A Simple Protocol to Insert the Profinity eXact™ Tag Into Alternate Expression Vectors

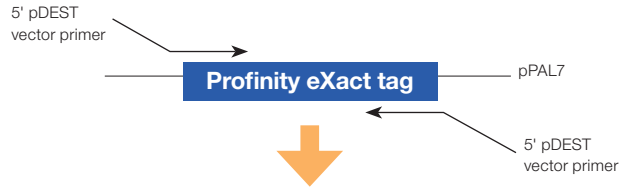
Introduction

The Profinity eXact affinity purification system yields purified, tag-free target protein in as little as 1 hour after lysate loading. This system utilizes an immobilized mutant subtilisin protease that binds the Profinity eXact tag with high affinity; upon addition of a fluoride-containing elution buffer, the protease is activated and the tag-free target protein is quickly released. The Profinity eXact tag is expressed as an N-terminal tag and contains an engineered cleavage recognition sequence (EEDKLFKAL) at its C-terminus. Cleavage occurs directly after this sequence. The majority of proteins are purified without any residual amino acids from the Profinity eXact tag. However, if cysteine or proline immediately follows the cleavage recognition site, cloning should be performed to introduce a two-amino acid spacer (Thr-Ser) prior to the starting amino acids of the target protein of interest. *E. coli* expression of a Profinity eXact fusion protein is easily accomplished with the Bio-Rad pPAL7 expression vector, which facilitates construction of an N-terminal Profinity eXact fusion tag with the protein expressed by the target DNA sequence. However, some users prefer to use other expression vectors in order to utilize alternative promoters, selection markers, or C-terminal affinity tags.

This article describes one method that can be used to quickly clone the Profinity eXact tag into a different expression vector. First, a PCR fragment containing the Profinity eXact tag is created with 5' and 3' sequences complementary to the insertion site of the expression vector to be modified. The two strands of the PCR fragment are then used as mutagenesis oligonucleotides to insert the new affinity tag into the expression vector or replace undesired sequences (for example, 6xHis tag) with the Profinity eXact tag. A modification of the manufacturer's instructions for the QuikChange XL site-directed mutagenesis kit (Stratagene Corporation) is used to create DNA sequences of up to 1 kb for insertion (Geiser et al. 2001). The example illustrated in this article describes the substitution of the Profinity eXact tag for the 6xHis tag within the Gateway pDEST17 vector (Invitrogen Corporation) (Figure 1).

Step 1: Design Primers

Primers are designed to produce the Profinity eXact tag with 5' ends that are complementary to the flanking regions of the insertion/ substitution site.

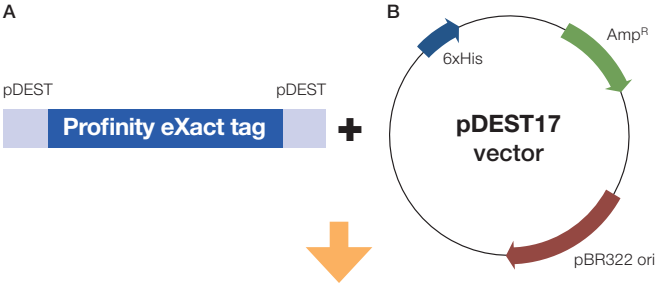


Step 2: Perform PCR

The resultant double-stranded PCR fragment is used as oligos for the mutagenesis reaction.

Step 3: Perform Mutagenesis

During the mutagenesis reaction, the Profinity eXact tag (A) replaces the original 6xHis (B) used in the pDEST17 vector.



Step 4: Create Modified pDEST17 Vector

The pDEST17 vector containing the Profinity eXact tag is created.

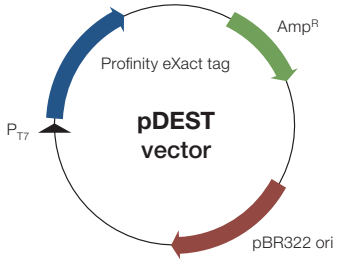


Fig. 1. Outline of strategy to replace 6xHis tag of Gateway pDEST17 recipient vector with the Profinity eXact tag.

Profinity eXact Tag Cloning Protocol

Use this method to insert the Profinity eXact tag into any expression vector quickly and easily:

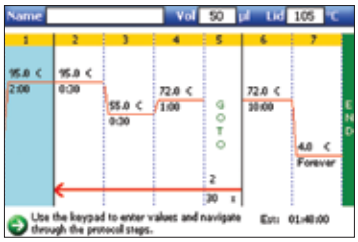
1. Use PCR to create the oligonucleotide fragments needed for use with the QuikChange XL site-directed mutagenesis kit (The fragments are too long — greater than 250 bases — for chemical synthesis).

   Figure 2 shows the primers containing the Profinity eXact tag (red sequences) flanked by the DNA sequences complementary to the region surrounding the pDEST17 insertion/replacement site (blue and green sequences in Figures 2 and 3).

Forward primer 5' GAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG GGAGGGAAATCAAACGGG

Reverse primer 5' CATTTTACGTTTCTCGTTCAGCTTTTTTGTACAAACTTGT CAAAGCTTTGAAGAGCTTGTC

PCR conditions



ddH_2O	34.5 µl
10× *Pfu* reaction buffer	5.0 µl
dNTP	1.0 µl
pPAL7 (100 ng/µl)	0.5 µl
Forward primer (10 µl)	4.0 µl
Reverse primer (10 µl)	4.0 µl
Pfu polymerase (1 U/µl)	1.0 µl

Fig. 2. Primer design and PCR setup to create mutagenesis oligos.

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                                    T7 promoter
1   AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG AGACCACAAC GGTTTCCCTC
    TCTAGAGCTA GGGCGCTTTA ATTATGCTGA GTGATATCCC TCTGGTGTTG CCAAAGGGAG

                                                 initiation ATG
                                          NdeI                6×His
61  TAGAAATAAT TTTGTTTAAC TTTAAGAAGG AGATATACAT ATGTCGTACT ACCATCACCA
    ATCTTTATTA AAACAAATTG AAATTCTTCC TCTATATGTA TACAGCATGA TGGTAGTGGT

        6×His                                attR1
121 TCACCATCAC CTCGAATCAA CAAGTTTGTA CAAAAAAGCT GAACGAGAAA CGTAAAATGA
    AGTGGTAGTG GAGCTTAGTT GTTCAAACAT GTTTTTTCGA CTTGCTCTTT GCATTTTACT

                                attR1
181 TATAAATATC AATATATTAA ATTAGATTTT GCATAAAAAA CAGACTACAT AATACTGTAA
    ATATTTATAG TTATATAATT TAATCTAAAA CGTATTTTTT GTCTGATGTA TTATGACATT

            attR1
241 AACACAACAT TCCAGTCAC  TATGGCGGCC GCATTAGGCA CCCCAGGCTT TACACTTTAT
    TTGTGTTGTA TAGGTCAGTG ATACCGCCGG CGTAATCCGT GGGGTCCGAA ATGTGAAATA
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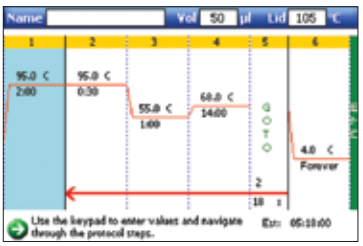
Fig. 3. Target pDEST17 vector DNA sequence. The region highlighted in blue represents the sequence that should be present on the 5'-end of the Profinity eXact forward primer. The region highlighted in green represents the sequence that should be present on the 5'-end of the Profinity eXact reverse primer. After the QuikChange XL mutagenesis kit reaction, the insertion site highlighted in yellow will be replaced by the Profinity eXact tag.

2. Purify the PCR product using a 1% ReadyAgarose™ gel. Figure 3 shows the complementary regions along with the insertion site in the pDEST17 vector DNA sequence.

3. Modify the pDEST17 vector using the QuikChange XL site-directed mutagenesis kit with the conditions illustrated in Figure 4.

PCR conditions



ddH_2O	16 µl
10× QuikChange XL reaction buffer	5 µl
dNTP mix (Stratagene)	1 µl
pDEST17 (100 ng/µl)	2 µl
Profinity eXact PCR product (84 ng/µl)	25 µl
Pfu polymerase (2.5 U/µl)	1 µl

Fig. 4. Mutagenesis reaction to create pDEST17 vector modification.

4. Treat the resulting PCR product with DpnI as described in the QuikChange XL protocol.
5. Use the DpnI-treated PCR product (5 µl) to transform *E. coli* cloning chemi-competent cells (50 µl, Bio-Rad catalog #156-3000).
6. Culture the putative transformant colonies.
7. Isolate the Profinity eXact tag-pDEST vector using mini prep DNA purification.

Summary

Benefits of using the Profinity eXact fusion tag system include time savings, increased lab efficiency, and the ability to generate unadulterated, tag-free protein in under an hour. The ability to obtain a purified target protein, free of its fusion partner, may still be realized regardless of its use with the pPAL7 expression vector. Whether due to user preference or increased familiarity with other expression vectors, there remain certain impediments for researchers when it comes to adoption of a new cloning system. The example presented here demonstrates how the Profinity eXact tag can easily be used to replace another tag (for example, 6xHis) by being inserted into a non-pPAL expression vector of choice. Depending on the vector to be modified, the user may instead choose to perform restriction enzyme cloning or recombination-mediated techniques, both common alternative strategies for DNA manipulation.

References

Geiser M et al. (2001). Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. Biotechniques 31, 88-90, 92.

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