Table of Contents

Purchaser's Agreement

Customer's order of SPP System™ will be accepted only when the Purchaser's Agreement is signed by a customer and is attached with an order.

- SPP System™ (hereinafter "PRODUCTS") are covered by U.S. Patents No. 6479260, which are owned by TAKARA BIO, and the U.S. Patents, 5981280, 6686174, 6333191, and other pending patent which are owned and issued by University of Medicine & Dentistry of New Jersey and are exclusively licensed to TAKARA BIO.
- HisTag sequences contained in pCold I, II DNA are covered by U.S. Patents No. 5284933 and 5310663 which are owned by Hoffmann-La Roche Inc. and are licensed to TAKARA BIO.
- When a customer uses SPP System™, component of SPP System™, derivatives of SPP System™, or products obtained through SPP System™ for commercial purposes other than internal research, commercialization license with TAKARA BIO shall be required.
  For this Agreement, please contact to TAKARA BIO's subsidiaries or local distributors.

I. Description

A Single Protein Production system (SPP system™) was proposed by Dr. M. Inouye's group at Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey. This system utilizes an *E. coli* protein MazF which was found by the same group and it was refered to as an mRNA Interferase™. The protein was determined to be a sequence-specific endoribonuclease which cleaves single strand RNAs at ACA sequences. In this system, the transcript of interest which should not contain any ACA sequences (i.e. ACA-less) , and MazF are co-expressed in a host *Escherichia coli*. Therefore the MazF does not cleave the transcript of interest, but cleave the ones derived from the host proteins or others at ACA sequences. So, only the transcript of interest is dominantly translated and only the protein of interest is dominantly expressed, with the SPP (Figure1). Instead of their dominant expression, some proteins of interest are expressed in lesser amount with this system than in other expression systems, for example, in Cold-shock expression vector system alone.

In order to construct SPP system, it is first necessary to prepare an ACA-less gene of interest by chemical synthesis or site-directed mutagenesis technology, etc. In designing the ACA-less gene, every ACA sequence in the gene of interest must be substituted to another keeping its amino acid sequence, and restriction sites for cloning must be also added to be in frame. Secondly the ACA-less gene is cloned into a ACA-less region of the pCold (SP-4) vector which has ACA-less transcription region. At this point the plasmid becomes ready for SPP of interest. Then a host *E. coli* is co-transformed with this expression plasmid for SPP and pMazF (Figure3) which is ready to express MazF in adequate quantity. The transformant with the both plasmids generates the SPP of interest.

Takara's SPP System ineludes four types of Cold-shock expression vectors for SPP, pCold I (SP-4), pCold II (SP-4), pCold III (SP-4) and pCold IV (SP-4), which vary in the existences of TEE (translation enhancing element), 6*His-Tag, and Factor Xa cleavage site (table & Figure4). pColdIV (SP-4) has no additional sequence at the upstream of the MCS.

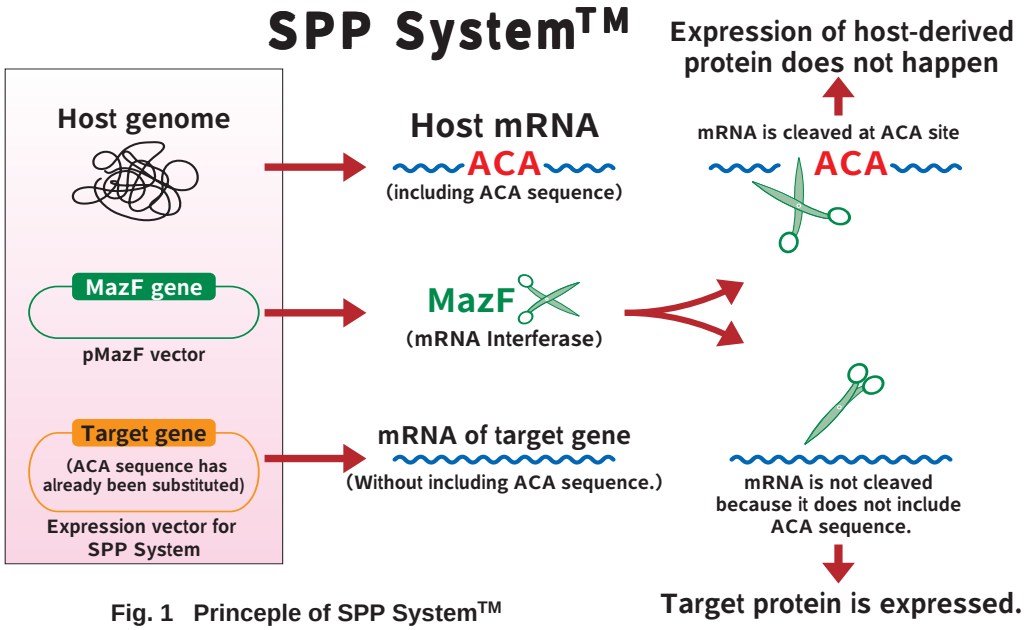


Fig. 1 Princeple of SPP System™

II. Components:

SPP System™ Set (Cat.# 3366)

1. Cold Shock Expression vectior for SPP System™ pCold™ I (SP-4) DNA, pCold™ II (SP-4) DNA, pCold™ III (SP-4) DNA, pCold™ IV (SP-4) DNA		ea. 20 µg (0.5 µg/µl)
2. MazF (mRNA Interferase™) expression plasmid	pMazF DNA	0.5 µg (20 ng/µl)
3. Positive Control pCold™ I (SP-4) envZB DNA		0.2 µg (20 ng/µl)

Expression plasmid prepared by inserting ORF of *E. coli*-derived protein envZB without ACA sequence into pCold™ I (SP-4) DNA
Estimated molecular weight of expressed protein 19.6 kDa

SPP System™ I (Cat.# 3367)

1. Cold Shock Expression vectior for SPP System™ pCold™ I (SP-4) DNA		20 µg (0.5 µg/µl)
2. MazF (mRNA Interferase™) expression plasmid	pMazF DNA	0.5 µg (20 ng/µl)
3. Positive Control pCold™ I (SP-4) envZB DNA		0.2 µg (20 ng/µl)

SPP System™ II (Cat.# 3368)

1. Cold Shock Expression vectior for SPP System™ pCold™ II (SP-4) DNA		20 µg (0.5 µg/µl)
2. MazF (mRNA Interferase™) expression plasmid	pMazF DNA	0.5 µg (20 ng/µl)
3. Positive Control pCold™ I (SP-4) envZB DNA		0.2 µg (20 ng/µl)

SPP System™ III (Cat.# 3369)

1. Cold Shock Expression vectior for SPP System™ pCold™ III (SP-4) DNA		20 µg (0.5 µg/µl)
2. MazF (mRNA Interferase™) expression plasmid	pMazF DNA	0.5 µg (20 ng/µl)
3. Positive Control pCold™ I (SP-4) envZB DNA		0.2 µg (20 ng/µl)

SPP System™ IV (Cat.# 3370)

1. Cold Shock Expression vectior for SPP System™ pCold™ IV (SP-4) DNA		20 µg (0.5 µg/µl)
2. MazF (mRNA Interferase™) expression plasmid	pMazF DNA	0.5 µg (20 ng/µl)
3. Positive Control pCold™ I (SP-4) envZB DNA		0.2 µg (20 ng/µl)

< Available *E.coli* expression strains >

As Cold Shock Expression vectors for SPP™ System utilize *csp*A promoter derived from *E.coli*, almost all *E.coli* strains can be used as a host for expression. However, pMazF DNA used for co-expression utilizes chloramphenicol as a selection marker, chloramphenicol-resistant *E.coli* strains, e.g. Rossetta™ (Novagen), cannot be used as a host.

III. Vector map:

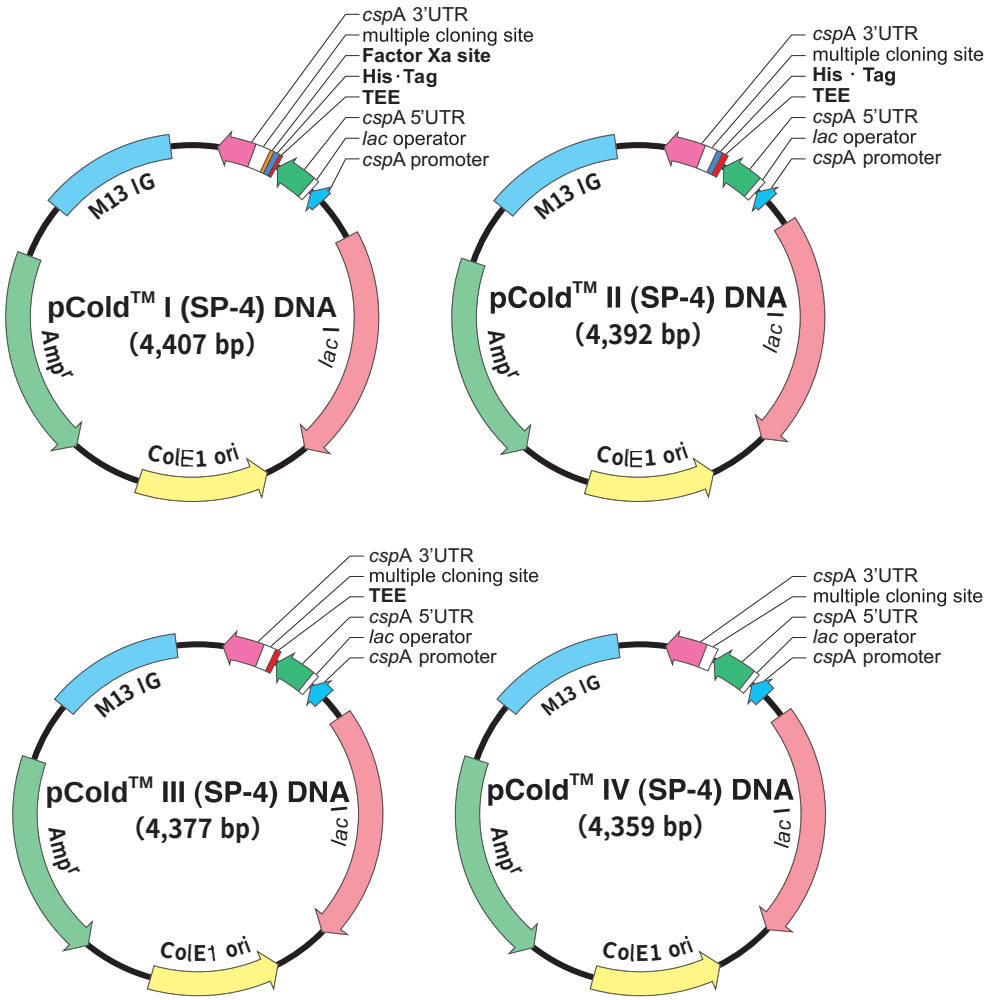


Fig. 2 Vector map of pCold I ~ IV (SP-4) DNAs

Vector	TEE Sequence	His-Taq Sequence	Factor Xa cleavage site
pCold™ I (SP-4) DNA	○	○	○
pCold™ II (SP-4) DNA	○	○	—
pCold™ III (SP-4) DNA	○	—	—
pCold™ IV (SP-4) DNA	—	—	—

Table: Various tags of pCold (SP-4) DNAs

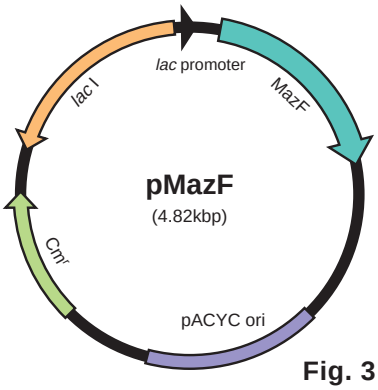


Fig. 3 Vector map of pMazF

IV. Form: 10mM Tris-HCl (pH8.0), 1mM EDTA solution

V. Purity: 1. Contains over 70% double-stranded covalently closed circular DNA (RF I)

For pCold™ (SP-4) DNA series:
2. Confirmed to maintain cloning sites by dideoxy sequencing method
3. Confirmed to be cleaved at a single site by restriction enzymes *Nde* I, *Sac* I, *Kpn* I, *Xho* I, *Bam*H I, *Eco*R I, *Hin*d III, *Sal* I, *Pst* I and *Xba* I.

VI. Storage: -20°C

VII. Protocol: 1. Construction of ACA-less gene expression plasmid

1) Design an ACA-less gene of interest by substituting every ACA sequence in its original sequence to another while keeping its amino acid sequence. This ACA-less gene may also have restriction sites at the each end to make it in frame when cloned in pCold (SP-4).
2) Synthesis and clone the ACA-less gene designed above by chemical synthesis or site-directed mutagenesis.
3) Prepare the ACA-less gene fragment from the clone in 2) and clone it into a pCold I ~ IV (SP-4) DNAs to obtain the ACA-less gene expression plasmid.

2. Building up SPP system

Transform a host *E. coli* with both components, pMazF and the ACA-less gene expression plasmid constructed above. The selection marker of for the pMazF, MazF expression plasmid is chloramphenicol resis tance and this transformant is resistant to both ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml).

50ng of each of the both plasmids is enough to use for transformation.

Do not keep the transformant obtained under cool condition until use. A promoter of a cold-shock protein *csp*A is utilized on in pCold (SP-4) to express the protein of interest and this promoter functions under cool condition.

Note that this system cannot be combined with chloramphenicol-resistant *E. coli* host strains or plasmids carrying chloramphenicol-resistance gene.

E. coli BL21 is a good candidate host strain appropriate for this system.

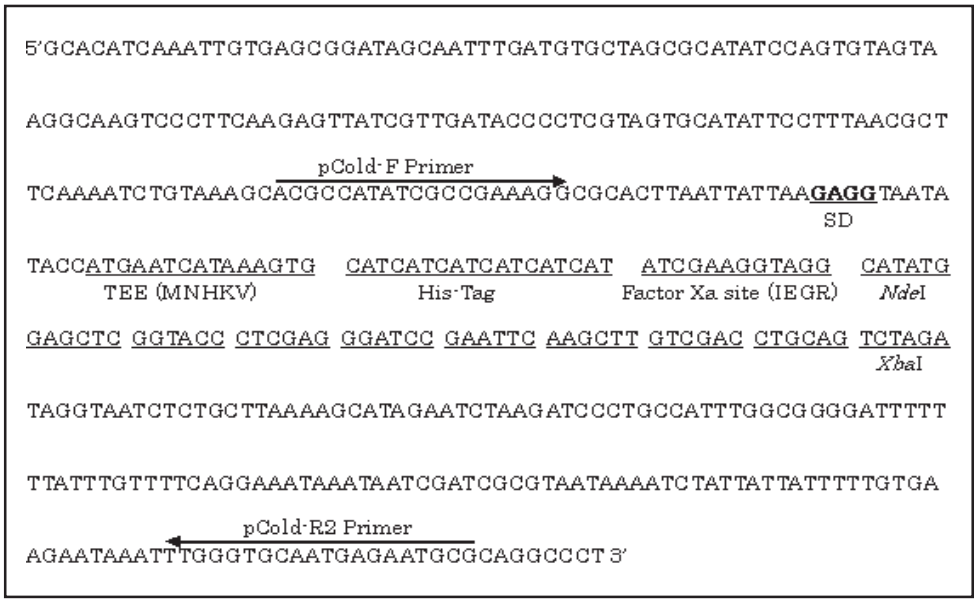
TaKaRa *E. coli* BL21 Competent Cell (Cat.#9126) is available.

3. SPP of interest: Pulse-labeling in M9-glucose medium culture
   1) Inoculate the transformant obtained at Section 2 into M9-glucose medium previously warmed at 37°C containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). Cultivate it shaking at 37°C and monitor its OD at 600nm.
   2) When the OD at 600 nm reaches about 0.5, cool down the culture to 15°C and leave it for 45minutes under this condition.
   3) Add IPTG (isopropyl-β-D-thiogalactoside) into the culture to be a final concentration of 1mM. And then cultivate it shaking at 15°C for 24hours.
   4) Incubate by shaking at 15°C for 24 hours.
   5) Add [^{35}S]-methionine to the culture and leave it at 15°C for 15 mins.
   6) Harvest these isotopic labeled cells and prepare them as a sample to be analyzed by autoradiography following SDS-PAGE.

   * LB medium contains a slight amount of some kind of an inducer for expression controlled by lac operator. Minimum media or M9-glucose medium is recommended to avoid this affect strictly.

VIII. Multicloning sites of pCold™ I ~ IV (SP-4) DNAs:

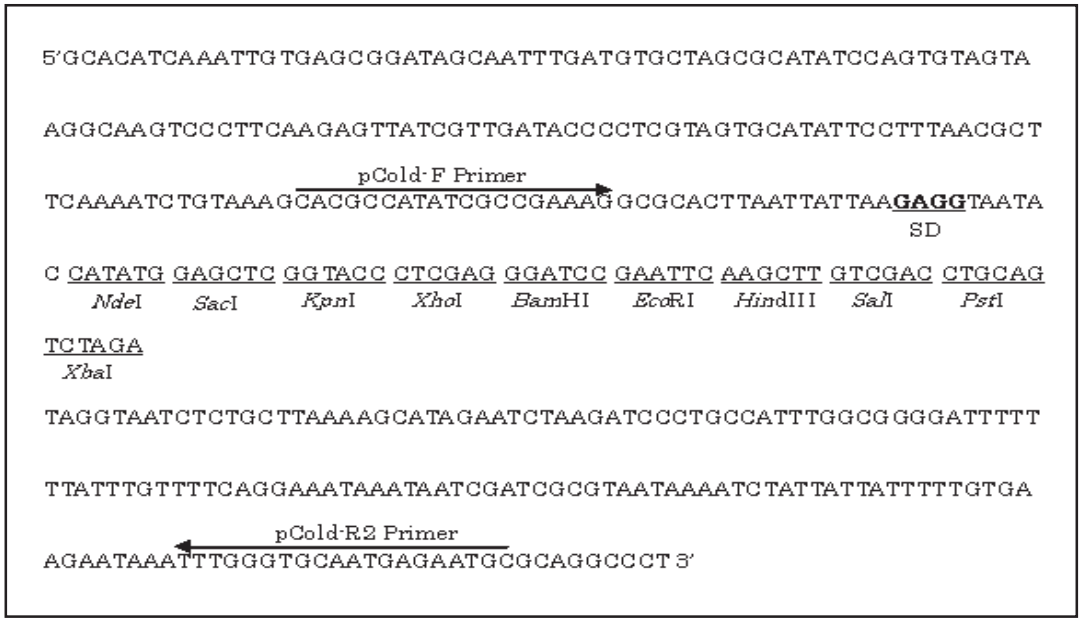
pCold™ I (SP-4) DNA



pCold™ II (SP-4) DNA

pCold™ III (SP-4) DNA

pCold™ IV (SP-4) DNA



IX. Application Example:

Expression of *E. coli* envZB with SPP system

1. Pulse labeling in M9-glucose medium culture

E. coli BL21 transformant with Positive Control pColdI (SP-4) envZB was tested with or without the effect of MazF. The pulse labeling results much difference in the background level derived from the synthesis of host pro teins. SPP system showed extremely low level of background. (Fig. 4)

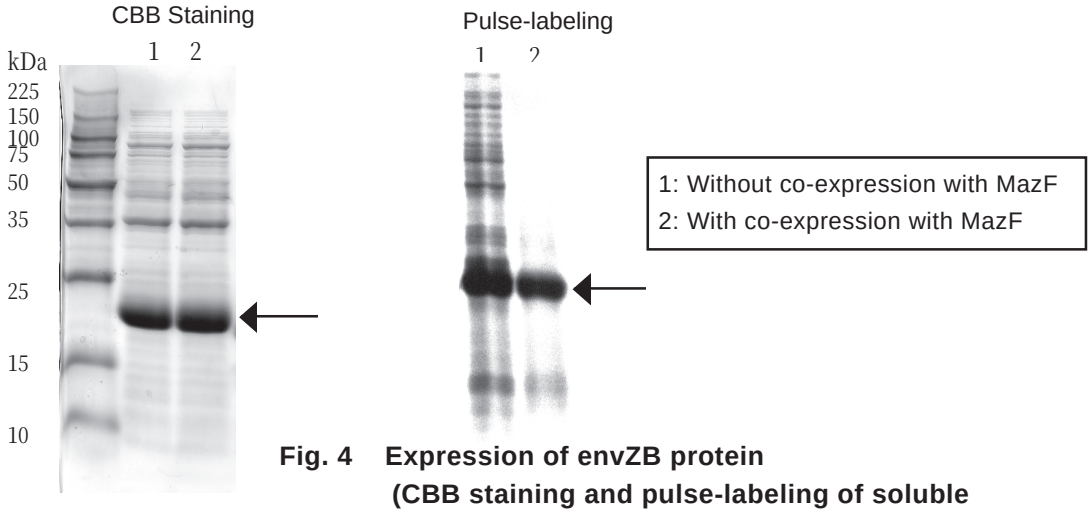


Fig. 4 Expression of envZB protein (CBB staining and pulse-labeling of soluble fraction derived from strain.)

2. *E. coli* envZB expression in LB medium culture

E. coli BL21 transformant with Positive Control pColdI (SP-4) envZB was tested with or without MazF co-expression.

*1: The whole process was proceeded by following the protocol, and the amount of 0.1 at OD_{600} was applied to gel electrophoresis. There was not significant difference in the production level with the protein of of interest between the transformant with pMazF (SPP system) and the one with a plasmid carrying no MazF gene. (Fig. 5)

*2: In the system of MazF co-expression, the expression of new protein except target protein is suppressed. This may result in growth stress against *E.coli* itself. Accordingly the expression yield of a target protein may decrease compared than general expression with pCold DNAs.

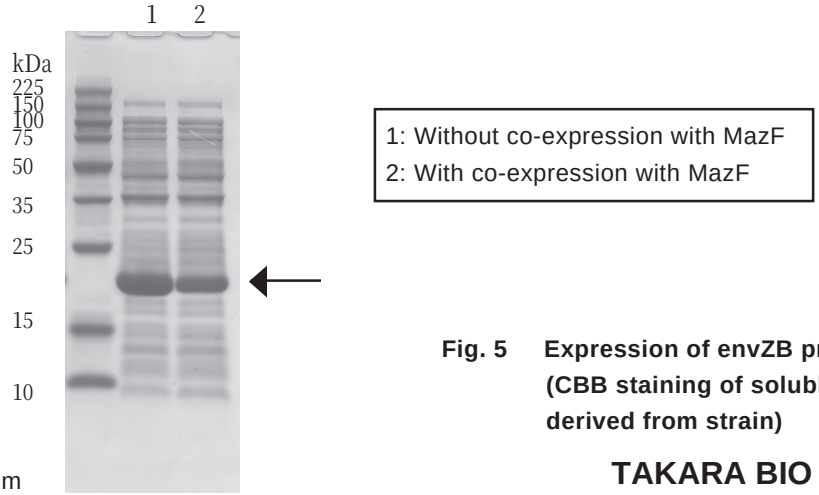


Fig. 5 Expression of envZB protein (CBB staining of soluble fraction derived from strain)

X. Q&A:

Q1. How is it possible to make more soluble the protein of interest more soluble which iswhen expressed in the insoluble form?

A1. Possible strategies are:
- Changing the timing to add IPTG to induce the expression (Some examination may be needed between early and late logarithmic phases of the culture.)
- Reducing the concentration of IPTG (down to 0.1mM)
- Changing the strain of host *E. coli*
- Extracting the cultured cells by sonication in a buffer which contains 0.1 to 1% of detergent (for example, octylglycoside, NP-40, Triton X-100, etc.)

Q2. What How to selects the type of Cold-shock expression vector for SPP to use?

A2. TEE facilitates translation of interest when using pCold™ I (SP-4), pCold™ II (SP-4) and pCold™ III (SP-4). Proteins expressed using pCold™ I (SP-4) and pCold™ II (SP-4) can be purified with Ni columns by means of its affinity to 6* His-Tag. If you do not desire to attach any excess amino acid sequences to the N-terminus of the protein of interest, it is recommended to use pCold™ I (SP-4) that allows cleavage of the Tag sequence with Factor Xa, or pCold™ IV (SP-4) that does not possess TEE nor Tag sequences.

Q3. What quantity of the protein of interest is gained for from 1L of culture?

A3. The expression level usually ranges from several mg to several tens of mg/L, although it is different for each protein of interest. An approximately 3-L culture can recover purified proteins in the mg scale, if the protein of interest can be detected by SDS-PAGE followed by CBB staining
In the system of MazF co-expression, the expression of new protein except target protein is suppressed. This may result in growth stress against *E.coli* itself. Accordingly the expression yield of a target protein may decrease com pared than general expression with pCold™ DNAs.

XI. References:

1. Suzuki, M. *et al.* (2005) *Molecular Cell* **18**, 253-261
Single Protein Production in Living Cells Facilitated by an mRNA Interferase
2. Zhang, Y. *et al.* (2004) *Journal of Biological Chemistry* **280**, 3143-3150
Insights into the mRNA cleavage mechanism by MazF, an mRNA interferase.
3. Zhang, Y. *et al.* (2003) *Molecular Cell* **12**, p913-923
MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*.
4. Qing, G. *et al.* (2004) *Nature Biotechnology* **22**, p877-882
Cold-shock induced high-yield protein production in *Escherichia coli*.

XII. Related Products:

1. mRNA Interferase™-MazF (Cat.#2415A)
2. pCold™ Vector Series (Cat.#3360, 3361, 3362, 3363, 3364)
3. TaKaRa Competent Cell BL21 (Cat.#9126)
4. IPTG (Cat.#9030)

XIII. License Notice:

* The use of this product is limited for research purposes. It must not be used for clinical purpose or in vitro diagnosis. Users must contact TAKARA when they plan to use this product for purposes other than research use.
* mRNA Interferase™ technology is sold under exclusive patent license from University of Medicine and Dentistry of New Jersey.
* pCold Vectors 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 and Dentistry of New Jersey and are exclusively licensed to TAKARA BIO. As a condition of the sales of the products, PUR CHASER should agrees to all of the Terms and Conditions in the purchaser's agreement, If PURCHASER agrees to the purchaser's agreement, please complete the agree ment and send it together with a purchase order.
* Protein Purification Technology of His•Tag used in some of pCold vectors is licensed from Hoffmann-La Roche, Inc., Nutley, NJ and/or Hoffmann-La Roche Ltd., Basel, Switzerland and is provided only for the use in resaerch. Information about licenses for commercial use is available from QIAGEN GmbH, Qiagen Strasse 1, D-40724 Hilden, Germany.

NOTE: For research use only.
Not for use in human and animal diagnostic or therapeutic.