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Purchaser's Agreement

Customer’s order of pCold DNAs will be accepted only when the Purchaser’s Agreement is signed by a customer and is attached with an order.

- pCold DNAs (hereinafter “PRODUCTS”) are covered by U.S. Patents No. 6479260, which are owned by TAKARA BIO, and the U.S. Patents, 5981280, 6686174, 6333191, which are owned by University of Medicine & Dentistry of New Jersey and are exclusively licensed to TAKARA BIO.
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I. Description

Elucidation of protein structure and function maintains an important role in post-genomic sequencing and analysis studies. An efficient protein production system is critical for obtaining large amounts of correctly folded recombinant protein for study. *E. coli* expression systems, which are used extensively for the production of recombinant proteins, offer two major advantages over other types of expression systems: (1) ease of use, and (2) low cost. However, some recombinant proteins do not fold correctly during expression in *E. coli*, and result in deposits of inactive insoluble protein termed "inclusion bodies".

In collaboration with Prof. Masayori Inouye (University of Medicine and Dentistry of New Jersey, USA), Takara Bio has developed the pCold DNA Vectors, a series of novel protein expression vectors. The pCold Vectors provide increased *in vivo* protein yield, purity, and solubility for expressed recombinant proteins using "cold shock" technology. More specifically, the *csp*A (cold shock protein A) promoter and related elements have been incorporated into these vectors to upregulate target protein production at lowered incubation temperatures (37°C-15°C). This temperature drop also suppresses expression of other cellular proteins and temporarily halts overall cell growth. This process allows expression of target proteins at high yield, high purity (up to 60% of cellular protein), and increased solubility as compared with conventional *E .coli* expression systems.

Co-expression of one or more chaperone proteins during expression of a heterologous target protein has proven effective for obtaining increased amounts of soluble recombinant protein in *E. coli* (see Takara's Chaperone Plasmid Set [Cat. # 3340]). This procedure, though, lacks the convenience of a single transformation step.

Takara's pCold TF DNA Vector is a fusion cold shock expression vector that expresses Trigger Factor (TF) chaperone as a soluble tag. Trigger Factor is a prokaryotic ribosome-associated chaperone protein (48 kDa) which facilitates co-translational folding of newly expressed polypeptides. Because of its *E. coli* origin, TF is highly expressed in *E. coli* expression systems. The pCold TF DNA Vector consists of the *csp*A promoter plus additional downstream sequences including a 5' untranslated region (5' UTR), a translation enhancing element (TEE), a His-Tag sequence, and a multicloning site (MCS). A *lac* operator is inserted downstream of the *csp*A promoter to ensure strict regulation of expression. Additionally, recognition sites for HRV 3C Protease, Thrombin, and Factor Xa are located between TF-Tag and the Multiple Cloning Site (MCS) and function to facilitate tag removal from the expressed fusion protein. Most *E. coli* strains can serve as expression hosts. The pCold TF DNA Vector provides cold shock technology for high yield protein expression combined with Trigger Factor (chaperone) expression to facilitate correct protein folding, thus enabling efficient soluble protein production for otherwise intractable target proteins.

II. Components

pCold TF DNA Vector 25 µg

< Available *E. coli* host strains >

Most *E. coli* strains can be used as expression hosts for Takara's pCold DNA Vector series since these vectors utilize the *E. coli csp*A (cold shock protein gene) promoter.

III. Vector map:

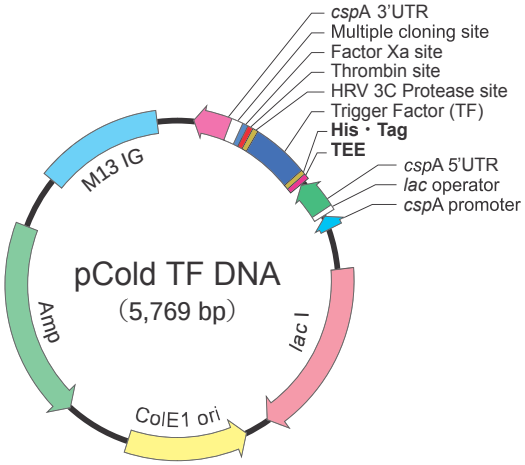

Fig.1 pCold TF DNA : Vector Map

GeneBank Accession No. AB213654

IV. Storage: -20°C (for shipping and storage)

V. Protocol: How to express the target gene;

The cultivation / induction conditions (culture medium, culture temperature, aeration, timing of induction, concentration of an inducer, cultivation time after induction) should be examined for each target protein.

The example of general method is shown below.

1) Insert the target gene to the multicloning site of pCold DNA to construct the plasmid for expression.
2) Transform the *E.coli* host strain (e.g. BL21) with the plasmid of expression, and select the transformants on the selection plate including ampicillin.
3) Inoculate the transformant in the medium including 50 µg/ml of ampicillin, and culture at 37°C with shaking.
4) At OD_{600}= 0.4 - 0.5, refrigerate the culture solution at 15°C and leave to stand for 30 minutes.
5) Add IPTG at the final concentration of 0.1- 1.0 mM, and continue the culture with shaking at 15°C for 24 hours.
6) Collect the cells, and confirm the expression of target protein with SDS-PAGE in soluble and insoluble fractions or activity assay.

By selection of the *E.coli* host strains for expression and optimization of cultivation / induction conditions (culture medium, culture temperature, aeration, timing of induction, concentration of an inducer, cultivation time after induction), the expression level and the degree of soluble expression are improvable. The tag sequence at the N-termini can be cut and removed by Factor Xa, Thrombin, and HRV 3C Protease.

VI. Multiple cloning site:

pCold TF DNA (Code. 3365)

5' TAACGCTTCAAAATCTGTAAAGCACGCCATATCGCCGAAAG

TEE — His-Tag

GCACACTTAATTATTAAGAGGTAATACACCATGAATCACAAAGTGCATCATCATCATCATCAC

SD Met Asn His Lys Val His His His His His His

HRV 3C Protease

ATGCAA..Trigger Factor (1296 bp)...CAGGCGTCCGCGGGTCTGGAAGTTCTGTTCCAGGGGCCCTCC

MetGln..Trigger Factor (432 aa)..Gln Ala Ser Ala Gly Leu Glu Val Leu Phe Gln Gly Pro Ser

Thrombin — **Factor Xa**

GCGGGTCTGGTGCCACGCGGTAGTGGTGGTATCGAAGGTAGG

Aly Gly Leu Val Pro Arg Gly Ser Gly Gly Ile Glu Gly Arg

Nde I *Sac* I *Kpn* I *Xho* I *Bam*H I *Eco*R I *Hin*d III *Sal* I *Pst* I *Xba* I

CATATG GAGCTC GGTACC CTCGAG GGATCC GAATTC AAGCTT GTCGAC CTGCAG TCTAGA TAGGTAATCTCTGCT

His Met Glu Leu Gly Thr Leu Glu Gly Ser Glu Phe Lys Leu Val Asp Leu Gln Ser Arg End

pCold-R Primer

TAAAAGCACAGAATCTAAGA**TCCCTGCC**ATTT**GGCGGGGA**TTTTTTTATTTGTTTTCAGGAAATAAATAATCGAT 3'

transcription terminator

VII. Application

Protein expression using pCold TF DNA was compared against expression using (1) the pCold DNA I Vector alone, (2) co-expression using the pCold DNA I Vector with Takara's Chaperone Plasmid pTf16, and (3) a T7 promoter expression system which included experiments using other tags for solubilization. pCold DNA I and pCold TF DNA Vectors were transformed separately into *E.coli* BL21 cells, cells were then cultured, and expression was performed according to each of their protocols. Expression from T7 promoter-driven vectors was additionally conducted using a general procedure involving addition of IPTG and subsequent culturing at 37°C.

(1) Example 1: Successful protein expression resulting in soluble form

The expression of enzyme protein A (estimated molecular weight 29 kDa) was not verified as an exact band around at the estimated molecular weight, 29 kDa, with the expression system utilizing T7 promoter or even with pCold I DNA (either individual expression or co-expression with chaperone). On the other hand, the expression of target protein (29 kDa and 52 kDa) was verified in case of using pCold TF DNA, and most of the obtained protein was in soluble form. It was confirmed that the expressed enzyme protein A has the enzyme activity even in the form of a fusion protein.

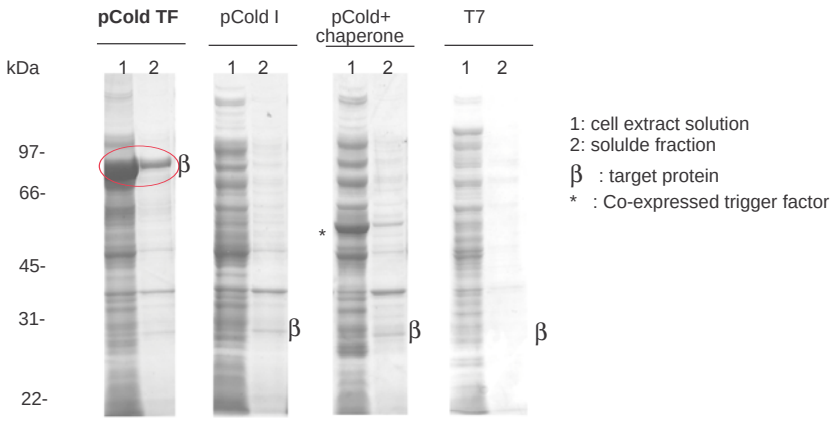

Fig.2 Expression of enzyme protein A

2) Example 2: Expression resulting in improved levels of soluble protein.

Expression of soluble enzyme protein B (M.W: ~63 kDa) was not able to be obtained using either pCold DNA I alone or co-expressed with chaperone proteins, nor with a T7 expression vector that included other tags for solubilization (Trx Tag [~12 kDa], Nus Tag [~55 kDa], and GST Tag [~26 kDa]). Alternatively, when pCold TF DNA Vector was used, most of the expressed target protein was verifiable as a soluble fraction and present at an expression level much higher than with other tags. (Note: The molecular weight of the target protein was observed as larger than its real size due to fused expression with each tag.)

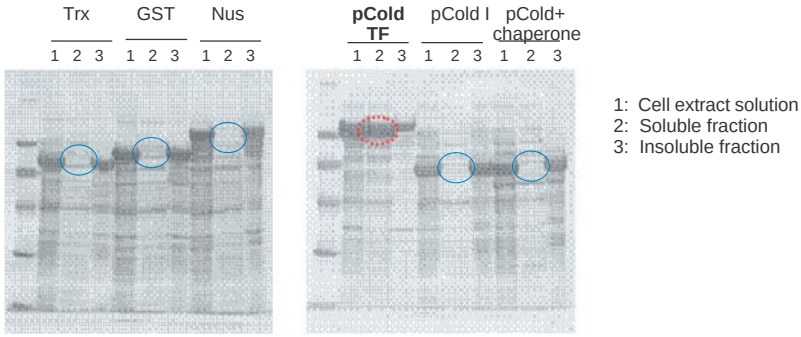

Fig.3 Expression of enzyme protein B

VIII. Q&A

Q1: What parameters should be examined when the expressed protein is insoluble?

A1: The optimal conditions for cultivation and induction vary depending on a kind of expressed protein. The cultivation and induction conditions should be determined by referring to the following points:

- Change the induction timing. It should be examined within early and late logarithmic growth phase.
- Change the concentration of inducer (IPTG) within 0.1-1 mM.
- Examine the cultivation time after induction (Generally 15°C, 24 hours is the most appropriate.)
- Change the conditions for aeration.

Q2: What remedial procedures should be taken in case that no protein is expressed or the expression level is low?

A2: It is recommended to examine again the conditions for cultivation and induction (Refer to the above Q1.), or change the host *E.coli* strain.

Q3: What host strains have been confirmed to work with pCold Vectors?

A3: BL21, Rosetta™, Origami™ from Novagen, Inc. BL21 is most commonly used as host. Origami™ lacks the *trx/gor* gene and allows the formation of disulfide bond in cytoplasm at high level. Accordingly, the solubility and refolding of expressed protein are facilitated. Rosetta™ contains a plasmid which supplies tRNAs corresponding to the codons that are rarely used in *E.coli*. It enables the universal transcription of the genes which are restricted by the codon usage of *E.coli*.

Q4: Can *E. Coli* retaining a pCold TF vector that contains target gene be stored at 4°C on a plate?

A4: We don't recommend the 4°C storage on a plate because it causes a possible leak of target protein in the cell. Pick the colony from the plate promptly, prepare a glycerol stock and store at -80°C.

Q5: Is it possible to express a large gene in pCold TF DNA?
A5: It was confirmed that human gene of 125 kDa can be expressed successfully in a soluble form. (The band of 125 + 52 kDa was was verified in CBB staining.)

IX. Appendix

Expression Plasmid Construction - Example using the thioredoxin gene

1) Overview of pCold TF expression vector construction

a) Select a restriction enzyme site such that the DNA fragment to be inserted will have the sequence of its target gene positioned in a continuous reading frame with that of the pCold TF DNA Vector.
b) Prepare the DNA fragment to be inserted into the vector.
c) Cut the vector with the desired restriction enzymes.
d) After ligating the digested vector with the insert DNA, transform it into an appropriate *E. coli* strain.
e) Prepare purified plasmid from the appropriate colonies containing the target insert.
f) Purified plasmid may be used for protein expression experiments.

There are several ways in which the insert DNA may be prepared, including PCR amplification, excision of a cloned gene by restriction enzyme digestion, and gene synthesis. Transfer of inserts already cloned into Takara's pCold I-IV Vectors can be easily accomplished since the pCold TF DNA multicloning site (MCS) is identical to the pCold I-IV MCS. Presented below is an example experiment which uses PCR amplification as the insert DNA preparation method.

2) Example Plasmid preparation for expression of the *E. coli* thioredoxin gene

a) Guidelines for primer design

Protocol and points to consider when designing primers:

i) Select two restriction enzymes whose sites are contained within the MCS of pCold TF DNA that have additionally been verified not to cut the insert DNA sequence.
ii) Construct a primer for the target sequence, adding the selected restriction sites from Step 2a) i) to the 5' terminus of each primer. Adjust the base number between the insert DNA sequence and N-terminal restriction sites such that the frame of the insert matches the reading frame of pCold TF DNA. "Either a restriction site or a stop codon can be directly added to the C-terminus if required.").
iii) Add four or more bases to sequences directly flanking the restriction sites. Most restriction enzymes require that several bases lie outside of the recognition site for efficient digestion to occur. Without the presence of this extra sequence, digestion efficiency will be lowered.

[Example - Primer Design]

Insertion of the thioredoxin gene into the pCold TF DNA *Nde* I/*Xho* I MCS restriction enzyme cloning sites

Nde I site: Primer 1 (normal direction primer)

Nde I
5' -GCCGCATATGAGCGATAAAATTATTCAC
extra sequence thioredoxin-origin sequence*1

Xho I site: Primer 2 (reverse direction primer)

Xho I
5' - GCCGCTCGAGTTAGGCCAGGTTAGCGTC
extra sequence thioredoxin-origin sequence *2

*1: When using *Nde* I site, adjust the position of the thioredoxin gene start codon (ATG) to correspond with the ATG site of *Nde* I

*2: Complementary Thioredoxin sequence with stop codons

b) Insert DNA Preparation

[Example - PCR amplification of the thioredoxin gene (~ 350 bp)]

i) PCR amplification of the insert DNA.

Prepare the reaction mixture by combing the following reagents. (use of a PCR Enzyme, such as *TaKaRa Ex Taq*™ (Cat. # RR001A) is recommended).

Template DNA (5 ng)*1	1 µl
10x *Ex Taq*™ Buffer*2	5 µl
dNTP Mixture (2.5 mM each)*2	4 µl
Primer 1 (10-50 pmol/µl)	1 µl
Primer 2 (10-50 pmol/µl)	1 µl
TaKaRa Ex Taq™ (5 units/µl)	0.25 µl
Sterilized distilled water	37.75 µl
Total	50 µl

Amplify the insert DNA using the following PCR cycling parameters (30 cycles):
When using Takara Thermal Cycler Dice (Cat. # TP600)

98°C, 10 sec.
55°C, 30 sec.
72°C, 1 min.

*1 For plasmid DNA, use 1-10 ng; for cDNA or genomic DNA, use 50-500 ng.

*2 10x *Ex Taq*™ Buffer and dNTP Mixture is supplied with TaKaRa *Ex Taq*™ (Cat. # RR001A).

ii) Verification of amplified product

Verify that the amplified insert DNA fragment is a single band of the correct expected size by performing agarose gel electrophorsis using 5 µl of the PCR product.

iii) PCR product purification

For DNA which is amplified and appears as a single band, purification using SUPREC™-02 (Cat. # 9041) is suggested. When multiple PCR products are generated, first isolate the band of interest from the agarose gel and then further purify using SUPREC™-01 (Cat.#9040) or TaKaRa RECOCHIP (Cat.#9039) or other similar method.

iv) Restriction enzyme digestion of amplified products

Digest the purified insert DNA with *Nde* I and *Xho* I restriction enzymes.

1) Prepare the following restriction enzyme digest mixture:

Insert DNA; 0.5-1 µg	X µl
10x K Buffer	3 µl
Nde I (10 units/ul)	1 µl
Xho I (10 units/ul)	1 µl
Sterilized distilled water	X µl
Total	30 µl

2) Incubate at 37°C for 1 hour.
3) Ethanol precipitate the digested DNA to purify it.*
4) Verify fragment purity using agarose gel electrophoresis or by measuring absorbance (OD_{260}).

* Both *Nde* I and *Xho* I can be inactivated by ethanol precipitation. However, when restriction enzymes which are not completely inactivated by ethanol precipitation are used, the digestion reaction should be treated with phenol. In addition, further purification and recovery of digested DNA by agarose gel electrophoresis can completely remove all short fragments generated by the digestion.

[Ethanol precipitation protocol]

1) Add 3M sodium acetate, pH 5.2, to the restriction enzyme digest mixture in a 1:10 ratio (e.g. 3 µl 3M sodium acetate added to 30 µl digest mixture), and mix well.
2) Add 2-2.5 times the volume of 100% cold ethanol to the above solution (e.g. add 66 µl 100% cold ethanol to 33 µl sodium acetate-digest mixture), and mix well. Chill at -20°C for 30 minutes.
3) Centrifuge at 4°C, 12,000 rpm, for 10-15 minutes. Discard the supernatant.
4) Add 70% cold ethanol and centrifuge again at 4°C, 12,000 rpm, for 5 minutes.
5) Discard the supernatant and air dry.
6) Dissolve the precipitate in 10-50 µl of TE buffer.

b) Restriction Enzyme Digestion of pCold TF DNA

Digest pCold TF DNA with the same restriction enzymes that were used for the digestion of amplified insert DNA, and purify. Dissolve the purified DNA in TE buffer, and measure the DNA concentration by measuring absorbance.

i) Prepare the following reaction mixture:

pCold TF DNA	1 µg
10 X K Buffer	3 µl
Nde I (10 units/µl)	1 µl
Xho I (10 units/µl)	1 µl
Sterilized distilled water	X µl
Total	30 µl

ii) Incubate at 37°C for 1-2 hours.
iii) Ethanol precipitate the digested vector DNA to purify.
iv) Dissolve the precipitated vector DNA pellet in TE buffer.
v) Measure the absorbance (OD260) and calculate the DNA concentration. For dsDNA (double-stranded DNA), calculate the DNA concentration assuming 1 OD_{260} = 50 µg/ml.
vi) Adjust the DNA concentration to100 ng/µl.

* After digestion with restriction enzymes, the vector DNA may be de-phosphorylated with *E. coli* Alkaline Phosphatase (BAP)(Cat.# 2120A), or Calf Intestinal Alkaline Phosphase (CIAP (Cat.# 2250A). Note that de-phosphorylation is essential if only a single restriction enzyme was used for digestion. In addition, complete removal of short fragments generated by restriction enzyme digestion is recommended. Purify the vector from any resulting short fragments using agarose gel electrophoresis, then further isolate and purify the vector from the gel.

c) Ligation of the DNA fragment and pCold TF DNA vector and transformation

i) Ligation reaction

Mix together the digested pCold TF DNA and the insert DNA fragment, and use this mixture for performing a ligation reaction using Takara's DNA Ligation Kit <Mighty Mix> Cat.# 6023). A 1:3-1:10 molar ratio of vector:insert DNA is recommended.

Prepare the following ligation reaction mixture on ice:

Digested pCold TF DNA ; 100 ng (- 0.03 pmol)	1 µl
Insert DNA fragment (0.1-0.3 pmol)	4 µl
Ligation Mix (from DNA Ligation Kit <MightyMix>)	5 µl
Total	10 µl

Incubate at 16°C for 1 hour.

ii) Transformation

Transform 100 µl *E. coli* JM109 Competent Cells (Cat.# 9052) with 10 µl ligated DNA mixture. Plate transformed cells on LB-ampicillin agar (100 µg/ml ampicilin) and grow at 37°C overnight.

1) Thaw E. coli JM109 competent cells on ice just before use.
2) Add 10 ul ligated DNA mixture to 100 µl competent cells, and mix gently.
3) Chill on ice for 30 minutes.
4) Incubate at 42°C for 45 seconds.
5) Chill on ice for 1-2 minutes.
6) Add warm (37°C) SOC medium to a final volume of 1 ml.
7) Shake at 37°C for 1 hour.
8) Plate on LB-ampicillin agar (100 µg/ml ampicilin) and incubate at 37°C overnight .

d) Plasmid preparation and verification

Inoculate a colony obtained in Step 4 ii) above into LB-ampicillin broth (100 µg/ml ampicilin) and incubate with gentle shaking at 37°C overnight. Use the resulting culture for plasmid maxi- or mini-preps (Takara's Mini Prep DNA Purification Kit [Cat.# 9085] is recommended).

After obtaining isolated plasmid DNA, digest the plasmid with the restriction enzymes *Nde* I and *Xho* I. Verify insertion of the correct DNA fragment by checking insert DNA fragment size using agarose gel electrophoresis.

When the vector construct has been verified, confirm the sequence of the inserted DNA fragment by sequencing analysis. This plasmid can be used as an expression plasmid for subsequent experiments. Sequencing primers should be designed such that they lie upstream and downstream of multicloning site by 50-100 bases. The following primer can be used as a downstream primer:

Downstream primer: pCold-R 5'-GGCAGGGATCTTAGATTCTG

X. Related Products

< *E.coli* Competent Cells >

E. coli DH5α Competent Cells (TaKaRa Cat.#9057)
E. coli HB101 Competent Cells (TaKaRa Cat.#9051)
E. coli JM109 Competent Cells (TaKaRa Cat.#9052)
E. coli DH5α Electro-Cells (TaKaRa Cat.#9027)
E. coli HB101 Electro-Cells (TaKaRa Cat.#9021)
E. coli JM109 Electro-Cells (TaKaRa Cat.#9022)

< Other >

IPTG (Isopropyl-β-D-thiogalactopyranoside) (TaKaRa Cat.#9030)
Chaperone Plasmid Set (TaKaRa Cat.# 3340)
pCold DNA vector series (TaKaRa Cat.# 3360-3364)

XI. References

1) Qing, G. *et al* (2004) *Nature Biotechnology* **22**, 877-2004.
2) Gerlined, S., *et al* (1995) *EMBO J.***14**, 4939-4948.

NOTE: (1) For research use only. Not for use in therapeutic or diagnostic use.

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