



PinPoint™ Xa Protein Purification System

Technical Manual No. 028

INSTRUCTIONS FOR USE OF PRODUCT V2020. PLEASE DISCARD PREVIOUS VERSIONS.
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I. Description

The PinPoint™ Xa Protein Purification System^(a,b) is designed for the production and purification of fusion proteins that are biotinylated in vivo. Biotinylated fusion proteins are produced in *E. coli* and are affinity-purified using the SoftLink™ Soft Release Avidin Resin^(c). This proprietary resin allows elution of the fusion protein under nondenaturing conditions. The PinPoint™ Vectors feature the encoded endo-proteinase Factor Xa (pronounced “ten a”) proteolytic site that provides a way to separate the purification tag from the native protein. These vectors also carry a convenient

multiple cloning region for ease in construction of fusion proteins. The schematic diagram in Figure 1 outlines the expression and purification system procedure.

The system contains vectors in all possible sense reading frames (see Figure 2 and Figure 3), an avidin-conjugated resin, Streptavidin-Alkaline Phosphatase, a purification column and biotin. The PinPoint™ Xa Control Vector^(b,d) (Figure 4) contains the chloramphenicol acetyltransferase (CAT) gene and is provided as a means of monitoring protein expression, purification and processing conditions.

II. Product Components

Product	Cat.#
PinPoint™ Xa Protein Purification System ^(a,b)	V2020
Includes:	
• 10µg PinPoint™ Xa-1 Vector ^(b,d)	
• 10µg PinPoint™ Xa-2 Vector ^(b,d)	
• 10µg PinPoint™ Xa-3 Vector ^(b,d)	
• 5µg PinPoint™ Xa Control Vector ^(b,d)	
• 3ml SoftLink™ Soft Release Avidin Resin ^(c)	
• 20µl Streptavidin-Alkaline Phosphatase	
• 1 PinPoint™ Purification Column	
• 1ml Biotin, 100mM (pH 7.2)	
• 1 Protocol	

Storage Conditions: Store the PinPoint™ Purification Column at room temperature. Store all remaining components at 4°C. The vectors may be stored at –20°C.

III. General Considerations

The PinPoint™ Xa Protein Purification System exploits the well-understood interaction of biotin with avidin for purification of biotinylated fusion proteins. The biotinylation reaction in *E. coli* is catalyzed by biotin ligase holoenzyme and results in a fusion purification tag that carries a single biotin specifically on one lysine residue (1–6). The biotin moiety is accessible to avidin or streptavidin, as demonstrated by binding to resins containing either molecule, and serves as a tag for detection and purification. *E. coli* produce a single endogenous biotinylated protein that, in its native conformation, does not bind to avidin rendering the downstream affinity purification highly specific for the recombinant fusion protein.

Avidin-biotin interactions are so strong that elution of biotin-tagged proteins from avidin-conjugated resins usually requires denaturing conditions. In contrast, SoftLink™ Soft Release Avidin Resin, monomeric avidin that is supplied with the system, allows the protein to be eluted by incubation in a nondenaturing 5mM biotin solution. The rate of dissociation of the monomeric avidin-biotin complex is sufficiently fast to effectively allow for the recovery of all of the bound protein in neutral pH and low salt conditions.

The SoftLink™ Soft Release Avidin Resin consists of monomeric avidin covalently attached to a polymethacrylate resin, which is highly resistant to many chemical reagents (e.g., 0.1N NaOH, 50mM acetic acid and nonionic detergents), permitting quite stringent cleaning conditions. The TetraLink™ Tetrameric Avidin Resin^(e) (Cat.# V2591) can be used in place of the SoftLink™ Soft Release Avidin Resin to

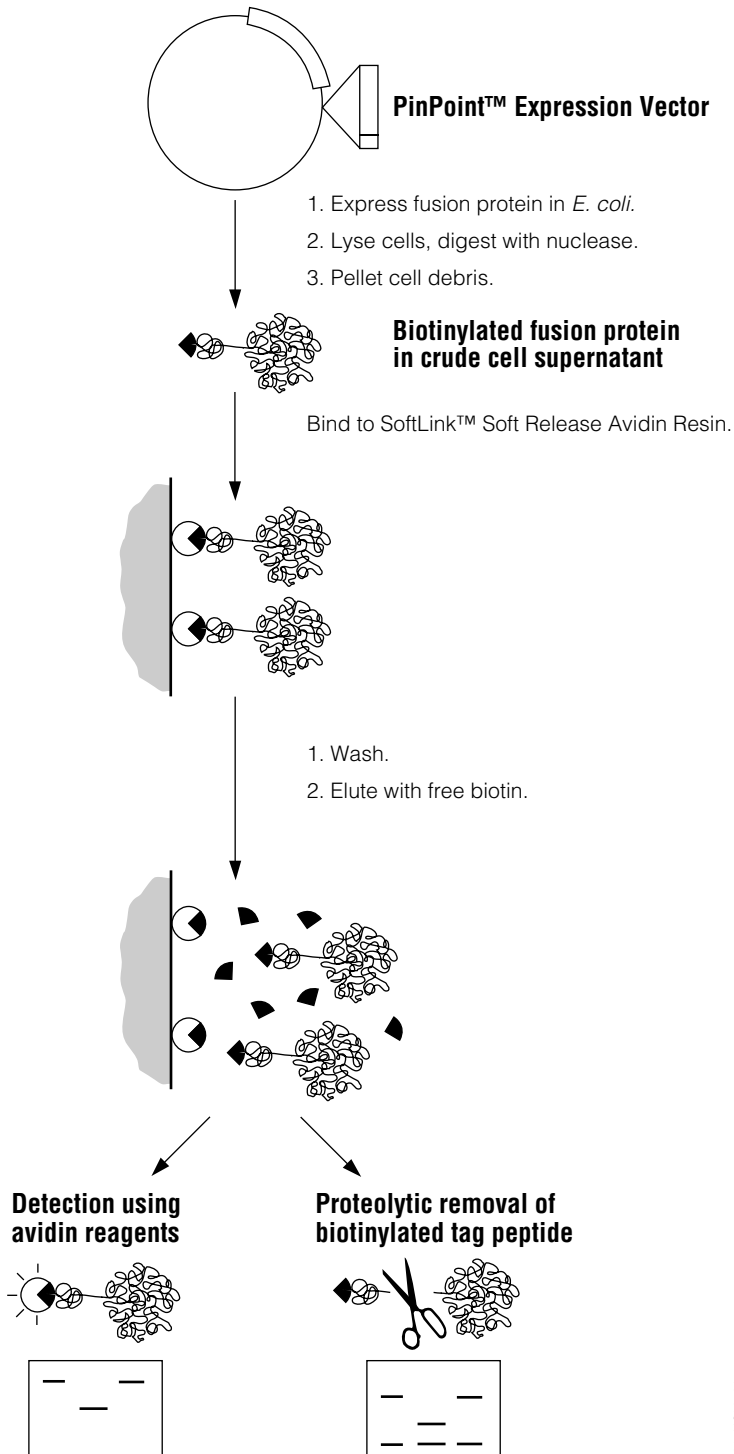


Figure 1. Schematic diagram of recombinant protein expression and purification using the PinPoint™ Xa Protein Purification System.

create affinity resins for purifying antibodies that recognize the antigenic portion of a fusion protein. Therefore, the TetraLink™ Resin is useful for immobilization and not necessarily the recovery of the fusion protein.

The PinPoint™ Xa Protein Purification System provides three vectors in three possible sense reading frames and multiple cloning sites downstream of the encoded biotinylated target sequence to facilitate the creation of an in-frame fusion protein. The PinPoint™ Xa Vectors also contain an *Nru* I site downstream of the Factor Xa cleavage recognition site located at the carboxy terminus of the biotinylated segment (Figure 2). The terminal arginine of the Factor Xa site is encoded within the *Nru* I restriction site that marks the 5'-end of the multiple cloning region.

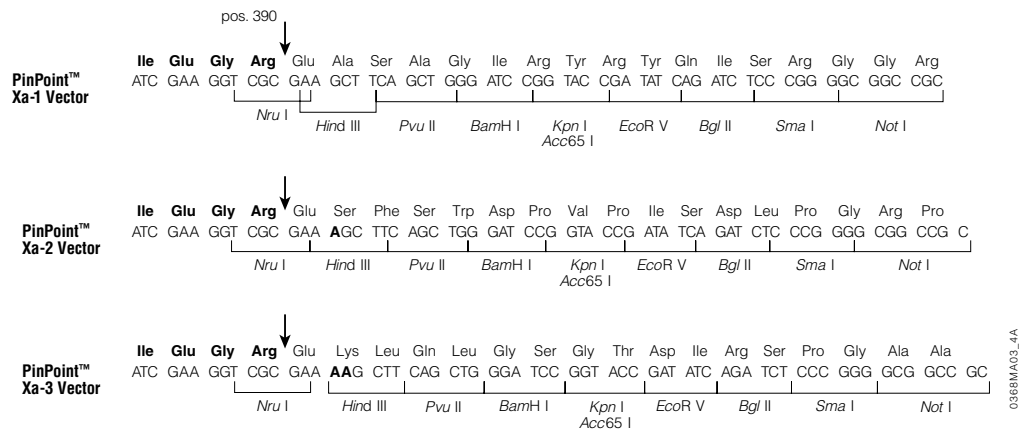


Figure 2. The multiple cloning regions of the three PinPoint™ Vectors. The codons, the corresponding amino acid residues and the restriction sites of the multiple cloning regions are aligned. The recognition sequence for Factor Xa Protease and the additional adenosines (A) are indicated in bold type; the cleavage site is indicated by the arrow.

Insertion of the desired gene into the *Nru* I site can produce a fusion protein that contains no additional amino acids between the Factor Xa site and the start of the protein of interest. As with many protein purification systems that rely on purification tags, the effectiveness of cleavage will depend upon the nature of the fusion partners and the protease. It is best to test both Factor Xa Protease and less specific proteases, such as Trypsin (Cat.# V5111 and V5113), for their ability to process the fusion protein. Additional recommendations concerning reaction times and proteases can be found in Section VII.

The PinPoint™ Xa Control Vector may be used to monitor protein expression and purification. The Control DNA contains the CAT gene fused to the biotinylated protein tag. After induction, cells containing the PinPoint™ Xa Control Vector produce a 40kDa protein that can be cleaved by Factor Xa Protease to yield the 27kDa CAT peptide and a 13kDa biotinylated peptide.

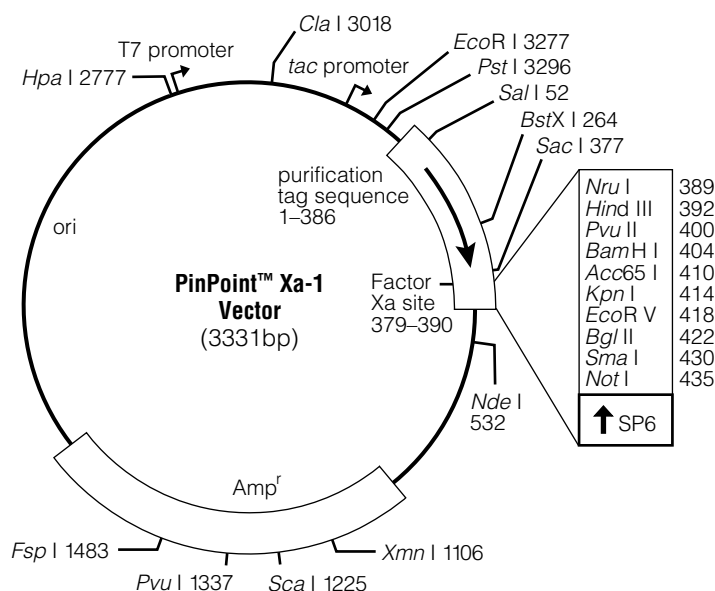


Figure 3. The PinPoint™ Xa-1 Vector circle map and sequence reference points. Base #1 is the translation start site for the purification tag sequence. The three PinPoint™ Xa Vectors below are identical except for an additional coding-strand adenosine at position 394 in the PinPoint™ Xa-2 Vector, and two additional coding-strand adenosines, at the same position, in the PinPoint™ Xa-3 Vector.

Sequence reference points:	Xa-1 Vector	Xa-2 Vector	Xa-3 Vector
<i>tac</i> promoter	3202–3282	3203–3283	3204–3284
biotin purification tag coding region	1–386	1–386	1–386
Factor Xa Protease recognition site	379–390	379–390	379–390
multiple cloning region	387–441	387–442	387–443
PinPoint™ Vector Sequencing Primer binding site	325–343	325–343	325–343
SP6 Sequencing Primer binding site	451–468	452–469	453–470
SP6 RNA polymerase promoter (–17 to +3)	449–468	450–469	451–470
T7 RNA polymerase promoter (–17 to +3)	2796–2815	2797–2816	2798–2817
biotinylated lysine codon	262–264	262–264	262–264
β-lactamase (Amp ^r) coding region	919–1778	920–1779	921–1780

Note: The PinPoint™ Vector Sequencing Primer^(d) (Cat.# V4211) and the SP6 Promoter Primer (Cat.# Q5011) can be used to sequence across the cDNA-encoded fusion site. A mismatch with the 5'-terminal base of the SP6 Promoter Primer does not interfere with sequencing.

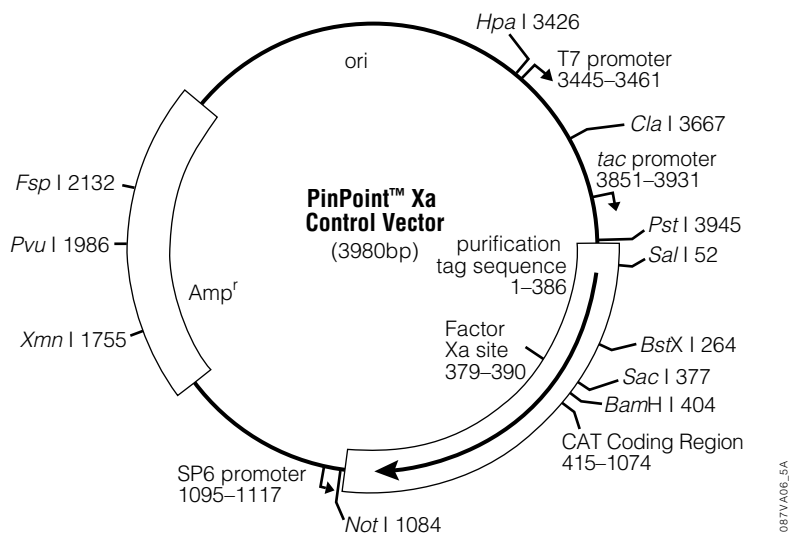


Figure 4. PinPoint™ Xa Control Vector circle map and reference points. Base #1 is the translation start site for the purification tag sequence.

Sequence reference points:	
<i>tac</i> promoter	3851–3931
biotin purification tag coding region	1–386
Factor Xa Protease recognition site	379–390
PinPoint™ Vector Sequencing Primer binding site	325–343
SP6 Sequencing Primer binding site	1100–1118
CAT coding region	415–1074
SP6 RNA polymerase promoter	1095–1117
T7 RNA polymerase promoter	3445–3461
biotinylated lysine codon	262–264
β-lactamase (<i>Amp^r</i>) coding region	1568–2427

IV. Expression of the Fusion Protein

Prior to purification, check for correct expression of the fusion product. The simplest way to detect the fusion protein is by SDS-polyacrylamide gel electrophoresis (PAGE) analysis followed by localization of the biotinylated protein using streptavidin-alkaline phosphatase conjugates.

Materials to Be Supplied by the User

(Solution compositions are provided in Section XII.A)

- ampicillin stock solution
- IPTG, 100mM
- LB medium
- NBT/BCIP (or Promega's Western Blue® Stabilized Substrate for Alkaline Phosphatase; Cat.# S3841)
- Ponceau S solution (Sigma Cat.# P7170)
- sample 1X buffer
- SDS-polyacrylamide minigel
- Streptavidin-Alkaline Phosphatase (Cat.# V5591)
- TBST buffer

A. Small-Scale Culture and Induction

Fusion proteins generated from PinPoint™ Xa Vectors have been biotinylated and expressed in JM109 and HB101 *E. coli* bacterial strains. *E. coli* strains that do not contain the *birA* mutation produce biotin ligase (1,2) and should be compatible with this system.

1. Start three individual cultures of the host strain carrying either the PinPoint™ Xa Vector with the desired insert DNA, the PinPoint™ Xa Control DNA, or containing no plasmid DNA. Inoculate 1–5ml of LB containing biotin (2µM final concentration) and ampicillin (100µg/ml) with a freshly isolated bacterial colony. Omit ampicillin for the culture lacking plasmid. Incubate the cultures overnight at 37°C with shaking.
2. Dilute the overnight cultures 1:100 in 25–50ml of fresh LB containing biotin (2µM final concentration) and ampicillin in a 250ml flask. Again, omit ampicillin in cultures lacking plasmid. Incubate an additional hour at 37°C with shaking.
3. Induce protein expression by adding IPTG (100µM final concentration) to all cultures. Incubate 4–5 hours at 37°C with shaking. Proceed directly to Section IV.B.

B. Detection of the Fusion Protein

1. Transfer 100µl of each culture to separate 1.5ml microcentrifuge tubes. Centrifuge at top speed in a microcentrifuge for 5 minutes. Remove the supernatant.
2. Add 50µl of sample 1X buffer to each tube and vortex to resuspend cells. Heat the resuspended cells at 95°C for 5 minutes with occasional vortexing. This treatment will lyse the cells and coat the proteins with SDS.

Note: The samples can be stored at –20°C at this point, but will require retreating with heat prior to SDS-PAGE analysis. Repeated heating of these samples, however, can result in the destruction of some proteins.

3. Load 5µl of the heat-treated samples on a 12% SDS polyacrylamide minigel (4,5) along with molecular weight markers in the flanking lanes. Run the gel as recommended by the manufacturer of the electrophoresis unit.
4. Following electrophoresis, transfer the proteins by electroblotting onto a membrane (such as Immobilon® PVDF or nitrocellulose). Refer to the instructions provided with the electroblotting apparatus and the membrane for recommendations on buffer compositions and transfer times.
5. Following the transfer, stain the protein blot with Ponceau S solution for 30 seconds and destain with water for one minute. Indicate the location of the molecular weight markers with a pencil.

Alternatively, separate duplicate samples by SDS-PAGE (as in Step 3), transfer to a membrane, excise and stain with Amido Black for nitrocellulose membranes or with Coomassie® Blue for PVDF membranes.

6. Locate the biotinylated protein bands in the lanes containing the cell lysates.
 - a. Incubate the membrane in TBST buffer for 30–60 minutes at room temperature with gentle agitation to block protein binding sites. Pour off the solution.

Note: An *Experienced User's Protocol* can be found at the end of this Technical Bulletin.

Note: Frequently, pre-stained markers are used since they require no subsequent staining for visual detection. If pre-stained markers are not used, load an amount of molecular weight markers to achieve >1µg of protein per band. Visualize the markers using Ponceau S staining (Steps 5 and 6).



Do not use sequencing membranes, such as Immobilon® SEQ or PROBLOTT®, which can generate high background.

- b. Combine 3 μ l of the Streptavidin-Alkaline Phosphatase and 15ml of TBST buffer. Place the membrane in this solution and incubate for 30 minutes at room temperature with gentle agitation. Pour off the solution.
- c. Wash the membrane for 5 minutes with TBST buffer. Repeat this wash two more times and rinse briefly with deionized water. Pour off the water.
- d. Add freshly prepared NBT/BCIP solution or Promega's Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841). Incubate at room temperature with gentle agitation until bands appear. Dark purple bands will indicate the location of the biotinylated protein species in the lanes containing cellular extracts.

Notes:

1. Strains of *E. coli* normally synthesize a single biotinylated protein of 22.5kDa (apparent molecular mass), which is made in relatively small amounts, and that will appear in the cellular extracts from the host strain. *E. coli* containing the PinPoint™ Xa Control Vector will produce the endogenous 22.5kDa protein and a fusion protein of approximately 40kDa. *E. coli* containing the recombinant PinPoint™ Xa Vector will produce the 22.5kDa protein and a fusion protein of size determined by the insert portion of the fusion construct. The size of the full fusion protein will be 13kDa larger than the fusion partner due to the presence of the biotin tag portion derived from the plasmid. Also, fusion proteins made by the PinPoint™ Xa Vectors are produced in much greater amounts than the endogenous, biotinylated 22.5kDa *E. coli* protein.
2. Cellular proteases may be degrading the recombinant protein if several bands are seen in the lanes containing the fusion protein extract. In this case, we strongly recommend adding a protease inhibitor, such as phenylmethylsulfonyl fluoride (PMSF), to all cell extracts during purification.

C. Large-Scale Culture and Induction

Large-scale cultures typically are needed for isolating large quantities of the fusion protein using the SoftLink™ Soft Release Avidin Resin. Typical yields using the PinPoint™ Xa Control Vector are 1–5mg of purified fusion protein per liter of culture. Yields of fusion constructs using PinPoint™ Xa Vectors may vary depending upon the fusion protein and growth conditions.

1. Scale up the growth of the cells, using the protocols in Section IV.A, to produce the amount of culture needed. Keep the proportions of the inoculates and the culture components equivalent to those suggested above to achieve equivalent results.
2. Harvest the cells by centrifugation at 8,000 \times *g* for 10 minutes. Immediately proceed with purification of the protein (Sections V and VI) or freeze the cells at –20°C for later use.

V. Cell Lysis

We recommend sonication for recovering the fusion protein from culture. Alternative methods include lysis by enzyme/detergent or French press. Section V.B is a protocol for cell lysis by lysozyme and detergent (6). For cell lysis by French press, please refer to the instructions provided by the manufacturer.

It is important to know the approximate cell mass produced in culture. On average, one liter of culture will produce 4–5 grams of pelleted, wet cells. Resuspend the cells in cell lysis buffer as described below.

A. Sonication

Materials to Be Supplied by the User

(Solution compositions are provided in Section XII.A)

- cell lysis buffer
 - sonicator
1. Resuspend the cells by stirring in 10 volumes (ml/gram cell paste) of cell lysis buffer. Perform this step at 4°C, or on ice, until no clumped cells appear.
 2. Transfer the cell suspension to a container and place on ice. The container should be at least as wide as the volume of the cell solution is deep.
Note: Perform the subsequent steps on ice to prevent excessive heating of the solution.
 3. Sonicate the suspension according to the instructions provided by the manufacturer. In the absence of specific instructions, sonicate by using ten, 15-second pulses with a 15-second pause between pulses. Lower the sonicator probe into the solution about one-half the depth of the solution prior to initiating the pulse. Two minutes of sonication usually is sufficient for lysis. Again, take care not to heat the extract.
 4. Centrifuge the crude lysate at 10,000 × *g* for 15 minutes at 4°C to remove cellular debris. The amount of debris should be significantly smaller in volume than that of the original cell pellet used. Proceed to Section VI.

B. Lysozyme and Detergent Alternative Lysis Protocol

An alternative protocol for protein recovery by cell lysis involving lysozyme and detergent solubilization is as follows. This procedure may be used in place of sonication (Section V.A) or other methods of cell lysis.

Materials to Be Supplied by the User

(Solution compositions are provided in Section XII.A)

- cell lysis buffer
 - DNase I (Sigma, Cat.# D4527)
 - lysozyme (Sigma Grade VI, Cat.# L2879)
 - sodium deoxycholate (DOC)
 - Triton® X-100
1. Resuspend the cells by stirring in 10 volumes (ml/gram cell paste) of cell lysis buffer. Perform this step at 4°C, or on ice, until no clumped cells appear.

Note: Use mechanical stirring for large solutions or use rapid vortexing for small solutions.

Note: Use mechanical stirring for large solutions or use rapid vortexing for small solutions.

Note: An equivalent concentration of Triton® X-100 can be substituted for DOC.

2. Add lysozyme to a final concentration of 1 mg/ml. Stir the solution at 4°C for 20 minutes.
3. Add sodium deoxycholate (DOC) to a final concentration of 0.1% and continue stirring at 4°C for five minutes. The solution should be highly viscous.
4. Add 200u of DNase I to reduce the viscosity of the solution. Continue stirring for an additional 10 minutes.
5. Centrifuge the crude lysate at 10,000 × g for 15 minutes at 4°C to remove cellular debris. The amount of debris should be significantly smaller in volume than that of the original cell pellet.
6. Carefully transfer the supernatant to a clean tube and proceed to Section VI.

VI. Resin Preparation and Protein Purification

The SoftLink™ Resin must be prepared before using it for the first time. Two methods of purifying the fusion protein, column capture and batch capture followed by elution, are provided. The column protocol works well for purification of large amounts of material. The alternative batch method may be more suitable for the simultaneous purification of several fusion proteins. The SoftLink™ Resin has a binding capacity of 20–40nmol of biotinylated protein per milliliter of resin.

The buffers used for washing the SoftLink™ Resin and eluting the fusion protein should stabilize the activity of the fusion protein. If stabilizing solutions are not known, use the cell lysis buffer (Sections V and XII.A) for washing the resin. For elution, use a solution of cell lysis buffer containing 5mM biotin.

Materials to Be Supplied by the User

(Solution compositions are provided in Section XII.A)

- acetic acid, 10%
- cell lysis buffer
- ethanol, 20%
- phosphate buffer, 100mM (pH 7.0)

A. Preparation and Regeneration of the SoftLink™ Resin

Preparation

Before using the SoftLink™ Resin for the first time, pre-adsorb the nonreversible binding sites by adding two resin-bed volumes of a compatible stabilizing buffer containing 5mM biotin. Allow the buffer/resin mixture to sit at room temperature for 15 minutes. Remove the buffer and proceed to the regeneration of the resin.

Regeneration

Regenerate the SoftLink™ Resin after each use by washing with 10% acetic acid and phosphate buffer. This cycle of elution and regeneration can be repeated at least ten times with no loss of biotin binding capacity. The resin may be regenerated in the column or in a beaker.

1. Wash the resin with eight resin-bed volumes of 10% acetic acid.
2. Wash the resin with eight resin-bed volumes of 100mM phosphate buffer (pH 7.0).



Do not expose the resin to NaOH concentrations greater than 0.1N. This will significantly reduce the binding capacity of the resin.

3. Monitor the pH of the eluate until it reaches pH 6.8. At this point, stop the column flow for a minimum of 30 minutes to allow the avidin to refold.
4. Store the resin in 20% ethanol at 4°C for up to six months without loss of binding capacity.

B. Column Capture

1. Pour the regenerated resin into a column that allows the bed height of the settled resin to be at least three times the diameter of the column.
2. Equilibrate the resin in the column using a stabilizing buffer compatible with the biotinylated molecule to be purified.
3. Slowly apply the cell extract (<1ml/minute) to allow efficient capture. In some instances, improved capture and yield can be obtained by passing the extract over the resin several times by use of a peristaltic pump.
4. Wash the column with at least five column-volumes of buffer.
5. To elute the protein, add a stabilizing buffer containing 5mM biotin. Immediately begin collecting fractions of 0.5ml. When a volume of elution buffer equal to one-half the volume of resin in the column has been applied, stop the flow from the column. Wait 15 minutes to allow for release of the fusion protein. This step yields a higher concentration of protein in the eluate.
6. Reinitiate the elution and continue to collect fractions.
7. Test the fractions for the presence of protein by measuring the activity of the protein of interest by quantitating protein using the Bradford assay or by monitoring the absorbance at A₂₈₀. Alternatively, detect biotinylated protein by using the Streptavidin-Alkaline Phosphatase assay (see Section IV.B).
8. Pool the fractions that contain the fusion protein. We recommend dialyzing pooled fractions of protein against a stabilizing buffer to remove excess biotin that may interfere with subsequent assays.

C. Batch Capture

1. Equilibrate the SoftLink™ Resin in a buffer compatible with the biotinylated molecule to be purified. Suggested buffers include lysis buffer, PBS or TBS. The resin is compatible with Triton® X-100, DTT or EDTA.
2. Mix equilibrated SoftLink™ Resin with the cell lysate at a ratio of 3ml of resin per liter of starting culture. Gently rock the mixture for 1–4 hours at 4°C.
3. Carefully aspirate the cell lysate from the resin mixture. Add 10 volumes of buffer per milliliter of resin and rock gently for 10 minutes at 4°C. Repeat the wash at least once. Allow the resin to settle between washes and then carefully aspirate the buffer. Alternatively, centrifuge at ≤500 × g at 4°C for five minutes between washes to sediment the resin.
4. To elute the purified protein, add a stabilizing buffer containing 5mM biotin to the resin at a ratio of 2:1 (v/v) biotin solution to resin. The buffer also should stabilize the protein's activity. Gently rock the resin suspension for a minimum of one hour at 4°C.
5. Allow the resin to settle. Transfer the biotin solution, which contains the released fusion protein to a clean tube.

Note: If desired, the resin can be regenerated directly in the column.

Note: Better yields may be obtained using longer incubation times.

Note: Better results are obtained with an overnight elution.

Note: Addition of a second equivalent volume of biotin solution may improve the yield.

VII. Cleaving the Fusion Protein

Conditions for the efficient cleavage at the Factor Xa site, or any other engineered proteolytic site, without inactivating the protein of interest will depend upon the properties of the fusion protein.

Determination of Optimal Protease Cleavage Conditions

In general, use 2–10% (w/w) of Factor Xa Protease to target protein. Digest the protein at room temperature or 37°C. In most cases, an overnight digestion will be necessary.

To determine the optimal level of protease to use, perform the following study. The example provided uses Factor Xa Protease for cleavage.

Materials to Be Supplied by the User

(Solution compositions are provided in Section XII.A)

- Factor Xa Protease (Cat.# V5581)
 - Factor Xa 10X reaction buffer
 - sample 1X buffer
 - sodium deoxycholate (DOC)
 - trichloroacetic acid (TCA)
 - Tris-HCl, 20mM (pH 8.0)
1. Add 25µg of the purified fusion protein to four separate microcentrifuge tubes. The volume added should be no more than 50µl (i.e., the starting concentration of the protein should be no less than 0.5µg/µl). In addition, add 25µg of purified protein derived from the PinPoint™ Xa Control Vector to four additional microcentrifuge tubes.
 2. For each fusion protein of interest, prepare the following digestion reactions in sterile 1.5ml microcentrifuge tubes. Repeat using the same amount of control protein.

	Tube 1	Tube 2	Tube 3	Tube 4
Factor Xa 10X reaction buffer	10µl	10µl	10µl	10µl
diluted Factor Xa Protease	—	2.5µl	6µl	12µl
target protein	25µg	25µg	25µg	25µg
sterile, deionized water to	100µl	100µl	100µl	100µl
 3. Incubate the tubes at room temperature or at 37°C, if desired. Remove a 20µl sample at one hour, at three hours and after an overnight incubation. In addition, take a 10µl sample at each time point to monitor the activity of the isolated protein. Store these samples at –20°C.
 4. Stop the reaction in the 20µl samples by drying under a vacuum. Alternatively, add DOC to a final concentration of 0.02%. Incubate at room temperature for 10 minutes. Then add TCA to a final concentration of 10%. Incubate at 4°C for 15 minutes. Centrifuge at top speed in a microcentrifuge for five minutes. Pour off the TCA and dry the pellet.
 5. Resuspend the dried samples in 20µl of sample 1X buffer and separate the samples by SDS-PAGE using 10–12% acrylamide. Stain and destain the gel using standard protocols (3). The biotinylated purification tag will appear as a 13kDa protein, and the proteolytically cleaved fusion protein partner should appear as a new protein species with an apparent molecular weight 13kDa less than that of the original fusion protein. The proteins expressed

by the PinPoint™ Xa Control Vector will include the 13kDa constitutive species and the 27kDa CAT protein.

- Determine the incubation time that allowed complete or nearly complete digestion of the fusion protein(s). Use the saved 10µl sample from the appropriate time point (Steps 3) and determine whether the incubation time affected the activity of the protein. If the activity is still acceptable, use this incubation time and appropriate amount of protease in larger digestion reactions.

Notes:

- If excessive degradation is apparent in the cleavage reaction with no added protease (Tube 1), a cellular protease(s) is co-purifying with the fusion protein. The appearance of bands other than the 13kDa biotin tag and the intact purified protein following the addition of another protease may indicate that the recombinant protein contains recognition sequences for this other protease. The use of protease inhibitors during the purification of the protein may inhibit these proteases.
- Other less specific proteases, such as Trypsin (Cat.# V5111 and V5113), may be used usually at much lower protease levels and using much shorter incubation times. To test if these proteases will process the fusion protein effectively, use the protocol as described, but decrease the additions of protease to 1, 3 and 5µl, in tubes 2, 3 and 4, respectively, of the diluted working enzyme stock, and remove samples at 15 minutes, one hour and two hours. Stop the reaction by precipitation with TCA in the presence of 0.02% DOC (Step 4). Analyze the samples as described in Step 5.

VIII. Troubleshooting

Symptoms	Possible Causes	Comments
Expressed protein is insoluble	Due to properties of the experimental protein	Try decreasing the induction temperature from 37°C to room temperature. Solubilize the cell debris in SDS and determine if protein is present by SDS-PAGE. Try the alternative lysozyme and detergent cell lysis procedure or add 0.1% Triton® X-100 (or Tween® 20) to the cell lysate after sonication.
Biotinylated proteins observed at expected size and unexpected smaller sizes	Possible proteolytic activity	Try decreasing the induction temperature from 37°C to room temperature. Add a protease inhibitor, such as PMSF or EDTA, to cell lysis buffer. Try an alternate <i>E. coli</i> host strain that is deficient in proteases (e.g., BL21).

For questions not addressed here, please contact your local Promega Branch Office or distributor. Contact information available at: www.promega.com.

E-mail: techserv@promega.com

VIII. Troubleshooting (continued)

Symptoms	Possible Causes	Comments
Low protein yield	Not all protein captured	Biotin contamination of the lysate.
	Low biotinylation	Examine the protein input and flowthrough using streptavidin-alkaline phosphatase and Western blotting to determine whether capture is complete.
	Protein not eluted	Need to preblock irreversible binding sites on the Soft Link™ Resin.
	Expression is low	Check expression on gel. Some fusions are toxic; therefore, optimize induction parameters.

IX. References

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5. Cronan, J.E., Jr. (1990) Biotinylation of proteins in vivo. A post-translational modification to label, purify, and study proteins. *J. Biol. Chem.* **265**, 10327.
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X. Appendix A: General Information for Constructing Fusion Proteins

A. Vectors and Cloning Considerations

General guidelines for cloning DNA inserts into plasmid vectors are described in Promega's *Protocols and Applications Guide* (3). Specific considerations for creating such constructs are provided below.

Although most genes can be expressed at high levels in *E. coli*, this may be lethal to bacterial cells. Preventing expression until a culture of the clone has grown for several generations can reduce or moderate these effects. Expression of the biotinylated protein segment in the PinPoint™ Xa Vectors is under the control of the *tac* promoter. Using strains, such as JM109, that overproduce the *lac* I protein can prevent expression by inhibiting this promoter. If you suspect that expression of a particular protein is or would be detrimental to the host, add an inducer of the *lac* promoter to host cultures 2–3 hours after, rather than one hour after, initiating growth (as described in Section IV.A).

B. Engineering Fusion Proteins

A correct reading frame between the tag in the vector and the cloned gene insert is crucial for expression of the desired fusion protein. Each PinPoint™ Xa Vector provides one of three possible reading frames to aid in the construction of the fusion protein. A proper gene fusion should maintain the reading frame of the fusion partner protein and should not introduce a stop codon.

To construct gene fusions, analyze the reading frames and positions of the restriction sites in the multiple cloning regions of the three vectors, and determine which site(s) in which vector(s) can be used for the direct ligation of the gene of interest (see Figure 2). You may want to incorporate a restriction site of choice by DNA amplification or other means should no unique sites be available in your gene. One sure way to determine if the construct's reading frame would be maintained is to determine which amino acids are encoded by the DNA comprising the restriction sites.

For example, the multiple cloning site in the PinPoint™ Xa-1 Vector contains a *Bam*H I restriction site that translates as follows:

Encoded Amino Acids	Gly Ile Arg
DNA Sequence	GGG ATC CGG
	<i>Bam</i>H I

If a *Bam*H I, *Bcl* I or *Bgl* II site is located at the beginning of the protein's coding region and the ATC nucleotides of the restriction site encode an isoleucine (Ile), then ligation of the gene insert and vector using these sites will generate an in-frame fusion between the segments.

If a proper gene fusion cannot be made as described above, then the reading frame of the genes may be aligned by one of the following manipulations.

1. Remove the bases in the restriction site overhang in the PinPoint™ Xa Vector and in the gene of interest, and ligate the resulting blunt ends in the correct reading frame. This should produce the desired construct, but we recommend sequencing for confirmation.
2. Insert a DNA linker that provides sufficient bases, or a unique restriction site, to maintain the reading frame of the two sequences. The linker segment also may encode a peptide segment that is recognized by another highly specific endoproteinase, such as enterokinase, for subsequent cleavage of the fusion protein between the biotinylated purification tag and the protein of interest.

Once a construct is made, we recommend confirming the sequence of the clone at the fusion site to ensure the fidelity of the reading frame by sequencing both strands. Sequencing primers for this purpose are available from Promega (see Section XII.B).

C. Constructs Containing a Proteolytic Cleavage Site

The PinPoint™ Xa Vectors contain a unique *Nru* I site at the 5'-end of the multiple cloning region. This site contains the terminal arginine codon of the preferred Factor Xa recognition site. Gene fusions made at this site may place the first amino acid of the recombinant gene adjacent to the arginine codon. Cleavage of this purified fusion protein with Factor Xa Protease (or another protease that will cut on the carboxy-terminal side of an arginine residue) should produce the desired protein containing no additional amino acids.

Digestion of the vector with *Nru* I produces a blunt end; the cut site interrupts the Factor Xa terminal arginine codon between the second and third base. To produce an in-frame gene fusion that does not contain additional amino-terminal residues, the gene fragment must be blunt-ended and engineered such that it contains one additional base (either A, G, T, or C) before the first codon of the protein of interest. This can be accomplished by taking advantage of a naturally

XI. Appendix B: PinPoint™ Xa Vector Sequence and Restriction Sites
A. PinPoint™ Xa-1 Vector Sequence

Note: Sequences for the PinPoint™ Xa-2 and Xa-3 Vectors differ only by the addition of one and two adenosine bases, respectively, directly after position 393 of this sequence.

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1  ATGAAACTGA  AGGTAACAGT  CAACGGCACT  GCGTATGACG  TTGACGTTGA
51  CGTCGACAAG  TCACACGAAA  ACCCGATGGG  CACCATCCTG  TTCGGCGGCG
101 GCACCGGCGG  CGCGCCGGCA  CCGGCAGCAG  GTGGCGCAGG  CGCCGGTAAG
151 GCCGGAGAGG  GCGAGATTCC  CGCTCCGCTG  GCCGGCACCG  TCTCCAAGAT
201 CCTCGTGAAG  GAGGGTGACA  CGGTCAAGGC  TGGTCAGACC  GTGCTCGTTC
251 TCGAGGCCAT  GAAGATGGAG  ACCGAGATCA  ACGCTCCAC  CGACGGCAAG
301 GTCGAGAAGG  TCCTGGTCAA  GGAGCGTGAC  GCGGTGCAGG  GCGGTCAGGG
351 TCTCATCAAG  ATCGGGGATC  TCGAGCTCAT  CGAAGGTCGC  GAAGCTTCAG
401 CTGGGATCCG  GTACCGATAT  CAGATCTCCC  GGGGCGGCCG  CGATCTGGTT
451 CTATAGTGTC  ACCTAAATCG  TATGTGTATG  ATACATAAGG  TTATGTATTA
501 ATTGTAGCCG  CGTTCTAACG  ACAATATGTC  CATATGGTGC  ACTCTCAGTA
551 CAATCTGCTC  TGATGCCGCA  TAGTTAAGCC  AGCCCCGACA  CCCGCCAACA
601 CCCGCTGACG  CGCCCTGACG  GGCTTGTCTG  CTCCCGGCAT  CCGCTTACAG
651 ACAAGCTGTG  ACCGTCTCCG  GGAGCTGCAT  GTGTCAGAGG  TTTTCACCGT
701 CATCACCGAA  ACGCGCGAGA  CGAAAGGGCC  TCGTGATACG  CCTATTTTTA
751 TAGGTTAATG  TCATGATAAT  AATGGTTTCT  TAGACGTCAG  GTGGCACTTT
801 TCGGGGAAAT  GTGCGCGGAA  CCCCTATTTG  TTTATTTTTC  TAAATACATT
851 CAAATATGTA  TCCGCTCATG  AGACAATAAC  CCTGATAAAT  GCTTCAATAA
901 TATTGAAAAA  GGAAGAGTAT  GAGTATTCAA  CATTTCCGTG  TCGCCCTTAT
951 TCCCTTTTTT  GCGGCATTTT  GCCTTCCTGT  TTTTGCTCAC  CCAGAAACGC
1001 TGGTGAAAGT  AAAAGATGCT  GAAGATCAGT  TGGGTGCACG  AGTGGGTTAC
1051 ATCGAACTGG  ATCTCAACAG  CGGTAAGATC  CTTGAGAGTT  TTCGCCCGA
1101 AGAACGTTTT  CCAATGATGA  GCACTTTTAA  AGTTCTGCTA  TGTGGCGCGG
1151 TATTATCCCG  TATTGACGCC  GGGCAAGAGC  AACTCGGTCG  CCGCATAAC
1201 TATTCTCAGA  ATGACTTGGT  TGAGTACTCA  CCAGTCACAG  AAAAGCATCT
1251 TACGGATGGC  ATGACAGTAA  GAGAATTATG  CAGTGCTGCC  ATAACCATGA
1301 GTGATAACAC  TCGCGCCAAC  TTACTTCTGA  CAACGATCGG  AGGACCGAAG
1351 GAGCTAACCG  CTTTTTTGCA  CAACATGGGG  GATCATGTAA  CTCGCCTTGA
1401 TCGTTGGGAA  CCGGAGCTGA  ATGAAGCCAT  ACCAAACGAC  GAGCGTGACA
1451 CCACGATGCC  TGTAGCAATG  GCAACAACGT  TCGCAAACCT  ATTAACCTGG
1501 GAACTACTTA  CTCTAGCTTC  CCGGCAACAA  TTAATAGACT  GGATGGAGGC
1551 GGATAAAGTT  GCAGGACCAC  TTCTGCGCTC  GGCCCTTCCG  GCTGGCTGGT
1601 TTATTGCTGA  TAAATCTGGA  GCCGGTGAGC  GTGGGTCTCG  CGGTATCATT
1651 GCAGCACTGG  GGCCAGATGG  TAAGCCCTCC  CGTATCGTAG  TTATCTACAC
1701 GACGGGGAGT  CAGGCAACTA  TGGATGAACG  AAATAGACAG  ATCGCTGAGA

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1751 TAGGTGCCTC ACTGATTAAG CATTGGTAAC TGTCAGACCA AGTTTACTCA
1801 TATATACTTT AGATTGATTT AAAACTTCAT TTTTAATTTA AAAGGATCTA
1851 GGTGAAGATC CTTTTTGATA ATCTCATGAC CAAAATCCCT TAACGTGAGT
1901 TTTCTGTTCCA CTGAGCGTCA GACCCCGTAG AAAAGATCAA AGGATCTTCT
1951 TGAGATCCTT TTTTTCTGCG CGTAATCTGC TGCTTGCAA CAAAAAACC
2001 ACCGCTACCA GCGGTGGTTT GTTTGCCGGA TCAAGAGCTA CCAACTCTTT
2051 TTCCGAAGGT AACTGGCTTC AGCAGAGCGC AGATACCAA TACTGTTCTT
2101 CTAGTGTAGC CGTAGTTAGG CCACCACTTC AAGAACTCTG TAGCACCGCC
2151 TACATACCTC GCTCTGCTAA TCCTGTTACC AGTGGCTGCT GCCAGTGGCG
2201 ATAAGTCGTG TCTTACCGGG TTGGACTCAA GACGATAGTT ACCGGATAAG
2251 GCGCAGCGGT CGGGCTGAAC GGGGGGTTTCG TGCACACAGC CCAGCTTGGA
2301 GCGAACGACC TACACCGAAC TGAGATACCT ACAGCGTGAG CTATGAGAAA
2351 GCGCCACGCT TCCCGAAGGG AGAAAGGCGG ACAGGTATCC GGTAAGCGGC
2401 AGGGTCGGAA CAGGAGAGCG CACGAGGGAG CTTCCAGGGG GAAACGCCTG
2451 GTATCTTTAT AGTCCTGTCG GGTTCGCCA CCTCTGACTT GAGCGTCGAT
2501 TTTTGTGATG CTCGTCAGGG GGGCGGAGCC TATGGAAAAA CGCCAGCAAC
2551 GCGGCCTTTT TACGGTTCCT GGCCTTTTGC TGGCCTTTTG CTCACATGTT
2601 CTTTCCTGCG TTATCCCCTG ATTCTGTGGA TAACCGTATT ACCGCCTTTG
2651 AGTGAGCTGA TACCGCTCGC CGCAGCCGAA CGACCGAGCG CAGCGAGTCA
2701 GTGAGCGAGG AAGCGGAAGA GCGCCCAATA CGCAAACCGC CTCTCCCCGC
2751 GCGTTGGCCG ATTCATTAAT GCAGGTTAAC CTGGCTTATC GAAATTAATA
2801 CGACTACTA TAGGGAGACC GGCTCGAGC AGCAAGGAGA TGGCGCCCAA
2851 CAGTCCCCCG GCCACGGGGC CTGCCACCAT ACCCACGCCG AAACAAGCGC
2901 TCATGAGCCC GAAGTGGCGA GCCCGATCTT CCCCATCGGT GATGTCGGCG
2951 ATATAGGCGC CAGCAACCGC ACCTGTGGCG CCGGTGATGC CGGCCACGAT
3001 GCGTCCGGCG TAGAGGATCG ATCCGGGCTT ATCGACTGCA CGGTGCACCA
3051 ATGCTTCTGG CGTCAGGCAG CCATCGGAAG CTGTGGTATG GCTGTGCAGG
3101 TCGTAAATCA CTGCATAATT CGTGTCGCTC AAGGCGCACT CCCGTTCTGG
3151 ATAATGTTTT TTGCGCCGAC ATCATAACGG TTCTGGCAA TATTCTGAAA
3201 TGAGCTGTTG ACAATTAATC ATCGGCTCGT ATAATGTGTG GAATTGTGAG
3251 CGGATAACAA TTTCACACAG GAAACAGAA TCCCAGCTTG GCTGCAGAAC
3301 CATTCCATTC GTTGATCCGG GAGTAACTCA C

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B. PinPoint™ Xa Vectors Restriction Sites

The following restriction enzyme tables were constructed using DNASTAR® sequence analysis software. Please note that we have not verified this information by restriction digestion with each enzyme listed. The location given specifies the 3'-end of the cut DNA (the base to the left of the cut site). For more information on the cut sites of these enzymes or if you identify a discrepancy, please contact your local Promega Branch or Distributor. In the U.S., contact Promega Technical Services at 800-356-9526. Vector sequences are also available on the Internet at www.promega.com/vectors/ and in the GenBank® database (GenBank®/EMBL Accession Numbers U47626, U47627 and U47628 for PinPoint™ Xa-1, Xa-2 and Xa-3 Vectors, respectively).

Table 1. Restriction Enzymes That Cut the PinPoint™ Xa-1 Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
Aat II	2	53, 787	EcoR V	1	418
Acc I	1	53	Ehe I	4	141, 2844, 2958, 2979
Acc65 I	1	410	Fok I	5	70, 624, 1267, 1554, 1735
Afl III	1	2594	Fsp I	1	1483
Alw44 I	4	537, 1034, 2280, 3043	Hind III	1	392
AlwNI	1	2185	Hpa I	1	2777
Asc I	1	110	Kas I	4	139, 2842, 2956, 2977
Ava I	4	250, 370, 428, 2824	Kpn I	1	414
Ava II	3	309, 1342, 1564	Mae I	3	1513, 1848, 2101
BamH I	1	404	Nae I	3	116, 183, 2991
Ban II	3	377, 2909, 2923	Nar I	4	140, 2843, 2957, 2978
Bbe I	4	143, 2846, 2960, 2981	Nde I	1	532
Bgl I	2	148, 1588	NgoM IV	3	114, 181, 2989
Bgl II	1	422	Not I	1	435
Bsa I	4	262, 355, 1640, 2809	Nru I	1	389
BsaO I	5	438, 1188, 1337, 2260, 2684	Nsp I	2	681, 2598
BsaJ I	5	428, 429, 2434, 2856, 2862	PaeR7 I	1	301
BspH I	4	761, 866, 1874, 2901	Ple I	4	1715, 2218, 2703, 2796
BspM I	3	118, 2762, 3087	PpuM I	1	309
BssH II	1	110	Psp5 II	1	309
BssS I	4	202, 730, 1037, 2421	PspA I	1	428
BstO I	5	313, 2435, 2448, 2569, 2781	Pst I	1	3296
BstX I	1	264	Pvu I	1	1337
BstZ I	1	435	Pvu II	1	400
Cla I	1	3018	Rsa I	3	412, 549, 1225
Dra I	3	1128, 1820, 1839	Sac I	1	377
Dra II	3	309, 726, 2867	Sal I	1	52
Drd I	4	49, 298, 623, 2492	Sca I	1	1225
Dsa I	1	2862	SgrA I	2	103, 2980
Eag I	1	435	Sin I	3	309, 1342, 1564
Ear I	2	907, 2711	Sma I	1	430
Ecl/HK I	2	525, 1706	Ssp I	2	901, 3191
Eco47 III	1	2898	Tfi I	3	165, 2620, 2760
Eco52 I	1	435	Tth111 I	1	220
EcoCR I	1	375	Vsp I	5	498, 1531, 2766 2795, 3215
EcoN I	2	205, 316	Xho I	3	250, 370, 2824
EcoR I	1	3277	Xma I	1	428
			Xmn I	1	1106

Note: The enzymes listed in boldface type are available from Promega.

Table 2. Restriction Enzymes That Do Not Cut the PinPoint™ Xa-1 Vector.

AccB7 I	Bcl I	<i>Bst</i> 1107 I	I-Ppo I	<i>Ppu</i> 10 I	<i>Srf</i> I
Acc III	<i>Blp</i> I	Bst98 I	Mlu I	<i>Psh</i> A I	<i>Sse</i> 8387 I
<i>Afl</i> II	<i>Bpu</i> 1102 I	BstE II	Nco I	<i>Rsr</i> II	Stu I
Age I	<i>Bsa</i> A I	Bsu36 I	Nhe I	Sac II	Sty I
Apa I	<i>Bsa</i> B I	Csp I	Nsi I	Sfi I	<i>Swa</i> I
<i>Avr</i> II	BsaM I	Csp45 I	<i>Pac</i> I	Sgf I (f)	Xba I
Bal I	<i>Bsm</i> I	<i>Dra</i> III	<i>Pfi</i> M I	Sna B I	<i>Xcm</i> I
<i>Bbr</i> P I	<i>Bsp</i> 120 I	<i>Eco</i> 72 I	<i>Pin</i> A I	Spe I	
<i>Bbs</i> I	Bsr BR I	<i>Eco</i> 81 I	<i>Pme</i> I	Sph I	
Bbu I	<i>Bsr</i> G I	<i>Fse</i> I	<i>Pml</i> I	<i>Spl</i> I	

Table 3. Restriction Enzymes That Cut the PinPoint™ Xa-1 Vector 6 or More Times.

<i>Aci</i> I	<i>Bsr</i> I	<i>Eae</i> I	Hpa II	<i>Mse</i> I	<i>Scr</i> F I
Acy I	BsrS I	<i>Fnu</i> 4H I	<i>Hph</i> I	Msp I	<i>Sfa</i> N I
Alu I	Bst71 I	Hae II	Hsp92 I	MspA1 I	Taq I
Alw26 I	<i>Bst</i> U I	Hae III	Hsp92 II	Nci I	Tru 9 I
<i>Asp</i> H I	Cfo I	<i>Hga</i> I	<i>Mae</i> II	Nde II	Xho II
Ban I	<i>Cfr</i> 10 I	Hha I	<i>Mae</i> III	<i>Nla</i> III	
<i>Bbv</i> I	Dde I	Hinc II	Mbo I	<i>Nla</i> IV	
<i>Bsa</i> H I	Dpn I	<i>Hind</i> II	Mbo II	Sau 3A I	
Bsp1286 I	<i>Dpn</i> II	Hinf I	<i>Mnl</i> I	Sau 96 I	

Table 4. Restriction Enzymes That Cut the PinPoint™ Xa-2 Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
Aat II	2	53, 788	<i>Bsp</i> M I	3	118, 2763, 3088
Acc I	1	53	BssH II	1	110
Acc65 I	1	411	<i>Bss</i> S I	4	202, 731, 1038, 2422
<i>Afl</i> III	1	2595	BstO I	5	313, 2436, 2449, 2570, 2782
Alw44 I	4	538, 1035, 2281, 3044	BstX I	1	264
<i>Alw</i> N I	1	2186	BstZ I	1	436
<i>Asc</i> I	1	110	Cla I	1	3019
Ava I	4	250, 370, 429, 2825	Dra I	3	1129, 1821, 1840
Ava II	3	309, 1343, 1565	<i>Dra</i> II	3	309, 727, 2868
BamH I	1	405	<i>Drd</i> I	4	49, 298, 624, 2493
Ban II	3	377, 2910, 2924	<i>Dsa</i> I	1	2863
<i>Bbe</i> I	4	143, 2847, 2961, 2982	<i>Eag</i> I	1	436
Bgl I	2	148, 1589	<i>Ear</i> I	2	908, 2712
Bgl II	1	423	Ecl HK I	2	526, 1707
<i>Bsa</i> I	4	262, 355, 1641, 2810	Eco 47 III	1	2899
BsaO I	5	439, 1189, 1338, 2261, 2685	Eco 52 I	1	436
<i>Bsa</i> J I	5	429, 430, 2435, 2857, 2863	Eco CR I	1	375
<i>Bsp</i> H I	4	762, 867, 1875, 2902	<i>Eco</i> N I	2	205, 316
			Eco R I	1	3278
			Eco R V	1	419
			<i>Ehe</i> I	4	141, 2845, 2959, 2980

Note: The enzymes listed in boldface type are available from Promega.

Table 4. Restriction Enzymes That Cut the PinPoint™ Xa-2 Vector Between 1 and 5 Times (continued).

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
Fok I	5	70, 625, 1268, 1555, 1736	<i>Psp5</i> II	1	309
<i>Fsp</i> I	1	1484	<i>PspA</i> I	1	429
Hind III	1	393	Pst I	1	3297
Hpa I	1	2778	Pvu I	1	1338
<i>Kas</i> I	4	139, 2843, 2957, 2978	Pvu II	1	401
Kpn I	1	415	Rsa I	3	413, 550, 1226
<i>Mae</i> I	3	1514, 1849, 2102	Sac I	1	377
Nae I	3	116, 183, 2992	Sal I	1	52
Nar I	4	140, 2844, 2958, 2979	Sca I	1	1226
Nde I	1	533	<i>SgrA</i> I	2	103, 2981
NgoM IV	3	114, 181, 2990	Sin I	3	309, 1343, 1565
Not I	1	436	Sma I	1	431
Nru I	1	389	Ssp I	2	902, 3192
<i>Nsp</i> I	2	682, 2599	<i>Tfi</i> I	3	165, 2621, 2761
<i>PaeR7</i> I	1	301	Tth111 I	1	220
<i>Ple</i> I	4	1716, 2219, 2704, 2797	Vsp I	5	499, 1532, 2767 2796, 3216
<i>PpuM</i> I	1	309	Xho I	3	250, 370, 2825
			Xma I	1	429
			Xmn I	1	1107

Table 5. Restriction Enzymes That Do Not Cut the PinPoint™ Xa-2 Vector.

AccB7 I	Bcl I	<i>Bst1107</i> I	I-Ppo I	<i>Ppu10</i> I	<i>Srf</i> I
Acc III	<i>Blp</i> I	Bst98 I	<i>Mlu</i> I	<i>PshA</i> I	<i>Sse8387</i> I
<i>Afl</i> II	<i>Bpu1102</i> I	BstE II	Nco I	<i>Rsr</i> II	Stu I
Age I	<i>BsaA</i> I	Bsu36 I	Nhe I	Sac II	Sty I
Apa I	<i>BsaB</i> I	Csp I	Nsi I	Sfi I	<i>Swa</i> I
<i>Avr</i> II	BsaM I	Csp45 I	<i>Pac</i> I	Sgf I	Xba I
Bal I	<i>Bsm</i> I	<i>Dra</i> III	<i>PfiM</i> I	SnaB I	<i>Xcm</i> I
<i>BbrP</i> I	<i>Bsp120</i> I	<i>Eco72</i> I	<i>PinA</i> I	Spe I	
<i>Bbs</i> I	BsrBR I	<i>Eco81</i> I	<i>Pme</i> I	Sph I	
Bbu I	<i>BsrG</i> I	<i>Fse</i> I	<i>Pml</i> I	<i>Spl</i> I	

Table 6. Restriction Enzymes That Cut the PinPoint™ Xa-2 Vector 6 or More Times.

<i>Aci</i> I	<i>Bsr</i> I	<i>Eae</i> I	Hpa II	<i>Mse</i> I	<i>ScrF</i> I
Acy I	BsrS I	<i>Fnu4H</i> I	<i>Hph</i> I	Msp I	<i>SfaN</i> I
Alu I	Bst71 I	Hae II	Hsp92 I	MspA1 I	Taq I
Alw26 I	<i>BstU</i> I	Hae III	Hsp92 II	Nci I	Tru9 I
<i>AspH</i> I	Cfo I	<i>Hga</i> I	<i>Mae</i> II	Nde II	Xho II
Ban I	<i>Cfr10</i> I	Hha I	<i>Mae</i> III	<i>Nla</i> III	
<i>Bbv</i> I	Dde I	Hinc II	Mbo I	<i>Nla</i> IV	
<i>BsaH</i> I	Dpn I	<i>Hind</i> II	Mbo II	Sau3A I	
Bsp1286 I	<i>Dpn</i> II	Hinf I	<i>Mnl</i> I	Sau96 I	

Note: The enzymes listed in boldface type are available from Promega.

Table 7. Restriction Enzymes That Cut the PinPoint™ Xa-3 Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location	Enzyme	# of Sites	Location
Aaf II	2	53, 789	EcoR V	1	420
Acc I	1	53	Ehe I	4	141, 2846, 2960, 2981
Acc65 I	1	412	Fok I	5	70, 626, 1269, 1556, 1737
Afl III	1	2596	Fsp I	1	1485
Alw44 I	4	539, 1036, 2282, 3045	Hind III	1	394
AlwN I	1	2187	Hpa I	1	2779
Asc I	1	110	Kas I	4	139, 2844, 2958, 2979
Ava I	4	250, 370, 430, 2826	Kpn I	1	416
Ava II	3	309, 1344, 1566	Mae I	3	1515, 1850, 2103
BamH I	1	406	Nae I	3	116, 183, 2993
Ban II	3	377, 2911, 2925	Nar I	4	140, 2845, 2959, 2980
Bbe I	4	143, 2848, 2962, 2983	Nde I	1	534
Bgl I	2	148, 1590	NgoM IV	3	114, 181, 2991
Bgl II	1	424	Not I	1	437
Bsa I	4	262, 355, 1642, 2811	Nru I	1	389
BsaO I	5	440, 1190, 1339, 2262, 2686	Nsp I	2	683, 2600
BsaJ I	5	430, 431, 2436, 2858, 2864	PaeR7 I	1	301
BspH I	4	763, 868, 1876, 2903	Ple I	4	1717, 2220, 2705, 2798
BspM I	3	118, 2764, 3089	PpuM I	1	309
BssH II	1	110	Psp5 II	1	309
BssS I	4	202, 732, 1039, 2423	PspA I	1	430
BstO I	5	313, 2437, 2450, 2567, 2783	Pst I	1	3298
BstX I	1	264	Pvu I	1	1339
BstZ I	1	437	Pvu II	1	402
Cla I	1	3020	Rsa I	3	414, 551, 1227
Dra I	3	1130, 1822, 1841	Sac I	1	377
Dra II	3	309, 728, 2869	Sal I	1	52
Drd I	4	49, 298, 625, 2494	Sca I	1	1227
Dsa I	1	2864	SgrA I	2	103, 2982
Eag I	1	437	Sin I	3	309, 1344, 1566
Ear I	2	909, 2713	Sma I	1	432
EclHK I	2	527, 1708	Ssp I	2	903, 3193
Eco47 III	1	2900	Tfi I	3	165, 2622, 2762
Eco52 I	1	437	Tth111 I	1	220
EcoCR I	1	375	Vsp I	5	500, 1533, 2768, 2797, 3217
EcoN I	2	205, 316	Xho I	3	250, 370, 2826
EcoR I	1	3279	Xma I	1	430
			Xmn I	2	395, 1108

Note: The enzymes listed in boldface type are available from Promega.

Table 8. Restriction Enzymes That Do Not Cut the PinPoint™ Xa-3 Vector.

AccB7 I	Bcl I	<i>Bst1107 I</i>	I-Ppo I	<i>Ppu10 I</i>	<i>Srf I</i>
Acc III	<i>Blp I</i>	Bst98 I	Mlu I	<i>PshA I</i>	<i>Sse8387 I</i>
<i>Afl II</i>	<i>Bpu1102 I</i>	BstE II	Nco I	<i>Rsr II</i>	Stu I
Age I	<i>BsaA I</i>	Bsu36 I	Nhe I	Sac II	Sty I
Apa I	<i>BsaB I</i>	Csp I	Nsi I	Sfi I	<i>Swa I</i>
<i>Avr II</i>	BsaM I	Csp45 I	<i>Pac I</i>	Sgf I	Xba I
Bal I	<i>Bsm I</i>	<i>Dra III</i>	<i>PflM I</i>	SnaB I	<i>Xcm I</i>
<i>BbrP I</i>	<i>Bsp120 I</i>	<i>Eco72 I</i>	<i>PinA I</i>	Spe I	
<i>Bbs I</i>	BsrBR I	<i>Eco81 I</i>	<i>Pme I</i>	Sph I	
Bbu I	<i>BsrG I</i>	<i>Fse I</i>	<i>Pml I</i>	<i>Spl I</i>	

Table 9. Restriction Enzymes That Cut the PinPoint™ Xa-3 Vector 6 or More Times.

<i>Aci I</i>	<i>Bsr I</i>	<i>Eae I</i>	Hpa II	<i>Mse I</i>	<i>ScrF I</i>
Acy I	Bsr S I	<i>Fnu4H I</i>	<i>Hph I</i>	Msp I	<i>SfaN I</i>
Alu I	Bst71 I	Hae II	Hsp92 I	MspA1 I	Taq I
Alw26 I	<i>BstU I</i>	Hae III	Hsp92 II	Nci I	Tru9 I
<i>AspH I</i>	Cfo I	<i>Hga I</i>	<i>Mae II</i>	Nde II	Xho II
Ban I	<i>Cfr10 I</i>	Hha I	<i>Mae III</i>	<i>Nla III</i>	
<i>Bbv I</i>	Dde I	Hinc II	Mbo I	<i>Nla IV</i>	
<i>BsaH I</i>	Dpn I	<i>Hind II</i>	Mbo II	Sau3A I	
Bsp1286 I	<i>Dpn II</i>	Hinf I	<i>Mnl I</i>	Sau96 I	

Note: The enzymes listed in boldface type are available from Promega.

XII. Appendix C: Reference Information

A. Composition of Buffers and Solutions

alkaline phosphatase buffer

100mM NaCl
 5mM MgCl₂
 100mM Tris-HCl (pH 9.5)

ampicillin stock solution

Dissolve ampicillin in deionized water (100mg/ml). Filter-sterilize (0.2µm filter) and store at -20°C.

BCIP stock solution

Dissolve 0.5g of 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) in 10ml of 100% dimethylformamide. Store at 4°C.

cell lysis buffer

50mM Tris-HCl (pH 7.5)
 50mM NaCl
 5% glycerol

Factor Xa 10X reaction buffer

200mM Tris-HCl (pH 7.4)
 1M NaCl

IPTG, 100mM

1.2g isopropyl β-D-thiogalactopyranoside (IPTG) (Cat.# V3951)

Add deionized water to 50ml final volume. Filter-sterilize (0.2µm filter) and store at 4°C.

LB (Luria-Bertani) medium

10g Bacto®-tryptone
 5g Bacto®-yeast extract
 5g NaCl

Add deionized water to 1L. Adjust pH to 7.5 with NaOH and autoclave.

A. Composition of Buffers and Solutions (continued)

lysozyme (prepare fresh)

5mg chicken egg white lysozyme
(Sigma Grade VI, Cat.# L2879)
1ml TEN buffer (see below)

Prepare fresh immediately before use.

TEN buffer

40mM Tris-HCl (pH7.5)
1mM EDTA (pH8.0)
150mM NaCl

NBT stock solution

Dissolve 0.5g of nitro blue tetrazolium chloride (NBT) in 10ml of 70% dimethylformamide. Store at 4°C.

NBT/BCIP solution (prepare fresh)

10ml alkaline phosphatase buffer
66µl NBT stock solution
33µl BCIP stock solution

Combine the NBT stock solution and alkaline phosphatase buffer. Mix well, and add the BCIP stock solution. Use within one hour.

PMSF

Dissolve 1.74mg of phenylmethylsulfonyl fluoride (PMSF) per milliliter of isopropanol (10mM). Aliquot and store at -20°C. A stock concentration of 100mM may be prepared if needed.

B. Related Products

Systems

Product	Size	Cat.#
PinPoint™ Xa-1 T-Vector System I(a,b,g)	20 reactions	V2610
PinPoint™ Xa-1 T-Vector System II plus Competent Cells(a,b,g)	20 reactions	V2850

Items Available Separately

Product	Size	Cat.#
IPTG, Dioxane-Free	1g	V3955
PinPoint™ Xa-1 Vector(b,d)	10µg	V2031
PinPoint™ Xa-2 Vector(b,d)	10µg	V2051
PinPoint™ Xa-3 Vector(b,d)	10µg	V2061
PinPoint™ Xa Control Vector(b,d)	5µg	V2041
PinPoint™ Vector Sequencing Primer(d)	2µg	V4211

sample 1X buffer

25% stacking gel 4X buffer
2% SDS
5% β-mercaptoethanol
20% glycerol
0.0025% bromophenol blue

stacking gel 4X buffer

6.06g Tris base
4ml 10% SDS

Adjust to pH 6.8 with 12N HCl, and add water to a 100ml final volume. Store at room temperature.

sodium deoxycholate (DOC)

Prepare a 10% (v/v) stock solution in cell lysis buffer.

TBST buffer

10mM Tris-HCl (pH 8.0)
150mM NaCl
0.05% Tween® 20

B. Related Products (continued)

Product	Size	Cat.#
SoftLink™ Soft Release Avidin Resin ^(c)	1ml	V2011
	5ml	V2012
TetraLink™ Tetrameric Avidin Resin ^(e)	1ml	V2591
	5ml	V2592
Streptavidin Alkaline Phosphatase	0.5ml	V5591
Western Blue® Stabilized Substrate for Alkaline Phosphatase	100ml	S3841
BCIP/NBT Color Development Substrate	1.25ml/2.50ml	S3771
Sequencing Grade Modified Trypsin	100µg	V5111
Factor Xa Protease	50µg	V5581

(a)For research purposes only. Not for diagnostic or therapeutic use. For nonresearch uses of the portion of the vector encoding the biotinylation sequence, please contact Promega Corporation for licensing information. For bulk purchases of the SoftLink™ Resin, contact TosoHaas, 156 Keystone Drive, Montgomeryville, PA 18936, 1-800-366-4875 or 215-283-5000.

(b)U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different bacteriophage RNA polymerase promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

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(f)U.S. Pat. No. 5,391,487 has been issued to Promega Corporation for Restriction Endonuclease *Sgf I*.

(g)Licensed under one or both of U.S. Pat. No. 5,487,993 and European Pat. No. 0 550 693.

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PinPoint™ Xa Protein Purification System: *Experienced User's Protocol*

This quick protocol is intended as an easy-to-follow reminder for experienced users. Please follow the complete protocol (Sections IV through VII) the first time you use the PinPoint™ Xa Protein Purification System.

<p>Small-Scale Culture and Induction (Section IV.A)</p>	<ol style="list-style-type: none"> 1. Inoculate 1–5ml of LB containing 2µM biotin and ampicillin (100µg/ml) with a freshly isolated bacterial colony carrying either the PinPoint™ Xa Vector with the desired insert DNA, the Control Plasmid, or no plasmid. Omit ampicillin for the culture lacking plasmid. Incubate the cultures overnight at 37°C with shaking. 2. Dilute the overnight cultures 1:100 in 25–50ml of fresh LB containing biotin and ampicillin in a 250ml flask. Again, omit ampicillin in cultures lacking plasmid. Incubate an additional hour at 37°C with shaking. 3. Induce protein expression by adding IPTG (100µM final concentration) to all cultures. Incubate 4–5 hours at 37°C with shaking.
<p>Detection of the Fusion Protein (Section IV.B)</p>	<ol style="list-style-type: none"> 1. Transfer 100µl of each culture to separate 1.5ml microcentrifuge tubes. Centrifuge for 5 minutes. Remove the supernatant. 2. Add 50µl of sample 1X buffer to each tube and vortex to resuspend cells. Heat the resuspended cells at 95°C for 5 minutes with occasional vortexing. This treatment will lyse the cells and coat the proteins with SDS. 3. Load 5µl of the heat-treated samples on a 12% SDS polyacrylamide minigel along with molecular weight markers in the flanking lanes. 4. Following electrophoresis, transfer the proteins by electroblotting onto a membrane (such as Immobilon® PVDF or nitrocellulose). 5. Stain the protein blot with Ponceau S solution for 30 seconds and destain with water for one minute. Indicate the location of the molecular weight markers with a pencil. Alternatively, separate duplicate samples by SDS-PAGE (as in Step 3), transfer to a membrane, excise and stain with Amido Black for nitrocellulose membranes or with Coomassie® Blue for PVDF membranes. 6. Locate the biotinylated protein bands in the lanes containing the cell lysates. <ol style="list-style-type: none"> a. Incubate the membrane in TBST buffer for 30–60 minutes at room temperature with gentle agitation to block protein binding sites. Pour off the solution. b. Combine 3µl of the Streptavidin-Alkaline Phosphatase and 15ml of TBST buffer. Place the membrane in this solution and incubate for 30 minutes at room temperature with gentle agitation. Pour off the solution. c. Wash the membrane for 5 minutes with TBST buffer. Repeat this wash two more times and rinse briefly with deionized water. Pour off the water. d. Add freshly prepared NBT/BCIP solution. Incubate at room temperature with gentle agitation until bands appear. Dark purple bands will indicate the location of the biotinylated protein species in the lanes containing cellular extracts.
<p>Large-Scale Culture and Induction (Section IV.C)</p>	<ol style="list-style-type: none"> 1. Grow the desired volume of culture in LB containing biotin (2µM) and ampicillin (100µg/ml). 2. Harvest the cells by centrifugation at 8,000 × g for 10 minutes. Immediately proceed with cell lysis or freeze at –20°C for later use.

**PinPoint™ Xa Protein Purification System:
Experienced User's Protocol (continued)**

Cell Lysis by Sonication (Section V.A)	<ol style="list-style-type: none"> Resuspend the cells on ice by stirring in 10ml of cell lysis buffer per gram of cell paste. Transfer the cell suspension to a container that is as wide as the cell suspension is deep. Place on ice. Sonicate the suspension on ice using 15 second pulses. Remove the cellular debris by centrifugation at 10,000 × g for 15 minutes at 4°C. Proceed with protein purification. 																									
Fusion Protein Purification (Section VI)	<p>Regeneration of SoftLink™ Resin</p> <ol style="list-style-type: none"> Prepare the resin as described in Section VI.A before using the resin for the first time. Wash the resin with eight resin-bed volumes of 10% acetic acid. Wash the resin with eight resin-bed volumes of 100mM phosphate buffer (pH 7.0). Once the pH of the wash buffer reaches pH 6.8, wait at least 30 minutes to allow the avidin to refold. The resin can be stored in 20% ethanol at 4°C for up to six months. <p>Batch Capture (see Section VI.B for Column Capture protocol)</p> <ol style="list-style-type: none"> Equilibrate the SoftLink™ resin in a buffer that is compatible with the biotinylated molecule being purified. Mix cell lysate and resin at a ratio of 3ml of resin per liter of starting culture. Gently rock the mixture for 1–4 hours at 4°C. Aspirate the cell lysate from the resin. Add 10 volumes of buffer per 1ml of resin and rock gently for 10 minutes at 4°C. Allow resin to settle, then carefully aspirate the buffer. Repeat the wash at least once. Elute the purified protein by adding stabilizing buffer containing 5mM biotin to the resin at a ratio of 2:1 (v/v) biotin solution to resin. Gently rock for at least one hour at 4°C. Allow the resin to settle and remove the biotin solution containing the released protein. 																									
Cleaving the Fusion Protein (Section VII)	<ol style="list-style-type: none"> Prepare the following digestion reactions: <table border="1" data-bbox="386 1312 1253 1480" style="margin-left: 20px;"> <thead> <tr> <th></th> <th>Tube 1</th> <th>Tube 2</th> <th>Tube 3</th> <th>Tube 4</th> </tr> </thead> <tbody> <tr> <td>Factor Xa 10X reaction buffer</td> <td>10µl</td> <td>10µl</td> <td>10µl</td> <td>10µl</td> </tr> <tr> <td>diluted Factor Xa Protease</td> <td>—</td> <td>2.5µl</td> <td>6µl</td> <td>12µl</td> </tr> <tr> <td>target protein (≥0.5µg/µl)</td> <td><u>25µg</u></td> <td><u>25µg</u></td> <td><u>25µg</u></td> <td><u>25µg</u></td> </tr> <tr> <td>sterile, deionized water to</td> <td>100µl</td> <td>100µl</td> <td>100µl</td> <td>100µl</td> </tr> </tbody> </table> Incubate the samples at room temperature or at 37°C. Remove a 20µl aliquot and a 10µl aliquot at one hour, three hours and after an overnight incubation. Store these samples at –20°C. Stop the reaction in the 20µl aliquots by drying the samples under vacuum. Analyze these samples by SDS-PAGE for complete cleavage. Determine the incubation time that allowed complete or nearly complete digestion of the fusion protein(s). Use the saved 10µl sample from the appropriate time point and determine whether the incubation time affected the activity of the protein. If the activity is still acceptable, use this incubation time and appropriate amount of protease in larger digestion reactions. 		Tube 1	Tube 2	Tube 3	Tube 4	Factor Xa 10X reaction buffer	10µl	10µl	10µl	10µl	diluted Factor Xa Protease	—	2.5µl	6µl	12µl	target protein (≥0.5µg/µl)	<u>25µg</u>	<u>25µg</u>	<u>25µg</u>	<u>25µg</u>	sterile, deionized water to	100µl	100µl	100µl	100µl
	Tube 1	Tube 2	Tube 3	Tube 4																						
Factor Xa 10X reaction buffer	10µl	10µl	10µl	10µl																						
diluted Factor Xa Protease	—	2.5µl	6µl	12µl																						
target protein (≥0.5µg/µl)	<u>25µg</u>	<u>25µg</u>	<u>25µg</u>	<u>25µg</u>																						
sterile, deionized water to	100µl	100µl	100µl	100µl																						