Technical Bulletin

HIS-Select® Nickel Affinity Gel

P6611

Product Description

HIS-Select® Nickel Affinity Gel is an immobilized metal-ion affinity chromatography (IMAC) product. The HIS-Select® Nickel Affinity gel is a proprietary quadridentate chelate on beaded agarose charged with nickel that is designed specifically to bind histidine-containing proteins, or His-tagged proteins. The matrix for this affinity gel is 6% beaded agarose. HIS-Select® Nickel Affinity Gel is selective for recombinant proteins with histidine tags (His-tag) and exhibits low non-specific binding of other proteins. The selectivity can be modulated with the inclusion of imidazole during chromatography.

HIS-Select® Nickel Affinity Gel may be used to capture recombinant His-tagged proteins at a high flow rate. Recombinant proteins with His-tags are bound using either native or denaturing conditions. The capacity of this affinity gel is > 15 mg/mL of packed gel, as determined with a ~30 kDa His-tagged protein. HIS-Select® Nickel Affinity Gel is supplied as a 50% suspension (gel:solvent), where the solvent is 30% aqueous ethanol. The maximum linear flow rate is 150 cm/hr or 5 psi.

It is recommended that the entire Technical Bulletin be read before use, especially the Reagent Compatibility Chart on Page 8.

Several theses[4-8] and dissertations[9-32] have cited use of this P6611 product in their research protocols.

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Reagent

HIS-Select® Nickel Affinity Gel is supplied as a 50% suspension in 30% ethanol. Maximum linear flow rate is 150 cm/hr or 5 psi.

Binding capacity: > 15 mg/mL of packed gel

Reagents and Equipment Required but Not Provided

Suggested Cat. Nos. are provided as appropriate.

- Appropriate centrifuge
- CelLytic™ Cell Lysis and Protein Extraction Reagent, available for various cell types:
  - Bacteria (B7435, B7310, C8740, C1990, C5491, CB0050, CB0500)
  - Mammalian cells (C2978) and tissues (C3228)
  - Plant cells (C2360)
  - Yeast (C4482)
- Appropriate column or centrifuge tubes
- Imidazole, such as Cat. No. I5513 (for molecular biology)
- Sodium chloride (NaCl), such as Cat. No. S3014 (for molecular biology)
- Sodium phosphate, such as Cat. No. S3139 (monobasic, for molecular biology) and S3264 (dibasic, for molecular biology)

Storage/Stability

HIS-Select® Nickel Affinity Gel is stable for at least one year when stored properly. The affinity gel should be cleaned after each use (see Procedure, General Cleaning). An antimicrobial agent such as 20-30% ethanol should then be added to the storage buffer.

Do not allow the HIS-Select® Nickel Affinity Gel to remain in any buffer for extended periods of time (> 24 hours) unless some type of antimicrobial agent (such as 20-30% ethanol) is added to the buffer.

Note: Buffers or reagents that chelate metal ions, such as EDTA, should **not** be used with this product, since chelating agents may strip the metal ions off the column. Strong reducing agents should also be avoided, since they may reduce the bound nickel, and thus eliminate the binding of His-tagged proteins. See the Reagent Compatibility Chart for more information.

Preparation Instructions

- The HIS-Select® Nickel Affinity Gel is stored in 30% ethanol. The ethanol must be removed just prior to use.
- Thoroughly resuspend the affinity gel with gentle inversion. Remove an appropriate aliquot for use. Take only the amount of affinity gel that is necessary to do the purification.
- The affinity gel may then be poured into a clean chromatography column using standard techniques, or handled batch-wise for trial-scale or large-scale preparations.
- The ethanol storage solution must be removed, as it may cause precipitation of some buffer salts. In general, the affinity gel is first washed with 1-2 volumes of deionized water to remove the ethanol, and then equilibrated with 3-5 volumes of equilibration buffer.

Procedure

Prepare the following buffers for use in procedures for purification of His-tagged recombinant proteins. For native conditions, the Equilibration Buffer and the Wash Buffer are the same.

***Note**: Batch adsorption must be performed in the absence of imidazole*.

Equilibration and Wash Buffer: 50 mM sodium phosphate (pH 8.0), with 0.3 M sodium chloride and 10 mM imidazole. A typical equilibration buffer consists of 50 mM sodium phosphate, 1-20 mM imidazole, and 0.15-0.5 M NaCl, pH 8.0.

Elution Buffer: 50 mM sodium phosphate (pH 8.0), with 0.3 M NaCl and 250 mM imidazole

I. Extract Preparation

- The recombinant protein with a His-tag / histidine-containing tag may be extracted from a crude cell extract or a partially purified protein fraction prepared by standard techniques. The protein sample preparation steps should be empirically determined by the end user, since the conditions may vary depending on the nature of the recombinant protein and the host organism.
- CelLytic™ products are available for various expression systems, and are recommended for use with the HIS-Select® Nickel Affinity Gel.
- Prior to application to the affinity gel, the recombinant protein sample must be clarified by centrifugation or filtration.
- For optimal results, the pH of the sample buffer must be between pH 7.0-8.0. The equilibration and sample buffer should be supplemented with 1-20 mM imidazole and 0.15-0.5 M NaCl to reduce non-specific protein binding. Consult the Reagent Compatibility Chart for the use of other reagents.

II. Trial-Scale Purification (mini-prep)

A trial-scale experiment (< 1 mg of target protein) should be performed before attempting a large-scale purification, to determine if the standard operating conditions will work for the recombinant protein of interest.

All steps may be performed at room temperature or at 2-8 °C.

1. Add 25-50 µL of HIS-Select® Nickel Affinity Gel suspension to a microcentrifuge tube. Centrifuge for 30 seconds at 5,000 × *g.*
2. Carefully remove the supernatant. This supernatant may be discarded.
3. Add 200 µL of Equilibration Buffer without imidazole. Mix well.
4. Centrifuge for 30 seconds at 5,000 × *g*. Remove and discard the supernatant.
5. Add 100 µL of clarified recombinant protein solution. Gently mix for 1 minute. Centrifuge the mixture for 30 seconds at 5,000 × *g*. Save the supernatant.
6. Wash the affinity gel two times with at least 500 µL of Wash Buffer without imidazole. Gently mix the affinity gel for 10 seconds. Then centrifuge for 30 seconds at 5,000 × *g*. Save the Wash Buffer solutions for analysis either as a single pool or three fractions.
7. Elute the target protein with 50 µL of Elution Buffer. Add the buffer and mix the affinity gel well.
8. Centrifuge for 30 seconds at 5,000 × *g*.
9. Repeat Steps 7 and 8 to recover more of the protein. Most of the protein will elute off in the first 50 µL fraction, but some residual protein may be eluted in the second cycle. Save the two fractions as a single pool or separate fractions.

10. Analyze all the fractions by SDS-PAGE to determine if the target protein bound to the affinity gel and was eluted. It is useful to perform a Western blot to determine where the His-tag / histidine-containing proteins are fractionated during the purification trial. If the target protein did **not** bind and elute from the affinity gel, refer to the Troubleshooting Guide. It may be necessary to repeat the trial under denaturing conditions.
11. Set the tube with the bead pellet on ice.

III. Large Scale Purification

All steps may be performed at room temperature or at 2-8 °C.

A. Native Conditions - Column Chromatography

1. Transfer the appropriate amount of HIS-Select® Nickel Affinity Gel to a chromatography column. Wash the affinity gel with 2 volumes of deionized water and then 3 volumes of Equilibration Buffer. Remove most of the Equilibration Buffer from the top of the column before use. Do not allow the affinity gel to remain in Equilibration Buffer for extended periods of time (> 24 hours) without antimicrobial agents.
2. The amount of affinity gel required depends upon the amount of target proteins in the extract. The target protein concentration should not exceed the capacity of the affinity gel in the column. This should be determined for each protein to be purified.
3. Load the clarified crude extract onto the column at a flow rate of 2-10 column volumes per hour. It is recommended that the cell extract be loaded as soon as it is made and that the loading time should not exceed 6 hours. If loading time will be excessive, the protein binding may be performed using the batch format (Section III-B, Steps 1-8). The affinity gel is then placed in a column. Wash and Elution are performed using Steps 4 and 5, which follow immediately in this section.
4. After all the extract is loaded, wash the column with Wash Buffer. The flow rate of the Wash Buffer should be about 10-20 column volumes per hour. The column should be extensively washed until the A_{280} of the material eluting from the column is stable and near that of the Wash Buffer.
5. The histidine-containing / His-tagged protein is eluted from the column using 3-10 column volumes of Elution Buffer. Collect fractions. Assay for the target protein. The flow rate of the Elution Buffer should be 2-10 column volumes per hour.

B. Native Conditions - Batch Purification Method

***Note**: Batch adsorption must be performed in the absence of imidazole.*

1. Add the appropriate amount of affinity gel suspension to a large centrifuge tube. Centrifuge the mixture at 5,000 × *g* for 5 minutes to pellet the affinity gel, and then discard the supernatant. Alternatively, remove the supernatant by filtration.
2. Resuspend the affinity gel in 10 gel volumes of Equilibration Buffer.
3. Centrifuge or filter the affinity gel to collect it after equilibration.
4. Remove the supernatant. This supernatant may be discarded.
5. Add the cell extract to the affinity gel. Gently mix the material on an orbital shaker (~175 rpm) for 15 minutes. **Do not use a stir plate, as the stir bar will break the affinity gel beads**.
6. Centrifuge the mixture at 5,000 × *g* for 5 minutes or filter. Remove and save the supernatant for SDS-PAGE analysis.
7. Add 10 volumes of Wash Buffer to the affinity gel.
8. Mix the affinity gel suspension on an orbital shaker (~175 rpm) for 4 minutes. Centrifuge the suspension at 5,000 × *g* for 5 minutes or filter.
9. Repeat Steps 7 and 8 to wash the affinity gel again.
10. The affinity gel can be washed further until the A_{280} of the eluate no longer decreases. Discard the washes.
11. Add 2 gel volumes of Elution Buffer. Mix the affinity gel on an orbital shaker (~175 rpm) for 10 minutes.
12. Centrifuge the mixture at 5,000 × *g* for 5 minutes or filter. Remove and save the supernatant or filtrate. The histidine-containing / His-tagged protein will be in this fraction.
13. Repeat Steps 11 and 12 to elute more protein. Save the eluted fractions as a single pool or separate fractions.

IV. Denaturing Conditions

- HIS-Select® Nickel Affinity Gel can be used to purify histidine-tagged proteins under denaturing conditions. If denaturing conditions must be used, the protein must first be solubilized with 6 M guanidine hydrochloride (such as Cat. No. G3272) or 8 M urea (such as Cat. No. U5378).
- Make sure the pH of the denatured cell extract is between pH 7.0 to 8.0 before applying it to the affinity gel.
- The same purification procedures previously described can be used with denaturing buffers. Reducing agents can also be added, such as DTT (up to 5 mM) or 2-mercaptoethanol (up to 20 mM).

Note: Any buffers that contain urea must be made fresh daily.

- An example of a urea denaturing system is described below:
  - Equilibration Buffer: 0.1 M sodium phosphate, pH 8.0, with 8 M urea
  - Wash Buffer: 0.1 M sodium phosphate, pH 6.3, with 8 M urea
  - Elution Buffer: 0.1 M sodium phosphate, pH 4.5-6.0, with 8 M urea
- The Elution Buffer pH may have to be varied because some recombinant His-tagged proteins will not elute in the pH range of 5.0-6.0. If the His-tagged recombinant proteins do not elute in this range, try a pH as low as 4.5.

V. Cleaning HIS-Select® Nickel Affinity Gel for Reuse

- The affinity gel should be cleaned after every run to ensure that it will function properly in the next use.
- If the same crude extract is used and has been made using CelLytic™ B, the column can usually be regenerated simply with Equilibration Buffer.
- The detergent in CelLytic™ B prevents most non-specific protein binding to the affinity gel, if used as directed. This has been done over 20 times with a crude *E. coli* extract with no loss of binding capacity or purity of the final product.
- All steps may be performed at room temperature or at 2-8 °C.

A. General Cleaning

1. Wash the affinity gel with 2 column volumes of deionized water.
2. Clean the affinity gel with 5 column volumes of 6 M guanidine HCl (such as Cat. No. G3272), pH 7.5. The flow rate should be no more than 5 column volumes per hour.
3. Remove the guanidine HCl solution by washing with 2-3 column volumes of deionized water.
4. The affinity gel can now be re-equilibrated with 2-3 column volumes of Equilibration Buffer for immediate use, or it can be washed with 1-2 column volumes of 30% ethanol and then resuspended in 30% ethanol for storage at 2-8 °C.

Note: The affinity gel can also be cleaned with 0.2 M acetic acid, 1-2% SDS, or ethanol. The ethanol can be used up to 100%, but the concentration percentage must be gradually increased and decreased in increments of no more than 25%, to prevent rapid volume changes of the affinity gel. One example of such increments is as follows:

$$25\% \rightarrow 50\% \rightarrow 75\% \rightarrow 100\% \rightarrow 75\% \rightarrow 50\% \rightarrow 25\% \rightarrow 0\%$$

B. Recharging HIS-Select® Nickel Affinity Gel

If the HIS-Select® Nickel Affinity Gel turns from a blue to a brown or gray color, the oxidation state of the nickel has been reduced. The reduced nickel must be removed, and the affinity gel recharged, using the following procedure.

1. Wash the affinity gel with 2 column volumes of deionized water.
2. Clean the affinity gel with 5 column volumes of 6 M guanidine HCl (such as Cat. No. G3272), pH 7.5. The flow rate should be no more than 5 column volumes per hour.
3. Remove the guanidine HCl solution by washing with 2-3 column volumes of deionized water.
4. Wash the affinity gel with 5 column volumes of 0.1 M EDTA (such as Cat. Nos. EDS, E6511, or 03699), pH 7.0-8.0, to remove the reduced nickel.
5. Wash the affinity gel with 2 column volumes of deionized water.
6. Recharge the column with 2 column volumes of 10 mg/mL of nickel(II) sulfate hexahydrate (such as Cat. Nos. N4882 or 227676).
7. Wash the affinity gel with 2 column volumes of deionized water.
8. The affinity gel can now be re-equilibrated with 2-3 column volumes of Equilibration Buffer for immediate use. Alternatively, the gel can be washed with 1-2 column volumes of 30% ethanol and then resuspended in 30% ethanol for storage at 2-8 °C.

VI. Results

SDS-PAGE analysis of eluted recombinant proteins should give nearly single-banded material under most circumstances. The affinity gel should bind at least 15 mg of protein per mL of gel. The capacity is dependent on the nature and size of the His-tagged recombinant protein being purified and the conditions used for the purification. Modification of conditions may enhance the binding capacity as well as the purity of the final product. See the Troubleshooting Guide for more recommendations.

When running SDS-PAGE on samples that contain guanidine HCl, a protein precipitation kit may be of use, such as Cat. No. 539180.

Alternatively, to remove guanidine HCl, the following general protocol for TCA precipitation can be used. This TCA procedure will also concentrate protein samples.

- Add 100% TCA solution (such as Cat. No. T0699) to the protein sample to give a final concentration of 10% TCA.
- Incubate the sample on ice for 15 minutes.
- Centrifuge the sample at full speed for 15 minutes.
- Carefully remove the supernatant with a pipette. Resuspend the pellet in SDS-PAGE sample buffer.

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Troubleshooting Guide

Problem	Possible Cause	Solution
Recombinant protein with His-tag will not bind to affinity gel	Incorrect conditions for binding	• Verify the pH and composition of sample and equilibration buffers. • Make sure there are no chelating or reducing agents present in the extraction buffer. If working in batch mode, remove imidazole.
	Recombinant protein is not present.	Run a Western blot of the extract to verify that the recombinant protein is present.
	The His-tag is buried within the protein structure	Run the affinity purification under denaturing conditions.
	Cells not extracted	Make sure that the cell extract contains target protein.
Protein elutes in the wash buffer before the elution buffer is even introduced	Wash stringency is too high	Lower the concentration of imidazole and verify that the pH is 7-8.
	The His-tag is buried within the protein structure	• Make sure the wash conditions are not too stringent. • Run the affinity purification under denaturing conditions.
Protein precipitates during purification	Temperature is too low.	Run the column at room temperature.
	Protein aggregates	• Add stabilizing agents such as 5-10% glycerol, 0.1% TWEEN® 20, or 0.1% TRITON® X-100. • Increase the sodium chloride concentration up to 2 M. • Add reducing agent, such as 2-mercaptoethanol (up to 20 mM) or DTT (up to 5 mM). • Add metals or cofactors to stabilize protein.
Pressure problems with column	Extract contains insoluble material	The protein extract must be free of insoluble material before it is loaded into the column. Insoluble material may be removed by centrifugation or filtration through a 0.45 µm membrane.
Affinity gel changes color	Extract exposure	During purification, many protein extracts tend to discolor an affinity gel during the loading step. The original color will return after the wash or elution step.
	Needs to be recharged	The affinity gel was used and cleaned numerous times. It is thus time to recharge the affinity gel with nickel.
	Loses color during run and does not regain it by the end of the run	• Do not use oxidizing or high concentrations of reducing agents in any of the buffers or extracts. • Strip and recharge the affinity gel with nickel. Reduced metal may not be fully removed from the resin.
Recombinant protein with His-tag will not elute off the affinity gel	Elution conditions are too mild	• Increase the amount of imidazole. • For a denaturing purification, make sure the pH is low enough to elute the tagged recombinant protein, such as to adjust the elution buffer to pH 4.5. • Perform batch purification so that high protein concentrations are avoided.
Non-specific proteins elute with the His-tagged recombinant protein	Binding and wash conditions are not strict enough	Increase the amount of imidazole in the extract and wash buffers up to 20 mM.
	Target protein is being degraded by proteases	Add protease inhibitor cocktail (such as Cat. Nos. P8849, MSSAFE, P8340, PIC0004).
	Material is linked by disulfide bonds	Add reducing agent, such as 2-mercaptoethanol (up to 20 mM) or DTT (up to 5 mM).

Reagent Compatibility Table

Reagent	Effect	Comments
Imidazole	Binds to the nickel-charged affinity gel and competes with the recombinant proteins with His-tags / histidine-containing tags	• For column chromatography, no more than 20 mM is suggested in the extract, equilibration, and wash buffers to prevent non-specific binding of proteins. • No more than 250 mM is suggested for the elution buffers. • Many proteins will elute with imidazole levels as low as 100-200 mM. For batch methods, the imidazole concentration may have to be reduced or eliminated.
Histidine	Binds to the nickel-charged affinity gel and competes with the histidine-containing proteins	• Can be used in place of imidazole in the extraction, equilibration, wash, and elution buffers. • No more than 250 mM is suggested for the elution buffers.
Chelating agents, such as EDTA or EGTA	Strips nickel ions from the affinity gel	• **Not recommended** as a buffer component, because of their ability to remove nickel ions. • Can be used to strip and recharge the affinity gel with fresh metal ions.
Guanidine HCl	Solubilize proteins	Used to denature proteins and for cleaning of the affinity gel.
Urea	Solubilize proteins	Use 8 M urea for purification under denaturing conditions.
Sodium phosphate	Used in equilibration, wash, and elution buffers to help prevent non-specific binding and buffer the solution	• Recommended buffer at 50-100 mM for purification with the affinity gel. • The pH of any buffer should be between 7-8 with the higher capacity at the higher pH.
Sodium chloride	Prevents ionic interactions	• Used in equilibration, wash, and elution buffers to help prevent binding of non-specific proteins to the affinity gel. • Recommended levels are 0.15-0.5 M. However, up to 2 M can be used.
2-Mercaptoethanol	A reducing agent used to prevent disulfide bonds formation	• Add up to 20 mM in the extract buffer to break disulfide bonds. • Higher levels may reduce the nickel ions.
Ethanol	Antimicrobial. Also eliminates hydrophobic bonds between proteins	The binding, washing, eluting and storage buffers may contain up to 20% ethanol.
Glycerol	Can help stabilize proteins	The binding, washing, eluting and storage buffers may contain up to 50% glycerol.
DTE and DTT	Reduces protein disulfide bonds	Up to 5 mM DTT or DTE can be used. Higher amounts may reduce the nickel in the resin.
Nonionic detergents (TRITON®, TWEEN®, IGEPAL® CA-630)	Helps prevent non-specific binding of proteins to the affinity gel	Up to 2% may be used.
Glycine	Binds weakly to affinity gel and competes weakly with histidine-containing proteins	**Not recommended**. Use histidine or imidazole instead.