ProteinExpress

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Remarkable Yield Translation System Kit 【RYTS Kit】

Instruction Manual

(Version 1.2)

For research purposes only.

Before using this product, please read carefully this instruction manual.

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1. Product overview

The Remarkable Yield Translation System (RYTS) Kit is a cell-free protein synthesis system. This kit includes an *E.coli* extract and all essential components for coupled transcription/translation reaction.

The RYTS Kit contains the *E.coli* Lysate, which is prepared according to unique method developed for highly efficient production of extensive protein by RIKEN (1-4). Protein*Express* Co., Ltd. has been licensed to distribute this method by RIKEN under JP patent No. 4477923.

2. Product features

Synthesis of recombinant protein using RYTS Kit is highly efficient, convenient and quick. Maximum yield of protein can reach 150 μg per the 300 μl reaction.

Furthermore, this kit is available for the expression of protein with unnatural amino acid by exploitation of Clover*Direct* ™ tRNA Reagents for Site-Directed Protein Functionalization. Clover*Direct*™ allows the incorporation of unnatural amino acids at defined positions of proteins using *in vitro* translation (see page 8 for details). If you need more information on Clover*Direct*™, please visit our web site. (http://www.proteinexpress.co.jp/e/products/index.html).

3. Kit contents

No.	cap color	Contents	Q'ty	Storage
1	white	*E.coli* Lysate	5 tubes	−80 °C
2	pink	2× Reaction Mix	5 tubes	
3	blue	Methionine	1 tube	
4	orange	Enzyme Mix	1 tube	−20 °C
5	green	CAT Control Vector 250ng/μl	1 tube	
6	clear	Nuclease Free Water	1 tube	

Storage condition

Store at appropriate temperature indicated in the Kit contents. Avoid multiple freeze-thawing cycles and vortex, which may cause for decrease of protein productivity.

4. Before using this kit

4.1 Template considerations

It is able to use circular DNA, linear DNA and mRNA for the protein synthesis using RYTS Kit. Use a DNA template and mRNA with high purity (Abs260 / Abs280 > 1.8).

4.2 Requirement of nuclease free environment

To eliminate nuclease contamination, you should wear gloves when preparing reaction mixture, reaction tubes and pipette tips should be nuclease-free. Avoid a template DNA or mRNA solution from contamination of nucleases.

5. Construction and preparation of expression template

5.1 DNA template

The DNA template (circular DNA or linear DNA) for protein synthesis should be contained that a protein-coding sequence be under the control of a T7 promoter and located downstream of a ribosomal binding site (RBS) sequence. Additionally, T7 terminator sequence is located at downstream of the coding sequence. Distance between T7 promoter and start ATG should not exceed 100 base pairs, and also distance between the RBS sequence and start ATG should not be more than 5-8 base pairs. Please refer to Figure 1 for an example of the DNA template.

Figure 1. An example of template

In case of very low protein yield, optimization of nucleotide sequence (codon usage, addition of N-terminal tags, etc.) is required to improve protein productivity. We recommend the use of ProX tag, which is original N-terminal peptide tag developed for efficient expression in *E.coli* cell-free protein synthesis system. ProX tag sequence is indicated at Figure 2.

(A)

5' -	AUG	UCU	AAA	CAA	AUC	GAA	GUA	AAC	UUU	UCU	AAU	GAG	- 3'
	Met	Ser	Lys	Gln	Ile	Glu	Val	Asn	Phe	Ser	Asn	Glu	

(B)

Figure 2. (A) ProX tag sequence, and (B) an example of template

5.2 mRNA template

Messenger RNA, which is generally synthesized from circular or linear DNA template using commercial transcription kit or RNA polymerase, is also available for RYTS reaction. In such case, the DNA template for transcription of mRNA should be also constructed according to section 5.1 DNA template. But, the T7 promoter, which is recognized by T7 RNA polymerase, can be replaced to other promoter recognized by other RNA polymerase (e.g., SP6 promoter recognized by SP6 RNA polymerase).

5.3 Related products

Cloning vectors for the construction of circular template DNA with ProX tag

- pROX-FL92.1ttt / Product Code: # TS013

Linear template generation kits for the construction of DNA template with or without ProX tag and His-tag

- RYTS Linear Template Set for *E.coli* (His-tag) / Product Code: #TS001
- RYTS Linear Template Set for *E.coli* (ProX-tag) / Product Code: #TS002

Transcription kit for preparation of mRNA

- T7 RNA polymerase / Product Code: #RP6T7

6. Protein synthesis

6.1 Additional material and equipment required

- DNA template (circular or linear) or mRNA encoding the protein of interest
- Nuclease-free microcentrifuge tubes and pipette tips
- Incubator, water bath or shaker with thermal controller

6.2 Standard procedure

1. Remove the reagents of this kit from freezer, and allow them to thaw on ice.

2. Set up the following reaction on ice in a nuclease-free microcentrifuge tube.

Cap No. / Cap color / Regents	volume
1 / white / *E.coli* Lysate	100 μl
2 / pink / 2× Reaction Mix	150 μl
3 / blue / Methionine	3 μl
4 / orange / Enzyme Mix	15 μl
a) circular DNA or linear DNA b) mRNA	a) 0.4 – 1.0 μg b) 50 – 100 μg
6 / clear / Nuclease Free Water	up to 300μl

3. Mix thoroughly by pipetting several times, then incubate the reaction at 30°C for two hours.

4. After reaction, store the reaction mixture at 4°C for short term storage.

Note;

We recommend performing additional reaction using "CAT control vector" in this kit for positive control of the reaction. CAT assay, an enzymatic assay for quantification of chloramphenicol acetyl transferase (CAT) activity, is available for measuring typical productivity of this kit. If you wish to use CAT assay, please refer to the previous released article (5).

6.3 Evaluation of produced protein activity

To evaluate performance of RYTS Kit in comparison to a competitor's cell-free protein synthesis kit (Kit A), activities of Green Fluorescent Protein (GFP) resulting from protein syntheses by these two kits were determined. After these reactions, the fluorescent intensities of each reaction mixtures were measured by a fluorescence scanner. It was shown that the obtained fluorescent intensity from RYTS reaction mixture was 3-fold greater than from Kit A reaction mixture (see Figure 3).

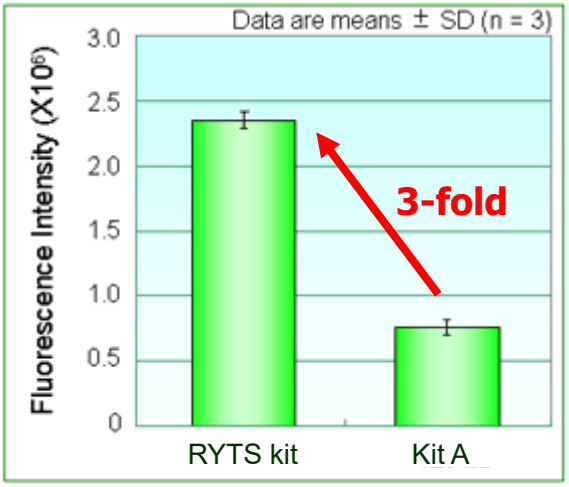

Figure 3. Comparison of produced GFP activity

Applied volume : 50 μl of translational reaction mix

Fluorescence detection (Ex : 488nm / Em : 520 nm)

7. Combined application of RYTS and Clover*Direct*™

7.1 About Clover*Direct*™

Clover*Direct*™ tRNA Reagent for Site-Directed Protein Functionalization allows the incorporation of unnatural amino acids at defined positions of proteins using a cell-free protein synthesis system (6-10). The protein with unnatural amino acid will be obtained within a few hours just by adding Clover*Direct*™ reagents and DNA template having an amber stop codon (UAG) or a four-base codon (CGGG) to a cell-free protein synthesis system. In our product lineup, transfer RNAs charged with unnatural amino acids containing fluorescent groups, biotin, PEG, photo-cross-linker, etc. are available (see page 10). For more information about Clover*Direct*™, please visit our web site.

(http://www.proteinexpress.co.jp/e/products/reagent_e/reagent_top_page_e.htm)

7.2 Standard procedure using with Clover*Direct*™

Clover*Direct*™ reagent contains lyophilized unnatural aminoacyl-tRNA and tRNA buffer. Dissolve unnatural aminoacyl-tRNA with tRNA buffer, and mix with template DNA (or mRNA) and reagents of RYTS Kit. Typical reaction using with Clover*Direct*™ is indicated below.

Cap No. / Cap color / Reagents	Volume
1 / white / *E.coli* Lysate	100 μl
2 / pink / 2× Reaction Mix	150 μl
3 / blue / Methionine	3 μl
4 / orange / Enzyme Mix	15 μl
a) circular DNA or linear DNA b) mRNA	a) 0.4 – 1.0 μg b) 50 – 100 μg
Dissolved unnatural aminoacyl-tRNA	10 μl
6 / clear / Nuclease Free Water	up to 300μl

7.3 Expression of site-directly labeled proteins

A typical result of the protein labeling using RYTS and Clover*Direct*™ TAMRA (Product Code, #CLD02 and #CLD06) is shown in Figure 4.

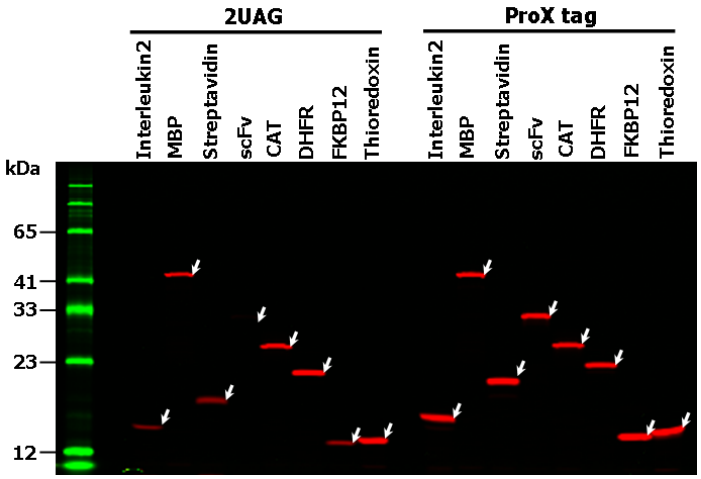

Figure 4. In-gel detection of produced TAMRA labeled proteins

2UAG: UAG codon is inserted after initiator AUG codon **ProX tag*:** ProX tag is fused to the N-terminus

Applied volume: 0.25 µl of RYTS translational reaction mix **White arrow:** TAMRA labeled proteins

*ProX tag is original N-terminal peptide tag developed for incorporation of unnatural amino acids (11).

7.4 Related products to Site-Directed Protein Functionalization

Clover*Direct*™ tRNA Reagents for Site-Directed Protein Functionalization

Site-Directed Fluorescence Labeling

Clover*Direct*™ CR110-X-AF-tRNA	[5-CR110-X : Abs/Em = 498/521nm]
Clover*Direct*™ HiLyte Fluor™ 488-AF-tRNA	[HiLyte Fluor™ 488 : Abs/Em = 497/525nm]
Clover*Direct*™ TAMRA-X-AF-tRNA	[5(6)-TAMRA-X : Abs/Em = 546/575nm]
Clover*Direct*™ ATTO 633-AF-tRNA	[ATTO633 : Abs/Em = 629/657nm]
Clover*Direct*™ ATTO 655-X-AF-tRNA	[ATTO655-X : Abs/Em = 633/684nm]

Site-Directed Biotin Labeling

Clover*Direct*™ Biotin-AF-tRNA	[Biotin]
Clover*Direct*™ Biotin-X-AF-tRNA	[Biotin-X]
Clover*Direct*™ Biotin-XX-AF-tRNA	[Biotin-XX]

Site-Directed Post-translational Modification

Clover*Direct*™ Lys(Me)-tRNA	[*ε*-methyl-Lys]
Clover*Direct*™ Lys(Me_2)-tRNA	[*ε*-dimethyl-Lys]
Clover*Direct*™ Lys(Ac)-tRNA	[*ε*-acetyl-Lys]

Site-Directed Unnatural Mutagenesis

PEGylated amino acids

Clover*Direct*™ PEG4-AF-tRNA	[Methyl-PEG4]
Clover*Direct*™ PEG8-AF-tRNA	[Methyl-PEG8]
Clover*Direct*™ PEG12-AF-tRNA	[Methyl-PEG12]

Closs-linking amino acids

Clover*Direct*™ BPA-tRNA	[*p*-benzoyl-phenylalanine]
Clover*Direct*™ AcPhe-tRNA	[*p*-acetyl-phenylalanine]

Photo-isomerizable amino acid

Clover*Direct*™ azoAla-tRNA	[*p*-phenylazophenyl-alanine]

For more information about Clover*Direct*™, please visit our web site.
(http://www.proteinexpress.co.jp/e/products/reagent_e/reagent_top_page_e.htm)

8. Troubleshooting

Low or no protein production	
1) Expired Kit	Use before expiration date.
2) Inappropriate storage condition	Store this product at appropriate temperature, and avoid multiple freeze-thawing.
3) Nuclease contamination	Nucleases markedly influence on productivity of protein (See section4.2, page 3).
4) Difficult to produce the target protein using *E.coli* cell-free system	Addition of ProX tag to the protein N-terminus may be effective (See page 4).

Low or no activity of protein	
1) Disulfide bond is essential for the protein activity	Addition of oxidized glutathione may be effective. The adequate concentration of oxidized glutathione is ten millimolar.
2) Post-translational modification is required for the protein activity	In *E.coli* cell-free system, post-translational modification activity such as glycosylation and phosphorylation is lacked.
3) Protein folding is not correct	Lower reaction temperature may be effective for correct folding. Otherwise, please try to investigate refolding condition.

If your trouble is not improved according to the troubleshooting, please co ntact us (See page 14, Contact and support).

9. References

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10. Contact and support

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