with CBB and detect the band of the protein of interest.**
   - The band of the positive control (DHFR) is around 18 kDa.

2-3 Evaluation of solubility

Centrifuge the PURE*frex*® reaction mixture containing the synthesized protein to separate it into supernatant and pellet fractions. Evaluate the solubility of the synthesized product by detecting the product in the supernatant with SDS-PAGE.

What is needed

PURE*frex*® reaction mixture after reaction

High-speed refrigerated microcentrifuge

3x Sample Buffer for SDS-PAGE (see Appendix for composition on page 33)

Water

95ºC heat block or water bath

SDS-PAGE gel

Molecular weight marker

Protocol

1. **Add <u>equal volume</u> of water to the reaction mixture after protein synthesis.**

2. **Withdraw an aliquot from the diluted reaction mixture. → "Total"**
3. **Centrifuge at 20,000 x*g* for 30 minutes at 4ºC.**
4. **Withdraw an aliquot from the supernatant. → "Sup"**
5. **Analyze the Total and Sup samples with SDS-PAGE.**

2-4 Evaluation of disulfide bond formation

Evaluate disulfide bond formation by analyzing the synthesized protein with SDS-PAGE under non-reducing condition. Proteins with disulfide bonds migrate faster under non-reducing condition than under reducing condition. The product with disulfide bond may also be stuck in the gel or detected as a smear band under non-reducing condition. Confirm that disulfide bonds are correctly formed by assaying the activity of the synthesized product.

What is needed

PURE*frex*® reaction mixture after reaction

3x Sample Buffer for SDS-PAGE (see Appendix for composition on page 33 **without** reducing agent)

Water

95ºC heat block or water bath

SDS-PAGE gel

Molecular weight marker

Protocol

1. **Add equal volume of water to the reaction mixture after protein synthesis.**
2. **Add equal volume of 3x Sample Buffer (without reducing agent) to the diluted reaction mixture.**
3. **Heat for 5 minutes at 95ºC.**
4. **Perform SDS-PAGE and analyze the bands of the synthesized product.**

3. Addition of supplement

If the protein of interest aggregates or contains disulfide bond, we recommend addition of molecular chaperones or additive to promote disulfide bond formation to PURE*frex*® reaction mixture. See “Other Related Products and Ordering Procedures” (page 34) for information on related products.

Examples of Protein Synthesis with PURE*frex*®

Examples of the synthesis of various proteins are also shown in the following web site:

https://www.genefrontier.com/en/cases/purefrex/

Posters presented by GeneFrontier at conferences can be downloaded below:

https://www.genefrontier.com/en/downloads/purefrex-poster/

1. Synthesis of DHFR

In this section, the preparation of template DNA for DHFR (the positive control in the kit) by PCR and synthesis of DHFR with PURE*frex*® *2.0* are described.

What was used

DNA containing ORF of DHFR

Primer (DHFR-F, DHFR-R3, T7PRO-SD Primer)

Water

PCR reagents (e.g., KOD-Plus-Neo (Toyobo, Japan))

Thermal cycler

PCR product purification kit (e.g., NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel))

6x Sample Buffer for agarose gel electrophoresis

Agarose gel (1%)

DNA size marker

UV gel imager

PURE*frex*® *2.0* (#PF201-0.25-EX)

Nuclease-free water

Heat block or water bath (37°C and 95°C)

3x Sample Buffer for SDS-PAGE (see Appendix for composition on page 33)

SDS-PAGE gel (10-20%)

Molecular weight marker

CBB stain

- PCR enzymes and DNA purification kits from other manufacturers can be used. Be sure, however, that PCR enzymes with high fidelity is used.

DHFR gene and primer sequences

DHFR gene

ATGATCAGTCTGATTGCGGCGTTAGCGGTAGATCGCGTTATCGGCATGGAAAACGCCATGCCGTGGAACCTGCCTGC
CGATCTCGCCTGGTTTAAACGCAACACCTTAAATAAACCCGTGATTATGGGCCGCCATACCTGGGAATCAATCGGTC
GTCCGTTGCCAGGACGCAAAAATATTATCCTCAGCAGTCAACCGGGTACGGACGATCGCGTAACGTGGGTGAAGTCG
GTGGATGAAGCCATCGCGGCGTGTGGTGACGTACCAGAAATCATGGTGATTGGCGGCGGTCGCGTTTATGAACAGTT
CTTGCCAAAAGCGCAAAAACTGTATCTGACGCATATCGACGCAGAAGTGGAAGGCGACACCCATTTCCCGGATTACG
AGCCGGATGACTGGGAATCGGTATTCAGCGAATTCCACGATGCTGATGCGCAGAACTCTCACAGCTATTGCTTTGAG
ATTCTGGAGCGGCGGTAA

Primer

FOR Primer（DHFR-F）

AAGGAGATATACCAATGATCAGTCTGATTG

REV Primer（DHFR-R3）

GGATTAGTTATTCATTACCGCCGCTCCAGAAT

T7PRO-SD Primer

GAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAG
ATATACCA

Preparation of template DNA by 2-step PCR

1. **The DHFR gene DNA and primer were diluted to 1 ng/μL and 2 μM, respectively, with water.**
2. **The PCR reagents other than the enzymes were thawed at room temperature.**
3. **The PCR reaction mixture was prepared as follows:**

water	3.5 μL
10x buffer	1.0 μL
2 mM dNTPs	1.0 μL
25 mM $MgSO_4$	0.6 μL
2 μM FOR Primer (DHFR-F)	1.5 μL
2 μM REV Primer (DHFR-R3)	1.5 μL
DMSO	0.2 μL
Enzyme (KOD-Plus- Neo)	0.2 μL
1 ng/μL DHFR gene DNA	0.5 μL
Total	10.0 μL

4. **PCR was performed as below: (Step 1)**

94ºC	2 min.	
94ºC	15 sec.	x 30 cycles
58ºC	30 sec.	
68ºC	1 min.	
68ºC	2 min.	
10ºC		

5. **The PCR reaction mixture (1 µL) was analyzed with 1% agarose gel electrophoresis to confirm the presence of the amplification products.**
6. **The PCR products were purified with a PCR product purification kit (NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel)). The bound DNA was eluted from the column with 20 µL of water.**
7. **The PCR product purified in step 6 (PCR-1 Product) was diluted 50-fold with water.**
8. **The PCR reaction mixture was prepared as follows:**

water	3.5 µL
10x buffer	1.0 µL
2 mM dNTPs	1.0 µL
25 mM $MgSO_4$	0.6 µL
2 µM FOR Primer (T7PRO-SD)	1.5 µL
2 µM REV Primer (DHFR-R3)	1.5 µL
DMSO	0.2 µL
Enzyme (KOD-Plus- Neo)	0.2 µL
50-fold diluted PCR-1 product	0.5 µL
Total	10.0 µL

9. **PCR was performed under the same condition used in step 4.**
10. **The PCR reaction mixture (1 µL) was analyzed with 1% agarose gel electrophoresis to confirm the presence of the amplification products.**
11. **The PCR products were purified with a PCR product purification kit (NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel)). The bound DNA was eluted from the column with 20 µL of water.**
12. **Absorbance at 260 nm was measured and the concentration of the purified PCR product was calculated.**
13. **The resulting DNA solution was adjusted to a concentration of 20 ng/µL.**

Result of 2-step PCR

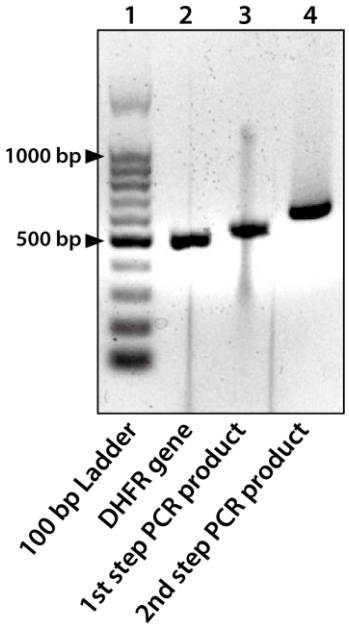

Figure 6. Preparation of the template DNA (DHFR DNA) by 2-step PCR

Synthesis with PURE*frex*® and detection of synthesized DHFR

1. **Solution I was warmed at room temperature for about 1 minute to thaw completely.**
2. **Solutions II and III were thawed on ice.**
3. **The thawed Solutions I, II, and III were vortexed to achieve uniform and then centrifuged to collect the contents at the bottom of the tubes.**
4. **The reaction mixture was prepared as follows:**

	(+) DNA	(-) DNA
Nuclease-free water	3.0 µL	3.5 µL
Solution I	5.0 µL	5.0 µL
Solution II	0.5 µL	0.5 µL
Solution III	1.0 µL	1.0 µL
DHFR DNA (20 ng/µL)	0.5 µL	0 µL
Total	10.0 µL	10.0 µL

5. **The protein was synthesized by incubating the tubes for 2 hours on a 37ºC heat block.**

6. **After incubation, 10 µL of water and 10 µL of 3x Sample Buffer were added to the reaction mixture.**
7. **The samples were heated for 5 minutes at 95ºC.**
8. **Three µL (equivalent to 1 µL of the reaction mixture) of samples were applied to 10-20% gradient gel, and electrophoresis was performed.**
9. **After electrophoresis, the gel was stained with CBB to detect the synthesized product.**

Result of synthesis of DHFR

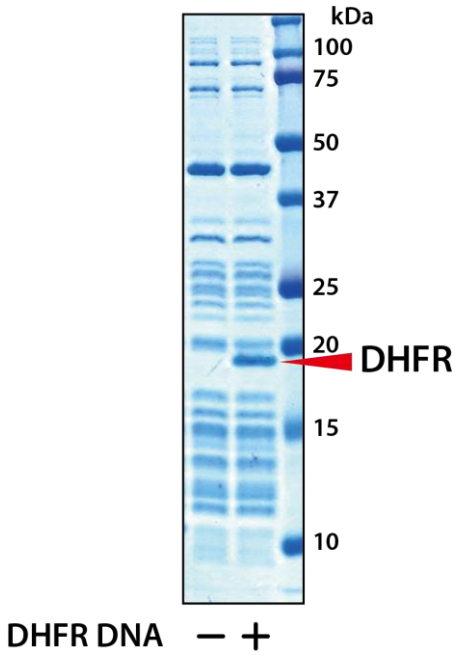

Figure 7. Synthesis of DHFR with PURE*frex*

2. Purification of His-tagged protein (DHFR-6xHis)

Since all of proteins involved in protein synthesis in PURE*frex*® reaction mixture are free of His-tag, the protein of interest can be synthesized as His-tagged protein and purified with metal affinity resin. An example of purification of His-tagged DHFR with a nickel affinity resin is shown below.

What was used

PURE*frex*® *2.0* (#PF201-0.25-EX)

Template DNA (containing DHFR-6xHis gene)

Nuclease-free water

Heat block or water bath (37°C and 95°C)

Ni-Sepharose 6 FF (GE Healthcare)

Binding Buffer: 50 mM Tris-HCl pH 8, 500 mM NaCl, 20 mM imidazole, 20 mM $Mg(OAc)_2$

Wash Buffer: 50 mM Tris-HCl pH 8, 500 mM NaCl, 20 mM imidazole

Elution Buffer: 50 mM Tris-HCl pH 8, 500 mM NaCl, 100 mM imidazole

High-speed refrigerated microcentrifuge

3x Sample Buffer for SDS-PAGE (see Appendix for composition on page 33)

SDS-PAGE gel (10-20%)

Molecular weight marker

CBB stain

Synthesis with PURE*frex*® and purification of synthesized DHFR-6xHis

1. **The reaction mixture was prepared as in "1. Synthesis of DHFR" (page 20) (volume: 25 µL).**
2. **The reaction mixture was incubated for 4 hours at 37ºC.**
3. **Five µL was withdrawn from the reaction mixture. (→ "Syn" in Figure 8)**
4. **The remaining reaction mixture (20 µL) was diluted with 80 µL of Binding Buffer.**
   - Diluting with buffer without magnesium ion may destabilize ribosomes, introducing ribosomal proteins into the eluted fraction. When purifying the synthesis product, dilute the reaction mixture with buffer containing magnesium ion.
5. **Ten µL of Ni-Sepharose 6 FF equilibrated with Binding Buffer was added to the diluted reaction mixture, and the resulting mixture was incubated for 1 hour at 4ºC.**
   - His-tagged proteins synthesized with PURE*frex*® tend to bind poorly to nickel affinity resin. Be sure to use more resin than recommended.

6. **The suspension was centrifuged briefly (about 5 seconds) to pellet the resin. The supernatant was isolated. (→ FT in Figure 8)**
7. **The resin was washed twice with 50 µL of Binding Buffer. (→ W1, W2 in Figure 8)**
8. **The resin was washed 3 times with 50 µL of Wash Buffer. (→ W3, W4, W5 in Figure 8)**
9. **Fifty µL of Elution Buffer was added to the resin to elute bound DHFR-6xHis. (→ E1, E2, E3 in Figure 8)**
10. **SDS-PAGE was performed with 10-20% gradient gel.**
11. **After electrophoresis, the gel was stained with CBB to detect the synthesized product.**

Result of purification of DHFR-6xHis

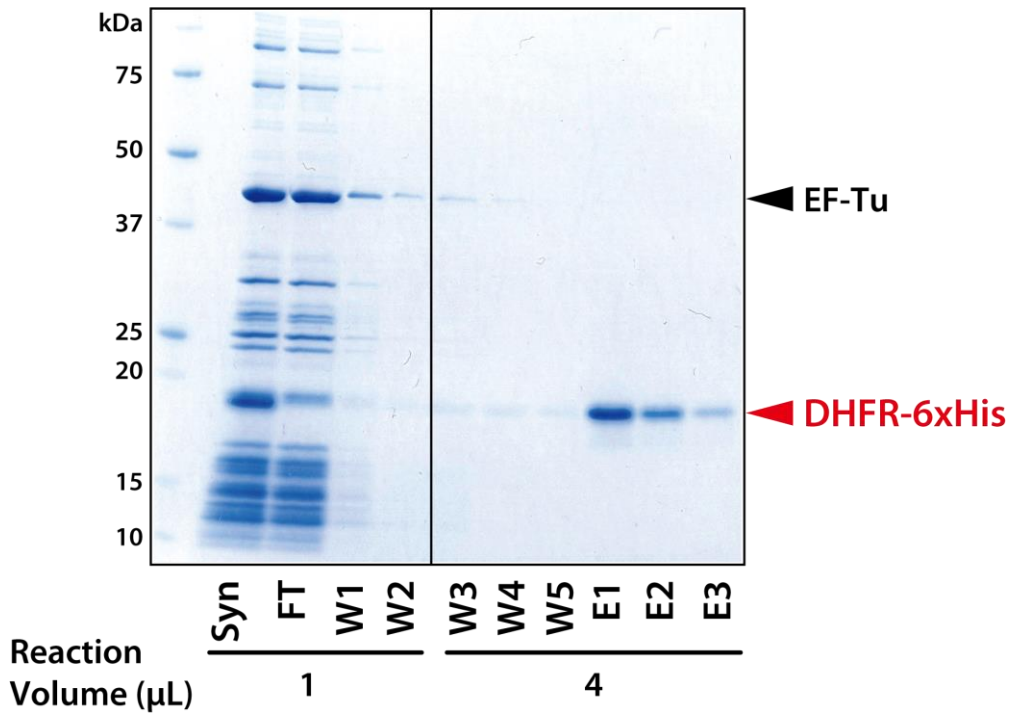

Figure 8. Simple purification of DHFR-6xHis synthesized with PURE*frex*®

An equivalent of 1 µL of the reaction mixture (Syn/FT/W1/W2) or 4 µL of the reaction mixture (W3-5/E1-3) was applied to SDS-PAGE.

3. Detection of the synthesized product with FluoroTect™ $Green_{Lys}$ (DHFR)

FluoroTect™ $Green_{Lys}$ (Promega) is a reagent that introduce fluorescently (BODIPY®) labeled lysine at lysine residues in the protein of interest. After SDS-PAGE, the synthesis of the target protein can be easily confirmed by detecting the fluorescently labeled band using a fluorescent imager.

What was used

DHFR DNA (positive control included in the kit)

PURE*frex*® *2.0* (#PF20-0.25-EX)

Nuclease-free water

37ºC heat block or water bath

FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System (Promega)

1 mg/mL RNase A

SDS-PAGE gel (10-20%)

3x Sample Buffer for SDS-PAGE (see Appendix for composition on page 33)

Molecular weight marker

Fluorescent gel imager (LAS)

Oriole™ stain (Bio-Rad)

Protein Synthesis in the presence of FluoroTect™ $Green_{Lys}$

1. **The reaction mixture was prepared as in "1. Synthesis of DHFR" (page 20). At the end, 0.5 µL of FluoroTect™ $Green_{Lys}$ was added per 10 µL of the reaction mixture.**
2. **The reaction mixture was incubated for 1 hour at 37ºC.**
3. **One µL of 1 mg/mL RNase A was added to the reaction mixture. The reaction mixture was additionally incubated for 15 minutes at 37ºC to digest unreacted FluoroTect™ $Green_{Lys}$.**
4. **Equal volume of water and 3x Sample Buffer was added to the reaction mixture, which was heated for 5 minutes at 95ºC.**
5. **Three µL (equivalent to 1 µL of the reaction mixture) of each sample was applied to SDS-PAGE gel, and electrophoresis was performed.**
6. **After electrophoresis, the band of the fluorescently labeled DHFR protein was detected by fluorescent gel imager.**
7. **The gel was stained with Oriole™ to detect all proteins in the reaction mixture.**

Result of synthesis in the presence of FluoroTect

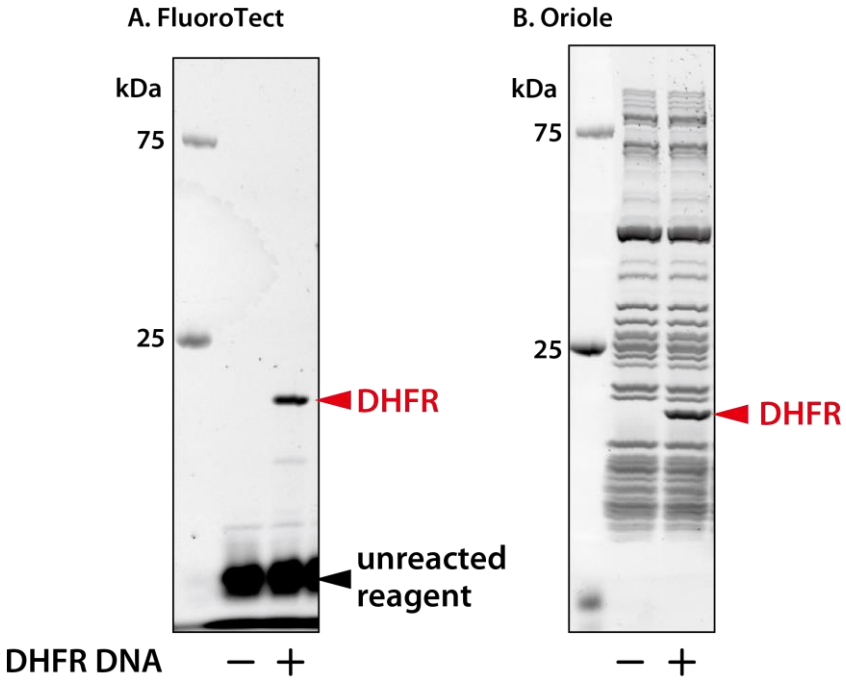

Figure 9. Synthesis of DHFR in the presence of FluoroTect™ Green$_{Lys}$

A. Detection of the fluorescence of FluoroTect™ Green$_{Lys}$ (derived from BODIPY®)

B. Staining of all proteins in the reaction mixture with Oriole™

4. Synthesis in the presence of molecular chaperones (luciferase)

Many proteins require molecular chaperones to achieve their proper conformation after synthesis. Since PURE*frex*® contains no molecular chaperones, add molecular chaperones to the reaction mixtures if the synthesized protein aggregate. We provide DnaK Mix (#PF003-0.5-EX) and GroE Mix (#PF004-0.5-EX) as supplement for PURE*frex*®. DnaK Mix is a mixture containing *E. coli* Hsp70 DnaK, and DnaJ and GrpE which help the function of DnaK. GroE Mix is a mixture containing *E. coli* Hsp60 GroEL and the helper protein GroES. Other molecular chaperones can be also used. Select the appropriate molecular chaperones because they depends on the synthesized protein.

Example of protein synthesis in the presence of DnaK Mix is shown below. Luciferase is an aggregate-prone protein and is tend to lose activity at 37ºC. When luciferase was synthesized in the presence of DnaK Mix, the activity was higher, indicating that addition of DnaK Mix had an effect on the activity of luciferase synthesized with PURE*frex*®.

What was used

Template DNA (Luciferase DNA)

PURE*frex*® *2.0* (#PF201-0.25-EX)

DnaK Mix (#PF003-0.5-EX)

Nuclease-free water

30ºC heat block or water bath

Luciferase Assay System (Promega)

Luminometer

Synthesis of luciferase in the presence of DnaK Mix and evaluation of its activity

1. **The reaction mixture was prepared as below.**

	(-) DnaK Mix	(+) DnaK Mix
Nuclease-free water	3.0 µL	2.5 µL
Solution I	5.0 µL	5.0 µL
Solution II	0.5 µL	0.5 µL
Solution III	1.0 µL	1.0 µL
DnaK Mix	0 µL	0.5 µL
Luciferase DNA (40 ng/µL)	0.5 µL	0.5 µL
Total	10.0 µL	10.0 µL

2. **The reaction mixture was incubated for 4 hours on 30ºC heat block.**
3. **One µL of the reaction mixture was diluted with 29 µL of water.**
4. **One µL of the diluted reaction mixture was added to 20 µL of Lumino-assay solution and mix by vortex.**

5. **Luminescence was measured by luminometer.**

Result of synthesis in the presence of DnaK Mix

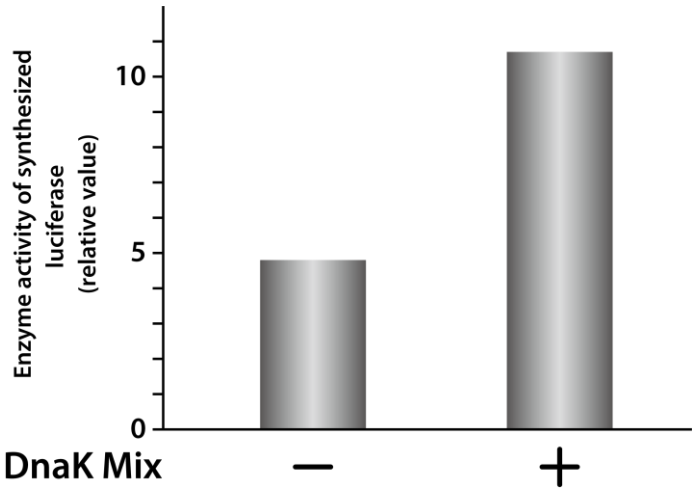

Figure 10. Effect of DnaK Mix on the activity of the synthesized luciferase

5. Synthesis of a protein with disulfide bonds (alkaline phosphatase)

PURE*frex*® *2.0* contains dithiothreitol (DTT) as a reducing agent, which inhibits formation of a disulfide bond. In contrast, PURE*frex*® *2.1* does not contain DTT and an appropriate reducing agent can be selected. DTT and reduced glutathione (GSH) solution are included in PURE*frex*® *2.1* kit. If the protein of interest requires disulfide bond formation, synthesize it with addition of DsbC Set (formerly DS supplement) (#PF005-0.5-EX) or PDI Set (#PF006-0.5-EX). DsbC Set includes oxidized glutathione (GSSG) and *E. coli* disulfide isomerase DsbC. PDI Set includes GSSG, human protein disulfide isomerase (PDI) and human ER oxidoreductin-1 (Ero1alpha).

An example of the synthesis of *E. coli* alkaline phosphatase (AP) with PURE*frex*® *2.1* is shown as below. AP has 2 disulfide bonds required for its activity. When AP was synthesized in the presence of GSSG, the synthesized product migrated in SDS-PAGE under non-reducing condition faster than reducing condition, allowing disulfide bond formation to be predicted. When GSH was used as a reducing agent, the migration rate was altered even when GSSG was not added. AP activity was detected when the migration rate of the synthesized product was altered in non-reducing SDS-PAGE. This indicates that AP with activity was synthesized.

What was used

PURE*frex*® *2.1*(#PF213-0.25-EX)

DsbC Set (formerly DS supplement) (#PF005-0.5-EX)

Template DNA (AP DNA)

Nuclease-free water

Heat block or water bath (37°C and 95°C)

3x Sample Buffer for SDS-PAGE (see Appendix for composition on page 33 (with and without a reducing agent))

SDS-PAGE gel (10-20%)

Molecular weight marker

Oriole™ stain (Bio-Rad)

Fluorescent gel imager (LAS)

Phosphatase Substrate Kit (Thermo Scientific)

96-well plate

Plate reader

Synthesis of AP

1. **The reaction mixture was prepared with PURE*frex*® *2.1* as follows: All volumes are in microliters.**

Sample no.	1	2	3	4	5	6
Nuclease-free water	2.5	1.5	1.5	2.5	1.5	1.5
Solution I	4.0	4.0	4.0	4.0	4.0	4.0
3 mM Cysteine	1.0	1.0	1.0	1.0	1.0	1.0
40 mM DTT	0.5	0.5	0.5	0	0	0
80 mM GSH	0	0	0	0.5	0.5	0.5
20 mM GSSG	0	1.0	0	0	1.0	0
40 mM GSSG	0	0	1.0	0	0	1
Solution II	0.5	0.5	0.5	0.5	0.5	0.5
Solution III	1.0	1.0	1.0	1.0	1.0	1.0
AP DNA (20 ng/μL)	0.5	0.5	0.5	0.5	0.5	0.5
Total	10.0	10.0	10.0	10.0	10.0	10.0

2. **The reaction mixture was incubated for 4 hours on 37ºC heat block.**
3. **Two μL of the reaction mixture was mixed with 10 μL of water and 6 μL of 3x Sample Buffer with reducing agent or without reducing agent.**
4. **The sample was heated for 5 minutes at 95ºC.**
5. **4.5 μL (equivalent to 0.5 μL of the reaction mixture) was applied to 10-20% gradient gel, and electrophoresis was performed.**
6. **After electrophoresis, the gel was stained with Oriole™ to detect the synthesized product.**

Measurement of activity of synthesized AP

1. **The reaction mixture containing the synthesized product was diluted 20-fold with water.**
2. **Assay solution containing PNPP was applied to a 96-well plate at 100 μL per well. The plate was warmed at 37ºC.**
3. **Two μL of the diluted reaction mixture was added to the assay solution in well.**
4. **The time course of absorbance change at 405 nm was measured at 37ºC.**
5. **AP activity was calculated based on the slope of the absorbance change.**

Result of AP synthesis

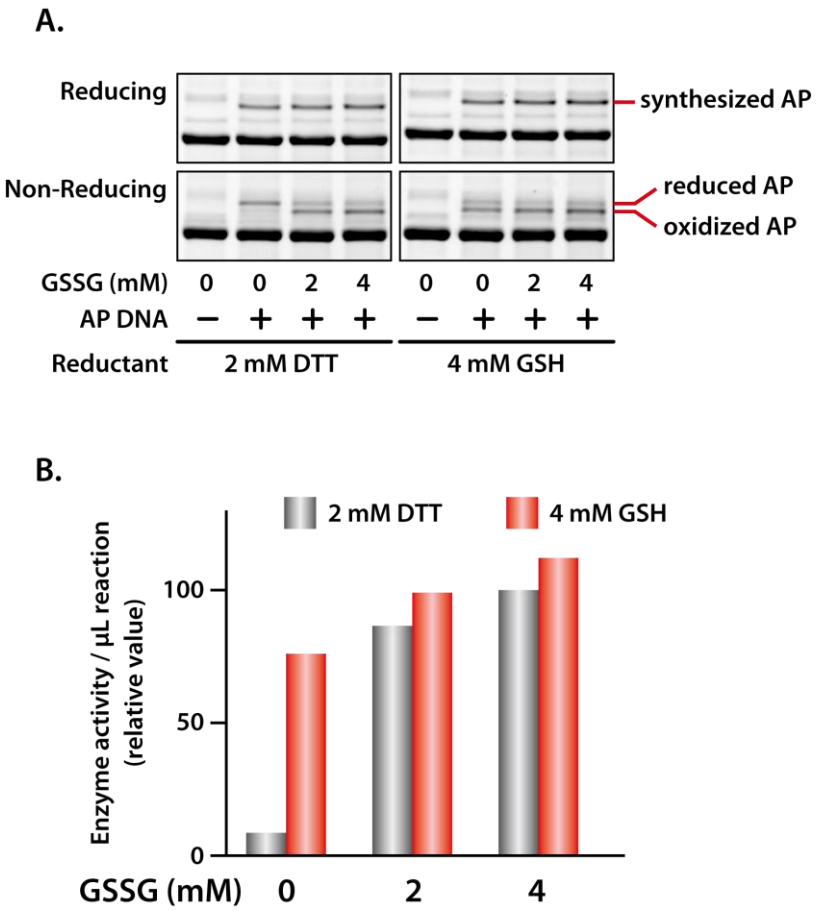

Figure 11. AP synthesis

A. SDS-PAGE under reducing and non-reducing conditions

B. Relative activity of synthesized AP. The activity of AP synthesized in the presence of 2 mM DTT and 4 mM GSSG is set to 100.

Troubleshooting

The positive control (DHFR) is not synthesized.

• Incubate the PURE*frex*® reaction mixture by directly heating the reaction tube with heat block or water bath. Gas-phase chamber (e.g., incubator) takes time to warm the reaction mixture and the yield is reduced.

• Some of the components of the kit may lose their activity. Store the kit at a proper temperature to prevent loss of activity. Divide the reagents into aliquots to avoid the repeated freeze-thaw cycles whenever possible.

The protein of interest is not synthesized.

The template DNA does not contain the minimum required sequences.

• Template DNA for PURE*frex*® must contain T7 promoter, ribosome binding site (SD sequence), start codon, and stop codon.

Too much or little DNA is added to the reaction mixture.

• Regardless of plasmid DNA or PCR product, the template DNA should be added at a concentration of 0.5 to 3 ng per 1 kbp for 1 µL of the reaction mixture. Adding too much may reduce the protein yield.

• The concentration of the template DNA added to the reaction mixture of PURExfrex® is defined as the number of DNA molecules (molar concentration) and it is added at a final concentration around 2 nM. About 2 nM is approximately 0.5 to 3 ng per 1 kbp for 1 µL of the reaction mixture. A 6 kbp plasmid DNA, for example, would be added at (0.5-3)×6 = 3-18 ng/µL, regardless of the length of the ORF.

The method for preparing the template DNA is inappropriate.

• See the tips described in “1. Preparation of the template DNA ” (page 5).

The template DNA contains a difficult-to-translate sequence.

• Confirm that the template DNA does not contain the sequences described in “Tips about the template DNA sequence ” (page 9).

The synthesized protein is insoluble.

Add molecular chaperones when synthesizing the protein.

• PURE*frex*® contains no molecular chaperones. When the protein of interest is synthesized in the presence of molecular chaperones, it may become soluble. Additionally, better results may be obtained with PURE*frex*® *1.0*, in which the yield of the product is lower than PURE*frex*® *2.0*. See "Other Related Products and Ordering Procedures" (page 34) about molecular chaperones and PURE*frex*® *1.0*.

Synthesize the protein at a lower translation speed.

• Proteins generally fold more slowly than they are translated. Reducing the translation speed by lowering the reaction temperature from 37ºC to 30ºC or 25ºC may increase the proportion of the target protein that is soluble. This will reduce the yield, so be sure to select conditions to efficiently produce the soluble protein.

The synthesized protein has no activity.

• The synthesized protein may be insoluble. Check its solubility.

• Factors required for proper activity (e.g., coenzymes, metal ions) may be missing. Since PURE*frex*® is a reconstituted system, it contains no small molecules not related to transcription and translation. Add the factors required for activity.

• If the protein of interest requires correct disulfide bond formation, synthesize it in the presence of an additive to promote this formation (e.g. GSSG, DsbC). In that case, use PURE*frex*® *2.1* in which reducing agent can be selected, because the efficiency of disulfide formation is affected by reducing agent.

The reaction mixture becomes turbid when sample buffer for SDS-PAGE is added.

• The PURE*frex*® reaction mixture has a relatively high salt concentration and may become turbid when heated after sample buffer containing SDS is directly added. To avoid turbidity, dilute the reaction mixture with at least equal volume of water before adding sample buffer. If this does not resolve the turbidity issue, heat the reaction mixture at a lower temperature (e.g., 37ºC) for a longer time (about 1 hour).

Appendix

DHFR DNA sequence information

The sequence is available in text format here:

https://www.genefrontier.com/en/solutions/sequence-of-dhfr-dna/

Compositions of SDS-PAGE sample buffer

3x Sample buffer (for 10 mL)

1 M Tris-HCl pH6.8	1.5 mL
Glycerol	3 mL
SDS	0.6 g
2-Mercaptoethanol	0.6 mL
Bromophenol Blue	As needed

(Bring to a volume of 10 mL with H_2O)

Publications

Publications on PURE*frex*® and PURE system are available here:

https://www.genefrontier.com/en/publications/purefrex/

Related Products and Ordering information

Kit for protein synthesis

Product no.	Product name	Volume	Price (without tax)
PF001-0. 25-EX	PURE*frex*® *1.0*	For 250 µL reactions	15,000 yen
PF001-0.25-5-EX	PURE*frex*® *1.0*	For 5x 250 µL reactions	67,500 yen
PF201-0.25-EX	PURE*frex*® *2.0*	For 250 µL reactions	24,000 yen
PF201-0.25-5-EX	PURE*frex*® *2.0*	For 5x 250 µL reactions	108,000 yen
PF213-0.25-EX	PURE*frex*® *2.1*	For 250 µL reactions	24,000 yen
PF213-0.25-5-EX	PURE*frex*® *2.1*	For 5x 250 µL reactions	108,000 yen

Supplement for protein synthesis

Product no.	Product name	Volume	Price (without tax)
PF003-0.5-EX	DnaK Mix	For 500 µL reactions	18,000 yen
PF004-0.5-EX	GroE Mix	For 500 µL reactions	18,000 yen
PF005-0.5-EX	DsbC Set	For 500 µL reactions	10,000 yen
PF006-0.5-EX	PDI Set	For 500 µL reactions	10,000 yen
PFS052-0.5-EX	EF-P	For 500 µL reactions	5,000 yen

Order support

Please contact here for requesting a quote and ordering information.

Cosmo Bio USA; https://www.cosmobiousa.com

Technical support

Please contact us for a technical information about PURE*frex*®.

https://www.genefrontier.com/en/contact/purefrex/

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