pCLIP$_f$ Vector

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N9215S 002120615061

N9215S

20 µg **Lot: 0021206**

Store at: –20°C **Exp: 6/15**

Introduction

pCLIP$_f$ Vector is a mammalian expression plasmid intended for the cloning and stable or transient expression of CLIP-tag® protein fusions in mammalian cells. This plasmid encodes *CLIP$_f$*, a CLIP-tag protein, which is expressed under control of the CMV promoter. The expression vector has an IRES (internal ribosome entry site) and a neomycin resistance gene downstream of the CLIP$_f$ for the efficient selection of stable transfectants. pCLIP$_f$ Vector contains two multiple cloning sites to allow cloning of the fusion partner as a fusion to the N- or C-terminus of the CLIP$_f$.

The CLIP-tag is a novel tool for protein research, allowing the specific, covalent attachment of virtually any molecule to a protein of interest. The CLIP-tag is a small polypeptide based on human O^6-alkylguanine-DNA-alkyltransferase (hAGT). CLIP-tag substrates are derivatives of benzyl cytosine (BC). In the labeling reaction, the substituted benzyl group of the substrate is covalently attached to the reactive cysteine of CLIP-tag forming a stable thioether link.

pCLIP$_f$ contains an improved version of CLIP-tag, termed CLIP$_f$. CLIP$_f$ displays faster kinetics in *in vitro* labeling and fast, specific and efficient labeling in live and fixed cell applications, thereby rendering it a desired research tool for analysis of protein dynamics.

Although CLIP-tag is based on the same protein as SNAP-tag®, the benzylcytosine substrates form a separate class of substrates, different from the benzylguanine substrates recognized by SNAP-tag. CLIP-tag and SNAP-tag can be used for orthogonal simultaneous labeling.

There are two steps to using this system: subcloning and expression of the protein of interest as a CLIP$_f$ fusion, and labeling of the fusion with the CLIP-tag substrate of choice. Cloning and expression of CLIP$_f$ fusion proteins are described in this document. The labeling of the fusion proteins with CLIP-tag substrates is described in the instructions supplied with the CLIP-tag substrates.

Materials Required but not Supplied:

Tissue culture reagents and media

Mammalian cell line(s)

Transfection reagents

Storage

pCLIP$_f$ Vector is supplied in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at a concentration of 0.5 µg/µl. Plasmid solutions can be stored at 4°C for up to one week. For long-term storage, –20°C is recommended.

Detailed Description

A plasmid map and the sequence of the cloning region can be found at the end of these instructions. The complete plasmid sequence can be downloaded at www.neb.com. This plasmid encodes the gene *CLIP$_f$* which is a mutant form of the human gene for O^6-alkylguanine-DNA-alkyltransferase (hAGT). The codon usage of the gene is optimized for expression in mammalian cells. In the plasmid sequence, the *CLIP$_f$* gene is encoded from 969 bp to 1514 bp.

This plasmid is intended for the cloning and stable or transient expression of CLIP-tag protein fusions in mammalian cells. It is suitable for the efficient production of stable cell lines expressing CLIP$_f$ gene fusions. The plasmid contains the CMV promoter followed by the genes for CLIP$_f$ and neomycin resistance separated by the IRES of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA; therefore after selection of stable mammalian cells for neomycin resistance, nearly all surviving colonies should stably express the CLIP$_f$ fusion protein. Unless the expression experiments require a pure population of cells, the pool of resistant cells can simply be used, otherwise cell clones can be isolated and characterized using standard procedures.

The plasmid contains the β-lactamase (Ampicillin resistance) gene for maintenance in bacteria. The gene of interest can be cloned upstream or downstream of the CLIP$_f$ coding sequence, as a fusion to the N- or C-terminus of the CLIP-tag. pCLIP$_f$ Vector can also be used as an expression control plasmid, expressing CLIP$_f$ alone, in which case the CLIP-tag protein is distributed throughout the cell. The *CLIP$_f$* gene can be isolated from the plasmid using PCR or direct cloning in order to subclone it into a different vector of choice.

Cloning of CLIP-tag Fusions in pCLIP$_f$

Cloning by PCR

To subclone the gene of interest into pCLIP$_f$ fused to the N-terminus of CLIP$_f$, use the available restriction sites: NheI, EcoRV (blunt), AscI, SwaI (blunt), BsrGI, AgeI or EcoRI which are located upstream of the CLIP-tag.

To subclone the gene of interest into pCLIP$_f$ fused to the C-terminus of CLIP$_f$, use the available restriction sites downstream of the CLIP-tag: SbfI, BamHI, PmeI (blunt), XhoI, PacI or NotI.

Note: When fusing the gene of interest to the C-terminus of CLIP$_f$, note that there is a stop codon between the PacI and NotI sites, so SbfI, BamHI, PmeI, XhoI or PacI must be used as the 5´ cloning site for the insert.

Note: PmeI and XhoI cannot be used together for cloning because they share a cytosine as part of their recognition sequences.

Primer Design and Cloning Considerations:

- Design the PCR primers to include a sufficient overlap (15–20 bp) with the sequence of the gene to be amplified.
- For fusion to the C-terminus of the CLIP-tag, a stop codon may be included at the C-terminus of the fusion (in front of the downstream cloning site) in order to terminate translation at this position.
- For fusions upstream of the CLIP$_f$, ensure that a start codon is included. The addition of a Kozak sequence (e.g. GCCRCCATG, where the start codon is underlined) may increase the translation efficiency.
- In general, any linker peptide between the proteins should be kept short to avoid degradation by proteases. If required, specific protease cleavage sites can be introduced into the linker peptide.
- Care should be taken to design the cloning strategy so that the fusion partners in the resulting construct are in frame.
- Perform the PCR reaction and subsequent cloning steps according to established protocols for molecular biology.
- After subcloning the gene of interest into pCLIP$_f$ as a fusion with the *CLIP$_f$* gene, the resulting plasmid can be used for stable or transient expression of the CLIP-tag fusion proteins in a suitable cell line.

Direct Cloning

Direct cloning can also be used to make fusions with the CLIP-tag. This is only possible if the fusion partner has compatible sites adjacent to the gene of interest.

Care should be taken to design the cloning so that the fusion partners in the resulting construct are in frame.

Note: When fusing the gene of interest to the C-terminus of CLIP$_f$, note that there is a stop codon between the PacI and NotI sites, so SbfI, BamHI, PmeI, XhoI or PacI must be used as the 5´ cloning site for the insert.

Note: PmeI and XhoI cannot be used together for cloning because they share a cytosine as part of their recognition sequences.

Expression of CLIP-tag Fusions

Transient Expression

Expression of the fusion protein cloned in pCLIP$_f$ can be achieved by transiently transfecting cells in culture with standard transfection protocols. The appropriate reagent and time to permit adequate expression must be empirically determined. We recommend using pCLIP$_f$-H2B (NEB #N9218) or pCLIP$_f$-Cox8A (NEB #N9217)as expression control plasmids. H2B-CLIP$_f$ fusion protein gives a nuclear localized signal and the COX8-2-CLIP$_f$ fusion protein gives a mitochondrial localized signal when labeled with CLIP-Cell substrates. If the empty pCLIP$_f$ plasmid is used as a control vector for transfection, an even distribution of the CLIP-tag in nucleus and cytoplasm should be seen. Both pCLIP$_f$ and the localization control plasmids have performed well in stable and transient transfection of CHO-K1, COS-7, U-2 OS and NIH 3T3 cells. Note that the intensity of the fluorescence may vary, depending on the cell line and labeling substrate used.

We recommend using TransPass D2 (NEB #M2554) in combination with TransPass V (NEB #M2561) or Roche's FuGENE® 6 Transfection Reagent for both transient and stable transfections.

Stable Expression

pCLIP$_f$ and the localization control plasmids can be transfected by standard transfection methods. Twenty-four to 48 hours after transfection, begin selecting mammalian cultures in 600–1,200 µg/ml G418 (geneticin) depending on the cell line. It is recommended that a kill curve be established for

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CERTIFICATE OF ANALYSIS

each cell line to determine optimal selection conditions. After 8–12 days of continuous selection, stable colonies will become visible. It is possible to use pools of stable cell populations for initial cell labeling to test for the presence of CLIP-tag expression. In addition, monoclonal cell lines can be isolated and characterized, if desired.

Troubleshooting

Cloning of the Gene of Interest

If subcloning of the gene of interest with the CLIP-tag does not work, reconfirm all the cloning steps (primer design, choice of restriction site, DNA isolation, ligation and transformation, etc.). If all steps are confirmed as being correct, then try the cloning using different restriction sites. Be sure to include a positive and negative control for the ligation reaction.

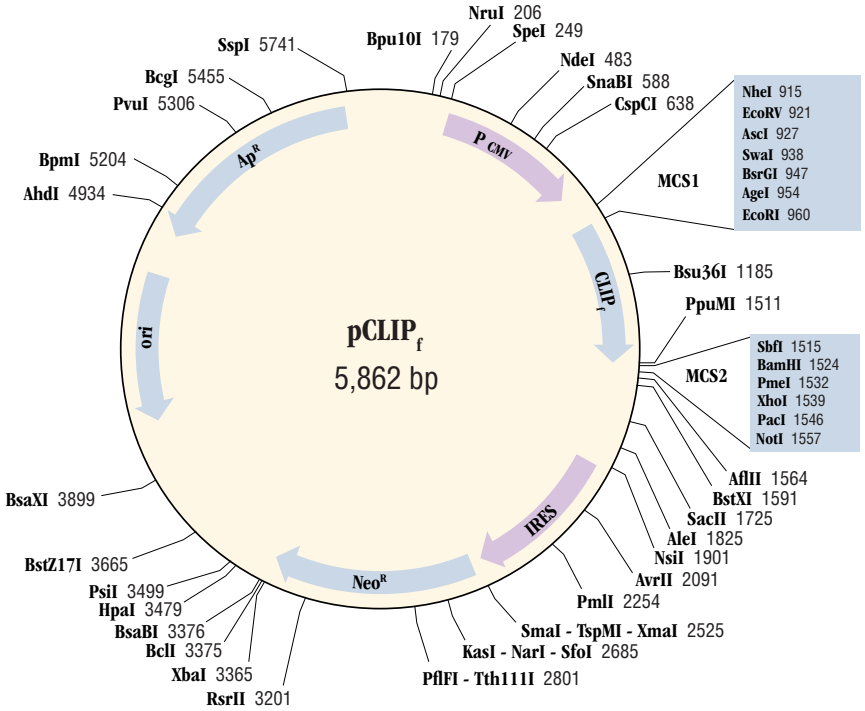
Alternatively, try to subclone the $CLIP_f$ gene into a mammalian expression vector already containing the gene of interest.

Expression

In general, we have not experienced problems expressing CLIP-tag protein fusions. However, if the fusion protein does not appear to be expressed, try expressing the H2B-$CLIP_f$ or COX8-2-$CLIP_f$ protein fusions as positive controls using cells transiently transfected with pCLIP$_f$-H2B or pCLIP$_f$-Cox8A. Labeling of such cells with a fluorescent CLIP-Cell substrate should show strong nuclear or mitochondrial localized fluorescence, respectively. The empty pCLIP$_f$ plasmid can also be used as a control (cytosolic and nuclear fluorescence). Note that the intensity of this fluorescence may vary depending on cell line and substrate used. If the localization controls are expressed but the fusion protein is not, then there are a variety of possible causes. It is possible that this fusion protein may be toxic for the cell line. It is difficult to troubleshoot such instances, but the use of a different expression plasmid or cell line or tagging the opposite end (N or C) of the protein may help. Signs of host cell toxicity could include slow proliferation or apoptosis. Counterstaining live cells with Hoechst 33342 or fixed cells with DAPI can be used to determine whether nuclei are healthy, if toxicity is suspected.

Plasmid Map of pCLIP$_f$ Vector

This map and the maps for the control plasmids can be downloaded at www.neb.com.



Cloning Region of pCLIP$_f$

Unique restriction sites in the regions flanking the *CLIP$_f$* gene are displayed above the coding strand. The complete sequence for pCLIP$_f$ and the control plasmids can be downloaded at www.neb.com

5´ MCS

```
   NheI  EcoRV AscI     SwaI    BsrGI  AgeI  EcoRI
...GCTAGC GATATCGGCG CGCCAGCATT TAAATCTGTA CAGACCGGTG AATTC
   CGATCG CTATAGCCGC GCGGTCGTAA ATTTAGACAT GTCTGGCCAC TTAAG...
```

3´ MCS

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                                  XhoI
   SbfI   BamHI    PmeI          PacI      NotI
...CCTGCA GGCGGATCCG CGTTTAAACT CGAGGTTAAT TAATGAGCGG CCGC
   GGACGT CCGCCTAGGC GCAAATTTGA GCTCCAATTA ATTACTCGCC GGCG...
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References:

1. Keppler, A. et al. (2003) *Nat. Biotechnol.* 21, 86.
2. Gautier, A. et al. (2008) *Chem. Biol.* 15, 128.
3. Keppler, A. et al. (2004) *Proc. Natl. Acad. Sci. USA* 101, 9955.
4. Maurel, D. et al. (2008) *Nat. Methods* 5, 561.
5. Jansen, L.E. et al. (2007) *J. of Cell Biol.* 176, 795.
6. Krayl, M., Guiard, B. Paal, K. and Vous, W. (2006) *Anal. Biol. Chem.* 355, 81–89.
7. Banala, S., Arnold, A. and Johnsson, K. (2008) *ChemBio Chem.* 9, 38–41.

Companion Products:

pCLIP$_f$-NKIR
#N9216S 20 µg

pCLIP$_f$-Cox8A
#N9217S 20 µg

pCLIP$_f$-H2B
#N9218S 20 µg