User Manual

His60 Ni Superflow Resin & Gravity Columns User Manual

Clontech

United States/Canada
800.662.2566
Asia Pacific
+1.650.919.7300
Europe
+33.(0)1.3904.6880
Japan
+81.(0)77.543.6116

Clontech Laboratories, Inc.
A Takara Bio Company
1290 Terra Bella Ave.
Mountain View, CA 94043
Technical Support (US)
E-mail: tech@clontech.com
www.clontech.com

NOTE FOR FIRST TIME USERS:

For **optimal results**, please **follow the written protocol** when performing the first purification. Each resin (e.g. Ni-IDA, Ni-NTA, Ni-TED, TALON) has a different chemistry. Optimal conditions for one resin are not optimal for another resin.

If you need to modify the protocol, please refer to Table II for compatible reagents and possible effects on the resin.

Cat. Nos. 635657, 635658, 635659, 635660, 635661, 635662, 635663, 635664, 635665, 635676, 635677, 631428, 631431

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Contact Us For Assistance	
Customer Service/Ordering:	**Technical Support:**
Telephone: 800.662.2566 (toll-free)	Telephone: 800.662.2566 (toll-free)
Fax: 800.424.1350 (toll-free)	Fax: 650.424.1064
Web: www.clontech.com	Web: www.clontech.com
E-mail: orders@clontech.com	E-mail: tech@clontech.com

I. Introduction

Clontech's **His60 Ni Superflow Resin** is a high-capacity Ni-IDA resin that has been optimized for the efficient purification of expressed his-tagged proteins from bacterial, mammalian, and baculovirus-infected cells. His-tagged proteins are purified from total soluble protein extract utilizing our high capacity His60 Ni resin charged with nickel immobilized onto Superflow 6 agarose beads.The combination of the high density of nickel (II) ion and high flow rates allow the efficient capture of target his-tagged proteins. Up to 60 mg of his-tagged protein can be adsorbed onto 1 ml of His60 Ni resin (data based on purification of AcGFP1).

His60 Ni resin is compatible with batch/gravity-flow applications, as well as with the major automated liquid chromatography systems or manual syringe processing. The resin enables fast, easy, and reproducible chromatographic separations under native or denaturing conditions, and can be regenerated for multiple uses. We recommend that you use one batch of resin to purify multiple preparations of the same protein.

His60 Ni Superflow resin is supplied as a 50% slurry in 20% ethanol and is available in a variety of sizes and in prepacked gravity columns.

The **His60 Ni Gravity Column Purification Kit** provides prepacked **His60 Ni Gravity Columns** (each containing 1 ml of resin), as well as all the buffers needed for protein extraction and purification (also available separately as the **His60 Ni Buffer Set**).

Table I: His60 Ni Superflow Resin Characteristics

Capacity[1] (mg protein/ml resin)	up to 60
Matrix	Superflow
Bead size (µm)	60–160
Maximum linear flow rate (cm/hr)	3,000
Maximum volume flow rate[2] (ml/min)	50
Maximum pressure	140 psi 10 bar 0.97 MPa
pH stability	2–14 (2 hr) 3–14 (24 hr)
Storage	20% ethanol
Storage temperature	4 to 30°C
Protein exclusion limit (Da)	4×10^6

[1]The binding capacity for individual proteins may vary.
[2]Determined on a 5 x 1 cm column.

His60 Ni Protocol Overview

The purification of his-tagged proteins consists of 4 steps: cell lysis, binding, washing, and elution (Figure 1).

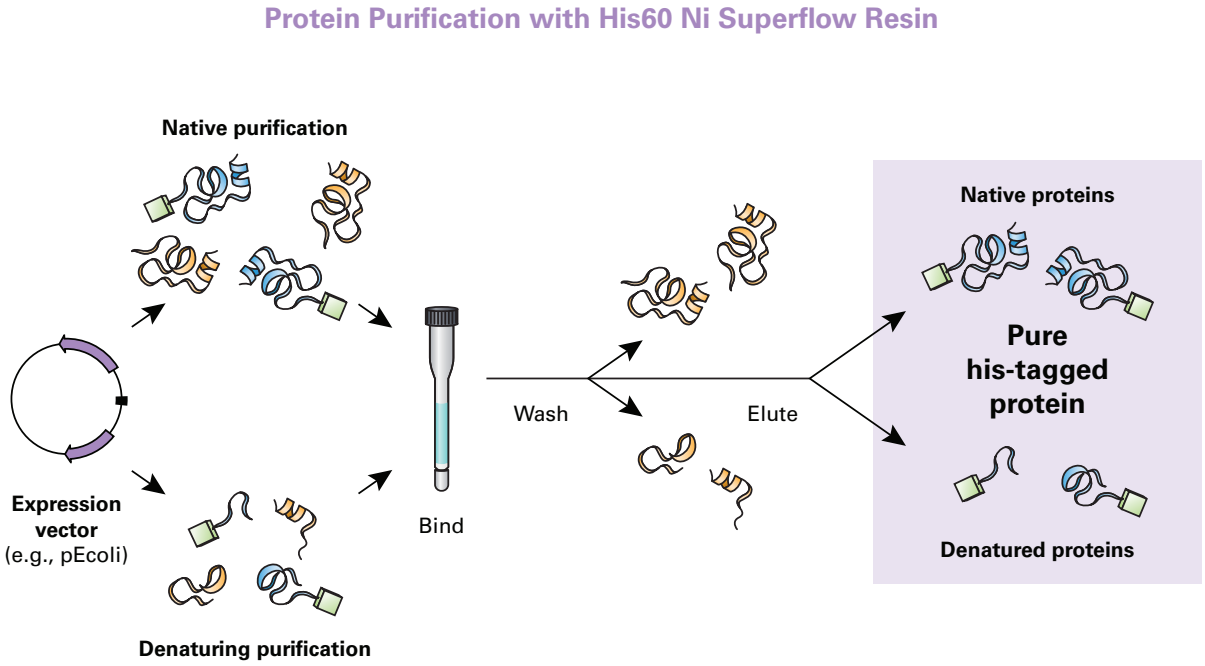

Figure 1. Purification of his-tagged proteins using His60 Ni Superflow Resin.

II. List of Components

Store all components at 4°C.

His60 Ni Gravity Columns (Cat. No. 635657)

- 5 His60 Ni Gravity Columns (1 ml each)

His60 Ni Buffer Set (Cat. No. 635665)

- 2 x 250 ml His60 Ni Equilibration Buffer
- 200 ml His60 Ni Elution Buffer
- 100 ml His60 Ni xTractor Buffer

His60 Ni Gravity Column Purification Kit (Cat. No. 635658)

- 1 His60 Ni Gravity Columns (5 x 1 ml) (Cat. No. 635657)
- 1 His60 Ni Buffer Set (Cat. No. 635665)

His60 Ni Superflow Resin

Amount	Cat. No.
10 ml	635659
25 ml	635660
4 x 25 ml	635661
250 ml	635662
2 x 250 ml	635663
4 x 250 ml	635664

Other

- His60 Ni Superflow Resin & Gravity Column User Manual (PT5017-1)

III. Additional Materials Required

A. Equipment

If you are using His60 Ni Resin for liquid chromatography, you will need the following equipment:

- A suitable liquid chromatography system (LC procedure only) and an appropriate column compatible with the system.

NOTE: For best results, process all buffers through a 0.45 µm filter and degas before use in LC applications.

III. Additional Materials Required continued

B. Buffers—Native Conditions

For your convenience, we provide a separate kit containing a set of His60 Ni Extraction, Equilibration, and Elution Buffers, the **His60 Ni Buffer Set** (Cat. 635665), sufficient for approximately 20 purifications on 1 ml **His60 Ni Gravity Columns** (Cat. 635657). The buffer set and gravity columns are also available together in the **His60 Ni Gravity Column Purification Kit** (Cat. No. 635658)—see Section II.

The following information is provided if you wish to prepare your own buffers for use with other applications. Please note that for FPLC and other automated applications, you need to filter the buffers through a 0.45 µm filter and degas them before use.

- **Equilibration Buffer**: 50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole; pH 7.4
- **Wash Buffer:** 50 mM sodium phosphate, 300 mM sodium chloride, 40 mM imidazole; pH 7.4
  - Wash Buffer is easily made on a binary pump LC system by mixing 7.1 parts of His60 Ni Elution Buffer and 92.9 parts of His60 Ni Equilibration Buffer. This buffer ratio can be achieved by running the LC system at 7.1% Pump B.
  - Alternatively, prepare manually by mixing 710 µl of His60 Ni Elution Buffer with 9.29 ml of His60 Ni Equilibration Buffer.
- **Elution Buffer:** 50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole; pH 7.4
- **Regeneration Buffer:** 20 mM MES (2-(N-morpholine)-ethanesulfonic acid), 0.3 M sodium chloride; pH 5.0
- **Imidazole:** Use a highly pure, low-absorbance imidazole ideal for LC applications (Fisher, Product No. BP 305-50).

C. Buffers—Denaturing Conditions—Guanidine-HCl or Urea

Denaturants, such as 5 M guanidine-HCl or 8 M Urea, enhance protein solubility. Because overexpressed proteins in prokaryotic systems are sometimes insoluble, you may need to purify proteins under denaturing conditions. When using high concentrations of guanidine-HCl or urea, protein unfolding takes place. On-column refolding or after elution refolding is protein dependent. When purifying proteins under denaturing conditions, we recommend preparing buffers as indicated below.

Buffers with 6M Guanidine-HCl

- **Equilibration Buffer**: 50 mM sodium phosphate, 6 M guanidine-HCl, 300 mM NaCl, 20 mM imidazole; pH 7.4
- **Wash Buffer:** 50 mM sodium phosphate, 6 M guanidine-HCl, 300 mM NaCl, 40 mM imidazole; pH 7.4
- **Elution Buffer:** 50 mM sodium phosphate, 6 M guanidine-HCl, 300 mM NaCl, 300 mM imidazole; pH 7.4

Buffers with 8M Urea

- **Equilibration Buffer**: 50 mM sodium phosphate, 8 M urea, 300 mM NaCl, 20 mM imidazole; pH 7.4
- **Wash Buffer:** 50 mM sodium phosphate, 8 M urea, 300 mM NaCl, 40 mM imidazole; pH 7.4
- **Elution Buffer:** 50 mM sodium phosphate, 8 M urea, 300 mM NaCl, 300 mM imidazole; pH 7.4

NOTE: Samples containing guanidine-HCl cannot be analyzed by SDS-PAGE. A buffer exchange to a buffer containing urea must be performed before SDS-PAGE analysis. Samples containing urea can be analyzed directly by SDS-PAGE.

D. Enzymes

- **Benzonase** (Sigma, Cat. No. E8263-5KU)
- **Recombinant DNase I** (TaKaRa, Cat. No. 2270A)

E. Optional

- **PD-10 desalting columns** (GE Healthcare, Cat. No. 17-0851-01) to remove excess imidazole from the final sample when required for downstream applications

IV. Related Products: extraction buffers, protease inhibitors, and his-tag antibodies

xTractor Buffer Kit (Cat. No. 635623)

Applications: extraction of insoluble protein from inclusion bodies, efficient extraction of high molecular weight proteins, complete disruption of bacterial cell wall and membrane

- 200 ml xTractor Buffer
- 400 µl DNase I
- 2.5 ml Lysozyme

xTractor Buffer (Cat. Nos. 635656, 635671, 635625)

Applications: bacterial lysis, extraction of proteins from yeast cells without the use of glass beads, mammalian cell pellet extraction, purification of affinity-tagged proteins

Amount	Cat. No.
• 100 ml	635656
• 250 ml	635671
• 500 ml	635625

ProteoGuard EDTA Free Protease Inhibitor Cocktail (Cat. No. 635673)

Complete, easy-to-use protease inhibitor cocktail that is EDTA-free (can be used on IMAC resins without interfering with protein binding).

Amount	Cat. No.
• 10 x 100 µl	635673

Antibodies for detection of tagged proteins

Product	Amount	Cat. No.
• 6xHis mAb-HRP conjugate (albumin-free)	100 µl	631210
• 6xHis Monoclonal Antibody (albumin-free)	200 µg	631212
• 6xHN Polyclonal Antibody	200 µl	631213

V. General Considerations

Use of His60 Ni Gravity Columns and His60 Ni Resin

Please note the following recommendations when using His60 Ni Gravity Columns and His60 Ni Resin:

- Do not use chelator-containing protease inhibitors or other additives, EDTA, or strong reducing agents (see Table II and the note below regarding the use of reducing agents).
- For automated liquid-chromatography (LC) applications, use highly pure, low-absorbance imidazole (Fisher, Product No. BP 305-50). Always filter buffers through a 0.45 µm filter and degas before use.
- His60 Ni allows protein purification under either native or denaturing conditions. The resin is compatible with multiple denaturants and detergents (Table II).

NOTE: Using βME as a reducing agent with His60 Ni Superflow Resin sharply reduces protein yield, however, βME permits high yields at concentrations up to 30 mM with TALON® resin.

V. General Considerations continued

Table II: Reagent Compatibility with His60 Ni Superflow Resin (Based on Literature References)

Reagent	Notes	Acceptable Concentrations
Amino Acids		
Arginine, Glycine, Glutamine		Not recommended
Histidine	Binds to His60 Ni and competes with histidine residues in the his-tag.	Can be used at low concentrations (20 mM) to inhibit nonspecific binding; and, at a higher concentration (up to 100 mM), to elute his-tagged proteins
Buffers		
HEPES, MOPS	Amine groups that are present in these buffers can interact with Ni^{2+} ions, diminishing the resin's binding capacity.	Up to 100 mM (with caution)
Sodium acetate		Up to 100 mM (with caution)
Sodium phosphate		Up to 50 mM can be used
Tris	Coordinates weakly with metal ions, causing a decrease in binding capacity.	Up to 50 mM (with caution). Loss in binding capacity can be seen.
Chelating Agents		
EDTA, EGTA	Will strip metal ions from the resin, resulting in protein elution and a resin color change.	Not recommended
Denaturing Agents		
Gu-HCl	With high concentrations, protein unfolding generally takes place. Protein refolding on-column (or after elution) is protein-dependent.	6 M
Urea	With high concentrations, protein unfolding generally takes place. Protein re-folding on-column (or after elution) is protein dependent.	8 M
Detergents[1]		
CHAPS	Ionic detergents like CHAPS, SDS, and sarkosyl are compatible at concentrations up to 1%. Even at low concentrations you should expect interference with binding.	1% (with caution)
NP-40	Has high absorbance at 280 nm.	2%
SDS	Ionic detergents such as CHAPS, SDS, and sarkosyl are compatible at concentrations up to 1%. Even at low concentrations you should expect interference with binding.	1% (with caution)
Triton X-100	Has high absorbance at 280 nm.	1%
Tween 20		2%
Reducing Agents		
βME	Use the resin immediately after equilibrating with buffers containing βME. Otherwise the resin will change color. Do not store the resin in buffers containing βME. **A slight change in color (yellowing of the resin) will occur.**	20 mM (with caution)
DTT	Since DTT is a reducing agent, low concentrations will reduce the metal ions in His60 Ni Superflow resin. Although enough of these ions may remain unaffected to allow protein purification, please use it with caution. Do at least 20 column volumes of washes, preferably with low concentrations of imidazole (40 mM) to wash out any reduced metal ions.	1 mM (with caution)
DTE		Not recommended
Others		
$MgCl_2$		4 M
$CaCl_2$		5 mM
Ethanol	May precipitate proteins, causing low yields and column clogging.	20%
Glycerol		20%

[1]Detergents cannot be easily removed by buffer exchange.

VI. Sample Preparation & Purification

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Use this procedure to (A) prepare your his-tagged protein sample for (B) purification using batch/gravity-flow or (C) purification using a liquid chromatography system.

Protocol
15 min

A. PROTOCOL: Sample Preparation

Use our His60 Ni xTractor Buffer as follows to prepare your protein sample for either batch/gravity-flow-based purification or purification on a liquid chromatography system:

1. Add 2 ml of His60 Ni xTractor Buffer per 100 mg of cell pellet. Gently pipet up and down until the cell pellet is fully resuspended. We recommend using between 200 mg and 1 g of pellet from *E. coli*- or baculovirus-infected cells expressing the target protein.

NOTE: His60 Ni xTractor Buffer is supplied in the His60 Ni Gravity Column Purification Kit (Cat. No. 635658) and the His60 Ni Buffer Set (Cat. No. 635665). xTractor Buffer suitable for use with His60 Ni products is also supplied separately in 100 ml (Cat. No. 635656) and 500 ml (Cat. No. 635625) sizes, as well as in the xTractor Buffer Kit (Cat. No. 635623).

2. To the resuspended pellet, add 1 µl of Benzonase or DNase I (Section III.C) for every 2 ml of extract (i.e., every 100 mg of cell pellet), and mix gently.
3. Incubate on ice, with intermittent mixing, for 15 min. Centrifuge for 20 min at 10,000 x g at 4°C.
4. Carefully collect the clear supernatant—this is your starting sample.

Protocol
15 min

B. PROTOCOL: Batch/Gravity-Flow Column Purification

This procedure is appropriate for our 1 ml His60 Ni Gravity Columns (Cat. No. 635657), which contain 1 ml of prepacked resin.The required volumes can easily be adjusted using the appropriate scaling factor if a batch/gravity-flow column of different volume is used.

1. Equilibrate the His60 Ni Gravity Column (1 ml) and all buffers to the working temperature. (Perform purifications at room temperature or at 4°C.)

NOTE: Before opening the column, fully suspend the matrix (to prevent loss of resin that may have settled near the top cap).

2. Wash the column with 5–10 column volumes of His60 Ni Equilibration Buffer. Put the bottom stopper on the outlet of the column.
3. Follow the sample preparation instructions in Section VI.A.
4. Add the clarified sample to the column and carefully connect the top stopper to the top of the column. Allow target protein to bind by slowly inverting the column for 1 hour (preferably at 4°C).
5. Install the column in a vertical position and let the resin settle at the bottom of the column.
6. Put a stand containing clean empty tubes under the outlet of the column.
7. Carefully remove the top stopper. Remove the bottom stopper and start collecting 1 ml fractions.
8. Wash the column with 10 column volumes of His60 Ni Equilibration Buffer followed by 10 column volumes of His60 Ni Wash Buffer.

NOTE: If you are using the buffers supplied in the His60 Ni Buffer Set (Cat. No. 635665) or the His60 Ni Gravity Column Purification Kit (Cat. No. 635658), prepare the Wash Buffer by mixing 710 µl of His60 Ni Elution Buffer with 9.29 ml of His60 Ni Equilibration Buffer (see Section III.B).

9. Elute the target protein with approximately 10 column volumes of Elution Buffer and collect 1 ml fractions.

VI. Sample Preparation & Purification continued

10. Monitor protein elution by measuring the absorbance of the fractions at 280 nm or performing a Bradford protein assay. The collected fractions can be analyzed by SDS-PAGE.
11. If necessary for downstream applications, remove excess imidazole by gel filtration on a PD-10 column (Section III.E).
12. The His60 Ni Gravity Column can be quickly regenerated by adding 20 ml of His60 Ni Equilibration Buffer or by washing with 10 column volumes of 20 mM MES, 0.3 M NaCl; pH 5.0 buffer. Regeneration allows the column to be reused to purify the same protein multiple times without significant loss of binding capacity.
13. For extended storage (over 1 week), wash the column with five column volumes of water after each use and store in 20% ethanol. Attach supplied bottom stopper, followed by the top stopper. Store the column at 4°C.

Protocol
15 min

C. PROTOCOL: Purification on a Liquid Chromatography System

1. Pack the resin in an appropriate column according to the manufacturer's recommendations.
2. Equilibrate the column and all buffers to the working temperature. Perform purifications at room temperature or at 4°C.
3. Degas all solutions.

ATTENTION: If you monitor the purification by UV absorbance, you may want to consider preparing the buffers with LC purity grade imidazole (Fisher, Product No. BP 305-50), especially if your protein is expressed at low levels. Otherwise you may not see a distinct peak at 280 nm, since the imidazole in the His60 Ni Buffer Set shows a weak absorbance at that wavelength. If your protein is expressed at higher levels, you can use our His60 Ni Buffer Set (Cat. No. 635665).

4. Set up the LC System as follows:
   a. Prepare the LC system by filling the tubing with buffer. On a binary pump LC system, fill Pump A and B with 50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole; pH 7.4 (His60 Ni Equilibration Buffer) and 50 mM sodium phosphate, 300 mM sodium chloride, 300 mM imidazole; pH 7.4 (His60 Ni Elution Buffer), respectively.
   b. Loosely connect the column to the pump outlet and start pumping His60 Ni Equilibration Buffer at a flow rate of 1 ml/min until a few drops fill in the top inlet.
   c. Pause the pump and tightly connect the column to the pump outlet.
   d. Start the pump. To avoid introducing air into the system, allow a few drops to emerge from the column before connecting to the LC UV monitor inlet port.
5. Equilibrate the column with 5–10 column volumes of His60 Ni Equilibration Buffer or its equivalent with low UV absorbance imidazole (at a flow rate of 1 ml/min for a 1 ml column).
6. For maximum extraction and binding, prepare the sample using our His60 Ni xTractor Buffer (Section V.A). If you used incompatible reagents (Section IV) during the extraction, desalt the sample on a PD-10 column (Section III.D) before proceeding to Step 7.
7. Load the clarified sample onto the column (at a flow rate of 0.5–1 ml/min for a 1 ml column). Collect fractions of appropriate volume (1 ml fractions for a 1 ml column).

VI. Sample Preparation & Purification continued

8. Wash the column with 10 column volumes of His60 Ni Equilibration Buffer followed by 10 column volumes of Wash Buffer (i.e., Equilibration Buffer containing 40 mM imidazole). See Section III.B for instructions on preparing Wash Buffer.
9. Elute with approximately 10 column volumes of His60 Ni Elution Buffer (containing 300 mM imidazole) and collect fractions. Monitor protein elution by measuring the absorbance of the fractions at 280 nm or performing a Bradford protein assay. The collected fractions can be analyzed by SDS-PAGE.
10. If necessary for downstream applications, remove excess imidazole by gel filtration on a PD-10 column (Section III.D).
11. The His60 Ni column can be regenerated quickly by washing with 20 ml of Equilibration Buffer or by washing with 10 column volumes of 20 mM MES, 0.3 M NaCl; pH 5.0 buffer. Regeneration allows the column to be reused to purify the same protein multiple times without significant loss of binding capacity. If you plan to purify multiple proteins using the same column, you must utilize the "Complete Regeneration" protocol described in Section D.
12. For extended storage (over 1 week), wash the column with five column volumes of water after each use and store in 20% ethanol. Attach supplied bottom cap, followed by the top plug. Store the column at 4°C.

Protocol

D. PROTOCOL: Complete Regeneration of His60 Ni Resin

If you plan to purify multiple proteins using the same column, you must use the following resin regeneration protocol before you purify a new protein:

1. Strip the resin of Ni ions by washing with 10 bed volumes of 0.2 M EDTA (pH 7.0) at room temperature.
2. Wash excess EDTA from the resin with an additional 10 bed volumes of double distilled H_2O (ddH_2O).
3. Charge the resin with 2 volumes of 100 mM $NiSO_4$ solution.
4. Wash resin with 7 bed volumes of ddH_2O, followed by 3 bed volumes of 300 mM NaCl and 3 bed volumes of ddH_2O, to remove excess Ni ions.
5. Equilibrate resin with Equilibration/Wash buffer (10 bed volumes).
6. Resin is ready to use. Please note that the resin may be regenerated up to 10 times.

VII. Troubleshooting Guide

Table III. Troubleshooting Guide for His60 Ni Gravity Columns & Resin

Description of Problem	Possible Explanation	Solution
Low target yield	Poor expression of target protein	Optimize bacterial expression conditions.
	Target protein forms inclusion bodies	• Decrease temperature to 25°C or lower during induction to minimize inclusion body formation. • Solubilize inclusion bodies and perform the purification in the presence of 8 M urea or 6 M guanidinium HCl.
	Inefficient target extraction	Use our His60 Ni xTractor Buffer.
	Inaccessible polyhistidine tag	Purify in presence of 6–8 M urea or 6 M guanidinium HCl.
Impurities in eluate	Insufficient washing	Increase wash volume or add intermediate wash at 60 mM imidazole. (This can result in partial loss of target protein.)
Low flow rate	Clogged column	Apply only clarified extract, and decrease the amount of loaded sample.
	Viscous sample	Treat sample with Benzonase or DNase I, as described in Section V.A.
Can not detect target protein by UV		• Use low UV absorbance imidazole in the buffers. • Perform a Bradford protein assay on collected fractions to identify target protein in eluate.