Ni-NTA Superflow BioRobot® Handbook

For
Automated medium and large-scale purification
of 6xHis-tagged proteins

September 2002

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Contents

Kit Contents

Ni-NTA Superflow 96 BioRobot® Kit for 2304 preparations	**(24)***
Catalog No.	**969263**
TurboFilter™ 96 Plates	24
QIAfilter™ 96 Plates	24
Ni-NTA Superflow	3 x 100 ml
Handbook	1
Ni-NTA Superflow 96 BioRobot Core Kit for 2304 preparations	**(24)***
Catalog No.	**969243**
QIAfilter 96 Plates	24
Ni-NTA Superflow	3 x 100 ml
Handbook	1
Ni-NTA Superflow Columns (12 x 1.5 ml)	
Catalog No.	**30622**
Polypropylene columns prepacked with 1.5 ml Ni-NTA Superflow	12
Handbook	1
Ni-NTA Superflow Columns (144 x 1.5 ml)	
Catalog No.	**30624**
Polypropylene columns prepacked with 1.5 ml Ni-NTA Superflow	144
Handbook	1

** If the Ni-NTA Superflow 96 BioRobot Kit or Core Kit is used exclusively for the high-yield medium-scale protocols an additional 200 ml Ni-NTA Superflow resin is required. See p. 60 for ordering information.*

Storage Conditions

Ni-NTA Superflow should be stored at 2–8°C (do not freeze). All other components of Ni-NTA Superflow 96 BioRobot® Kits should be stored dry at room temperature (15–25°C). Ni-NTA Superflow Columns should be stored upright at 2–8°C (do not freeze).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding Ni-NTA Superflow products or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please call one of the QIAGEN Technical Service Departments or local distributors (see inside front cover).

Product Use Limitations

QIA*express*® products are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see inside front cover).

Safety Information

Listed below are the relevant European Community risk and safety phrases for QIAGEN products contained in Ni-NTA Superflow 96 BioRobot Kits and Columns.

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/ts/msds.asp** where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

The following risk and safety phrases apply to the components of Ni-NTA Superflow 96 BioRobot Kits and Columns.

Ni-NTA Superflow

Contains nickel-nitrilotriacetic acid and ethanol: Harmful, sensitizer, flammable. Risk and safety phrases*: R10-22-40-42/43. S13-26-36-46

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

** R10: Flammable. R22: Harmful if swallowed. R40: Possible risk of irreversible effects. R42/43: May cause sensitization by inhalation and skin contact. S13: Keep away from food, drink, and animal feeding stuffs. S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. S36: Wear suitable protective clothing. S46: If swallowed seek medical advice immediately and show the container or label.*

Introduction

Ni-NTA Superflow 96 BioRobot Kits are designed for rapid, automated purification of 6xHis-tagged proteins from small-volume bacterial cultures using the BioRobot 9600, 3000 or 8000 workstations. The procedure has been developed for high-throughput applications where relatively large amounts (up to 2 mg) of highly pure recombinant proteins are needed.

Ni-NTA Superflow Columns are used for purification of larger amounts of 6xHis-tagged protein (up to 15 mg per column) on BioRobot 3000 workstations. The increased yields are highly suited for applications such as protein crystallization where milligram amounts of protein from a single batch are needed.

The QIA*express* System is a powerful and versatile tool for working with recombinant 6xHis-tagged proteins and other biomolecules. The system is based on the remarkable selectivity of unique, patented Ni-NTA (nickel-nitrilotriacetic acid) for proteins with an affinity tag of six consecutive histidine residues — the 6xHis tag. This technology allows purification, detection, and assay of almost any 6xHis-tagged protein from any expression system. The unique features of the QIA*express* System provide a number of significant advantages (Table 1, see next page) that are not available with other affinity-tag and chromatography methods. The 6xHis tag rarely affects protein structure or function, and for most applications does not need to be removed from the purified protein. In cases where native proteins are desired, such as protein crystallization for structural determination, the TAGZyme™ system offers an extremely efficient solution for exoproteolytic removal of N-terminal affinity tags (1).

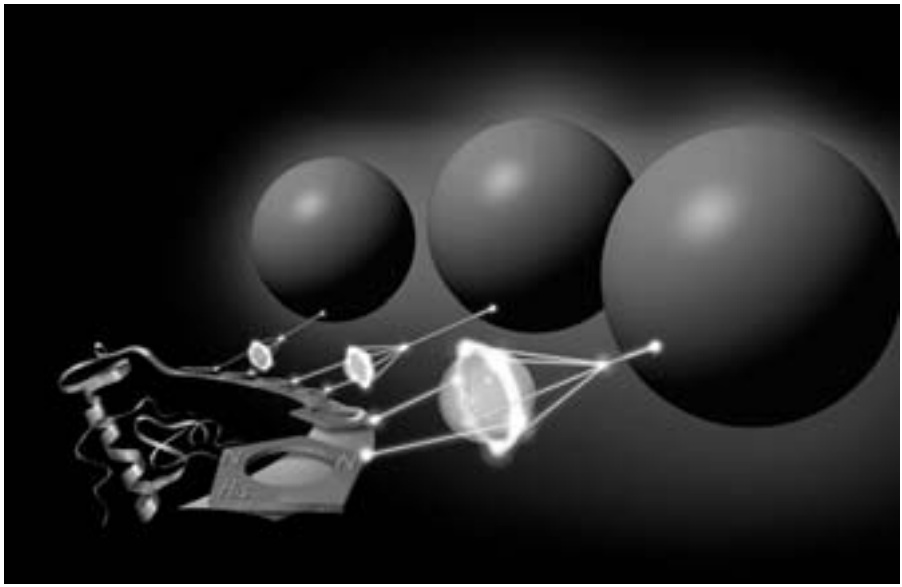


Table 1. Features and benefits of the QIA*express* System

Features	Benefits
The interaction of the 6xHis tag with Ni-NTA matrices is conformation independent.	One-step purification can be carried out under native or denaturing conditions.
Mild elution conditions can be used.	Binding, washing, and elution are highly reproducible, and have no effect on protein structure.
	Pure protein products are ready for direct use in downstream applications.
The 6xHis tag is much smaller than other commonly used tags.	6xHis tags can be used in any expression system.
	Tag does not interfere with the structure or function of the recombinant protein.
	Tag removal by protease cleavage is not necessary.
The 6xHis tag is uncharged at physiological pH.	The 6xHis tag does not interfere with secretion.
The 6xHis tag is poorly immunogenic.	The recombinant protein can be used without prior removal of the tag as an antigen to generate antibodies against the protein of interest.
Some QIA*express* vectors feature a 6xHis dihydrofolate reductase tag (6xHis-DHFR tag)	Small peptides fused to the 6xHis DHFR tag are stabilized while being expressed.
	The 6xHis-DHFR tag is poorly immunogenic in mouse or rat, so peptides fused to the tag can be used directly for immunizations or epitope mapping.

Ni-NTA technology

Immobilized-metal affinity chromatography (IMAC) was first used to purify proteins in 1975 (2) using the chelating ligand iminodiacetic acid (IDA, Figure 1). IDA has only 3 metal-chelating sites and cannot tightly bind metal ions. This results in considerable leaching of metal ions from the chromatography matrix, reducing the efficiency of protein purification (3). Therefore proteins that bind the metal ions are weakly bound to the metal-chelating matrix. A new, improved ligand for metal-chelate chromatography, Ni-NTA, was developed at Hoffmann-La Roche and is exclusively available from QIAGEN. NTA occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag (Figure 1). NTA binds metal ions far more stably than other available chelating resins (4,5) and retains the ions under a wide variety of stringent conditions. The 6xHis tag binds strongly to the immobilized nickel ion allowing optimum separation. For optimal performance, both the 6xHis tag–nickel and the nickel–NTA interactions are important (Figure 2).

The QIA*express* Protein Expression and Purification Systems are described in detail in *The QIA*expressionist™, a comprehensive manual for the high-level expression and purification of 6xHis-tagged proteins. QIA*express* Detection and Assay Systems further extend the range of applications made possible by the 6xHis tag. For details, refer to the *QIA*express *Detection and Assay Handbook* and to the *Ni-NTA Magnetic Beads Handbook* — or contact QIAGEN.

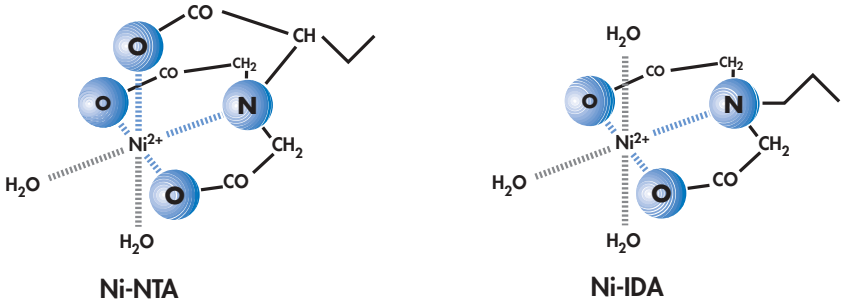


Figure 1. Comparison of the interactions of different metal chelate matrices with nickel ions.

Figure 2. *The capture of 6xHis-tagged proteins by metal-chelate affinity matrices relies on two interactions. Both are important for optimal performance. If interaction A is weak, there is no binding of the 6xHis-tagged proteins. If interaction A is strong, but interaction B weak, protein is lost as protein–metal complexes during wash steps. When NTA ligand and nickel are used to bind 6xHis-tagged molecules, both interactions are stronger, providing advantages over systems that rely on other ligands or metals.*

Automated protein purification

Its broad applicability and robustness make Ni-NTA IMAC technology suitable for automation on laboratory robotic workstations. QIAGEN offers solutions for multiparallel purification of 6xHis-tagged proteins over a wide range of yields by automating Ni-NTA applications on BioRobot workstations (6). Ni-NTA Superflow technology was first established as an automated, standardized procedure on the BioRobot 9600 workstation. This procedure offers cost-effective, high-throughput protein purification, and certified protocols and chemistry. The process has been further developed to allow automated protein purification using both the BioRobot 3000 and 8000 series.

The modular, customizable design of the BioRobot 3000 series allows almost unlimited flexibility to match various application requirements, and the BioRobot 8000 offers fully automated, walk-away liquid and plate handling for high-throughput applications, in combination with QIAGEN certified chemistries. Table 2 gives an overview of the protocols and products available for automated protein purification. Every protocol can be run under native or denaturing conditions.

Table 2. Automated protocols for 6xHis-tagged protein applications

Application	Culture volume	Samples per run	Yield per well or column	Workstation	Product
Micro-scale purification and assay	1 ml (96-well blocks)	96	1–15 µg	BioRobot 3000 and 8000	Ni-NTA Magnetic Agarose beads
Medium-scale purification	5 ml (24-well blocks)	96	15–300 µg	BioRobot 9600, 3000, and 8000	Ni-NTA Superflow 96 BioRobot Kit Ni-NTA Superflow 96 BioRobot Core Kit
Medium-scale purification	Up to 25 ml (shake flask)	96	Up to 2 mg	BioRobot 3000 and 8000	Ni-NTA Superflow 96 BioRobot Kit Ni-NTA Superflow 96 BioRobot Core Kit
Large-scale purification	50–1000 ml (shake flask/ fermenter)	24*	Up to 15 mg	BioRobot 3000	Ni-NTA Superflow Columns

**12 per vacuum manifold in multiples of 4 samples*

Using Ni-NTA Magnetic Agarose Beads up to 15 µg of 6xHis-tagged protein can be purified under both native and denaturing conditions from 96 *E. coli* cultures. Ni-NTA Magnetic Beads are also well suited for various assay procedures. See the *Ni-NTA Magnetic Agarose Beads Handbook* for more details.

The Ni-NTA Superflow 96 BioRobot Kit can be used to purify up to 300 µg of recombinant 6xHis-tagged protein per well — depending upon the protein's expression rate. The Ni-NTA Superflow 96 BioRobot procedure provides high reproducibility, purity, and speed (just 120–150 min for a 96-well protein purification), as well as providing a high degree of automation. For applications demanding higher protein yields QIAGEN has adapted the Ni-NTA Superflow 96 BioRobot protocols, allowing processing of larger culture volumes and purification of milligram amounts of 6xHis-tagged protein per well. Increasing the amount of Ni-NTA Superflow resin used in the procedure and an optimized lysis buffer formulation enables up to 25 ml (purification under native conditions) or 15 ml (purification under denaturing conditions) of cell culture medium (LB) to be processed. Cultivation of the expression cultures in shake flasks is recommended. Figure 3 and Table 3 show the high reproducibility and increased yields obtainable using the adapted protocols.

Milligram Amounts of Pure 6xHis-tagged Protein Per Well

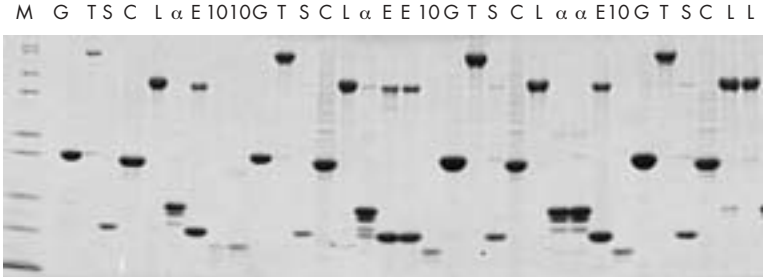


Figure 3. *Purification of milligram amounts of 6xHis-tagged protein. 25 ml expression cultures were processed using the optimized Ni-NTA Superflow BioRobot protocol. 5 µl (0.9%) of the first elution fraction was loaded for SDS-PAGE and proteins were visualized by Coomassie® staining.* ***G****: Green Fluorescent Protein (29 kDa);* ***T****: T7 RNA Polymerase (100 kDa);* ***S****:* E. coli *GroES (12 kDa). Some endogenous GroEL is copurified;* ***C****:* E. coli *chloramphenicol acetyltransferase (28 kDa);* ***L****:* E. coli *GroEL (60 kDa);* ***a****: human tumor necrosis factor α (18 kDa);* ***E****:* E. coli *GroES purified as a complex with co-overexpressed nontagged GroEL (12 and 60 kDa);* ***10****:* Saccharomyces cerevisiae *Cpn-10 (10 kDa).* ***M****: markers.*

Table 3. Yields of 6xHis-tagged proteins using the High-yield Ni-NTA Superflow 96 BioRobot protocol

6xHis-tagged protein	Total yield per well (µg)	Protein concentration (mg/ml)
Green fluorescent protein	4000	6.0
T7 RNA polymerase	1000	1.4
GroES	300	0.4
Chloramphenicol acetyltransferase	2400	4.4
GroEL	740	1.0
Tumor necrosis factor α	1600	2.5
GroES/GroEL	1200	1.5
Cpn-10	170	0.3

The latest additions to QIAGEN's portfolio of automated solutions for purification of 6xHis-tagged proteins are large-scale protocols using Ni-NTA Superflow Columns on the BioRobot 3000 workstation. Twelve culture samples, each up to 1 liter in volume can be processed per vacuum manifold. Using a BioRobot 3000 equipped with 2 vacuum manifolds, 24 samples can be processed within approximately 150 minutes. The procedure provides yields of up to 15 mg 6xHis-tagged protein per column.

Automated protocols have been developed for purification under both native and denaturing conditions. When purified under native conditions, 6xHis-tagged proteins usually retain their full biological activity due to the small size of the 6xHis tag. The catalytic and structural activity of 6xHis-tagged proteins has been shown in numerous examples (see references 7–12).

When immobilized via their 6xHis tag, proteins usually retain their active conformation and thus, 6xHis-tagged proteins are ideal for assays that require structurally intact, biologically active proteins. 6xHis-tagged proteins can be immobilized on magnetic beads or on the well-surfaces of Ni-NTA HisSorb Plates in 96- or 384-well format. Fully automated protocols are available for use with Ni-NTA Magnetic Agarose Beads. For further information, contact QIAGEN.

Purification of 6xHis-tagged proteins can also be performed under denaturing conditions. In 96-well format this is particularly useful for initial screening of expression libraries and expression studies.

A strategy for high-throughput protein research projects using the QIA*express* system

The following strategy can be used for studies in proteomics and functional and structural genomics.

As a starting point, fast, fully automated Ni-NTA Magnetic Bead protocols can be performed in parallel under native and denaturing conditions for expression-clone screening and solubility studies.

Standard Ni-NTA Superflow 96 protocols process 5 ml *E. coli* cultures grown in 24-well blocks, and are suitable for quantification of protein expression, enabling estimation of expression rates in larger culture volumes, and delivering 20–300 µg of protein; sufficient for most functional studies.

If more protein is required, the adapted Ni-NTA Superflow 96 protocols can be used to provide yields of up to 2 mg per protein, enabling screening of crystallization conditions, immunization of animals, or selection of aptamers to generate capture molecules for coating of microarray surfaces. Alternatively, protein can be directly spotted onto microarray surfaces. Milligram amounts of purified protein are sufficient to generate a large number of uniform chips, allowing highly reproducible analyses.

For even higher protein yields, which allow a higher number of chips to be spotted, or protein structural analysis by NMR or by crystallization and x-ray diffraction, large-scale Ni-NTA Superflow Column protocols can be used.

The Ni-NTA Superflow 96 BioRobot and Core Kits can also be used manually for protein purification using the QIAvac 96 vacuum manifold or a suitable centrifuge. For more information and a protocol, contact QIAGEN.

Table 4. Compatibility of reagents with Ni-NTA matrices

Reagent	Effect	Comments
Buffer reagents		
Tris, HEPES, MOPS	Buffers with secondary or tertiary amines will reduce nickel ions	Up to 100 mM has been used in some cases Sodium phosphate or phosphate-citrate buffer is recommended
Chelating reagents		
EDTA, EGTA	Strip nickel ions from resin	Up to 1 mM has been used in some cases, but care must be taken
Sulfhydril reagents		
β-mercaptoethanol	Prevents disulphide cross-linkages Can reduce nickel ions at high concentration	Up to 20 mM can be used
DTT, DTE	Low concentrations will reduce nickel ions	A maximum of 1 mM may be used, but β-mercaptoethanol is recommended
Detergents		
Nonionic detergents (Triton®, Tween®, Nonidet® P-40, etc.)	Remove background proteins and nucleic acids	Up to 2% can be used
Cationic detergents		Up to 1% can be used
Anionic detergents (SDS, sarcosyl)		Not recommended, but up to 0.3% has been successfully used in some cases
Denaturants		
Gu·HCl	Solubilize proteins	Up to 6 M can be used
Urea	Solubilize proteins	Up to 8 M can be used

Table 4 continued on the next page

Reagent	Effect	Comments
Amino acids		
Glycine		Not recommended
Glutamine		Not recommended
Arginine		Not recommended
Histidine	Binds to Ni-NTA and competes with histidine residues in the 6xHis tag	Can be used at low concentrations (20 mM) to inhibit nonspecific binding and, at higher concentrations (>100 mM), to elute the 6xHis-tagged protein from the Ni-NTA matrix
Other additives		
NaCl	Prevents ionic interactions	Up to 2 M can be used; should use at least 150 mM
$MgCl_2$		Up to 4 mM
$CaCl_2$		Up to 5 mM
Glycerol	Prevents hydrophobic interaction between proteins	Up to 50% (v/v) can be used
Ethanol	Prevents hydrophobic interactions between proteins	Up to 20% (v/v) can be used
Imidazole	Binds to Ni-NTA and competes with histidine residues in the 6xHis tag	Can be used at low concentrations (20 mM) to inhibit nonspecific binding and, at higher concentrations (>100 mM), to elute the 6xHis-tagged protein from the Ni-NTA matrix
Sodium bicarbonate		Not recommended
Hemoglobin		Not recommended
Ammonium		Not recommended
Citrate		Up to 60 mM has been used successfully

Establishing the immobilized-metal affinity chromatography technique on-site

For detailed background information and recommendations on immobilized-metal affinity chromatography (IMAC) refer to The *QIA*expressionist, our handbook for standard purification of 6xHis-tagged proteins by Ni-NTA chromatography.

We recommend optimizing culture conditions with respect to optical density and 6xHis-tagged protein expression rates following the guidelines given on page 24.

You may wish to modify the recommended buffers used (see protocols, pages 30 to 53) by the addition of certain chemicals, for example, when screening for proteins with particular properties and requirements. The robustness of the QIA*express* System for purification of 6xHis-tagged proteins allows a wide range of buffer compositions to be used for purification. However, certain limitations have to be considered (for details, refer to Table 4). For instance, Ni-NTA matrices should not be exposed to high concentrations of strong reducing agents such as DTT or DTE; these reagents reduce the nickel ions and thereby prevent them from binding 6xHis-tagged proteins. Ni-NTA resins that have been reduced turn brown in color. β-mercaptoethanol can be used as a reducing agent at concentrations up to 20 mM.

EDTA, EGTA, or other strong chelating agents bind nickel ions and strip them from the Ni-NTA matrices. Ni-NTA resins turn white in the absence of nickel ions.

Use any reducing or chelating agent with care, and if in doubt, test it on a small amount of Ni-NTA resin. High concentrations of buffer components containing strong electron donating groups (e.g., NH^{4+}), or amino acids such as arginine, glutamine, glycine, or histidine in the lysate should also be avoided.

The Ni-NTA Superflow 96 BioRobot Procedure

Important notes before starting

Both the standard (15–300 µg) and adapted (up to 2 mg) medium-scale Ni-NTA Superflow 96 protocols are designed for automated high-throughput purification of 6xHis-tagged proteins from bacterial cultures in 96-well format using the BioRobot 9600, 3000, or 8000.

Cell growth

- If cells are grown in 24-Well Blocks, a volume of 5 ml LB medium is recommended. Culture volume may be decreased if cell density significantly exceeds the recommendation (see below). Do not exceed 5 ml medium per well.
- If cells are grown in shake flasks, add medium to between 1/10 and 1/5 of the shake flask's total volume (for example, incubate expression cultures of 25 to 50 ml in a 250 ml flask).
- While LB medium is recommended for culture, richer media (e.g., 2xYT, SM, TB) can be used with culture volumes up to 5 ml; for use in 96-well purification procedures care should be taken, however, not to exceed a final OD_{600} of 6 at the time of harvesting. For detailed information on growing bacterial cultures, see page 24.
- The Ni-NTA Superflow 96 BioRobot Procedure procedures works optimally when the same vector–host strain combination and culture volume is used for all expression cultures processed in parallel (see page 25 for details of the optimized conditions). Growth conditions for different vector–host strain combinations should be individually optimized, especially with respect to the final OD_{600} reached.

Ni-NTA Superflow 96 BioRobot Kits

- The Ni-NTA Superflow 96 BioRobot Kit contains two different types of filter plates. The TurboFilter™ 96 Plate contains an asymmetric laminar membrane with a gradation of pore sizes for removal of cell debris and insoluble components. The Ni-NTA Superflow resin is distributed into the individual wells of the QIAfilter™ 96 Plate. The wells of the QIAfilter 96 Plate serve as micro-chromatography columns holding back the Ni-NTA Superflow resin during the chromatographic purification steps.
- The Ni-NTA Superflow 96 BioRobot Core Kit contains QIAfilter 96 plates only and is for purification of 6xHis-tagged proteins from precleared lysates and in vitro translation extracts.

Lysis and elution vessels

- If four 24-Well Blocks are used for lysis, the samples are processed in order as if they were a single 96-well plate (see Figure 4).
- Single 14 ml tubes can only be used for cell lysis with the BioRobot 9600 and 3000 workstations. On the BioRobot 8000, only 24-Well Blocks can be processed.
- Collection Microtubes or an S-Block are recommended for elution volumes ≤500 µl. Note that Collection Microtubes are only suitable for a single elution. If using Collection Microtubes do not exceed 500 µl elution volume.
- An S-Block is recommended for elution volumes of 500–650 µl.

Notes on buffers

- For details of buffer compositions, see the protocols.
- Store the buffers for protein purification under denaturing conditions (Buffer B–4 M urea, Buffer C–4 M urea, and Buffer E–8 M urea) at room temperature. Check for precipitation of urea before use and, if necessary, redissolve by stirring and warming. Check the pH of the buffers immediately before use.
- If Buffer B–8 M urea does not sufficiently dissolve the recombinant protein of interest, Buffer A–6 M Gu·HCl may be used for cell lysis under denaturing conditions (see page 37).
- Lysozyme solution (10 mg/ml in water) should be either freshly prepared or stored frozen (at –20°C in aliquots) and thawed immediately before use.

Equipment to be supplied by user

Square-Well Blocks for bacterial preculture growth

24-Well Blocks RB for bacterial expression culture growth

AirPore™ Tape Sheets

14 ml polypropylene tubes (e.g., Greiner, Cat. No. 187261) — two or four are required for the Ni-NTA Superflow suspension; they can also be used for culturing bacteria (BioRobot 9600 and 3000 only)

Centrifuge with a rotor for 96-well plates (e.g., QIAGEN Centrifuge 4K15C, Cat. No. 81220; equipped with the Plate Rotor 2 x 96, Cat. No. 81031)

Tape Pads (optional; for sealing unused wells, BioRobot 9600 and 3000 only)

S-Blocks or Collection Microtubes (racked) and Caps for elution of purified 6xHis-tagged proteins

Benzonase®* (adapted Ni-NTA Superflow BioRobot 96 protocol only)

For ordering information for the above items, see page 60

** We recommend Merck Benzonase purity grade I (=99 %), ≥25 Units/µl, 10,000 Units/vial. Cat. No. 1.01694.0001, 1.01694.0002 (US & Canada).*

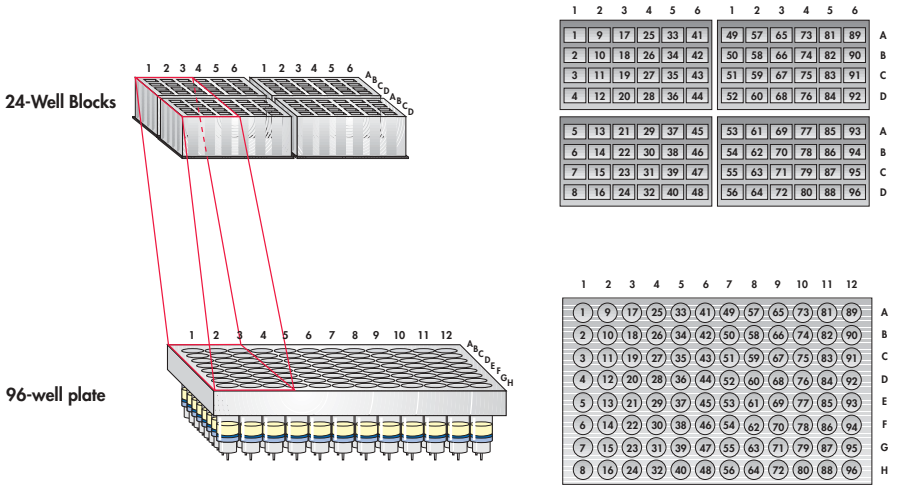
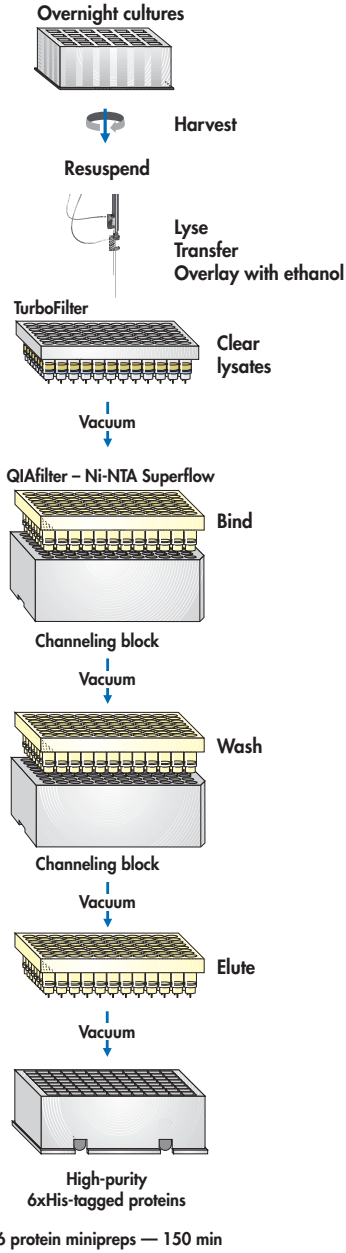


Figure 4. *Cell lysates are transferred from the wells of 24-Well Blocks to the corresponding wells of the 96-well plate as shown. If proteins are purified from fewer than 96 samples, then the wells of the 96-well plate are used in columns from left to right (starting with column 1). The samples must be provided in the corresponding wells of the 24-Well Blocks. For example, if 24 samples are processed, they should be provided in the first three columns on the left-hand side of two 24-Well Blocks placed in the shaker mtp back/left and front/left positions. The samples are transferred one row at a time starting with row A.*

Ni-NTA Superflow 96 BioRobot Procedure



The Ni-NTA Superflow Column Procedure

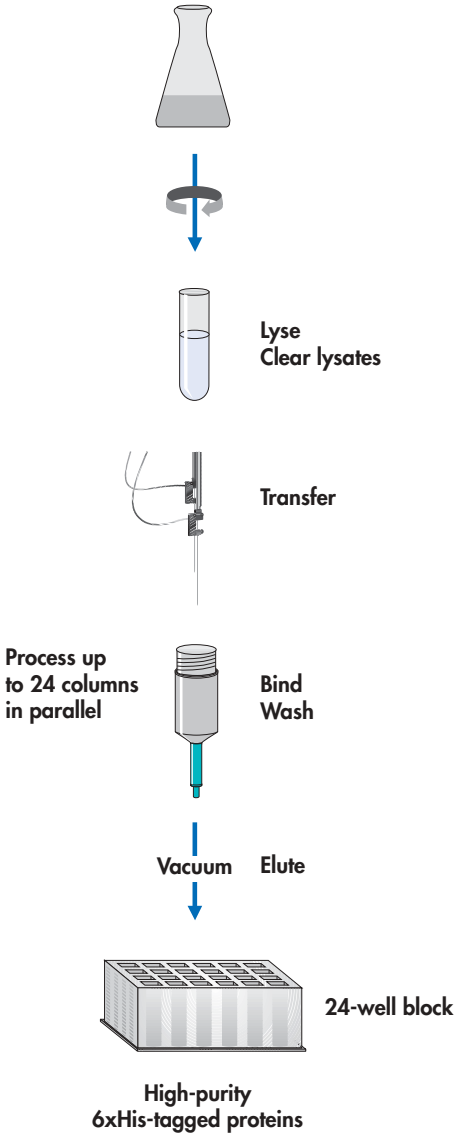
Important notes before starting

Ni-NTA Superflow Columns (1.5 ml) are used for purification of up to 15 mg 6xHis-tagged protein in an automated procedure on the BioRobot 300 workstation or in a manual gravity-flow procedure. For the manual procedure, columns can be placed in a QIArack (cat. no. 19015) for convenient processing. Each column can be used to purify protein from cleared lysate derived from up to 1 liter of bacterial culture.

- If cells are grown in shake flasks, add medium to between 1/10 and 1/5 of the shake flask's total volume (for example, incubate expression cultures of 100 to 200 ml in a 1 liter flask.)
- Ni-NTA Superflow Columns can be processed in multiples of four on the BioRobot vacuum manifold (see Figure 5). All unused positions on the QIAvac 6S top plate should be sealed using the plugs supplied with the QIAvac Luer Adapter Set (cat. no. 19541).

Figure 5. *Ni-NTA Superflow Columns on the BioRobot vacuum manifold. Columns are processed in multiples of 4; up to 12 columns can be processed per manifold.*

Ni-NTA Superflow 96 Column Automated Procedure



Growing Bacterial Cultures for Protein Purification

Choice of host strain and vector

This cultivation guideline has been developed using the QIA*express Escherichia coli* host strain M15[pREP4] transformed with pQE-derived constructs.

The pQE series of expression vectors provides high-level expression of 6xHis-tagged proteins in *E. coli* based on the T5 promoter transcription-translation system. Vectors can be chosen where the sequences coding for the 6xHis tag are at the 5' or 3' end of the cloning region. Furthermore, QIAGEN offers vectors for cloning into all three reading frames — especially important for subcloning of libraries.

The high transcription rate from the T5 promoter is efficiently repressed by the presence of high levels of the *lac* repressor protein. *E. coli* host strains used in the QIA*express* System contain the low-copy number plasmid pREP4 that constitutively expresses the *lac* repressor encoded by the *lacI* gene and is maintained by kanamycin selection. The new pQE-80L series of expression vectors include a *lacI*^{q} gene that represses 6xHis-tagged protein expression. This allows use of any *E. coli* host strain.

Expression of recombinant proteins encoded by pQE vectors is rapidly induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which binds to the *lac* repressor protein and inactivates it. Once the repressor is inactivated, the host cell's RNA polymerase transcribes the sequences downstream from the promoter. The transcripts produced are then translated into the recombinant protein. The special double operator system present in pQE expression vectors, in combination with the high levels of the *lac* repressor protein generated by pREP4, ensures tight control at the transcriptional level. The pREP4 plasmid is present in the QIA*express E. coli* strains M15[pREP4] and SG13009[pREP4].

In principle, any *E. coli* strain containing both the expression (pQE) and the repressor (pREP4) plasmids can be used for the production of recombinant proteins. The QIA*express E. coli* strain M15[pREP4] permits high-level expression, is well established, and is easy to handle.

E. coli strains that harbor the *lacI*^{q} mutation, such as XL1Blue, JM109, and TG1, produce enough *lac* repressor to efficiently block transcription, and are ideal for maintenance and propagation of pQE vectors. These strains can also be used as hosts for expressing nontoxic proteins. However, because expression is regulated less tightly than in strains harboring pREP4, if the expressed protein is toxic to the cell, "leaky" expression before induction may result in poor culture growth or in the selection of deletion mutants which grow faster than bacteria containing the correct plasmid.

Selection for pREP4 in *E. coli* strains M15 and SG13009 is maintained by the inclusion of kanamycin in the culture medium.

Cultivation of *E. coli* M15[pREP4] Harboring pQE Expression Constructs

Important note before starting

For growth in 24-Well Blocks or in polypropylene tubes, pilot experiments are recommended to optimize cultivation conditions on-site (see below).

1. **1. Transform expression construct into M15[pREP4] and plate onto selective medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin.**
2. **Incubate overnight at 37°C.**
3. **Prepare LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, and transfer 0.5 ml aliquots into each of the 96 wells of an Square-Well Block for preculture.**

   Higher preculture volumes are required for cultivation in shake flasks, e.g., a 100 ml culture medium is inoculated with 5 ml preculture with an OD_{600} of 10 to give a starting OD_{600} of 0.05 (see below).
4. **Pick single colonies using a sterile tooth pick, inoculate a single well or vessel, allow to stand for 5 minutes, and shake briefly. Alternatively, inoculate precultures from glycerol stocks.**
5. **Remove the toothpick, and cover the vessel.**

   Square-Well Blocks can be sealed with an AirPore Tape sheet.
6. **Incubate precultures overnight at 37°C, shaking at 150 rpm. Incubate shake flask precultures at 20–25°C, shaking at 150 rpm.**

   The OD_{600} of the precultures after overnight growth should be between 1 and 5. To measure optical density at 600 nm (OD_{600}) dilute a sample of the culture with medium and measure in a spectrophotometer to give a value between 0.1 and 0.5. Calculate the OD_{600} of the culture using the dilution used.

7a. **Cultures in 24-Well Blocks (5 ml): Prepare four 24-Well Blocks containing 5 ml LB medium with appropriate antibiotics under sterile conditions.**

   If you wish to work with fewer than 96 samples, note that the individual wells of 24-Well Blocks are processed by the BioRobot workstation in the order described on page 20 (see Figure 4). Note that the BioRobot 8000 always processes all 96 wells on a plate. If the number of sample-containing wells is less than 96, all remaining wells will also be processed.

   Alternatively, 14 ml polypropylene tubes (e. g., Greiner, Cat. No. 187261) can be used with the BioRobot 9600 and 3000. To ensure good aeration, the tubes should be incubated at an angle of 45° with vigorous shaking.

7b. Shake flask cultures (≥15 ml): Prepare the required number of shake flasks containing LB medium with appropriate antibiotics under sterile conditions.

For optimal aeration, add medium to between 1/10 and 1/5 of the shake flask's total volume (for example, incubate expression cultures of 25 to 50 ml in a 250 ml flask).

8a. Cultures in 24-Well Blocks: Inoculate expression cultures in 24-Well Blocks by adding 25–100 µl of overnight preculture from the Square-Well Block to obtain an OD_{600} of approximately 0.05.

To ensure proper adjustment of the OD_{600} after inoculation, we recommend performing pilot experiments to verify the average OD_{600} of precultures under the particular conditions. Adjust the inoculation volume or shaker speed during overnight incubation to achieve an OD_{600} of ~0.05.

8b. Shake flask cultures: Inoculate expression cultures in shake flasks by adding a volume of preculture to obtain an OD_{600} of approximately 0.05.

9a. Cultures in 24-Well Blocks: Cover 24-Well Blocks with an AirPore Tape sheet and incubate cultures at 37°C, shaking at 150 rpm, for 2–3 h or until an OD_{600} of 0.6–0.8 is reached.

9b. Shake flask cultures: Incubate the cultures at 37°C, shaking at 150 rpm, for 2–3 h or until an OD_{600} of 0.6–0.8 is reached.

10a. Cultures in 24-Well Blocks: Induce expression of 6xHis-tagged proteins by adding 25 µl of 0.1M IPTG (final concentration: 0.5 mM IPTG) and incubate the cultures overnight or for 16 h at 37°C and 150 rpm, or until an OD_{600} of 6 is reached.

To avoid exceeding a final OD_{600} of 6 we recommend monitoring growth in several wells of the 24-Well Blocks in pilot experiments under the chosen growth conditions. Shorten the induction phase of the expression cultures if overnight growth leads to OD_{600} values greater than 6. If the cultures are grown to higher optical densities, the TurboFilter Plate can become blocked during clearing of the crude lysate.

10b. Shake flask cultures: Induce expression of 6xHis-tagged proteins by addition of IPTG to a final concentration of 1 mM and incubate cultures for 4 h at 37°C and 150 rpm.

11a. Cultures in 24-Well Blocks: Pellet cells by centrifuging 24-Well Blocks at 3000–1000 x *g* for 10 min in a centrifuge suitable for 96-well plates, remove supernatant, and freeze cell pellets at –20°C.

11b. Culture volume 15–25 ml: Pellet cells by repeated centrifugation into the wells of 24-Well Blocks, and freeze cell pellets at –20°C (e.g., to process 15 ml culture volume, centrifuge 7.5 ml, discard supernatant, add and centrifuge the remaining 7.5 ml, and discard supernatant).

11c. Culture volume >50 ml: Pellet cells by centrifugation in a suitable tube/bottle, transfer the wet cell paste into a 50 ml tube (e.g., Falcon™ tubes) using a spatula, and briefly spin cells to the bottom of the tube. Freeze cell pellets at –20°C.

Cells from frozen pellets are lysed much more effectively than fresh cells. Pellets should be frozen for at least 1 hour at –20°C before starting the purification procedure.

Medium-Scale Purification of 6xHis-tagged Proteins under Native Conditions

This section gives an overview of the protein purification procedure under native conditions, and the order of the different operations, rather than a detailed, stepwise description of the protocol. User interactions necessary during a run on the BioRobot 9600 and 3000 systems are indicated. For a detailed description and follow-up of the current operations, please follow the messages and information given on the screen during the run of an individual protocol.

Materials

Bacterial cultures — for guidelines on the growth of bacterial expression cultures for this protocol, see page 24.

Elution vessel (S-Block or Collection Microtube Rack) — for ordering information see page 60.

2 x 14 ml polypropylene tubes (4 x 14 ml polypropylene tubes for adapted protocol)

Channeling Block

TurboFilter 96 Plate

QIAfilter 96 Plate

Preparation of buffers and reagents

Buffer NPI-10	**for 500 ml:**
50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$
300 mM NaCl	8.77 g NaCl
10 mM imidazole	0.34 g imidazole

Adjust pH to 8.0 using NaOH. Prepare 500 ml in a 500 ml bottle.

Buffer NPI-10/Benzonase®

Prepare a volume of Buffer NPI-10 and add the required units of Benzonase as given in the display message, mix, and provide in a 60 ml bottle.

Buffer NPI-20	**for 500 ml:**
50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$
300 mM NaCl	8.77 g NaCl
20 mM imidazole	0.68 g imidazole

Adjust pH to 8.0 using NaOH. Prepare 500 ml in a 500 ml bottle.

Buffer NPI-250	**for 500 ml:**
50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$
300 mM NaCl	8.77 g NaCl
250 mM imidazole	8.51 g imidazole

Adjust pH to 8.0 using NaOH. Provide 200 ml in a 250 ml bottle.

Provide 2 ml lysozyme solution (10 mg/ml) in a 2 ml microcentrifuge tube.

Provide a 250 ml bottle containing 150 ml analytical grade ethanol (100%).

Table 5. Tubing/position on work table for purification under native conditions

Buffers/solutions/accessories	BioRobot 9600/3000	BioRobot 8000
Buffer NPI-10	Tubing 1	Rotor Slot 2
Buffer NPI-10/Benzonase*	Reagent Slot B9	Reagent Slot B9
Buffer NPI-20	Tubing 2	Rotor Slot 4
Buffer NPI-250	Tubing 8	Rotor Slot 3
Lysozyme solution	Reagent Slot B5	Therm Subslot C, Position A
Ethanol	Tubing 7	Therm Subslot A, Trough 20ml
Ni-NTA Superflow suspension	Reagent Slot B1+B2	Therm Subslot B, Position A+B
TurboFilter 96 Plate	Plate Holder/MP Slot 1	Plate Slot 6
QIAfilter 96 Plate	Vacuum Manifold Top	Plate Slot 6/2
Plate Holder	–	MP Slots 6, 11, 12, 16
Filtration Adapter	–	Plate Slot 16
Channeling Block	Vacuum Manifold Base†	Plate Slot 11
96-well elution vessel:		
one vessel	MP Slot 3	MP Slot 8
two vessels	MP Slot 3	MP Slot 8+9
24-well blocks	Shaker back/left	Shaker back/left
	Shaker front/left	Shaker front/left
	Shaker back/right	Shaker back/right
	Shaker front/right	Shaker front/right

** Used for cell lysis in the adapted protocol.*

† The Channeling Block should be placed in this position during user interaction 2.

Protocol for the BioRobot 9600 and 3000

Note: If the Ni-NTA Superflow 96 BioRobot Kit or Core Kit is used exclusively for the adapted high-yield medium-scale protocols, both Benzonase and an additional 200 ml Ni-NTA Superflow resin are required.

1. **Prepare all reagents required for the protocol (see page 28).**
2. **Make sure that the High-Speed Pipetting System and the workstation are switched on.**

   Note: Always switch on the High-Speed Pipetting System before the workstation.
3. **Switch on the computer and monitor.**
4. **Select "Execute" environment in the "Main Menu" (if necessary).**
5. **Select the "Protein Applications" package in the toolbar drop-down list and from this package select "Ni-NTA Superflow 96 native" or "Ni-NTA Superflow native 1 mg".**
6. **Click "RUN" on the toolbar.**

   The "Run protocol: slot config." dialog box appears.
7. **Select the cultivation vessel type to be processed: 24-Well Blocks or 14 ml polypropylene tubes.**
8. **Click "OK".**

   The "Run protocol: no. of samples" dialog box appears.
9. **Enter the number of samples to be processed (24–96).**

   The final arrangement of the samples in four 24-Well Blocks placed on the shaker mtp is as if they were a single 96-well plate (see Figure 4, page 20). If using 14 ml tubes, place them into the corresponding positions in the appropriate shaker rack.
10. **Click "OK".**

    The first protocol message box appears.
11. **Make sure that the system liquid bottle is filled with distilled water.**
12. **Make sure that the peristaltic-pump cassettes are fitted.**
13. **Empty the vacuum trap.**
14. **Empty the waste bottle.**
15. **Click "Continue".**
16. **Using the information given in the following protocol message boxes:**

    Connect the buffer bottles in the external buffer rack to the tubing.

    Seal any unused wells of the 96-well modules with tape from a Tape Pad.
17. **Click "Continue".**

    The next protocol message box appears.
18. **Place the 24-Well Blocks or 14 ml tube shaker rack onto the shaker mtp.**

    Note: For the position of the 24-Well Blocks, refer to Figure 4, page 20.

19. **Click "Continue".**

    Wait for the workstation to initialize and calibrate.

20. **Click the speaker icon to stop the beeper.**

21. **Provide the required volume of Ni-NTA Superflow suspension.**

    The required volume is calculated from the number of samples to be processed and appears on the screen (a safety reserve is included).

22. **Resuspend the resin thoroughly, transfer the required volume of suspension into 1 x, 2 x, or 4 x 14 ml polypropylene tubes, and place the tube(s) in the appropriate position (see Table 5).**

23. **Click "Continue".**

24. **Enter the elution volume.**

    An elution volume between 350 and 600 µl (450 and 800 µl for the adapted protocol) can be chosen. The standard elution volume is 450 µl (550 µl) per well. Reducing the elution volume may result in lower protein recovery.

    Approximately 70% of bound 6xHis-tagged protein elutes within the first elution. A second elution step can be performed.

25. **Choose a 96-well elution vessel from list.**

    Depending upon the elution vessel and volume chosen, 1 or 2 eluates may be recovered in a single 96-well vessel.

    A message appears as to where to position the 96-well elution vessel(s) on the working table.

26. **Click "Continue".**

    The system probes and the dilutor syringes are flushed.

    The volumes of the solutions provided are checked.

    Buffer NPI-10/Buffer NPI-10 Benzonase and lysozyme solution are distributed for resuspension of bacterial pellets.

    The vessels containing the cells are shaken at 750 rpm for 30 min. For efficient resuspension, the shaker changes the direction of rotation every 30 seconds.

    The QIAfilter 96 Plate is washed with Buffer NPI-10. 1 ml is pipetted into each well and drawn through by vacuum application.

    Ni-NTA Superflow resin is resuspended and distributed to the QIAfilter Plate.

    200 µl/400 µl of Ni-NTA Superflow suspension (corresponding to 100 µl/200 µl bed volume) is pipetted per well followed by 600 µl of Buffer NPI-10 for equilibration of binding conditions. Buffer is drawn through by application of a vacuum.

    A beeper sounds to let you know that equilibration of the Ni-NTA QIAfilter Plate is complete.

User interaction 1

1. **Place the Ni-NTA QIAfilter Plate within a Plate Holder into accessory slot 2.**
2. **Click "Continue".**

   A beeper sounds.
3. **Place a TurboFilter 96 Plate into the vacuum manifold top position.**

   Seal any unused wells with tape from a Tape Pad.
4. **Click "Continue".**

   The TurboFilter 96 Plate is washed. 1 ml of Buffer NPI-10 is pipetted into each well and drawn through by vacuum application.
5. **Place the Plate Holder and the Ni-NTA QIAfilter Plate into the vacuum manifold base position.**

   Note: Take care that the outlets of the TurboFilter Plate correctly fit into the wells of the Ni-NTA QIAfilter Plate below. Check by pressing on the TurboFilter Plate; the TurboFilter Plate should move downwards slightly. If the plates do not fit together correctly, the pump cannot create the desired vacuum.
6. **Click "Continue".**

   The BioRobot workstation transfers the cell lysates from the 24-Well Blocks on the shaker to the TurboFilter Plate. Each TurboFilter well is overlayed with 100 µl ethanol to reduce frothing. The lysates are then cleared by vacuum filtration and pass directly into the wells of the Ni-NTA QIAfilter Plate.

   A beeper sounds to let you know that the process is finished.

User interaction 2

1. **Discard the TurboFilter Plate.**
2. **Place Channeling Block into the vacuum manifold base position and place the Ni-NTA QIAfilter Plate into the vacuum manifold top position.**

   Fit the outlets of the Ni-NTA QIAfilter Plate into the channels of the Channeling Block.
3. **Click "Continue".**

   A weak vacuum of –200 mbar is applied and the 6xHis-tagged proteins bind to the Ni-NTA Superflow affinity matrix while slowly passing through the Ni-NTA QIAfilter Plate. Finally, the vacuum is increased briefly to ensure completion of binding in each well. The BioRobot workstation washes each well of the Ni-NTA QIAfilter plate twice with 800 µl Buffer NPI-20.

   A beeper sounds to let you know that the step is finished.

User interaction 3

1. **Place the chosen elution vessel into the vacuum manifold base position.**
2. **Click "Continue".**

   The BioRobot workstation distributes elution buffer (Buffer NPI-250) into the Ni-NTA QIAfilter Plate and elutes the 6xHis-tagged proteins into the chosen elution vessel.
3. **A beeper sounds to let you know that the purification process is finished.**

Protocol for the BioRobot 8000

Note: If the Ni-NTA Superflow 96 BioRobot Kit or Core Kit is used exclusively for the adapted high-yield medium-scale protocols, both Benzonase and an additional 200 ml Ni-NTA Superflow resin are required.

1. **Prepare all reagents required for the protocol (see above).**
2. **Make sure that the workstation and computer are switched on.**
3. **Select "Execute" environment in the "Main Menu" (if necessary).**
4. **Select the "Protein Applications" package in the toolbar drop-down list and from this package select "Ni-NTA Superflow 96 native" or "Ni-NTA Superflow native 1 mg".**
5. **Click "RUN" on the toolbar.**

   The "Run protocol: slot config." dialog box appears.
6. **Click "OK".**

   The first protocol message box appears.
7. **Make sure that the system liquid bottle is filled with distilled water.**
8. **Empty vacuum trap and vacuum condensate trap.**
9. **Empty the waste bottle.**
10. **Using the information given in the following protocol message boxes:**

    Place bottles in the relevant position (given in Table 5).

    Place the 24-Well Blocks onto the shaker.

    Note: The BioRobot 8000 always processes all 96 wells on a plate. If the number of sample-containing wells is less than 96, all remaining wells will also be processed. For the position of the 24-Well Blocks, refer to Figure 4, page 20.
11. **Provide the required volume of Ni-NTA Superflow suspension.**

    Resuspend the resin thoroughly. Take 2 x 14 ml polypropylene tubes, transfer 10 ml of suspension into each tube, and place the tubes in the appropriate positions (see Table 5).
12. **Enter the elution volume.**

    An elution volume between 350 and 600 µl (450 and 800 µl for the adapted protocol) can be chosen. The standard elution volume is 450 µl/550 µl per well. Reducing the elution volume may result in lower protein recovery.

    Approximately 70% of bound 6xHis-tagged protein elutes within the first elution. A second elution step can be performed.

13. **Choose a 96-well elution vessel from list.**

    Depending upon the elution vessel and volume chosen, 1 or 2 eluates may be recovered in a single 96-well vessel.

    A message appears as to where to position the 96-well elution vessel(s) on the worktable.

14. **Click "Continue".**

    The system probes and the dilutor syringes are flushed.

    The volumes of the solutions provided are checked.

    Buffer NPI-10/Buffer NPI-10 Benzonase and lysozyme solution are distributed for resuspension of bacterial pellets.

    The vessels containing the cells are shaken at 750 rpm for 30 min. For efficient resuspension, the shaker changes the direction of rotation every 30 s.

    The BioRobot workstation assembles the vacuum chamber system.

    The QIAfilter 96 Plate is washed with Buffer NPI-10. 1 ml is pipetted into each well and drawn through by vacuum application. Ni-NTA Superflow resin is resuspended and distributed to the QIAfilter Plate. 200 µl/400 µl of Ni-NTA Superflow suspension (corresponding to 100 µl/200 µl bed volume) is pipetted per well followed by 600 µl of Buffer NPI-10 for equilibration of binding conditions. Buffer is drawn through by application of a vacuum.

    The TurboFilter 96 Plate is washed. 1 ml of Buffer NPI-10 is pipetted into each well and drawn through by vacuum application.

    The BioRobot workstation transfers the cell lysates from the 24-Well Blocks on the shaker to the TurboFilter Plate. Each TurboFilter well is overlayed with 100 µl ethanol to reduce frothing. The lysates are then cleared by vacuum filtration and pass directly into the wells of the Ni-NTA QIAfilter Plate.

    The Turbo Filter plate is removed and the Ni-NTA QIAfilter plate is moved to the vacuum manifold top position. A weak vacuum of –200 mbar is applied and the 6xHis-tagged proteins bind to the Ni-NTA Superflow affinity matrix while slowly passing through the Ni-NTA QIAfilter Plate. Finally, the vacuum is increased briefly to ensure completion of binding in each well. The BioRobot workstation washes each well of the Ni-NTA QIAfilter plate twice with 800 µl Buffer NPI-20.

    The elution vessel is moved to the vacuum manifold base position.

    Elution buffer (Buffer NPI-250) is dispensed into the Ni-NTA QIAfilter Plate and elutes the 6xHis-tagged proteins into the chosen elution vessel.

    A beeper sounds to let you know that the purification process is finished.

Medium-Scale Purification of 6xHis-tagged Proteins under Denaturing Conditions

This section gives an overview of the protein purification procedure under denaturing conditions, and the order of the different operations, rather than a detailed, stepwise description of the protocol. User interactions necessary during a run on the BioRobot 9600 and 3000 systems are indicated. For a detailed description and follow-up of the current operations, please follow the messages and information given on the screen during the run of an individual protocol.

Materials

Bacterial cultures — for guidelines on the growth of bacterial expression cultures for this protocol, see page 24.

Elution vessel (Square-well Block or Collection Microtube Rack) – for ordering information see page 60.

2 x 14 ml polypropylene tubes (4 x 4 ml polypropylene tubes for the adapted protocol)

Channeling Block

TurboFilter 96 Plate

QIAfilter 96 Plate

Preparation of buffers

Buffer B–8 M urea	**for 500 ml:**
100 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$
10 mM Tris·Cl	0.61 g Tris base
8 M urea	240.24 g urea

Adjust pH to 8.0 using HCl. Provide 250 ml in a 250 ml bottle.

Buffer B–7 M urea/Benzonase	**for 500 ml:**
100 mM NaH2 PO4	6.90 g $NaH_2\ PO_4 \cdot H_2\ O$
10 mM Tris·Cl	0.61 g Tris base
7 M urea	210.21 g urea

Adjust pH to 8.0 using HCl. Prepare a volume of Buffer B–7 M urea and add the required units of Benzonase as given in the display message, mix, and provide in a 60 ml bottle.

Buffer B–4 M urea	**for 500 ml:**
100 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$
10 mM Tris·Cl	0.61 g Tris base
4 M urea	120.12 g urea

Adjust pH to 8.0 using HCl. Provide 500 ml in a 500 ml bottle.

Buffer C–4 M urea	**for 500 ml:**
100 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$
10 mM Tris·Cl	0.61 g Tris base
4 M urea	120.12 g urea

Adjust pH to 6.3 using HCl. Provide 500 ml in a 500 ml bottle.

Buffer E–8 M urea	**for 500 ml:**
100 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$
10 mM Tris·Cl	0.61 g Tris base
8 M urea	240.24 g urea

Adjust pH to 4.5 using HCl. Provide 200 ml in a 250 ml bottle.

Provide a 250 ml bottle containing 150 ml analytical grade ethanol (100%).

Provide a 500 ml bottle containing 250 ml distilled water.

Buffer A–6 M Gu·HCl	**for 500 ml:**
100 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$
10 mM Tris·Cl	0.61 g Tris base
6 M Gu·HCl	286.5 g guanidine hydrochloride

Adjust pH to 8.0 using HCl.

Buffer A–6 M Gu·HCl can be used instead of Buffer B–8 M urea for cell lysis under denaturing conditions. Note that Gu·HCl inactivates Benzonase, and therefore cannot be used for the adapted protocol.

Table 6. Tubing/position on work table for purification under denaturing conditions

Buffers/solutions/accessories	BioRobot 9600/3000	BioRobot 8000
Buffer B-8M urea	Tubing 8	Rotor Slot 1
Buffer B-7M urea/Benzonase*	Reagent Slot B9	Reagent Slot B9
Buffer B-4M urea	Tubing 1	Rotor Slot 2
Buffer C-4M urea	Tubing 2	Rotor Slot 4
Buffer E-8M urea	Tubing 9	Rotor Slot 3
Distilled water	Tubing 3	Rotor Slot 6
Ethanol	Tubing 7	Therm Subslot A, Trough 20ml
Ni-NTA Superflow suspension	Reagent Slot B1+B2	Therm Subslot B, Position A+B
Turbofilter 96 Plate	Plate holder/ MP Slot 1	Plate Slot 6
QIAfilter 96 Plate	Vacuum manifold top	Plate Slot 6/2
Plate Holder	–	MP Slots 6, 11, 12, 16
Filtration Adapter	–	Plate Slot 16
Channeling Block	Vacuum Manifold Base†	Plate Slot 11
96-well elution vessel:		
one vessel	MP Slot 3	MP Slot 8
two vessels	MP Slot 3	MP Slot 8+9
24-well blocks	Shaker back/left	Shaker back/left
	Shaker front/left	Shaker front/left
	Shaker back/right	Shaker back/right
	Shaker front/right	Shaker front/right

** Used for cell lysis in adapted protocol.*

† The Channeling Block should be placed in this position during user interaction 2.

Protocol for the BioRobot 9600 and 3000

Note: If the Ni-NTA Superflow 96 BioRobot Kit or Core Kit is used exclusively for the adapted high-yield medium-scale protocols, both Benzonase and an additional 200 ml Ni-NTA Superflow resin are required.

1. **Prepare all reagents required for the protocol (see above).**

   Due to the dissociation of urea, the pH of the buffers should be adjusted immediately prior to use.

2. **Make sure that the High-Speed Pipetting System and the BioRobot are switched on.**

   Note: Always switch on the High-Speed Pipetting System before the BioRobot.

3. **Switch on the computer and monitor.**
4. **Select "Execute" environment in the "Main Menu" (if necessary).**
5. **Select the "Protein Applications" package in the toolbar drop-down list and from this package select "Ni-NTA Superflow 96 denaturing" or "Ni-NTA Superflow denaturing 1 mg".**
6. **Click "RUN" on the toolbar.**

   The "Run Protocol: slot config." dialog box appears.

7. **Select the cultivation vessel type to be processed: 24-Well Blocks or 14 ml polypropylene tubes.**
8. **Click "OK".**

   The "Run protocol: No. of Samples" dialog box appears.

9. **Enter the number of samples to be processed (24–96).**

   The final arrangement of the samples in four 24-Well Blocks placed on the shaker mtp is as if they were a single 96-well plate (see Figure 4, page 20). If using 14 ml tubes, place them into the corresponding positions in the appropriate shaker rack.

10. **Click "OK".**

    The first protocol message box appears.

11. **Make sure that the system liquid reservoir is filled with distilled water.**
12. **Make sure that the peristaltic-pump cassettes are fitted.**
13. **Empty the vacuum trap.**
14. **Empty the waste container.**
15. **Click "Continue".**
16. **Using the information given in the following protocol message boxes:**

    Connect the buffer bottles in the external buffer rack to the tubing.

    Seal any unused wells of the 96-well modules with tape from a Tape Pad.

17. **Click "Continue".**

    The next protocol message box appears.

18. **Place the 24-Well Blocks or the 14 ml tube shaker rack onto the shaker mtp.**

    Note: For the position of the 24-Well Blocks, refer to Figure 4, page 20.

19. **Click "Continue".**

    Wait for the BioRobot to initialize and calibrate.

20. **Click the speaker icon to stop the beeper, and provide the required volume of Ni-NTA Superflow suspension.**

    The required volume is calculated from the number of samples processed (a safety reserve is included).

21. **Resuspend the resin thoroughly, transfer the required volume of suspension into 1 x, 2 x, or 4 x 14 ml polypropylene tubes, and place the tube(s) in the appropriate position (see Table 6).**

22. **Enter the elution volume.**

    An elution volume between 350 and 600 µl (400 and 800 µl for the adapted protocol) can be chosen. The standard elution volume is 450 µl/650 µl per well. Reduced recovery may result from reducing the elution volume.

    Approximately 70% of bound 6xHis-tagged protein elutes within the first elution. A second elution step can be performed.

23. **Choose a 96-well elution vessel from list.**

    Depending upon the elution vessel and volume chosen, 1 or 2 eluates may be recovered in a single 96-well vessel.

    A message appears as to where to position the 96-well elution vessel(s) on the worktable.

24. **Click "Continue".**

    The system probes and dilutor syringes are flushed.

    The volumes of the solutions provided are checked.

    Buffer B–8 M urea/Buffer B-7M urea/Benzonase is distributed for resuspension and lysis of bacterial pellets.

    The vessels containing the cells are shaken at 750 rpm for 30 min. For efficient resuspension, the shaker changes the direction of rotation every 30 s.

    After completion of buffer distribution the system is flushed.

    The QIAfilter 96 Plate is washed with water. 1 ml is pipetted into each well and drawn through by application of a vacuum.

    Ni-NTA Superflow resin is resuspended and distributed to the QIAfilter Plate.

    200 µl/400 µl of Ni-NTA Superflow suspension corresponding to 100 µl/200 µl bed volume is pipetted per well, followed by 600 µl of water. The water is removed by vacuum application.

    A beeper sounds to let you know that the Ni-NTA QIAfilter Plate is ready.

User interaction 1

1. **Place the Ni-NTA QIAfilter Plate within a Plate Holder into accessory slot 2.**
2. **Click "Continue".**

   A beeper sounds.
3. **Place a TurboFilter 96 Plate into the vacuum manifold top position.**

   Seal any unused wells with tape from a Tape Pad.
4. **Click "Continue".**

   The TurboFilter 96 Plate is washed. 1 ml of distilled water is pipetted into each well and drawn through by vacuum application.
5. **Place the Plate Holder and the Ni-NTA QIAfilter Plate into the vacuum manifold base position.**

   Note: Take care that the outlets of the TurboFilter Plate correctly fit into the wells of the Ni-NTA QIAfilter Plate below. Check by pressing on the TurboFilter Plate; the TurboFilter Plate should move downwards slightly. If the plates do not fit together correctly, the pump cannot create the desired vacuum.
6. **Click "Continue".**

   The BioRobot workstation transfers the cell lysates from the 24-Well Blocks on the shaker to the TurboFilter Plate. Each TurboFilter well is overlayed with 100 µl ethanol to reduce frothing. The lysates are then cleared by vacuum filtration and pass directly into the wells of the Ni-NTA QIAfilter Plate.

   A beeper sounds to let you know that the process is finished.

User interaction 2

1. **Discard the TurboFilter Plate.**
2. **Place Channeling Block into the vacuum manifold base position and place the Ni-NTA QIAfilter Plate into the vacuum manifold top position.**

   Fit the outlets of the Ni-NTA QIAfilter Plate into the channels of the Channeling Block.
3. **Click "Continue".**

   A weak vacuum of –250 mbar is applied and the 6xHis-tagged proteins bind to the Ni-NTA Superflow affinity resin while slowly passing through the filter.

   Finally, the vacuum is increased briefly to ensure completion of binding and flow through of the samples in each well. The BioRobot washes each well of the Ni-NTA QIAfilter Plate with 800 µl Buffer B-4 M urea, followed by two washes with 800 µl Buffer C-4 M urea.

   A beeper sounds to let you know that the step is finished.

User interaction 3

1. **Place the chosen elution vessel into the vacuum manifold base position.**
2. **Click "Continue".**

   The BioRobot distributes elution buffer (Buffer E–8 M urea) into the Ni-NTA QIAfilter Plate and elutes the 6xHis-tagged proteins into the chosen elution vessel.

   A beeper sounds to let you know that the purification process is finished.

Protocol for the BioRobot 8000

Note: If the Ni-NTA Superflow 96 BioRobot Kit or Core Kit is used exclusively for the adapted high-yield medium-scale protocols, both Benzonase and an additional 200 ml Ni-NTA Superflow resin are required.

1. **Prepare all reagents required for the protocol (see above).**

   Due to the dissociation of urea, the pH of the buffers should be adjusted immediately prior to use.

2. **Make sure that workstation and computer are switched on.**
3. **Select "Execute" environment in the "Main Menu" (if necessary).**
4. **Select the "Protein Applications" package in the toolbar drop-down list and from this package select "Ni-NTA Superflow 96 denaturing" or "Ni-NTA Superflow 96 denaturing 1mg".**
5. **Click "RUN" on the toolbar.**

   The "Run Protocol: slot config." dialog box appears.

6. **Click "OK".**

   The first protocol message box appears.

7. **Make sure that the system liquid reservoir is filled with distilled water.**
8. **Empty the vacuum trap and vacuum condensate trap.**
9. **Empty the waste container.**
10. **Using the information given in the following protocol message boxes:**

    Place bottles in the relevant position (given in Table 6).

    Place the 24-Well Blocks onto the shaker.

    Note that the BioRobot 8000 always processes all 96 wells on a plate. If the number of sample-containing wells is less than 96, all remaining wells will also be processed. For the position of the 24-Well Blocks, refer to Figure 4, page 20.

11. **Provide the required volume of Ni-NTA Superflow suspension.**

    Resuspend the resin thoroughly. Take 2 x 14 ml (4 x 14 ml for the adapted protocol) polypropylene tubes, transfer 10 ml of suspension into each tube, and place the tubes in the appropriate positions (see Table 6).

12. **Enter the elution volume.**

    An elution volume between 450 and 800 µl can be chosen. The standard elution volume is 650 µl per well. Reduced recovery may result from reducing the elution volume.

    Approximately 70% of bound 6xHis-tagged protein elutes within the first elution. A second elution step can be performed.

13. Choose a 96-well elution vessel from list.

Depending upon the elution vessel and volume chosen, 1 or 2 eluates may be recovered in a single 96-well vessel.

A message appears as to where to position the 96-well elution vessel(s) on the worktable.

14. Click "Continue".

The system probes are flushed using the peristaltic pump. The dilutor syringes are flushed.

The volumes of the solutions provided are checked.

Buffer B–8 M urea/Buffer B-7M urea/Benzonase is distributed for resuspension and lysis of bacterial pellets.

The vessels containing the cells are shaken at 750 rpm for 30 min. For efficient resuspension, the shaker changes the direction of rotation every 30 s.

After completion of buffer distribution the system is flushed.

The robot assembles the vacuum chamber system.

The QIAfilter 96 Plate is washed with water. 1 ml is pipetted into each well and drawn through by vacuum application.

Ni-NTA Superflow resin is resuspended and distributed to the QIAfilter Plate.

200 µl/400 µl of Ni-NTA Superflow suspension corresponding to 100 µl/200 µl bed volume is pipetted per well, followed by 600 µl of water. The water is removed by application of a vacuum.

The BioRobot transfers the cell lysates from the shaker to the TurboFilter Plate. Each TurboFilter well is overlayed with 100 µl ethanol to reduce frothing. The lysates are cleared by vacuum filtration and pass directly into the wells of the Ni-NTA Superflow QIAfilter Plate. The TurboFilter Plate is removed. The Ni-NTA QIAfilter Plate is placed into the vacuum manifold top position.

A weak vacuum of –250 mbar is applied and the 6xHis-tagged proteins bind to the Ni-NTA Superflow affinity resin while slowly passing through the filter.

Finally, the vacuum is increased briefly to ensure completion of binding and flow through of the samples in each well. The BioRobot washes each well of the Ni-NTA QIAfilter Plate with 800 µl Buffer B-4 M urea, followed by two washes with 800 µl Buffer C-4 M urea.

The elution vessel is placed into the vacuum manifold base position.

The BioRobot distributes elution buffer (Buffer E–8 M urea) into the Ni-NTA QIAfilter Plate and elutes the 6xHis-tagged proteins into the chosen elution vessel.

A beeper sounds to let you know that the purification process is finished.

Large-Scale Purification of 6xHis-tagged Proteins under Native Conditions

Large-scale Ni-NTA Superflow protocols are designed for automated purification of 6xHis-tagged proteins using the BioRobot 3000 workstation. Up to 12 cleared lysates from up to 1 liter culture volume can be processed per vacuum manifold. A protocol for the efficient preparation of cleared lysates is provided below.

Ni-NTA Superflow Columns are designed to process up to 1 liter *E. coli* culture volume with an OD_{600} ~6. Therefore, it should be possible to process up to 100 ml cell culture from a small-scale high-density fermenter with a final OD_{600} of 50–60.

The Ni-NTA Superflow large-scale procedure using Ni-NTA Superflow Columns (1.5 ml) can only be performed on the BioRobot 3000 workstation. In addition to Ni-NTA Superflow Columns, the following equipment is required to run these protocols:

- Set of short steel probes
- QIAvac 6S top plate
- QIAvac Luer Adapter Set
- Shaker Adapter (96-tube, 13 ml).

See page 60 for ordering information.

This section gives an overview of the protein purification procedure under native conditions, and the order of the different operations, rather than a detailed, stepwise description of the protocol. User interactions necessary during a run on the BioRobot 3000 system are indicated. For a detailed description and follow-up of the current operations, please follow the messages and information given on the screen during the run of an individual protocol.

Preparation of cleared lysates from *E. coli* cell pellets under native conditions

This protocol describes the generation of cleared cell lysate from *E. coli* cells. The entire lysis and purification protocols are performed at room temperature to avoid repeated temperature shifts that may be harmful to protein activity and structure.

1. **Place frozen bacterial cells (in 50 ml tubes) at room temperature and allow to thaw for 15 min.**
2. **Add 10 ml Buffer NPI-10, X ml lysozyme solution (10 mg/ml), and 3 units Benzonase for every ml of the original cell culture volume (for example, for a 100 ml cell culture, add 300 units Benzonase) to the cell pellet.**
3. **Resuspend the pellet by pipetting up and down.**
4. **Incubate for 30 min at room temperature.**

5. **Transfer crude lysates into appropriate tubes and centrifuge for 15 min at 15°C and 15,000 x *g*. Insoluble cell components are pelleted at the bottom of the tube.**
6. **Collect supernatants containing soluble 6xHis-tagged proteins, transfer into 14 ml polypropylene tubes, and place into the relevant position of the Shaker Adapter (see Purification Procedure).**

Purification procedure

Materials

Cleared cell lysates — see above for a protocol for efficient generation of cleared lysates from *E. coli* cell pellets

Ni-NTA Superflow Columns (1.5 ml) — for ordering information see page 60

Elution vessel (24-well block) — for ordering information see page 60

14 ml polypropylene tubes

Channeling Block

Set of 4 short steel probes

QIAvac 6S top plate

QIAvac Luer Adapter Set (6 strips of 4 adapters each)

Shaker Adapter (96-tube, 13 ml)

Preparation of buffers and reagents

Buffer NPI-10	**for 500 ml:**
50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$
300 mM NaCl	8.77 g NaCl
10 mM imidazole	0.34 g imidazole

Adjust pH to 8.0 using NaOH. Prepare 500 ml in a 500 ml bottle.

Buffer NPI-20	**for 500 ml:**
50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$
300 mM NaCl	8.77 g NaCl
20 mM imidazole	0.68 g imidazole

Adjust pH to 8.0 using NaOH. Prepare 500 ml in a 500 ml bottle.

Buffer NPI-250	**for 500 ml:**
50 mM NaH_2PO_4	3.45 g $NaH_2PO_4 \cdot H_2O$
300 mM NaCl	8.77 g NaCl
250 mM imidazole	8.51 g imidazole

Adjust pH to 8.0 using NaOH. Provide 200 ml in a 250 ml bottle.

Optional: Protease inhibitors can be added to all buffers. Strong chelators such as EDTA should be avoided, as they will strip nickel ions from the Ni-NTA matrix. For ease of use we recommend EDTA-free complete protease inhibitor cocktail tablets, for example Roche Applied Science, cat. no. 1 873 580.

Table 7. Tubing/position on worktable for purification under native conditions

Buffers/solutions/accessories	**BioRobot 3000**
Buffer NPI-10	Tubing 1
Buffer NPI-20	Tubing 2
Buffer NPI-250	Tubing 8
Channeling Block	Vacuum Manifold Base
Luer Adapter strips	Vacuum Manifold Top
24-well elution vessel:	
one vessel	MP Slot X
two vessels	MP Slot Y
Shaker Adapter (96-tube, 13 ml)	shaker

Positioning of Columns on the Vacuum Manifold

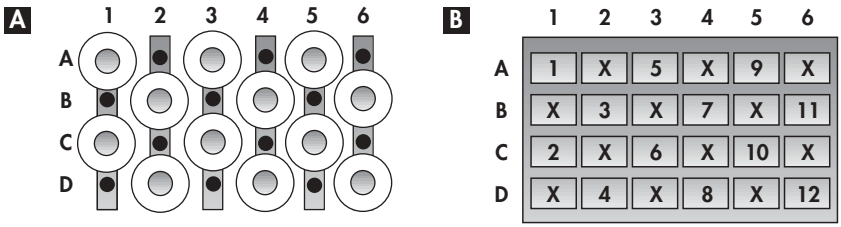


Figure 6. **A** *Positioning of Ni-NTA Superflow Columns on Luer Adapter Strips on the QIAvac 6S Top Plate.* **B** *Elution fractions (1–12) are collected in the indicated wells of a 24-well block.*

Protocol for the BioRobot 3000

1. **Prepare all reagents required for the protocol (see above).**
2. **Make sure that the High-Speed Pipetting System and the BioRobot are switched on.**

   Note: Always switch on the High-Speed Pipetting System before the BioRobot.
3. **Switch on the computer and monitor.**
4. **Select "Execute" environment in the "Main Menu" (if necessary).**
5. **Select the "Protein Applications" package in the toolbar drop-down list and from this package select "Ni-NTA Superflow native large-scale".**
6. **Click "RUN" on the toolbar.**

   The "Run protocol: No. of Samples" dialog box appears.
7. **Enter the number of samples to be processed (1–24).**
8. **Click "OK".**

   The first protocol message box appears.
9. **Make sure that the system liquid reservoir is filled with distilled water.**
10. **Make sure that the peristaltic-pump cassettes are fitted.**
11. **Empty the vacuum trap.**
12. **Empty the waste container.**
13. **Click "Continue".**
14. **Using the information given in the following protocol message boxes:**

    Connect the buffer bottles in the external buffer rack to the tubing.

    Seal any unused Luer adapters on the strips in the QIAvac 6S top plate.
15. **Click "Continue".**

    The next protocol message box appears.
16. **Place the 14 ml tubes containing cleared lysates into the relevant positions in the appropriate shaker rack.**
17. **Place a corresponding number of Ni-NTA Superflow Columns (1.5 ml) in the odd positions (starting at position A1, see Figure 6, page 47) of the Luer adapter strips in the top plate of the vacuum manifold.**

    Note: Break the seals at the outlet of the columns before opening the screw cap! Fit the outlets of the columns into the channels of the Channeling Block.
18. **Click "Continue".**

    Wait for the BioRobot to initialize and calibrate.

19. **Click the speaker icon to stop the beeper, and enter the elution volume.**

    An elution volume of between 2 and 3 ml can be chosen. The standard elution volume is 3 ml per column. Reduced recovery may result from reducing the elution volume. Approximately 70% of bound 6xHis-tagged protein elutes within the first elution. A second elution step can be performed and both eluates may be recovered in a single 24-well block. A message appears as to where to position the 24-well elution vessel(s) on the worktable.

20. **Click "Continue".**

    The system probes and dilutor syringes are flushed.

    The volumes of the solutions provided are checked.

    Storage buffer is drawn through the Ni-NTA Superflow columns and Buffer NPI-10 is distributed for equilibration of the columns. After completion of Buffer distribution the system is flushed. Equilibration buffer is drawn through the Ni-NTA Superflow Columns.

    Samples are loaded onto the Ni-NTA Superflow columns. The BioRobot workstation transfers 2 ml aliquots of cleared lysate from the Shaker Adapter to the corresponding column on the vacuum manifold. A weak vacuum of –10 mbar is applied and the samples slowly pass through the columns allowing the 6xHis-tagged proteins to bind to the Ni-NTA resin.

    Columns are washed twice by application of Buffer NPI-20.

User interaction 1

1. **Remove the Channeling Block and place a 24-well block in the vacuum manifold base position for elution of the 6xHis-tagged proteins. Place the outlets of the Ni-NTA Superflow Columns (1.5 ml) above the corresponding wells of the 24-well block.**

2. **Click "Continue".**

    Elution buffer NPI-250 is distributed to the columns and a weak vacuum is applied. Elution fractions are collected in the wells of the 24-well block as described in Figure 6.

    A beeper sounds to let you know that the purification process is finished.

Large-Scale Purification of 6xHis-tagged Proteins under Denaturing Conditions

This section gives an overview of the protein purification procedure under native conditions, and the order of the different operations, rather than a detailed, stepwise description of the protocol. User interactions necessary during a run on the BioRobot 3000 system are indicated. For a detailed description and follow-up of the current operations, please follow the messages and information given on the screen during the run of an individual protocol.

Preparation of cleared lysates from *E. coli* cell Pellets under denaturing conditions

This protocol describes the generation of cleared cell lysate from *E. coli* cells. The entire lysis and purification protocols are performed at room temperature to avoid repeated temperature shifts that may be harmful to protein activity and structure.

1. **Place frozen bacterial cells (in 50 ml tubes) at room temperature and allow to thaw for 15 min.**
2. **Add 10 ml Buffer B-7M urea, and 3 units Benzonase for every ml of the original cell culture volume (for example, for a 100 ml cell culture, add 300 units Benzonase) to the cell pellet.**
3. **Resuspend the pellet by pipetting up and down.**
4. **Incubate for 30 minutes at room temperature.**
5. **Transfer crude lysates into appropriate tubes and centrifuge for 15 min at 15°C and 15,000 x *g*. Insoluble cell components are pelleted at the bottom of the tube.**
6. **Collect supernatants containing soluble 6xHis-tagged proteins, transfer into 14 ml polypropylene tubes, and place into the relevant position of the Shaker Adapter (see Purification Procedure).**

Purification procedure

Materials

Cleared cell lysates — see above for a protocol for efficient generation of cleared lysates from *E. coli* cell pellets

Ni-NTA Superflow Columns (1.5 ml) — for ordering information see page 60

Elution vessel (24-well Block) — for ordering information see page 60

14 ml polypropylene tubes

Channeling Block

Set of 4 short steel probes

QIAvac 6S top plate

QIAvac Luer Adapter Set (6 strips of 4 adapters each)

Shaker Adapter (96-tube, 13 ml)

Preparation of buffers and reagents

Buffer B–7 M urea for 500 ml Buffer B–7 M urea:

100 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$
10 mM Tris·Cl	0.61 g Tris base
7 M urea	210.21g urea

Adjust pH to 8.0 using HCl. Provide 500 ml in a 500 ml bottle.

Buffer C–7 M urea	**for 500 ml:**
100 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$
10 mM Tris·Cl	0.61 g Tris base
7 M urea	210.21g urea

Adjust pH to 6.3 using HCl. Provide 500 ml in a 500 ml bottle.

Buffer E–8 M urea	**for 500 ml:**
100 mM NaH_2PO_4	6.90 g $NaH_2PO_4 \cdot H_2O$
10 mM Tris·Cl	0.61 g Tris base
8 M urea	240.24 g urea

Adjust pH to 4.5 using HCl. Provide 200 ml in a 250 ml bottle.

Table 8. Tubing/position on work table for purification under denaturing conditions

Buffers/solutions/accessories	**BioRobot 3000**
Buffer B–7 M urea	Tubing 8
Buffer C–7 M	Tubing 2
Buffer E–8 M	Tubing 9
Channeling Block	Vacuum Manifold Base
Luer Adapter strips	Vacuum Manifold Top
24-well elution vessel:	
one vessel	MP Slot X
two vessels	MP Slot Y
Shaker Adapter (96-tube, 13 ml)	shaker

Protocol for the BioRobot 3000

1. **Prepare all reagents required for the protocol (see above).**

   Due to the dissociation of urea, the pH of the buffers should be adjusted immediately before use.

2. **Make sure that the High-Speed Pipetting System and the BioRobot are switched on.**

   Note: Always switch on the High-Speed Pipetting System before the BioRobot.

3. **Switch on the computer and monitor.**
4. **Select "Execute" environment in the "Main Menu" (if necessary).**
5. **Select the "Protein Applications" package in the toolbar drop-down list and from this package select "Ni-NTA Superflow denaturing large-scale".**
6. **Click "RUN" on the toolbar.**
7. **The "Run protocol: No. of Samples" dialog box appears.**
8. **Enter the number of samples to be processed (1–24).**
9. **Click "OK".**

   The first protocol message box appears.

10. **Make sure that the system liquid reservoir is filled with distilled water.**
11. **Make sure that the peristaltic-pump cassettes are fitted.**
12. **Empty the vacuum trap.**
13. **Empty the waste container.**
14. **Click "Continue".**
15. **Using the information given in the following protocol message boxes:**

    Connect the buffer bottles in the external buffer rack to the tubing.

    Seal any unused Luer adapters on the strips in the QIAvac 6S top plate.

16. **Click "Continue".**

    The next protocol message box appears.

17. **Place the 14 ml tubes containing cleared lysates into the relevant positions in the appropriate shaker rack.**
18. **Place a corresponding number of Ni-NTA Superflow Columns (1.5 ml) in the odd positions (starting at position A1, see Figure 7) of the Luer adapter strips in the top plate of the vacuum manifold.**

    Note: Break the seals at the outlet of the columns before opening the screw cap! Fit the outlets of the columns into the channels of the Channeling Block.

19. **Click "Continue".**

    Wait for the BioRobot to initialize and calibrate.

20. Click the speaker icon to stop the beeper, and enter the elution volume.

An elution volume of between 2 and 3 ml can be chosen. The standard elution volume is 3 ml per column. Reduced recovery may result from reducing the elution volume.

Approximately 70% of bound 6xHis-tagged protein elutes within the first elution. A second elution step can be performed, and both eluates may be recovered in a single 24-well block.

A message appears as to where to position the 24-well elution vessel(s) on the worktable.

21. Click "Continue".

The system probes and dilutor syringes are flushed.

The volumes of the solutions provided are checked.

Storage buffer is drawn through the Ni-NTA Superflow columns and Buffer B–7 M urea is distributed for equilibration of the columns. After completion of buffer distribution the system is flushed. Equilibration buffer is drawn through the Ni-NTA Superflow Columns.

Samples are loaded onto the Ni-NTA Superflow columns. The BioRobot workstation transfers 2 ml aliquots of cleared lysate from the Shaker Adapter to the corresponding column on the vacuum manifold. A weak vacuum of –10 mbar is applied and the samples slowly pass through the columns allowing the 6xHis-tagged proteins to bind to the Ni-NTA resin.

Columns are washed in two steps by sequential application of Buffer B–7 M urea and Buffer C–7 M urea.

User interaction 1

1. Remove the Channeling Block and place a 24-Well Block in the vacuum manifold base position for elution of the 6xHis-tagged proteins. Place the outlets of the Ni-NTA Superflow Columns (1.5 ml) above the corresponding wells of the 24-Well Block.

2. Click "Continue".

Elution buffer C–8 M urea is distributed to the columns and a weak vacuum is applied. Elution fractions are collected in the wells of the 24-well block as described in Figure 6, page 47.

A beeper sounds to let you know that the purification process is finished.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists at QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications (see inside front cover).

	Comments and Suggestions
Eluates contain no protein	
Protein does not bind to the Ni-NTA resin	
a) 6xHis tag is not present	Sequence ligation junctions to ensure that the reading frame is correct. Check for possible internal translation starts (N-terminal tag) or premature termination sites (C-terminal tag).
b) 6xHis tag is inaccessible	Purify protein under denaturing conditions. In some rare cases, recombinant protein may not dissolve in Buffer B–8 M urea. Buffer A–6 M Gu·HCl can be used instead of Buffer B–8 M urea for cell lysis. Move tag to opposite end of protein.
c) 6xHis tag has been degraded	Check that the 6xHis tag is not associated with a portion of the protein that is processed.
d) Binding conditions incorrect	Check pH and composition of all buffers and solutions. Dissociation of urea often causes a shift in pH. pH values should be checked immediately prior to use. Ensure that the concentration of chelating or reducing agents, or imidazole is not too high (refer to Table 4, page 15).
Protein elutes in the wash buffer	
a) Wash stringency is too high	Lower the concentration of imidazole or increase the pH slightly.
b) 6xHis tag is partially hidden	Reduce wash stringency. Purify under denaturing conditions.
c) Buffer conditions incorrect	Check pH and composition of wash buffer. Ensure that there are no chelating or reducing agents present.

Protein precipitates during purification

a) Temperature is too low	Perform purification at room temperature.
b) Protein forms aggregates	Try adding solubilization reagents such as 0.1% Triton X-100 or Tween 20, up to 20 mM β-mercaptoethanol, up to 2 M NaCl, or stabilizing cofactors such as Mg^{2+}. These may be necessary in all buffers to maintain protein solubility.

Protein does not elute

a) Elution conditions are too mild (protein may be in an aggregate or multimer form)	Elute with a pH or imidazole step-gradient to determine the optimal elution conditions.
b) Protein has precipitated in the column	Elute under denaturing conditions.

Yields are low

Increase expression rates by optimizing the conditions (e.g., by increasing induction time) or increase culture volumes.

Check the concentration of IPTG used for induction. Optimization of the IPTG concentration may increase expression rates.

Check the conductance of the buffers, especially Buffer NPI-250. Buffer NPI-250 should contain sufficient imidazole to elute the protein. When correctly made up the buffers should have the following conductances:

NPI-10: 30 mS/cm
NPI-20: 32 mS/cm
NPI-250: 40 mS/cm

Check the pH of denaturing buffers:

Buffer A–6 M Gu·HCl: pH 8.0
Buffer B–8 M urea: pH 8.0
Buffer B–4 M urea: pH 8.0
Buffer C–4 M urea: pH 6.3
Buffer E–8 M urea: pH 4.5

	Comments and Suggestions
Two or more proteins copurified	Two bacterial colonies may have been picked. Make sure that bacterial cells are sufficiently dilute prior to spreading on plates to allow single colonies to be picked. An interacting protein may have been copurified with the 6xHis-tagged protein. Add β-mercaptoethanol to a maximum of 20 mM to reduce disulphide bonds. Increase salt and/or detergent concentrations, or add ethanol/glycerol to wash buffer to disrupt nonspecific interactions.
Protein not sufficiently pure	Collection Microtubes were too full. Elution volumes that are too high can lead to cross-contamination between wells — the maximum recommended volume for elution into Collection Microtubes is 500 µl. When proteins are eluted twice into one vessel, or using elution volumes greater than 500 µl, use S-Blocks. Wells were not tightly sealed with AirPore Tape during cultivation leading to cross-contamination. Seal wells with AirPore Tape. Low expression rates can lead to a suboptimal ratio of Ni-NTA resin to 6xHis-tagged protein to be purified. Increase expression rate (e.g., by increasing induction time) or increase culture volumes. Too little buffer in reservoir bottles to efficiently wash each well. Before starting the procedure always fill each bottle. Check the pH of denaturing buffers: Buffer A–6 M Gu·HCl: pH 8.0 Buffer B–8 M urea: pH 8.0 Buffer B–4 M urea: pH 8.0 Buffer C–4 M urea: pH 6.3 Buffer E–8 M urea: pH 4.5

Samples were not completely drawn through the TurboFilter plate in the allotted time although the usual vacuum pressure was achieved	If the cultures are too dense, the lysates may be too viscous to pass through the TurboFilter Plate. Reduce final OD_{600} of the cultures. Do not allow cultures to reach an OD_{600} higher than 6. Inactive Benzonase led to clogging of filters. Be sure to add the correct amount of Benzonase as given in the display message. Be sure that the correct lysis buffer had been provided (NPI-10/Benzonase or Buffer B-7 M urea/Benzonase). Benzonase is inactive in the presence of >100 mM Gu·HCl.
Lysate cleared using the wrong plate	If the QIAfilter Plate is used for clearing lysates then the wells may become blocked. The QIAfilter Plate should only be used for the affinity chromatography steps.
Insufficient vacuum during vacuum steps	Check that the connection between the waste container and the pump is airtight. The lid should be screwed tight to ensure that a vacuum can be built up. Make sure that all components on the lid are correctly assembled. Check the integrity of the TurboFilter or QIAfilter 96 Plate. If the edges of the plate are broken it will prevent vacuum build up. If the outlet collars of the upper plate (TurboFilter Plate or QIAfilter Plate) do not fit into the inlets of the lower plate (QIAfilter Plate or Channeling Block), or the upper plate cannot be pressed into the flexible seal on the vacuum chamber, then the vacuum may be insufficient. Ensure that all plates are correctly positioned.

Large-scale protocols

Desired vacuum not reached	Columns were not fitted tightly into Luer adapters. Ensure that columns fit tightly by twisting when inserting them into the adapters.
Samples are drawn through columns with unequal speed	Uneven vacuum was applied to different columns, because columns were not fitted equally tightly into adapters. Ensure that columns fit tightly by twisting when inserting them into the adapters.
Samples are not drawn through columns	Lysate is too viscous. Be sure to add Benzonase to lysis buffers. Residual genomic DNA/RNA may cause high viscosity, especially when large culture volumes with a high final OD_{600} are processed.
Cell debris was recovered with the supernatant	Be sure not to disturb parts of the cell debris pellet after centrifugation of cell lysates.
Air bubbles introduced at bottom of column	Break seal at outlet of the column first and insert into Luer adapter or gravity flow stand, then open screw cap. This ensures that air caught at the outlet during production of the column is released in direction of the outlet and not through the frit and into the resin.
Columns run dry during vacuum-based purification process	Even if columns run dry, this will not interfere with protein purification and recovery. The weak vacuum applied will not overdry the resin. Protocols have been optimized to the described buffer conditions and to "normal" protein concentrations. If columns are regularly seen to run dry, shorten vacuum times in the individual steps affected.
Protein is eluted into adjoining wells	Fit column outlets above corresponding wells of the 24-well block before the elution step.

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Ordering Information

Product	Contents	Cat. No.
Ni-NTA Superflow 96 BioRobot Kit (24)	For 24 x 96 rapid purification of 6xHis-tagged proteins: 24 QIAfilter 96 Plates, 24 TurboFilter 96 Plates, 3 x 100 ml Ni-NTA Superflow	969263
BioRobot 9600*	System includes: robotic workstation with 2 dilutor drives; microprocessor-controlled shaker and vacuum pump; vacuum manifold; QIAsoft 3.0 Operating System; computer, cables; installation and training; 1-year warranty on parts and labor	900200
BioRobot 3000*	System includes: custom-designed robotic workstation comprised of 2–4 dilutor units and selected system components; QIAsoft 4.0 Operating System; computer; installation and training; 1 year warranty on parts and labor; worktables of various sizes are available	900400
BioRobot 8000*	System includes: robotic workstation comprised of 8 dilutor units and selected system components; variable spacing system; QIAsoft 4.0 Operating System; computer; installation and training; 1 year warranty on parts and labor	900500
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Square-Well Blocks (24)	96-well blocks with 2.2 ml wells, 24 per case	19573
24-Well Blocks RB (24)	24-well blocks with 10 ml wells, 24 per case	19583
AirPore Tape Sheets (50)	Microporous tape sheets for covering multiwell blocks: 50 sheets per pack	19571
Tape Pads (5)	Adhesive tape sheets for sealing multiwell plates and blocks: 25 sheets per pad, 5 pads per pack	19570

** QIAGEN Robotic Systems are not available in all countries; please inquire.*

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Collection Microtubes (racked)	Nonsterile polypropylene tubes (1.2 ml), 960 in racks of 96	19560
Collection Microtube Caps	Nonsterile polypropylene caps for collection microtubes (1.2 ml) and round-well blocks, 960 in strips of 8	19566
Collection Microtubes (racked)	For use with the BioRobot 8000	Inquire
Related products		
96-Well Microplates RB (24)	96-well microplates with round-bottom wells plus lids, 24 per case, for use with QIAvac manifolds and BioRobot Systems	19581
Ni-NTA Magnetic Agarose Beads (2 x 1 ml)	2 x 1 ml nickel-charged magnetic agarose beads (5% suspension)	36111
Ni-NTA Magnetic Agarose Beads (6 x 1 ml)	6 x 1 ml nickel-charged magnetic agarose beads (5% suspension)	36113
96-Well Magnet Type A	Magnet for separating magnetic beads in wells of 96-well plates, 2 x 96-Well Microplates FB	36915
Ni-NTA HisSorb Plates (5)	5 Ni-NTA–coated, transparent 96-well plates	35061
Ni-NTA HisSorb Plates (25)	25 Ni-NTA–coated, transparent 96-well plates	35063
Ni-NTA HisSorb Plates, white (5)	5 Ni-NTA–coated, opaque, white 96-well plates	35081
Ni-NTA HisSorb Plates, white (25)	25 Ni-NTA–coated, opaque, white 96-well plates	35083
Ni-NTA Agarose (25 ml)	25 ml nickel-charged resin (max. pressure: 2.8 psi)	30210
Ni-NTA Agarose (100 ml)	100 ml nickel-charged resin (max. pressure: 2.8 psi)	30230

Ordering Information

Product	Contents	Cat. No.
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Ni-NTA Superflow (25 ml)	25 ml nickel-charged resin (max. pressure: 140 psi)	30410
Ni-NTA Superflow (100 ml)	100 ml nickel-charged resin (max. pressure: 140 psi)	30430
Ni-NTA Superflow (500 ml)	500 ml nickel-charged resin (max. pressure: 140 psi)	30450

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