Technical Bulletin

HIS-Select® Cobalt Affinity Gel

For mammalian expression systems

H8162

Product Description

The histidine tag (known also as the His-tag), which commonly consists of six histidine (His) residues in succession, is a widely used protein tag in recombinant protein purification.[1-3] The HIS-Select® Cobalt Affinity Gel is an immobilized metal-ion affinity chromatography (IMAC) product. The HIS-Select® Cobalt Affinity gel is a proprietary quadridentate chelate, on beaded agarose charged with cobalt, that is designed specifically to bind His-tagged proteins. The matrix for this affinity gel is 6% beaded agarose.

HIS-Select® Cobalt 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® Cobalt Affinity Gel is durable and can capture His-tagged recombinant proteins at a high flow rate. Recombinant proteins with His-tags are bound using native, denaturing, or mild reducing conditions.

Cobalt has a different affinity for histidine residues compared to nickel. This may make cobalt a preferable choice of metal ion in the resin for purification of His-tagged proteins in some instances.[1,4] Several dissertations cite use of this H8162 product in their research protocols.[5-8]

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

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.

Reagents

HIS-Select® Cobalt Affinity Gel is pink and is supplied as a 50% suspension (of gel to solvent), in 30% ethanol. The capacity of this affinity gel is on the order of 15 mg/mL of packed gel, as determined with a His-tagged protein of molecular mass ~30 kDa.

Reagents and Equipment Required but not provided

(Cat. Nos. have been suggested where appropriate)

- Centrifuge
- Chromatography column or centrifuge tubes
- CelLytic™ B (Cat. Nos. B7435, B7310, or C8740), CelLytic™ B Plus Kit (Cat. No. CB0500), or CelLytic™ Express (Cat. No. C1990), for bacterial lysis
- CelLytic™ M (Cat. No. C2978), for mammalian cell lysis
- Imidazole, such as Cat. No. I5513 (for molecular biology)
- Sodium chloride, such as Cat. No. S3014 (for molecular biology)
- Sodium phosphate, such as Cat. No. S3139 (monobasic, BioReagent, for molecular biology)
- Guanidine HCl, such as Cat. No. G3272 (for molecular biology)
- Urea, such as Cat. No. U5378 (BioReagent, for molecular biology)

Storage/Stability

HIS-Select® Cobalt Affinity Gel is stable for at least one year when stored properly. The HIS-Select® Cobalt Affinity Gel should be cleaned after each use as described, and an antimicrobial agent such as 30% ethanol should be added to the storage buffer.

Do not allow the affinity gel to remain in any buffer for extended periods of time (>24 hours) unless it contains an antimicrobial agent (such as 30% ethanol).

Note: Buffers or reagents that chelate metal ions should **not** be used with this product, because chelators may strip the cobalt from the gel matrix. Strong reducing agents should also be avoided, since they may reduce the bound cobalt and eliminate the binding of His-tagged proteins. See the Reagent Compatibility Chart for more information.

Preparation Instructions

The HIS-Select® Cobalt Affinity Gel is stored in 30% ethanol. The ethanol from the storage solution should be removed just prior to use. Resuspend the affinity gel with gentle inversion and remove an aliquot for use. Take only the amount of affinity gel necessary to do the purification. The affinity gel may then be poured into a chromatography column for protein purification using standard techniques, or handled batch-wise for trial-scale or large-scale preparations. If not removed, the ethanol 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.

Prepare the following buffers for use in procedures for purification of His-tagged recombinant proteins:

- Equilibration and Wash Buffer: 50 mM sodium phosphate (pH 8.0), 0.3 M NaCl, 10 mM imidazole

A typical equilibration buffer consists of 50 mM sodium phosphate (pH 8.0), 1-20 mM imidazole, and 0.15-0.5 M NaCl.

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

A typical elution buffer consists of 50 mM sodium phosphate (pH 8.0), 100-250 mM imidazole, and 0.15-0.5 M NaCl.

Procedures

Extract Preparation

- The His-tagged recombinant protein may be extracted from a crude cell extract or from a partially purified protein fraction prepared by standard techniques. The researcher should empirically determine the optimal protein sample preparation procedure, because conditions may depend on the nature of the recombinant protein and the host organism.
- CelLytic™ B or CelLytic™ Express supplemented with 1-20 mM imidazole is recommended for lysing *E. coli* cells.
- 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 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 other reagents that can be used.

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® Cobalt Affinity Gel suspension to a microcentrifuge tube and centrifuge for 30 seconds at 5,000 × *g.*
2. Carefully remove, and then discard, the supernatant.
3. Add 200 µL of Equilibration Buffer and 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 and gently mix for 1 minute. Centrifuge the mixture as in Step 4. Save the supernatant.
6. Wash the affinity gel twice with at least 500 µL of Wash Buffer. 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 separate fractions.
7. Elute the target protein with 50 µL of Elution Buffer. Add the buffer to the affinity gel and mix 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 be eluted 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 was bound to the affinity gel and was eluted. It is useful to perform a Western blot to determine where the His-tagged proteins are fractionated during the purification trial. If the target protein did **not** bind or elute from the affinity gel, refer to the Troubleshooting Guide. It may be necessary to repeat the trial under denaturing conditions.

Large-Scale Purification

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

Native Conditions: Column Chromatography

1. Transfer the appropriate amount of HIS-Select® Cobalt Affinity Gel to a chromatography column. Wash the affinity gel with 2 volumes of deionized water and then 3 volumes of Equilibration Buffer. The surface of the Equilibration Buffer should not go below the top of the gel bed. Do not allow the affinity gel to remain in Equilibration Buffer for extended periods of time (>24 hours) without the addition of antimicrobial agents.
2. The amount of affinity gel required depends upon the amount of target His-tagged proteins in the extract. The capacity of the affinity gel for the target protein 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/hour. It is recommended to load the cell extract 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 (see the Native Conditions: Batch Purification Methods section, Steps 1-8). Place the batch-loaded affinity gel in a column. Proceed with the Wash and Elution Steps, 4 and 5.
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/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 His-tagged protein is eluted from the column using 3-10 column volumes of Elution Buffer. Collect fractions and assay for the target protein. The flow rate of the elution buffer should be 2-10 column volumes/hour.

Native Conditions: Batch Purification Method

1. Add the appropriate amount of affinity gel suspension to a large centrifuge tube. Centrifuge the mixture at 5,000 × *g* for 2 minutes to pellet the affinity gel. Then discard the supernatant. Alternatively, remove the supernatant by filtration.
2. Resuspend the affinity gel in 10 gel volumes of Equilibration Buffer.
3. Centrifuge the mixture at 5,000 × *g* for 2 minutes or filter the affinity gel to collect it after equilibration.
4. Remove and discard the supernatant.
5. Add the cell extract to the affinity gel. Gently mix the material on an orbital shaker (~175 rpm) for 30 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 2 minutes or filter. Remove and save the supernatant for SDS-PAGE analysis.
7. Add 10 volumes of Wash Buffer to the affinity gel.
8. Fully resuspend the affinity gel in the Wash Buffer. Centrifuge the suspension at 5,000 × *g* for 2 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 30 minutes.
12. Centrifuge the mixture at 5,000 × *g* for 2 minutes or filter. Remove and save the supernatant or filtrate. The His-tagged protein will be in this fraction.
13. Repeat Steps 11 and 12 to elute additional protein. Save the eluted fractions as a single pool or separate fractions.

Denaturing Conditions

HIS-Select® Cobalt Affinity Gel can be used to purify proteins under denaturing conditions. If denaturing conditions must be used, the protein must first be solubilized with 6 M guanidine hydrochloride or 8 M urea. Make sure the pH of the denatured cell extract is between pH 7.0–8.0 before applying it to the affinity gel. The same purification procedures employed with native conditions can be used with denaturing buffers.

Note: Buffers containing urea must be prepared fresh daily.

An example of a urea denaturing system is described below:

Equilibration/Wash Buffer:

- 0.1 M sodium phosphate (pH 8.0), 8 M urea

Elution Buffer:

- 0.1 M sodium phosphate (pH 4.5-6.0), 8 M urea

or

- 0.1 M sodium phosphate (pH 8.0), 8 M urea, 250 mM imidazole

For pH-dependent elution, the pH of the elution buffer may have to be varied, because some recombinant proteins with His-tags will not elute in the range of pH 5.0-6.0. If the His-tagged recombinant proteins will not elute in this range, try a pH as low as 4.5.

Reducing Conditions

HIS-Select® Cobalt Affinity Gel can be used to purify proteins under mild reducing conditions. If reducing conditions must be used, do not exceed a concentration of 20 mM 2-mercaptoethanol (such as Cat. No. M3148) or 5 mM dithiothreitol (DTT, such as Cat. No. D5545).

Note: When performing purifications in the presence of reductants, a decrease in binding capacity is often observed. Therefore, it is recommended to increase the amount of affinity gel used when purifying in reducing conditions.

Cleaning HIS-Select® Cobalt Affinity Gel

The affinity gel should be cleaned after every run to ensure proper functioning on the next use. All steps may be performed at room temperature or at 2-8 °C.

General Cleaning

1. Wash the affinity gel with 2 column volumes of deionized water.
2. Clean with 5 column volumes of 6 M guanidine HCl (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. For immediate use, re-equilibrate the affinity gel with 2-3 column volumes of Equilibration Buffer. If the gel is to be stored, wash with 1-2 column volumes of 30% ethanol and store at 2-8 °C.

Notes: The affinity gel can also be cleaned with 0.2 M acetic acid, 1-2% SDS, or ethanol.

- In the case of ethanol, the ethanol concentration can be as high as 100%.
- However, the ethanol concentration must be gradually increased or decreased in stages.
- Each stage should have an increase in ethanol concentration of no more than 25% (v/v), to prevent rapid volume changes of the affinity gel.
    - For example, one series of stages may run: 25% → 50% → 75% → 100% → 75% → 50% → 25% → 0%.

If the HIS-Select® Cobalt Affinity Gel turns from a pink to a brown or gray color, the cobalt has been reduced. Reduced cobalt cannot be stripped with EDTA. Fresh gel must be used.

Results

Recombinant proteins purified by this procedure should, under most circumstances, show essentially a single band when assayed by SDS-PAGE. The affinity gel should bind >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, one must first TCA-precipitate the sample. This TCA procedure will also concentrate a protein sample.

- 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.

Reagent Compatibility Chart

Reagent	Effect	Comments
Imidazole	Binds to the cobalt-charged affinity gel and competes with the recombinant proteins with histidine tags	• For column chromatography, no more than 20 mM imidazole 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 cobalt-charged affinity gel and competes with the histidine-tagged proteins	Not recommended. Use imidazole instead.
Chelating agents, such as EDTA, EGTA	Strips cobalt ions from the affinity gel	• Not recommended as buffer components, because they can remove cobalt ions. • Used to strip the affinity gel before recharging 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, but up to 2 M NaCl can be used.
2-Mercaptoethanol	A reducing agent used to prevent disulfide bond formation	• Add up to 20 mM in the extract buffer to break disulfide bonds. • Higher levels may reduce the cobalt ions and decrease binding capacity.
Ethanol	Antimicrobial. Also eliminates hydrophobic interactions between proteins	• The binding, washing, eluting, and storage buffers may contain up to 30% ethanol. • Note: Ethanol may cause precipitation of some buffer salts. Buffers should be prepared and checked for salt precipitation before use.
Glycerol	Can help stabilize proteins	The binding, washing, eluting, and storage buffers may contain up to 50% glycerol.
DTE, DTT	Prevent disulfide bond formation, may reduce cobalt ion	Add up to 5 mM in the extract buffer to break disulfide bonds. Reduction in binding capacity is often observed when using DTT.
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 imidazole instead.

Troubleshooting Guide

Problem	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.
	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	Verify the presence of the target protein in the cell extract.
Protein elutes in the wash buffer before the elution buffer is introduced	Wash stringency is too high	Lower the imidazole concentration. Verify that the pH is between 7-8.
	The His-tag is buried within the protein structure	• Verify that 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% TRITON® X-100 or TWEEN® 20. • Increase the sodium chloride concentration up to 2 M. • Add reducing agent, such as 2-mercaptoethanol up to 20 mM.
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 previously used and cleaned numerous times. The affinity gel can be recharged with cobalt, such as Cat. No. C8661, Cobalt(II) Chloride.
	Loses color during run and does not regain it by the end of the run	• Do not use strong oxidizing or reducing agents in any of the buffers or extracts. • Reduced cobalt ion cannot be stripped from the affinity matrix. • Use new affinity gel.
Recombinant protein with histidine tag will not elute from the affinity gel	Elution conditions are too mild	• Increase the amount of imidazole. • For a denaturing purification with pH elution, make sure the pH is low enough to elute the tagged recombinant protein. For example, adjust the elution buffer to pH 4.5. • Perform batch purification so that high protein concentrations are avoided.
Non-specific proteins elute with the histidine-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 a Protease Inhibitor Cocktail **without chelating agents**, such as Cat. Nos. P8849 or MSSAFE.
	Material is linked by disulfide bonds	Add a reducing agent, such as 2-mercaptoethanol up to 20 mM.

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