

pPEPTIDE Cloning Vector

Product Information and Instructions
June 2003



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pPEPTIDE Cloning Vector

Small Peptide Expression Vector System

1. Summary

This new *E. coli* vector system is ideally suited for the over-expression of proteins but in particular of polypeptides as small as five residues. The vector is a unique fusion construct based on the pET-3a expression system containing a novel sequence which promotes efficiently the expression of recombinant target proteins as well as very small peptides in *E. coli*. Inherent in this novel sequence is an optimized affinity tag which allows for facile high-efficient affinity purification of the expressed product. A unique chemical cleavage site has also been engineered into this vector for easy removal of the fusion sequence from the target protein/peptide.

2. Introduction

Very often researchers are confronted with the problem that expression of a recombinant target protein is rather poor even when trying a variety of commercially available expression vectors. Furthermore, if the expressed target proteins are e.g. very basic, insoluble at physiological pH and ionic strength and can be easily degraded by proteolytic enzymes, only low yields of the intact proteins are achieved.

At the other end of the spectrum there is the difficulty or impossibility of expressing and purifying small peptides. Attempts by many laboratories to produce peptides reproducibly in high yields have proven to be futile up to now. Although not documented, one popular explanation for lack of success is that the smaller the peptide, the greater the chances that the final product may be degraded or incorporated into and interfere with various aspects of the bacterial growth and/or metabolic systems. Poor expression of peptide and proteins, with negligible yield of the final pure product, very often hampers and delays progress in proteomics research. For example, to complete the solution structures of crucial target regions of specific proteins or their complex with ligands, and finalizing their amino acid assignments, ¹⁵N- and /or ¹³C-labelled small peptides are

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required in sufficient amounts for NMR studies. With the elucidation of a growing number of genome sequences, protein structure/function studies will increase dramatically. To accommodate such needs, an expression vector system is required that can be applied to a variety of peptides/proteins possessing different chemical properties.

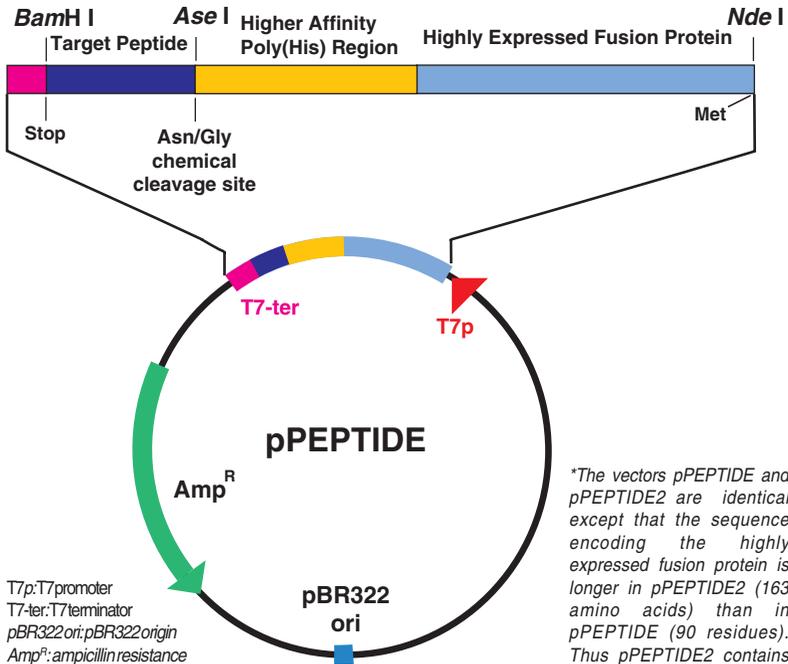
The novel expression vector pPEPTIDE has specifically been designed for producing consistently high levels of protein expression, while simultaneously streamlining the number of steps involved in the purification procedure. This new vector also overcomes the above mentioned problems with low yields of recombinantly expressed peptides by initially incorporating the smaller peptide within a longer fusion product which is highly expressed only after induction and non-toxic to the bacterial cells.

pPEPTIDE possesses a DNA sequence encoding a very unusual metal-chelating cluster of residues which exhibits stronger metal-binding properties than the present commercially available poly(His)-tag, even in the presence of denaturing buffer conditions such as 3 M GuHCl (guanidine HCl) - a feature which could be utilized for overcoming solubility problems associated with some proteins. pPEPTIDE is the optimal vector system for over-expression and affinity purification of small peptides as well as other target proteins. Peptides/proteins, which are difficult or impossible to express independently using presently available means, can now be juxtaposed downstream and expressed as a fusion product, regardless of their size or nature.

To release the target peptide/protein from the fusion protein, hydroxylamine chemical cleavage was chosen rather than enzymic digestion (e.g. Factor Xa or thrombin) for several reasons: (1) enzymes have a limited active life; (2) they must be stored under stringent temperature conditions (-20°C or -80°C); (3) they are not as specific in their site of cleavage. For example, Factor Xa and thrombin may produce unwanted cuts at similar amino acid sequences within the target protein, necessitating site-directed mutagenesis to change the amino acid sequence in that region of the protein. (4) Additionally, in some cases, the activity of commercially available Factor Xa may be too low for efficient cleavage, necessitating purification of a supply of the enzyme from horse blood, involving protracted and expensive procedures. The traditional conditions for hydroxylamine cleavage at Asn-Gly bond (which previously resulted in only 25-50% cleavage), was refined in this protocol to achieve 70-80% cleavage, and in some cases up to 90%.

3. The pPEPTIDE Vector

3.1. Vector Map pPEPTIDE/pPEPTIDE2*



3.2. Unique Features Engineered into the Vector

The vector is composed of three sections as follows:

(1) Highly Expressed Fusion Protein;- after the Met initiation codon the vector encodes either 89 [pPEPTIDE] or 162 [pPEPTIDE2] amino acids which have been demonstrated to be highly expressed in the pET expression vector system;

(2) poly(His)-Tag;- immediately downstream is a naturally-occurring metal binding cluster of amino acids with much higher affinity than the usual poly(His)-tag sequence to facilitate isolation of the fusion protein by metal chelating affinity chromatography (MCAC), and which is engineered to end in a rarely occurring (average 1 in 400) hydroxylamine chemical cleavage site which, in turn, is

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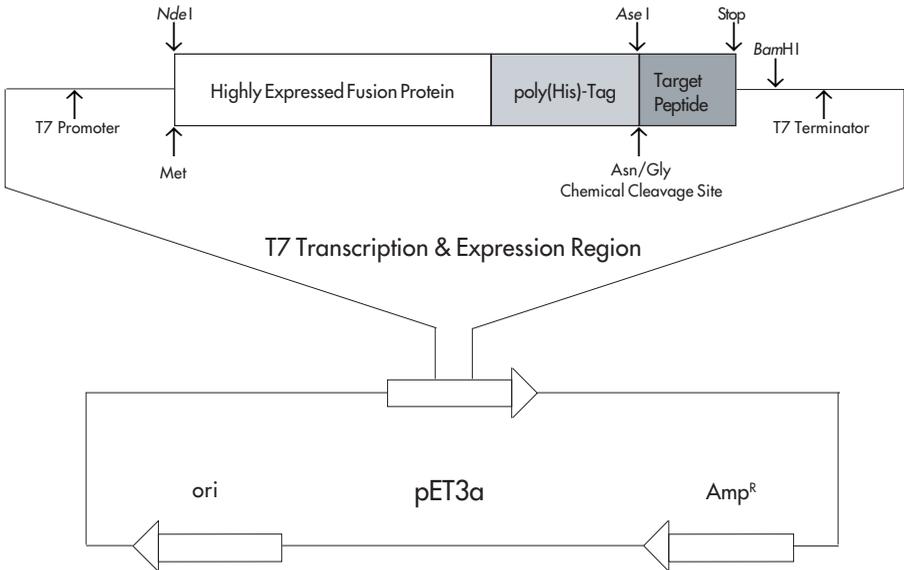


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juxtaposed to the first amino acid of

(3) The Target Peptide:- the final target peptide/protein (from 5 amino acid peptide to full length protein), with N-terminal Gly resulting from the chemical cleavage, is purified using the same affinity column.

3.3. Schematic of the pPEPTIDE Expression Vector



- 90 amino acids (pPEPTIDE) or 163 amino acids (pPEPTIDE2*)
- 50 amino acids
- From 5 amino acid peptide to full length protein

The DNA sequence encoding the target peptide in pPEPTIDE/pPEPTIDE2 encompasses the codons for a very basic 38 residue peptide which will be cut out using *Ase I*/*Bam* HI and subsequently replaced by a DNA sequence encoding any other desired peptide or protein.

* pPEPTIDE2 can be used alternatively if longer fusion proteins are desired!

3.4. Advantages Over Present Commercially Available Systems

1. Some proteins have proven to be toxic to *E. coli* cells when low levels of protein are expressed prior to induction in "leaky" pET expression vectors, resulting in very poor to negligible yields of the final product. To offset this problem, in this vector a polypeptide which is highly expressed only after induction and therefore does not interfere with cell growth, is located upstream of the target protein.

2. The fusion peptide/protein as well as the final cleaved target peptide/protein can be rapidly purified from *E. coli* lysates using the same metal affinity chromatography column, which can be freshly charged with metal and reused many times.

3. The new metal-chelating region exhibits stronger binding properties than the present commercially available poly(His)-Tag, even in the presence of 3 M GuHCl (a mild denaturant which helps to increase the solubility of some proteins).

4. No cloning system incorporating chemical release of the target peptide is presently available on the market. The release of target protein by enzymic digestion has several disadvantages including instability of enzyme and stringent storage temperature, low activity, and unwanted cuts at similar sequence sites within the protein. In this system, the conditions for hydroxylamine cleavage have been refined to achieve 70-80% efficiency. The target peptide is subsequently recovered in the flow-through fraction upon re-application to the same metal chelating affinity column, since the truncated fusion segment remains bound to the resin. At this point the product is ~90-95% pure as assessed by SDS-PAGE (polyacrylamide gel electrophoresis). If greater purity is required, a final purification step by HPLC achieves >99% purity as assessed by SDS-PAGE, mass spectroscopy, and amino acid analysis.

5. If chemical release of the peptide is not desired, additional flexibility can be achieved by incorporating any of a variety of enzyme cleavage sites (e.g. amino acid sequence IEGR, the Factor Xa site) into the oligonucleotide primer immediately prior to the first amino acid of the peptide. This expression system will still have the inherent properties of over-expression coupled with stronger affinity purification, providing a unique advantage over other expression systems currently on the market.

6. A major advantage of this technique is the ability to prepare polypeptides uniformly labeled with ^{13}C and/or ^{15}N (by incorporating ^{13}C -glucose and ^{15}N -ammonium sulfate in the minimal media) for NMR structural and other investigations at reasonable cost, and in adequate yield. The alternative method of chemically synthesizing labeled peptides is prohibitively expensive since labeling is achieved by incorporating specific ^{13}C and/or ^{15}N -amino acid residues at each step of the synthesis.



7. This new construct also offers an alternative to chemical synthesis of non-labelled peptides for laboratories already equipped for using molecular biology protocols. In some cases ordering even a small quantity of chemically synthesized peptide may not be feasible due to its properties. For example, the Trp-rich hydrophobic nature of membrane proteins may cause unwanted side reactions during chemical synthesis, a feature which should not affect this expression system.

8. In chemical synthesis, peptides longer than ~35 residues may also present problems and therefore take much longer to produce and show lower yields.

For example, it may take several months and considerable cost (>\$3000) to produce ~13 mg of a peptide >50-residues synthetically, of which only 69% by weight may be actual protein when analyzed by amino acid analysis. Furthermore, a repeat synthesis of the same peptide may not be reproducible using the same side-chain blocking chemistry as in the initial synthesis, necessitating the search for an alternate blocking protocol which will result in a further delay. In contrast, once the oligonucleotides are obtained for the initial PCR reaction, the peptide/protein can be reproducibly over-expressed and purified using this vector system in a short period of time (~a few weeks) and in much higher yield (see data section - chapter 6).

9. Often the amino acid sequence of the desired peptide is known, but the cDNA is not available. In such cases, the optimal codons for expression of these residues in *E. coli* can be used to synthesize a stretch of DNA to be used as a template for the initial PCR reaction;- such was the case for the acidic peptide (see "Examples" - chapter 6).

3.5. Application of the Technology

1. Production of potentially any polypeptide or protein, regardless of size, but particularly useful for the preparation of ^{13}C and/or ^{15}N uniformly labeled peptides for NMR research purposes.
2. Potential for commercial production of peptides/proteins of medical interest.
3. Alternative to chemical peptide synthesis.

4. Kit Components

PP001 5 µg pPEPTIDE vector DNA
 5 µg pPEPTIDE2 vector DNA
 500 pmole sequencing primers (forward and reverse)*

Shipped at room temperature (RT), store at 4°C.

* 5' TAATACGACTCACTATAGGG 3' (forward primer, T7)
 5' AAACCCCTCAAGACCCG 3' (reverse primer, T7terbis)



5.2. Cloning Peptide Insert into Expression Vector

It takes ~6 hours from start of PCR reaction to start of transformation step.

1. Perform PCR reaction to produce target peptide insert using cDNA or synthetic DNA template and primers¹;
2. Cleave PCR product with *Ase I/BamH I* (ensure final product is >100 bp)²;
3. Purify insert (e.g. use Edge Biosystems QuickStep2 PCR Purification Kit)³;
4. Ligate target peptide insert + *Ase I/BamH I*/phosphatased expression vector;
5. Transform ligation mix into supercompetent *E. coli* cells (e.g. XL-1 Blue or JM101);
6. Plate transformed cells onto LB agar plate supplemented with Ampicillin (50 µg/mL) and incubate at 37°C for ~16 hours;
7. Screen⁴ for presence of plasmid (optional); prepare miniprep of desired clone;
8. Sequence purified plasmid DNA to ensure correctness (i.e. no unwanted mutations) e.g. by using the provided forward and reverse primers.

5.3. Preparative Expression and Purification of Target Peptide

1. Transform cloned plasmid vector into competent BL21(DE3)pLysS cells (Invitrogen, Novagen; chloramphenicol-resistant strain, carries the gene coding for the phage T7 polymerase enabling T7 promoter-induced transcription of the cloned DNA). Spread onto LB + Amp + Chlor plate⁵ and incubate at 37°C overnight.
2. Inoculate one colony from plate (or 4 µL of "continuing" culture if test expression⁵ was carried out) into 5 mL 2XTY media (supplemented with 100 µg/mL ampicillin and 25 µg/mL chloramphenicol). Vortex the cell suspension, then inoculate 100 µL into each of 8 x 1 L of the same media (in 2-L Erlenmeyer flasks). Incubate at 37°C with shaking and monitor growth of cells at 600 nm.
3. At OD_{600nm} ~0.8 (~15 hours), remove 1 mL of "noninduced" cells from one flask for a gel sample and leave at RT for 3 hours. Induce the cultures (8 x 1 L) by adding 1 mL 0.4 M IPTG (isopropyl-β-D-thiogalactopyranoside, 0.4 mM final) and continue incubation with shaking for further 3 hours to allow for protein expression.
4. Remove a sample (1 mL) of "induced" cells from one flask, and along with the previously removed "noninduced" sample, prepare for analysis by SDS-



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PAGE (see test expression protocol). Harvest the 8 L cell cultures by centrifugation (e.g. Damon/IEC DPR/1600 at 4°C, 4200 rpm, 20 minutes), and discard supernatant. Resuspend cells in ~50 mL fresh 2XTY media + 100 µL β-mercaptoethanol, transfer into 4 X 250 mL polypropylene bottles (i.e. each 250-mL bottle contains the resuspension and washings from 2 L cell culture). Centrifuge to remove the buffer (e.g. Sorvall GSA rotor, 7000 rpm, 10 minutes, 4°C). The cell pellet can be either stored at -20°C overnight, or processed further to the acetone powder stage⁶.

5. Thaw cell pellet and make acetone powder (to remove any lipid material)⁶, while lysing the cells via homogenization (acetone powder can be frozen at this stage);
6. Extract⁷ the fusion protein (from the acetone powder) into an appropriate buffer via homogenization, and centrifuge to obtain clear supernatant;
7. Apply supernatant to metal chelating affinity chromatography (MCAC)⁸ column, allowing unbound *E. coli* proteins to flow through with the equilibration buffer;
8. Preferentially elute the bound fusion protein (~99% pure at this stage);
9. Cleave fusion protein with hydroxylamine⁹ to release target peptide (~70-80% cleavage);
10. Re-apply cleaved mixture to the same MCAC column¹⁰ and collect the released peptide in the initial flow-through fractions, leaving any uncleaved fusion protein (~20-30%) plus the cleaved fusion segment still bound to the resin;
11. Remove buffer salts from the peptide (e.g. via dialysis using the correct Mr dialysis tubing, or gel filtration) and freeze-dry (~95% pure at this stage);
12. If greater purity is desired, include a final purification step using HPLC¹¹ (e.g. TSK G3000SW gel filtration or C18 reverse phase) to achieve >99% purity.

Footnotes:

¹To check the size, quantity and purity of the PCR product, 5 µL can be run on a 1.7% agarose gel along with DNA standard ladder (e.g. MoBiTec DNA Ladders 100 bp, 200 bp, 1000 bp and 100 bp extended).

²Both *Ase I* and *BamH I* can be used in the same New England Biolab buffer #3 (100 mM NaCl, 50 mM Tris.HCl, 10 mM MgCl₂, 1mM DTT, pH 7.9, 37°C). *Ase I* is included in the digestion reaction at the start, and *BamH I* added one hour later. The digestion is then allowed to continue for another hour at 37°C.

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³The DNA should be eluted in H₂O (since the EDTA in TE buffer may interfere with the enzyme reaction in the following ligation step), but must then be stored at -20°C to prevent autocatalytic degradation.

⁴Rapid screening of colonies was performed routinely to ensure presence of the plasmid. Using sterile toothpicks, streak ~7 colonies from the overnight LB + Amp plate onto one new LB + Amp plate and incubate overnight at 37°C. The next day, aliquot into each of 7 Eppendorf tubes, 40 µL TE buffer (10 mM Tris.HCl, pH 7.5, 1 mM EDTA), then 40 µL 1:1 chloroform:phenol. Using the flat end of sterile toothpicks, scoop up as much of the smear from each of the colonies in turn, and transfer to the sides of the Eppendorf tubes. Spin for 1 second to pellet the colonies to the bottom of the tubes. Vortex the mixture for 10 seconds. Spin tubes 2 minutes to separate the phases. Into another set of 7 tubes, add 4 µL of 10x loading buffer (to run on a 0.6% agarose gel), and then carefully and slowly draw up 25 µL of the top TE buffer phase containing the plasmid DNA, taking care not to include the interphase which contains contaminating *E. coli* chromosomal DNA. Load 10 µL slowly into the sample wells (sample may swirl out of the well if loaded too quickly) along with a DNA standard ladder. The plasmid (~4 kb depending on size of the target DNA insert) runs slower than the RNA bands of *E. coli*.

⁵Test expression of target peptide is optional at this stage, but may routinely be carried out prior to the preparative culture growth since not all colonies express protein equally. To ensure the highest yield in the subsequent preparative culture, this ~7 hour procedure was included in each new peptide preparation, especially when more expensive chemicals such as (¹⁵NH₄)₂SO₄ and/or ¹³C-glucose were used in minimal media to produce labeled peptides for NMR studies.

Protocol for Test Expression:

Inoculate six colonies into six sterile tubes (~ 10 mL capacity), each containing 2 mL 2X-TY medium supplemented with ampicillin plus chloramphenicol, and incubate at 37°C with shaking (~225 rpm) for ~ 3 hours until moderately turbid (OD_{600 nm} ~0.3).

For induction, aliquot 1 mL of the growing culture into a fresh tube containing IPTG (0.4 mM), and allow both "noninduced" and "induced" cultures to grow for 2.5 hours.

In order to maintain a log-phase "continuing" culture of cells for later use, freshly inoculate 2 µL from each of the six "noninduced" growing cultures into another set of six tubes containing antibiotic-supplemented 2X TY medium, and allow growth to continue at 37°C with shaking.



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To harvest the "noninduced" and "induced" cells, transfer the 1 mL cultures to 1.5 mL conical tubes and centrifuge at maximum speed in an Eppendorf centrifuge for 10 minutes. Discard the supernatant.

For analysis of expression levels using SDS-PAGE (15%), resuspend the centrifuged samples in 100 μ L 2X TY buffer + 100 μ L Laemmli gel sample buffer + 5 μ L β -mercaptoethanol, boil 5 minutes, and recentrifuge for 10 minutes prior to application of 10 μ L on the gel. On the same gel, run a protein standard (e.g. MoBiTec's Protein Molecular Weight Marker; order number: PS-101JB) to check Mr of the expressed target fusion protein.

Acetone Powder of Cell Pellet:

If cells were frozen at -20°C overnight, thaw at RT \sim 1 hour. To minimize proteolysis during preparation of acetone powder, keep the four bottles in an ice bath and work with one bottle at a time.

Weigh a Whatman #3 filter paper disk, fit into a buchner funnel (\sim 165 cm diameter), wet with deionized water, and connect to in-house vacuum to "seal" the paper onto the funnel. Leave on benchtop until needed.

To each pellet (from 2 L cells) in 250-mL polypropylene (ethanol/acetone resistant) centrifuge bottle, add 120 mL cold 95% ethanol. Homogenize at #3 setting (low speed) using large probe (Brinkman Polytron Homogenizer) until pellet is uniformly suspended, then increase setting to #4-5 (high speed) for 30-40 seconds. Repeat homogenization 2-3 times.

Centrifuge the four bottles in GSA rotor at 16,300 rpm for 10 minutes. If supernatant still contains particulate matter, respin. Discard supernatant.

Repeat ethanol extraction twice more.

Extract pellet 3 times with cold acetone as above, but for the third wash, use only 60 mL cold acetone. Instead of removing the acetone by centrifugation, filter slowly, \sim 2 mL at a time using a Pasteur pipette, onto the center of the "wetted" Whatman filter disk prepared previously. Allow the powder to be sucked dry before applying the next aliquot. Repeat until all the suspension has been applied, leaving the vacuum on for a further 0.5-1 hour until the pellet turns gray-white and "cracks". Transfer the filter paper with damp acetone powder to a clean dish, cover loosely with pin-pricked plastic wrap, and dry overnight in a fume hood.

The next morning, transfer the powder into a tared tube and determine the weight of the acetone powder. The powder can be stored at -20°C , or extracted immediately.

⁷Extraction of Fusion Protein from Acetone Powder of Cell Pellet:

The concentration of powder used for extraction is ~7 mg/mL in the particular benign buffer used for the initial column chromatography.

Transfer the powder into a cylindrical glass bottle which will be used for homogenization, and with a pestle break up the chunks.

Add the required volume of *benign* buffer (e.g. 50 mM MOPS (4-morpholine-propanesulfonic acid), 0.01% sodium azide, pH 6.5) and a stirring bar, and allow mixture to stir at RT for ~ 1 hour to soften the powder.

Remove stirring bar, add a few drops of octanoic acid to prevent excess foaming during homogenization, and homogenize with the large probe at high speed (~#7-10 setting) for ~ 1 minute. Repeat homogenization two-three times. Remove 25 μ L for a gel sample.

Aliquot into 30 mL bottles and centrifuge at 48,000g in SS-34 rotor (20,000 rpm, 30 minutes, 4°C). Decant supernatant into flask and if still cloudy, re-spin or filter through Millipore "low protein binding" 5 μ m filter.

To determine the yield from extraction, remove 25 μ L of the supernatant for a gel sample. Resuspend the pellet in the same volume of buffer used previously, homogenize to disperse the particulate matter, and immediately remove 25 μ L for analysis by SDS-PAGE. To the three gel samples, add an equal volume of Laemmli sample buffer and prepare as described previously (by addition of β -mercaptoethanol, boiling and spinning), loading 10 μ L onto the gel. The clear supernatant is ready for purification by column chromatography.

⁸Metal Chelating Affinity Column (MCAC):

Wash Chelating Sepharose Fast Flow Resin (50 mL, 17-0575-01 from Pharmacia Biotech), pack into a 2.5 x 20 cm glass column, and charge with Zinc metal as per their instructions. Equilibrate the column with buffer A (50 mM MOPS, 0.5 M NaCl, 3M GuHCl, 0.01% azide, pH 6.5).

Add solid NaCl and a solution of 6 M GuHCl, 0.01% azide, pH 6.5 (equal in volume to the clear supernatant containing the extracted fusion protein⁷) to the supernatant, so that the final buffer composition is 25 mM MOPS, 0.5 M NaCl, 3 M GuHCl, 0.01% azide, pH 6.5 (Adjust pH slightly if necessary).

Load supernatant onto the column and wash with buffer A until all the *E. coli* proteins are eluted in the flow-through peak, and a flat baseline is seen.

Wash column with buffer B (50 mM MOPS, 0.5 M NaCl, 10 mM imidazole, 0.01% azide, pH 6.5). Very little protein matter is eluted with this wash due to the strong affinity of this particular His tag segment to the resin.



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Elute the fusion protein with buffer C (same as buffer B but with 100 mM imidazole). Pool the desired fraction, dialyze versus pH 6.5 deionized water + β -mercaptoethanol to remove salts etc., and lyophilize to obtain ~99% pure fusion protein.

Wash the column with buffer D (50 mM MOPS, 0.5 M NaCl, 50 mM EDTA, pH 6.5) to strip the chelating zinc off the column, and to illustrate that no fusion protein remains bound to the column.

The MCAC column can be recharged with fresh metal solution and re-used many times.

Alternative protocol using Vivapure Metal Chelate Mini Spin Columns

Buffers

Lysis-, Wash- and Equilibration buffer: 100 mM NaH_2PO_4 ; 10 mM Tris; pH 8.0

Elution buffer: 100 mM NaH_2PO_4 ; 10 mM Tris; 250 mM imidazol; pH 8.0

Metal chelate-membrane

Vivapure Metal Chelate Mini Spin Columns (see page 26)

Sample

His-tagged protein/peptide from pPEPTIDE

Metal chelate-membrane assay

Optional clarification:

1. Pipette up to 400 μl of the sample onto the clarification mini spin column. Spin for 5 minutes at 2,000 x g. The flow-through represents the clarified sample.

(For sample volumes higher than 400 μl , the clarification mini spin column can be re-loaded until the whole sample is clarified.) Store the clarified sample on ice or under appropriate conditions.

Purification:

1. Pre-wet the Vivapure Metal Chelate Mini Spin Column with 400 μl distilled water. Spin for 1 minute at 2,500 x g and discard the flow-through.

2. Load 400 μl of a 0.5 M NiSO_4 solution. Spin for 1 minute at 2,500 x g and discard the flow-through. Repeat this step.

3. Fill in 400 μl distilled water and spin for 1 minute at 2,500 x g to remove unbound metal ions. Repeat this step.

4. Equilibrate the membrane with 400 μl equilibration buffer. Spin for 1 minute at 2,500 x g and discard the flow-through. Repeat this step.

5. Load up to 400 μl sample solution onto the membrane. Centrifuge for 3 minutes at 2,500 x g. For sample volumes higher than 400 μl , the Vivapure Metal Chelate

Mini Spin Column can be re-loaded to bind your complete sample as long as the membrane capacity is considered.

6. Wash the membrane with 400 μ l washing buffer. Spin for 3 minutes at 2,500 x g and discard the flow-through. Repeat this step.

Note: An optional third washing step might be included to increase purity.

7. Pipette 200 μ l elution buffer onto the membrane to elute the protein. Spin for 3 minutes at 2,500 x g. The flow-through contains the purified protein.

8. A second and third elution step with 400 μ l elution buffer may be necessary to elute all of the desired protein.

^oHydroxylamine Chemical Cleavage of Fusion Protein:

Use protective eye goggles, gloves and mask, and prepare this solution in a fume hood, fresh, just prior to using. Prepare a 40 mL solution of 4 M NH_2OH (Hydroxylamine.HCl from Fisher Scientific), 0.4 M K_2CO_3 , pH 9, as follows:

(1) in a 50 mL beaker embedded in an ice bath, dissolve 11.12 g NH_2OH in 12 mL cold deionized H_2O ;

(2) slowly add in small aliquots while stirring and monitoring the pH carefully, a total of ~4 mL 12.5 N NaOH;

(3) then add 8 mL ice-cold 2 M K_2CO_3 . The solution becomes clear, and pH~5.7;

(4) adjust pH slowly back up to 9.0 (need ~8 mL 12.5 N NaOH), being careful not to overshoot it.

Protein solution (using 350 mg fusion protein as an example; final concentration to be ~5 mg/mL) is prepared for cleavage as follows:

(1) dissolve 350 mg protein in 35 mL 6 M GuHCl, pH 6.5, prewarmed to 45°C;

(2) cover tightly and shake protein solution in an environmental shaker at 45°C for ~10 minutes.

Cleavage reaction:

(1) add 35 mL ice-cold 4 M NH_2OH , 0.4 M K_2CO_3 , pH 9.0 solution to the prewarmed protein solution, cover tightly and shake to mix thoroughly (final buffer for cleavage is 2 M NH_2OH , 0.2 M K_2CO_3 , 3 M GuHCl, pH 9.0);

(2) shake at 45°C, ~250 rpm for ~5.25 hours;

(3) in a fume hood, terminate reaction by decreasing the pH to 2-3 (need ~1.5 mL 12 N HCl) and continue stirring for a further ~10 minutes; (4) neutralize reaction mixture by increasing pH back up to 6.5 (need ~7 mL 12.5 N NaOH). The cleaved products can be applied directly at this stage to the MCAC column. Generally cleavage of the fusion protein is ~70-80%, leaving ~20-30% still uncleaved.



¹⁰MCAC column purification of cleaved peptide:

The mixture of cleaved and uncleaved products is applied to the same MCAC column equilibrated in buffer A (50 mM MOPS, 0.5 M NaCl, 3 M GuHCl, pH 6.5) and monitored at 229 nm. The released peptide, which now has no affinity for the chelating resin, is eluted in the flow-through fraction (~95% pure at this stage). Fractions are pooled, dialyzed against a volatile liquid such as deionized water, or 1% formic acid, or dilute ammonium bicarbonate (depending on the solubility and chemical property of the particular peptide) and using the appropriate Mr cut-off membrane tubing, then lyophilized.

¹¹Further purification of released peptide: Data regarding the column conditions and elution profiles, as well as SDS-PAGE gels, can be found in the following chapter.

6. Examples

The vector was used successfully to over-express the following three peptides: (1) a very basic 38-residue peptide ($pI \cong 12.5$) in enriched media; (2) a 45-residue basic peptide in ¹⁵N-supplemented minimal media; (3) a 20-residue acidic peptide in enriched media.

Initially, a peptide was chosen which posed a challenge for purification due to its extremely basic nature ($pI \cong 12.5$). Numerous 8 L cell cultures having the desired construct were reproducibly grown and harvested in order to test various column purification methods, buffer compositions, and chemical cleavage conditions.

To ensure that over-expression was also obtained in minimal media supplemented with ¹⁵N-ammonium sulfate, a longer version (45 residues) of the initial basic peptide was chosen.

The 20-residue acidic peptide was tested in order to illustrate the diverse application of this novel expression construct.

1. Expression and Purification of Basic 39-Residue Gly-Peptide :

1. The induced expression of fusion protein is shown in Fig. 1a, lanes 1 versus 2, with "u" indicating its position on the 15% SDS-PAGE gel. A comparison of lanes 3, 4 and 5 indicates that ~99% of the total fusion protein (lane 3) is extracted from the acetone powder of the harvested cell pellet into the supernatant fraction (lane

4) using 50 mM MOPS (4-morpholine-propanesulfonic acid), 0.01% sodium azide, pH 6.5 buffer, leaving only unwanted *E. coli* proteins in the pellet (lane 5).

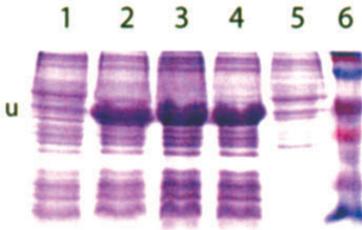


Fig. 1a. 15% SDS-PAGE showing induced over-expression of fusion protein ($M_r \sim 28$ kd) and extraction from acetone powder. Non-induced (1) and induced (2) cells, respectively; total acetone powder (3); supernatant (4) and pellet (5), respectively, of acetone powder extract in 50 mM MOPS, 0.01% Na-azide, pH 6.5 buffer; M_r standards (6, Kaleidoscope, M_r 18-200 kd). "u" indicates location of fusion.

2. After addition of GuHCl and NaCl (final buffer composition 25 mM MOPS, 0.5 M NaCl, 3 M GuHCl, 0.01% azide, pH 6.5), the supernatant is loaded onto a metal chelating affinity chromatography (MCAC) column equilibrated with buffer A (50 mM MOPS, 0.5 M NaCl, 3 M GuHCl, 0.01% azide, pH 6.5). Although very little protein material is observable in fractions 22 and 61 under the flow-through peak eluted with buffer A, (Fig. 1b, lanes 5 and 6), concentration of the pooled fractions across this peak and reanalysis on SDS-PAGE (after dialysis and lyophilization) indicates the presence of ~99% of the total *E. coli* proteins.

3. A subsequent wash of the column with buffer B (50 mM MOPS, 0.5 M NaCl, 10 mM imidazole, 0.01% azide, pH 6.5) results in little or no elution of proteins in fractions 91 and 96 (see Fig. 1b, lanes 7 and 8), illustrating the strong affinity of this particular poly(His)-tag segment to the resin.

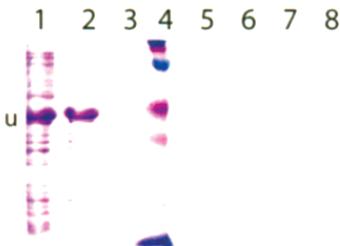


Fig. 1b. 15% SDS-PAGE of fractions from MCAC column. 1, 2 and 3 are the same acetone powder samples as lanes 3, 4 and 5 respectively in Fig. 1a; 4, M_r standards; 5 and 6, fractions 22 and 61 eluted with buffer A; 7 and 8, fractions 91 and 96 eluted with buffer B. "u" indicates location of fusion protein.



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4. The fusion protein is then eluted with buffer C (same as buffer B but with 100 mM imidazole) in fractions 107-114 (Fig. 1c, lanes 3-9), dialyzed and lyophilized. At this stage, after only one column, the fusion protein is ~99% pure.

5. A final wash of the MCAC column with buffer D (50 mM MOPS, 0.5 M NaCl, 50 mM EDTA, pH 6.5), which strips the chelating metal off the column, indicates that no fusion protein remains bound to the resin (Fig. 1c, lanes 12-15). The MCAC column can be recharged with fresh metal solution at this stage and re-used many times.

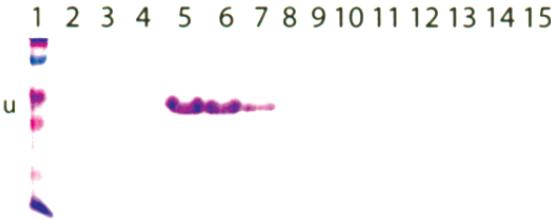


Fig. 1c. Continuation of fractions from MCAC column. 1, Mr standards; 2-11, fractions 106, 107, 108, 109, 110, 111, 113, 115, 117 and 119 respectively, buffer C (100 mM imidazole); 12-15, fractions 125, 128, 130 and 133 respectively, EDTA wash. "u" indicates location of fusion protein.

6. The fusion protein is chemically cleaved using hydroxylamine to release the target peptide. The products of a typical cleavage are shown in Fig. 1d. The preparative cleavage indicates ~20-30% of the starting fusion protein material is left uncleaved (Fig. 1d, lane 5, "u"), while ~70-80% is cleaved (Fig. 1d, lane 5, "c"), resulting in a fusion segment and released target peptide (Fig. 1d, lane 5, "p").

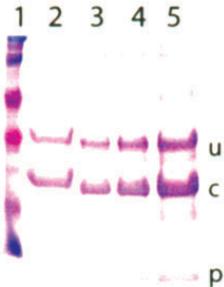


Fig. 1d. 15% SDS-PAGE of products from chemical cleavage of fusion protein. 1, Mr standards; 2, trial cleavage; 3-5, 3, 5 and 10 μ L respectively of a diluted preparative cleavage sample. "u", uncleaved fusion protein; "c" cleaved fusion portion; "p", released target peptide.

7. Purification of the target peptide is achieved by application of the mixture of cleavage products to the same MCAC column equilibrated with 50 mM MOPS, 0.5 M NaCl, 3 M GuHCl, pH 6.5 (buffer A). The peptide (p), which has no affinity for the resin, is eluted in fractions 5-17 under the flow-through peak (Fig. 2a, lanes 2-8), indicating ~90-95% purity at this stage. The uncleaved (u) and cleaved (c) proteins, both containing the fusion portion and therefore still bound to the resin, are subsequently eluted in fractions 26-30 (Fig. 2a, lanes 12-14) with buffer B (50 mM MOPS, 0.5 M NaCl, 100 mM imidazole, pH 6.5). A final wash with buffer C (50 mM MOPS, 0.5 M NaCl, 50 mM EDTA) is used to strip off the chelating metal, in preparation for regeneration of the resin. Fractions 5-17 containing the peptide are pooled, dialyzed against 1% formic acid using 1000 Mr cut-off membrane tubing, then lyophilized.

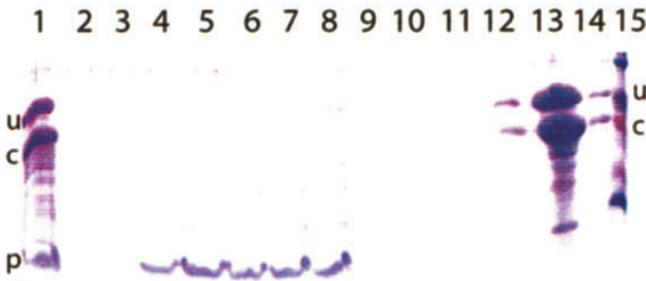


Fig. 2a. 15% SDS-PAGE of fractions from MCAC column. 1, chemical digest applied to column; 2-10, fractions 5, 6, 7, 9, 11, 13, 15, 18 and 19 eluted with buffer A in flow-through portion; 11-14, fractions 23, 26, 28 and 30 eluted with buffer B. "u", uncleaved fusion protein; "c" cleaved fusion protein; "p", released target peptide.

8. In order to achieve >99% purity of this basic peptide, various methods utilizing its solubility properties were tested. In the first option, an aliquot of the lyophilized sample is dissolved in 0.1% TFA (trifluoroacetic acid) and the pellet (after centrifugation) analyzed by SDS-PAGE. A 10-fold concentrated loading of the pellet (Fig. 3a, lane 3) indicates the presence of the higher Mr contaminants with

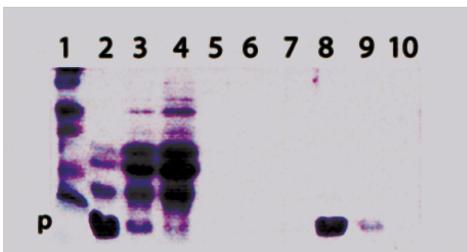


Fig. 3a. 15% SDS-PAGE of fractions from TSK G3000SW column. 1, Mr standards; 2, supernatant loaded onto column; 3 and 4, precipitate of sample from 0.1% TFA and 10 mM ammonium bicarbonate respectively; 5-10, pooled fractions 17-22, 23-26, 27-29, 30-34, 35-45 and 46-60 respectively. "p" is target peptide.

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minimal loss of peptide. Utilizing the other end of the pH spectrum, a 5-fold concentrated loading of the pellet fraction from 10 mM NH_4HCO_3 (Fig. 3a, lane 4) indicates that the higher Mr contaminants can also be eliminated using a basic solution. Both of these trials illustrate a simple step for purification of the target peptide. The supernatant fraction from 0.1% TFA (Fig. 3a, lane 2) is applied to a TSK G3000SW gel filtration column for final purification by HPLC. The eluted fractions are pooled into six sections and analyzed by SDS-PAGE as follows: Fig. 3a, lane 5, fractions 17-22; lane 6, fractions 23-26; lane 7, fractions 27-29; lane 8, fractions 30-34; lane 9, fractions 35-45; lane 10, fractions 46-60. The peptide from fractions 27-45 (Fig. 3a, lanes 7-9) is now ~99% pure.

9. Using the solubility properties gleaned from the previous results, a second option was also tested for use as the final purification step. A sample of the peptide is dissolved in 2 mM NH_4HCO_3 , pH 6.0 (solution A), clarified by centrifugation, and the supernatant (Fig. 4a, lane 2) applied to a TSK G3000SW gel filtration column equilibrated with the same solution A. Fractions across the flow-through peak (Fig. 4a, lanes 3-10) indicate the presence of higher Mr contaminants (the presence also of some peptide material was proven in a subsequent run with less material to be due to overloading the capacity of the resin). The resin is washed with water to remove any trace of NH_4HCO_3 , and the peptide subsequently eluted with 0.1% TFA. As seen in Fig. 4a, lane 11, the target peptide is >99% pure. Purity of the peptide was also confirmed by amino acid analysis, mass spectroscopy, and amino acid sequence analysis of the first five residues.

10. From a typical 8 L cell culture preparation, the yield of peptide after MCAC (~95% pure at this stage) was 132 mg (29.28 μmoles of Mr = 4507). After an additional HPLC purification step, the yield of 99% pure peptide was 106.4 mg (23.61 μmoles).

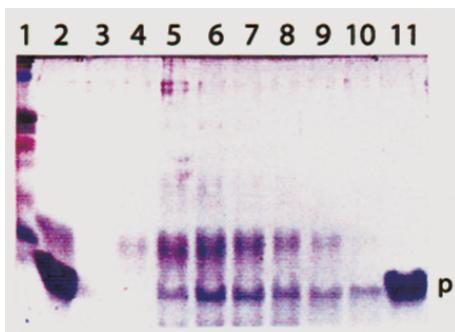


Fig. 4a. 15% SDS-PAGE of fractions from TSK G3000SW column. 1, Mr standards; 2, supernatant loaded onto column; 3-10, fractions eluted with buffer A (2 mM ammonium bicarbonate) in flow-through portion; 11, pooled fractions 29 + 30 eluted with 0.1% TFA. "p" is target peptide.

2. Expression and Purification of Basic 45-Residue Gly-Peptide:

1. To ensure that over-expression of the fusion protein could also be obtained when the cells are grown in ^{15}N -supplemented minimal media, a 45-residue peptide (a longer version of that used in example 1) was chosen as the test case for use in NMR studies. As seen in Fig. 5a, good expression of ^{15}N -fusion protein is obtained in both the initial trial (Fig. 5a, lanes 2 versus 3) as well as preparative experiments (Fig. 5a, lanes 4 versus 5). An acetone powder of the cell pellet fraction (Fig. 5a, lane 6) is prepared, and the ^{15}N -fusion protein extracted into the supernatant fraction (Fig. 5a, lane 7).

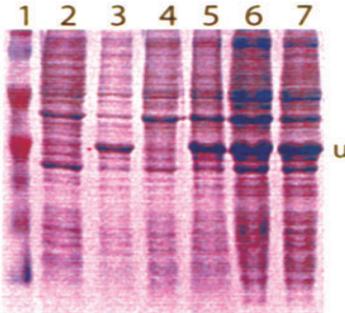


Fig. 5a. 15% SDS-PAGE showing induced over-expression of fusion protein containing target peptide. Cells were grown on ^{15}N -ammonium sulphate-supplemented minimal media. 1, Mr standards; 2 and 3, non-induced and induced cells respectively of test expression; 4 and 5, non-induced and induced cells respectively of preparative expression; 6, acetone powder of cell pellet fraction; 7, supernatant of the extracted acetone powder. "u", uncleaved fusion protein.

2. In order to avoid the higher costs of ^{15}N -labelled chemicals, the subsequent purification steps were performed using the unlabelled fusion protein isolated from cells grown in enriched media. Similar to the result observed in Fig. 5a with ^{15}N -labelled product, the unlabelled fusion protein is completely extracted into the supernatant fraction from the acetone powder of the cell pellet (Fig. 5b, lanes 2-4).

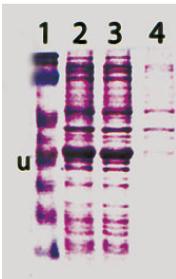


Fig. 5b. 15% SDS-PAGE of extraction of fusion protein from acetone powder of cell pellet. 1, Mr standards; 2, acetone powder of cell pellet; 3 and 4, supernatant and pellet respectively of acetone powder extract; "u", uncleaved fusion protein.

3. Application of the supernatant (Fig. 5c, lane 2) to the MCAC column achieves ~99% purity of the fusion protein (Fig. 5c, lane 3).



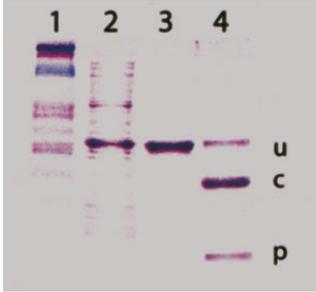


Fig. 5c. 15% SDS-PAGE showing chemically-cleaved products of purified fusion protein. 1, Mr standards; 2 and 3, fusion protein before and after purification on MCAC column; 4, products of chemical cleavage; "u", uncleaved fusion protein; "c", cleaved fusion portion; "p", released target peptide.

4. Chemical digestion of the fusion protein indicates ~80% cleavage (Fig. 5c, lane 4 and Fig. 5d, lane 2).

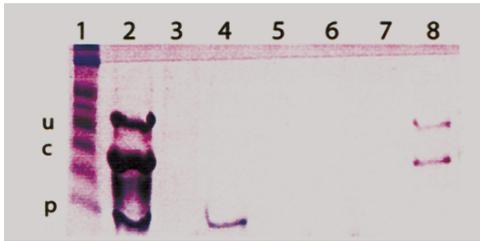


Fig. 5d. 15% SDS-PAGE showing purification of released target peptide on MCAC column. 1, Mr standards; 2, supernatant of chemical digest applied to column ("u", "c" and "p" as in Fig. 5c); 3, pellet of sample in 2; 4, fraction 11 eluted with buffer A in flow-through portion; 5 and 6, fractions 26 and 28 eluted with buffer B; 7 and 8, fractions 33 and 37 eluted with buffer C.

5. The released peptide was subsequently purified on the same MCAC column equilibrated with 30 mM MOPS, 0.67 M NaCl, 4 M GuHCl, pH 6.5 (buffer A). As in the previous case, the ~95% pure peptide (p) having no affinity for the resin is eluted in fractions 8-14 under the flow-through peak (Fig. 5d, lane 4). Washing the column with 25 mM MOPS, 0.5 M NaCl, 3 M GuHCl, 10 mM imidazole, pH 6.5 (buffer B) does not elute the tightly bound uncleaved (u) and cleaved (c) proteins having the fusion portion (Fig. 5d, lanes 5 and 6, fractions 26 and 28). These are both subsequently eluted in fractions 36-41 (Fig. 5d, lane 8) with buffer C (buffer B with 100 mM imidazole). A final wash with buffer D (50 mM MOPS, 1 M NaCl, 50 mM EDTA, pH 6.5) is used to strip the chelating metal off the resin.

3. Over-expression and Purification of a 20-Residue Acidic Peptide:

1. An acidic peptide illustrates the diverse application of this novel expression vector. Over-expression of the fusion protein is observed in both the test (Fig. 6a, lane 2 versus 3) and comparative (Fig. 6a, lane 4 versus 5) experiments.

2. Application of the fusion protein to an MCAC column and its subsequent elution in 100 mM imidazole buffer are the same as described previously (see Fig. 1). Purity to ~99% is achieved at this stage (Fig. 6b, lanes 1-3).

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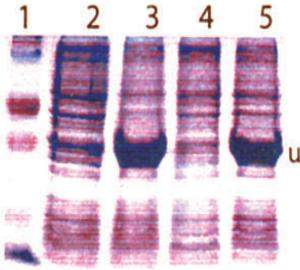


Fig. 6a. 15% SDS-PAGE showing induced over-expression of fusion protein containing an acidic 19-residue target peptide. 1, Mr standards; 2 and 3, non-induced and induced cells, respectively, of test expression; 4 and 5, non-induced and induced cells, respectively, of preparative expression. "u", fusion protein.



Fig. 6b. 15% SDS-PAGE of fusion protein after purification on MCAC column. 1-3, fractions under the peak eluted with 100 mM imidazole buffer. "u", fusion protein.

3. After chemical cleavage (Fig. 6c, lane 2), purification of the released 20-residue acidic peptide is accomplished by application of the cleaved mixture to the same MCAC column. Buffers A, B and C are the same as in Fig. 2. Although the presence of GuHCl in the gel samples results in skewed bands (Fig. 6c), it is still possible to track the locations of the peptide (~95% pure) in fractions 11-24 under the flow-through peak (Fig. 6c, lanes 3-5) and the bound uncleaved (u) and cleaved (c) fusion proteins, which are eluted with 100 mM imidazole buffer in fractions 37-40 (Fig. 6c, lane 6).

4. A final purification step on a Vydac C18 HPLC column (data not included; buffer A, 0.05% TFA; buffer B, 0.05% TFA/CH₃CN; buffer gradient 0.1% B/minutes) results in ~99% pure peptide eluting at ~55% buffer B (Fig. 6c, lane 9), confirmed by amino acid analysis.

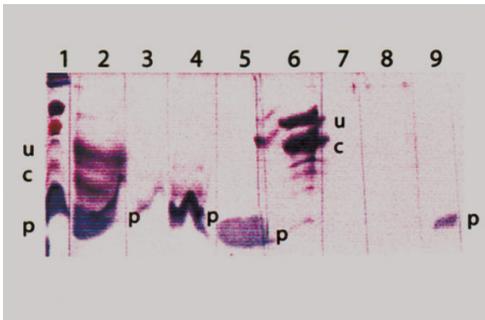


Fig. 6c. 15% SDS-PAGE of purification of released target peptide on MCAC column (2-6) followed by final HPLC step using TSK G3000SW column (7-9). 1, Mr standards; 2, cleaved product loaded onto MCAC column; 3-5, fractions 12, 14 and 18 eluted in flow-through peak with buffer A; fraction 38 eluted with buffer B; 7-9, fractions of Vydac C18 HPLC column (see text for buffer compositions). 7, 25% buffer B; 8, ~32% buffer B; 9, ~55% buffer B. "u", uncleaved fusion protein; "c", cleaved fusion portion; "p", released target peptide.

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7. Order Information, Shipping & Storage

order #	description	amount
PP001	pPEPTIDE vector DNA	5 µg
	pPEPTIDE2 vector DNA	5 µg
	sequencing primers (forward and reverse)	500 pmole

Shipped at room temperature (RT). Lyophilized plasmid DNA can be stored at 4°C. Once the DNA has been dissolved in sterile water or buffer we recommend storage at -20°C.

Related MoBiTec Products:

order #	description	amount
PP002	pPEPTIDE Cloning Vector Kit (includes pPEPTIDE/pPEPTIDE2 vector DNA, 5 µg each; forward and reverse sequencing primers, 500 pmole each; 3 Vivapure Metal Chelate Mini Spin columns "m"; 3 clarification mini spin columns; protocol)	1
MC01MC12-VS	Vivapure Metal Chelate Mini Spin Columns (includes 12 Vivapure Metal Chelate Columns "m"; 12 clarification mini spin columns; 36 collection tubes; instruction manual)	1