TECHNICAL MANUAL

PinPoint™ Xa Protein Purification System

Instructions for Use of Product
V2020

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TM028

PinPoint™ Xa Protein Purification System

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1. Description

The PinPoint™ Xa Protein Purification System 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. This proprietary resin allows elution of the fusion protein under nondenaturing conditions. The PinPoint™ Vectors feature the encoded endoproteinase 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 diagram in Figure 1 outlines the expression and purification system procedure.

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

2. Product Components and Storage Conditions

PRODUCT	CAT.#
PinPoint™ Xa Protein Purification System	**V2020**

Includes:

- 10µg PinPoint™ Xa-1 Vector
- 10µg PinPoint™ Xa-2 Vector
- 10µg PinPoint™ Xa-3 Vector
- 5µg PinPoint™ Xa Control Vector
- 3ml SoftLink™ Soft Release Avidin Resin
- 20µl Streptavidin-Alkaline Phosphatase
- 1 PinPoint™ Purification Column
- 1ml Biotin, 100mM (pH 7.2)

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.

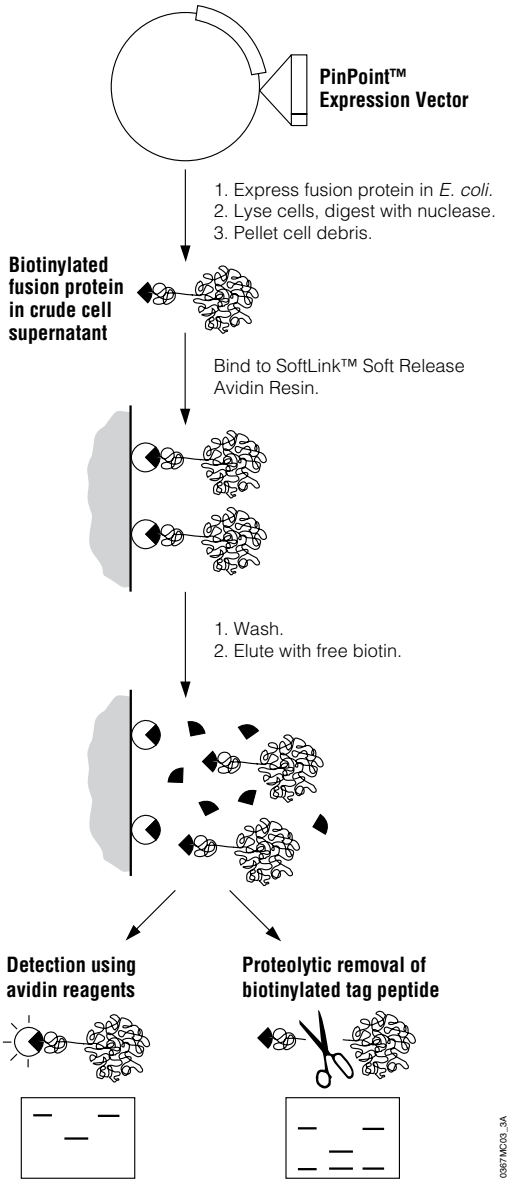

Figure 1. Schematic diagram of recombinant protein expression and purification using the PinPoint™ Xa Protein Purification System.

3. General Considerations

The PinPoint™ Xa Protein Purification System exploits the well understood interaction of biotin with avidin to purify 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, the SoftLink™ Soft Release Avidin Resin that is supplied with this system allows the protein to be eluted in a nondenaturing 5mM biotin solution. The rate of dissociation of the monomeric avidin-biotin complex is sufficiently fast to effectively allow 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 PinPoint™ Xa Protein Purification System provides three vectors in each possible reading frame with multiple cloning regions downstream of the encoded biotinylated target sequence to facilitate creating an in-frame fusion protein. The PinPoint™ Xa Vectors also contain an NruI 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 NruI restriction site that marks the 5´-end of the multiple cloning region.

Insertion of the desired gene into the NruI 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 7.

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.

3. General Considerations (continued)

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.

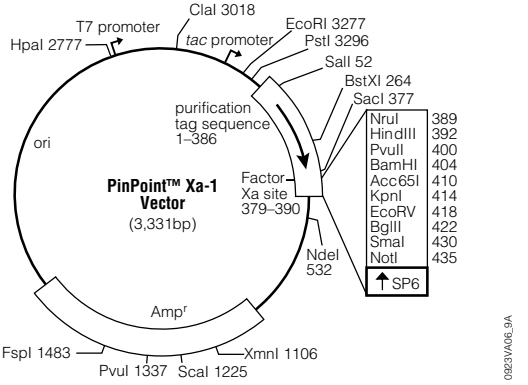

Figure 3. The PinPoint™ Xa-1 Vector circle map and sequence reference points. Base #1 is the translation start site for the purification tag sequence. The three PinPoint™ Xa Vectors below are identical except for an additional coding-strand adenosine at position 394 in the PinPoint™ Xa-2 Vector, and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

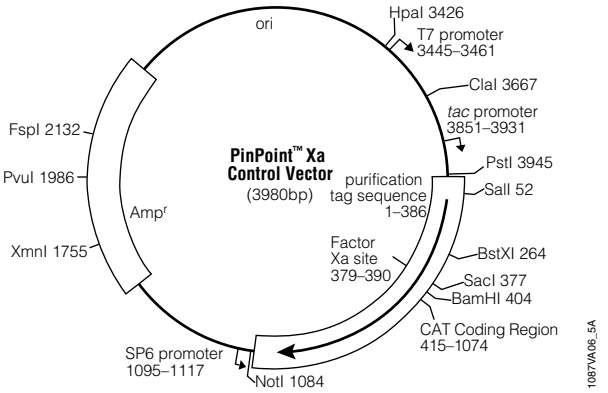

Figure 4. PinPoint™ Xa Control Vector circle map and reference points. Base #1 is the translation start site for the purification tag sequence.

Sequence reference points:	
tac 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 (Amp^r) coding region	1568–2427

4. 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 12.A.)

- ampicillin stock solution
- IPTG, 100mM
- LB medium
- NBT/BCIP (or 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

4.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 *bir*A 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 4.B.

4.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.

   Notes:

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

   Frequently, prestained markers are used since they require no subsequent staining for visual detection. If you do not use prestained markers, load an amount of molecular weight markers to acheive >1µg of protein per band. Visualize the markers using Ponceau S staining (Steps 5 and 6).
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.

   Note: Do not use sequencing membranes such as Immobilon™ Psq, which can generate high background.
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.
   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.

4.B. Detection of Fusion Protein (continued)

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 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 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.

4.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 4.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 × *g* for 10 minutes. Immediately proceed with purification of the protein (Sections 5 and 6) or freeze the cells at –20°C for later use.

5. Cell Lysis

We recommend sonication for recovering the fusion protein from culture. Alternative methods include lysis by enzyme/detergent or French press. Section 5.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.

5.A. Sonication

Materials to Be Supplied by the User
(Solution compositions are provided in Section 12.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.

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

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 before 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 6.

5.B. Lysozyme and Detergent Alternative Lysis Protocol

An alternative protocol for protein recovery by cell lysis involving lysozyme and detergent solubilization follows. This procedure may be used in place of sonication (Section 5.A) or other methods of cell lysis.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 12.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.

2. Add lysozyme to a final concentration of 1mg/ml. Stir the solution at 4°C for 20 minutes.
3. Add sodium deoxycholate 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 6.

6. 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. Cell Lysis Buffer may be used for washing the resin, and Cell Lysis Buffer containing 5mM biotin may be used for elution, if a stabilizing buffer is not available for the protein of interest.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 12.A.)

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

6.A. Preparation and Regeneration of SoftLink™ Resin

Preparation (Preadsorption of SoftLink™ Resin with Biotin): Perform at 4°C or room temperature.

Note: Preparation and regeneration must be performed before using the SoftLink™ Resin for the first time.

1. Pour a column with appropriate column volume.
2. Equilibrate the column in 0.1M $NaPO_4$ (pH 7.0).
3. Preadsorb nonreversible binding sites on fresh SoftLink™ Resin by washing with 5mM biotin in the phosphate equilibration buffer. Wash with a flow rate of 6ml biotin/hour/ml of SoftLink™ Resin until two column volumes have passed through.
4. Stop the flow for 15 minutes to allow biotin binding.
5. Regenerate the column before attempting to purify biotinylated molecules.

Regeneration: Perform at 4°C or room temperature.

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

1. Wash the column with 8 column volumes of 10% acetic acid.
2. Wash the column with 8 column volumes of 100mM $NaPO_4$ (pH 7.0).
3. Monitor pH of eluate until it reaches pH 6.8. Stop the flow for a minimum of 30 minutes to allow the avidin to refold.
4. Equilibrate the column in a buffer compatible with the biotinylated molecule to be purified.

   Suggested Equilibration Buffers: 50mM Tris-HCl (pH 8.0) or 50mM Tris-phosphate (pH 7.8) with or without one or more of the following additives: 50–200mM NaCl, 4mM dithiothreitol, 2mM EDTA, 10% glycerol or 0.1% Triton® X-100.
5. Store the column in 20% ethanol for up to six months without loss of binding capacity.

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.

6.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.

   Note: If desired, the resin can be regenerated directly in 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 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 A_{280}. Alternatively, detect biotinylated protein by using the Streptavidin-Alkaline Phosphatase assay (see Section 4.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.

6.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.

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

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.

   Note: Better results are obtained with an overnight elution.

5. Allow the resin to settle. Transfer the biotin solution, which contains the released fusion protein, to a clean tube.

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

7. 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 12.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. Prior to digestion, dilute the Factor Xa Protease (Cat.# V5581) 1:10 in Tris-HCl, 20mM (pH 8.0), on ice.
2. 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.

7. Cleaving the Fusion Protein (continued)

3. 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**

4. Incubate the tubes at room temperature or at 37°C, if desired. Remove a 20µl sample at 1 hour, at 3 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.

5. 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.

6. 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.

7. 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 (Step 4) 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:

1. If excessive degradation is apparent in the cleavage reaction with no added protease (Tube 1), cellular protease(s) is copurifying 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 protein purification may inhibit these proteases.
2. 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 5). Analyze the samples as described in Step 6.

8. Troubleshooting

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**

Symptoms	Causes andComments
Expressed protein is insoluble	The properties of the experimental protein may contribute to solubility problems. 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 at 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 *E. coli* host strain that is deficient in proteases (e.g., BL21).

8. Troubleshooting (continued)

Symptoms	Causes and Comments
Low protein yield	Not all of the protein was captured. The lysate may have been contaiminated with biotin.
	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 SoftLink™ Resin.
	Expression is low. Check expression on gel. Some fusions are toxic; therefore, optimize induction parameters.

9. References

1. Wilson, K.P. *et al.* (1992) *Escherichia coli* biotin holoenzyme synthetase/bio repressor crystal structure delineates the biotin- and DNA-binding domains. *Proc. Natl. Acad. Sci. USA* **89**, 9257–61.
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10. Appendix A: General Information for Constructing Fusion Proteins

10.A. Vectors and Cloning Considerations

General guidelines for cloning DNA inserts into plasmid vectors are described in the *Protocols and Applications Guide* (3). Specific considerations for creating such constructs are 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 4.A).

10.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 BamHI restriction site that translates as follows:

Encoded Amino Acids	Gly	Ile	Arg
DNA Sequence	G**GG**	**ATC**	**C**GG
		BamHI	

If a BamHI, BclI or BglII 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.

10.B. Engineering Fusion Proteins (continued)

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 12.B).

10.C. Constructs Containing a Proteolytic Cleavage Site

The PinPoint™ Xa Vectors contain a unique NruI 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 NruI 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 (A, G, T, or C) before the first codon of the protein of interest. This can be accomplished by taking advantage of a naturally occurring restriction site (or an artificially incorporated one) spanning the first amino acid of the protein, or by use of a DNA linker placed between the fusion tag and the gene coding region such that the first amino acid following the cleavage site is the first amino acid found in the protein of interest. Table 1 provides the recognition sequences of some common proteases that may be considered for processing the fusion proteins, and Figure 5 illustrates a typical engineering strategy.

Table 1. Common Proteases and Their Cleavage Recognition Sites.

Protease	Recognition Site
Endoproteinase Factor Xa	Ile Glu Gly Arg↓
Enterokinase	Asp Asp Asp Asp Lys↓
Endoproteinase Arg-C	Arg↓
Trypsin	Arg↓ or Lys↓

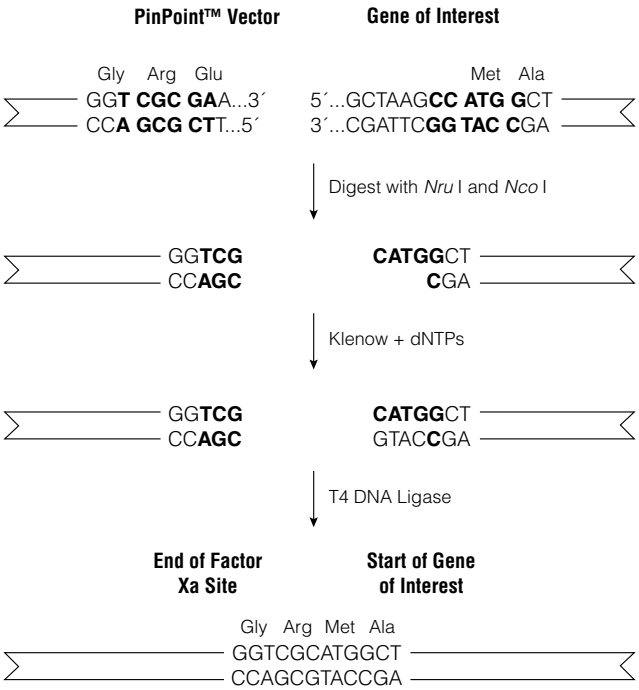

Figure 5. Engineering strategy to produce PinPoint™ Vector fusion constructs.

11. Appendix B: PinPoint™ Xa Vector Sequence Accession Numbers and Restriction Enzyme 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 available in the GenBank® database (GenBank®/EMBL Accession Numbers U47626, U47627 and U47628 for PinPoint™ Xa-1, Xa-2 and Xa-3 Vectors, respectively) and on the Internet at: **www.promega.com/vectors/**

11. Appendix B: PinPoint™ Xa Vector Sequence Accession Numbers and Restriction Enzyme Sites (continued)

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

Enzyme	# of Sites	Location
AatII	2	53, 787
AccI	1	53
Acc65I	1	410
AflIII	1	2594
Alw44I	4	537, 1034, 2280, 3043
AlwNI	1	2185
AscI	1	110
AvaI	4	250, 370, 428, 2824
AvaII	3	309, 1342, 1564
BamHI	1	404
BanII	3	377, 2909, 2923
BbeI	4	143, 2846, 2960, 2981
BglI	2	148, 1588
BglII	1	422
BsaI	4	262, 355, 1640, 2809
BsaOI	5	438, 1188, 1337, 2260, 2684
BsaJI	5	428, 429, 2434, 2856, 2862
BspHI	4	761, 866, 1874, 2901
BspMI	3	118, 2762, 3087
BssHII	1	110
BssSI	4	202, 730, 1037, 2421
BstOI	5	313, 2435, 2448, 2569, 2781
BstXI	1	264
BstZI	1	435
ClaI	1	3018
DraI	3	1128, 1820, 1839
DraII	3	309, 726, 2867
DrdI	4	49, 298, 623, 2492
DsaI	1	2862
EagI	1	435
EarI	2	907, 2711
EclHKI	2	525, 1706
Eco47III	1	2898
Eco52I	1	435
EcoICRI	1	375
EcoNI	2	205, 316
EcoRI	1	3277
EcoRV	1	418
EheI	4	141, 2844, 2958, 2979
FokI	5	70, 624, 1267, 1554, 1735
FspI	1	1483
HindIII	1	392
HpaI	1	2777
KasI	4	139, 2842, 2956, 2977
KpnI	1	414
MaeI	3	1513, 1848, 2101
NaeI	3	116, 183, 2991
NarI	4	140, 2843, 2957, 2978
NdeI	1	532
NgoMIV	3	114, 181, 2989
NotI	1	435
NruI	1	389
NspI	2	681, 2598
PaeR7I	3	250, 370, 2824
PleI	4	1715, 2218, 2703, 2796
PpuMI	1	309
Psp5II	1	309
PspAI	1	428
PstI	1	3296
PvuI	1	1337
PvuII	1	400
RsaI	3	412, 549, 1225
SacI	1	377
SalI	1	52
ScaI	1	1225
SgrAI	2	103, 2980
SinI	3	309, 1342, 1564
SmaI	1	430
SspI	2	901, 3191
TfiI	3	165, 2620, 2760
Tth111I	1	220
VspI	5	498, 1531, 2766 2795, 3215
XhoI	3	250, 370, 2824
XmaI	1	428
XmnI	1	1106

Table 3. Restriction Enzymes That Do Not Cut the PinPoint™ Xa-1 Vector.

AccB7I	Bpu1102I	Csp45I	PinAI	SplI
AccIII	BsaAI	DraIII	PmeI	SrfI
AflII	BsaBI	Eco72I	PmlI	Sse8387I
AgeI	BsaMI	Eco81I	Ppu10I	**StuI**
ApaI	BsmI	FseI	PshAI	StyI
AvrII	Bsp120I	**I-PpoI**	RsrII	SwaI
BalI	BsrGI	**MluI**	**SacII**	**XbaI**
BbrPI	Bst1107I	**NcoI**	SfiI	XcmI
BbsI	Bst98I	**NheI**	**SgfI**	
BbuI	**BstEII**	**NsiI**	**SnaBI**	
BclI	Bsu36I	PacI	**SpeI**	
BlpI	CspI	PflMI	**SphI**	

Table 4. Restriction Enzymes That Cut the PinPoint™ Xa-1 Vector 6 or More Times.

AciI	BsrSI	**HaeII**	**Hsp92II**	NdeII
AcyI	Bst71I	**HaeIII**	MaeII	NlaIII
AluI	BstUI	HgaI	MaeIII	NlaIV
Alw26I	**CfoI**	**HhaI**	MboI	Sau3AI
AspHI	Cfr10I	**HincII**	MboII	Sau96I
BanI	**DdeI**	HindII	MnlI	ScrFI
BbvI	**DpnI**	**HinfI**	MseI	SfaNI
BsaHI	DpnII	**HpaII**	**MspI**	**TaqI**
Bsp1286I	EaeI	HphI	MspA1I	Tru9I
BsrI	Fnu4HI	**Hsp92I**	**NciI**	XhoII

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

11. Appendix B: PinPoint™ Xa Vector Sequence Accession Numbers and Restriction Enzyme Sites (continued)

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

Enzyme	# of Sites	Location
AatII	2	53, 788
AccI	1	53
Acc65I	1	411
AflIII	1	2595
Alw44I	4	538, 1035, 2281, 3044
AlwNI	1	2186
AscI	1	110
AvaI	4	250, 370, 429, 2825
AvaII	3	309, 1343, 1565
BamHI	1	405
BanII	3	377, 2910, 2924
BbeI	4	143, 2847, 2961, 2982
BglI	2	148, 1589
BglII	1	423
BsaI	4	262, 355, 1641, 2810
BsaOI	5	439, 1189, 1338, 2261, 2685
BsaJI	5	429, 430, 2435, 2857, 2863
BspHI	4	762, 867, 1875, 2902
BspMI	3	118, 2763, 3088
BssHII	1	110
BssSI	4	202, 731, 1038, 2422
BstOI	5	313, 2436, 2449, 2570, 2782
BstXI	1	264
BstZI	1	436
ClaI	1	3019
DraI	3	1129, 1821, 1840
DraII	3	309, 727, 2868
DrdI	4	49, 298, 624, 2493
DsaI	1	2863
EagI	1	436
EarI	2	908, 2712
EclHKI	2	526, 1707
Eco47III	1	2899
Eco52I	1	436
EcoICRI	1	375
EcoNI	2	205, 316
EcoRI	1	3278
EcoRV	1	419
EheI	4	141, 2845, 2959, 2980
FokI	5	70, 625, 1268, 1555, 1736
FspI	1	1484
HindIII	1	393
HpaI	1	2778
KasI	4	139, 2843, 2957, 2978
KpnI	1	415
MaeI	3	1514, 1849, 2102
NaeI	3	116, 183, 2992
NarI	4	140, 2844, 2958, 2979
NdeI	1	533
NgoMIV	3	114, 181, 2990
NotI	1	436
NruI	1	389
NspI	2	682, 2599
PaeR7I	3	250, 370, 2825
PleI	4	1716, 2219, 2704, 2797
PpuMI	1	309
Psp5II	1	309
PspAI	1	429
PstI	1	3297
PvuI	1	1338
PvuII	1	401
RsaI	3	413, 550, 1226
SacI	1	377
SalI	1	52
ScaI	1	1226
SgrAI	2	103, 2981
SinI	3	309, 1343, 1565
SmaI	1	431
SspI	2	902, 3192
TfiI	3	165, 2621, 2761
Tth111I	1	220
VspI	5	499, 1532, 2767, 2796, 3216
XhoI	3	250, 370, 2825
XmaI	1	429
XmnI	1	1107

Table 6. Restriction Enzymes That Do Not Cut the PinPoint™ Xa-2 Vector.

AccB7I	Bpu1102I	Csp45I	PinAI	SplI
AccIII	BsaAI	DraIII	PmeI	SrfI
AflII	BsaBI	Eco72I	PmlI	Sse8387I
AgeI	BsaMI	Eco81I	Ppu10I	**StuI**
ApaI	BsmI	FseI	PshAI	StyI
AvrII	Bsp120I	**I-PpoI**	RsrII	SwaI
BalI	BsrGI	**MluI**	**SacII**	**XbaI**
BbrPI	Bst1107I	**NcoI**	SfiI	XcmI
BbsI	Bst98I	**NheI**	**SgfI**	
BbuI	**BstEII**	**NsiI**	**SnaBI**	
BclI	Bsu36I	PacI	**SpeI**	
BlpI	CspI	PflMI	**SphI**	

Table 7. Restriction Enzymes That Cut the PinPoint™ Xa-2 Vector 6 or More Times.

AciI	BsrSI	**HaeII**	**Hsp92II**	NdeII
AcyI	Bst71I	**HaeIII**	MaeII	NlaIII
AluI	BstUI	HgaI	MaeIII	NlaIV
Alw26I	**CfoI**	**HhaI**	MboI	Sau3AI
AspHI	Cfr10I	**HincII**	MboII	Sau96I
BanI	**DdeI**	HindII	MnlI	ScrFI
BbvI	**DpnI**	**HinfI**	MseI	SfaNI
BsaHI	DpnII	**HpaII**	**MspI**	**TaqI**
Bsp1286I	EaeI	HphI	MspA1I	Tru9I
BsrI	Fnu4HI	**Hsp92I**	**NciI**	XhoII

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

11. Appendix B: PinPoint™ Xa Vector Sequence Accession Numbers and Restriction Enzyme Sites (continued)

Table 8. Restriction Enzymes That Cut the PinPoint™ Xa-3 Vector Between 1 and 5 Times.

Enzyme	# of Sites	Location
AatII	2	53, 789
AccI	1	53
Acc65I	1	412
AflIII	1	2596
Alw44I	4	539, 1036, 2282, 3045
AlwNI	1	2187
AscI	1	110
AvaI	4	250, 370, 430, 2826
AvaII	3	309, 1344, 1566
BamHI	1	406
BanII	3	377, 2911, 2925
BbeI	4	143, 2848, 2962, 2983
BglI	2	148, 1590
BglII	1	424
BsaI	4	262, 355, 1642, 2811
BsaOI	5	440, 1190, 1339, 2262, 2686
BsaJI	5	430, 431, 2436, 2858, 2864
BspHI	4	763, 868, 1876, 2903
BspMI	3	118, 2764, 3089
BssHII	1	110
BssSI	4	202, 732, 1039, 2423
BstOI	5	313, 2437, 2450, 2567, 2783
BstXI	1	264
BstZI	1	437
ClaI	1	3020
DraI	3	1130, 1822, 1841
DraII	3	309, 728, 2869
DrdI	4	49, 298, 625, 2494
DsaI	1	2864
EagI	1	437
EarI	2	909, 2713
EclHKI	2	527, 1708
Eco47III	1	2900
Eco52I	1	437
EcoICRI	1	375
EcoNI	2	205, 316
EcoRI	1	3279
EcoRV	1	420
EheI	4	141, 2846, 2960, 2981
FokI	5	70, 626, 1269, 1556, 1737
FspI	1	1485
HindIII	1	394
HpaI	1	2779
KasI	4	139, 2844, 2958, 2979
KpnI	1	416
MaeI	3	1515, 1850, 2103
NaeI	3	116, 183, 2993
NarI	4	140, 2845, 2959, 2980
NdeI	1	534
NgoMIV	3	114, 181, 2991
NotI	1	437
NruI	1	389
NspI	2	683, 2600
PaeR7I	3	250, 370, 2826
PleI	4	1717, 2220, 2705, 2798
PpuMI	1	309
Psp5II	1	309
PspAI	1	430
PstI	1	3298
PvuI	1	1339
PvuII	1	402
RsaI	3	414, 551, 1227
SacI	1	377
SalI	1	52
ScaI	1	1227
SgrAI	2	103, 2982
SinI	3	309, 1344, 1566
SmaI	1	432
SspI	2	903, 3193
TfiI	3	165, 2622, 2762
Tth111I	1	220
VspI	5	500, 1533, 2768, 2797, 3217
XhoI	3	250, 370, 2826
XmaI	1	430
XmnI	2	395, 1108

Table 9. Restriction Enzymes That Do Not Cut the PinPoint™ Xa-3 Vector.

AccB7I	Bpu1102I	Csp45I	PinAI	SplI
AccIII	BsaAI	DraIII	PmeI	SrfI
AflII	BsaBI	Eco72I	PmlI	Sse8387I
AgeI	BsaMI	Eco81I	Ppu10I	**StuI**
ApaI	BsmI	FseI	PshAI	StyI
AvrII	Bsp120I	**I-PpoI**	RsrII	SwaI
BalI	BsrGI	**MluI**	**SacII**	**XbaI**
BbrPI	Bst1107I	**NcoI**	SfiI	XcmI
BbsI	Bst98I	**NheI**	**SgfI**	
BbuI	**BstEII**	**NsiI**	**SnaBI**	
BclI	Bsu36I	PacI	**SpeI**	
BlpI	CspI	PflMI	**SphI**	

Table 10. Restriction Enzymes That Cut the PinPoint™ Xa-3 Vector 6 or More Times.

AciI	BsrSI	**HaeII**	**Hsp92II**	NdeII
AcyI	Bst71I	**HaeIII**	MaeII	NlaIII
AluI	BstUI	HgaI	MaeIII	NlaIV
Alw26I	**CfoI**	**HhaI**	MboI	Sau3AI
AspHI	Cfr10I	**HincII**	MboII	Sau96I
BanI	**DdeI**	HindII	MnlI	ScrFI
BbvI	**DpnI**	**HinfI**	MseI	SfaNI
BsaHI	DpnII	**HpaII**	**MspI**	**TaqI**
Bsp1286I	EaeI	HphI	MspA1I	Tru9I
BsrI	Fnu4HI	**Hsp92I**	**NciI**	XhoII

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

Promega

12. Appendix C: Reference Information

12.A. Composition of Buffers and Solutions

alkaline phosphatase buffer

100mM	NaCl
5mM	$MgCl_2$
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

D-biotin, 100mM

Dissolve desired amount of biotin in 100mM Na_2HPO_4. Adjust pH to 7.2 with 100mM NaH_2PO_4. Bring to desired volume with 100mM sodium phosphate buffer (pH 7.2). Filter-sterilize (0.22µm filter) and dispense under aseptic conditions.

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.

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 (pH 7.5)
1mM	EDTA (pH 8.0)
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 phenylmethylsulfonyl fluoride (PMSF) per milliliter of isopropanol (10mM). Aliquot and store at –20°C. A stock concentration of 100mM may be prepared if needed.

sample 1X buffer

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

0.1M $NaPO_4$ Buffer (pH 7.0 at 25°C)

57.7ml	1M Na_2HPO_4
42.3ml	1M NaH_2PO_4
900ml	distilled H_2O

stacking gel 4X buffer

6.06g	Tris base
4ml	10% SDS

Adjust to pH 6.8 with 12N HCl; 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

12.B.Related Products

Product	Size	Cat.#
IPTG, Dioxane-Free	1g	V3955
PinPoint™ Vector Sequencing Primer	2µg	V4211
SoftLink™ Soft Release Avidin Resin	1ml	V2011
	5ml	V2012
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
Flexi® System, Entry/Transfer		C8640
Flexi® System, Transfer		C8820
Carboxy Flexi® System, Transfer		C9320
10X Flexi® Enzyme Blend (SgfI and PmeI)	25µl	R1851
	100µl	R1852

12.B. Related Products (continued)

Product	Size	Cat.#
pF1A T7 Flexi® Vector	20µg	C8441
pF1K T7 Flexi® Vector	20µg	C8451
pF4A CMV Flexi® Vector	20µg	C8481
pF4K CMV Flexi® Vector	20µg	C8491
pFN2A (GST) Flexi® Vector	20µg	C8461
pFN2K (GST) Flexi® Vector	20µg	C8471
pF3A WG (BYDV) Flexi® Vector	20µg	L5671
pF3K WG (BYDV) Flexi® Vector	20µg	L5681
pFN6A (HQ) Flexi® Vector	20µg	C8511
pFN6K (HQ) Flexi® Vector	20µg	C8521
pFC7A (HQ) Flexi® Vector	20µg	C8531
pFC7K (HQ) Flexi® Vector	20µg	C8541
pFC8A (HaloTag®) CMV Flexi® Vector	20µg	C3631
pFC8K (HaloTag®) CMV Flexi® Vector	20µg	C3641

13. Summary of Changes

The following change was made to the 6/17 revision of this document:

1. Expired patent statements were removed.