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TC-FlAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit with Mammalian Gateway™ Expression Vectors

USER GUIDE

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Revision history

Revision	Date	Description
A.0	30 August 2016	Corrected Kit contents, updated Documentation and support, and rebranded.
2.0	17 August 2011	Basis for the current revision.

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Contents

Kit contents and storage

Kit components

The TC-FlAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit (with Mammalian Gateway™ expression vectors) contains the components listed below.

Component	Concentration	Volume
FlAsH-EDT_2 labeling reagent	2 mM in DMSO	40 µL
ReAsH-EDT_2 labeling reagent	2 mM in DMSO	40 µL
BAL wash buffer	100X in ddH_2O (25 mM)	2.4 mL
pcDNA™6.2/cTC-Tag-DEST	150 ng/µL in TE, pH 8.0	40 µL
pcDNA™6.2/nTC-Tag-DEST	150 ng/µL in TE, pH 8.0	40 µL
pcDNA™6.2/nTC-Tag-p64 Control Plasmid	Lyophilized in TE, pH 8.0	10 µg

Molecular weights of the labeling reagents

The table below lists the molecular weights and micrograms supplied for each labeling reagent.

Reagent	Molecular weight	Amount
FlAsH-EDT_2 labeling reagent	664.50 g/mol	53.2 µg
ReAsH-EDT_2 labeling reagent	545.38 g/mol	43.6 µg

Storage

Upon receipt, store the components of the TC-FlAsH™ and TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit as detailed below.

Component	Storage
FlAsH-EDT_2 labeling reagent	≤–20°C, protected from light
ReAsH-EDT_2 labeling reagent	≤–20°C, protected from light
pcDNA™6.2/cTC-Tag-DEST	≤–20°C
pcDNA™6.2/nTC-Tag-DEST	≤–20°C
pcDNA™6.2/nTC-Tag-p64 control plasmid	≤–20°C
BAL wash buffer	**2–6°C, air sensitive**

Introduction

Overview

Description

The TC-FlAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit contains Gateway™-adapted destination vectors designed with the protein labeling technology based on the binding of biarsenical ligands to tetracysteine motifs.

The pcDNA™6.2/TC-Tag-DEST vectors supplied with each kit facilitate *in vivo* fluorescence labeling and detection of recombinant proteins when used with a TC-FlAsH™ or TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit.

Features

The pcDNA™6.2/cTC-Tag-DEST and pcDNA™6.2/nTC-Tag-DEST vectors contain the following elements:

- Human cytomegalovirus immediate-early (CMV) promoter/enhancer for high-level expression in a wide range of mammalian cells
- TC-Tag for C-terminal (pcDNA™6.2/cTC-Tag-DEST) or N-terminal (pcDNA™6.2/nTC-Tag-DEST) fusion to the gene of interest for fluorescence detection
- Two recombination sites, *att*R1 and *att*R2, downstream of the CMV promoter for recombinational cloning of the gene of interest from an entry clone
- Chloramphenicol resistance gene located between the two *att*R sites for counterselection
- The *ccd*B gene located between the two *att*R sites for negative selection
- The Herpes Simplex Virus thymidine kinase polyadenylation signal for proper termination and processing of the recombinant transcript
- f1 intergenic region for production of single-strand DNA in F plasmid-containing *E. coli*
- SV40 early promoter and origin for expression of the Blasticidin resistance gene and stable propagation of the plasmid in mammalian hosts expressing the SV40 large T antigen
- Blasticidin resistance gene for selection of stable cell lines
- The pUC origin for high copy replication and maintenance of the plasmid in *E. coli*
- The ampicillin resistance gene for selection in *E. coli*

For a map of pcDNA™6.2/cTC-Tag-DEST and pcDNA™6.2/nTC-Tag-DEST, see pages 21 and 23.

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Overview, continued

The Gateway™ Technology

The Gateway™ Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda[1] to provide a rapid and highly efficient way to move your gene of interest into multiple vector systems. To express your gene of interest in mammalian cells using Gateway™ Technology, simply:

1. Clone your gene of interest into a Gateway™ entry vector to create an entry clone.
2. Generate an expression clone by performing an LR recombination reaction between the entry clone and a Gateway™ destination vector (e.g. pcDNA™6.2/ cTC-Tag-DEST or pcDNA™6.2/nTC-Tag-DEST).
3. Transfect your expression clone into the cell line of choice for transient or stable expression of your gene of interest.

For more information on Gateway™, refer to the Gateway™ Technology with Clonase™ II manual. This manual is available for download at **thermofisher.com** or by contacting Technical Support (page 27).

Advantages of the labeling technology

The TC-FlAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit uses biarsenical labeling reagents to bind and detect proteins containing a tetracysteine motif (i.e. TC-Tag).[2] Using the TC-FlAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits for fluorescence labeling of recombinant proteins provides the following advantages:

- Small size of the TC-Tag (6 amino acids, 585 Da) is less likely to interfere with the structure or biological activity of the protein of interest.
- FlAsH-EDT2 and ReAsH-EDT2 labeling reagents are membrane-permeable and readily cross the cell membrane, allowing labeling and detection of recombinant proteins in live mammalian cells.
- FlAsH-EDT2 and ReAsH-EDT2 labeling reagents bind the TC-Tag with high specificity and high affinity (nanomolar or lower dissociation constant), allowing targeted labeling of the protein of interest.[5]
- FlAsH-EDT2 and ReAsH-EDT2 labeling reagents become strongly fluorescent (green and red, respectively) only upon binding the TC-Tag, allowing specific detection of TC-tagged proteins.
- FlAsH-EDT2 and ReAsH-EDT2 labeling reagents can be applied sequentially on the same sample, allowing temporal detection of protein turnover and trafficking.[3]
- ReAsH-EDT2 labeling reagent can be used for both fluorescence-based microscopy and electron microscopy.[3]
- FlAsH-EDT2 labeling reagent provides a superior alternative to yellow-fluorescent protein (YFP) when coupled with cyan-fluorescent protein (CFP) for FRET-based cellular analysis.[4]

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Overview, continued

System components

The TC-FlAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit consists of two major components:

- The tetracysteine TC-Tag (Cys-Cys-Pro-Gly-Cys-Cys) in the pcDNA™6.2/ TC-Tag-DEST vector. When fused to a gene of interest, the TC-Tag allows the expressed fusion protein to be specifically recognized by a biarsenical labeling reagent. For more information on the tetracysteine motif, see below.
- A biarsenical labeling reagent, FlAsH-EDT2 or ReAsH-EDT2, which becomes fluorescent upon binding to recombinant proteins containing the TC-Tag. The FlAsH-EDT2 or ReAsH-EDT2 labeling reagents are supplied pre-complexed to the dithiol EDT (1,2-ethanedithiol) which stabilizes and solubilizes the biarsenic reagents.

Tetracysteine motif

Both the FlAsH-EDT_2 and ReAsH-EDT_2 reagents bind a tetracysteine motif consisting of Cys-Cys-Xaa-Xaa-Cys-Cys, where Cys equals cysteine and Xaa equals any amino acid other than cysteine. This motif is rarely seen in naturally occurring proteins allowing specific fluorescence labeling of recombinant proteins fused to the TC-Tag.

In the TC-FlAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit, the optimized Cys-Cys-Pro-Gly-Cys-Cys tetracysteine motif is used. This motif has been shown to have a higher affinity for and more rapid binding to biarsenic compounds as well as enhanced stability compared to other characterized motifs. [5]

In-gel tetracysteine tag detection

For sensitive and specific in-gel detection of TC-Tagged fusion proteins, we recommend the Lumio™ Green Detection Kit available from Thermo Fisher Scientific (Cat. No. LC6090). The Lumio™ Green Detection Kit enables immediate visualization of TC-Tagged proteins in polyacrylamide gels using a UV transilluminator or a visible light laser-based scanner and without the need for staining or western blotting. In addition, the BenchMark™ Fluorescent Protein Standard (Cat. No. LC5928) allows you to easily visualize molecular weight ranges of proteins labeled with Lumio™ Green Detection Reagent.

Methods

Generate an entry clone

Introduction

To recombine your gene of interest into pcDNA™6.2/cTC-Tag-DEST or pcDNA™6.2/nTC-Tag-DEST, you will need an entry clone containing the gene of interest. Many entry vectors including pENTR/D-TOPO™ (Cat. No. K2400-20) to facilitate generation of entry clones. For more information, refer to our website (**thermofisher.com**) or contact Technical Support. Refer to the manual for the specific entry vector you are using for detailed instructions to construct an entry clone.

Tag-On-Demand™ system

The pcDNA™6.2/cTC-Tag-DEST vector is compatible with the Tag-On-Demand™ System which allows expression of both native and C-terminally-tagged recombinant protein from the same expression construct.

The System is based on stop suppression technology originally developed by RajBhandary and colleagues[6] and consists of a recombinant adenovirus expressing a $tRNA^{ser}$ suppressor. When an expression vector encoding a gene of interest with the TAG (amber stop) codon is transfected into mammalian cells, the stop codon will be translated as serine, allowing translation to continue and resulting in production of a C-terminally-tagged fusion protein.

For more information, refer to the Tag-On-Demand™ Suppressor Supernatant manual. This manual is available for download at **thermofisher.com**.

NOTE: If you wish to express a human or mouse gene of interest, we recommend using an Ultimate™ Human ORF (hORF) or Ultimate™ Mouse ORF (mORF) Clone available from Thermo Fisher Scientific. Each Ultimate™ ORF Clone is a fully sequenced clone provided in a Gateway™ entry vector that is ready-to-use in an LR recombination reaction with pcDNA™6.2/cTC-Tag-DEST. In addition, each clone contains a **TAG** stop codon, making it fully compatible for use in the Tag-On-Demand™ System. For more information about the Ultimate™ ORF Clones available, refer to our website (**thermofisher.com**) or contact Technical Support.

Kozak consensus sequence

If you will be expressing your protein from pcDNA™6.2/cTC-Tag-DEST, your insert in the entry clone should contain a Kozak translation initiation sequence with an ATG initiation codon for proper initiation of translation.[7–9] An example of a Kozak consensus sequence is provided below. The ATG initiation codon is shown underlined.

(G/A)NNATGG

Other sequences are possible, but the G or A at position –3 and the G at position +4 are the most critical for function (shown in bold).

continued on next page

Generate an entry clone, continued

Points to consider for pcDNA™6.2/ cTC-Tag-DEST

pcDNA™6.2/cTC-Tag-DEST allows expression of recombinant proteins with a C-terminal peptide containing the V5 epitope and the TC-Tag; however, you may use this vector to express a native protein, if desired. You may also use this vector in the Tag-On-Demand™ System (see page 5). Consider the following when generating your entry clone.

If you wish to...	Then your insert...
include the V5 epitope and TC-Tag	• should contain a Kozak initiation sequence (see page 5) • should **not** contain a stop codon • should be in frame with the V5 epitope and TC-Tag after recombination (see page 8 for a diagram)
include the V5 epitope and TC-Tag for use in the Tag-On-Demand™ System	• should contain a Kozak initiation sequence (see previous page) • should contain a **TAG** stop codon • should be in frame with the V5 epitope and TC-Tag after recombination (see page 8 for a diagram)
not include the V5 epitope and TC-Tag	• should contain a Kozak initiation sequence (see page 5) • should contain a stop codon

Points to consider for pcDNA™6.2/ nTC-Tag-DEST

pcDNA™6.2/nTC-Tag-DEST allows expression of recombinant proteins with an N-terminal peptide containing the TC-Tag and V5 epitope tag and contains an ATG initiation codon within the context of a Kozak consensus sequence (see page 5). To include the TC-Tag and V5 epitope tag, your insert in the entry clone should:

- **not** contain a Kozak initiation sequence
- be in frame with the TC-Tag and V5 epitope tag after recombination (see page 9 for a diagram)
- contain a stop codon

Create an expression clone

Introduction

After you have generated an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into your pcDNA™6.2/TC-Tag-DEST vector to create your expression clone. To ensure that you obtain the best possible results, we recommend that you read this section and "Perform the LR recombination reaction" (page 10) before beginning.

Experimental outline

To generate an expression clone, you will:

1. Perform an LR recombination reaction using the *att*L-containing entry clone and the *att*R-containing pcDNA™6.2/TC-Tag-DEST vector.
2. Transform the reaction mixture into a suitable *E. coli* host.
3. Select for expression clones (refer to pages 8–9 for diagrams of the recombination region of the resulting expression clones).

Propagate the vectors

If you wish to propagate and maintain the pcDNA™6.2/TC-Tag-DEST vectors, we recommend using One Shot™ *ccd*B Survival T1^R Chemically Competent *E. coli* (Cat. No. C7510-03) for transformation. The *ccd*B Survival T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain the integrity of the vector, select for transformants in media containing 50–100 µg/mL ampicillin and 15–30 µg/mL chloramphenicol.

Note: Do not use general *E. coli* cloning strains including TOP10 or DH5 ☐ for propagation and maintenance of the pcDNA™6.2/TC-Tag-DEST vectors as these strains are sensitive to CcdB effects.

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Create an expression clone, continued

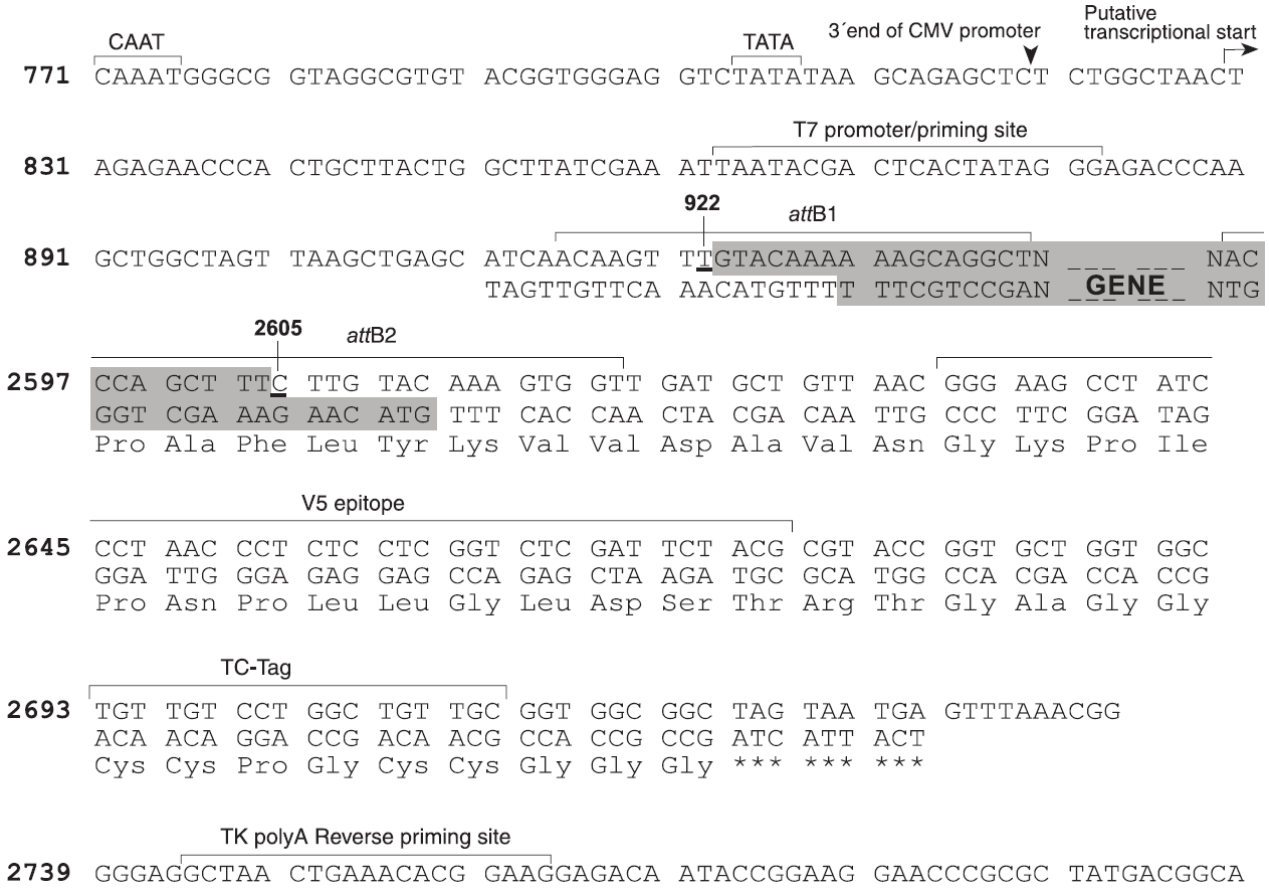
Recombination region of pcDNA™6.2/ cTC-Tag-DEST

The recombination region of the expression clone resulting from pcDNA™6.2/ cTC-Tag-DEST × entry clone is shown below.

Note: If you are using pcDNA™6.2/cTC-Tag-DEST in the Tag-On-Demand™ System, your gene of interest must contain a TAG stop codon (see page 5 for more information).

Features of the Recombination Region:

- Shaded regions correspond to DNA sequences transferred from the entry clone into pcDNA™6.2/cTC-Tag-DEST by recombination. Non-shaded regions are derived from the pcDNA™6.2/cTC-Tag-DEST vector.
- Bases 922 and 2605 of the pcDNA™6.2/cTC-Tag-DEST vector sequence are marked.



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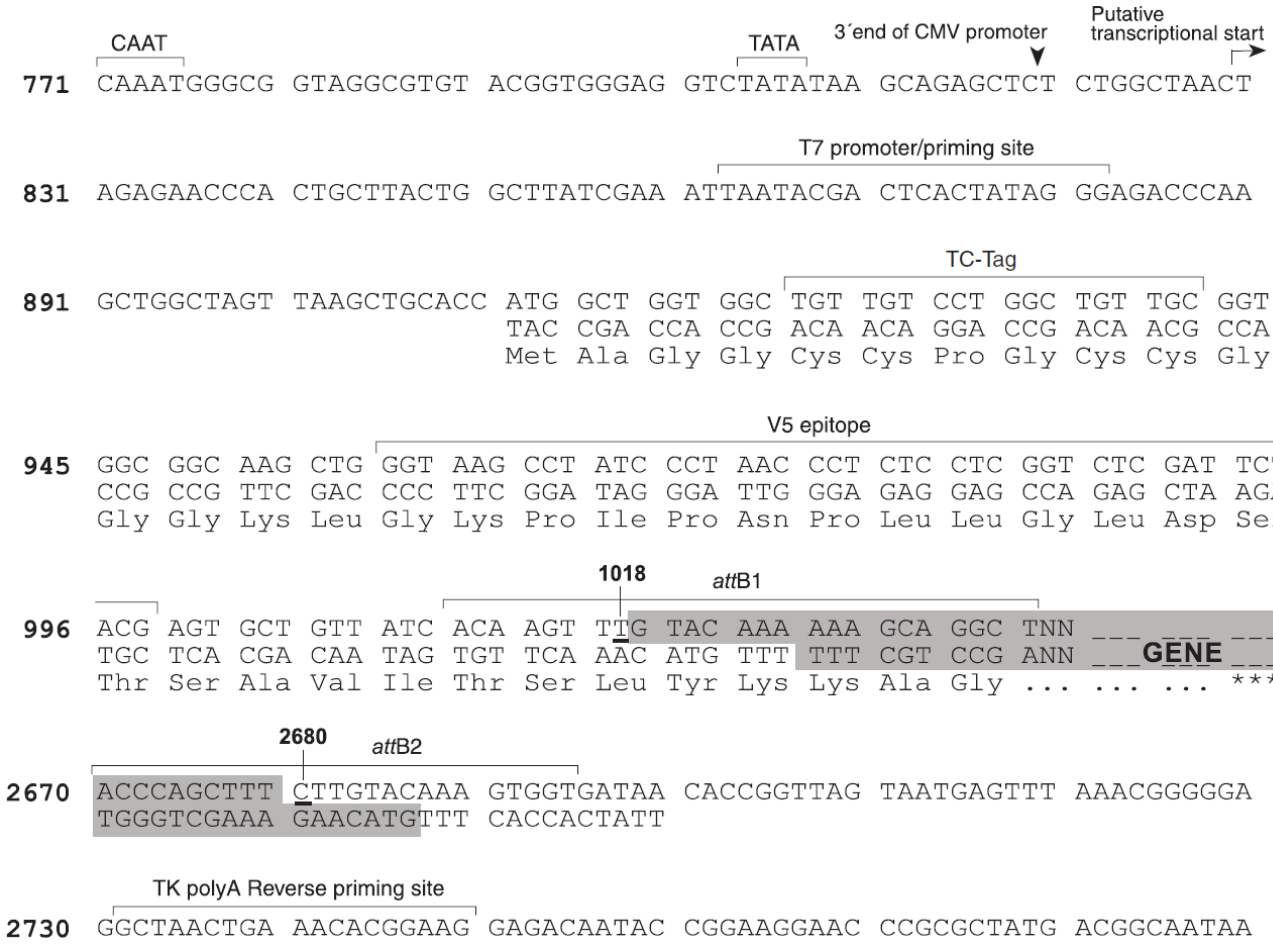
Create an expression clone, continued

Recombination region of pcDNA™6.2/ nTC-Tag-DEST

The recombination region of the expression clone resulting from pcDNA™6.2/ nTC-Tag-DEST × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to DNA sequences transferred from the entry clone into pcDNA™6.2/nTC-Tag-DEST by recombination. Non-shaded regions are derived from the pcDNA™6.2/nTC-Tag-DEST vector.
- Bases 1018 and 2680 of the pcDNA™6.2/nTC-Tag-DEST vector sequence are marked.



Perform the LR recombination reaction

Introduction

Once you have obtained an entry clone containing your gene of interest, you may perform an LR recombination reaction between the entry clone and your pcDNA™6.2/TC-Tag-DEST vector, and transform the reaction mixture into a suitable *E. coli* host (see below) to select for an expression clone. We recommend including the pENTR™-gus positive control supplied with the LR Clonase™ II enzyme mix in your experiments to help you evaluate your results.

E. coli host

You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™ or equivalent for transformation. **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (e.g. TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

NOTE: The presence of the EM7 promoter and the Blasticidin resistance gene in the pcDNA™6.2/TC-Tag-DEST vectors allows for selection of *E. coli* transformants using Blasticidin. For selection, use Low Salt LB agar plates containing 100 µg/mL Blasticidin (see the **Appendix**, page 19). For Blasticidin to be active, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.0.

Blasticidin is available separately from Thermo Fisher Scientific (50 mg, Cat. No. R210-01). Refer to the **Appendix** for instructions on how to prepare and store Blasticidin.

LR Clonase™ II enzyme mix

LR Clonase™ II enzyme mix is available separately (Cat. No. 11791-020) to catalyze the LR recombination reaction. The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase™ reaction buffer previously supplied as separate components in LR Clonase™ enzyme mix into an optimized single-tube format for easier set-up of the LR recombination reaction. Use the protocol provided on page 11 to perform the LR recombination reaction using LR Clonase™ II enzyme mix.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired. To use LR Clonase™ enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for LR Clonase™ II enzyme mix provided in this manual as reaction conditions differ.

continued on next page

Perform the LR recombination reaction, continued

Materials needed

You should have the following materials on hand before beginning:

- Purified plasmid DNA of your entry clone (50–150 ng/µL in TE, pH 8.0)
- pcDNA™6.2/cTC-Tag-DEST or pcDNA™6.2/nTC-Tag-DEST vector (150 ng/µL in TE, pH 8.0)
- LR Clonase™ II enzyme mix (Cat. No. 11791-020; keep at ≤–20°C until immediately before use)
- pENTR™-gus positive control, optional (50 ng/µL in TE, pH 8.0; supplied with the LR Clonase™ II enzyme mix)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µL Proteinase K solution (supplied with the LR Clonase™ II enzyme mix; thaw and keep on ice until use)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- LB agar plates with the appropriate antibiotic to select for expression clones

Set up the LR recombination reaction

1. Add the following components to 1.5 mL microcentrifuge tubes at room temperature and mix.

   Note: To include a negative control, set up a second sample reaction and substitute TE buffer, pH 8.0 for the LR Clonase™ II enzyme mix (see Step 4).

Component	Sample	Positive control
Entry clone (50–150 ng/reaction)	1–7 µL	—
Destination vector (150 ng/µL)	1 µL	1 µL
pENTR™-gus (50 ng/µL)	—	2 µL
TE Buffer, pH 8.0	to 8 µL	5 µL

2. Remove the LR Clonase™ II enzyme mix from ≤–20°C and thaw on ice (~2 minutes).
3. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
4. To each sample above, add 2 µL of LR Clonase™ II enzyme mix. Mix well by pipetting up and down.

   Reminder: Return LR Clonase™ II enzyme mix to ≤–20°C immediately after use.
5. Incubate reactions at 25°C for 1 hour.

   Note: Extending the incubation time to 18 hours typically yields more colonies.
6. Add 1 µL of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Transform 1 µL of the LR recombination reaction into a suitable *E. coli* host (follow the manufacturer's instructions) and select for expression clones.

   Note: You may store the LR reaction at ≤–20°C for up to 1 week before transformation.

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Perform the LR recombination reaction, continued

What to expect

If you use *E. coli* cells with a transformation efficiency of ≥1 × 10^8 cfu/µg, the LR reaction should give >5000 colonies if the entire reaction is transformed and plated.

Confirm the expression clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be ampicillin-resistant and chloramphenicol-sensitive. Transformants containing a plasmid with a mutated *ccd*B gene will be both ampicillin- and chloramphenicol-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/mL chloramphenicol. A true expression clone will not grow in the presence of chloramphenicol.

Sequence analysis

You may sequence your construct to confirm that your gene is in frame with the appropriate N-terminal or C-terminal fusion tag, if desired. We suggest using the T7 Promoter and TK polyA Reverse primer sequences (see below). See the diagram on page 8 or page 9 for the location of the primer binding sites.

Primer	Sequence
T7 Promoter Primer	5´-TAATACGACTCACTATAGGG-3´
TK polyA Reverse Primer	5´-CTTCCGTGTTTCAGTTAGC-3´

Transfect cells

Introduction

This section provides general information for transfecting your expression clone into the mammalian cell line of choice. We recommend that you include the pcDNA™6.2/nTC-Tag-p64 positive control vector and a non–TC-Tag vector control in your transfection experiment to help you evaluate your results. The non–TC-Tag control should contain cells transfected with a vector that does not encode the TC-Tag. Using untransfected cells (no transfection reagent, no DNA) as a control is not optimal since transfection may introduce fluorescent artifacts or alter cell morphology that could affect the labeling and detection of your protein.

Plasmid preparation

Once you have generated your expression clone, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be clean and free from contamination with phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HQ Mini Plasmid Purification Kit (Cat. No. K2100-01). Other plasmid purification kits or methods are suitable.

Transfection methods

For established cell lines (e.g. HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend that you pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology*.[10]

Methods for transfection include calcium phosphate[11,12], lipid-mediated[13,14], and electroporation.[15,16] For high efficiency transfection in a broad range of mammalian cell lines, we recommend using Lipofectamine™ 2000 Reagent (Cat. No. 11668-027) available from Thermo Fisher Scientific. For more information about Lipofectamine™ 2000 and the other transfection reagents available from Thermo Fisher Scientific, refer to our website (**thermofisher.com**) or contact Technical Support.

Cell density

If you plan to label your recombinant protein using the TC-FlAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit, we recommend designing your transfection experiment so that cells will be at optimal density at the time of labeling. Suspension cells typically label most efficiently at a density of 1×10^6–2×10^6 cells/mL. Adherent cells label most efficiently when they are 60–90% confluent at the time of labeling.

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Positive control

pcDNA™6.2/nTC-Tag-p64 is provided as a positive control vector for mammalian cell transfection and expression (see page 25 for a map) and may be used to optimize recombinant protein expression levels in your cell line. This vector allows expression of the human c-myc (p64) protein with an N-terminal fusion to the TC-Tag and V5 epitope tag.

To propagate and maintain the plasmid:

1. Resuspend the vector in 10 µL sterile water to prepare a 1 µg/µL stock solution. Use the stock solution to transform a *rec*A, *end*A *E. coli* strain like TOP10, DH5α, or equivalent.
2. Select transformants on LB agar plates containing 50–100 µg/mL ampicillin.
3. Prepare a glycerol stock of a positive transformant for long-term storage.

Create stable cell lines

Introduction

The pcDNA™6.2/TC-Tag-DEST vectors contain the Blasticidin resistance gene to allow selection of stable cell lines. If you wish to create stable cell lines, transfect your construct into the mammalian cell line of choice and select for foci using Blasticidin. General information and guidelines are provided below.

NOTE: The TC-FlaSH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit works best for labeling proteins that are expressed at high levels or are concentrated in a subcellular region. If you are expressing proteins at low levels (e.g. from a weak promoter) or if you are expressing a cytoplasmic protein, we recommend conducting initial labeling and detection studies using the TC-FlAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits in transiently transfected cells where protein expression levels are higher. Once labeling conditions are optimized, you may analyze your protein in stable cell lines.

To obtain stable transfectants, we recommend that you linearize your pcDNA™6.2/TC-Tag-DEST construct before transfection. While linearizing the vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts elements necessary for expression in mammalian cells. To linearize your construct, cut at a unique site that is not located within a critical element or within your gene of interest.

Determine Blasticidin sensitivity

To successfully generate a stable cell line expressing your protein of interest, you need to determine the minimum concentration of Blasticidin required to kill your untransfected host cell line by performing a kill curve experiment (see below).

Typically, concentrations ranging from 2.5–10 µg/mL Blasticidin are sufficient to kill most untransfected mammalian cell lines. Blasticidin is available separately from Thermo Fisher Scientific (50 mg, Cat. No. R210-01). See the **Appendix**, page 20, for instructions on how to prepare and store Blasticidin.

1. Plate cells at approximately 25% confluence. Prepare a set of 6 plates.
2. On the following day, replace the growth medium with fresh growth medium containing varying concentrations of Blasticidin (e.g. 0, 1, 3, 5, 7.5, and 10 µg/mL Blasticidin).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of Blasticidin that prevents growth within 10–14 days after addition of Blasticidin.

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Create stable cell lines, continued

Generate stable cell lines

Once you have determined the appropriate Blasticidin concentration to use for selection, you can generate a stable cell line expressing your pcDNA™6.2/TC-Tag-DEST construct.

1. Transfect the mammalian cell line of interest with the pcDNA™6.2/cTC-Tag-DEST or pcDNA™6.2/nTC-Tag-DEST expression construct using your transfection method of choice.
2. 24 hours after transfection wash the cells and add fresh growth medium.
3. 48 hours after transfection, split the cells into fresh growth medium such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the cells. Antibiotics work best on actively dividing cells.
4. Incubate the cells at 37°C for 2–3 hours until they have attached to the culture dish.
5. Remove the growth medium and replace with fresh growth medium containing Blasticidin at the predetermined concentration required for your cell line.
6. Feed the cells with selective media every 3–4 days until Blasticidin-resistant colonies can be identified.
7. Pick at least 10 Blasticidin-resistant colonies and expand them to assay for recombinant protein expression.

Detect TC-tag fusion proteins

Introduction

Once you have transfected your expression clone into mammalian cells, you may:

- Detect protein expression and localization in live cells by fluorescence microscopy using the TC-FlAsH™ or TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits. For detailed guidelines and protocols, refer to the TC-FlAsH™ or TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits instruction manual.
- Detect protein expression directly in polyacrylamide gels using the Lumio™ Green Detection Kit (see below).
- Detect protein expression by Western blot analysis using Anti-V5 Antibodies available from Thermo Fisher Scientific.

In-gel tetracysteine tag detection

The Lumio™ Green Detection Kit (Cat. No. LC6090) enables immediate and specific visualization of TC-tagged proteins in polyacrylamide gels using a UV transilluminator or a visible light laser-based scanner and without the need for staining or western blotting.

Refer to the Lumio™ Green Detection Kit manual for detailed protocols to prepare lysate samples specifically for detection with the Lumio™ Green Detection Reagent. This manual is available for download at **thermofisher.com**.

Western blot

You may detect expression of your recombinant fusion protein using the Anti-V5 Antibody (Cat. No. R960-25), Anti-V5-HRP Antibody (Cat. No. R961-25), or Anti-V5-AP Antibody (Cat. No. R962-25) available from Thermo Fisher Scientific. You may use any method of choice to prepare your mammalian cell lysates for Western blot analysis. We recommend the following guidelines:

- If you plan to analyze your samples using the Lumio™ Green Detection Kit (see above) in addition to Western blotting, you will need to prepare your samples using lysis buffer. Lysates containing standard LaemmLi SDS-PAGE sample buffer will not be suitable for in-gel detection with the Lumio™ Green Detection Kit. Refer to the Lumio™ Green Detection Kit manual for a protocol to prepare cell lysates that are compatible with both in-gel detection and Western blot analysis.
- For cells transfected with the pcDNA™6.2/nTC-Tag-p64 positive control vector, you will need to prepare lysates using RIPA or SDS-PAGE sample buffer to adequately release p64 from the nucleoli. If you are preparing samples using lysis buffer, you may sonicate your samples to release p64.
- To detect p64 (human c-myc) expression, you may use any of the Anti-V5 Antibodies or the Anti-myc Antibodies available from Thermo Fisher Scientific.

  Note: The *c-myc* gene encodes a protein with an expected molecular weight of 48 kDa, however, the native protein actually runs at a range of 55–64 kDa on an SDS-PAGE gel.

continued on next page

Detect TC-tag fusion proteins, continued

Polyacrylamide gels available from Thermo Fisher Scientific

To facilitate separation and visualization of your recombinant protein by polyacrylamide gel electrophoresis, a wide range of pre-cast NuPAGE™ and Novex™ Tris-Glycine polyacrylamide gels and electrophoresis apparatus are available from Thermo Fisher Scientific. In addition, Thermo Fisher Scientific carries a large selection of molecular weight protein standards and staining kits. For more information, refer to our website (**thermofisher.com**) or contact Technical Support.

NOTE: Expression of your protein fused to the TC-Tag and V5 epitope tags will increase the size of your recombinant protein. The table below lists the expected size increase in molecular weight for your recombinant protein. Note that the expected sizes take into account any additional amino acids between the gene of interest and the fusion peptide (see pages 8 and 9 for a diagram).

Vector	Fusion	Expected size increase
pcDNA™6.2/cTC-Tag-DEST	C-terminal	4 kDa
pcDNA™6.2/nTC-Tag-DEST	N-terminal	4 kDa

Appendix A: Media and reagents

Media and plates

LB (Luria-Bertani) medium and plates

Composition:

1.0% Tryptone

0.5% Yeast Extract

1.0% NaCl

pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic (100 µg/mL ampicillin) if needed.
4. Store at room temperature or at 2–6°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (100 µg/mL of ampicillin), and pour into 10 cm plates.
4. Let harden, then invert and store at 2–6°C.

Low-salt LB medium with Blasticidin

Low-salt LB medium:

10 g Tryptone
5 g NaCl
5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 mL. Adjust pH to 7.0 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the Blasticidin to 100 µg/mL final concentration.
4. Store plates at 2–6°C in the dark. Plates containing Blasticidin are stable for up to 2 weeks.

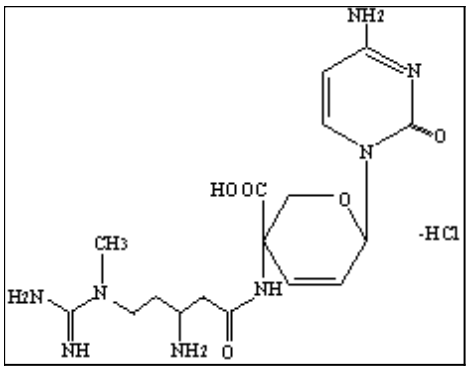
Blasticidin

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells.[17, 18] Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *bsd* from *Aspergillus terreus*[19] or *bsr* from *Bacillus cereus*.[20] These deaminases convert Blasticidin S to a non-toxic deaminohydroxy derivative.[20]

Molecular weight, formula, and structure

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of Blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (e.g. a laboratory coat) when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Prepare and store stock solutions

Blasticidin may be obtained separately (Cat. No. R210-01). Use sterile water to prepare Blasticidin stock solutions of 5–10 mg/mL.

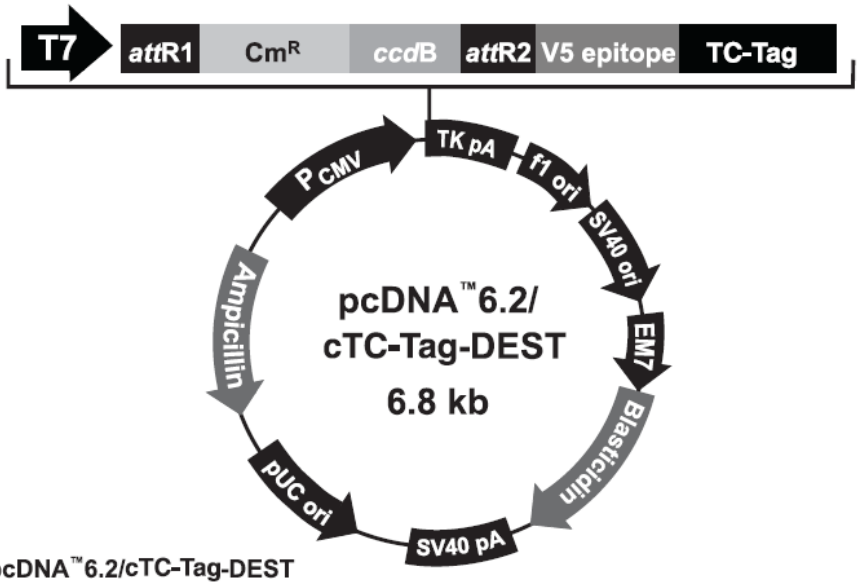
- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see next to last point below) and freeze at ≤–20°C for long-term storage or store at 2–6°C for short-term storage.
- Aqueous stock solutions are stable for 1–2 weeks at 2–6°C and 6–8 weeks at ≤–20°C.
- pH of the aqueous solution should be 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store the thawed stock solution at 2–6°C for up to 2 weeks.
- Medium containing Blasticidin may be stored at 2–6°C for up to 2 weeks.

Appendix B: Vectors

Map and features of pcDNA™6.2/cTC-Tag-DEST

Map

The map below shows the elements of pcDNA™6.2/cTC-Tag-DEST. DNA from the entry clone replaces the region between bases 922 and 2605. The complete sequence of this vector is available for download at **thermofisher.com** or by contacting Technical Support.



Comments for pcDNA™6.2/cTC-Tag-DEST
6809 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
*att*R1 site: bases 915-1039
Chloramphenicol resistance gene: bases 1148-1807
*ccd*B gene: bases 2149-2454
*att*R2 site: bases 2495-2619
V5 epitope: bases 2633-2674
TC-Tag: bases 2693-2710
TK polyadenylation signal: bases 2737-3008
TK polyA reverse priming site: bases 2744-2762
f1 origin: bases 3044-3472
SV40 early promoter and origin: bases 3499-3807
EM7 promoter: bases 3862-3928
Blasticidin resistance gene: bases 3929-4327
SV40 early polyadenylation signal: bases 4485-4615
pUC origin (c): bases 4998-5668
Ampicillin (*bla*) resistance gene (c): bases 5813-6673
bla promoter (c): bases 6674-6772

(c) = complementary strand

continued on next page

Map and features of pcDNA™6.2/cTC-Tag-DEST, continued

Features

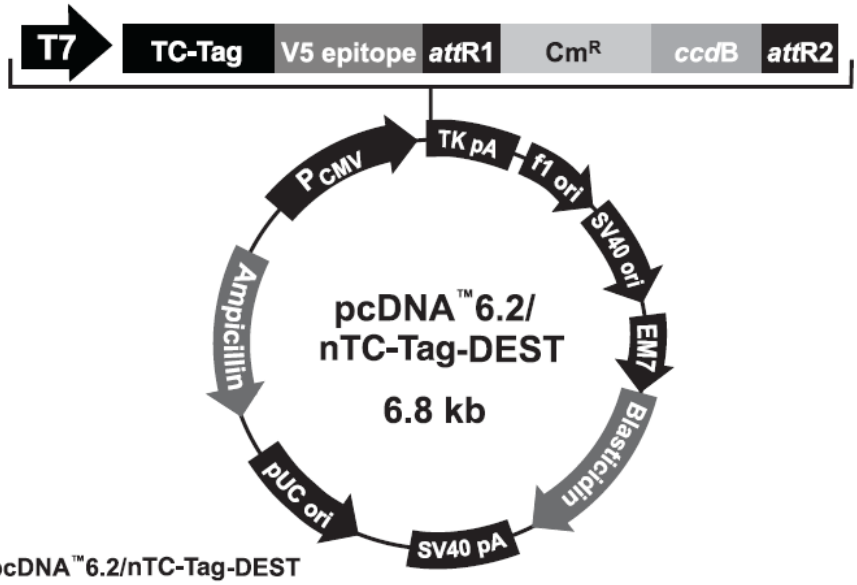
pcDNA™6.2/cTC-Tag-DEST (6809 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein [21–23]
T7 promoter/priming site	Allows *in vitro* transcription in the sense orientation and sequencing through the insert
*att*R1 and *att*R2 sites	Allows recombinational cloning of the gene of interest from an entry clone
Chloramphenicol resistance gene	Allows counterselection of plasmid
*ccd*B gene	Allows negative selection of plasmid
V5 epitope	Allows detection of the recombinant fusion protein by the Anti-V5 antibodies [24]
TC-Tag	Allows binding of the FlAsH-EDT_2 and ReAsH-EDT_2 labeling reagents to facilitate *in vivo* fluorescence detection of the recombinant fusion protein [5]
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA [25]
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
EM7 promoter	Allows expression of the Blasticidin resistance gene in *E. coli*
Blasticidin (*bsd*) resistance gene	Allows selection of stable transfectants in mammalian cells [19]
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
Ampicillin resistance gene	Allows selection of transformants in *E. coli*

Map and features of pcDNA™6.2/nTC-Tag-DEST

Map

The map below shows the elements of pcDNA™6.2/nTC-Tag-DEST. DNA from the entry clone replaces the region between bases 1018 and 2680. The complete sequence of this vector is available for download at **thermofisher.com** or by contacting Technical Support.



Comments for pcDNA™6.2/nTC-Tag-DEST
6796 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
TC-Tag: bases 924-941
V5 epitope: bases 957-998
*att*R1 site: bases 1011-1135
Chloramphenicol resistance gene: bases 1244-1903
*ccd*B gene: bases 2224-2529
*att*R2 site: bases 2570-2694
TK polyadenylation signal: bases 2724-2995
TK polyA reverse priming site: bases 2731-2749
f1 origin: bases 3031-3459
SV40 early promoter and origin: bases 3486-3794
EM7 promoter: bases 3849-3915
Blasticidin resistance gene: bases 3916-4314
SV40 early polyadenylation signal: bases 4472-4602
pUC origin (c): bases 4985-5655
Ampicillin (*bla*) resistance gene (c): bases 5800-6660
bla promoter (c): bases 6661-6759

(c) = complementary strand

continued on next page

Map and features of pcDNA™6.2/nTC-Tag-DEST, continued

Features

pcDNA™6.2/nTC-Tag-DEST (6796 bp) contain the following elements. All features have been functionally tested.

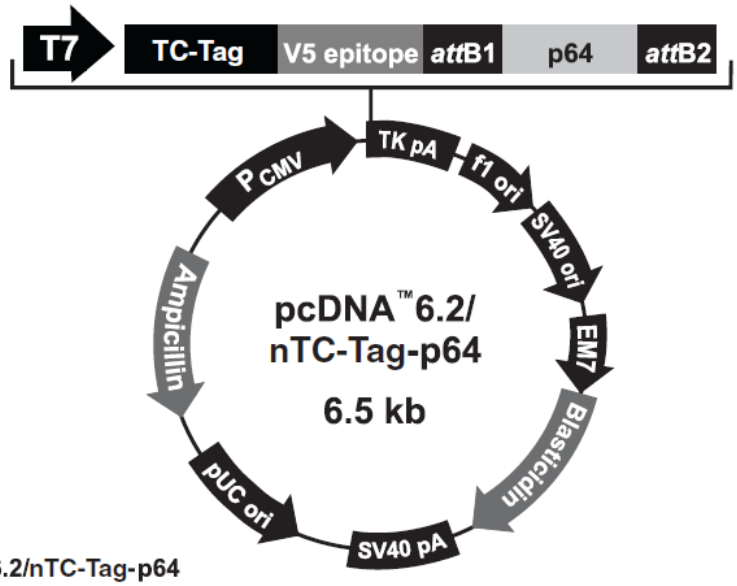
Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein [21–23]
T7 promoter	Allows *in vitro* transcription in the sense orientation
TC-Tag	Allows binding of the FlAsH-EDT_2 and ReAsH-EDT_2 labeling reagents to facilitate *in vivo* fluorescence detection of the recombinant fusion protein [5]
V5 epitope	Allows detection of the recombinant fusion protein by the Anti-V5 antibodies [24]
*att*R1 and *att*R2 sites	Allows recombinational cloning of the gene of interest from an entry clone
Chloramphenicol resistance gene	Allows counterselection of plasmid
*ccd*B gene	Allows negative selection of plasmid
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA [25]
TK polyA reverse priming site	Allows sequencing through the insert
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the Blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen
EM7 promoter	Allows expression of the Blasticidin resistance gene in *E. coli*
Blasticidin (*bsd*) resistance gene	Allows selection of stable transfectants in mammalian cells [19]
SV40 early polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA
pUC origin	Allows high-copy number replication and growth in *E. coli*
Ampicillin resistance gene	Allows selection of transformants in *E. coli*

Map of pcDNA™6.2/nTC-Tag-p64

Description

pcDNA™6.2/nTC-Tag-p64 (6485 bp) is a control vector expressing the p64 gene, and was generated using the Gateway™ LR recombination reaction between an entry clone containing the human *c-myc* gene [26–28] and pcDNA™6.2/nTC-Tag-DEST. The complete sequence of this vector is available for download at **thermofisher.com** or by contacting Technical Support.

Note: The *c-myc* gene encodes a protein with an expected molecular weight of 48 kDa, however, the native protein actually runs at a range of 55–64 kDa on an SDS-PAGE gel.



Comments for pcDNA™6.2/nTC-Tag-p64
6485 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
TC-Tag: bases 924-941
V5 epitope: bases 957-998
*att*B1 site: bases 1011-1035
p64 gene: bases 1038-2357
*att*B2 site: bases 2359-2383
TK polyadenylation signal: bases 2413-2684
TK polyA reverse priming site: bases 2420-2438
f1 origin: bases 2720-3148
SV40 early promoter and origin: bases 3175-3483
EM7 promoter: bases 3538-3604
Blasticidin resistance gene: bases 3605-4003
SV40 early polyadenylation signal: bases 4161-4291
pUC origin (c): bases 4674-5344
Ampicillin (*bla*) resistance gene (c): bases 5489-6349
bla promoter (c): bases 6350-6448

(c) = complementary strand

Appendix C: Ordering information

In-Cell Tetracysteine Tag Detection Kits

Ordering information for the TC-FlAsH™ and TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits is provided below.

Product	Amount	Catalog No.
TC-FlAsH™ TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit (with Mammalian Gateway™ expression vectors)	1 kit	T34563
TC-FlAsH™ II In-Cell Tetracysteine Tag Detection Kit	1 kit	T34561
TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit	1 kit	T34562

Documentation and support

Obtaining support

Technical support

For the latest services and support information for all locations, visit **www.thermofisher.com**.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**thermofisher.com/support)**
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at **thermofisher.com/support**.

IMPORTANT! For the SDSs of chemicals not distributed by Thermo Fisher Scientific contact the chemical manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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1. Annu Rev Biochem 58, 913 (1989); 2. Science 281, 269 (1998); 3. Science 296, 503 (2002); 4. Nature Methods 2, 171 (2005); 5. J Am Chem Soc 124, 6063 (2002); 6. EMBO J 4, 213 (1985); 7. J Cell Biol 115, 887 (1991); 8. Proc Natl Acad Sci U S A 87, 8301 (1990); 9. Nucleic Acids Res 15, 8125 (1987); 10. *Current Protocols in Molecular Biology*, Ausubel FM, et al., Eds. (1994); 11. Mol Cell Biol 7, 2745 (1987); 12. Cell 11, 223 (1977); 13. Proc West Pharmacol Soc 32, 115 (1989); 14. Nature 337, 387 (1989); 15. Nucleic Acids Res 15, 1311 (1987); 16. Biotechniques 6, 742 (1988); 17. J Antibiot (Tokyo), Series A 11, 1 (1958); 18. J. Biochem 57, 667 (1965); 19. Biochim Biophys Acta 1219, 653 (1994); 20. Exp. Cell Res 197, 229 (1991); 21. J Biol Chem 264, 8222 (1989); 22. Cell 41, 521 (1985); 23. Mol Cell Biol 7, 4125 (1987); 24. J Gen Virol 72, 1551 (1991); 25. Mol Cell Biol 5, 2104 (1985); 26. EMBO J 2, 2375 (1983); 27. Nature 301, 722 (1983); 28. Nature 303, 725 (1983).