HEPARIN AGAROSE HP RESIN
USER GUIDE

- Protein Ark's Heparin agarose High Performance (HP) resin is designed for rapid one-step purification and is ideal for preparative purification and contaminant removal.
- Heparin resin is optimised for affinity purification of Heparin binding proteins such as growth factors and nucleic acid-binding proteins.
- The Heparin agarose resin provides high binding capacity with high stability, chemical compatibility and re-use.
- It is also offered pre-packed in 1 ml and 5 ml AKTA-compatible HiFliQ columns.

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Features of the Heparin Resin:

- Fast and reliable affinity purification.
- Highly stable 6% cross linked agarose with coupled Heparin ligand provides high buffer stability and broad compatibility.
- High binding capacity for growth factors and nucleic acid binding proteins.
- Simple bind-wash-elute procedure.
- Available as pre-packed AKTA-compatible HiFliQ columns (1 ml and 5 ml)

Specification:

Item:	Heparin Agarose
Volumes:	10 ml, 25 ml, 100 ml, 500 ml
Resin:	Heparin Agarose HP
Base Matrix:	6% cross-linked Agarose
Coupled ligand:	Porcine Heparin
Ligand density:	>5 mg/ml
Typical Binding Capacity:	3-5 mg
Mean Bead Size:	20-50 µm (35 µm mean; HP)
Recommended flow rate:	1-10 ml/min
Max. operating pressure:	0.5 MPa (72 psi)
pH stability:	pH 4-13 (short term) pH 4-12 (long term)
Storage (2-8°C):	50 mM Sodium Acetate in 20% Ethanol

Chemical compatibility:

Buffer compatibility	
Standard buffers:	Common aqueous buffers and salts
pH range:	pH 4-13 (short term) pH 4-12 (long term)
Chelating agents	
EDTA:	1 mM
Sulfhydryl reagents	
β-mercaptoethanol:	20 mM
DTT:	20 mM
Denaturants	
Urea:	8 M
Guanidinium hydrochloride:	6 M
Detergents	
DDM (n-Dodecyl-β-D-maltoside):	0.1%
OG (n-Octyl-β-D-glucopyranoside):	5%
Triton® X-100:	Up to 2 %
Tween® 20:	Up to 2%
NP-40:	0.2%
SDS:	Up to 0.03% (w/v)
Cetyltrimethylammonium bromide (CTAB):	UP to 1%
C12E8:	0.05%
Brij 35:	0.1%
Cholate:	5%
Deoxycholate:	2%
CHAPS :	1%
Other additives	
NaCl	1 M
NaOH	0.1 M (long term) 0.5 M (short term; 1 hour)
Ethanol:	70% (v/v)

Principles of Heparin affinity chromatography:

Heparin is a highly sulphated glycosaminoglycan with an average molecular weight of 12-15 kDa. It consists of alternating 2-O-sulphated hexauronic acid and D-glucosamine residues. Heparin has a number of chemically reactive functional groups. Each disaccharide repeating unit contains a carboxyl group. All repeating units contain one or more 1° or 2° hydroxyl groups and an average of 2-2.5 sulfo groups.

It is sourced from porcine intestinal mucosa. When immobilized to agarose, it can bind often through two modes of interaction with proteins:- as a cation exchanger due to the high degree of sulphation and by specific affinity interaction through its carbohydrate sequence. Both interactions can be weakened with salt. Immobilized heparin is used to purify native or recombinant proteins eg. enzymes such as lipases, plasma coagulation proteins, lipoproteins, growth factors, nucleic acid binding proteins such as transcription factors, DNA & RNA polymerase, hormone receptors, serine proteases inhibitors and extracellular matrix proteins such as fibronectin, laminin and collagens etc.

Heparin affinity HP resin:

Porcine heparin covalently immobilised on to 6% cross-linked agarose beads via a short linker spacer through reductive amination.

General considerations for selecting optimal binding conditions for the Heparin resin:

The key parameters affecting the binding of the Heparin binding protein to the heparin resin are the flow rate over the column and the buffer conditions. Heparin shows slow kinetics for its binding partners and it's important to maintain a low flow rate over the resin during loading of the lysate to achieve the maximum binding capacity. Binding is also dependent upon the buffer conditions. Binding is most effective under physiological conditions (or neutral buffers) such as Tris-HCl buffer, 0.1-0.3 M NaCl pH 7.4-8.0.

If the binding efficiency is found to be poor and the lysis buffer (assuming that you are purifying a recombinant protein) differs significantly from the pre-equilibration buffer, it is recommended that the lysate be dialysed, titrated with a concentrated stock solution, or buffer exchanged using an ultrafiltration device with a more appropriate pre-equilibration buffer.

It is imperative that the sample e.g. plasma, lysate, extract is clarified prior to loading on the column as any particulate matter (e.g. cell debris) may partially foul and clog-up the resin resulting in an increased back pressure and reduced flow rates. This will significantly increase the binding, washing and elution times and effect the final purify of the eluted protein. It is recommended that the cleared sample is filtered (0.2 µm) just prior to loading even if it has been previously filtered several days before. Ideally samples should be processed rapidly and, if the need arises, the protein purified at 4°C. It is also recommended that number of freeze/thaw cycles be minimised during storage to reduce the amount of protein aggregation/precipitation.

Optimal buffer conditions for binding the target molecule to a resin are critical for successful purification of the protein. If the binding conditions are not optimal with respect to pH, salt concentration, detergent ...etc., purification can be adversely affected.

Protein purification conditions:

This protocol describes the purification of recombinant Heparin binding protein from an *E.coli* cell pellet under native conditions using Protein Ark Heparin resin. Reagent amounts given apply to IPTG-induced bacterial culture of a well-expressed protein (approximately 10-50 mg/l). Cells are lysed with lysozyme because it is an inexpensive and efficient method for cells that have been frozen. However other lysis methods based on physical disruption (e.g. sonication or homogenization) or detergents (e.g. CHAPS) can also be used. The Heparin binding protein is then purified from the cleared lysate under native conditions in a bind-wash-elute procedure. It is recommended that the bind-wash-elute conditions be tested and optimised in order to achieve the optimum conditions for purification. All volumes are given in column bed volume (CV).

Example: Buffers for purifications using NaCl elution.

Lysis buffer:
100 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA pH 7.5
Wash buffer:
100 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA pH 7.5
Elution buffer:
100 mM Tris-HCl, 1-2 M NaCl, 1 mM EDTA, pH 7.5

NOTE: Optimal buffer conditions may vary depending on the protein of interest. Proteins may require addition of protease inhibitor cocktail, EDTA, 1-5 mM DTT, 1% BSA, or detergents such as 0.5-1% Igepal CA-630 (Nonindet P-40) or 0.5-1% Tween-20.

Procedure

1. Thaw the *E. coli* cell pellet on ice.

NOTE: Freezing the cell pellet at –20°C for 30 min prior to incubation at room temperature improves lysis by lysozyme.

2. Resuspend the cell pellet in 'Lysis buffer' (50 ml / litre cell media) supplemented with 1 mg/ml Lysozyme.

NOTE: Benzonase® is recommended to reduce the viscosity caused by the nucleic acid (3 U/ml bacterial culture) if required.
NOTE: Add 1 protease inhibitor tablet to reduce protease activity and protein cleavage.

3. Incubate at room temperature for >30 min (or > 1 hour at 4°C) on an end-over-end shaker.

NOTE: Mechanical lysis can also be used by repeated freeze/thaw, vortexing, homogenization, sonication or French press if required.

4. Centrifuge the lysate for 30 min at 4°C and 10,000 x g. Collect the supernatant.

NOTE: If the supernatant remains cloudy then repeat step 4.

5. Connect the Heparin column to an FPLC/syringe/pump and wash with 3-5 CV's of distilled water to remove the storage buffer before equilibrating with 10 CV's of 'Wash buffer'.

6. Filter the cleared lysate through a 0.2 µm syringe filter directly before loading onto the column at the recommended flow rate.

NOTE: For optimal binding reduce the flow rate over the column to maximise the interaction time with the resin.
1 ml Heparin column= flow rate 0.2-1 ml/min
5 ml Heparin column= flow rate 1-5 ml/min

7. After loading wash the column with 'Wash buffer' until the measured absorbance (A_{280}) reaches a stable baseline.

NOTE: This will typically take 10-15 CV's.
NOTE: Keep the wash fractions for SDS-PAGE analysis if required.

8. Elute the target protein with a linear or stepwise gradient of 'elution buffer' over 15-20 CV or until the measured absorbance (A_{280}) reaches a stable baseline.

NOTE: Additional elutions with increased NaCl concentrations may be required. Up to 2M NaCl is some circumstances.
NOTE: Collect the eluate in separate tubes for SDS PAGE analysis and protein concentration determination.

9. Analyse all fractions by SDS-PAGE.

NOTE: Western Blot experiment can be performed if the primary Ab is available.

NOTE: Volumes and buffer conditions are protein dependent and may require scouting and optimising.
NOTE: It is recommended that each column is re-used only for identical protein samples to avoid any cross-contamination.

Desalting and concentrating the purified protein:

NaCl can be removed by diafiltration using ultrafiltration concentrators or rapid dialysis against an appropriate buffer for your downstream application.

Column washing and regeneration conditions:

Protein Ark Heparin columns should be carefully washed with TBS (wash buffer) then with distilled water and stored in 20% ethanol after each run. If the column shows increased back pressure or loss of binding capacity then this may be due to the accumulation of precipitated, denatured, or non-specifically bound substances and proteins. This protocol delineates washing and regeneration procedures for the Heparin column to remove these substances and proteins. All volumes are given in column bed volume (CV).

Example: Buffers washing and regeneration.

Regeneration buffer #1:	6M Guanidine hydrochloride
Regeneration buffer #2:	70% Ethanol
Regeneration buffer #3:	1% Triton™ X-100
Wash buffer:	Tris buffered saline
Storage buffer:	20% Ethanol

Wash and regeneration procedure 1:
Removal of precipitated and denatured substances and proteins.

1. Connect the HiFliQ Heparin column to an FPLC/syringe/pump and wash with 5 CV's of 'Wash buffer'.

2. Wash the column with 2 CV's 'Regeneration buffer #1'.

3. Immediately wash the column again with 5 CV's of 'Wash buffer'.

> NOTE: The column is now ready for re-use if required.

4. Wash the column with 3-5 CV's of 'storage buffer' for long term storage at 4°C.

> NOTE: If wash procedure 1 does not fully resolve the problem then repeat or proceed to wash procedure 2.

Wash and regeneration procedure 2:
Removal of hydrophobic substances and proteins.

1. Connect the HiFliQ Heparin column to an FPLC/syringe/pump and wash with 5 CV's of 'Wash buffer'.

2. Wash the column with 3-4 CV's of 'Regeneration buffer #2' (or 2 CV's of 'Regeneration buffer #3').

3 Immediately wash the column again with 5 CV's of 'Wash buffer'.

> NOTE: The column is now ready for re-use if required.

4. Wash the column with 3-5 CV's of 'storage buffer' for long term storage at 4°C.

> NOTE: If wash procedure 2 does not fully resolve the problem then repeat the procedure with the alternative 'Regeneration buffer'.

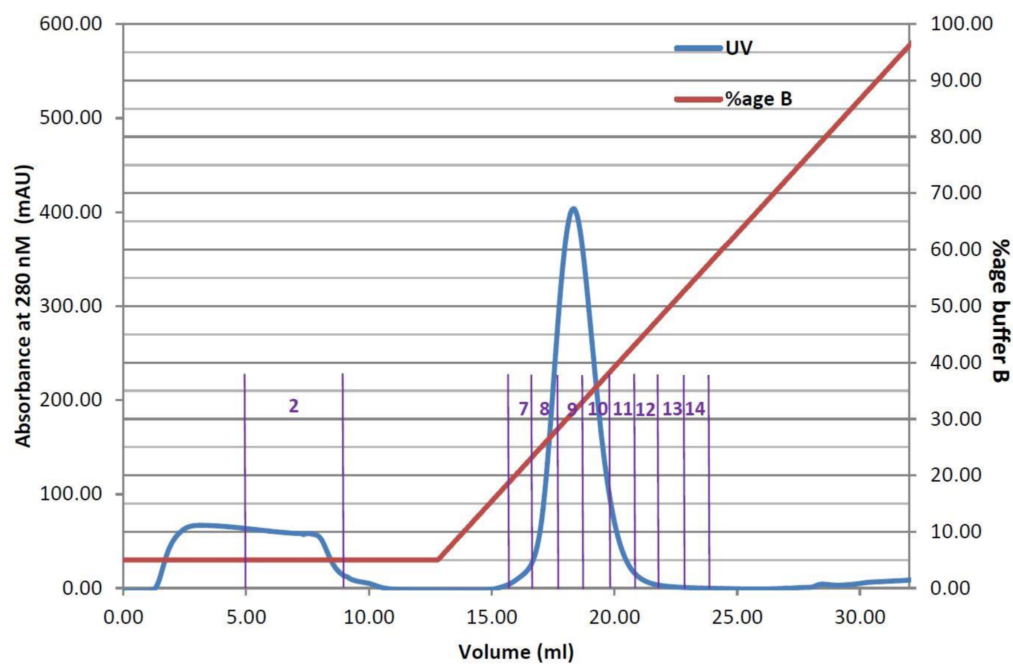
Performance data:

Purification of 25 kDa DNA binding protein from *E.coli* lysate.

Heparin affinity HP purification of recombinant protein from 1 ml cleared *E.coli* lysate using a 1 ml HiFliQ Heparin FPLC column on an FPLC. The eluted fractions contained >95% pure protein according to SDS-PAGE analysis (Figure 1B).

Sample:	1 ml *E.coli* lysate
Column:	1 ml HiFliQ Heparin FPLC Column
Instrument:	FPLC
Flow rate:	1 ml/min at 16° C
Binding buffer:	25 mM Tris-HCl, pH 7.5, 1% glycerol, 1 mM EDTA
Elution Buffer:	25 mM Tris-HCl, pH 7.5, 1% glycerol, 1 mM EDTA, 1 M NaCl
Eluted Protein:	3.0 mg

A)



B)

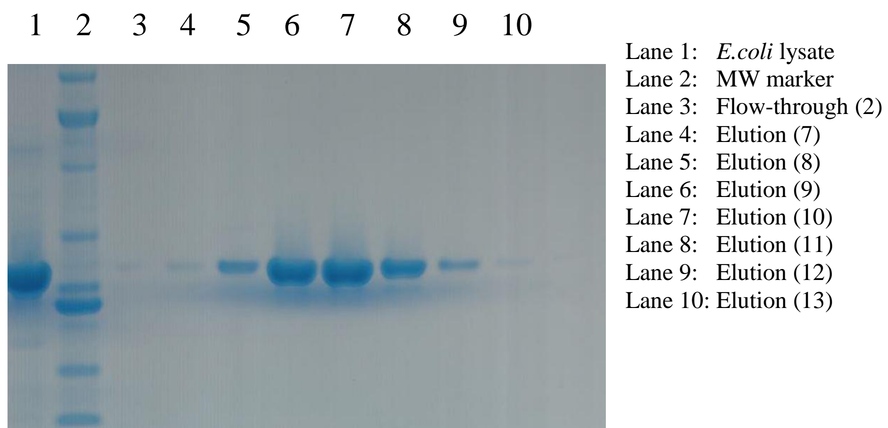


Figure 1. Purification of DNA-binding protein from *E.coli* lysate on a 1 ml HiFliQ HEPARIN FPLC column. (A) FPLC chromatogram (B) SDS-PAGE of purification fractions using NuPage Gel (4-12% gradient), stained with Quick Coomassie.

Storage conditions:

Item:	Heparin Resin
Shipping:	50 mM Sodium Acetate in 20% Ethanol at room temperature
Short-term storage:	Equilibration buffer
Long-term storage:	20% Ethanol at 4°C

NOTE: Do not freeze or store the column in buffer or water for long periods. Each column is stored in 20 % ethanol and reusable for up to 2 years from the date of resin manufacture. Please see the label for the recorded expiry date.

Questions and Answers:

1. *What is the shelf-life of the Heparin Resin?*
   The resin is guaranteed for 2 years after the date of manufacture provided it is stored at 2-8°C.

2. *Do I need to filter the buffers prepared in my laboratory?*
   It is good laboratory practice to filter all buffers.

3. *How should I prepare my sample for Heparin affinity separation?*
   Many chromatographic procedures demand that the sample is pre-conditioned prior to loading. We recommend that all samples are filtered to at least 0.45 µm (preferably 0.20 µm) pore size. High viscosity is mostly attributed to contaminating DNA or RNA. The intrinsic viscosity of a lysate can be reduced by either drawing it through a syringe needle several times or by adding appropriate amounts of DNase and/or RNase (5-10 µg/ml) to the lysis buffer and incubating the mix on ice for 15 mins.

4. *How can I regenerate the Heparin resin?*
   We recommend that you wash the column with PBS and store in 20% ethanol between each run. If column performance and binding capacity becomes reduced then we recommend washing with harsher conditions. See 'column washing and regeneration conditions' for further details.

5. *Should I be concerned if the column partially dries out during the chromatographic steps?*
   The resin is robust although we recommend flushing out as much air as possible from the column before continuing. Partially dried resin rehydrates rapidly however the performance of the column (binding capacity and running pressure) may be affected.

6. *Can I load purified protein immediately on to an SDS-gel?*
   Proteins purified from the Heparin resin under the recommended conditions can be loaded on to an SDS-polyacrylamide gel.

7. *Under what circumstances can I re-use the Heparin column?*
   We recommend regular washing and cleaning between purifications in order to maintain performance. Should you observe a slowdown in flow rate or increase in back pressure then we recommended washing and regenerating the column prior to further use. See 'Column washing and regeneration conditions' for further details.

Troubleshooting assistance:

Bubbles or cracks appear in the resin bed:

- The resin has been stored at a cool temperature and then rapidly warmed up. The resin should be warmed slowly to room temperature before use.

The sample does not flow easily through the column:

- The resin is clogged with particulates. Pre-filter the sample just before loading it on to the column.
- If the column is not stored at 2-8°C, or it has been used more than once and stored in the absence of a bacteriostat, microbial growth may restrict flow through the column.

No binding or elution of the target protein is observed from the column:

- Extensive sonication can denature the protein resulting in loss of structural integrity and preventing the protein from binding to the column.
- Check the pH of the 'Lysis buffer'. If the pH is not within the range of 6-8 then binding of the Heparin to the Heparin resin will be affected. If required dialyse or titrate with a concentrated stock solution, or buffer exchanged the lysate to within the correct pH range.
- Test the binding conditions and Heparin resin are working correctly using just the expressed sample for bind-wash-elution.
- The retention time may not be sufficient for binding. Reduce the flow rate of the loading stage down to 0.2-0.5 ml/min (1 ml Heparin column) or 1-3 ml/min (5 ml Heparin column).
- The column may contain a build-up of precipitated, denatured, or hydrophobic substances and proteins which may impede binding. See 'Column washing and regenerating conditions' for details of washing and regeneration protocols or use a new HiFliQ Heparin FPLC column.

The recovery of target protein is low:

- Increase the elution time and volume of the 'Elution buffer'.
- The Elution buffer may not be strong enough for elution. Try increasing the salt concentration to 2 M NaCl in the 'Elution buffer'.
- Hydrophobic interactions from the fused protein maybe affecting elution. These may require disrupting using 0.1% Triton X-100 or 2% N-octylglucoside to improve elution of some Heparin binding proteins.

Poor resolution of the target protein:

- This maybe the result of partial degradation of the heparin binding protein. Try including protease inhibitor tablets in the 'Lysis buffer'.
- In order to minimize degradation try reducing the expression, lysis and purification times.
- The procedure may require performing at 4°C to reduce degradation and stabilise the heparin binding protein.
- Target the recombinant protein to the periplasm region of the *E.coli* cell during expression using a signal peptide sequence (for example pelB, OmpA, DsbA, TolB and MalE). This may aid folding and stability whilst reducing degradation. This will however reduce the expression levels considerably.

Glossary:

affinity chromatography - chromatographic separation based on a specific interaction between an immobilized ligand and a binding site on a macromolecule.

chaotropic agent - a molecule which interferes with hydro-phobic interactions by disrupting the ordered structure of water molecules. Examples include urea and guanidine hydrochloride.

chelating agent – a compound such as EDTA or EGTA that is able to combine with a metal ion to form a structure with one or more rings.

cleared lysate – the soluble cell extract after the cell debris and other particulates have been removed by centrifugation.

column bed volume (CV) - the total volume occupied by the chromatographic packed bed. It is also referred to as the column volume or CV.

Dithiothreitol (DTT) – reducing agent used to break disulphide bonds.

French pressure cell – a device that uses high shear forces to rupture microbial cells. The suspension is poured into a chamber, which is closed at one end by a needle valve and at the other end by a piston. Pressures of up to 16,000 lb/in2 are applied by a hydraulic press against a closed needle valve. When the desired pressure is attained, the needle valve is fractionally opened to marginally relieve the pressure. The cells subsequently expand and rupture, thereby releasing the cellular components through the fractionally open valve.

freeze-thawing – a method that is sometimes used to break open cells by successive periods of slow freezing and thawing. Ice crystals are generated during the freezing stage, which disrupt the cells when they melt during thawing. The method, however, is slow and releases a limited amount of subcellular components.

Heparin - heparin is a member of the glycosaminoglycan family of carbohydrates and consists of a variably sulfated repeating disaccharide unit.

inclusion bodies – quite a lot of proteins form insoluble crystalline aggregates known as inclusion bodies when they are expressed at high levels inside bacteria. The proteins can be solubilized using denaturants such as 8 M urea or 6 M guanidine hydrochloride.

isoelectric point - the pH at which the protein has no net charge.

lysozyme – an enzyme than hydrolyzes β-1,4-linkages between N-acetylmuramic acid and 2-acetamido-2-deoxy-D-glucone in peptidoglycan heteroploymers of prokaryotic cell walls. An example is egg white lysozyme and this enzyme is used to disrupt cells in order to liberate expressed proteins. 1 mg/ml lysozyme is normally added to *E.coli* cells in lysis buffer and incubated for 30 min to aid cell disruption. The pH optimum for lysozyme is pH 9.2 (Davies *et al* 1969).

sonication – this technique uses ultrasonic energy to generate high transient pressures that are believed to disrupt the cells.

tris(2-carboxyethyl)phosphine (TCEP) – strong reducing agent used to break disulphide bonds.

Literature:

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HiFliQ Heparin Ordering information:

Product	Quantity	Order Code
1 ml HiFliQ Heparin FPLC column (1 x 1 ml)	1 x 1 ml	HiFliQ1-HEP-1
1 ml HiFliQ Heparin FPLC columns (5 x 1 ml)	5 x 1 ml	HiFliQ1-HEP-5
5 ml HiFliQ Heparin FPLC column (1 x 5 ml)	1 x 5 ml	HiFliQ5-HEP-1
5 ml HiFliQ Heparin FPLC columns (5 x 5 ml)	5 x 5 ml	HiFliQ5-HEP-5

Heparin Agarose Ordering Information:

Product	Quantity	Order Code
10 ml Super Heparin Agarose HP Resin (1 x 10 ml)	1 x 10 ml	Super-HEP-10
25 ml Super Heparin Agarose HP Resin (1 x 25 ml)	1 x 25 ml	Super-HEP-25
100 ml Super Heparin Agarose HP Resin (1 x 100 ml)	1 x 100 ml	Super-HEP-100
500 ml Super Heparin Agarose HP Resin (1 x 500 ml)	1 x 500 ml	Super-HEP-500

Technical support:

Contact the Protein Ark technical support and sales centre for assistance:

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Email: info@proteinark.com
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Disclaimer:

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