Cat. # 3371

For Research Use

TaKaRa

pCold™ ProS2 DNA

Product Manual

v201909Da

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I. Description

Elucidation of protein structure and function has 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.
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 inactive insoluble protein accumulates in particles called "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 *cspA* (cold shock protein A) promoter has been incorporated into these vectors and up-regulates target protein production at low incubation temperatures. This temperature shift also suppresses expression of other cellular proteins and temporarily halts overall cell growth. These effects allow expression of target proteins at high yield and high purity (up to 60% of cellular protein), and increased solubility as compared with conventional *E. coli* expression systems.

pCold ProS2 DNA is a fusion cold shock expression vector, which utilizes Protein S of the gram-negative myxobacteria *Myxococcus xanthus* as a solubilization tag. Protein S, which is comprised of 173 amino acid residues, is a stable soluble protein component of the spore coat of *Myxococcus xanthus*. Fusion of the protein of interest with the ProS2 tag, a tandem repeat of the Protein S N-terminal domain (NTD), improves the stability and solubility of the fusion protein. In addition, it is possible to easily separate the protein of interest and the ProS2 Tag, because this tag has a low affinity for proteins to which it is fused.

The pCold ProS2 DNA Vector contains the *cspA* promoter, a 5' untranslated region (5' UTR), a translation enhancing element (TEE), a His-Tag sequence, the ProS2 tag, and a Multiple cloning site (MCS). A *lac* operator is inserted downstream of the *cspA* promoter to ensure tight regulation of expression. Additionally, recognition sequences for HRV 3C protease, thrombin, and factor Xa are located between ProS2 Tag and the Multiple Cloning Site (MCS), allowing tag removal from the expressed fusion protein. Most *E. coli* strains can serve as expression hosts. The pCold ProS2 DNA Vector uses cold shock technology to provide high yield protein expression and to facilitate correct protein folding, thus enabling efficient soluble protein production for target proteins, including those that are insoluble when expressed using other systems.

II. Components

pCold ProS2 DNA Vector 25 μg

【Plasmid storage buffer】
10 mM Tris-HCl, pH 8.0
1 mM EDTA

< *E. coli* host strains >
Most *E. coli* strains can be used as expression hosts with the pCold DNA Vector series since these vectors utilize the *E. coli cspA* (cold shock protein gene) promoter.

III. Vector Map

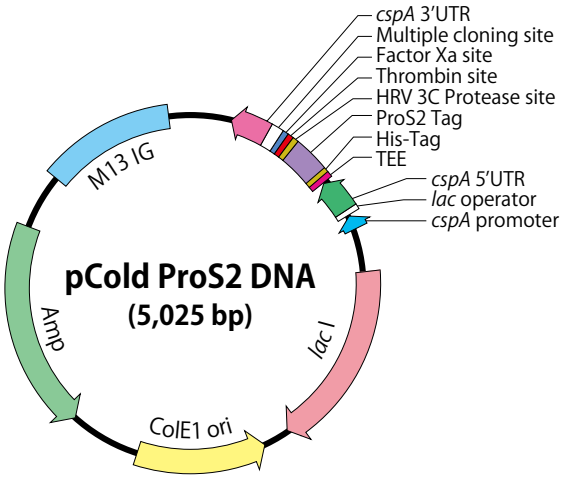


Figure 1. pCold ProS2 DNA

IV. Storage

-20℃

* Use within 2 years from date of receipt under proper storage conditions.

V. Protocol

How to express the target gene

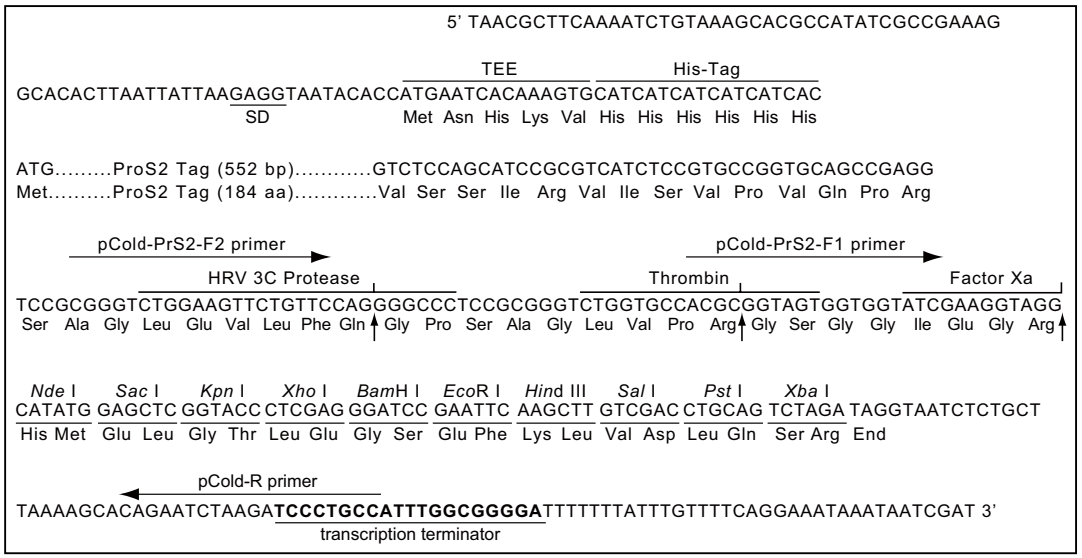
The growth/induction conditions (culture medium, culture temperature, aeration, timing of induction, concentration of inducer, incubation time after induction) should be optimized for each target protein.
A general description of the protocol is provided below.

1) Insert the target gene fragment into the multiple cloning site of pCold ProS2 DNA. Be sure that the sequence of the fragment is inserted in-frame with the ProS2 tag sequence.
2) Transform the *E. coli* host strain (e.g, BL21) with this plasmid, and select transformants on an agar plate containing ampicillin.
3) Inoculate LB medium containing 50 - 100 μg/ml of ampicillin with Amp^+ transformant clones, and incubate with shaking at 37℃.
4) When the OD_{600} of the culture reaches 0.4 - 0.8, quickly cool the culture to 15℃ in ice water, and let stand for 30 minutes.
5) Add IPTG to a final concentration of 0.1 - 1.0 mM, and incubate with shaking at 15℃ for 24 hours.
6) Collect the cells, and confirm the presence, amount, and solubility of the target protein using SDS-PAGE or an activity assay.

By optimizing the host strain, culture, and expression induction conditions (e.g., culture medium and temperature, degree of aeration and agitation, timing of induction, IPTG concentration, culture conditions after induction, etc.), it may be possible to increase the expression level and solubility of the target protein. The tag sequence at the N-termini can be removed by factor Xa, thrombin, and HRV 3C protease (Cat. #7360). To purify a his-tagged fusion protein, use TALON® Metal Affinity Resins (Cat. #635501, etc.).

VI. Multiple Cloning Site

pCold ProS2 DNA



VII. Experimental Examples

Expression of a target gene cloned into pCold ProS2 DNA was compared with expression of the same target gene cloned into pCold I DNA. The pCold I DNA and pCold ProS2 DNA constructs were each transformed into *E. coli* BL21 cells, and protein expression was induced according to the respective protocol for each vector.

(1) Example 1 : Protein expression in soluble form

Expression of enzyme protein A (estimated molecular weight 52.4 kDa) was not detected as a distinct band using pCold I DNA. In contrast, expression of a ~75 kDa target protein (52.4 kDa protein+23 kDa tag) was detected using pCold ProS2 DNA. Most of the protein was present in the soluble fraction.

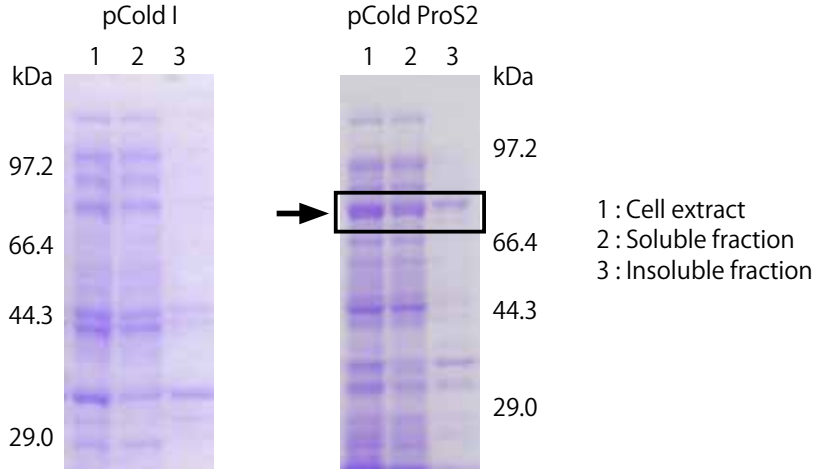


Figure 2. Expression of enzyme protein A

(2) Example 2 : Improvement of soluble protein yield.

With pCold I, expression of soluble protein B (molecular weight 43.3 kDa) was not detected. However, when the pCold ProS2 DNA Vector was used, most of the expressed target protein (~66 kDa; 43.3 kDa protein + 23 kDa tag) was present in the soluble fraction.

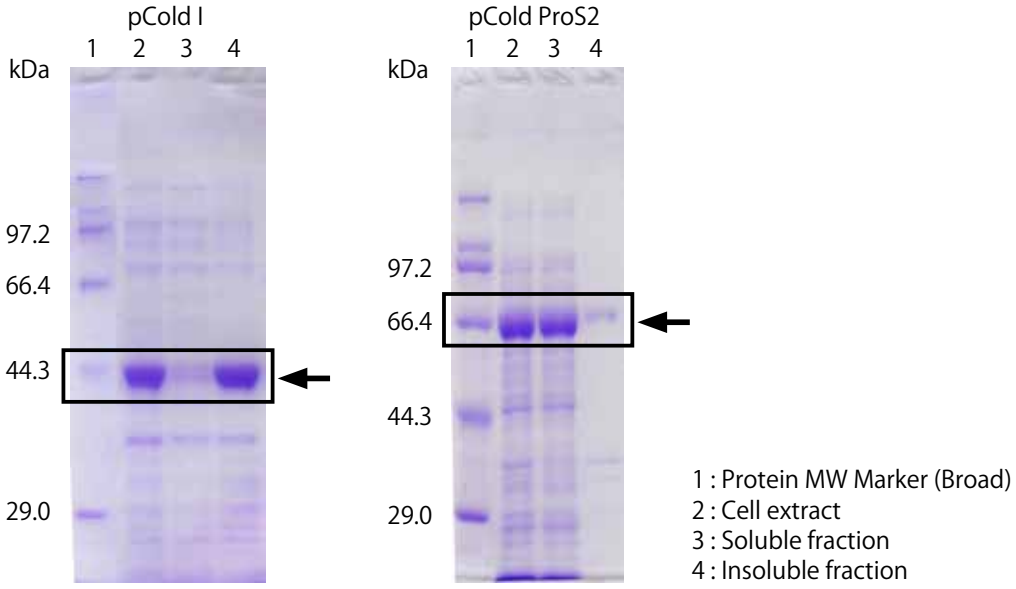


Figure 3. Expression of protein B

(3) Example 3 : Tag removal from a fusion protein.

After purification using a Ni-chelate affinity column, the fusion protein in Example 2 was digested with HRV 3C protease. The target protein (43.3 kDa) was then separated from the tag region by running the digestion mixture through a Ni-chelate affinity column, which yielded purified target protein in the pass-through fraction.

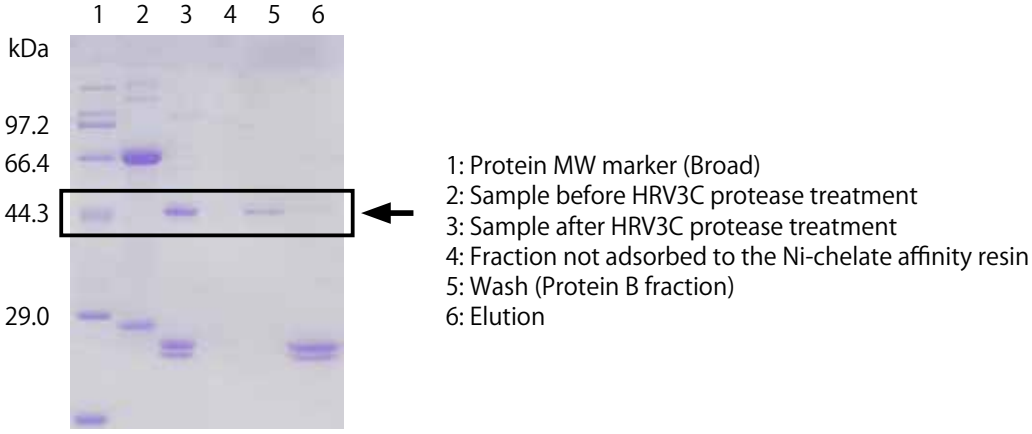


Figure 4. Separation of the target protein and the tag

VIII. Appendix

Expression Plasmid Construction

1) Overview of pCold ProS2 expression vector construction

a) Select a restriction enzyme site such that the DNA fragment will be in-frame with the pCold ProS2 DNA Vector.
b) Prepare the DNA fragment.
c) Digest the vector with the desired restriction enzymes.
d) After ligating the digested vector with the insert DNA, transform into an appropriate *E. coli* strain.
e) Purify the plasmid from the appropriate transformants containing the target insert.
f) The purified plasmid can be used for protein expression experiments.

The insert DNA may be prepared by PCR amplification, excision of a cloned gene by restriction enzyme digestion, or gene synthesis. The inserts cloned into pCold I - IV and pCold TF Vectors can be easily transferred into pCold ProS2 since the multiple cloning site (MCS) of these vectors are identical. In-Fusion® Cloning Kits can also be used for rapid directional cloning, and in cases where appropriate restriction enzyme sites are not present in a target gene. An example using PCR amplification for insert DNA preparation is provided below.

2) Example of plasmid preparation for *E. coli* thioredoxin expression

a) Guidelines for primer design

i) Select two restriction enzymes that are absent in the insert DNA but present in the MCS of pCold ProS2 DNA.

ii) Construct PCR primers for the target sequence, adding the restriction sites selected above to the 5' terminus of each primer. Adjust the nucleotide number between the insert DNA sequence and N-terminal restriction sites such that the reading frame of the insert matches the frame of ProS2 tag in pCold ProS2 DNA. A restriction site can be directly added to a stop codon at the C-terminus.

iii) Include four or more bases of random sequence outside of the restriction sites. Most restriction enzymes require several bases outside of the recognition site for efficient digestion. In the absence of this additional sequence, digestion efficiency may decrease.

[Primer Design]

Insertion of the thioredoxin gene into the pCold ProS2 DNA *Nde* I/*Xho* I restriction enzyme sites

Nde I site : Primer 1 (forward primer)

Nde I
5' - GCCGCATATGAGCGATAAAATTATTCAC
extra sequence thioredoxin N terminal sequence*1

Xho I site : Primer 2 (reverse primer)

Xho I
5' - GCCGCTCGAGTTAGGCCAGGTTAGCGTC
extra sequence thioredoxin C terminal sequence*2

*1 When using the *Nde* I site, adjust the position of the start codon (ATG) to correspond with the ATG site of *Nde* I.
*2 Complementary sequence with stop codon

b) Insert DNA Preparation

[PCR amplification of the thioredoxin gene (approx. 350 bp)]

i) PCR amplification

Prepare the reaction mixture by combining the following reagents. (use of a high-fidelity PCR enzyme, such as PrimeSTAR® HS DNA Polymerase (Cat. #R010A), is recommended).

Template DNA (5 ng)*1	1 µl
5X PrimeSTAR Buffer*2	10 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
PrimeSTAR DNA Polymerase (5 units/µl)	0.5 µl
Sterile purified water	32.5 µl
Total	50 µl

Amplify the insert DNA using the following PCR conditions when using TaKaRa PCR Thermal Cycler Dice™ *Touch*/Gradient (Cat. #TP350/TP600)*3.

98℃ 10 sec
55℃ 5 - 15 sec
72℃ 1 min
] 30 cycles

*1 For plasmid DNA, use 10 pg - 1 ng; for cDNA or genomic DNA, use 5 - 200 ng.
*2 5X PrimeSTAR Buffer and dNTP Mixture are supplied with PrimeSTAR HS DNA Polymerase (Cat. #R010A).
*3 Not available in all geographic locations. Check for availability in your area.

ii) Verification of amplified product
Verify that the amplified DNA fragment is a single band of the expected size by agarose gel electrophoresis using 5 μl of the PCR product.

iii) PCR product purification
For PCR products that appear as a single band, purify the DNA by phenol/chloroform extraction, etc. When multiple PCR products are generated, isolate a band of interest from the agarose gel and then purify using NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250) or other methods.

iv) Restriction enzyme digestion of the purified product
Digest the purified insert DNA with *Nde* I and *Xho* I restriction enzymes.

1) Prepare the following digestion mixture :

Insert DNA; 0.5 - 1 μg	x μl
10X K Buffer	3 μl
Nde I (10 U/μl)	1 μl
Xho I (10 U/μl)	1 μl
Sterile purified water	y μl
Total	30 μl

2) Incubate at 37℃ for 1 hour.
3) Purify the digested DNA by ethanol precipitation etc.*
4) Verify size and concentration of the purified fragment using agarose gel electrophoresis and absorbance (OD_{260}).

* Both *Nde* I and *Xho* I can be inactivated by ethanol precipitation. However, when restriction enzymes that are not completely inactivated by ethanol precipitation are used, purify the reaction by phenol extraction.

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

c) Restriction Enzyme Digestion of pCold ProS2 DNA

Digest pCold ProS2 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 ProS2 DNA	1 μg
10X K Buffer	3 μl
Nde I (10 U/μl)	1 μl
Xho I (10 U/μl)	1 μl
Sterile purified water	x μl
Total	30 μl

ii) Incubate at 37℃ for 1 - 2 hours.
iii) Purify the digested vector DNA by ethanol precipitation.*
iv) Dissolve the precipitated DNA in TE buffer.
v) Measure the absorbance (OD_{260}) 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 to 100 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 Phosphatase (CIAP) (Cat. #2250A). Note that de-phosphorylation is essential if only a single restriction enzyme was used for digestion.

d) Ligation and transformation

i) Ligation reaction

Mix the digested pCold ProS2 DNA and the insert DNA, and ligate using a ligation kit, such as DNA Ligation Kit < Mighty Mix > (Cat. #6023). A 1 : 3 - 1 : 10 molar ratio of vector : insert DNA is recommended.

Prepare the following ligation mixture on ice :

Digested pCold ProS2 DNA ; 100 ng (about 0.03 pmol)	1 μl
Insert DNA (0.1 - 0.3 pmol)	4 μl
Ligation Mix (from DNA Ligation Kit <Mighty Mix>)	5 μl
Total	10 μl

Incubate at 16℃ for 1 hour.

ii) Transformation

1) Thaw *E. coli* HST08 Premium competent cells (Cat. #9128) 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℃ for 45 sec.
5) Chill on ice for 1 - 2 minutes.
6) Add warm (37℃) SOC Medium to a final volume of 1 ml.
7) Incubate with shaking at 37℃ for 1 hour.
8) Plate on LB-ampicillin agar plates (100 μg/ml ampicillin) and incubate at 37℃ overnight .

e) Plasmid preparation and verification

Inoculate a colony obtained in ii) into LB-ampicillin broth (100 μg/ml ampicillin) and incubate with shaking at 37℃ overnight. Purify the plasmid from the culture.
Verify the correct DNA fragment by digesting an aliquot of the plasmid with restriction enzymes.
After the vector construct is verified, analyze DNA sequence of the inserted DNA fragment using the following sequencing primer. This plasmid can be used as an expression plasmid for subsequent experiments.

Upstream primer :	pCold-PrS2-F1 primer	5′-CACGCGGTAGTGGTGGTATC
	pCold-PrS2-F2 primer	5′-CGGGTCTGGAAGTTCTGTTC
Downstream primer :	pCold-R	5′-GGCAGGGATCTTAGATTCTG

IX. References

1) Qing G, *et al. Nature Biotechnology.* (2004) **22**: 877-882.
2) Inouye M, *et al. Proc Natl Acid Sci USA*. (1979)**76**: 209-213
3) Kobayashi H, Yoshida T, and Inouye M. *Appl Environ Microbiol*. (2009) **75(16)**: 5356-5362.

X. Related Products

Protein expression and purification-related products:

[Induction of target protein expression]
TaKaRa Competent Cells BL21 (Cat. #9126)
IPTG (Isopropyl-β-D-thiogalactopyranoside) (Cat. #9030)

[His-tagged fusion protein purification]
TALON® Metal Affinity Resin (Cat. #635501 - 635504/635652/635653)
TALON® Superflow Metal Affinity Resin (Cat. #635506/635507/635668 - 635670)
HisTALON™ Superflow Cartridge Purification Kit (Cat. #635649/635681)

[pCold vector series]
pCold™ DNA Series (Cat. #3360 - 3364)*
pCold™ TF DNA (Cat. #3365)*
pCold™ GST DNA (Cat. #3372)*

Cloning-related products:

[PCR amplification of target genes]
PrimeSTAR® Max DNA Polymerase (Cat. #R045A)
PrimeSTAR® GXL DNA Polymerase (Cat. #R050A/B)
PrimeSTAR® HS DNA Polymerase (Cat. #R010A/B)
Tks Gflex™ DNA Polymerase (Cat. #R060A/B)*

[Purification of target gene fragments]
NucleoSpin Gel and PCR Clean-up (Cat. #740609.10/.50/.250)

[Insertion of a DNA fragment into a vector and transformation]
In-Fusion® HD Cloning Plus (Cat. #638909)
In-Fusion® HD EcoDry™ Cloning Plus (Cat. #638912)
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)

[Plasmid preparation from *E. coli*]
NucleoSpin Plasmid EasyPure (Cat. #740727.10/.50/.250)

* Not available in all geographic locations. Check for availability in your area.

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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