Cat. # 3365

For Research Use

TaKaRa

pCold™ TF DNA

Product Manual

v201909Da

II. Components

pCold TF DNA Vector 25 μg

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

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

III. Vector Map

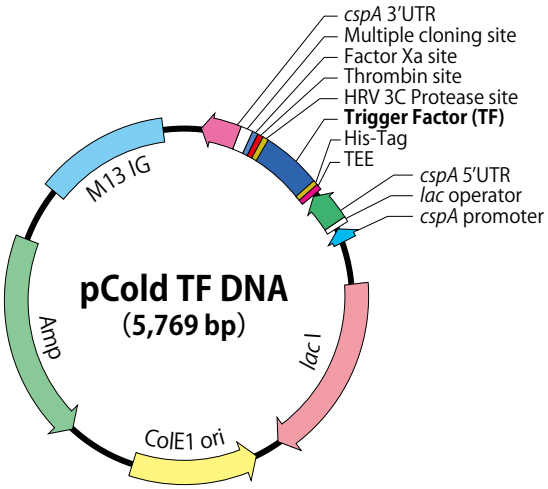

Figure 1. pCold TF DVA :Vector Map
GenBank Accession No. AB213654

IV. Storage

-20℃

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

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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. *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 accumlates 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 and related elements have been incorporated into these vectors and up-regulate 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, 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 Chaperone Plasmid Set, Cat. #3340). This procedure, though, lacks the convenience of a single transformation step.

Takara Bio'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 *cspA* 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 *cspA* 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), allowing tag removal from the expressed fusion protein. Most *E. coli* strains can serve as expression hosts. The pCold TF DNA Vector uses cold shock technology to provide high yield protein expression combined with Trigger Factor (chaperone) expression to facilitate correct protein folding, thus enabling efficient soluble protein production for target proteins that are insoluble when expressed using other systems.

V. Protocol

How to express the target gene;
The growth/induction conditions (culture medium, culture temperature, aeration, timing of induction, concentration of an inducer, and incubation time after induction) should be optimized for each target protein.
A general description of the protocol is shown below.

1) Insert the target gene fragment into the multicloning site of pCold TF DNA. Be sure that the sequence of the fragment is inserted in-frame with the TF tag sequence.
2) Transform the *E. coli* host strain (e.g. BL21) with this plasmid, and select the 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 at the 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 taq sequence at the N-termini can be removed by factor Xa, thrombin, and HRV 3C protease (Cat. #7360).

VI. Multiple Cloning Site

pCold TF DNA

VII. Application

Expression of a target gene cloned into pCold TF DNA was compared with (1) expression of the same target gene cloned into the pCold DNA I Vector alone, (2) co-expression of this pCold DNA I construct together with Takara Bio's Chaperone Plasmid pTf16, and (3) expression of the same target gene with a T7 promoter expression system, using other solubilization tags. These pCold DNA I and pCold TF DNA constructs were transformed separately into *E. coli* BL21 cells, cells were then cultured, and protein expression was induced according to the respective protocol for each vector. Expression from T7 promoter-driven vectors was also performed using a general procedure involving addition of IPTG and subsequent incubation at 37℃.

(1) Example 1: Protein expression in soluble form

Expression of enzyme protein A (estimated molecular weight 29 kDa) was not detected as a distinct band with the expression system utilizing a T7 promoter or even with the pCold I DNA construct (whether expressed alone or co-expressed with chaperone proteins). In contrast, expression of a target protein (81 kDa=29 kDa + 52 kDa) was detected using pCold TF DNA, and most of the obtained protein was in soluble form. Moreover, the expressed enzyme protein A showed enzymatic activity, even though it was part of a fusion protein.

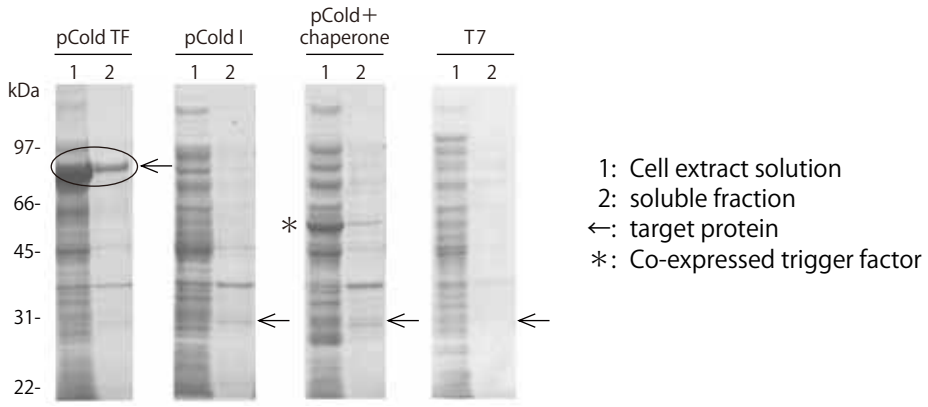
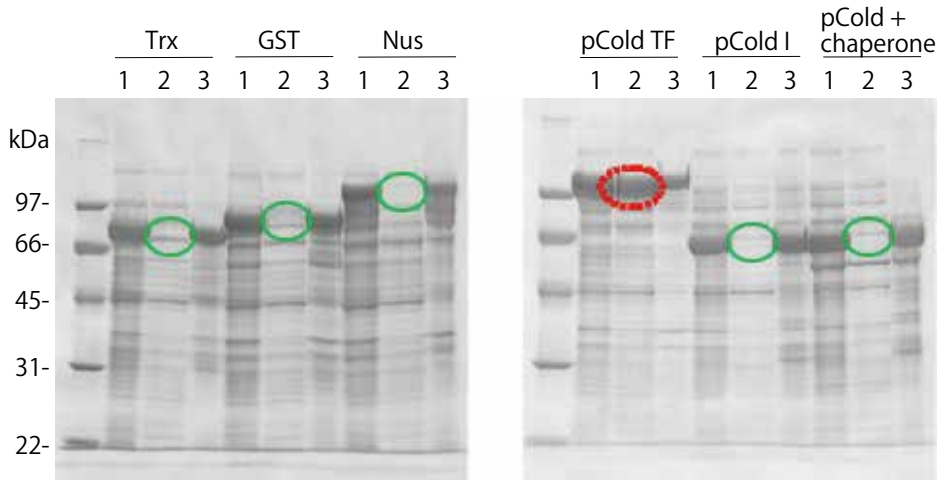


Figure 2. Expression of enzyme protein A

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

Expression of soluble enzyme protein B (M.W.: ~63 kDa) was not detected using either pCold DNA I alone or co-expressed with chaperone proteins, nor with a T7 expression vector that included other solubilization tags (Trx Tag [~12 kDa], Nus Tag [~55 kDa], and GST Tag [~26 kDa]). Alternatively, when pCold TF DNA was used, most of the expressed target protein was detected in the soluble fraction and expressed at a much higher level than with other tags. (Note: The observed molecular weight of the target proteins was greater than their actual size because they were expressed as fusions to solubilization tags.)



1: Cell extract solution
2: Soluble fraction
3: Insoluble fraction

Figure 3. Expression of enzyme protein B

VIII. Q&A

Q1: What parameters should be optimized when the expressed protein is insoluble?
A1: The optimal conditions for growth and induction vary, depending on the type of protein expressed, and should be determined by changing the following parameters:
- Vary the timing of induction within the early and late logarithmic growth phase.
- Vary the concentration of inducer (IPTG) from 0.1 - 1 mM.
- Vary the incubation time after induction (Generally 15℃, 24 hours provides the best results.)
- Change the aeration conditions.

Q2: What changes should be made to the protocol if no protein is expressed or the expression level is low?
A2: Try reoptimizing the growth and induction conditions (refer to the above Q1.), or changing the host *E. coli* strain.

Q3: What host strains have been shown to work with pCold Vectors?
A3: BL21, Rosetta, and Origami cells from Merck Millipore.
- BL21 is most commonly used as the host strain.
- Origami host strains lack the *trx/gor* gene, greatly enhancing disulfide bond formation in the cytoplasm and thus facilitating the solubility and refolding of the expressed protein.
- Rosetta host strains contain a plasmid which supplies tRNAs corresponding to codons that are rarely used in *E. coli*. It enables the "universal" translation of genes which is otherwise limited by the codon usage of *E. coli*.

Q4: Can an *E. coli* strain harboring a pCold TF vector containing a target gene be stored at 4℃ on a plate?
A4: We don't recommend storage at 4℃ on a plate because it may cause the target protein to leak from the cells. Pick the colony from the plate promptly, prepare a glycerol stock, and store at -80℃.

Q5: Is it possible to express a large gene in pCold TF DNA?
A5: It was confirmed that a human gene coding for a 125 kDa protein can be expressed successfully in a soluble form. [A band of 125 + 52 kDa (including the TF tag) was detected using CBB staining.]

Q6: Why would an expressed target protein digested with a protease to remove the TF tag continue to associate with the TF tag, even though the digestion has been shown to be complete using SDS-PAGE?
A6: This depends on the properties of the target protein, and is more likely to occur if the cleaved target protein has a lower solubility. Adding a reagent that improves the solubility of the cleaved protein might provide a solution. For example, 1% Triton X-100, 0.5 M arginine, 5 mM DTT or 20 mM CHAPS may be effective, but there is no single set of conditions that is effective in all cases. Therefore it is necessary to optimize solubilization conditions for individual target proteins.
If the interaction between a target protein and the TF-tag is extremely strong, the target protein may not be successfully separated from the TF tag because the complex does not dissociate.
In such a case, we recommend using pCold ProS2 DNA (Cat. #3371). It is possible to efficiently separate a protein of interest from the ProS2 Tag, because this tag has a low affinity for proteins to which it is fused.

Q7: Under what conditions should antibodies be raised against a protein expressed using the pCold ProS2 DNA vector?
A7: In many cases, the TF tag shows strong antigenicity in rabbits. Therefore, we recommend that antibodies against a TF-tagged fusion protein be raised in a guinea pig host. When immunizing a rabbit, we recommend digesting the TF-tagged protein with protease, then isolating the cleaved target protein from the tag, and using it to produce antibody.

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 be in-frame with the pCold TF 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 it into an appropriate *E. coli* strain.
e) Prepare purified plasmid from the appropriate transformants containing the target insert.
f) The purified plasmid may 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 TF DNA 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 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 that are absent in the insert DNA but present in the MCS of pCold TF 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 TF DNA. A restriction site can be directly added to a stop codon at the C-terminus.
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 may decrease.

[Example - Primer Design]

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

Nde I
Primer 1 (normal direction primer) 5' -GCCGCATATGAGCGATAAAATTATTCAC
extra sequence thioredoxin-origin sequence＊1

Xho I
Primer 2 (reverse direction primer) 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 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 HS DNA Polymerase (5 U/μl)	0.5 μl
Sterile purified water	32.5 μl
Total	50 μl

*1 For plasmid DNA, use 10 pg - 1 ng; for cDNA or genomic DNA, use 5 - 200 ng.

*2 5X PrimeSTAR Buffer Buffer and dNTP Mixture is supplied with PrimeSTAR HS DNA Polymerase (Cat. #R010A)

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

98℃	10 sec	┐
55℃	5 - 15 sec	├ 30 cycles
72℃	1 min	┘

ii) Verification of amplified product

Verify that the amplified insert DNA fragment is a single band of the expected size by performing 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 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 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 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 3 M sodium acetate, pH 5.2, to the restriction enzyme reaction mixture in a 1 : 10 ratio (e.g. 3 μl of 3 M sodium acetate to 30 μl reaction 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℃ for 30 minutes.
3) Centrifuge at 4℃, 12,000 rpm, for 10 - 15 minutes. Discard the supernatant.
4) Add 70% cold ethanol and centrifuge again at 4℃, 12,000 rpm, for 5 minutes.
5) Discard the supernatant and air dry the pellet.
6) Dissolve the precipitate in 10 - 50 μl of TE buffer.

(3) 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
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 DNA by ethanol precipitation etc.*
iv) Dissolve the precipitated vector DNA pellet 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 (*E. coli* C75)(Cat. #2120A), or Alkaline Phosphase (Calf intestine) (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.

(4) Ligation 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 DNA Ligation Kit <Mighty Mix> (Cat. #6023). A 1 : 3 - 1 : 10 molar ratio of vector : insert DNA is recommended.

1. 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

2. 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) Shake at 37℃ for 1 hour.
8) Plate on LB-ampicillin agar (100 μg/ml ampicillin) and incubate at 37℃ overnight .

(5) 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℃ overnight. Purify the plasmid from the culture.

Digest the purified 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 using the following sequencing primers. This plasmid can be used as an expression plasmid for subsequent experiments.

Upstream primer:	pCold-TF-F1	5'-CCACTTTCAACGAGCTGATG
	pCold-TF-F2	5'-GCGAAAGTGACTGAAAAAG
Downstream primer:	pCold-R	5'-GGCAGGGATCTTAGATTCTG

X. References

1) Qing G, *et al. Nature Biotechnology*. (2004) **22**: 877-882.
2) Gerlined S, *et al. EMBO J*. (1995) **14**: 4939-4948.

XI. 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™ ProS2 DNA (Cat. #3371)*
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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