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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.
- HisTag sequences contained in pCold™ I, II and TF 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.
- PRODUCTS are for research or laboratory use only. PURCHASER understands and agrees that PRODUCTS shall not be administered to humans or animals, and shall not be used for pharmaceutical, in vitro diagnostic, or any commercial purposes other than internal research.
- PURCHASER shall not modify pCold™ Vector DNA sequences located between and including the CspA 3’UTR and CspA Promoter. The adjacent multicloning site (MCS) is exempt from this restriction.
- PURCHASER shall not utilize any partial sequences from PRODUCTS for the purpose of new plasmid construction using the Cold Shock Expression System.
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URL: http://www.takara-bio.com
I. Description

Elucidation of protein structure and function is an important subject for post-genome study and an efficient protein production system is an indispensable fundamental technology to study these subjects. Expression systems with *E. coli* as host are extensively used in the production of recombinant proteins. *E. coli* expression systems have advantages of ease to use and low cost. For some genes, however, expression is difficult and expressed proteins are insoluble.

In order to solve these problems, TaKaRa conducted a joint research with Professor Masayori Inouye (University of Medicine and Dentistry of New Jersey, USA) to develop an efficient protein expression vector based on the low-temperature expression gene (cold-shock gene) of *E. coli*. This product, pCold™ DNA Series has the above advantages and provides an important tool for functional and structural analyses as well as other areas in protein research.

< Product overview and features >

When the culture temperature of *E. coli* is reduced sufficiently, the growth is temporarily halted and almost of protein expression decrease, while expression of a group of proteins called "cold-shock proteins" is specifically induced. Cold-shock expression vectors, pCold™ DNA I-IV, are designed to perform efficient protein expression utilizing the promoter derived from *cspA* gene, which is one of the cold-shock genes. At the downstream of the *cspA* promoter, lac operator is inserted so that the expression is strictly controlled. In addition, 5' untranslated region (5' UTR), translation enhancing element (TEE), His-Tag sequence, Factor Xa cleavage site, and multicloning site (MCS) are located at the downstream of the *cspA* promoter. As this product utilizes the promoter derived from *E. coli*, most *E. coli* strains can be utilized as an expression host. There are four kinds of pCold™ vectors, whose arrangements vary in the existence of TEE, His Tag Sequence and Factor Xa cleavage site.

II. Components

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Code</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCold™ Vector Set</td>
<td>3360</td>
<td>1 Set</td>
</tr>
<tr>
<td>1) pCold™ I DNA</td>
<td>3361</td>
<td>25 μg</td>
</tr>
<tr>
<td>2) pCold™ II DNA</td>
<td>3362</td>
<td>25 μg</td>
</tr>
<tr>
<td>3) pCold™ III DNA</td>
<td>3363</td>
<td>25 μg</td>
</tr>
<tr>
<td>4) pCold™ IV DNA</td>
<td>3364</td>
<td>25 μg</td>
</tr>
</tbody>
</table>

<Available *E. coli* host strains>

Most *E. coli* strains can be used as an expression host for pCold™ DNA series, because these vectors utilize the promoter of a cold-shock gene, *cspA*, derived form *E. coli*. 

URL: http://www.takara-bio.com
Cold Shock Expression System pCold™ DNA

### III. Vector map

**Fig. 1 pCold™ Vector Map**

<table>
<thead>
<tr>
<th>Vector</th>
<th>TEE</th>
<th>His Tag</th>
<th>Factor Xa Cleavage Site</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCold™ I DNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>AB186388</td>
</tr>
<tr>
<td>pCold™ II DNA</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>AB186389</td>
</tr>
<tr>
<td>pCold™ III DNA</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>AB186390</td>
</tr>
<tr>
<td>pCold™ IV DNA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>AB186391</td>
</tr>
</tbody>
</table>

URL: http://www.takara-bio.com
**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 - 100 μg/ml of ampicillin, and culture at 37°C with shaking.
4) At OD₆₀₀= 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. Moreover, when the expressed protein is insoluble, the combined use with Chaperone Plasmid Set (Cat. #3340) is effective.

**VI. Multiple cloning site**

pCold™ I DNA (Cat. #3361)
pCold™ II DNA (Cat. #3362)

\[
\begin{align*}
\text{pCold-F Primer} & \\
5' & \text{TAACGCTTCAAAATCTGTAAAGC} \\
\text{SD} & \text{TEE His \cdot Tag} \\
& \text{Met Asn His Lys Val His His His His}
\end{align*}
\]

CACGCCATATCGCAGGAAGGACCACCTTAATTATTAAGGTAATACCCATGCACTAAAGG
CATATG GAGCTC GGTACC CTGGAG GGATCC GAATTC AAGCTT GTCGAC CTGCAG TCTAGA
TAGGTAATCTCTGCT
TAAAAGCACAGAATCTAAGCATCCCTGCGGCGGATTTTTTATTTTTTACAGGAATAAAATAATCGAT 3'
transcription terminator

\[
\begin{align*}
pCold-R Primer & \\
5' & TAACGCTTCAAAATCTGTAAAGC \\
\text{SD} & \text{TTE His • Tag} \\
& \text{Met Asn His Lys Val His His His His}
\end{align*}
\]

\[
\begin{align*}
\text{Nde I Sac I Kpn I Xho I BamH I EcoR I Hind III Sal I Pst I Xba I} \\
\text{CATATG GAGCTC GGTACC CTGGAG GGATCC GAATTC AAGCTT GTCGAC CTGCAG TCTAGA TAGGTAATCTCTGCT}
\end{align*}
\]

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

\[
\begin{align*}
\text{pCold-F Primer} & \\
5' & AAAATCTGTAAAGCACGCCATATCGCAGGAAGGACCACCTTAATTATTAAGGTAATACCCATGCACTAAAGG
\text{SD} & \text{TTE His • Tag} \\
& \text{Met Asn His Lys Val His His His His}
\end{align*}
\]

\[
\begin{align*}
pCold-R Primer & \\
5' & AAAATCTGTAAAGCACGCCATATCGCAGGAAGGACCACCTTAATTATTAAGGTAATACCCATGCACTAAAGG
\text{SD} & \text{TTE His • Tag} \\
& \text{Met Asn His Lys Val His His His His}
\end{align*}
\]

\[
\begin{align*}
\text{Nde I Sac I Kpn I Xho I BamH I EcoR I Hind III Sal I Pst I Xba I} \\
\text{CATATG GAGCTC GGTACC CTGGAG GGATCC GAATTC AAGCTT GTCGAC CTGCAG TCTAGA TAGGTAATCTCTGCT}
\end{align*}
\]

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

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\end{align*}
\]

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5' & AAAATCTGTAAAGCACGCCATATCGCAGGAAGGACCACCTTAATTATTAAGGTAATACCCATGCACTAAAGG
\text{SD} & \text{TTE His • Tag} \\
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\end{align*}
\]

\[
\begin{align*}
\text{Nde I Sac I Kpn I Xho I BamH I EcoR I Hind III Sal I Pst I Xba I} \\
\text{CATATG GAGCTC GGTACC CTGGAG GGATCC GAATTC AAGCTT GTCGAC CTGCAG TCTAGA TAGGTAATCTCTGCT}
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\text{SD} & \text{TTE His • Tag} \\
& \text{Met Asn His Lys Val His His His His}
\end{align*}
\]

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5' & AAAATCTGTAAAGCACGCCATATCGCAGGAAGGACCACCTTAATTATTAAGGTAATACCCATGCACTAAAGG
\text{SD} & \text{TTE His • Tag} \\
& \text{Met Asn His Lys Val His His His His}
\end{align*}
\]

\[
\begin{align*}
\text{Nde I Sac I Kpn I Xho I BamH I EcoR I Hind III Sal I Pst I Xba I} \\
\text{CATATG GAGCTC GGTACC CTGGAG GGATCC GAATTC AAGCTT GTCGAC CTGCAG TCTAGA TAGGTAATCTCTGCT}
\end{align*}
\]

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

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\begin{align*}
\text{pCold-F Primer} & \\
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\text{SD} & \text{TTE His • Tag} \\
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\end{align*}
\]

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\begin{align*}
pCold-R Primer & \\
5' & AAAATCTGTAAAGCACGCCATATCGCAGGAAGGACCACCTTAATTATTAAGGTAATACCCATGCACTAAAGG
\text{SD} & \text{TTE His • Tag} \\
& \text{Met Asn His Lys Val His His His His}
\end{align*}
\]

\[
\begin{align*}
\text{Nde I Sac I Kpn I Xho I BamH I EcoR I Hind III Sal I Pst I Xba I} \\
\text{CATATG GAGCTC GGTACC CTGGAG GGATCC GAATTC AAGCTT GTCGAC CTGCAG TCTAGA TAGGTAATCTCTGCT}
\end{align*}
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His Met Glu Leu Gly Thr Leu Glu Gly Ser Glu Phe Lys Leu Val Asp Leu Gln Ser Arg End

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\text{pCold-F Primer} & \\
5' & AAAATCTGTAAAGCACGCCATATCGCAGGAAGGACCACCTTAATTATTAAGGTAATACCCATGCACTAAAGG
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\text{SD} & \text{TTE His • Tag} \\
& \text{Met Asn His Lys Val His His His His}
\end{align*}
\]
VII. Application

As described below, expression of genes that showed poor expression level or poor solubility in T7 promoter expression system were attempted with the cold-shock expression system. The pCold™ I DNA was used as a cold-shock vector and E. coli BL21 strain was used as a host for expression. Expression from T7 promoter-driven vectors was conducted with the common procedure of adding IPTG and culturing at 37℃.

(1) The expression became possible.

For human gene A (estimated molecular weight: 31 kDa), expression was not observed in the T7 expression system, but was observed in the cold-shock expression system (Fig. 2).

(2) The expression level increased.

For thermophile gene B (estimated molecular weight: 30 kDa), solubility was improved and expression level was increased compared to the T7 expression system (Fig. 3).

Expression level and solubility was improved.

---

Fig. 2  Expression of human gene A was compared using pCold™ and T7 Vector followed by CBB staining.

Fig. 3  Expression of thermophile gene B was compared with total protein and soluble protein using pCold™ and T7 Vector followed by CBB staining.
(3) The soluble expression level was increased.

For human gene C (estimated molecular weight: 80 kDa), expression was mostly insoluble in the T7 expression system. In the cold-shock expression system, however, the expression level of soluble fraction was increased remarkably (Fig. 4). Cold-shock expression systems are expected to improve the expression level and solubility of the target protein compared to the T7 expression system.

Cold Shock Expression System pCold™ DNA

(4) Comparative study with pulse-labeling experiment

Human gene D (estimated molecular weight: 12 kDa) was pulse-labeled to compare both expression systems (Fig. 5). In the T7 expression system, E. coli proteins other than the target protein were also labeled. In contrast, most of labeled proteins in the cold-shock expression system were the expression product of the target gene, indicating that the expression of the target gene was specifically induced.

Fig. 4 Expression of human gene C was compared using pCold™ and T7 Vector in soluble or insoluble fraction.

Fig. 5 Pulse labeling of human gene D
Q1 Why does pCold™ DNA allow efficient expression of proteins at low temperatures?

A1 pCold™ DNA, which was developed based on a cspA gene that encodes a cold shock protein, contains the cspA promoter, 5′ UTR, and the N-terminus region of CspA.

It is possible to achieve transcription from the cspA promoter at 37°C; however, translation is not efficient because of the extreme instability of the downstream 5′ UTR at 37°C. By lowering the temperature from 37°C to 15°C, the structure of 5′ UTR becomes highly stable. This results in improved translation efficiency, allowing extremely efficient protein synthesis at a low temperature (15°C).1)

Moreover, when the mRNA that encodes some of the N-terminus of CspA is formed by transcription, ribosomes are preferentially used for translation of the formed mRNA and rarely supplied for translation of other mRNAs (ribosome trapping)2).

As described above, pCold™ DNA enables extremely efficient protein expression at low temperatures by virtue of a cspA promoter that does not cause decrease in transcriptional activity at low temperatures, structural stability of 5′ UTR at low temperatures, and ribosome trapping. Although protein expression at low temperatures has been performed before, innovative features of pCold™ DNA provide a suitable, unique expression vector for protein expression at low temperatures.


Q2 What should be considered if no expression is observed?

A2 Consider the type of vector, host, culture, and induction conditions.
- Change the type of vector (pCold™ I to IV DNA). In some cases, proteins can be expressed by attaching the TEE or His-Tag sequence to the N-terminus.
- Examine the frequency of codon usage. Some genes are influenced by the frequency of codon usage. In some cases, the expression level can be improved by using commercially available strains of E. coli that support rare codons (such as Rosetta™2).
- Expression may be influenced by pre-culture and storage conditions of expression clones (see Q8).
- Consider the timing for cold-shock induction. The expression level sometimes decreases if induction is late. In this case, early induction may improve the expression level.
- The cooling process prior to addition of IPTG should be sufficient (normally 30 min or longer at 15°C). The culture process following addition of IPTG should also be performed at 15°C.
Q3 What should be considered if expressed proteins are insolubilized?

A3 Appropriate culture and induction conditions are different for each target protein. Consider culture and induction conditions, and the strain of *E. coli* used as a host and the extraction method in reference to the following recommendations:

- Change the timing for induction (examine between early and late logarithmic phases).
- Change the concentration of the inducer (IPTG) (0.1 to 1 mM).
- Consider the temperature and duration of culture following induction (15°C for 24 hours is usually appropriate).
- Consider the strain of host *E. coli* and use of chaperone. Try chaperone co-expression using the Chaperone Plasmid Set or commercially available strains of host *E. coli* (such as Origami™) that facilitate solubilization of expressed proteins.
- Change the extraction method. Some proteins are not sufficiently solubilized with commercially available *E. coli*-lysis agent. It is also effective to perform sonication with 0.1 to 1% of detergent (octylglycoside, NP-40, or Triton X-100).

Q4 What molecular weight range of proteins can be expressed?

A4 We have expressed proteins from several kDa to 100 kDa.

Q5 Which species of genes have been expressed so far?

A5 We have expressed genes of *E. coli*, thermophiles, hyperthermophiles, human, mouse, and plants.

Q6 What are the criteria for selecting pCold™ vectors?

A6 The TEE sequence, which is contained in pCold™ I, II, and III DNA, facilitates translation of the target gene. Proteins expressed using vectors with the His-Tag sequence (pCold™ I, II DNA) can be purified with Ni columns. If you do not desire to attach excess amino acids sequences to the N-terminus of the target protein, it is recommended to use pCold™ I DNA that allows cleavage of the Tag sequence with Factor Xa, or pCold™ IV DNA that does not possess the TEE and Taq sequences.

Q7 What is the expression level for 1 L of medium?

A7 The expression level usually ranges from several mg to several tens of mg/L, although it is different for each target gene. An approximately 3-L culture can recover purified proteins in the mg scale, if expression of the target protein can be detected by SDS-PAGE followed by CBB staining.

Q8 Is it possible to store *E. coli* that was transformed with the pCold™ vector containing a target gene on a plate at 4°C?

A8 It is not recommended to store *E. coli* on a plate at 4°C. If *E. coli* that was transformed with the pCold™ vector into which a target gene was inserted is stored on a plate at 4°C, the target protein may leak and the pCold™ vector cannot be maintained. It is recommended to remove the bacterium from the plate as soon as possible to prepare a glycerol stock and store the stock at -80°C.
Q9 How should one select from among the 5 chaperone plasmids to perform coexpression with the Chaperone Plasmid Set?

A9 Expression systems based on pCold™ vectors tend to produce better results by coexpression with chaperone teams containing the tig sequence. It is recommended to start by studying coexpression with pG-Tf2 or pTf16.

Q10 To what extent does OD$_{600}$ increase in the case of culture at 15°C for 24 hours after induction?

A10 OD$_{600}$ of culture using this system is different for each strain of host *E. coli* and each type of inserted target genes. OD$_{600}$ is around 1.2 using BL21 as a host.

Q11 Which strains of host *E. coli* have been used so far?

A11 We have expressed many protein using BL21 as a host. Origami™ and Rosetta™ from Novagen are also available. It is recommended to start with BL21, while most of *E. coli* strains can be used as hosts for pCold™ vectors that use the cspA promoter derived from *E. coli*.

Q12 The pCold™ vector expression system induces expression of target proteins by culturing at low temperatures. Why is IPTG added prior to induction?

A12 Since pCold™ vectors use the promoter of a cold-shock protein, they hardly express target proteins at 37°C intrinsically. However, some inserts induce a small amount of leakage. Therefore, we employed a regulatory system based on the lac operon supplementary.

In the case of pET vectors that turn expression on and off only on the addition of IPTG, whether expression is regulated or not without addition of IPTG is an important question. In the case of pCold™ vectors that control induction by changing temperature, whether expression is regulated by the lac operon or not after the temperature is lowered is not important. Nevertheless, it is necessary to add IPTG, since any regulation may cause inconvenience.

The strength of regulation by the lac operon is different for each type of host *E. coli*. It is considered that those that have lac Iq (such as JM109) and those that are regulated only with lac I (such as BL21) inherent to *E. coli* have different strengths of regulation. However, it may not necessary to select bacterial strains with lac Iq, since the strength of regulation after lowering the temperature is not important.
IX. Appendix

Expression Plasmid Construction - Example using the thioredoxin gene

1) Overview of pCold™ 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™ 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. Alternatively, In-Fusion™ HD PCR Cloning Kit (Clontech Lab., Inc.) is available for directional cloning easily and rapidly, in case without proper restriction enzyme site in a target gene also. 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™ 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™. "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™ Nde I/Xho I MCS restriction enzyme cloning sites

   \[ Nde I \text{ site: Primer 1 (normal direction primer)} \]

   \[ Nde I \]

   \[ 5'-\text{GCCGCATATGAGCGATTTATTTCAC} \]

   thioredoxin derived sequence*1

   \[ Xho I \text{ site: Primer 2 (reverse direction primer)} \]

   \[ Xho I \]

   \[ 5'-\text{GCCGCTCGAGTTAGGCCAGGTTAGCGTC} \]

   thioredoxin derived 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 PrimeSTAR® HS DNA Polymerase (Cat. #R010A) is recommended).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA (5 ng)*1</td>
<td>1</td>
</tr>
<tr>
<td>5X PrimeSTAR® Buffer*2</td>
<td>10</td>
</tr>
<tr>
<td>dNTP Mixture (2.5 mM each)*2</td>
<td>4</td>
</tr>
<tr>
<td>Primer 1 (10 - 50 pmol/μl)</td>
<td>1</td>
</tr>
<tr>
<td>Primer 2 (10 - 50 pmol/μl)</td>
<td>1</td>
</tr>
<tr>
<td>PrimeSTAR® HS DNA Polymerase (5 units/μl)</td>
<td>0.5</td>
</tr>
<tr>
<td>Sterilized distilled water</td>
<td>32.5</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
</tr>
</tbody>
</table>

* 1 : For plasmid DNA, use 1 - 10 ng; for cDNA or genomic DNA, use 50 - 500 ng.
* 2 : 5X PrimeSTAR® Buffer and dNTP Mixture is supplied with PrimeSTAR® HS DNA Polymerase (Cat. #R010A).

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

- 98℃, 10 sec.
- 55℃, 30 sec.
- 72℃, 1 min.

30 cycle

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, phenol/chloroform extraction is suggested to remove PrimeSTAR® HS DNA Polymerase. When multiple PCR products are generated, first isolate the band of interest from the agarose gel and then further purify using 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:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert DNA</td>
<td>0.5 - 1</td>
</tr>
<tr>
<td>10X K Buffer</td>
<td>3</td>
</tr>
<tr>
<td>Nde I (10 units/μl)</td>
<td>1</td>
</tr>
<tr>
<td>Xho I (10 units/μl)</td>
<td>1</td>
</tr>
<tr>
<td>Sterilized distilled water</td>
<td>up to 30</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
</tr>
</tbody>
</table>

2) Incubate at 37℃ 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\textsubscript{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 3 M 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.

c) Restriction Enzyme Digestion of pCold™ DNA

Digest pCold™ 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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCold™ Vector</td>
<td>1 μg</td>
</tr>
<tr>
<td>10 X K Buffer</td>
<td>3 μl</td>
</tr>
<tr>
<td>Nde I (10 units/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Xho I (10 units/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Sterilized distilled water</td>
<td>up to 30 μl</td>
</tr>
<tr>
<td>Total</td>
<td>30 μl</td>
</tr>
</tbody>
</table>

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 (OD\textsubscript{260}) and calculate the DNA concentration. For dsDNA (double-stranded DNA), calculate the DNA concentration assuming 1 OD\textsubscript{260} = 50 μg/ml.
vi) Adjust the DNA concentration to 100 ng/μl.

*: After digestion with restriction enzymes, the vector DNA may be de-phosphorylated with \textit{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.

URL:http://www.takara-bio.com
d) Ligation of the DNA fragment and pCold™ DNA vector and transformation

i) Ligation reaction
Mix together the digested pCold™ 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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested pCold™ DNA ; 100 ng (- 0.03 pmol)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Insert DNA fragment (0.1 - 0.3 pmol)</td>
<td>4 μl</td>
</tr>
<tr>
<td>Ligation Mix (from DNA Ligation Kit &lt;MightyMix&gt;)</td>
<td>5 μl</td>
</tr>
<tr>
<td>Total</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

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 ampicillin) and grow at 37°C overnight.

1) Thaw E. coli JM109 competent cells on ice just before use.
2) Add 10 μl 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 ampicillin) and incubate at 37°C overnight.

e) Plasmid preparation and verification
Inoculate a colony obtained in Step 4 ii) above into LB-ampicillin broth (100 μg/ml ampicillin) and incubate with gentle shaking at 37°C overnight. Use the resulting culture for plasmid maxi- or mini-preps.

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. The following primers' sequence can be used for sequencing.

Upstream primer :  pCold™-F 5'-ACGCCATATCGCCGAAAGG
Downstream primer :  pCold™-R 5'-GGCAGGGATCTTAGATTCTG

This primer pair cannot be used as PCR primers. When it is used in PCR reaction, the extra band of approximately 500 bp appears.
X. Related Products

< For soluble expression of recombinant protein >
Chaperone Competent Cells BL21 Set (Cat. #9120)
Chaperone Competent Cell pG-KJE8/BL21 (Cat. #9121)
Chaperone Competent Cell pGro7/BL21 (Cat. #9122)
Chaperone Competent Cell pKE7/BL21 (Cat. #9123)
Chaperone Competent Cell pG-Tf2/BL21 (Cat. #9124)
Chaperone Competent Cell pTF16/BL21 (Cat. #9125)
TaKaRa Competent Cell BL21 (Cat. #9126)
Chaperon Plasmid Set (Cat. #3340)

< E. coli Competent Cells >
E. coli HST08 Premium Competent Cells (Cat. #9128)
E. coli DH5α Competent Cells (Cat. #9057)
E. coli JM109 Competent Cells (Cat. #9052)
E. coli HST08 Premium Electro-Cells (Cat. #9028)
E. coli DH5α Electro-Cells (Cat. #9027)
E. coli JM109 Electro-Cells (Cat. #9022)

< Other >
IPTG (Isopropyl-β-D-thiogalactopyranoside) (Cat. #9030)
pCold™ TF DNA (Cat. #3365)
pCold™ ProS2 DNA (Cat. #3371)

XI. References

NOTICE TO PURCHASER: LIMITED LICENSE

[L13a] pCold™ vectors
This product is covered by the claims of U.S. Patent No. 5,981,280, 6,686,174 and their foreign counterpart patent claims, assigned to the UMDNJ.

[L16] His-Tag Sequence
This product is covered by the claims of U.S. Patent No. 5,284,933, 5,310,663 and their foreign counterpart patent claims.
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 research. Information about licenses for commercial use is available from QIAGEN GmbH, Qiagen Strasse 1, D-40724 Hilden, Germany.

[M9] pCold™ vectors
This product is covered by the claims of U.S. Patent No. 6,479,260 and its foreign counterpart patent claims.

NOTE: This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc.
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