Using the Profinity eXact™ Fusion-Tag System: Strategies for Success

Introduction

The Profinity eXact fusion-tag system is a novel *E. coli*–based system for the expression, detection, purification, and on-column cleavage of affinity-tagged proteins without the addition of protease. The system utilizes an immobilized, extensively engineered protease that both recognizes and avidly binds to the small N-terminal (K_D < 100 pM) coexpressed affinity tag in the fusion protein. Subsequent to column washing, the protease performs a specific, controlled cleavage and removal of the tag from the fusion protein directly on the column. The result is the release of highly purified recombinant protein with a native N-terminus. This article presents helpful tips and techniques to apply when working with the Profinity eXact fusion-tag system to ensure purification success and the generation of a native, tag-free protein in a single step (Figure 1).

Cloning Using pPAL7 Expression Vectors

Fusion proteins with an N-terminal Profinity eXact tag are expressed with the 5.9 kb pPAL7 expression vector. This inducible expression vector utilizes the strong, tightly regulated T7lac promoter. The pPAL7 plasmid has been designed to facilitate cloning of a target gene through several methods, including restriction-based cloning and restriction-independent cloning (RIC).

When using RIC methods, the following conditions can be optimized:

- **T4 DNA polymerase/dGTP reaction** — use a thermal cycler to accurately obtain the desired 12°C reaction temperature. An alternative is to place your tube in a 12 °C water bath or heating block — this can be achieved by setting a water bath or a heating block to 12 °C in a refrigerator or in a cold room.
- **Cloning efficiencies** — if reduced cloning efficiencies are observed, ensure that the PCR primers are 5'-phosphorylated. Primers must be phosphorylated at the 5' end because the RIC-ready vector has been alkaline phosphatase-treated
- **Ligation** — when using a quick ligase with the RIC vector, incubate the ligase reaction mixture for 20 min at room temperature. The SapI-generated overhang of the RIC vector has only three bases, so using a quick ligase for only 5 min does not allow time for proper ligation. Standard ligases should be incubated for 16 hr at 16°C
- ***E. coli* transformation** — achieve highest transformation efficiencies by incubating the chemical competent cells and the RIC ligation on ice for 30 min prior to the 30 sec, 42°C heat shock

When using general cloning methods, the following techniques can help achieve best results:

Use of threonine-serine (Thr-Ser) spacer — A Thr-Ser spacer can help overcome problems related to binding and cleavage. A Thr-Ser spacer is recommended when:

- **Proteins exhibit significant N-terminal structure** — protein binding to the resin may be affected by significant N-terminal structure. A Thr-Ser spacer may be introduced at the cloning stage between the Profinity eXact tag and the target proteins to generate an imprecise fusion when poor binding of the protein occurs
- **Undesirable P1'–P2' amino acids are in the target protein** — when designing the construct, consider the P1' and P2' amino acids and their effects on purification. If either or both of these amino acids in the target protein

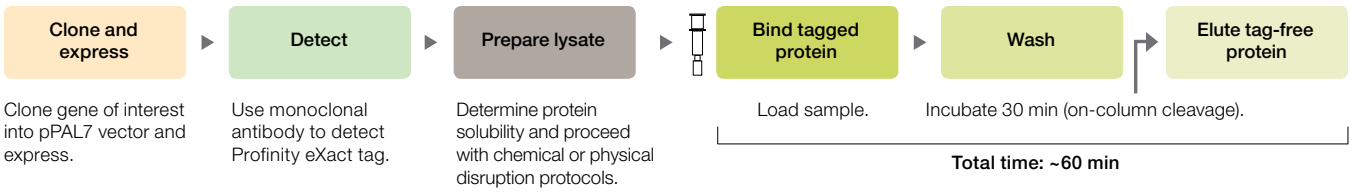

Fig. 1. Protein expression and purification workflow. The Profinity eXact system offers parallel purification and on-column cleavage. Availability of immobilized protease on the column appreciably shortens the purification process.

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(immediately downstream of the Profinity eXact cleavage site) are Pro, then the fusion will not cleave; introduce a Thr-Ser spacer (Figure 2, Table 1)

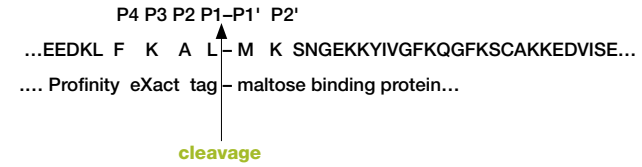

Fig. 2. Cloning considerations for P1' – P2' amino acids.

Table 1. Troubleshooting problems with cleavage and P1'–P2' amino acids.

Position	Amino Acid	Problem	Solution
P1'	Proline	No cleaving	Spacer (Thr-Ser)
	Cysteine	Premature cleaving	Cold wash buffer Spacer
	Aspartate	Very slow cleaving	Spacer
P2'	Proline	Cleaving may be very slow	Spacer

Use of alternative vectors —The Profinity eXact tag can be placed into a customer's vector of choice. Simply isolate the tag sequence from the pPAL7 vector using NdeI and a downstream restriction enzyme corresponding to a site in the multiple cloning site (for example, NcoI, BamHI, EcoRI, XhoI, or NotI). The complete vector sequence is available online at **www.bio-rad.com/profinityexact/** (from this page, select "Expression and Cloning Products" to download the sequence file). Another alternative method is described in bulletin 5813.

Purification Using Profinity eXact Resin

The Profinity eXact system utilizes an extensively engineered subtilisin protease that is immobilized directly onto a chromatography support. The tag is a modified form of the subtilisin prodomain, a 75-amino acid sequence (8 kD) fused to the N-terminus of the target protein. Both the mature protease and its prodomain tag bind strongly to one another. Upon incubation (30 min) with a fluoride-containing buffer, a controlled and highly specific cleavage occurs directly after the 9-amino acid cleavage recognition site (EEDKLFKAL) to generate a tag-free protein containing its native N-terminal amino acid sequence.

The following tips can be useful to improve experimental conditions in the cell lysis, sample application, and washing steps.

- **Improve protein binding** — allow the lysate to incubate with resin for up to 1 hr at 4°C or for 30 min at room temperature. Lysates with fusion proteins >75 kD often benefit from a longer incubation period
- **Substitute with acetate buffers** — ensure lysis and wash buffers do not contain triggering ions, such as Cl^- or F^-. Chloride ions from additives such as NaCl, KCl, and Tris-HCl act as slower cleavage/elution-triggering anions. Substitute sodium acetate (NaOAc) or potassium acetate (KOAc) for NaCl or KCl
- **Improve ionic strength** — if using the 0.1 M $NaPO_4$ Profinity eXact bind/wash buffer, use a higher $NaPO_4$ concentration (0.3–1.0 M, pH 7.2) to raise the ionic concentration of the lysis and wash buffers
- **Minimize intrinsic cleavage** — use a lysis buffer with a pH of <7.0, but do not use HCl to adjust the pH of the buffer
- **Improve solubility/protein denaturation** — use 8 M urea instead of guanidine-HCl for denaturation, but dilute lysates to 2–4 M urea before loading onto the column; high concentrations of urea affect performance of the resin
- **Chill buffers** — maintain the lysate at 4°C prior to loading to reduce intrinsic cleavage. If the P1' amino acid is Cys, prechill the lysate and use cold buffers; otherwise, the fusion protein may cleave during sample loading and resin washing steps

Elution of Target Proteins

Elution of target proteins is typically conducted by incubating the resin in 100 mM NaF, 100 mM $NaPO_4$, pH 7.2, at room temperature for 30 min. For best results:

- **Increase elution incubation times** — if the P1' amino acid is Asp, the fusion will cleave very slowly. Perform an overnight elution incubation at room temperature, or introduce a Thr-Ser spacer
- **Use azide in the elution buffers** — as an alternative to F^-, use azide in the elution buffer as the triggering ion. A much lower concentration of azide is required (10 mM vs. 100 mM fluoride), and the purified protein can be used directly in downstream applications without the need for desalting or buffer exchange

If contaminants are observed in the eluate:

- Dilute the lysate (for multimeric proteins)
- Reduce the load of fusion protein
- Incubate the lysate with resin for up to 1 hr at 4°C to increase binding capacity of the target fusion protein
- Perform an additional wash step
- Reduce nonspecific, electrostatic binding by increasing the ionic concentration of the wash buffer; use up to 0.3 M $NaPO_4$, NaOAc, or $(NH_4)_2SO_4$ (pH 7.2), or amend the wash buffer with any of the aforementioned salts. Do not use NaCl
- Reduce hydrophobic interactions by decreasing the salt concentration of the wash buffer
- Supplement the wash buffer with a suitable detergent (Table 2)

Table 2. Chemical compatibility.*

Reagent Type	Compatible With Profinity eXact System
Lysis solutions	Bacterial lysis and extraction reagent (Bio-Rad) B-PER protein extraction reagent in Pi buffer (Thermo Fisher Scientific) B-PER protein extraction reagent in Tris buffer** BugBuster protein extraction reagent** (Novagen) FastBreak cell lysis reagent** (Promega Corporation)
Protease inhibitors	1x Protease inhibitor cocktail (BD Pharmingen) 2x Protease inhibitor cocktail set 1 (Calbiochem) Complete protease inhibitor tablets (Roche Diagnostics) 0.5 mM PMSF 0.1 mM TLCK 0.1 mM TPCK
Detergents	5% (v/v) Triton X-100 5% (v/v) NP-40 5% (v/v) Tween-20 5% (w/v) octylthioglucoside 5% (w/v) n-dodecyl β-D-maltoside 5% (w/v) CHAPS 5% (w/v) CHAPSO
Reducing reagents	20 mM β-mercaptoethanol 10 mM DTT 5 mM TCEP
Chelating reagents	20 mM EDTA 20 mM EGTA
Buffer reagents	50 mM Tris-acetate, pH 7.2 50 mM Tris-phosphate, pH 7.2 50 mM HEPES, pH 7.2 50 mM PIPES, pH 7.2 50 mM MOPS, pH 7.2 50 mM MES, pH 7.2
Additives	20% (v/v) glycerol 20% (v/v) ethylene glycol 20% (v/v) ethanol 20% (w/v) sorbitol 20% (w/v) sucrose 200 mM imidazole 200 mM sodium acetate 100 mM sodium borate 100 mM sodium citrate 100 mM sodium sulfate 15% (w/v) ammonium sulfate 5% (v/v) DMSO 20 mM β-mercaptoethanol 5 mM $MgCl_2$** 5 mM $CaCl_2$**

* Compatibilities determined using Profinity eXact control lysate; some reagents, like ammonium sulfate, are protein dependent.

** Chloride ions trigger slow cleavage of target proteins from the column.

Confirming Generation of Target Protein

Use mass spectrometry (MS) methods to confirm the generation of a tag-free protein. The following experimental results are included to illustrate a suggested method.

Purified maltose binding protein (MBP) from a Profinity eXact column is free of extraneous amino acids, as evidenced by MS data (data not shown). MBP eluates purified from a Profinity eXact column were analyzed by LC/MS using a QSTAR quadrupole-time of flight mass spectrometer. The expected mass of the purified, tag-free MBP (calculated from the amino acid sequence using the ExPASy compute MW tool) was 40,339.89 Da; experimental mass from spectra was 40,343.9 Da.

The terminal residue of the tag is a leucine, which has a molecular mass of 113 Da. The first residue of MBP is methionine, which has a molecular mass of 131 Da (Figure 2). If any miscleavage occurred, the mass spectrometer-measured mass of the purified protein would be off by greater than 100 Da — compared to the 4 Da difference demonstrated by these results (observed vs. expected mass difference is within the 100 ppm mass accuracy of the instrument).

Resin Storage and Cleaning

To ensure maximum shelf life of the Profinity eXact resin:

- Store it at 4°C. However, the subtilisin mutant is stable in incubations of 30 min at 60°C and 5 days at 37°C
- Regenerate it by stripping off the cleaved Profinity eXact tag from the mutant subtilisin ligand by incubating the resin in 0.1 M H_3PO_4. This also effectively removes contaminants from the resin
- Immediately after cleaning, re-equilibrate the resin with bind/wash buffer or storage buffer (100 mM $NaPO_4$, 0.02% sodium azide, pH 7.2) to prevent loss of activity
- Remove any other residual contaminants by washing the resin with 0.1 M NaOH (the resin is base stable). After cleaning with NaOH, equilibrate the resin with bind/wash buffer
- Do not store the resin in 0.1 M H_3PO_4, 0.1 M NaOH, or water for long periods of time (>1 hr)

For more information, visit **www.bio-rad.com/profinityexact/**.

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