

**Xpress™ System
Protein Expression
pEBVHis**
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**Xpress™ System
Protein Expression
pEBVHis**

**A Manual of Methods for Expression of
Polyhistidine - Containing Recombinant Proteins
Using the pEBVHis Vector System in Mammalian Cells**

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UNPACKING INSTRUCTIONS

The Xpress™ Protein Mammalian Expression System (pEBVHis)

PLACE ALL COMPONENTS AT +4°C

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Instructions: Xpress System™ Protein Expression

1. pEBVHis Vectors

The pEBVHis A, B, and C vectors are derived from the Epstein Barr Virus (EBV) expression vector pREP4. They are designed for efficient protein expression and purification in mammalian tissue-cultured cells.

High levels of expression of DNA sequences cloned into the pEBVHis vectors are made possible by the presence of the EBV origin of replication, designated ori-P, and the EBNA-1 protein (EBV-*encoded nuclear antigen*) which *trans*-activates ori-P. The inclusion of these elements in the EBV vectors enables them to replicate episomally when transfected into an appropriate mammalian cell line (e.g. 293, 293-EBNA, COS, or CV-1). They will not replicate in rodent cell lines (e.g. CHO or NIH-3T3). The vectors can be maintained as high copy number plasmids for up to 3 months, if selective pressure is maintained, making it no longer necessary to produce a stably transformed clonal derivative.

Transcription of the gene of interest is directed by the Rous Sarcoma Virus long terminal repeat (RSV-LTR). The vectors carry the Hygromycin B drug resistance marker under the control of the Thymidine Kinase (TK) promoter, enabling selection in mammalian cells with hygromycin.

The vector contains a sequence which codes for (in 5' to 3' direction from N-terminal to C-terminal) an ATG translation initiation codon, a series of six histidine residues that function as a metal binding domain in the translated protein, a transcript stabilizing sequence from gene 10 of phage T7, and an enterokinase cleavage recognition sequence. A multiple cloning site positioned downstream of this sequence allows insertion of the foreign gene in the correct reading frame relative to the initiation codon.

The metal binding domain of the fusion peptide allows simple one step purification of recombinant proteins by Immobilized Metal Affinity Chromatography, which is made possible with Invitrogen's ProBond™ resin (available as prepackaged disposable columns or in bulk). The enterokinase cleavage recognition site in the fusion peptide between the metal binding domain and the recombinant protein allows for subsequent removal of this N-terminal fusion peptide from the purified recombinant protein.

2. Cloning into the Expression Vector

In pEBVHis, downstream of the 5' sequences described above, there is a multiple cloning site (MCS) that has nine unique restriction sites: *BamH I*, *Xho I*, *Bgl II*, *Pvu II*, *Kpn I*, *Hind III*, *Not I*, *Sfi I*, and *Cla I* (refer to vector map for details). In order to generate recombinant proteins that include the correct N-terminal fusion peptide, it is necessary to clone the DNA fragment into one of the sites of the MCS in frame with the ATG. To accomplish this there are three different versions of this vector (pEBVHis A, pEBVHis B, and pEBVHis C) that differ only in the spacing between the sequences that code for the N-terminal peptide and the MCS. For proper expression of the sequence, first determine which restriction site is appropriate for ligation, and then choose which vector will preserve the reading frame between the 5' sequences and the insert when ligated into that site. This will vary depending on which restriction site in the MCS is chosen for fragment insertion - not all cloning sites are in the same frame in each vector. Consult the detailed sequence map of pEBVHis A, B, and C provided in this manual.

Choose wisely!

To propagate the pEBVHis expression plasmid, transform the pEBVHis construction into competent *E. coli* strain (see Appendix A for strain selection and transformation procedure). Select for ampicillin-resistant transformants and confirm the correct construction by restriction enzyme analysis and sequencing. For details relating to DNA ligations, *E. coli* transformation, screening for proper inserts, and subsequent manipulations of plasmid DNA, consult *Molecular Cloning: A Laboratory Manual*, Second Edition (1989), J. Sambrook, E. F. Fritsch, and T. Maniatis, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, or another comparable source.

3. Transfection of a Cell Line and Production of Recombinant Protein

The procedure outlined here for introduction of DNA into mammalian cells involves the use of the calcium phosphate transfection method first developed by Graham and van der Ebb (*Virology*, 1973) and later modified by Wigler (*Cell*, 1977). The procedure is routinely used to transfect a wide variety of cell types for either transient expression or production of stable transformants. However the use of other standard transfection methods including DEAE-dextran or liposomes may be substituted. At Invitrogen, the technique of electroporation has not been as efficient as calcium phosphate transfection, probably due to the large size of the vector.

3.1 Calcium Phosphate Transfection

The basis of this method of introduction of DNA into mammalian cells is the formation of a calcium phosphate-DNA precipitate. The precipitate is then added to the cells where the calcium phosphate is thought to facilitate the binding of the DNA to the cell surface. It is thought that the DNA then enters the cell by endocytosis.

The DNA is mixed directly with a concentrated solution of CaCl_2 . This solution is then added dropwise to a phosphate buffer to form a fine precipitate. Aeration of the phosphate buffer, while adding the DNA- CaCl_2 solution, helps to ensure that the precipitate which forms is as fine as possible. This is important because clumped DNA will not adhere to or enter the cell as efficiently.

3.1.1 General Considerations

- Generally, use a final CaCl_2 concentration of 60mM for calcium phosphate transfections.
- The final volume used is not critical but should not exceed 1/10th of the volume of media in which the cells are plated.
- Transfections can be carried out in either 60mm or 100mm dishes.
- Cells should be seeded at a density such that on the day of transfection they are no more than 50% confluent. The optimal seeding density produces a nearly confluent dish of cells when they are harvested or split into selective media 48 hours after the transfection. This will vary for each cell line and is dependent upon their doubling time.
- Generally, seed cells at a density of 5×10^5 /60mm dish or $1-2 \times 10^6$ /100mm dish.
- As a rule, transfect no less than 10 μg of DNA (as much as 100 μg can be used).
- Transfection efficiencies may be increased in some cell lines when the transfection is followed by either a glycerol (3-4) or Dimethylsulfoxide [DMSO] shock (4-6). These chemicals are toxic to cells and thus conditions for individual cell types must be optimized. The exact mechanism by which these chemicals work is unknown, but it may involve an alteration of the cell membrane to further facilitate DNA uptake.

3.1.2 Standard Transfection Procedure

Day 1

Plate cells in 100mm or 60mm dishes at the required density. Incubate overnight at 37°C in a humidified CO_2 incubator.

Day 2

1. Change the media on the dishes 3-4 hours prior to transfection.

2. Transfection Mixture:

For a 100mm dish containing 10ml of media:

To a tube labeled A add:

36µl 2M CaCl₂

20µg DNA

Volume to 300µl with Sterile H₂O

To a tube labeled B add;

300µl of 2x Hepes Buffered Saline (HBS), pH 7.1

For a 60mm dish containing 5ml of media:

To a tube labeled A add:

18µl 2M CaCl₂

10µg DNA

Volume to 150µl with Sterile H₂O

To a tube labeled B add;

150µl of 2x Hepes Buffered Saline (HBS), pH 7.1

Note: The pH of the 2x Hepes Buffered Saline must lie between 7.05 and 7.12 if a precipitate is to form.

3. Using a pasteur pipette, slowly add A to B dropwise while bubbling air (filtered with an auto pipettor or from a sterile air tank) through solution B. This process should take at least 1-2 minutes.
4. A fine precipitate should be formed. Incubate the tube at room temperature for 30 minutes.
5. Add the precipitate dropwise through the media to the cells in either a 60mm or 100mm dish. Gently rock the plate back and forth (do **not** move the plate in a circular motion).
6. Incubate overnight at 37°C in a humidified CO₂ incubator.

Day 3

1. Remove the media from the cells.
2. Wash the cells twice with Phosphate Buffered Saline (PBS).
Optional: If a glycerol or DMSO shock is to be carried out, proceed with it now.
3. Add fresh media and incubate the dishes at 37°C in a humidified CO₂ incubator for a further 24-48 hours.

Glycerol Shock:

1. Prepare a fresh 15% glycerol shock solution in 1x HBS.
2. Wash the cells once with 1x PBS.
3. Add 2ml of glycerol solution per 60mm dish or 3ml per 100mm dish.
4. Incubate the dishes at room temperature for exactly 2 minutes and remove the glycerol (if multiple plates are being done, stagger them so that the glycerol can be removed from each plate after exactly 2 minutes at room temperature).
5. Wash once with 1x PBS.
6. Add fresh media and incubate the dishes at 37°C in a humidified CO₂ incubator for a further 24-48 hours.

DMSO Shock:

1. Prepare a fresh stock of 10% DMSO in 1x PBS.
2. Remove the media from the plate and add 2ml of DMSO solution per 60mm dish or 3ml per 100mm dish.
3. Incubate the dishes at room temperature for exactly 2.5 minutes and remove the DMSO (if multiple plates are being done, stagger them so that the DMSO can be removed from each plate after exactly 2.5 minutes at room temperature).
4. Add fresh media.
5. Incubate the dishes at 37°C in a humidified CO₂ incubator for a further 24-48 hours.

3.2 Selection of Stable Transformants

In generating stable long-term clones it is necessary to select for individual clones in which the plasmid is replicating episomally or in which the DNA has integrated into the chromosome. Since the vectors carry the hygromycin B drug resistance marker, this is accomplished by selection with hygromycin.

The concentration of the drug used for selection will vary with the cell line used and should be determined before the experiment is undertaken.

Growth of the cells in increasing concentrations of hygromycin, typically in the range of 200-800 μ g will enable you to plot a killing curve for that cell line. The optimal concentration is one that kills the cells within 10 to 14 days.

3.2.1 Hygromycin Selection

Basis for selection:

Hygromycin-B is an aminocyclitol that inhibits protein synthesis by disrupting translocation and promoting mistranslation. The Hygromycin-B-Phosphotransferase (HPH) gene (isolated from *E.coli* plasmid pJR225; Gritz and Davies, 1983) detoxifies hygromycin-B by phosphorylation.

The HPH gene has only recently been used in mammalian systems and vectors which efficiently express the gene are not widespread. The level of hygromycin-B needed for selection can vary from 10 to 400 μ g/ml with many cell lines requiring 200 μ g/ml.

Determining Hygromycin Concentration for Selection:

1. Prepare the mock-transfected cells.
2. Seed the cells at low-density confluence (2.5% for fast growing cell lines and up to 10% for slow growing cell lines) in six well dishes.
3. Add complete media with a hygromycin concentration ranging from 0 to 600 μ g/ml at 50 to 100 μ g increments.
4. Change the media every 4 days.
5. After 10-14 days, assess the cell viability. Visual inspection will usually suffice, but trypan blue may be used.
6. Choose the lowest concentration of hygromycin that resulted in complete killing.

Selection with Hygromycin:

1. Maintain the cells in non-selective media for 48 hours post-transfection.
2. Remove the media and replace with media containing hygromycin at the concentration determined to be optimal for the cell line.
3. Maintain the selection for 2-3 weeks, changing the media every 2-3 days to eliminate dead cells, until discreet colonies can be visualized.
4. Remove the media and wash the cells twice in 1x PBS.
5. Harvest the cells by treatment with trypsin-EDTA for 2-5 minutes.

Note: The production of single clonal derivatives as cell lines has not been found to be necessary.

Should you wish to do so however, detailed description of procedures used for selection of individual stable clones is described in References 8 and 9.

6. Add media containing 10% Fetal Bovine Serum to inactivate the trypsin. Transfer the cells to a sterile microcentrifuge tube.
7. Pellet the cells in a microcentrifuge at 1500 rpm for 5 minutes.
8. Replate all of the cells in T-75 flasks in media without hygromycin and allow them to attach. The time necessary for the cells to attach varies for each cell line, but as a general rule, most cells will attach within 3 hours after plating.

9. Following cell attachment, remove the media and replace with selective media.
10. Maintain the cells in selective media until they form a confluent monolayer. Harvest the cells as described above and expand in several T-75 flasks in selective media. Once a stable hygromycin resistant cell line is established in several flasks, prepare frozen stocks.

3.2.2 Confirmation of the Presence of an Episomally Replicating Vector in the Cell Line

To ensure that the cell line generated contains the plasmid of interest and that it is replicating episomally, a simple alkaline lysis miniprep of the mammalian cells will produce sufficient DNA to allow visualization on an agarose gel and transformation into bacteria. The DNA can then be isolated from the bacterial transformants for analysis by restriction enzyme digestion.

Procedures for alkaline lysis minipreps and chemical transformation are outlined in Appendix G and B, respectively.

3.3 Production of Recombinant Protein

1. Seed cells in either five T-75 flasks or two to three T-150 flasks.
2. Grow the cells in selective media until they are 80-90% confluent.
3. Harvest the cells by trypsinization as described in Section 3.2.1.
4. The pellet of cells can then be frozen on liquid nitrogen and stored at -70°C until needed. Each cell pellet should contain 5×10^6 - 1×10^7 cells.

4. Purification of Recombinant Protein

The recombinant Xpress protein produced by expression from pEBVHis contains six tandem histidine residues in the N-terminal peptide which have a high affinity for Invitrogen's ProBond™ resin. Xpress proteins may be isolated under either native or denaturing conditions. Optimal purification parameters will vary with each protein being purified. In general, Xpress recombinant proteins will bind to ProBond™ in 20mM phosphate buffer, 500mM NaCl pH 7.8 (\pm 8M Urea), while the majority of the host cell proteins will not bind to the ProBond™ resin. Host cell proteins which do have some affinity for the ProBond™ resin under these binding conditions can be removed by washing the protein-bound ProBond™ resin with a lower pH buffer (e.g. pH 6.0). The Xpress recombinant protein is eluted from ProBond™ by washing with pH 4.0 buffer in denaturing conditions or with an imidazole gradient in pH 6.0 buffer under native conditions. The imidazole elution is the method of choice to competitively displace the polyhistidine-containing fusion proteins from the matrix if the expressed protein is unstable or denatures at low pH.

The **Xpress System™** has been designed to provide the researcher with a convenient, one step purification of recombinant proteins. However, due to the unique characteristics of each individual protein being purified, some difficulties may be encountered in reaching the level of purity that suits the individual needs of the researcher. In these cases, the **Xpress System™** can provide the initial parameters for the development of a purification scheme for a particular application, as well as provide a convenient first step toward scale-up. If the development of a customized purification regime is necessary, an Appendix is included which provides the formulations of all the buffers required to perform the separation, as well as suggestions for modifying the protocol.

4.1 Buffer Preparation

Begin by diluting the kit-provided Stock Solution A (10x) and Stock Solution B (10x) 1:10 with sterile distilled water. Prepare 100ml each of 1x Stock Solution A and 1x Stock Solution B for each purification.

Native Binding Buffer:

20mM Sodium Phosphate
500mM Sodium Chloride
pH 7.8

For 50ml: Combine 2.9ml of 1x Stock Solution A with 47.1ml of 1x Stock Solution B. Adjust the pH to 7.8 by using 1x Stock Solution B to raise and 1x Stock Solution A to lower the pH. This buffer is used as the binding buffer in the native purification protocol.

Native Wash Buffer:

20mM Sodium Phosphate
500mM Sodium Chloride
pH 6.0

For 50ml: Combine 37ml of 1x Stock Solution A with 13ml of 1x Stock Solution B. Adjust the pH to 6.0 by using 1x Stock Solution B to raise and 1x Stock Solution A to lower the pH. This buffer is used as the wash buffer in the native purification protocol.

Native-Imidazole Elution Buffers:

To generate the four imidazole elution buffers used for the imidazole gradient combine the following volumes of Native Wash Buffer and 3M Imidazole.

	3M Imidazole	Wash Buffer
50mM	0.08ml	4.92ml
200mM	0.33ml	4.67ml
350mM	0.58ml	4.42ml
500mM	0.83ml	4.17ml

Guanidinium Lysis Buffer:

This buffer is supplied in the Xpress Purification Kit. Check the pH and adjust it to 7.8 using 1N NaOH or 1N HCl if necessary. This buffer is used to lyse cells under denaturing conditions.

Denaturing Binding Buffer:

This buffer is supplied in the Xpress Purification Kit. Check the pH and adjust it to 7.8 using 1N NaOH or 1N HCl if necessary. This buffer is used as the binding buffer for purification under denaturing conditions.

Denaturing Wash Buffer 6.0:

Adjust the pH of a 10ml aliquot of the kit-supplied Denaturing Wash Buffer to 6.0 using 1N NaOH or 1N HCl. This buffer is the low-stringency wash under denaturing conditions.

Denaturing Wash Buffer 5.3:

Adjust the pH of a 10ml aliquot of the kit-supplied Denaturing Wash Buffer to 5.3 using 1N NaOH or 1N HCl. This buffer is the high-stringency wash under denaturing conditions.

Denaturing Elution Buffer:

Adjust the pH of a 10ml aliquot of the kit-supplied Denaturing Wash Buffer to 4.0 using 1N NaOH or 1N HCl. This buffer is the elution buffer for purification under denaturing conditions.

4.2 Preparation of ProBond™ Columns

Note: Do not use DTT with ProBond™ columns. DTT destroys the resin. In addition, do not use EDTA in the loading buffers or wash buffers as it strips the nickel from the columns.

1. Remove one spin column from the kit and resuspend the resin by inverting and gently tapping the column repeatedly.
2. Place the column into a Falcon 2059 tube (or equivalent) and centrifuge at 800 x g in a swinging bucket rotor for 2 minutes to pack the resin. Alternatively, clamp the column in a vertical position and allow the resin to settle completely by gravity (5 to 10 minutes).
3. Carefully remove the column. Take the top closure off and gently aspirate the buffer, being careful not to remove any of the resin. Add 7ml of sterile, distilled water and replace the top closure. Resuspend the resin as in Step 1 by alternately inverting and gently tapping the column.
4. Centrifuge the column at 800 x g in a swinging bucket rotor for two minutes or clamp in a vertical position and allow the resin to settle completely. Gently aspirate the water. Repeat this water wash one additional time.
5. If a native purification is to be done, add 7ml of Native Binding Buffer; or for purifications done under denaturing conditions, add 7ml Denaturing Binding Buffer. Resuspend the resin as described in Step 1. Repeat the centrifugation or gravity settling and remove the buffer by aspiration. Repeat this Native Binding Buffer or Denaturing Binding Buffer wash two additional times. The column is now equilibrated and is ready for sample application.

4.3 Preparation of Cell Lysates/Sample Application

1. If the recombinant protein is to be isolated under native conditions, resuspend the cell pellet (generated in Section 3.3) in 4ml of Native Binding Buffer. Addition of protease inhibitors such as bestatin and leupeptin may be necessary depending on the cell line and expressed protein. Lyse the cells by two freeze-thaw cycles using a liquid nitrogen or dry ice/ethanol bath and a 42°C water bath. Shear the DNA by passing the preparation through an 18-gauge needle four times.
If the protein is to be isolated under denaturing conditions, resuspend the cell pellet in 4ml of Guanidinium Lysis Buffer and pass the preparation through an 18-gauge needle four times.
2. Batch bind protein to be purified by resuspending the pre-equilibrated column with the 4ml lysate solution. Gently rock the column for 10 minutes to keep the resin resuspended and allow the poly His containing protein to fully bind. Settle the resin by gravity or low speed centrifugation and carefully aspirate the supernatant.

4.4 Column Washing and Elution Under Native Conditions

1. Wash the column with 4ml of Native Binding Buffer by resuspending the resin, rocking for two minutes and then separating the resin from the supernatant by gravity or centrifugation.
2. Wash the column with 4ml of Native Washing Buffer by resuspending the resin, rocking for two minutes and then separating the resin from the supernatant by gravity or centrifugation. Repeat the Native Washing procedure two more times for a total of three washes.
3. Clamp the column in a vertical position and snap off the cap on the lower end. Elute the protein by applying consecutively 5ml of each of the four Imidazole Elution Buffers in increasing imidazole concentration (i.e. 50mM, 200mM, 350mM, 500mM). Collect 1ml fractions. Monitor the elution by taking OD₂₈₀ readings of the fractions. Pool the fractions which contain the peak absorbance and concentrate them by any standard method (i.e. commercially available 10,000 MW cutoff low-protein binding centrifugal units or vacuum concentration units). An alternative procedure which usually requires less time is to do the four imidazole elutions in batch mode. Sequentially resuspend the resin in the increasing imidazole Native Elution Buffers, gently rock for 5 minutes, then separate the resin from the supernatant by gravity or centrifugation. Save all of the supernatants and assay them for the presence of the desired protein. One drawback to the batch elution method is that the purified protein is recovered in a larger volume than in the drip column method.

Note: It may be desirable to collect all of the flow through fractions for analysis after purification.

4.5. Column Washing and Elution Under Denaturing Conditions

1. Wash the column with 4ml of Denaturing Binding Buffer by resuspending the resin, rocking for two minutes and then separating the resin from the supernatant by gravity or centrifugation.
2. Wash the column with 4ml of Denaturing Washing Buffer 6.0 by resuspending the resin, rocking for two minutes and then separating the resin from the supernatant by gravity or centrifugation. Repeat this wash procedure once more for a total of two pH 6.0 washes.
3. Wash the column with 4ml of Denaturing Washing Buffer 5.3 by resuspending the resin, rocking for two minutes and then separating the resin from the supernatant by gravity or centrifugation. Repeat this wash procedure once more for a total of two pH 5.3 washes.
4. Clamp the column in a vertical position and snap off the cap on the lower end. Elute the protein by applying 5ml of Denaturing Elution Buffer. Collect 1ml fractions and monitor the elution by taking OD₂₈₀ readings of the fractions. Pool the fractions which contain the peak absorbance and dialyze against 10mM Tris, pH 8.0, 0.1% Triton X-100 overnight at 4°C to remove the urea. Concentrate the dialyzed material by any standard method (i.e. commercially available 10,000 MW cut off low-protein binding centrifugal units or vacuum concentration units).

Note: It may be desirable to collect all of the flow through fractions for analysis after purification.

4.6 Cleavage Of The Fusion Peptide Using Enterokinase

Digestion of Xpress™ Fusion Proteins with Enterokinase

After obtaining purified recombinant fusion protein, you may wish to cleave the Xpress™ tag away from your protein. Invitrogen has a recombinant preparation of the catalytic subunit of enterokinase (EnterokinaseMax™). This enzyme recognizes -Asp-Asp-Asp-Asp-Lys and cleaves after the lysine. It has high specific activity, leading to more efficient cleavage and requires less enzyme. For more information, please contact Technical Service, 1-800-955-6288 (U. S. and Canada) or +31 (0) 594 515 175 (Europe).

Description	Catalog no.
EnterokinaseMax™, 250 units	E180-01
EnterokinaseMax™, 1000 units	E180-02

5. Troubleshooting the Xpress System™ Protein Purification

Problem	Probable Cause	Possible Solution
<ul style="list-style-type: none"> No recombinant protein recovered following elution 	<ul style="list-style-type: none"> Nothing bound because of protein "folding" 	<ul style="list-style-type: none"> Try denaturing conditions
	<ul style="list-style-type: none"> Expression levels too low Too stringent washing 	<ul style="list-style-type: none"> Check expression levels Raise pH of wash buffer in high-stringency wash step. Wash less extensively in high-stringency wash step
	<ul style="list-style-type: none"> Not enough sample loaded 	<ul style="list-style-type: none"> Increase amount of sample loaded or lysate used
	<ul style="list-style-type: none"> Recombinant protein has very high affinity for ProBond™ resin 	<ul style="list-style-type: none"> Increase stringency of elution (decrease pH or increase imidazole)
<ul style="list-style-type: none"> Good recombinant-protein recovery but contaminated with non-recombinant proteins 	<ul style="list-style-type: none"> Wash conditions not stringent enough 	<ul style="list-style-type: none"> Lower pH of wash buffer in high-stringency wash step. Wash more extensively in proteins high-stringency wash step
<ul style="list-style-type: none"> Good recombinant-protein recovery but contaminated with non-recombinant proteins 	<ul style="list-style-type: none"> Recombinant protein has low affinity for resin comes off in wash with many contaminating proteins 	<ul style="list-style-type: none"> Try denaturing conditions Try an imidazole step gradient elution Try a pH gradient with decreasing pH
<ul style="list-style-type: none"> Low recombinant protein-recovery and contaminated with non-recombinant proteins 	<ul style="list-style-type: none"> Recombinant protein not binding tightly to resin 	<ul style="list-style-type: none"> Try denaturing conditions Try "reverse-chromatography": bind lysate, including recombinant protein; allow recombinant protein to come off in low stringency washes; collect these fractions; re-do chromatography on saved fractions on new or stripped and recharged column. Works for native purification only.
<ul style="list-style-type: none"> Low recombinant protein recovery and contaminated with non-recombinant proteins 	<ul style="list-style-type: none"> Expression levels too low 	<ul style="list-style-type: none"> Consider an additional high stringency wash at a lower pH (i.e., between pH 6 and pH 4) before elution step
<ul style="list-style-type: none"> Column turns reddish brown 	<ul style="list-style-type: none"> DTT is present in of the buffers 	<ul style="list-style-type: none"> Use β-Mercaptoethanol as a reducing agent

6. Appendix

A. Strain

E. coli strain TOP10

F⁻, *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL endA1 nupG*

Strain Selection

The *E. coli* strain TOP10 has been provided with the Xpress™ Kit and is recommended for propagation of the pEBVHis vector. *E. coli* strains with comparable genotypes may be substituted.

B. Protocol for the Preparation and Transformation of Competent *E. coli*

1. Take the TOP10 stab provided and streak out a small portion of it on an LB media plate (without ampicillin). Incubate at 37°C overnight or longer. Store the stab at 4°C in the dark - it should stay viable for several months. It is also advisable to prepare a frozen glycerol stock for long-term storage (see Appendix C for freezing instructions).
2. Pick a single colony and transfer it into 100ml of SOB media in a 1 liter flask (see Appendix D for media recipes). Incubate the flask for approximately 3 hours at 37°C with vigorous shaking (> 200 cycles/minute in a rotary shaking incubator). Monitor growth by determining the OD₆₀₀.
3. When the OD₆₀₀ of the culture reaches approximately 0.5, collect the cells by centrifugation (4000 rpm, 4°C, 10 minutes, Sorvall GSA rotor, or equivalent) and resuspend the pellet in 10ml ice-cold 50mM CaCl₂. Incubate cells on ice for at least 30 minutes.
4. Centrifuge the CaCl₂-treated cells in a 4°C rotor (4000 rpm, 4°C, 5 minutes, Sorvall SS-34 rotor, or equivalent). Gently resuspend the cells in 4ml of ice-cold 50mM CaCl₂. Keep the cells on ice.
5. Aliquot 100μl of CaCl₂-treated cells for each transformation into a pre-chilled (on ice) Falcon 2059 tube (or equivalent). Add transforming DNA (10 to 100ng) and incubate on ice for 30 minutes.
6. After 30 minutes on ice, heat shock the cells at 42°C for 45 seconds (in a water bath). After the heat shock, return the tube(s) to ice for an additional 2 minutes.
7. Add 1ml of SOC media and incubate the cultures for 45 minutes at 37°C with vigorous shaking (> 200 cycles/minute in a rotary shaking incubator).
8. Plate appropriate amounts of cells onto SOB or LB plates containing ampicillin (50μg/ml).

C. Making Frozen Glycerol Stock of *E. coli* Strain

Grow 1 to 2ml of the strain to be frozen in rich bacterial media (e.g. SOB) overnight with antibiotic selection when appropriate. Combine 0.85ml of the overnight culture with 0.15ml of sterile glycerol (sterilized by autoclaving). Mix the culture well by vortexing. Transfer to an appropriate freezing vial (preferably, a screw cap, air-tight gasket). Freeze in an ethanol-dry ice bath or liquid nitrogen and then transfer to -70°C for long-term storage.

D. Media and Solution Recipes

SOB (For 1 Liter)

To 950ml of deionized water add:

20.0g	Tryptone
5.0g	Yeast Extract
0.5g	NaCl
186.0mg	KCl

Mix the solution until dissolved. Adjust the pH to 7.0 with 5N NaOH (approximately 0.2ml). If making solid media (for plates or top agar), add 15g of agar after adjusting the pH. Adjust the volume to 1000ml and sterilize by autoclaving. Once autoclaved, add 10ml of sterile 2M Mg⁺⁺ (e.g. 10ml of either sterile 1M MgCl₂ or sterile 1M MgSO₄).

SOC (For 1 Liter)

Follow recipe as per SOB. After autoclaving, let cool to about 60°C (can be touched by hand) and add 10ml of 50% glucose. Mix the media well.

LB (For 1 Liter)

Component	liquid	plates	top agar
Tryptone	10g	10g	10g
Yeast Extract	5g	5g	5g
NaCl	10g	10g	10g
Agar	-----	15g	7g

Combine the tryptone, yeast extract, and NaCl with 950ml of deionized water. Mix the solution until dissolved. Adjust the pH to 7.0 with 5N NaOH (will take about 0.2ml). If making solid media (for plates or top agar) add the appropriate amount of agar after adjusting the pH. Adjust volume to 1 liter with water. Sterilize by autoclaving.

Ampicillin

Prepare a stock solution of 50mg/ml in deionized water and filter sterilize it with a 0.22 μ filter. To select for plasmids that confer ampicillin-resistance, cool media to ~ 50°C, add 1ml of the ampicillin stock per liter of media (both liquid and solid). Store the stock solution at -20°C.

50mM CaCl₂

(calcium chloride, MW = 111) For 100ml of a 50mM solution, combine 0.56g of anhydrous CaCl₂ and 100ml of deionized water. Filter sterilize (0.22 μ m filter). Use this solution ice cold for competent cell preparation.

E. Purification Reagents

Stock Solution A (10x):

200mM monobasic sodium phosphate (NaH_2PO_4), 5M NaCl. Prepare by dissolving 27.6g of monobasic sodium phosphate and 292.9g of NaCl in 1000ml of deionized water.

Stock Solution B (10x):

200mM dibasic sodium phosphate (Na_2HPO_4), 5M NaCl. Prepare by dissolving 28.4g of dibasic sodium phosphate and 292.9g of NaCl in 1000ml of deionized water.

3M Imidazole Buffer (10x):

500mM NaCl, 20mM NaPO_4 buffer, pH 6.0. Prepare an additional 100ml of this solution by combining 20.6g of imidazole, 8.77ml of Stock Solution A, 1.23ml of Stock Solution B, and water to 90ml. Adjust the pH to 6.0 with HCl or NaOH as necessary. Bring the final volume to 100ml with water. If the solution forms a precipitate, heat it until the precipitate dissolves.

Phosphate Buffers.

Mixing different predetermined amounts of 1x Stock Solution A and 1x Stock Solution B is the method for making phosphate buffers of differing pH. The following table gives the appropriate amounts of 1x Stock Solution A and 1x Stock Solution B needed to arrive at pH values ranging from 7.8 to 4.0. Mixing the designated amount of each stock solution will give 100ml of 20mM phosphate buffer that is also 500mM NaCl. This information is provided to allow the researcher to modify the pH of the buffers used, if necessary, to meet the needs of specific application.

For 100ml of 20mM Phosphate Buffer 500mM NaCl

<u>ml sol'n A</u>	<u>ml sol'n B</u>	<u>predicted pH</u>	<u>ml Sol'n A</u>	<u>ml Sol'n B</u>	<u>predicted pH</u>
100	0	4.0	40.8	59.2	6.7
95.4	4.6	5.0	36.8	63.2	6.8
91.7	8.3	5.3	31.9	68.1	6.9
87.1	12.9	5.5	27.7	72.3	7.0
81.2	18.8	5.8	19.9	80.1	7.1
77.4	22.6	5.9	17.2	82.8	7.2
73.8	26.2	6.0	14.5	85.5	7.3
70.0	30.0	6.1	12.1	87.9	7.4
65.7	34.3	6.2	13.1	89.6	7.5
61.1	38.9	6.3	8.6	91.4	7.6
55.6	44.4	6.4	7.1	92.9	7.7
65.5	48.9	6.5	5.8	94.2	7.8
46.3	53.7	6.6			

Note: These data are based on titration of 1x Native Buffers A and B in nanopure water. Some variation may occur in which case A and B should be titrated to the proper pH empirically.

Guanidinium Lysis Buffer:

6M Guanidine Hydrochloride
20mM Sodium Phosphate
500mM Sodium Chloride
pH 7.8

For 100ml: Combine 0.58ml of Stock Solution A (10x) with 9.42ml of Stock Solution B (10x). Add 57.3g Guanidine Hydrochloride and H_2O to 90ml. Stir the solution until completely dissolved. Adjust the pH to 7.8 using 1N NaOH or 1N HCl. Bring the volume to 100ml and filter sterilize the buffer using a 0.45 μm filter (autoclaving has been found to alter the pH of the buffer).

Denaturing Binding Buffer:

8M Urea
20mM Sodium Phosphate
500mM Sodium Chloride
pH 7.8

For 100ml: Combine 0.58ml of Stock Solution A (10x) with 9.42ml of Stock Solution B (10x). Add 48.1g of Urea and H₂O to 90ml. Stir the solution with gentle heating (50 to 60°C maximum temperature - do not overheat) until completely dissolved. When cooled to room temperature, adjust the pH to 7.8 using 1N NaOH or 1N HCl. Bring the volume to 100ml and filter sterilize the buffer using a 0.45µm filter (autoclaving has been found to alter the pH of the buffer).

Denaturing Wash Buffer 6.0:

8M Urea
20mM Sodium Phosphate
500mM Sodium Chloride
pH 6.0

For 100ml: Combine 7.38ml of Stock Solution A (10x) with 2.62ml of Stock Solution B (10x). Add 48.1g of Urea and H₂O to 90ml. Stir the solution with gentle heating (50 to 60°C maximum temperature - do not overheat) until completely dissolved. Adjust the pH to 6.0 using 1N NaOH or 1N HCl. Bring the volume to 100ml and filter sterilize the buffer using a 0.45µm filter (autoclaving has been found to alter the pH of the buffer).

Denaturing Wash Buffer 5.3:

8M Urea
20mM Sodium Phosphate
500mM Sodium Chloride
pH 5.3

For 100ml: Combine 9.17ml of Stock Solution A (10x) with 0.83ml of Stock Solution B (10x). Add 48.1g of Urea and H₂O to 90ml. Stir the solution with gentle heating (50 to 60°C maximum temperature - do not overheat) until completely dissolved. Adjust the pH to 5.3 using 1N NaOH or 1N HCl. Bring the volume to 100ml and filter sterilize the buffer using a 0.45µm filter (autoclaving has been found to alter the pH of the buffer).

Denaturing Elution Buffer:

8M Urea
20mM Sodium Phosphate
500mM Sodium Chloride
pH 4.0

For 100ml: Combine 10ml of Stock Solution A (10x) with 48.1g of Urea, and H₂O to 90ml. Stir the solution with gentle heating (50 to 60°C maximum temperature - do not overheat) until completely dissolved. Adjust the pH to 4.0 using 1N NaOH or 1N HCl. Bring the volume to 100ml and filter sterilize the buffer using a 0.45µm filter (autoclaving has been found to alter the pH of the buffer).

F. Regeneration of ProBond™ Resin

1. Wash the column two times with 8ml of 50mM EDTA to strip away the chelated nickel ions.
2. Wash the column with 16ml of 0.5M NaOH.
3. Wash the column with 16ml of sterile, distilled water.
4. Recharge the column with 16ml of NiCl₂ (5mg/ml; prepared in sterile, distilled water).
5. Wash the column with 16ml of distilled water.
6. Store the charged column under 20% ethanol until use.

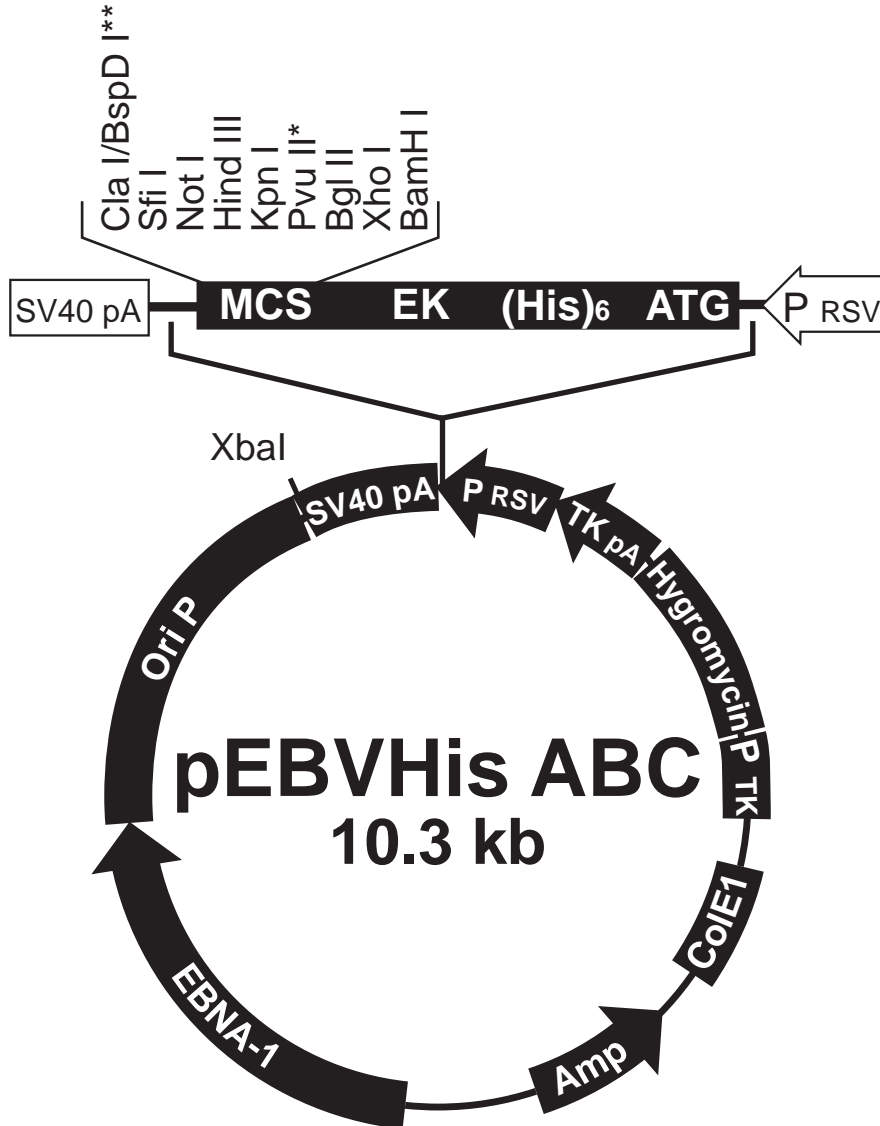
It is recommended that resin is not recharged more than three times and that it is only reused for purification of the same recombinant protein (such as for purification after enterokinase cleavage).

G. Bacterial Alkaline Lysis Miniprep

1. Grow 2ml of bacterial culture (L broth with the appropriate antibiotic) at 37°C overnight in a rotary shaking incubator.
2. Decant 1.5ml of the culture into a microcentrifuge tube and spin it for 10 seconds. Discard the supernatant, leaving 50-100µl of medium in the tube. Vortex the tube to completely resuspend the cells.
3. Add 300µl of TENS solution (10mM Tris-HCl, pH 7.5; 1mM EDTA; 0.1N NaOH; 0.5% SDS) then vortex the tube for 2-5 seconds or until the mixture becomes viscous.
4. Add 150µl of 3M sodium acetate, pH 5.2, then vortex the tube for 2-5 seconds to mix completely.
5. Spin the tube for 2 minutes in a microcentrifuge to pellet the cell debris and the chromosomal DNA. Transfer the supernatant to a fresh microcentrifuge tube, add 900µl of cold 100% ethanol and mix well. Freeze the solution on dry ice.
6. Spin the tube for 5 minutes to pellet the plasmid DNA and the RNA. The pellet should have a white appearance. Discard the supernatant and rinse the pellet twice with 1ml of 70% ethanol. Remove the residual ethanol after another quick spin.
7. Resuspend the pellet for further analysis in 20-50µl of TE buffer, pH8.0 (10mM Tris-HCl, pH 8.0; 1mM EDTA, pH8.0) or sterile water containing RNase A at a concentration of 100µg/ml.

H. pEBVHis Vector Map

The sequence of pEBVHisB is provided after page 17 for your convenience. The sequences of all pEBVHis vectors are available by downloading from our Web site (www.invitrogen.com) or from Technical Service (see front cover page).



Comments for pEBVHis B 10307 nucleotides

SV40 Poly A: bases 6-405
 Multiple cloning site: bases 408-592
 RSV 3' LTR: bases 593-1202
 TK poly A: bases 1367-1738
 Hygromycin resistance gene: bases 1739-2778
 TK promoter: bases 2779-3031
 Ampicillin resistance/Origin of replication: bases 3533-5427
 EBNA-1: bases 5430-8013
 Ori P: bases 8043-10230

* *Pvu* II is not present in the MCS of pEBVHis C.

** *Cla* I and *Bsp* D I are isoschizomers. They are both methyl sensitive and will not cut in most lab *E. coli* strains.

I. pEBVHis MCS Detail

pEBVHis A:

RSV LTR
TGTGACCTCC ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT
Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr **BamH I**
GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAT CGA TGG GGA
Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp Arg Trp Gly
Xho I **Kpn I**
Bgl II **Pvu II** **Hind III** **Not I**
TCC GAG CTC GAG ATC TGC AGC TGG TAC CAT ATG GGA ATT CGA AGC TTG CTA GCG GCC
Ser Glu Leu Glu Ile Cys Ser Trp Tyr His Met Gly Ile Arg Ser Leu Leu Ala Ala
Sfi I **Cla I***
GCT CGA TCG AGG CCG GCA AGG CCG GAT CGA TCC AGA
Ala Arg Ser Arg Pro Ala Arg Pro Asp Arg Ser Arg

pEBVHis B:

RSV LTR
TGTGACCTCC ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT
Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr
BamH I **Xho I**
GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAT CCG AGC TCG
Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp Pro Ser Ser
Kpn I
Bgl II **Pvu II** **Hind III** **Not I**
AGA TCT GCA GCT GGT ACC ATA TGG GAA TTC GAA GCT TGC TAG CGG CCG CTC GAT CGA
Arg Ser Ala Ala Gly Thr Ile Trp Glu Phe Glu Ala Cys ... Arg Pro Leu Asp Arg
Sfi I **Cla I***
GGC CGG CAA GGC CGG ATC GAT CCA GAC
Gly Arg Gln Gly Arg Ile Asp Pro Asp

pEBVHis C:

RSV LTR
TGTGACCTCC ATG GGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT
Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr
BamH I
GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT GAC GAT AAG GAT CGA TGG ATC
Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp Arg Trp Ile
Bgl II
Xho I **Kpn I** **Hind III** **Not I**
CGA CCT CGA GAT CTG CAG ATG GTA CCA TAT GGG AAT TCG AAG CTT GCT AGC GGC CGC
Arg Pro Arg Asp Leu Gln Met Val Pro Tyr Gly Asn Ser Lys Leu Ala Ser Gly Arg
Sfi I **Cla I***
TCG ATC GAG GCC GGC AAG GCC GGA TCG ATC
Ser Ile Glu Ala Gly Lys Ala Gly Ser Ile

* *Cla I* is methylation sensitive.

J. Extraction of Plasmid DNA from Transfected Mammalian Cells by Alkaline Lysis

This protocol is a modification of the bacterial alkaline lysis prep that can be used in place of a Hirt extract. Other resin based miniprep methods have also been found to work well.

1. Take one confluent 100mm plate of cells, remove media and wash two times with 1x PBS.
2. Add 5ml of 1x PBS and use a rubber policeman or similar device to scrape the cells gently off the bottom of the plate.
3. Pellet the cell by centrifugation in a microcentrifuge for 5 minutes at 1500 rpm.
4. Remove the supernatant and resuspend the cells in 1ml of 1x PBS and transfer to a sterile microcentrifuge tube.
5. Pellet the cells by centrifugation (as in Step 3), resuspend the pellet in 100 μ l of TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA) and incubate on ice for 5 minutes.
6. Add 200 μ l of freshly made 0.2N NaOH, 1% SDS mix by inversion then incubate on ice for 5 minutes.
7. Add 150 μ l of 3M Sodium Acetate, mix by inversion and incubate on ice for 5 minutes.
8. Pellet the cell debris by centrifugation in a microcentrifuge for 5 minutes at 1500 rpm.
9. Remove the supernatant to a fresh tube, add RNase A to a final concentration of 20ng/ml and incubate at 37°C for 30 minutes.
10. Extract with an equal volume of phenol-chloroform, spin for 5 minutes and then transfer the supernatant to a fresh tube.
11. Add 1ml of ice-cold 100% ethanol and incubate the tube on dry ice for 30 minutes.
12. Spin in a microcentrifuge for 15 minutes at 15,000 rpm. Wash with 200 μ l of 70% ethanol, spin for 5 minutes. Remove the ethanol and briefly spin. Remove any remaining traces of ethanol.
13. Resuspend the pellet in 40 μ l of sterile water.
14. With high copy numbers, 5 μ l of the DNA should be readily visualized on a 0.8% agarose gel, otherwise, transformation into bacteria is necessary for further use.

K. References

- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1991) *Current Protocols in Molecular Biology Vol. 1*. John Wiley and Sons, New York.
- Blochlinger, K. and Diggelmann, H. (1984) *Mol. Cell Biol.* **4**: 2929.
- Frost, E. and Williams, J. (1978) *Virology* **91**: 39.
- Goeddel, David V. ed. (1991) "Expression in Mammalian Cells." *Methods in Entymology Vol. 185*. Academic Press, San Diego, California.
- Graham, F.L. and van der Ebb, A.J. (1973) *Virology* **52**: 456.
- Laemmli, U.K. (1970) *Nature* **227**: 680-685.
- Lopata, M.A., Cleveland, D.W. and Sollner-Webb, B. (1984) *Nucl. Acid Res.* **12**: 5707.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. *J. Biol. Chem.*, **193**: 265-275.
- Lowy, D.R., Rands, E. and Scolnick, E.M. (1978) *J. Virology* **26**: 291.
- Lewis, W.H., *et al.*, (1980) *Somat. Cell Genet.* **6**: 333.
- Maniatis, T., Fritsch, E.F. and Sambrook, M.D. (1989) *Molecular Cloning (A Laboratory Manual)*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Wigler, M. *et al.*, (1977) *Cell* **11**: 223.
- Zhou *et al.*, (1990). *Biotechniques*, **8(2)**: 172.