CELL-FREE PROTEIN SYNTHESIS KIT (PSS3050)
TRANSLATION REAGENT SET (TLS4050)

PROTOCOL

Aug, 2018 / V1.7

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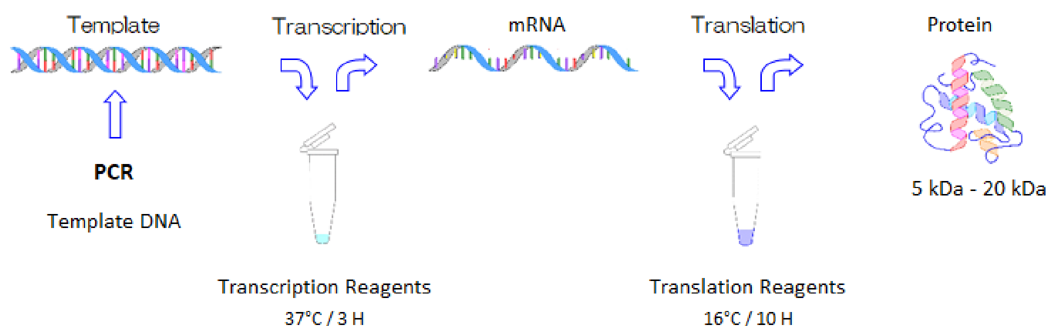
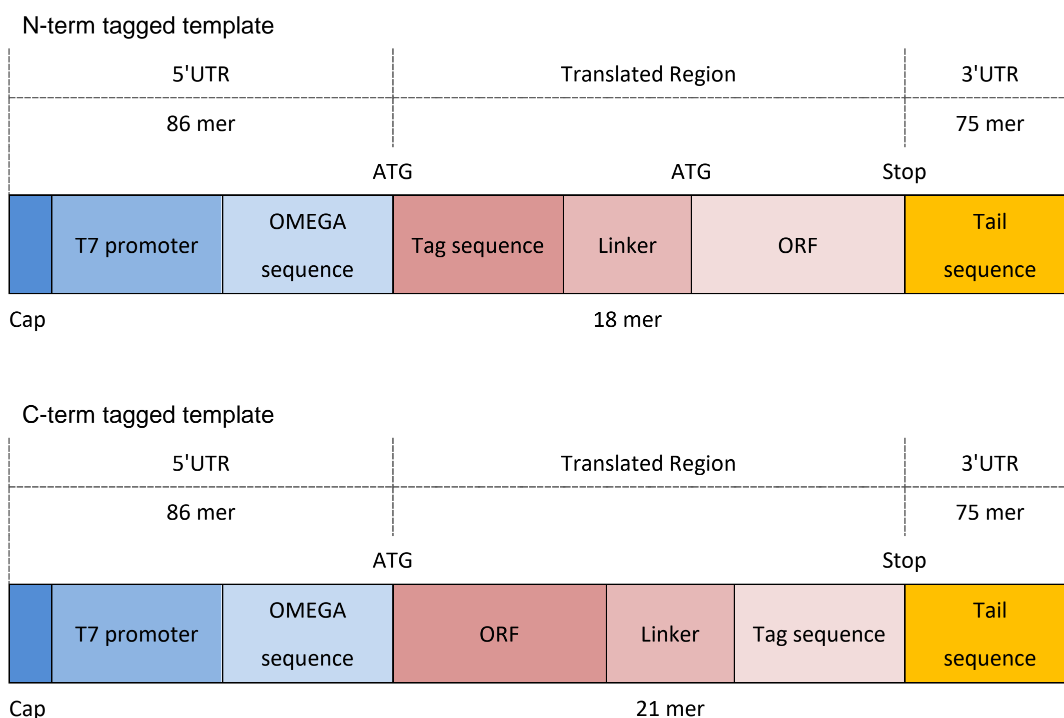
INTRODUCTION

Protein synthesis using cell-free protein synthesis reagent kit consists of three stages: preparation of transcription template, transcription and translation. For the use of the translation reagent set, please refer to section “Translation”.



In preparation of transcription template, it is necessary to prepare a primer in advance.

In the different attachment, five different tag sequences are shown, and by carrying out PCR twice, it is possible to efficiently create a transcription template containing multiple tag sequences. Also, it is possible to tag both N-terminal and C-terminal. In addition, this protocol and primer list can be downloaded from our website (https://nuprotein.jp/knowledge-base/).



In this protocol, we show the PCR program when used Toyobo Co., Ltd. KOD®-Plus-Neo, Takara Bio Inc. PrimeSTAR®, PrimeSTAR® Max as PCR enzymes.

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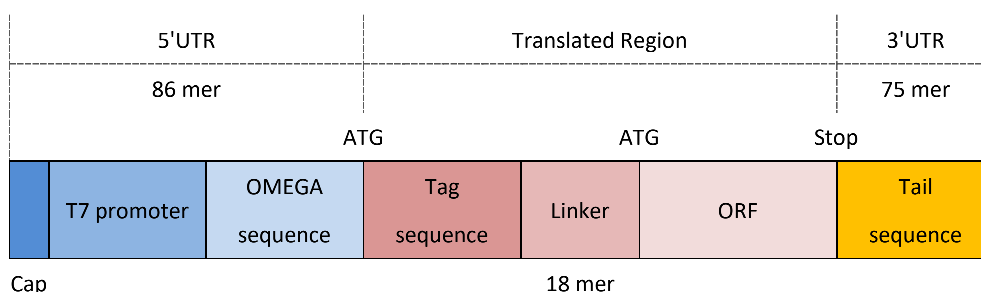
PRIMER DESIGN

STRUCTURE OF N-TERMINAL TAG DNA TEMPLATE

The structure of N-terminal Tag DNA template and the sequence of each part are shown in (Fig.1) and (Table 1).

Primer design and PCR are more complicated than usual because of the long sequence added to the prepared ORF.

(Figure.1) Structure of N-terminal Tag DNA Template



(Table.1) Sequences / Part

Region	Site	Sequence 5' to 3'	Tag
5'UTR	Cap	CCCGC GAAAT	
	T7 promoter	TAATA CGACT CACTA TAGGG	
	OMEGA sequence	CTCAC CTATC TCTCT ACACA AAACA TTTCC CTACA TACAA CTTTC AACTT CCTAT T	
Translated Region		ATG	
	Tag sequence	CAT CAT CAT CAT CAT CAT	6 x His
		GAC TAC AAG GAT GAC GAT GAC AAG	FLAG
		TAT CCT TAT GAC GTG CCT GAC TAT GCC AGC CTG GGA GGA CCT	HA
		GGC CTG AAC GAC ATC TTC GAG GCC CAG AAG ATC GAG TGG CAC GAA	Biotin
	Linker	CTC CAG CAG GGA GGT ACT (Leu-Gln-Gln-Gly-Gly-Thr)	
	ORF	ATG + NNNNN ･･･ ･･･NNNNN	
	Stop codon	TAA, TAG, TGA	
3'UTR	Tail sequence	AAAAA AAAAA GAGCT CTTGG ATCCG GCCAT AAGGT TGGAT CCGGC CATAA GGGCC TGATC CTTCG AGGGG GGGCC	

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PROCEDURE FOR PREPARATION OF PRIMER FOR N-TERMINAL TAG TRANSCRIPTION TEMPLATE

Since PCR is carried out in two stages, 1st PCR and 2nd PCR, multiple primers are required. (Figure 2).

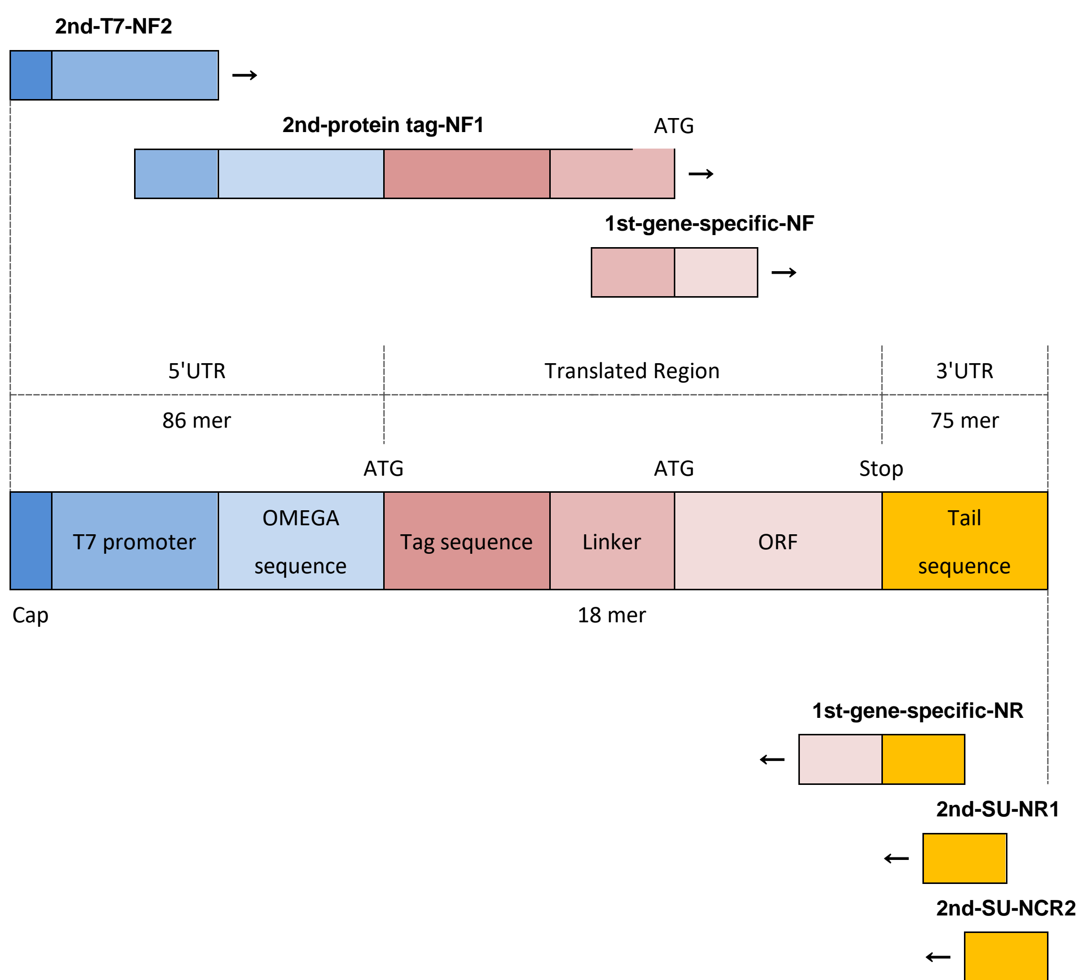
The Primer List (e.g His-tag insertion) is as shown in (Table 2). Tag refferred in red in (Table 2) can be change to other Tag .

Please note that Reverse primer is complementary to the sequence of (Table.1).

In the case of non-Tag, please leave the Linker as it is.

The Linker sequence can be changed to an appropriate protease recognition sequence.

(Figure.2) PCR of N-terminal tag DNA template



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(Table.2) Primer List / N-term His-tag Template DNA

1st PCR		
primer type/name	primer form	primer sequence 5' to 3'
1st-gene-specific-NF	Gene specific 1st Fw primer	CCAGC AGGGA GGTAC T＋an array of 18 mer at 5' end that start from ATG
1st-gene-specific-NR	Gene specific 1st Rv primer	CCTTA TGGCC GGATC CAAGA GCTCT TTTTT TTTTT TA + an array of 18 mer at 3' end excluding stop codon
2nd PCR		
primer type/name	primer form	primer sequence 5' to 3'
2nd-T7-NF2	Universal 2nd Fw primer	CCCGC GAAAT TAATA CGACT CACTA TAG
2nd-protein tag-NF1	2nd Fw primer-tag	CGACT CACTA TAGGG CTCAC CTATC TCTCT ACACA AAACA TTTCC CTACA TACAA CTTTC AACTT CCTAT TATGC ATCAT CATCA TCATC ATCTC CAGCA GGGAG GTACT ATG
2nd-SU-NCR2	Universal 2nd Rv primer	GGCCC CCCCT CGAAG G
2nd-SU-NR1		CCCTC GAAGG ATCAG GCCCT TATGG CCGGA TCCAA

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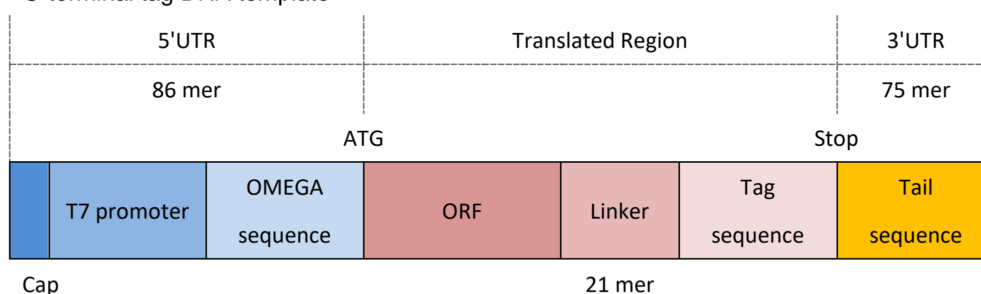
STRUCTURE OF C-TERMINAL TAG DNA TEMPLATE

The structure of C-terminal Tag DNA template and the sequence of each part are shown in (Fig.3) and (Table 3).
Same as with N-terminal tag, Primer design and PCR are more complicated than usual because of the long sequence added to the prepared ORF

(Figure.3) C-terminal tag DNA template



(Table.3)

Sequences / Part

Region	Site	Sequence 5' to 3'	Tag
5'UTR	Cap	CCCGC GAAAT	
	T7 promoter	TAATA CGACT CACTA TAGGG	
	OMEGA sequence	CTCAC CTATC TCTCT ACACA AAACA TTTCC CTACA TACAA CTTTC AACTT CCTAT T	
Translated Region	ORF	ATG + NNNNN ・・・ ・・・NNNNN	
	Linker	GGT CTC CAG CAG GGA GGT ACT (Gly-Leu-Gln-Gln-Gly-Gly-Thr)	
	Tag sequence	CAT CAT CAT CAT CAT CAT	6 x His
		GAC TAC AAG GAT GAC GAT GAC AAG	FLAG
		TAT CCT TAT GAC GTG CCT GAC TAT GCC AGC CTG GGA GGA CCT	HA
		GGC CTG AAC GAC ATC TTC GAG GCC CAG AAG ATC GAG TGG CAC GAA	Biotin
	Stop codon	TAA, TAG, TGA	
3'UTR	Tail sequence	AAAAA AAAAA GAGCT CTTGG ATCCG GCCAT AAGGT TGGAT CCGGC CATAA GGGCC TGATC CTTCG AGGGG GGGCC	

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PROCEDURE FOR PREPARATION OF PRIMER FOR C-TERMINAL TAG TRANSCRIPTION TEMPLATE

Since PCR is carried out in two stages, 1st PCR and 2nd PCR, multiple primers are required. (Figure 4).

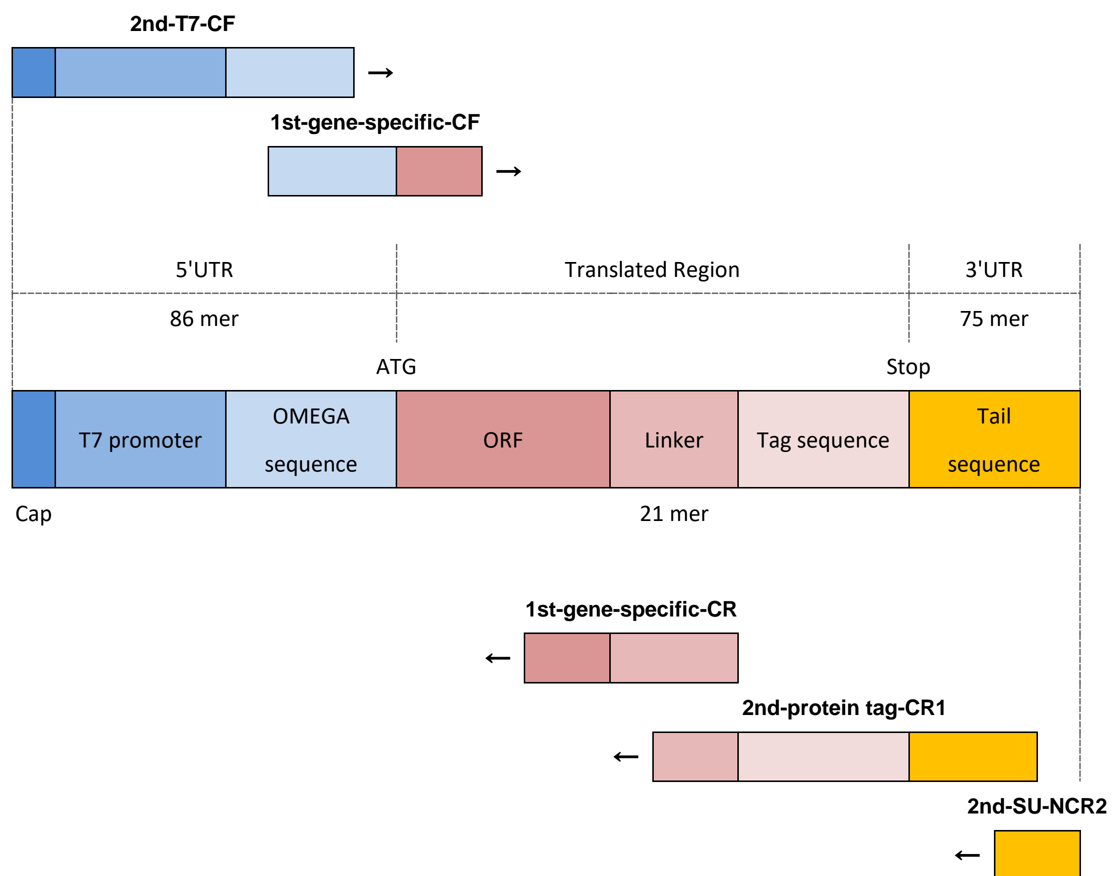
The Primer List (i.e. His-tag insertion) is shown in (Table 4). Tag in red (Table.3) can be changed to other Tag sequences.

Please note that Reverse primer is complementary to the sequence of (Table.3)

In the case of non-Tag, please leave the Linker as it is.

The Linker sequence can be changed to an appropriate protease recognition sequence.

(Figure.4) PCR of C-terminal tag DNA template



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(Table.4)

Primer List / C-term His-tag Template DNA

1st PCR		
primer type/name	primer form	primer sequence 5' to 3'
1st-gene-specific-CF	Gene specific 1st Fw primer	CACAA AACAT TTCCC TACAT ACAAC TTTCA ACTTC CTATT＋an array of 18 mer at 5' end that start from ATG
1st-gene-specific-CR	Gene specific 1st Rv primer	AGTAC CTCCC TGCTG GAGAC C＋an array of 18 mer at 3' end excluding stop codon
2nd PCR		
primer type/name	primer form	primer sequence 5' to 3'
2nd-T7-CF	Universal 2nd Fw primer	CCCGC GAAAT TAATA CGACT CACTA TAGGG CTCAC CTATC TCTCT ACACA AAACA TTTCC
2nd-SU-NCR2	Universal 2nd Rv primer	GGCCC CCCCT CGAAG G
2nd-protein tag-CR1	2nd Rv primer-tag	CCCTC GAAGG ATCAG GCCCT TATGG CCGGA TCCAA GAGCT CTTTT TTTTT TTTAA TGATG ATGAT GATGA TGAGT ACCTC CCTGC TGG

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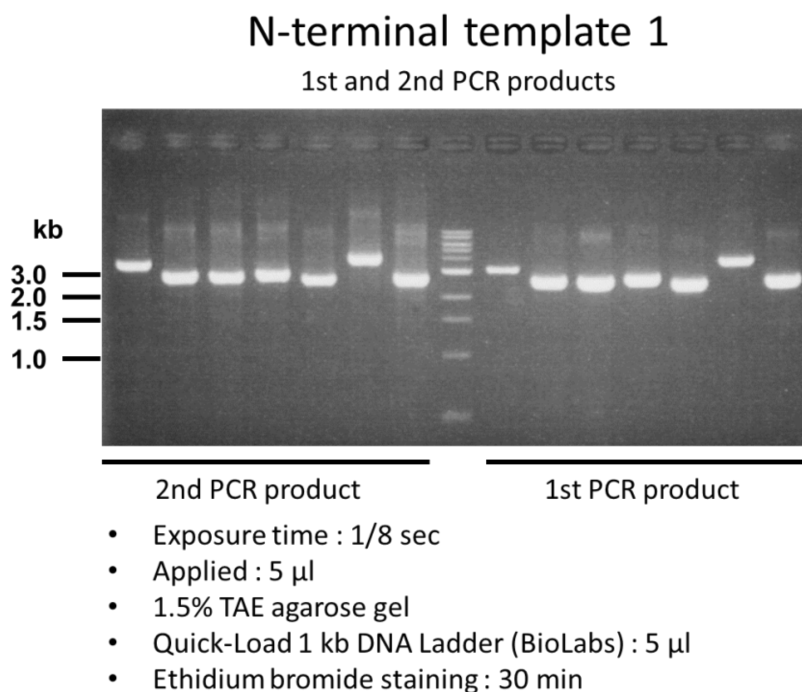
DETECTION OF 1st PCR AND 2nd PCR PRODUCT

Please check the PCR products after 1st PCR and 2nd PCR as follows.

1. Prepare a TAE agarose gel at a concentration according to the DNA size and set in electrophoresis.
2. Load the 3 μl sample PCR without purification into the agarose gel above.
3. After completion of electrophoresis, the agarose gel is stained with Ethidium Bromide for 30 minutes.
4. Fluorescence detection of DNA is performed by a transilluminator.

【Example of checking PCR product for N-terminal tag】



- Exposure time : 1/8 sec
- Applied : 5 μl
- 1.5% TAE agarose gel
- Quick-Load 1 kb DNA Ladder (BioLabs) : 5 μl
- Ethidium bromide staining : 30 min

As a result of confirmation of PCR Product, if problem occurs, please review again in the following order

A) Confirm PCR program
B) Check composition of the reaction solution preparation
C) Confirm PCR primer

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1st PCR

The composition and reaction program for one sample are shown in the table below.

It is also recommended to change the description program in consideration of the amplification efficiency etc.

After completion of 1st PCR reaction, check 1st PCR product (3 μl) using agarose gel electrophoresis.

In the following program, 1st-gene-specific-F and 1st-gene-specific-R indicate 1st-gene-specific-NF, 1st- gene-specifc-NR in the case of N-terminal tag transcription template, For C-terminal tag transcription template they are 1st-gene-specific-CF and 1st-gene-specific-CR respectively.

Composition and PCR program when using KOD-Plus-Neo

KOD-Plus-Neo ver.

Reagents	
10 X PCR buffer	5 μl
2 mM dNTPs	5 μl
25 mM $MgSO_4$	3 μl
10 μM 1st-gene-specific-F	1 μl
10 μM 1st-gene-specific-R	1 μl
Plasmid	1 ng
KOD	1 μl
Sterile water	X μl
Total	50 μl

PCR program

Temp.	Time	Cycle
94°C	5 min	1
98°C	10 sec	30
55°C	30 sec	
68°C	3 min (1 min/kb)	
72°C	2 min	1
20°C	-	

Composition and PCR program when using PrimeSTAR

PrimeSTAR ver.

Reagents	
5 X PrimeSTAR buffer	10 μl
2.5 mM dNTPs	4 μl
10 μM 1st-gene-specific-F	1 μl
10 μM 1st-gene-specific-R	1 μl
Plasmid	1 ng
Polymerase (2.5 U/μl)	0.5 μl
Sterile water	X μl
Total	50 μl

PCR program

Temp.	Time	Cycle
94°C	5 min	1
98°C	10 sec	30
55°C	30 sec	
68°C	(1 min./kb)	
72°C	2 min	1
20°C	-	

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Composition and PCR program when using PrimeSTAR Max

PrimeSTAR Max ver.

Reagents	
2 X PrimeSTAR Max Premix	25 μl
10 μM 1st-gene-specific-F	1 μl
10 μM 1st-gene-specific-R	1 μl
Plasmid	1 ng
Sterile water	X μl
Total	50 μl

PCR program

Temp.	Time	Cycle
94°C	5 min	1
98°C	10 sec	30
55°C	30 sec	
72°C	(5 sec./kb)	
72°C	2 min	1
20°C	-	

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2nd PCR

Please use 1st PCR Product in unpurified and unconcentrated state for 2nd PCR

After 2nd PCR reaction, check 2nd PCR product (3 μl) using agarose gel electrophoresis.

It is also recommended to change the description program in consideration of the amplification efficiency etc.

*Normally, 1 μl of 1st PCR product is used for 2nd PCR.

Each program of 2nd PCR

Following program is common for N-terminal tag and C-terminal tag.

PCR program (KOD or PrimeSTAR ver.)

Temp.	Time	Cycle
98°C	1 min	1
98°C	10 sec	10
60°C	1 min	
68°C	3 min (1 min/kb)	
98°C	10 sec	30
60°C	15 sec	
68°C	3 min (1 min/kb)	
72°C	2 min	1
20°C	-	

PCR program (PrimeSTAR Max ver.)

Temp.	Time	Cycle
98°C	1 min	1
98°C	10 sec	5
55°C	15 sec	
72°C	(5 sec./kb)	
98°C	10 sec	30
60°C	15 sec	
72°C	(5 sec./kb)	
72°C	2 min	1
20°C	-	

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Composition for N-terminal tag of 2nd PCR

KOD-Plus-Neo ver.

Reagents	
10 X PCR buffer	5 µl
2 mM dNTPs	5 µl
25 mM $MgSO_4$	3 µl
10 µM 2nd-T7-NF2	1 µl
100 nM 2nd-protein tag-NF1	1 µl
10 µM 2nd-SU-NCR2	1 µl
100 nM 2nd-SU-NR1	1 µl
1st PCR product	1-5 µl*
KOD	1 µl
Sterile water	X µl
Total	50 µl

PrimeSTAR ver.

Reagents	
5 X PrimeSTAR buffer	10 µl
2.5 mM dNTPs	4 µl
10 µM 2nd-T7-NF2	1 µl
100 nM 2nd-protein tag-NF1	1 µl
10 µM 2nd-SU-NCR2	1 µl
100 nM 2nd-SU-NR1	1 µl
1st PCR product	1-5 µl*
Polymerase (2.5 U/µl)	0.5 µl
Sterile water	X µl
Total	50 µl

PrimeSTAR Max ver.

Reagents	
2 X PrimeSTAR Max Premix	25 µl
10 µM 2nd-T7-NF2	1 µl
100 nM 2nd-protein tag-NF1	1 µl
10 µM 2nd-SU-NCR2	1 µl
100 nM 2nd-SU-NR1	1 µl
1st PCR product	1-5 µl*
Sterile water	X µl
Total	50 µl

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Composition for C-terminal tag of 2nd PCR

KOD-Plus-Neo ver.

Reagents	
10 X PCR buffer	5 µl
2 mM dNTPs	5 µl
25 mM $MgSO_4$	3 µl
10 µM 2nd-T7-CF	1 µl
100 nM 2nd-protein tag-CR1	1 µl
10 µM 2nd-SU-NCR2	1 µl
1st PCR product	1-5 µl*
KOD	1 µl
Sterile water	X µl
Total	50 µl

PrimeSTAR ver.

Reagents	
5 X PrimeSTAR buffer	10 µl
2.5 mM dNTPs	4 µl
10 µM 2nd-T7-CF	1 µl
100 nM 2nd-protein tag-CR1	1 µl
10 µM 2nd-SU-NCR2	1 µl
1st PCR product	1-5 µl*
Polymerase (2.5 U/µl)	0.5 µl
Sterile water	X µl
Total	50 µl

PrimeSTAR Max ver.

Reagents	
2 X PrimeSTAR Max Premix	25 µl
10 µM 2nd-T7-CF	1 µl
100 nM 2nd-protein tag-CR1	1 µl
10 µM 2nd-SU-NCR2	1 µl
1st PCR product	1-5 µl*
Sterile water	X µl
Total	50 µl

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TRANSCRIPTION

Preparation of Transcription Reaction Solution

Prepare the transcription reaction solution on ice in a 1.5 ml tube as follows.
Please be careful not to mix 2nd PCR Product directly with 10x Transcription Buffer

Reagents (one reaction)	
10 X Transcription buffer (TB)	2.5 μl
25 mM NTPs	2.5 μl
0.1 M DTT	1.25 μl
T7 RNA Polymerase	1 μl
2nd PCR product	2.5 μl
RNase-free water (DEPC)	15.25 μl
Total	25 μl

Transcription Reaction

1. Tap the prepared transcription reaction solution lightly and use tabletop centrifuge to drop the solution that attached to the wall gently.
2. Close the cap securely to prevent evaporation of the solution.
3. Incubate in water bath or incubator at 37 °C for 3 hours.

*The standard for reaction time is 3 hours up to 6 hours at most.

Transcription Purification

1. After completion of the transcription reaction, load 1 μl transcription sample to agarose gel electrophoresis.
2. Add 10 μl of 4 M ammonium acetate per one sample (25 μl), mix well, add 100 μl of 100% ethanol, inverted the tube, then use tabletop centrifuge for several seconds. Let stand for 20 minutes on ice.
   Ammonium acetate is essential as neutralizing reagent. Please note that mRNA is inactivated and proteins cannot be synthesized when purified with reagents for purification, trizole, ammonium sulfate, etc.
3. Centrifuge at 15,000 rpm for 20 minutes at 4 °C
4. Discard the supernatant with a pipete and use tabletop centrifuge for several seconds. Discard the supernatant again by careful aspiration with a pipette. At this time, be careful to not touch the pellet of the sample.
5. Leave the pellet until dries. Keep the lid of the tube open, please be careful not to contaminate impurities, dust, brushes.

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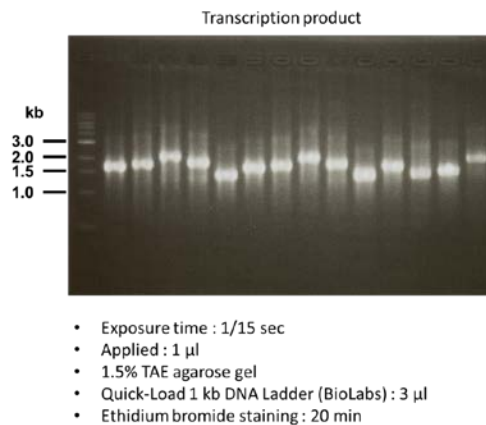
＊Nucleic acid dryer is not allowed

6. Add 70 μl of RNase-free water (DEPC water) per one sample (25 μl of transcription reaction solution) to dissolve the mRNA pellet.
   Suspend the pellet well and use it as an mRNA solution
   * Be careful as mRNA may adhere to the wall of the tube in a difficult state to check

Confirmation of Transcription

1． Confirmation of transcription (mRNA)
After completion of purification, confirm the mRNA by electrophoresis.



- Exposure time : 1/15 sec
- Applied : 1 μl
- 1.5% TAE agarose gel
- Quick-Load 1 kb DNA Ladder (BioLabs) : 3 μl
- Ethidium bromide staining : 20 min

2． Concentration measurement of transcription.
When measuring the concentration, purify phenol / chloroform system separately from the sample for translation.
Yield of one target mRNA from the transcription reaction is > 25 ug.

Depending on the target protein, mRNA may be synthesized in large quantities in the transcription reaction, but when mRNA is subjected to the translation reaction in a large amount (40 ug or more), on the contrary, the protein yield drops extremely, or the full length protein can not be obtained. Therefore, We suggest you can measure the concentration, and in the case of 110 μl of translation solution in the next chapter, as mRNA solution. Please prepare and supply to 25 ~ 40 ug / 70 ul, preferably 30 ~ 35 ug / 70 ul of mRNA for translation per 110ul sample size.

In the case of coexpression using heterologous mRNA such as heterodimer, for example, coexpression of protein with molecular weight A and molecular weight B, making the ratio of each mRNAs is A: B respectively and total amount of mRNA about 30 to 35 ug / 70 ul, improves multimer of them.

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TRANSLATION

Preparation of translation reaction solution

Prepare the translation reaction solution with 1.5 ml tube or PCR tube as follows.

Prepare reagents other than mRNA first and make it to room temperature, then added to the mRNA solution and resuspend gently so as not formed foam.

When mRNA concentration is unknown, it is suggested that whole mRNA synthesized by one transcription reaction is added to one translation reaction solution.

Reagents	
Wheat germ extract	20 µl
Amino acid mix	20 µl
mRNA	70 µl
Total	110 µl

Translation reaction (simple batch method)

1. Incubate the tube at 16 °C and let it react overnight (over 10 hours)
2. Gently mix the sample by pipete, collected in an Eppendorf tube, centrifuged at 15,000 rpm for 10 minutes at 4 ° C. The supernatant is stored at -80 ° C

Scale-Up

In this protocol, 1 translation reaction is 110 µl, but scale-up is possible.

For scaleup, please increase the ratio of translation reaction system as it is.

e.g.) When translation reaction 110 µl is scaled up to 220 µl, the composition is

Wheat germ extract	20ul	->	40ul
Amino acid mix	20ul	->	40ul
mRNA	70ul	->	140ul

If you scaled up, the yield of the target protein may be improved.

e.g.) In the case one translation reaction of 110 µl have a yield 10 µg, one translation reaction of 550 µl improves the yield per reaction solution with the target yield, about 70 µg

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Translation reaction (dialysis method)

Dialysis method can be used instead of simple batch method. In the dialysis method, insert the translation solution into the inside of the dialysis cup (sample solution), and use additional Amino acid mix as the dialysis external solution. Dialysis external solution is generally 20 times to 100 times the capacity.

When using Slide-A-Lyzer ™ Mini dialysis tool (sample 0.1 mℓ, molecular weight cut off 3.5 K) manufactured by Thermo Fisher Scientific Inc.

1. Place 2 ml of Amino Acid Mix into 1 well of a 24-well titer plate, diluted 4 times with ultrapure water into, and cover the plate. ＊ Since 2 ml Eppendorf tube matches the dialysis cup, the dialysis method can be do with 2 ml Eppendorf tube.
2. Squeeze the dialysis cup into the lid of the plate.
3. Take the total volume of 110 μℓ of the prepared translation reaction solution and transfer to dialysis cup gently so bubbles do not enter on to the dialysis cup, two-step pushing of the pipette can causes foaming. Then close the lid of the dialysis cup. At this time, please set the level of the sample solution (reaction layer) and the level of the external dialysate (Amino Acid Mix) are at the same level or the level of the sample solution is slightly lower.
4. Incubate at 16 °C and let it react overnight (over 10 hours)

Moreover, by drilling 9mm hole in 24-well titer plate can fit to dialysis cup, but if it is difficult to drill, use a floater to microtube.

- Since the volume of amino acid mix in the kit is not enough for dialysis method, please purchase the optional Amino acid mix (product name: AAMX004) for dialysis method 4 reaction when dialysis method is used.

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Confirmation of Protein

Confirm the translation product by acrylamide gel electrophoresis as follows.

1. After completion of the translation reaction, fill up the translation reaction solution with ultrapure water up to 3 volumes and stir gently.
2. Centrifuge at 15,000 rpm for 20 min at 4 °C, collect supernatant.
3. Load about 1-2 ul of sample to acrylamide gel, then electrophorese
4. Detect according the staining method.

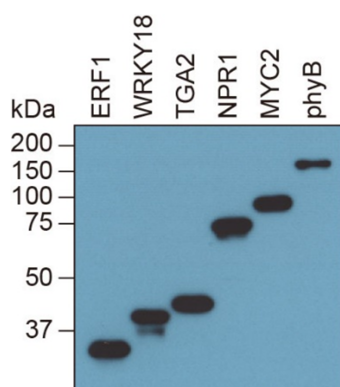
About the above procedure

Procedure 1. , The purpose of fill up is to save the detection sample, to avoid overloading, and to solubilize the nonsolubilized compound. If it is Fill up up to three times, the buffer capacity of the reaction solution is maintained, but be careful when filling beyond this. If you do not need it, skip this step.

Procedure 2. Regarding precipitates, in addition to components derived from Wheat Germ, non - solubilized objects may also be included. If necessary, resuspend the precipitate with an appropriate Buffer to recover the desired product. If there is any doubt about the amount of target product recovered from the supernatant, this will grasp the solubilization rate and improvisation by optimizing the translation reaction solution.

Procedure 3. For electrophoresis samples, Western blot, silver staining, CBB staining, both are detected at approximately 1-2 ul. In detection other than immunostaining, Wheat Germ components are also detected, so loading large amounts of samples is not recommended.

The following is the result of synthesizing FLAG® fusion protein using this kit and detecting these proteins by western blot using anti-FLAG® antibody.



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