New pET expression vectors and HRV 3C Protease for efficient fusion tag removal

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Use the combination of new pET vectors, HRV 3C Protease, and immobilized metal affinity chromatography (IMAC) for expression, fusion tag removal, and purification of expressed proteins. Cleave and remove fusion tags at low temperature from recombinant proteins with the highly specific and efficient HRV 3C Protease. This small recombinant enzyme also contains a His•Tag® sequence that enables its capture by IMAC.

Fusion tag technology has dramatically improved expression, purification, and detection of recombinant proteins produced in *E. coli* and other hosts (1). Sometimes it is necessary to remove fusion tags from recombinant proteins to accurately determine catalytic activity, protein:protein interactions, and crystal structures.

For this purpose, many vectors encode one or more recognition sites for specific proteases between the fusion tag and target open reading frame (ORF) cloning region. Ideally, the protease used for fusion tag removal has the following characteristics:

- Highly specific recognition of the cleavage site to minimize unwanted digestion of the target protein
- Small size to minimize steric hindrance
- High specific activity to minimize the amount of protease needed for efficient cleavage
- Activity at low temperatures to maximize target protein stability during the cleavage reaction
- Option for protease removal after the cleavage reaction to obtain protease-free, purified target protein

The human rhinovirus type 14 3C protease (HRV 3C protease) specifically cleaves the sequence LEVLFQ↓GP between the glutamyl and glycyl residues (2). This protease is active at low reaction temperatures (2), and exhibits enhanced activity at high ionic strengths (3). Its small size [20 kDa (4)] decreases the likelihood that steric effects will hinder access to the cleavage site (5). These characteristics make HRV 3C protease a useful enzyme for removing fusion tags and systematically producing recombinant proteins suitable for structural and functional proteomics. We have developed a recombinant HRV 3C protease (22 kDa) that contains a His•Tag sequence (HRV 3C Protease) to enable efficient removal of the protease post-reaction by immobilized metal affinity chromatography (IMAC). Here we describe a streamlined, automatable system for bacterial cell growth, recombinant protein expression, purification, and N-terminal fusion tag and HRV 3C Protease removal.

New pET vectors

The Novagen pET-47b(+), pET-48b(+), pET-49b(+), and pET-50b(+) vectors are designed for expression of fusion proteins with removable N- and C-terminal fusion tags. As described in Table 1 and illustrated in Figure 1, these vectors share several common features: they encode His•Tag and HRV 3C protease cleavage

continued on page 4

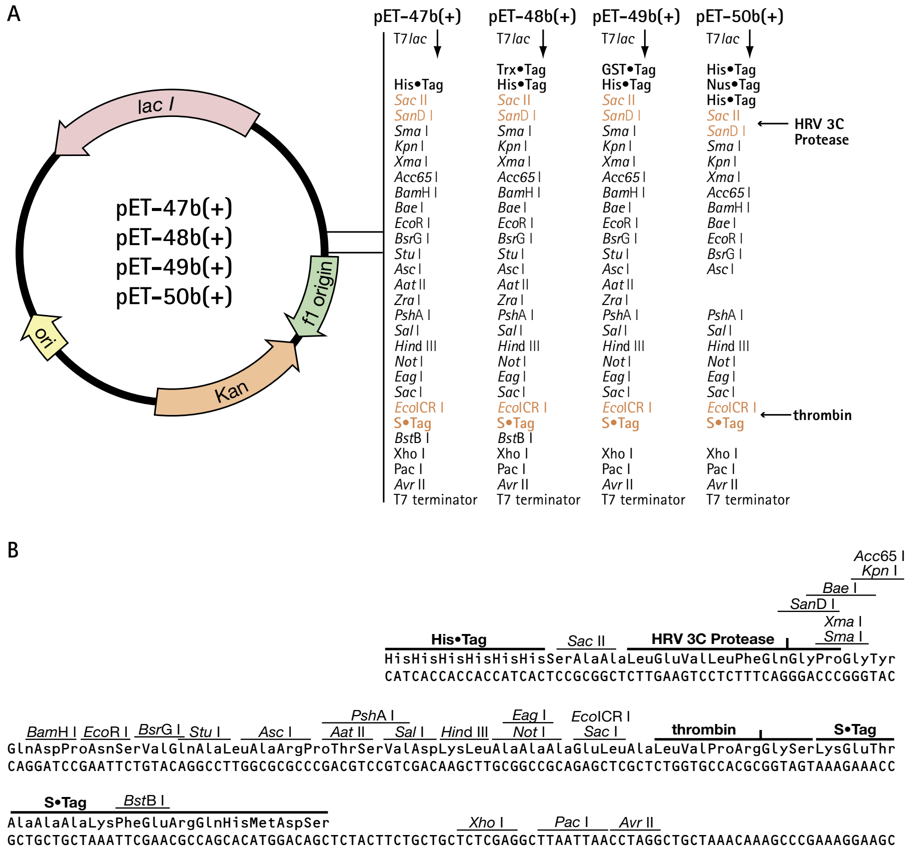


Figure 1. Features of the pET-47b(+), pET-48b(+), pET-49b(+), and pET-50b(+) vectors and common MCS region

A. pET-47–pET-50 Vector map and MCS region. **B.** MCS region sequence details the segment from the His•Tag sequence to the start of the S•Tag™ sequence, including protease cleavage sites.

continued from page 3

recognition sequences upstream from the multiple cloning site (MCS), and carry an optional C-terminal thrombin site and S•Tag™ coding sequence. The individual vectors also encode other frequently used fusion tags, such as glutathione S-transferase (6), thioredoxin (7), and NusA (8, 9) (see Table 1). Cloning an ORF for a target protein into the *San*D I or *Sma* I sites minimizes the number of vector-encoded N-terminal amino acids retained after HRV 3C Protease cleavage.

As a test target protein, we used the entire coding sequence (lacking a stop codon) from a human enolase 2 cDNA (I.M.A.G.E. Consortium 3629603) (10). To produce the constructs, we amplified the ORF with KOD Hot Start DNA Polymerase using primers that incorporated the *San*D I and *Not* I recognition sites for cloning into the corresponding sites in the vectors. The expressed proteins carried all the vector-encoded N-terminal fusion tags and C-terminal thrombin site and S•Tag sequences. Table 2 lists the predicted sizes of the fusion proteins.

Target proteins from the enolase constructs described in Table 2 were expressed in Rosetta™ 2(DE3) cells and purified using the RoboPop™ Ni-NTA His•Bind® Purification Kit. Figure 2 shows the SDS-PAGE analysis of the purified proteins.

Table 1. Features of the pET-47b(+), pET-48b(+), pET-49b(+), and pET-50b(+) vectors

Vector*	N-Terminal Tags	MCS and Protease Sites†	C-terminal Tag
pET-47b(+)	His•Tag	3C, MCS, Tb	S•Tag
pET-48b(+)	Trx•Tag/His•Tag	3C, MCS, Tb	S•Tag
pET-49b(+)	GST•Tag/His•Tag	3C, MCS, Tb	S•Tag
pET-50b(+)	His•Tag/Nus•Tag/His•Tag	3C, MCS, Tb	S•Tag

* All vectors share T7*lac* promoter, kanamycin (Kan) resistance, and the ColE1 replicon.
† 3C: HRV 3C Protease; MCS: multiple cloning sites; Tb: thrombin. See Figure 1.

Table 2. The pET-47b(+), pET-48b(+), pET-49b(+), and pET-50b(+) enolase constructs

Recombinant Vector*	Fusion Protein (designation)	Predicted M_r (kDa)
pET-47b enolase	His•Tag® enolase S•Tag (His•Tag enolase)	53.0
pET-48b enolase	Trx•Tag/His•Tag enolase S•Tag (Trx•Tag enolase)	66.8
pET-49b enolase	GST•Tag/His•Tag enolase S•Tag (GST•Tag enolase)	80.4
pET-50b enolase	His•Tag/Nus•Tag/His•Tag enolase S•Tag (Nus•Tag enolase)	109.4

* Target proteins from the enolase constructs were expressed in Rosetta 2(DE3) cells and purified using the RoboPop Ni-NTA His•Bind® Purification Kit. See Figure 2.

Site-specific cleavage and protease removal

We performed the cleavage reaction with the IMAC-purified protein derived from pET-50b(+) (see Table 2) using the recommended 1:50 ratio [protease unit:microgram (µg) target protein]. Additionally, we examined the possibility of non-specific cleavage by using 10 times more protease (1:5 ratio). As shown in lanes 2 and 3 in Figure 3, at either concentration, the HRV 3C Protease specifically cleaved > 95% of the N-terminal fusion tags during an overnight (16-hour) incubation at 4°C; the untagged enolase and the fusion tag are clearly visible. Lane 2 also reveals a faint band corresponding to the HRV 3C Protease in the 1:5 reaction mixture. Lanes 4 and 5 show that the untagged enolase was recovered in the flow-through after another IMAC step, while lanes 6 and 7 show the IMAC-captured fusion tag, small amounts of untagged enolase and fusion protein, and the IMAC-captured HRV 3C Protease. Significantly, even after a 16-hour HRV 3C Protease cleavage with the higher protease concentration (1:5 ratio), lanes 2 and 4 revealed no detectable cleavage at secondary sites.

We used two different detection methods to confirm that the enolase in the IMAC flow-through was essentially free of HRV 3C Protease. First, we analyzed the flow-through for presence of HRV 3C Protease by performing Western blot analysis with the His•Tag Monoclonal Antibody and Goat Anti-mouse Alkaline Phosphatase Conjugate. Although we readily detected a 5-ng positive control sample of HRV 3C Protease by this method, the flow-through exhibited no detectable HRV 3C Protease (data not shown). Second, we used a colorimetric, synthetic HRV 3C Protease substrate to assay for activity in the flow-through (11). Results of this assay also confirmed efficient protease removal; the IMAC flow-through yielded readings similar to the negative controls (data not shown).

One-hour proteolysis in solution

We performed an experiment to determine the extent of cleavage after 30 and 60 minutes, using equal masses of HRV 3C Protease and a comparable protease

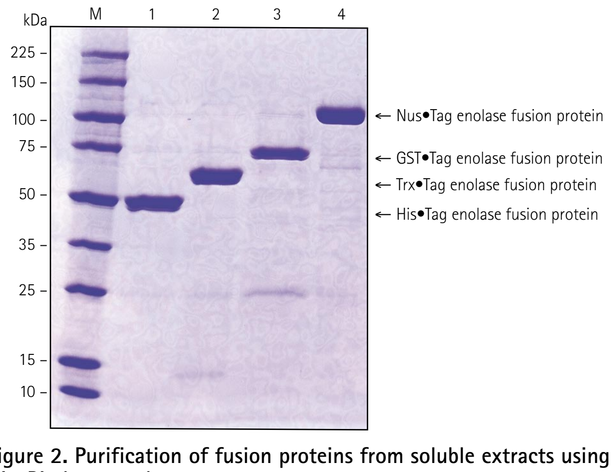
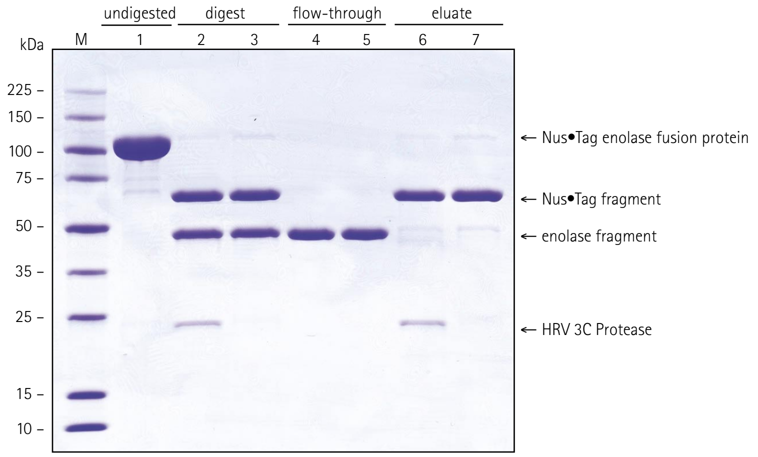


Figure 2. Purification of fusion proteins from soluble extracts using the RoboPop Ni-NTA His•Bind protocol

Cultures of Rosetta 2(DE3) strain, each carrying one of the pET-47b–pET-50b enolase constructs, were grown for 16 h at 30°C with shaking at 300 rpm in a 24-well plate containing 5 ml/well Terrific Broth (TB) plus Overnight Express™ Autoinduction System 1 components. The cultures (OD_{600} of 9 to 12) were processed using PopCulture® Reagent and Ni-NTA His•Bind chromatography on a PerkinElmer MultiPROBE® II liquid handling system. The purified enolase fusion proteins were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie™ blue staining. M: Perfect Protein™ Markers, 10–225 kDa.



Lane	Sample
M	Perfect Protein™ Markers, 10–225 kDa
1	3 µg purified Nus•Tag enolase fusion protein
2	3 µg from 1:5 reaction
3	3 µg from 1:50 reaction
4	3.5 µl flow-through from 1:5 reaction
5	3.5 µl flow-through from 1:50 reaction
6	3 µl from 1:5 reaction eluate
7	3 µl from 1:50 reaction eluate

Figure 3. HRV 3C Protease cleavage and Ni-NTA His•Bind® chromatography for removing fusion tags and protease

Two 1-ml reactions each containing 1 mg purified Nus•Tag™ enolase fusion protein were incubated with HRV 3C Protease at 4°C for 16 h at different ratios: 1:50 and 1:5 protease unit:µg target protein. A 750-µl sample of each reaction was combined with 250 µl equilibrated Ni-NTA His•Bind Resin and batch-adsorbed for 20 min at 4°C by repeated inversions. The mixture was loaded into a 2-ml Spin Filter and centrifuged at 1000 x g for 1 min. The cleaved enolase was collected in the flow-through, and the resin-adsorbed proteins were eluted with 750 µl Ni-NTA Elute Buffer. Samples were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie™ blue staining.

from a competitor (competitor's protease). In this experiment, we used purified Nus•Tag enolase as the target protein at a 1:100 weight-to-weight ratio (1 µg protease:100 µg target protein). SDS-PAGE analysis (Figure 4) demonstrated that the cleavage reaction with HRV 3C Protease was more than 50% complete after 30 minutes (lane 2) and nearly complete after only 60 minutes (lane 4). In contrast, the cleavage reaction with the competitor's protease was less than 50% complete after 60 minutes (lane 5).

On-column cleavage

We also tested the capabilities of the HRV 3C Protease to perform on-column cleavage, and thereby reduce the number of steps and time needed to remove fusion tags. For this experiment, we used GST•Tag™ enolase as the target protein. After the fusion protein was expressed and soluble proteins were extracted, we adsorbed GST•Tag enolase target to Ni-NTA His•Bind Resin in batch. Next we packed a column with the GST•Tag enolase-resin mixture, washed it with Ni-NTA Wash Buffer, and equilibrated it with HRV 3C Protease Cleavage Buffer. After adding HRV 3C Protease and incubating the reaction for 16 hours at 4°C, we collected the untagged enolase in the flow-through (Figure 5). As lane 4 shows, this on-column cleavage method efficiently removed the N-terminal fusion tag from the target protein and cleanly separated the untagged enolase from both its fusion tag and the HRV 3C Protease.

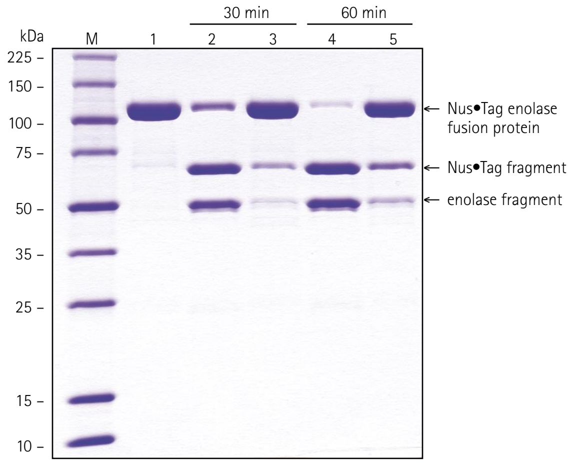
Combined dialysis and cleavage

After purification, fusion proteins are typically dialyzed to equilibrate them in the appropriate buffer for storage, additional purification steps, or further experiments. Because HRV 3C Protease activity is stable and enhanced by high ionic strength conditions (3), we tested whether the protease could successfully remove a fusion tag during dialysis from Ni-NTA Elute Buffer into HRV 3C Protease Cleavage Buffer. Before dialysis, we added the HRV 3C Protease in a 1:10 protease unit:µg target protein ratio directly to purified Nus•Tag enolase in Ni-NTA Elute Buffer. SDS-PAGE analysis indicated that the HRV 3C Protease successfully cleaved the fusion tag from the target protein in 16 hours (Figure 6). Performing simultaneous dialysis and cleavage reduces processing time. Further, because the target protein concentration can be accurately determined by standard methods after the IMAC step, this dialysis/cleavage approach enables the use of a known protease-to-target protein ratio in the cleavage reaction.

Summary

We have demonstrated application of the pET-47b(+), pET-48b(+), pET-49b(+),

continued on page 6



Lane	Sample
M	Perfect Protein Markers, 10–225 kDa
1	3 µg purified Nus•Tag enolase fusion protein
2	3 µg of 30-min HRV 3C Protease reaction
3	3 µg of 30-min competitor's protease reaction
4	3 µg of 60-min HRV 3C Protease reaction
5	3 µg of 60-min competitor's protease reaction

Figure 4. Cleavage efficiency comparison of HRV 3C Protease with a competitor's protease

Using a 1:100 (w/w) ratio of protease:target protein, 500 µg of purified Nus•Tag enolase fusion protein was incubated in a 500-µl reaction volume at 4°C under the same cleavage reaction conditions as the experiment described in Figure 3. The reaction was quenched by adding equal volumes to 4X SDS Sample Buffer and then immediately placing the samples into a water bath at 75°C for 5 min.

continued from page 5

and pET-50b(+) vectors, HRV 3C Protease, and IMAC to produce, cleave, and purify untagged target proteins. These new pET vectors direct the expression of fusion proteins that contain combinations of His•Tag® and Trx•Tag™, GST•Tag™, or Nus•Tag™ sequences upstream of an HRV 3C protease recognition site. The HRV 3C Protease promotes efficient N-terminal fusion tag removal by cleavage either in solution, on-column, or during dialysis at low temperatures.

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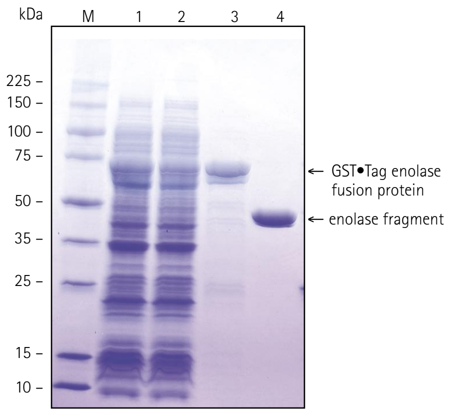
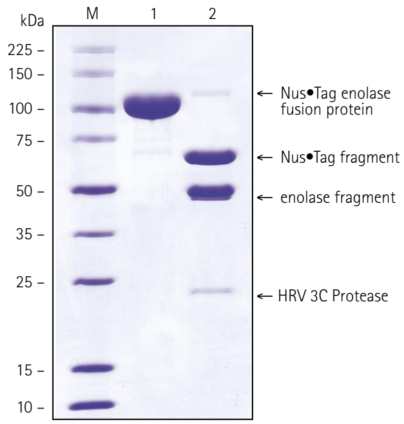


Lane	Sample
M	Perfect Protein Markers, 10–225 kDa
1	Total cell protein
2	Soluble cell protein
3	Purified GST•Tag enolase fusion protein
4	Flow-through

Figure 5. On-column cleavage and concomitant removal of fusion tag and HRV 3C Protease

Cultures of Rosetta™ 2(DE3) strain containing GST•Tag enolase fusion protein were grown in 500-ml baffled flasks containing 100 ml TB plus Overnight Express™ Autoinduction System 1 at 37°C with shaking at 300 rpm to OD_{600} 3.0. The temperature was reduced to 25°C with continued incubation for 14 h. The cultures had a final OD_{600} of 11.6. The cells were harvested by centrifugation at 9000 x g for 10 min and proteins were extracted from the resultant pellets with BugBuster® Protein Extraction Reagent plus Lysonase™ Solution. The extract was centrifuged at 7000 x g, and the soluble fraction was added to a 50% slurry of Ni-NTA His•Bind® Resin (1 ml; 0.5 ml settled volume) equilibrated with Ni-NTA Bind Buffer. After a 30-min reaction at 4°C with mixing, the mixture was poured into a chromatography column and washed three times (10 ml each) with Ni-NTA Wash Buffer followed by two washes (10 ml each) with HRV 3C Protease Cleavage Buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5). On-column cleavage was achieved by a 16-h incubation at 4°C in 1 ml HRV 3C Protease Cleavage Buffer containing 200 units HRV 3C protease. After the incubation, the cleaved enolase was collected in the flow-through. Samples were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie™ blue staining.



Lane	Sample
M	Perfect Protein™ Markers, 10–225 kDa
1	3 µg purified Nus•Tag enolase fusion protein
2	3 µg of 16-h reaction

Figure 6. Dialysis and cleavage of purified Nus•Tag enolase fusion protein

Nus•Tag enolase fusion protein (1.3 mg:1.5 ml) was purified with the RoboPop™ Ni-NTA His•Bind® Purification Kit, combined with HRV 3C Protease in a 1:10 protease unit:µg target protein ratio and then dialyzed (3.5 kDa MW cut-off) against approximately 650 ml HRV 3C Protease Cleavage Buffer for 16 h at 4°C. Samples were analyzed by SDS-PAGE (10–20% gradient gel) and Coomassie blue staining.

Product	Size	Cat. No.
HRV 3C Protease	500 U	71493-3
Components: • 1 × 500 U HRV 3C Protease • 1 × 10 µg HRV 3C Cleavage Control Protein • 1 × 10 ml 10X HRV 3C Cleavage Buffer		
pET-47b(+) DNA	10 µg	71461-3
pET-48b(+) DNA	10 µg	71462-3
pET-49b(+) DNA	10 µg	71463-3
pET-50b(+) DNA	10 µg	71464-3
pET Expression System 47b		71465-3
pET Expression System 47b plus Competent Cells		71466-3
pET Expression System 48b		71467-3
pET Expression System 48b plus Competent Cells		71468-3
pET Expression System 49b		71469-3
pET Expression System 49b plus Competent Cells		71470-3
pET Expression System 50b		71471-3
pET Expression System 50b plus Competent Cells		71472-3
RoboPop™ Ni-NTA His•Bind® Purification Kit		71188-3
Overnight Express™ Autoinduction System 1		71300-3 71300-4
Rosetta™ 2(DE3) Singles™ Competent Cells	11 rxn 22 rxn	71400-3 71400-4
Ni-NTA His•Bind Resin	10 ml 25 ml 100 ml	70666-3 70666-4 70666-5
Ni-NTA Buffer Kit		70899-3
Perfect Protein™ Markers, 10-225 kDa	100 lanes	69079-3
His•Tag® Monoclonal Antibody	100 µg 3 µg	70796-3 70796-4
KOD Hot Start DNA Polymerase*	200 U 5 × 200 U	71086-3 71086-4

*Manufactured by TOYOBO and distributed by Merck Biosciences. Not available from Merck Biosciences in Japan.