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# Profinity eXact™ Protein Purification System

## Instruction Manual

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# Section 1

## Introduction

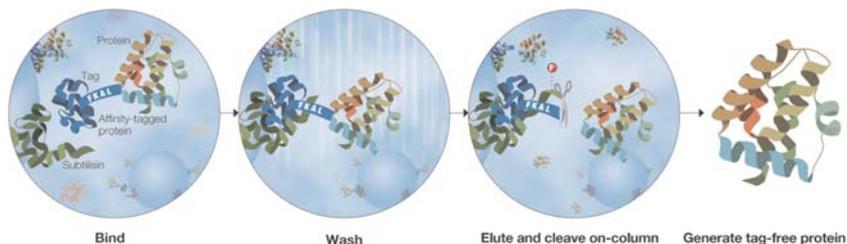


Fig. 1. One-step tag cleavage and elution: Pure and simple.

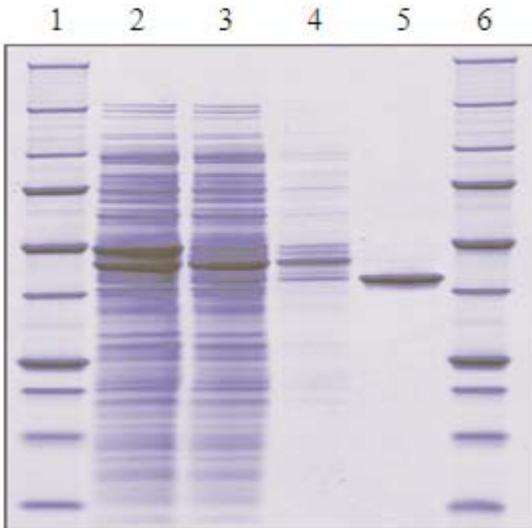
### 1.1 Introduction

Affinity tags are commonly used to facilitate the purification of recombinant proteins. Once purified, these proteins are often studied to further elucidate the protein's structure, function, and interaction with other molecules. However, just as a protein's intermolecular relationship with other proteins is complex, so is the intramolecular dynamic between a fusion protein's affinity tag and the target protein. An affinity tag or protease recognition sequence may negatively interfere with the protein's natural biochemical properties or characteristics (1, 2, 4, 5, 7–12, 15, 16, 18), yielding ambiguous experimental results. Consequently, researchers have utilized a variety of methods to remove the affinity tag from a purified protein.

The current technology for tag removal is tedious and time consuming, requiring a number of steps. After initial purification, the eluted tagged protein is exposed to a cleavage enzyme, which hydrolyzes the tag from the protein fusion. A subsequent step is then needed to isolate the protein from the tag and the protease, which can result in significant loss of product, additional investment of labor, and increased cost to the overall purification.

The Profinity eXact™ (exact affinity cleavage technology) protein purification system offers a novel alternative to existing affinity purification and cleavage tools (Figure 1). The system utilizes an immobilized, extensively engineered protease that both recognizes and avidly binds ( $K_D < 100$  pM) to the small N-terminal co-expressed affinity tag in the protein fusion. Subsequent to column washing, the protease performs a specific, controlled cleavage and removal of the tag from the fusion directly on the column, resulting in the

release of highly purified recombinant protein with a native N-terminus. During elution, the cleaved Profinity eXact tag remains tightly associated with the resin's immobilized protease, eliminating the need for additional steps to remove the protease and the tag. Addressing a key dissatisfaction with recombinant tag technology, the Profinity eXact system improves the efficiency of tag removal by generating a highly purified protein containing only its native amino acid sequence—all with one simple elution step (see Figure 2). The tag-free protein's crystal structure, protein function, and interactions with other proteins may be studied without concern for an interfering affinity tag. Additionally, this purification system is well suited for the expression of peptides, whose small size often complicates expression and purification.



**Fig. 2. Profinity eXact affinity-tagged maltose binding protein (MBP) purified with a 1 ml Bio-Scale Mini™ Profinity eXact cartridge on a Biologic Duoflow™ system.** Samples were run on a 4–12% Criterion™ Bis-Tris gel. Lanes 1 and 6, Precision Plus Protein™ standards; lane 2, crude *E. coli* extract; lane 3, host protein contaminants in the flow-through fraction; lane 4, host protein contaminants washed from the cartridge by Profinity eXact bind/wash buffer; and lane 5, eluted tag-free MBP.

## Section 2

# Product Description

### 2.1 Profinity eXact™ System Components

**Table 1. Profinity eXact System Components and Storage Conditions.**

<b>Cloning and Expression Starter Kit, 20 reactions</b>		<b>Catalog No. 156-3000</b>
pPAL7 RIC-ready vector	0.5 µg	-20°C
pPAL7 supercoiled vector	10 µg	-20°C
SOC medium	2 × 10 ml	Ambient/-70°C
<i>E. coli</i> BL21 (DE3) chemical competent expression cells	10 × 50 µl	-70°C
<i>E. coli</i> C-Max5α chemical competent cloning cells	10 × 50 µl	-70°C
pUC19 control (10 ng/µl)	10 µl	-70°C
<b>RIC-Ready Expression Vector, 20 reactions</b>		<b>Catalog No. 156-3001</b>
pPAL7 RIC-ready vector	0.5 µg	-20°C
<b>Supercoiled Expression Vector, 20 reactions</b>		<b>Catalog No. 156-3002</b>
pPAL7 Supercoiled vector	10 µg	-20°C
<b><i>E. coli</i> BL21 (DE3) Expression Competent Cells Kit, 10 reactions</b>		<b>Catalog No. 156-3003</b>
BL21 (DE3) expression cells	10 × 50 µl	-70°C
pUC19 control (10 ng/µl)	10 µl	-20°C /-70°C
SOC medium	1 × 10 ml	Ambient/-70°C
<b>Monoclonal Antibody</b>		<b>Catalog No. 156-3004</b>
Profinity eXact affinity-tagged antibody	1 vial	-20°C
<b>Bottled Resin</b>		<b>Catalog No. 156-3005</b>
Profinity eXact resin	10 ml	4°C
<b>Mini Purification Starter Kit, 10 pack</b>		<b>Catalog No. 156-3006</b>
Profinity eXact mini spin columns	10 each	4°C
2 mL capped collection tubes	50 each	
Lyophilized Profinity eXact MBP control lysate	1 vial	4°C
Bacterial lysis and extraction reagent	10 ml	ambient
Binding/washing buffer	50 ml	4°C/ambient
Elution buffer	20 ml	4°C/ambient
<b>Mini Spin Columns, 10 pack</b>		<b>Catalog No. 156-3007</b>
Profinity eXact mini spin columns	10 pack	4°C
2ml capped collection tubes	50 pack	
<b>Purification Cartridges, 2 pack or 4 pack</b>		<b>Catalog Nos. 732-4646 &amp; 732-4647</b>
Profinity eXact cartridges, 1 ml	2 or 4	4°C
<b>Purification Cartridge, 1 × 5 ml</b>		<b>Catalog No. 732-4648</b>
Profinity eXact cartridges, 5 ml	1	4°C
<b>Expression and Purification Starter Kit</b>		<b>Catalog Nos. 156-3000 &amp; 156-3006</b>
Cloning and expression starter kit	20 reactions	-70°C
Mini purification starter kit	10 each	4°C

## 2.2 Profinity eXact Expression Technology

Fusion proteins with an N-terminal Profinity eXact tag are expressed with the pPAL7 expression vector (5.9 kb). This inducible expression vector utilizes the strong, tightly regulated T7 lac promoter (6). The pPAL7 plasmid confers ampicillin resistance, constitutively expresses the lacI repressor, and has been designed to facilitate cloning of a target gene through several different methods.

Precise fusions—in which the resultant purified product does not contain any additional amino acids at the N-terminus—may be constructed using the HindIII restriction site and an appropriate restriction site in the multiple cloning site (MCS) or by cloning with the prepared restriction-independent cloning (RIC) vector. Use of the linearized, RIC-ready vector facilitates high-throughput cloning of DNA sequences, regardless of the restriction sites that may be present within a target gene.

Some proteins exhibit significant N-terminal structure that may affect binding to the resin (Appendix A: N-terminal Target Protein Structure and Cleavage Kinetics), or their first two amino acids result in non-ideal cleavage kinetics (Appendix B: Intrinsic Cleavage Rates and Appendix C: Triggered Cleavage Rates). The challenges presented by these proteins are easily addressed at the cloning stage by utilizing a two amino acid linker (such as the recommended Thr-Ser linker) between the Profinity eXact tag and the target protein—generating an *imprecise fusion*. A Thr-Ser spacer between the Profinity eXact tag and the target protein ensures optimal cleavage kinetics and purification, regardless of the target protein. These imprecise fusion proteins, once purified, retain the Thr-Ser linker at the N-terminus.

## 2.3 Profinity eXact Purification Technology

The Profinity eXact purification resin utilizes Superflow™ crosslinked agarose coupled to a unique subtilisin mutant. The Superflow support matrix exhibits low nonspecific protein binding while providing the high mechanical stability and flow dynamics desired for medium pressure liquid chromatography applications (see Table 2).

Mutant subtilisin protease—S189, the functional ligand immobilized to the support matrix—has been precisely engineered for affinity chromatography (3, 14). S189 subtilisin is extremely stable and efficiently refolds *in vitro*. The recombinant protease also is designed to specifically recognize and

cleave at the C-terminus of a nine amino acid sequence (EEDKLFKAL). Through a third set of mutations, this cleavage reaction has been changed from a self-initiating digestion to one that is triggered by 100 mM potassium fluoride; complete cleavage is most often achieved within 30 min, at room temperature.

The subtilisin exhibits extremely high binding affinity to the 8 kD Profinity eXact tag ( $K_D < 100$  pM). The Profinity eXact tag is a form of the wild-type subtilisin BPN' prodomain—genetically engineered for high stability and to contain the distinct cleavage recognition sequence of the subtilisin mutant. Additionally, the subtilisin prodomain serves as an exo-recognition sequence for the immobilized subtilisin, which significantly increases the system's binding affinity and cleavage specificity to the tag.

Once it has been captured by the Profinity eXact purification resin—and the cleavage reaction has been triggered with a sodium fluoride-containing buffer—the affinity tag is precisely cleaved at the C-terminus of the cleavage recognition sequence. The result is a quickly purified protein product that lacks any amino acid residues derived from the Profinity eXact tag.

## 2.4 Resin Characteristics

The resin attributes are summarized in Table 2.

**Table 2. Characteristics of Profinity eXact Purification Resin.**

<b>Functional ligand</b>	Mutant subtilisin, S189
<b>Base bead</b>	Superflow agarose (6% crosslinked)
<b>Format</b>	Spin column: 600 $\mu$ l 17% suspension (100 $\mu$ l settled resin)  Bottled: 50% suspension  Cartridge: 1 or 5 ml packed resin  Storage buffer: 100 mM sodium phosphate, 0.02% sodium azide, pH 7.2
<b>Particle size</b>	60–160 $\mu$ m
<b>Molecular exclusion limit</b>	6,000 kD
<b>Recommended linear flow rate</b>	<1,000 cm/hr at 25°C*
<b>pH stability</b>	2–13
<b>Chemical compatibility</b>	See Table 4 and Appendix D: Comprehensive Chemical Compatibility List
<b>Storage</b>	4°C
<b>Shelf life in storage buffer</b>	When unopened, 1 year from receipt; store at 4°C
<b>Operational temperature</b>	4–40°C

\* Recommended linear flow rate determined by the following factors:

The flow rate of 100 mM sodium phosphate, pH 7.2 was incrementally increased through a Bio-Scale Mini Profinity eXact 1 ml cartridge packed with a 20% compression factor. The pressure-flow curve for the Profinity eXact purification resin became nonlinear at linear velocities above 1,000 cm/hr.

## 2.5 Chemical Compatibility

The Profinity eXact purification system is compatible with a broad range of additives commonly used to purify proteins. Extensive information is listed in Appendix D: Comprehensive Chemical Compatibility List.

 **Highest purification yields are achieved by eliminating Cl<sup>-</sup> ions from all lysis and wash buffers.** The chloride ions from additives like NaCl, KCl, and Tris-HCl act as slower cleavage/elution-triggering anions (see Table 3). NaCl or KCl salt is commonly added to lysis and wash buffers to reduce electrostatic interactions between background proteins (or DNA) with a chromatographic support. When using the Profinity eXact purification resin, sodium acetate (NaOAc) and potassium acetate (KOAc) are

recommended substitutes for NaCl or KCl. If using the 0.1 M sodium phosphate Profinity eXact bind/wash buffer, a higher sodium phosphate concentration (e.g. 0.3–1.0 M, pH 7.2) may also be used to raise the ionic concentration of the lysis and wash buffers.

**Table 3. Triggering Anions.**

Triggering Anion	Compound	Concentration for Fast Cleavage	Concentration for Moderate Cleavage
F <sup>-</sup>	NaF, KF	100 mM	5 mM
N <sub>3</sub> <sup>-</sup>	NaN <sub>3</sub>	10 mM	1 mM
NO <sub>2</sub> <sup>-</sup>	NaNO <sub>2</sub>	5 mM	1 mM
HCO <sub>2</sub> <sup>-</sup>	NaHCO <sub>2</sub>	1,000 mM	25 mM
Cl <sup>-</sup>	NaCl, KCl	>1,000 mM	75 mM

Although the wild-type subtilisin BPN' is a serine protease, the purification resin is unaffected by most serine protease inhibitors due to the Profinity eXact subtilisin's active site mutations. The protease cocktails tested include combinations of the following inhibitors: AEBSF, aprotinin, benzamidine, E-64, EDTA, leupeptin, pepstatin A, phenanthroline, and PMSF. See Table 4 for additional information regarding the compatibility of protease inhibitors, common additives, and solutions typically used in affinity chromatography methods.

**Table 4. Chemical Compatibility (Partial List—See Appendix A for Complete List)**

Reagent Group	Reagent	Comments	Acceptable Concentration	
 Salts	Triggering Ions	F <sup>-</sup> , Cl <sup>-</sup> , N <sub>3</sub> <sup>-</sup> , NO <sub>2</sub> <sup>-</sup> , HCO <sub>2</sub> <sup>-</sup>	Triggers cleavage of fusion and elution of target protein	Remove from all <b>lysis and wash</b> buffers for highest yields See <b>Table 3</b> for ionic concentrations to use as trigger in elution buffer
		Sodium acetate	Reduces non-specific protein binding due to ionic interactions	≤3 M (an excellent substitute for NaCl in lysis and wash buffers)
 Buffers		<b>NaCl or KCl</b>	Triggers cleavage of fusion and elution of target protein	Remove from all <b>lysis and wash</b> buffers—use sodium acetate or sodium phosphate instead to reduce electrostatic interactions
		Tris-HCl	Cl <sup>-</sup> in lysis and wash buffers triggers cleavage of fusion and elution of target protein	Substitute with Tris-acetate or Tris-phosphate and adjust pH with acetic or phosphoric acid
		Tris-acetate		50 mM tested
		Tris-phosphate		50 mM tested
Acids	HCl	Cl <sup>-</sup> in lysis and wash buffers triggers cleavage of fusion and elution of target protein		Avoid use of HCl for highest yields; instead, use acids such as phosphoric or acetic acid
Detergents	Non-ionic (NP-40, Triton <sup>®</sup> X-100, Tween <sup>®</sup> -20)	Solubilize proteins		≤5% (v/v)
	Zwitterionic (CHAPS, CHAPSO, Dodecyl-β, D-maltoside, Octylthioglucoside)	Solubilize proteins		≤5% (v/v or w/v)
	Ionic (CTAB, Sarkosyl, SDS)	Significantly lower yields		Remove from all buffers
Protease Inhibitors	PMSF	F <sup>-</sup> triggers cleavage of fusion and elution of target protein		0.5 mM; no adverse effects when incubated with resin for up to 3 hr
	TLCK			100 μM
	TPCK			100 μM
	Pharmingen™ Protease Inhibitor Cocktail	F <sup>-</sup> may lower yield slightly		2×
	Calbiochem <sup>®</sup> Protease Inhibitor Cocktail Set I			1×

Reagent Group	Reagent	Comments	Acceptable Concentration
	Roche cOMplete Protease Inhibitor Cocktail Tablet	F <sup>-</sup> may lower yield slightly	1×
Lysis Solutions	Bacterial Lysis and Extraction (Bio-Rad)		1×
	B-PER <sup>®</sup> in phosphate buffer (Pierce)		1×
	B-PER <sup>®</sup> (Pierce)	Cl <sup>-</sup> from Tris-HCl results in slightly lower yield	1×
	ReadyPreps <sup>™</sup> Lysis solutions (Epicentre)		Do not use
	BugBuster <sup>®</sup> (EMD)		1×
	FastBreak <sup>™</sup> Cell Lysis (Promega)		1×
	CellLytic <sup>™</sup> Express (Sigma)		Do not use
Denaturants	Guanidine•HCl	Cl <sup>-</sup> triggers cleavage of fusion during load and wash steps	Remove from all <b>lysis</b> and <b>wash</b> buffers
	Urea	Solubilizes proteins	≤2 M; with 4 M, ~30% reduced control MBP binding capacity observed; with 8 M, ~70% reduced control MBP binding capacity and reduced purity observed
Other Additives	CaCl <sub>2</sub>	Essential component for purification of Ca <sup>2+</sup> -binding proteins  Cl <sup>-</sup> triggers cleavage during load and wash steps	≤5 mM; use with buffer such as MES, MOPS, or PIPES buffer to eliminate precipitation of Ca <sup>2+</sup> with either fluoride- or phosphate-containing Profinity eXact buffers
	MgCl <sub>2</sub>	Essential metal cofactor for nucleases  Cl <sup>-</sup> triggers cleavage during load and wash steps	≤5 mM; if higher Mg <sup>2+</sup> concentrations are desired, use magnesium acetate instead  Do not add to Profinity eXact elution buffer—Mg <sup>2+</sup> precipitates with F <sup>-</sup> ; instead, use an elution buffer with an alternative trigger, such as 10 mM Na <sub>3</sub> P <sub>3</sub> , with 100 mM sodium phosphate, pH 7.2

## Section 3

# Profinity eXact™ System Strategies

1. **Basic purification strategy**—create and purify an imprecise fusion that ensures initial purification success with minimal optimization by incorporating a Thr-Ser spacer.
  - a. Determine cloning method and construct fusion protein
    - RIC-ready pPAL7 (Section 4.6)
    - Supercoiled pPAL7 (Section 4.7)
  - b. Express Profinity eXact™ affinity-tagged fusion protein
    - E. coli* culturing and expression (Section 5.1)
    - Solubility check of expressed fusion protein (Section 5.2)
  - c. Lyse cells and store lysate at 4°C
    - Bacterial lysis and extraction reagent (provided with mini spin column purification kit)
    - Cell resuspension buffer (100 mM sodium phosphate, pH 7.2) and lyse using desired lysis method
  - d. Purify protein with chloride-free wash (100 mM sodium phosphate, pH 7.2; 4°C) and elution (100 mM sodium fluoride, 100 mM sodium phosphate, pH 7.2; room temperature) buffers using appropriate purification protocol
    - Spin column (Section 6.2)
    - Bio-Scale™ Mini cartridge with LC instrument (Section 6.3)
    - Bio-Scale Mini cartridge with syringe (Section 6.4)
    - Gravity column (Section 6.5)
2. **Precise purification strategy**—create a precise fusion to obtain a completely tag-free, purified protein
  - a. Consider, if known, the P1' and P2' (first two N-terminal amino acids of target protein) residues' effect on cleavage kinetics

Appendix A: N-terminal Target Protein Structure and Cleavage Kinetics

Appendix B: Intrinsic Cleavage Rates

Appendix C: Triggered Cleavage Rates

- b. Determine cloning method and construct fusion protein
  - RIC-ready pPAL7 (Section 4.6)
  - Supercoiled pPAL7 (Section 4.7)
- c. Express Profinity eXact™ affinity-tagged fusion protein
  - E. coli* culturing and expression (Section 5.1)
  - Solubility check of expressed fusion protein (Section 5.2)
- d. Lyse cells and store lysate at 4°C
  - Select appropriate chloride-free lysis buffer (Section 5.3.1)
  - Lyse using desired lysis method (Section 5.3.6)
- e. Identify appropriate buffers and conditions
  - Select appropriate chloride-free wash buffer (Section 6.1.1)
- f. Purify protein using appropriate purification protocol
  - Spin column (Section 6.2)
  - Bio-Scale Mini cartridge with LC instrument (Section 6.3)
  - Bio-Scale Mini cartridge with syringe (Section 6.4)
  - Gravity column (Section 6.5)

## Section 4

# Cloning and Expression Process

### 4.1 pPAL7 Expression Vector

The pPAL7 expression vector utilizes the T7lac promoter to strongly express a Profinity eXact fusion protein in *E. coli* cells producing T7 RNA polymerase. The pPAL7 plasmid constitutively expresses the lacI repressor and confers ampicillin resistance to the host cell (Figure 3). The Profinity eXact™ tag is an 8.2 kD affinity tag that functions as an N-terminal fusion tag (Figure 3).

### 4.2 *E. coli* C-Max5α Cloning Host Cell

The *E. coli* C-Max5α cells [ $F^- \phi 80 \text{ dlacZ}\Delta\text{M15 } \Delta(\text{lacZYA-argF})\text{U169 } \text{recA1 } \text{endA1 } \text{hsdR17}(r_k^-, m_k^+) \text{ } \text{phoA } \text{supE44 } \lambda^- \text{ } \text{thi-1 } \text{gyrA96 } \text{relA1}$ ] are chemical competent transformation cells that are specifically designed to maximize cloning success. This *E. coli* K12 derivative facilitates isolation of high-quality plasmid DNA and is an ideal host strain for transformation of ligation cloning reactions.

### 4.3 *E. coli* BL21(DE3) Expression Host Cell

*E. coli* BL21(DE3) cells [ $E. coli$  B  $F^- \text{ dcm } \text{ompT } \text{hsdS}(r_B^-, m_B^-) \text{ gal } \lambda(\text{DE3})$ ] are used for high-level protein expression with T7 RNA polymerase-based expression systems. The strain is a derivative of *E. coli* B, which is deficient in the *lon* protease as well as the *ompT* outer membrane protease, facilitating the isolation of intact recombinant proteins. The host is a lysogen of  $\lambda\text{DE3}$  and contains the T7 RNA polymerase gene—integrated into the chromosome—under control of the lacUV5 promoter. 1–2 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) is used to induce the expression of recombinant proteins cloned into vectors downstream of a T7lac promoter and transformed into the *E. coli* BL21(DE3) cells.

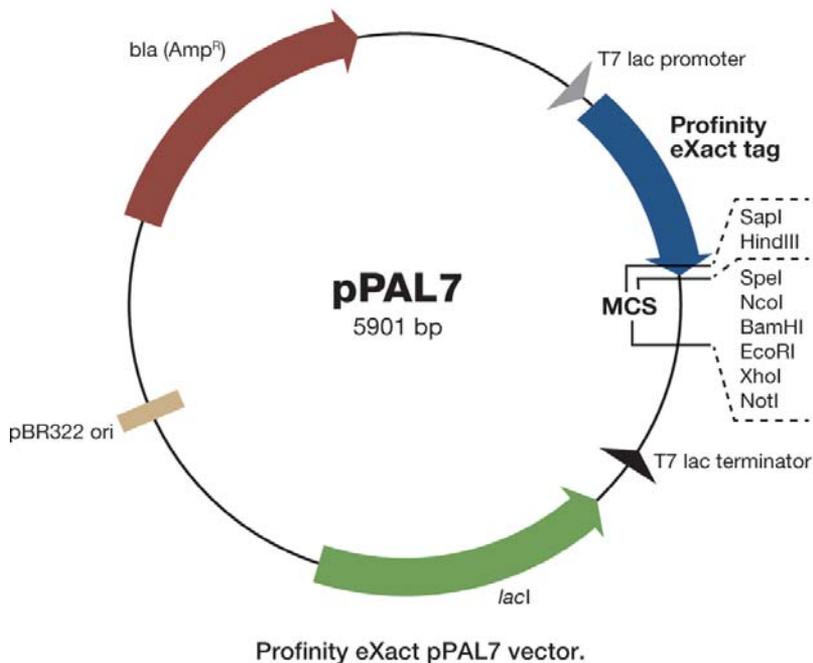


Fig. 3. pPAL7 vector map.

Table 5. Features of the pPAL7 Expression Vector.

Vector Feature	Vector Position
T7/lac promoter	1–17
Profinity eXact tag	92–316
MCS	
SpeI	318
NcoI	325
BamHI	333
EcoRI	345
XhoI	351
NotI	358
T7 terminator	413–460
<i>bla</i> (Amp <sup>R</sup> )	1,079–1,936
<i>Ori</i>	2,499
<i>lacI</i>	4,634–5,713

## 4.4 Cloning Region Sequence

```

      T7 Promoter          lac Operator
1 TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG
  ATTATGCTGA GTGATATCCC CTTAACACTC GCCTATTGTT AAGGGGAGAT CTTTATTAAA ACAAAATGAA ATTCTTCCTC

      Profinity eXact Tag (92-316)
      NdeI
81 ATATACATAT GGGAGGGAAA TCAAACGGGG AAAAGAAATA TATTGTCGG TCAAAACAGG GCTTTAAGAG CTGCGCTAAG
  TATATGTATA CCCTCCCTTT AGTTTGCCCC TTTTCTTTAT ATAAACGCC AAGTTTGTCC CGAAATCTCT GACGCGATTG

      Profinity eXact Tag (92-316)
161 AAGGAGGATG TCATTTCTGA AAAAGCGGG AACTCCAAA AGTGCTTCAA ATATGTAGAC GCAGCTAGCG CTACATTAAA
  TTCCTCTAC AGTAAAGACT TTTTCCGCC TTTGAGGTTT TCACGAAGTT TATACATCTG CGTCGATCGC GATGTAATTT

      Profinity eXact Tag (92-316)
      SpeI   HindIII   SpeI
      Cleavage Recognition Sequence
241 CGAAAAGCCT GTAGAGAAT TGA AAAAAGA TCCGAGCGC GCGTACGTAG AAGAAGACAA GCTCTTCAA GCTTTGACTA
  GCTTTTTCGA CATCTTCTTA ACTTTTTTCT AGGCTCGCAC CGCATGCATC TTCTCTGTT CGAGAAGTTT CGAAACTGAT
      Profinity eXact Cleavage Site

      SpeI   NcoI           BamHI           EcoRI           XhoI           NotI   STOP
      SThrMetAla GlySerGly CysGluPheLeu GluAlaAla Ala***
321 GTACCATGGC GGGATCCGGC TCGCAATTC TCGAGGCGGC CGCATAAGCC CGAAAGGAG CTGAGTTGGC TGCTGCCACC
  CATGTTACCC CCTAGGCCG ACGCTTAAGG AGCTCCGCCG GCGTATTCCG GCTTTCTTCC GACTCAACCG ACGACGGTGG

      T7 Terminator
401 GCTGAGCAAT AACTAGCATA ACCCCTTGGG GCCTCTAAAC GGGTCTTGAG GGGTTTTTTG CTGAAAGGAG GAACATATC
  CGACTCGTTA TTGATCGTAT TGGGGAACCC CGGAGATTTG CCGAGAACTC CCCAAAAAAC GACTTTCTCT CTTGATATAG

```

Fig. 4. Sequence of Profinity eXact tag and cloning region.

## 4.5 General Cloning Guidelines

The pPAL7 vector is provided in both linearized and supercoiled form in the Profinity eXact cloning and expression starter kit. The linearized plasmid (Profinity eXact RIC-ready pPAL7 vector) may be ligated with PCR products possessing compatible overhangs generated by either the restriction-independent cloning (RIC) or traditional restriction enzyme digestion method. Both the RIC-ready and the supercoiled pPAL7 vector may be used to generate either precise- or imprecise-fusion proteins:

*Precise fusions*—purifications result in a tag-free target protein without extra amino acids after cleavage of the Profinity eXact tag.

*Imprecise fusions*—purifications result in the retention of a spacer (e.g., Thr-Ser) at the N-terminus of the target protein. Presence of the Thr-Ser spacer ensures successful purifications with minimal optimization.

Optimal room-temperature purification of the target protein with the Profinity eXact resin is dependent upon the amino acid residues in the first two positions of the target protein. (Residues 1 and 2 are referred to as positions P1' and P2', respectively.) Target proteins with a P1' proline are not subject to resin cleavage and elution; on the other hand, proteins with a P1' cysteine have a particularly high intrinsic cleavage and elution rate with the Profinity eXact resin, even in the absence of a fluoride-containing elution buffer. Refer to Appendices A-C for more details. Target proteins with these challenging P1'-P2' amino acid residues should be cloned with a threonine-serine spacer. This spacer maximizes successful purification results, regardless of the target protein's P1'-P2' residues or any effect its N-terminal structure may have on the Profinity eXact tag's resin-binding properties.

If the presence of an additional Thr-Ser dipeptide (208 Da) at the N-terminus of the target protein is not an issue, cloning of a target gene and the spacer into the vector is the recommended strategy—requiring the least amount of purification optimization (either using the SpeI cloning site or obtaining a PCR target gene product with the Thr-Ser spacer at the N-terminus).

## 4.6 Cloning With the Linearized, RIC-Ready pPAL7 Vector

### 4.6.1 RIC Cloning

Because this cloning method is completely free of any use of restriction enzymes, it is independent of restriction enzyme sites within the target protein and is thus ideal for **high-throughput cloning**. The RIC-ready vector has been prepared to generate the *dephosphorylated* overhangs depicted in Figure 5.

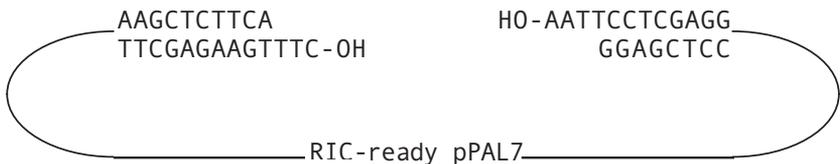


Fig. 5. RIC-ready pPAL7 vector.

Forward and reverse PCR primers starting with the predefined eight bases illustrated in Step 1 are used to PCR the target gene. Using the 3'→5' exonuclease activity of T4 DNA polymerase, the PCR product is treated with T4 DNA polymerase in the presence of dGTP to create overhangs compatible with the RIC-ready pPAL7 vector. The prepared insert and RIC-ready vector are then ligated using standard ligation techniques and finally transformed into an appropriate *Escherichia coli* cloning host such as the *E. coli* C-Max5α competent cells provided with the Profinity eXact system.

## Method

### 1. PCR-amplify target gene using iProof™ high-fidelity DNA polymerase.

Both forward and reverse primers **must be 5'-phosphorylated** to clone into the dephosphorylated vector, and they must contain the following DNA sequences (*N* indicates target DNA sequence):

Forward primer: 5' – **P**–**AAGCTTTG**(**ACTTCT**)NNNNNNNNN... – 3'  
*Thr-Ser*  
*Optional*

Reverse primer: 5' – **P**–**AATTCTT**ANNNNNNNNNN... – 3'  
stop

The optional ACTTCT (Thr-Ser) spacer of the forward primer can be included to minimize all target protein effects on cleavage of the Profinity eXact tag. If the template is an ampicillin-resistant plasmid, it may be DpnI-treated post PCR amplification to ensure background-free transformants.

2. Purify, if desired, PCR product by either excising and purifying band following agarose gel electrophoresis, or using PCR Kleen™ spin column kit (catalog #732-6000).
3. Generate RIC-ready pPAL7 vector-compatible overhangs by T4 DNA polymerase treatment of purified PCR product.

Amount of purified PCR product used in reaction mix: 0.2–1 pmol

T4 DNA polymerase: 5 U/pmol DNA fragment (Novagen; add DTT to 5 mM in reaction mix) or  
5 U/ $\mu$ g DNA fragment (Promega) or  
1 U/ $\mu$ g DNA fragment (NEB)

ddH <sub>2</sub> O	x $\mu$ l
PCR fragment	y $\mu$ l
10 × T4 DNA polymerase buffer	2 $\mu$ l
100 mM dGTP	0.5 $\mu$ l
T4 DNA polymerase	z $\mu$ l
Total	<hr/> 20 $\mu$ l

Incubate reaction 20 min at 12°C; then inactivate the polymerase by incubating 20 min at 75°C; store on ice until ready to use. Further purification of the reacted product is unnecessary.

**Note:** Shorter or longer incubations or use of higher temperatures during the T4 DNA polymerase treatment may result in reduced cloning efficiency.

**4. Ligate 2  $\mu$ l (20 ng) RIC-ready pPAL7 vector and RIC-prepared PCR product insert using T4 DNA ligase and standard ligation protocol.**

If using a quick ligase, incubate ligation mix for 20 min at room temperature. The slightly longer incubation time is recommended because of the short three-base overhang.

Ligations with molar ratios of (1–10)/1 (insert/vector) are typical.

**5. Transform *E. coli* C-Max5 $\alpha$  chemical competent cells within 30 min of completion of the ligation reaction.**

Add 1–5  $\mu\text{l}$  ligation mix to each 50  $\mu\text{l}$  aliquot of competent cells. Incubate on ice, 30 min. Heat shock at 42°C, **exactly** 30 sec. Quickly return to ice and incubate 2 min. Add 250  $\mu\text{l}$  SOC recovery medium to transformation mix. Shake tubes at 37°C, 1 hr; best results are obtained when recovery cultures are shaken horizontally, to maximize oxygen transfer rate. Dilute cells (e.g., 1/20 $\times$ ) with SOC medium. Plate undiluted and diluted cells on relatively dry, LB+100  $\mu\text{g}/\text{ml}$  ampicillin agar plates and incubate 37°C, 16–18 hr.

As an example, when a 10/1 molar ratio of a 1.1 kb MBP RIC-prepared insert to RIC-ready pPAL7 vector (37 ng insert/20 ng vector) is used with the described ligation and transformation protocols, transformation efficiencies of  $>1 \times 10^7$  CFU/ $\mu\text{g}$  DNA are typically achieved with  $>95\%$  correct clone yield.

#### **4.6.2 Restriction Enzyme Digestion Cloning**

Using appropriate PCR primers, the target gene may also be amplified and digested with a suitable Type IIS restriction enzyme and EcoRI to obtain a PCR product with overhangs compatible with the RIC-ready pPAL7 vector.

##### **Method**

#### **1. PCR-amplify target gene using iProof high-fidelity DNA polymerase.**

The forward primer must contain a recognition sequence for a suitable Type IIS restriction enzyme that generates a three-base, 5'-overhang (e.g., BspQI or Earl). The reverse primer must contain an EcoRI recognition sequence and an in-frame stop codon. Note that the uncut vector contains a stop codon after the NotI site, in frame with the Profinity eXact tag.

Restriction Enzyme (and Isoschizimers)	Enzyme Cleavage	Forward Primer
BspQI (SapI/ LguI/PciSI)	GCTCTTC (1/4)	5' - <b>XXGCTCTTCAAAGCTTTG</b> (ACTTCT)NNN... - 3'
EarI (Bst6I/ Eam1104I/Ksp632I)	CTCTTC (1/4)	5' - <b>XXCTCTTCAAAGCTTTG</b> (ACTTCT)NNNN... - 3'

Restriction Enzyme	Enzyme Cleavage	Reverse Primer
EcoRI	G/AATTC	5' - <b>XXGAATTC</b> TTA stop NNNNNNNNNNNNNN... - 3'

**Bolded** sequences must be present in primer.

*ACTTCT* sequence is optional; include to create a fusion protein with a Thr-Ser spacer for ideal binding and cleaving kinetics.

**AAG** represents 5'-three-base overhang generated upon BspQI or EarI digestion.

**X** represents additional bases to improve restriction enzyme digestion efficiency.

**N** represents target protein gene sequence.

If the template is an ampicillin-resistant plasmid, it may be DpnI-treated post PCR amplification to ensure background-free transformants.

- Digest PCR product with EcoRI and Type IIS restriction enzymes corresponding to forward primer.**
- Purify, if desired, PCR product by either excising and purifying band following agarose gel electrophoresis, or using PCR Kleen™ spin column kit (catalog #732-6000).**
- Ligate 2 µl (20 ng) RIC-ready pPAL7 vector and restriction enzyme-digested PCR product insert using T4 DNA ligase and standard ligation protocol.**

Ligations with molar ratios of (1–10)/1 (insert/vector) are typical.

- Transform *E. coli* C-Max5α chemical competent cells within 30 min of completion of ligation reaction.**

Add 1–5  $\mu\text{l}$  ligation mix to each 50  $\mu\text{l}$  singles aliquot of competent cells. Incubate on ice, 30 min. Heat shock at 42°C, **exactly** 30 sec. Quickly return to ice and incubate 2 min. Add 250  $\mu\text{l}$  SOC recovery medium to transformation mix. Shake tubes at 37°C, 1 hr; best results are obtained when recovery cultures are shaken horizontally, to maximize oxygen transfer rate. Dilute cells (e.g., 1/20 $\times$ ) with SOC medium. Plate undiluted and diluted cells on relatively dry, LB+100  $\mu\text{g/ml}$  ampicillin agar plates and incubate 37°C, 16–18 hr.

## 4.7 Cloning with the Supercoiled pPAL7 Vector

When using the supercoiled pPAL7 vector, a conventional cloning strategy is employed—using a PCR-amplified gene and standard restriction enzymes to create vector-compatible overhangs for ligation. Both the supercoiled pPAL7 vector and the PCR product are digested with HindIII (to create a precise fusion) or SpeI (for an imprecise fusion). A second restriction enzyme that digests the vector and the PCR product at one of the available downstream restriction sites (i.e., NcoI, BamHI, EcoRI, XhoI, or NotI) in the vector's MCS enables directional cloning. The plasmid and insert are then ligated and transformed into an appropriate host using standard techniques.

### Method

#### 1. PCR-amplify target gene using iProof high-fidelity DNA polymerase.

The forward primer must contain the bolded sequence below. The reverse primer must contain an appropriate enzyme recognition sequence and a stop codon. The following choices of primer sequences and enzymes may be used:

<b>Forward Primer</b> with HindIII sequence	Precise Fusion	5'- <b>XXAAGCTT</b> G( <i>ACTTCT</i> )NNN...-3'
<b>Forward Primer</b> with SapI sequence	Precise Fusion	5'- <b>XXGCTCTTCAAAGCTT</b> G( <i>ACTTCT</i> )NNN...-3'
<b>Forward Primer</b> with SpeI sequence	Imprecise Fusion	5' - <b>XXACTAGT</b> NNNNNNN... - 3'
<b>Reverse Primer</b> with/NotI sequence (BamHI, EcoRI, XhoI, or NcoI sequence may also be used)		5' - <b>XXCGGCGCGCTTA</b> NNNN... -3' Stop

**Bolded** sequences must be present in primer.

*ACTTCT* sequence is optional; include to create a fusion protein with a Thr-Ser spacer for ideal binding and cleaving kinetics.

X represents additional bases to improve restriction enzyme digestion efficiency.

N represents target protein gene sequence.

If an imprecise fusion is desired, but the spacer length is not critical, the forward PCR primer may be designed for cloning into NcoI, BamHI, EcoRI, or XhoI.

Should a target gene contain an internal HindIII site that would preclude cloning into the vector's HindIII site, a forward primer may be designed that utilizes a Type IIS restriction enzyme to generate a four-base, HindIII-compatible, 5'-overhang (e.g., BsaI, BsmAI, or BsmBI). The design of this forward primer is similar to the Type IIS restriction enzyme method described on page 18.

If the template is an ampicillin-resistant plasmid, it may be DpnI-treated post PCR amplification to ensure background-free transformants.

- Digest PCR product and supercoiled pPAL7 vector with appropriate upstream and downstream restriction enzymes.**
- Purify, if desired, PCR product by either excising and purifying band following agarose gel electrophoresis, or using PCR Kleen™ spin column kit (catalog #732-6000).**
- Ligate supercoiled pPAL7 vector and restriction enzyme-digested PCR product insert using T4 DNA ligase and standard ligation protocol.**

Ligations with molar ratios of (1–10)/1 (insert/vector) are typical.

- Transform *E. coli* C-Max5α chemical competent cells within 30 min of completion of ligation reaction.**

Add 1–5  $\mu\text{l}$  ligation mix to each 50  $\mu\text{l}$  aliquot of competent cells. Incubate on ice, 30 min. Heat shock at 42°C, **exactly** 30 sec. Quickly return to ice and incubate 2 min. Add 250  $\mu\text{l}$  SOC recovery medium to transformation mix. Shake tubes at 37°C, 1 hr; best results are obtained when recovery cultures are shaken horizontally, to maximize oxygen transfer rate. Dilute cells (e.g., 1/20 $\times$ ) with SOC medium. Plate undiluted and diluted cells on relatively dry, LB+100  $\mu\text{g}/\text{ml}$  ampicillin agar plates and incubate 37°C, 16–18 hr.

## **Section 5**

# **Protein Expression and Lysate Preparation**

### **5.1 Protein Expression**

Glucose is a catabolite repressor of the lac operon (19) and, in conjunction with the lacI repressor and T7lac promoter from pPAL7, affords tighter background control of target gene expression prior to induction. Maximum plasmid stability of pPAL7 constructs within *E. coli* BL21(DE3) bacteria is achieved by cultivating the cells and inoculum cultures on LB plates and in liquid medium—both supplemented with 0.5–1.0% glucose (13, 17).

#### **1. Purify cloned expression vector from cloning strain by DNA miniprep.**

The Aurum™ plasmid mini kit (catalog #732-6400) may be used to quickly obtain high purity plasmid DNA.

#### **2. Transform *E. coli* BL21(DE3) chemical competent cells with the purified plasmid DNA.**

Add 0.5  $\mu\text{l}$  purified DNA to a 50  $\mu\text{l}$  aliquot of competent cells. Incubate on ice, 30 min. Heat shock at 42°C, **exactly** 30 sec. Quickly return to ice and incubate 2 min. Add 250  $\mu\text{l}$  SOC recovery medium to transformation mix. Shake tubes at 37°C, 1 hr; best results are obtained when recovery cultures are shaken horizontally, to maximize oxygen transfer rate. Dilute cells (e.g., 1/20 $\times$ ) with SOC medium. Plate undiluted and diluted cells on relatively dry, LB+100  $\mu\text{g}/\text{ml}$  ampicillin+0.5% glucose agar plates and incubate 37°C, 16–18 hr.

**3. Prepare overnight seed culture by inoculating 2 ml LB+100 µg/ml ampicillin + 0.5% glucose.**

Shake culture at 37°C, 16–18 hours.

**4. Inoculate expression culture with overnight seed culture.**

After inoculation of suitable expression medium supplemented with 100 µg/ml ampicillin, shake expression culture at desired temperature until cell density ( $OD_{600}$ ) reaches 0.5–0.7. Induce expression of target protein by addition of 1–2 mM isopropyl-β-D-thiogalactopyranoside (IPTG); for cultures expressed at 37°C continue shaking for 3–4 hr. Collect culture sample for analysis in Step 5. Harvest cells by centrifugation. Lyse cells immediately or freeze cell paste.

**5. Analyze whole cell culture by Experion™ or SDS-PAGE to verify target protein expression.**

The Quick Solubility Screening Protocol, below, may be followed to determine whether the expressed protein is in the soluble or insoluble lysate fraction.

## **5.2 Quick Solubility Screening Protocol**

Before choosing a purification strategy, it is useful to determine the approximate expression level of a protein and to determine if the overexpressed target protein partitions into the soluble or insoluble lysate fraction. Soluble proteins are typically purified with the native purification procedure, while insoluble proteins may be solubilized with detergents or urea.

The following procedure provides a quick screen for solubility and expression level:

- 1. Pellet ~2 ml *E. coli* culture by centrifuging at 16,000 × g for 1 min at 4°C.**

Decant supernatant.

- 2. Resuspend pellet in 500 µl of bind/wash buffer and sonicate for 60 sec, on ice, in 10 sec pulses.**

Transfer 50 µl of the sonicate to a tube, and label as the *Total* sample.

- 3. Centrifuge lysate at 16,000 × g for 5 min at 4°C; transfer supernatant to a clean tube.**

Transfer 50 µl of the supernatant to a tube, and label *Soluble*.

- 4. Resuspend insoluble pellet in 500 µl of bind/wash buffer containing 4 M urea.**

- 5. Centrifuge lysate at 16,000 × g for 5 min at 4°C.**

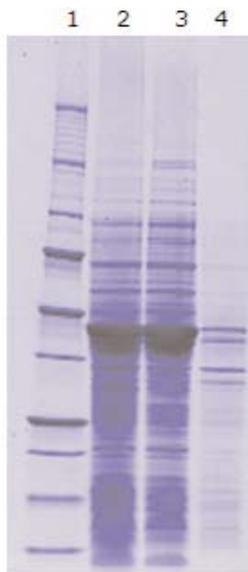
Transfer 50 µl of the supernatant to a tube, and label *Insoluble*

- 6. To each 50 µl Total, Soluble, and Insoluble samples, add 150 µl Laemmli sample buffer, and heat for 5 min at 95°C.**

- 7. Load 10 µl of each sample on SDS-PAGE gel.**

- 8. Examine soluble and insoluble fractions for target protein.**

Approximate the expression level, and determine partitioning of the target protein. A partitioning profile of a soluble target protein, with approximate expression level, can be seen in Figure 6.



**Fig. 6. Partitioning profile.** Precision Plus Protein™ molecular weight markers were loaded in lane 1, followed by the total, soluble, and insoluble fractions in lanes 2–4 respectively. The gel depicts Profinity eXact™ affinity-tagged maltose binding protein (MBP), which partitions into the soluble fraction and can be purified using the native purification protocol.

## 5.3 Lysate Preparation

### 5.3.1 Lysis/Cell Resuspension Buffer Selection (NaCl, Tris-HCl, and pH)

The Profinity eXact bind/wash buffer (100 mM sodium phosphate, pH 7.2; provided in the mini purification starter kit (catalog #156-3006) may be used to resuspend cells for lysis. To prevent target protein cleavage and elution during the lysate loading step, **resuspension and lysis buffers should not contain any chloride ions (e.g., NaCl)**. If a higher ionic concentration is desired to reduce possible electrostatic interactions between contaminant proteins and the resin, sodium acetate or a higher sodium phosphate concentration may be used (e.g., 300 mM).



Similarly, Tris-HCl buffers should be avoided because of the chloride present in the acid conjugate. Tris buffers still may be used by utilizing Tris base and acetic acid to produce a Cl-free, Tris-acetate buffer with the proper pH. A Tris-phosphate buffer may likewise be made with Tris base and phosphoric acid.

The Profinity eXact purification resin is fully functional at pH 6–8. Intrinsic cleavage of some proteins due to high OH<sup>-</sup> concentrations during the lysate loading and wash steps may be observed if the lysis and wash buffer pH are greater than 7.5. Lysis buffers close to pH 7.0 are therefore preferred. On the other hand, using a lysis buffer at a pH below 7.0 may help minimize intrinsic cleavage for those proteins with non-ideal P1'–P2' amino acids. When adjusting the buffer pH with an acid, avoid any use of HCl; instead, use a Cl<sup>-</sup>-free acid such as H<sub>3</sub>PO<sub>4</sub> or HOAc.

### **5.3.2 Nucleases and MgCl<sub>2</sub>**

Nucleases may be added to the lysate to reduce the viscosity caused by DNA. In most situations, Mg<sup>2+</sup> ions will not need to be added to the lysate for effective nuclease activity. However, if the addition of magnesium is desired, add magnesium acetate or magnesium sulfate, instead of magnesium chloride. Elimination of chloride ions will maximize final product yield.

### **5.3.3 Protease Inhibitors**

The Profinity eXact purification resin is unaffected by most protease inhibitors and protease inhibitor cocktails (see Table 4). The fully compatible protease cocktails tested include combinations of the following inhibitors: AEBSF, aprotinin, benzamidine, E-64, EDTA, leupeptin, pepstatin A, phenanthroline, and PMSF. Please refer to the manufacturer's literature for specific inhibitor combinations and concentrations. Exposure of the resin to 0.5 mM PMSF for greater than 3 hours is not recommended.

### **5.3.4 Urea and Guanidine-HCl**

Urea and guanidine-HCl are often used to solubilize proteins expressed in inclusion bodies or otherwise insoluble. Purifications with the Profinity eXact resin can be run with up to 2 M urea without affecting binding capacity or column performance. Proteins solubilized in 4 M urea can also be purified, although lower yields may be observed. Concentrations of urea above 4 M are not recommended, as both yield and target protein purity suffer. The chloride ions present in guanidine-HCl preclude the use of this denaturant.

### 5.3.5 Profinity eXact MBP Control Lysate

The Profinity eXact MBP control lysate is a lyophilized *E. coli* lysate that contains recombinant Profinity eXact affinity-tagged maltose binding protein (Met-Lys P1'-P2'). Upon purification, the 40 kD tag-free MBP protein is recovered.

For use with the Profinity eXact mini purification spin kit, add 10 mL Profinity eXact bind/wash buffer to the lyophilized control lysates, and gently swirl or mix the buffer until the lyophilized cake is completely resuspended. Store the vial on ice, and use within 30 min of preparation for best results. Load 0.5 ml of resuspended lysate into a Profinity eXact mini spin column or 5 ml lysate onto a 1 ml cartridge.

### 5.3.6 Lysis Protocol

#### 1. Lyse cells using either method a or b:

- a. **For cell lysis by sonication, or other mechanical method, resuspend cells in lysis buffer at buffer/wet cell paste ratio of 5–10 ml buffer/g wet cell paste.**
- b. **Lyse cells using sodium phosphate-based bacterial lysis and extraction reagent (pH 7.5).**

The bacterial lysis and extraction reagent was developed for quick lysis and extraction of recombinant proteins expressed in *E. coli* for purification with the Profinity eXact system. This lysis solution has the same formulation as the Profinia™ bacterial lysis/extraction reagent (250 ml, catalog #620-0220).

Using the volume of lysis and extraction reagent suggested in Table 6, completely resuspend the cell pellet with the lysis solution by vortexing or vigorously pipetting the lysate. Vortex for an additional minute. For lysis of cell pellets from larger bacterial cultures, a 10 ml/1 g ratio of lysis and extraction reagent solution to wet cell paste is recommended.

**Table 6. Recommended Lysis Volumes.**

Culture Volume (mL)	Cell Density (OD <sub>600</sub> )	Lysis and Extraction Reagent Recommended Volume	Lysis Method
1.5	1.5–3.0	300 µL	Vortex 1 min
40	1.5–3.0	5 mL	Incubate 10 min

- 2. Centrifuge lysate at  $\geq 10,000 \times g$ , 30 min. Transfer supernatant to separate container until ready to purify.**
- 3. Filter lysate with 0.45-µm (or 1.0-µm) filter.**

To preserve the flow characteristics of the Bio-Scale™ Mini Profinity eXact cartridges or any prepared gravity flow columns, it is recommended that the whole cell lysate be centrifuged at high speed and filtered (e.g., 0.45 µm membrane) prior to application.

- 4. Chill lysate to 4°C prior to loading on resin.**

Maintaining the lysate on ice reduces target protein degradation and minimizes potential cleavage and elution during the lysate loading step. Using lysate and wash buffers at 4°C is the easiest and most effective method of addressing proteins sensitive to intrinsic cleavage.



## Section 6 Purification Procedures

### 6.1 General Guidelines

#### 6.1.1 Wash Buffer Selection



**For maximum yield, prevent target protein cleavage and elution during the wash step by ensuring wash buffers do not contain any chloride ions (e.g., NaCl).** If a higher ionic concentration is desired to reduce possible electrostatic interactions between contaminant proteins and the resin, sodium acetate or a higher sodium phosphate concentration may be used (e.g., 300 mM).

Similarly, Tris-HCl buffers should be avoided because of the chloride present in the acid conjugate. Tris buffers may still be used by utilizing Tris base and

acetic acid to produce a chloride-free, Tris-acetate buffer with the proper pH. A Tris-phosphate buffer may likewise be made with Tris base and phosphoric acid.

The Profinity eXact™ purification resin is fully functional at pH 6–8. Some intrinsic cleavage due to high OH<sup>-</sup> concentrations during the wash steps may be observed if the wash buffer pH is greater than 7.5. Wash buffers close to pH 7.0 are therefore preferred. On the other hand, using a wash buffer at a pH below 7.0 may help to minimize intrinsic cleavage for those proteins with non-ideal P1'–P2' amino acids. When adjusting the buffer pH with an acid, avoid any use of HCl; instead, use a Cl<sup>-</sup>-free acid such as phosphoric acid or acetic acid.



Wash buffers should be prechilled to 4°C to minimize target elution during the wash step. The Profinity eXact bind/wash buffer may be stored at 4°C for ease of use.

### **6.1.2 Lysate Loading**

As mentioned previously, lysates should be maintained at 4°C to ensure maximum purified protein yields. Improved purification results of multimeric proteins are typically observed by loading a diluted lysate preparation and using substoichiometric amounts of the fusion protein, compared to the available number of Profinity eXact tag binding sites.

### **6.1.3 Profinity eXact MBP Control Lysate**

The Profinity eXact MBP control lysate is a lyophilized *E. coli* lysate that contains recombinant Profinity eXact affinity-tagged maltose binding protein (Met-Lys P1'–P2'). Upon purification, the 40 kD tag-free MBP protein is recovered.

For use with the Profinity eXact mini purification spin kit, add 10 ml Profinity eXact bind/wash buffer to the lyophilized control lysates, and gently swirl or mix the buffer until the lyophilized cake is completely resuspended. Store the vial on ice, and use within 30 min of preparation for best results. Load 0.5 ml of resuspended lysate into a Profinity eXact mini spin column or 5 ml lysate onto a 1 ml cartridge.

### 6.1.4 Cleavage and Elution

Elution of target proteins is typically conducted by incubating the resin in 100 mM sodium fluoride, 100 mM sodium phosphate buffer, pH 7.2, at room temperature, for 30 min. However, if elution at 4°C is desired, the resin should be incubated for at least 16 hr to ensure complete cleavage.

Elution of some slow-cleaving proteins may be accelerated by replacing the sodium fluoride with 10 mM sodium azide in 100 mM sodium phosphate, pH 7.2 buffer, or using prolonged elution incubation. The azide-containing buffer is also an excellent alternative for eluting proteins that would otherwise require a subsequent buffer-exchange step.

Finally, if purified *uncleaved* fusion protein is desired, the Profinity eXact affinity-tagged target protein may be eluted by applying 0.1 M H<sub>3</sub>PO<sub>4</sub> resin regeneration solution instead of fluoride-containing elution buffer.

### 6.1.5 Cleaning and Regeneration

The Profinity eXact resin can be regenerated with 0.1 M H<sub>3</sub>PO<sub>4</sub> by stripping off the cleaved Profinity eXact tag from the S189 ligand. Use of the acid cleaning solution also effectively removes contaminants from the resin. Immediately after the cleaning step, the resin should be re-equilibrated with bind/wash buffer or storage buffer (100 mM sodium phosphate, 0.02% sodium azide, pH 7.2) to prevent loss of activity. Because the resin is base stable, residual contaminants may also be removed by washing the resin with 0.1 M NaOH. However, after cleaning with NaOH, the resin should be equilibrated with bind/wash buffer. The Profinity eXact tag must then be removed using the aforementioned H<sub>3</sub>PO<sub>4</sub> regimen.

## 6.2 Profinity eXact Mini Spin Column Kit Purifications

Each prepacked spin column contains 100 µl settled resin. One column volume (CV) is equivalent to one volume of settled resin (i.e., 1 CV = 100 µl, for a spin column).

Prepare the Profinity eXact MBP control lysate as described on page 29.

Reserve a small amount of lysate prior to loading the sample onto the column. This will serve as a sample for the *lysate* lane for later analysis (e.g., SDS-PAGE). All centrifugation steps are carried out at room temperature at 1,000 × g for 30 sec. If a centrifuge is unavailable, the spin columns may be drained by gravity flow.

**1. Prechill bind/wash buffer on ice or at 4°C.**

**2. Remove storage buffer from packed spin column.**

Snap off end cap of spin column, and **retain** the cap for later use to plug the spin column. Place spin column in a 2 ml collection tube and centrifuge 1,000 × g, 30 sec. Decant filtrate and replace spin column in collection tube.

**3. Preequilibrate spin column with 2 × 500 µl wash buffer.**

Because the storage buffer contains 0.02% sodium azide as a bactericide, this pre-equilibration step **must** be performed to remove the azide triggering ion. Add the first 500 µl aliquot of wash buffer; centrifuge; decant filtrate. Repeat. Plug the spin column by inverting the snap-off cap and inserting it into the bottom of the spin column.



**4. Add up to 600 µl chilled, clarified *E. coli* lysate—depending on expression level—to each spin column.**

Use a pipet to gently mix lysate with the resin. Cap the column.

Use 500 µl of the resuspended Profinity eXact MBP control lysate as a purification positive control.

**5. Gently mix 5–20 min at 4°C on rocking platform or rotator.**

For quick screening, incubate the lysate and resin at room temperature for 1–5 min.

When using the spin column format, purification of larger proteins (>75 kD) may benefit from longer incubations at 4°C.

**6. Collect unbound, flow-through material.**

Carefully twist and remove end cap. Retain end cap, place the spin column in a clean collection tube. Centrifuge and collect the flowthrough; label the fraction *Flowthrough*.

When purifying proteins of low expression, higher volumes of lysate may be applied to the resin by repeating Steps 4–6.

**7. Insert spin column into new 2 ml collection tube.**

**8. Wash the resin with 500 µl wash buffer.**

Mix contents using a pipet or by inverting the capped spin column.

## 9. Centrifuge, repeat wash step.

Remove remaining unbound proteins by centrifuging. Save the flow through from both wash steps and label fractions *Wash 1* and *Wash 2*. Additional washes with more stringent wash buffers may be performed at this stage if required for the target protein being purified.

## 10. Plug spin column with inverted, snap-off end cap.

## 11. Elute tag-free protein by adding 500 µl elution buffer.

Mix contents with a pipet or by inverting the capped spin column. Incubate samples at room temperature for at least 30 min. with occasional mixing throughout. For maximum yield, the capped column should be placed on a rocking platform or rotary mixer. For cleavage incubations performed at 4°C, incubate at least 12 hr.

Carefully twist and remove end cap. At the end of the incubation, place column in a new collection tube, and centrifuge to obtain released, tag-free target protein. Save the eluates for further analysis.

## 12. Analyze collected fractions from above steps by $A_{280}$ , Experion, SDS-PAGE, ELISA, western blot, etc.

## 6.3 Bio-Scale™ Mini Cartridge Purifications with LC Instrumentation

Profinity eXact resin-packed 1 ml Bio-Scale Mini cartridges are typically run at up to 3 ml/min (720 cm/hr), but may be run at flow rates up to 4 ml/min (1,000 cm/hr). The 5 ml cartridges should be run at flow rates up to 10 ml/min (480 cm/hr). The back pressure at these flow rates should be less than 10 psi. To avoid over pressurization of the cartridges during 4°C chromatography purifications, linear velocities  $\leq 480$  cm/hr (240 cm/hr, for a 5 ml cartridge) are recommended if the lysate or wash buffer contains  $\geq 20\%$  glycerol or other viscous additives.

One column volume (CV) is equivalent to one volume of settled resin (i.e., 1 CV = 1 ml, for a 1 ml cartridge).

Reserve a small amount of lysate prior to loading sample onto the column. This will serve as sample for the lysate lane for later analysis (e.g., SDS-PAGE).

**Note:** Flow rates are given for a 1 ml cartridge. (Flow rates for a 5 ml cartridge are provided in parenthesis.)

## Part 1: Buffer Preparation

### 1. Prepare wash buffer (0.1 M sodium phosphate, pH 7.2).



Avoid NaCl in the wash buffer. See Wash Buffer Selection section, page 28. Store wash buffer at 4°C.

### 2. Prepare elution buffer (0.1 M sodium phosphate, 0.1 M sodium fluoride, pH 7.2).

### 3. Prepare cleaning solution (0.1 M H<sub>3</sub>PO<sub>4</sub>)

Be sure to prepare a 0.1 M phosphoric acid (cf. 0.1 N).

### 4. Prepare storage buffer (0.02% sodium azide, 0.1 M sodium phosphate, pH 7.2).

## Part 2: Sample Loading and Washing

### 5. Equilibrate cartridge with 10 CV wash buffer at 3 ml/min (10 ml/min).

Because the storage buffer contains 0.02% sodium azide as a bactericide, this equilibration step **must** be performed to remove the azide triggering ion.



### 6. Load cell extract/lysate at 1 ml/min (5 ml/min).

For a 1 ml cartridge, a 5 ml lysate load is typical.

### 7. Wash cartridge with at least 10 CV wash buffer at 3 ml/min (10 ml/min), or until UV trace has achieved baseline.

Applying wash buffer at a higher flow rate (compared to lysate loading) shortens the process and will reduce potential intrinsic cleavage. Additional washes with more stringent wash buffers may be performed at this stage if required for the target protein being purified.

## Part 3: Elution

### 8. Equilibrate cartridge with 2 CV Elution Buffer at 3 ml/min (10 ml/min) or until cartridge effluent conductivity indicates that cartridge is saturated with elution buffer; stop pump.

### 9. Incubate cartridge in elution buffer for at least 30 min, room temperature.

For 4°C cleavage, incubate the cartridge for at least 12 hr.

**10. Restart pumping at 3 ml/min (10 ml/min) of elution buffer and collect eluate containing purified target protein.**

The OD<sub>280</sub> may be used to monitor the elution of the purified protein and determine when to stop the elution process.

Alternatively, protein may be eluted by pumping elution buffer at 0.1 ml/min (0.5 ml/min). In this case, the slow flow rate is maintained until 5 CV elution buffer is applied.

**Part 4: Cleaning**

- 11. Wash cartridge with at least 5 CV equilibration buffer at 3 ml/min (10 ml/min).**
- 12. Clean cartridge with 3 CV cleaning solution at 3 ml/min (10 ml/min).**
- 13. Neutralize cartridge with 5 CV wash buffer at 3 ml/min (10 ml/min).**
- 14. Apply at least 2 CV storage buffer by pump or syringe to the cartridge; cap cartridge and store at 4°C.**

**6.4 Bio-Scale Mini Cartridge Purifications With Syringe**

If a liquid chromatography system is unavailable, a syringe may be used to apply lysate and buffers to a cartridge. A 20+ ml, luer-fitting syringe is suggested. One column volume (CV) is equivalent to one volume of settled resin (i.e., 1 CV = 1 ml, for a 1 ml cartridge).

Reserve a small amount of lysate prior to loading sample onto the column. This will serve as sample for the *lysate* lane for later analysis (e.g., SDS-PAGE).

**Part 1: Buffer Preparation**

- 1. Prepare wash buffer (0.1 M sodium phosphate, pH 7.2).**



**Avoid NaCl in the wash buffer.** See Wash Buffer Selection section, page 28. Store wash buffer at 4°C.

- 2. Prepare elution buffer (0.1 M sodium phosphate, 0.1 M sodium fluoride, pH 7.2).**

When using a syringe, the elution buffer should be kept at 4°C.

- 3. Prepare cleaning solution (0.1 M H<sub>3</sub>PO<sub>4</sub>).**

Be sure to prepare a 0.1 M phosphoric acid (cf. 0.1 N).

4. **Prepare storage buffer (0.02% sodium azide, 0.1 M sodium phosphate, pH 7.2).**

## Part 2: Sample Loading and Washing

5. **Remove plugs from each end of cartridge and *retain plugs* for later use (Steps 9 and 15).**
6. **Equilibrate cartridge with at least 10 CV wash buffer.**



Because the storage buffer contains 0.02% sodium azide as a bactericide, this equilibration step **must** be performed to remove the azide triggering ion. Fill syringe with Wash buffer, attach to cartridge and push full volume through the cartridge.

7. **Load chilled (4°C) cell extract/lysate.**

Fill syringe with desired volume of lysate, attach to cartridge and push full volume through the cartridge. For a 1 ml cartridge, a 5 ml lysate load is typical.

8. **Wash cartridge with at least 15 CV wash buffer.**

Fill syringe with wash buffer, attach to cartridge and push full volume through the cartridge. Additional washes with more stringent wash buffers may be performed at this stage if required for the target protein being purified.

## Part 3: Elution

9. **Fill syringe with 6 CV elution buffer and apply 1.5 CV to cartridge.**

Collect the 1.5 CV filtrate in 0.5 CV fractions and analyze fractions for target protein. Some purified protein is often collected in the second fraction.

The syringe may be left attached to the cartridge inlet end; plug the outlet using the retained luer plug from Step 5.

**10. Incubate cartridge in elution buffer for at least 30 min, room temperature.**

If performing the incubation at 4°C, incubate the cartridge for at least 12 hr.

**11. Apply remaining 5 CV elution buffer.**

The bulk of the target protein is typically collected in the first five elution fractions.

**Part 4: Cleaning**

**12. Wash cartridge with at least 5 CV wash buffer.**

**13. Clean cartridge with 3 CV cleaning solution.**

**14. Neutralize cartridge with 5 CV wash buffer.**

**15. Equilibrate the cartridge with 2 CV storage buffer; cap cartridge and store at 4°C.**

## **6.5 Gravity Column Purifications**

The Profinity eXact affinity resin may be packed in a Bio-Rad Econo-Pac® column for use in a gravity purification format. Columns packed with 1–4 ml resin exhibit the most practical flow characteristics. One column volume (CV) is equivalent to one volume of settled resin (i.e., 1 CV = 1 ml, for a column packed with 1 ml settled resin).

Reserve a small amount of lysate prior to loading sample onto the column. This will serve as sample for the lysate lane for later analysis (e.g., SDS-PAGE).

### **Part 1: Buffer and Column Preparation**

**1. Prepare wash buffer (0.1 M sodium phosphate, pH 7.2).**

 **Avoid NaCl in the wash buffer.** See Wash Buffer Selection section, page 28. Store wash buffer at 4°C.

**2. Prepare elution buffer (0.1 M sodium phosphate, 0.1 M sodium fluoride, pH 7.2).**

**3. Prepare cleaning solution (0.1 M H<sub>3</sub>PO<sub>4</sub>).**

Be sure that 0.1 M phosphoric acid is used (cf. 0.1 N).

4. **Prepare storage buffer (0.02% sodium azide, 0.1 M sodium phosphate, pH 7.2).**

#### **Part 2: Column Preparation**

5. **Mix resin gently to completely resuspend 50% (v/v) resin slurry.**
6. **Transfer 2–8 ml 50% resin slurry to Econo-Pac column; allow storage buffer to completely drain.**

#### **Part 3: Sample Loading and Washing**

7. **Equilibrate column with 10 CV Wash buffer.**



Because the storage buffer contains 0.02% sodium azide as a bactericide, this equilibration step **must** be performed to remove the azide triggering ion. Apply wash buffer to resin and allow buffer to gravity drain.

8. **Load chilled (4°C) cell extract/lysate.**

Apply lysate to resin and allow buffer to gravity drain.

9. **Wash cartridge with at least 15 CV cold wash buffer.**

Upon complete draining of the wash buffer, plug the Econo-Pac column. Additional washes with more stringent wash buffers may be performed at this stage if required for the target protein being purified.

#### **Part 4: Elution**

10. **Apply 5 CV elution buffer to plugged column.**

Mix the resin with the elution buffer by using a pipet.

11. **Incubate column in elution buffer for at least 30 min, room temperature.**

If performing the incubation at 4°C, incubate the column for at least 12 hr.

12. **Collect purified protein.**

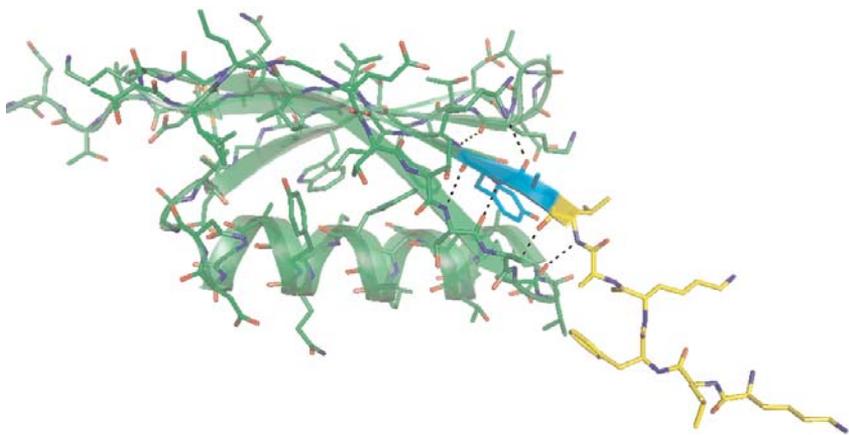
Remove the column's plug and allow eluate containing the purified target protein to gravity drain into collection tube.

## Part 5: Cleaning

13. Wash column with at least 5 CV wash buffer.
14. Clean column with 3 CV cleaning solution.
15. Neutralize resin with 5 CV wash buffer.
16. Equilibrate resin with 2 CV storage buffer; cap cartridge and store at 4°C.

## Appendix A N-terminal Target Protein Structure and Cleavage Kinetics

The N-terminal structure of a target protein may affect the binding of N-terminal affinity tags to their ligands. Protein  $G_B$  (6 kD) is one example of a protein with high N-terminal structure. To assess the effect of N-terminal structure on the cleavage properties of the Profinity eXact™ tag, a fusion protein was constructed to deliberately place the last two amino acids of the Profinity eXact tag (P1 = Leu, P2 = Ala) within the  $\beta$ 1-strand of the four-stranded  $G_B$   $\beta$ -sheet fold (Figure 7), thus obscuring the cleavage site in a construct termed -2. Binding of the eXact- $G_B$  fusion protein to S189 remains strong, even though it requires local unfolding of  $G_B$ .



**Fig. 7. N-terminal interactions of Profinity eXact affinity-tagged  $G_B$ .** The cognate sequence of the Profinity eXact tag is partially shown in yellow (KLFKAL); the P1' amino acid (Ala) and the P2' amino acid (Tyr)  $G_B$  are shown in cyan. Notice that the P2, P1, P1' and P2' amino acids are all part of the  $\beta$ -strand. The hydrogen bonds that would likely be broken for the fusion protein to bind to the affinity resin are shown by the dashed lines. The tyrosine side chain of the P2' amino acid is buried in the hydrophobic core of  $G_B$ .

The relative rates of cleavage of a series of -2  $G_B$  mutants as a function of P1' and P2' amino acids are similar to the GFP series (Figure 9 and Figure 10, see pages 42–43), but the absolute rates are about 10-fold slower than GFP. Even with the  $G_B$ -occluded processing site, all P1' amino acids—except E, I, D, and P—are >90% processed in 30 minutes (with P2' = Y). Apparently, the strong binding of the Profinity eXact tag to the S189 subtilisin ligand is able to disrupt the N-terminal structure of  $G_B$  enough to permit productive substrate binding interactions and processing at reasonable rates for almost all P1', P2' combinations. The processing of P1' = E (P2' = Y) is about 70% complete in 30 minutes with 0.1 M KF and P1' = I or D (P2' = Y) is 50% processed in 30 minutes. As mentioned previously, a fusion containing a proline residue in the P1' position is not processed.

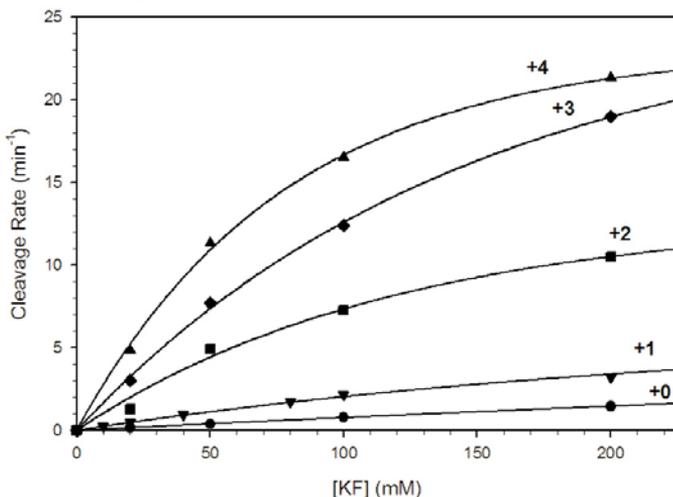
Although practical elution times are generally achieved with no spacer amino acids (+0 constructs), the use of a two amino acid spacer between the Profinity eXact tag and the target protein can minimize any negative impact of N-terminal target protein structure on cleavage kinetics. This effect is demonstrated by a series of Profinity eXact tag- $G_B$  mutants in which length of the connection between the Profinity eXact tag and  $G_B$  was varied (Table 7).

**Table 7. Profinity eXact Tag:  $G_B$  Fusion Constructs.**

Spacer Length (# Amino Acids)	Fusion Construct
+0	Profinity eXact tag - <u>M A Y</u> $G_B$
+1	Profinity eXact tag - M <u>A T Y</u> $G_B$
+2	Profinity eXact tag - M A <u>T T Y</u> $G_B$
+3	Profinity eXact tag - M A T <u>T T Y</u> $G_B$
+4	Profinity eXact tag - M A T G <u>T T Y</u> $G_B$

Amino acids that are structured in  $G_B$  are underlined and in red.

The kinetics of these constructs as a function of fluoride concentration are shown in Figure 8.



**Fig. 8. Cleavage rates vs. [fluoride] for Profinity eXact tag- $G_B$  fusions.**

The results show that while N-terminal structure can slow cleavage kinetics, the cleavage rate of the +2 construct is about five-times faster than the +1 construct. The effect of lengthening the insertion beyond +2 diminishes for a purification application.

Because the average rate of triggered cleavage using the EEDKLFKAL cognate sequence is quite fast relative to the requirements for convenient purification (cleavage rate  $> 0.1 \text{ min}^{-1}$ ), almost any target protein, except those with proline at the P1' positions, should work in the Profinity eXact system. A slow P1' amino acid (E, V, I, or D) in combination with extensive N-terminal structure may require some increase of the standard incubation time in fluoride for good recovery of the target protein.

## **Appendix B**

### **Intrinsic Cleavage Rates**

Intrinsic cleavage rates are dependent not only upon P1'–P2' residues. High N-terminal target protein structure and lower purification temperatures (relative to 25°C) will minimize intrinsic cleavage. Therefore, Figure 9 is presented as a relative guide to assist in determining cloning and purification strategies.

Intrinsic rates  $< 0.03 \text{ min}^{-1}$  are desired for minimum product loss during lysate loading and resin washing. **Except for targets with a P1'-cysteine, use of 4°C lysate and wash buffers will alleviate most intrinsic cleavage issues.** Intrinsic cleavage may also be reduced by addition of a Thr-Ser spacer during cloning.

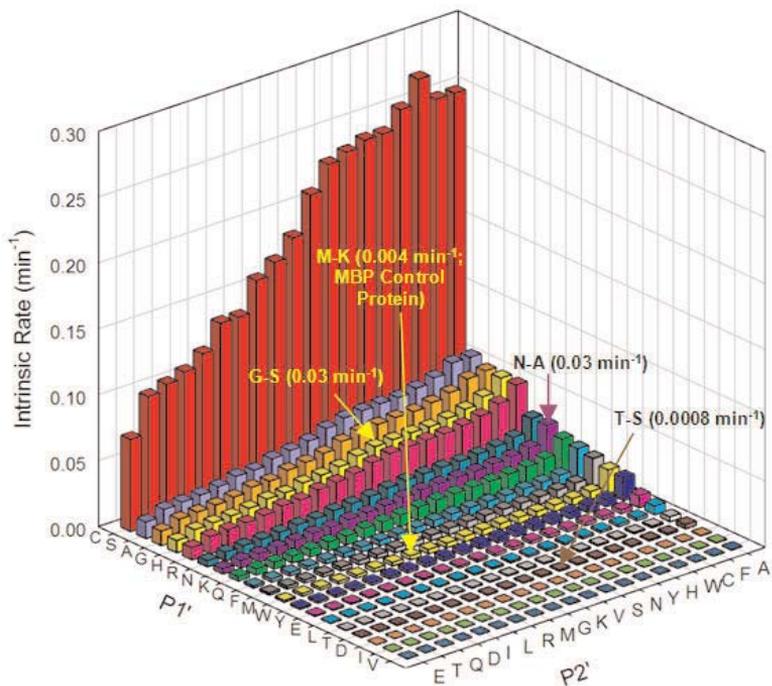


Fig. 9. Effect of P1'–P2' amino acids on intrinsic cleavage rate (rates derived from a green fluorescent protein (GFP) fusion in presence of 100 mM sodium phosphate buffer, pH 7.2; room temperature).

## Appendix C Triggered Cleavage Rates

For elution of target protein, fluoride ion-triggered cleavage rates  $>0.1 \text{ min}^{-1}$  will yield rates conducive to successful purification. Proteins with a P1'-proline will not be cleaved and must be cloned with a spacer (e.g., Thr-Ser) for proper purification. A spacer should also be considered for proteins with a P2'-Pro (with P1'- Asp, Ile, Val, or Glu) or a P1'-Asp.

Purification of fusion proteins with slower anticipated triggered cleavage rates should be incubated with elution buffer for at least 1 hour.

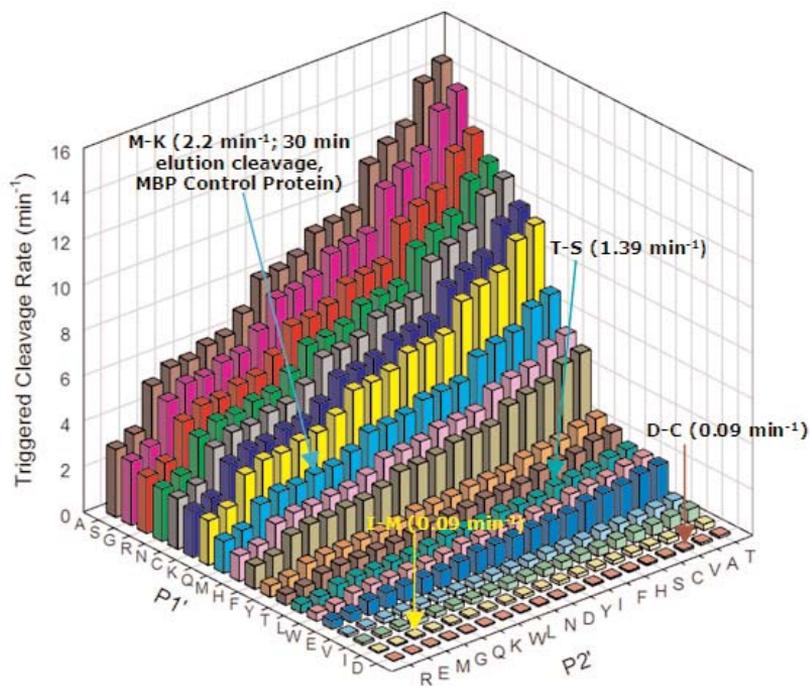


Fig. 10. Effect of P1'-P2' amino acids on triggered cleavage rate (rates derived from a GFP fusion in presence of 100 mM sodium fluoride, 100 mM sodium phosphate buffer, pH 7.2; room temperature).

# Appendix D

## Comprehensive Chemical Compatibility List

Reagent Group	Reagent	Comments	Acceptable Concentration
Triggering Ions	F <sup>-</sup> , Cl <sup>-</sup> , N <sub>3</sub> <sup>-</sup> , NO <sub>2</sub> <sup>-</sup> , HCO <sub>2</sub> <sup>-</sup>	Triggers cleavage of fusion and elution of target protein	Remove from all <b>lysis and wash</b> buffers for highest yields  See Table 3 for ionic concentrations to use as trigger in elution buffer
Salts	Sodium acetate	Reduces nonspecific protein binding due to ionic interactions	≤3 M (an excellent substitute for NaCl in lysis and wash buffers)
	<b>NaCl</b> or <b>KCl</b>	Triggers cleavage of fusion and elution of target protein	Remove from all <b>lysis and wash</b> buffers—use sodium acetate or sodium phosphate instead to reduce electrostatic interactions
Buffers	Tris-HCl	Cl <sup>-</sup> in lysis and wash buffers triggers cleavage and elution of target protein	Substitute with Tris-acetate or Tris-phosphate and adjust pH with acetic or phosphoric acid
	Tris-acetate		50 mM tested
	Tris-phosphate		50 mM tested
	HEPES		50 mM tested
	PIPES		50 mM tested
	MOPS		50 mM tested
	MES		50 mM tested
	Sodium acetate		200 mM tested
	Sodium bicarbonate		200 mM tested
Sodium borate		100 mM tested	
Acids	HCl	Cl <sup>-</sup> in lysis and wash buffers triggers cleavage of fusion and elution of target protein	Avoid use of HCl for highest yields; instead, use acids such as phosphoric or acetic acid

Reagent Group	Reagent	Comments	Acceptable Concentration
Detergents	Non-ionic (NP-40, Triton® X-100, Tween®-20)	Solubilizes proteins	≤5% (v/v)
	Zwitterionic (CHAPS, CHAPSO, Dodecyl-β, D-maltoside, Octylthioglucoside)	Solubilizes proteins	≤5% (v/v or w/v)
	Ionic (CTAB, Sarkosyl, SDS)	Significantly lowers yields	Remove from all buffers
Protease Inhibitors	PMSF	F <sup>-</sup> may lower yield slightly by triggering cleavage of fusion and elution of target protein	0.5 mM; no adverse effects when incubated with resin for up to 3 hr
	TLCK		100 μM
	TPCK		100 μM
	Pharmingen™ Protease Inhibitor Cocktail	F <sup>-</sup> may lower yield slightly	2×
	Calbiochem® Protease Inhibitor Cocktail Set I		1×
	Roche cOmplete Protease Inhibitor Tablet	F <sup>-</sup> may lower yield slightly	1×
	Lysis Solutions	Bacterial Lysis and Extraction Reagent (Bio-Rad)	
B-PER® in phosphate buffer (Pierce)			1×
B-PER		Cl <sup>-</sup> from Tris-HCl results in slightly lower yield	1×
ReadyPreps™ Lysis solutions (Epicentre)			Do not use
BugBuster® (EMD)			1×
FastBreak™ Cell Lysis (Promega)			1×
CellLytic™ Express (Sigma)			Do not use

Reagent Group	Reagent	Comments	Acceptable Concentration
Sulfhydryl (Reducing) Agents	$\beta$ -mercaptoethanol		20 mM
	Dithiothreitol (DTT)		10 mM
	Tris-(2-carboxyethyl) phosphine (TCEP)		5 mM
Denaturants	Guanidine•HCl	Solubilizes proteins	Remove from all <b>lysis and wash</b> buffers
	Urea	Cl <sup>-</sup> triggers cleavage of fusion during load and wash steps	$\leq 2$ M; with 4 M, ~30% reduced control MBP binding capacity observed; with 8 M, ~70% reduced control MBP binding capacity, and lower purity observed
Metal Chelators	EDTA	Metalloprotease inhibitor	20 mM
	EGTA	Metalloprotease inhibitor	20 mM
Other Additives	CaCl <sub>2</sub>	Essential component for purification of Ca <sup>2+</sup> -binding proteins	$\leq 5$ mM; use with buffer such as MES, MOPS, or PIPES buffer to eliminate precipitation of Ca <sup>2+</sup> with either fluoride- or phosphate-containing Profinity eXact buffers
		Cl <sup>-</sup> triggers cleavage of fusion during load and wash steps	
	MgCl <sub>2</sub>	Essential metal cofactor for nucleases	$\leq 5$ mM; if higher Mg <sup>2+</sup> concentrations are desired, use magnesium acetate instead
		Cl <sup>-</sup> triggers cleavage of fusion during load and wash steps	Do not add to Profinity eXact elution buffer—Mg <sup>2+</sup> precipitates with F <sup>-</sup> ; instead, use elution buffer with alternative trigger, such as 10 mM NaN <sub>3</sub> , 100 mM sodium phosphate, pH 7.2
	Glycerol	Stabilizer	20%; higher concentrations do not affect resin, but do increase viscosity
	Ethylene glycol	Stabilizer	20% (v/v)
Ethanol	Stabilizer	20% (v/v)	
Sorbitol	Stabilizer	20% (w/v)	
Sucrose		20% (w/v)	

Reagent Group	Reagent	Comments	Acceptable Concentration
	Imidazole		200 mM
	Ammonium sulfate		15%; use of higher concentrations is dependent upon target's solubility
	Sodium sulfate		100 mM
	DMSO	Stabilizer	5%
	DNase/RNase	Reduce lysate viscosity	200 U nuclease, 10 U Rnase I

## Appendix E Troubleshooting Guide

### Premature elution of cleaved, target protein is observed:

#### Fusion protein

- Insert Thr-Ser spacer between Profinity eXact™ tag and target protein by cloning into pPAL7's Spel site

#### Lysis

- For maximum yields, do not use lysis buffers and reagents that contain triggering ions, specifically Cl<sup>-</sup> or F<sup>-</sup>
- Maintain lysate at 4°C prior to loading
- Shorten incubation time
- Utilize lysis buffer with pH <7.0



#### Wash

- Pre-chill wash buffer to 4°C prior to use
- Do not use lysis buffers and reagents that contain triggering ions, specifically Cl<sup>-</sup> or F<sup>-</sup>. When preparing wash buffer, substitute sodium acetate (NaOAc) for NaCl; do not use HCl to adjust pH—phosphoric or acetic acid should be used
- Utilize wash buffer with pH <7.0



### Target protein remains uncleaved and/or does not elute completely:

#### Fusion protein

- Verify that first amino acid (P1') downstream of the Profinity eXact cleavage site is not proline
- If first amino acid of target protein is undesirable for cleavage, insert amino acid spacer (e.g., Thr-Ser) immediately downstream of cleavage site by site-directed mutagenesis or clone into Spel site of pPAL7

## Elution

- Incubate resin with elution buffer for longer time (try up to 1 hour at room temperature or overnight at 4°C)
- If performing elution at 4°C, incubate overnight
- Increase fluoride concentration in elution buffer (see Figure 8)
- Substitute 10 mM sodium azide for fluoride in elution buffer
- Elute with application of second volume of elution buffer to spin column resin

## Eluate contains significant contaminants:

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### Load

- Dilute lysate for multimeric proteins
- Reduce fusion protein load amount to achieve subsaturating level bound to resin
- Incubate lysate with resin for up to an hour at 4°C to increase binding capacity of target fusion protein

### Wash

- Perform additional wash step
- Reduce nonspecific, electrostatic binding by increasing ionic concentration of wash buffer; use up to 0.3 M Sodium phosphate, NaOAc, or  $(\text{NH}_4)_2\text{SO}_4$  (pH 7.2), or amend wash buffer with any of the aforementioned salts. Do not use NaCl
- Reduce hydrophobic interactions by decreasing salt concentration of wash buffer
- Supplement wash buffer with suitable detergent (see Appendix D: Comprehensive Chemical Compatibility List)

## Fusion protein binds poorly to the resin:

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Fusion Protein	<ul style="list-style-type: none"><li>• Proteins with high N-terminal structure may interfere with binding; adding amino acid spacer (i.e., Thr-Ser) between Profinity eXact tag and target protein will improve tag's accessibility</li></ul>
Lysis	<ul style="list-style-type: none"><li>• If using urea to help solubilize proteins, use [urea] &lt;4 M; higher urea concentrations reduce binding capacity</li></ul>
Load	<ul style="list-style-type: none"><li>• Allow lysate to incubate with resin for up to an hour at 4°C or for 30 minutes at room temperature; lysates with fusion proteins &gt;75 kD often benefit from a longer incubation period</li><li>• When purifying multimeric proteins, avoid saturating resin; diluting lysate may also improve yields</li></ul>
Resin	<ul style="list-style-type: none"><li>• Resin previously used must be regenerated to remove cleaved Profinity eXact tag; apply three column volumes of 0.1M H<sub>3</sub>PO<sub>4</sub> to resin</li></ul>

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## Appendix G

### Ordering Information

<b>Catalog #</b>	<b>Description</b>
156-3008	<b>Profinity eXact Expression and Purification Starter Kit</b>
156-3000	<b>Profinity eXact Cloning and Expression Starter Kit</b>
156-3006	<b>Profinity eXact Mini Spin Purification Starter Kit</b> , 10 pack
156-3001	<b>Profinity eXact RIC-Ready Expression Vector</b> , 20 reactions
156-3002	<b>Profinity eXact Supercoiled Expression Vector</b> , 20 reactions
156-3003	<b>BL21 (DE3) Chemi-Competent Expression Cells</b> , 10 reactions
156-3004	<b>Profinity eXact Antibody Reagent</b>
156-3005	<b>Profinity eXact Purification Resin</b> , 10 ml
156-3007	<b>Profinity eXact Mini Spin Columns</b> , 10 pack
732-4646	<b>Bio-Scale Mini Profinity eXact Cartridges</b> , 2 × 1 ml
732-4647	<b>Bio-Scale Mini Profinity eXact Cartridges</b> , 4 × 1 ml
732-4648	<b>Bio-Scale Mini Profinity eXact Cartridges</b> , 1 × 5 ml

## **Appendix H**

### **Legal Information**

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