Thermo Scientific

ProPac™ IMAC-10

Product Manual

P/N: 065090-05 July 2012

Thermo SCIENTIFIC

PRODUCT MANUAL

ProPac IMAC-10 Columns

(1 x 50 mm, P/N 063617)
(2 x 50 mm, P/N 063272)
(4 x 50 mm, P/N 063276)
(9 x 50 mm, P/N 063615)

ProPac IMAC-10 Analytical Columns

(2 x 250 mm, P/N 063274)
(4 x 250 mm, P/N 063278)

ProPac IMAC-10 Semi-prep Columns

(9 x 250 mm, P/N 063280)
(22 x 250 mm, P/N 063282)

Document No. 065090
Revision 05
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SECTION 1. INTRODUCTION TO IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC)

1.1. Features of the ProPac IMAC-10 Column

Immobilized Metal Affinity Chromatography (IMAC) is a specialty technique used primarily for the purification of proteins that contain surface-exposed histidine residues and for enrichment of phosphopeptides. It is based on a reversible interaction between metal-binding sites on the protein with metal bound to a stationary phase.

IMAC is typically performed using a capture/release method for the purpose of protein and peptide enrichment. Often, the fractions collected contain impurities in the form of other metal-binding proteins. These impurities are not resolved from the proteins of interest either because the resolution and efficiency of the resin is insufficient and/or because the cartridges are too short and not amenable to gradient methods. Thus, further purification of the collected fractions is required by reversed-phase HPLC, ion exchange, or size exclusion.

Dionex has developed a novel version of IMAC technology where the functionality is confined to the surface of an impermeable, spherical polymer substrate. The Thermo Scientific ProPac IMAC-10 analytical columns allow researchers to run gradient separations in the IMAC mode, resulting in the resolution of retained proteins and the collection of highly pure fractions. Features of the ProPac™ IMAC-10 column include:

- High efficiency resin enabling resolution of the protein of interest from impurity proteins.
- HPLC technique allowing high resolution separations.
- On-line detection of analytes facilitating collection of only the peak of interest.
- Automatable for faster throughput.

1.2. Architecture of the ProPac IMAC-10 Column

The ProPac IMAC-10 phase is engineered using 10μm, nonporous, polymeric beads as the substrate. A dense, hydrophilic intermediary layer shields the styrene-divinylbenzene substrate beads, eliminating hydrophobic interaction between the analyte and the substrate. This hydrophilic layer has linear acrylate chains with pendant iminodiacetate (IDA) groups grafted to it. When charged with transition-metal ions, the chains collapse into compact, metal-cross linked nanoparticles about 2–5 nm in diameter, as shown in the transmission electron microscopy (TEM) image in Figure 1. The image reveals the bead interior, the hydrophilic layer and the copper nanoparticles. It is these nanoparticles that act as the IMAC interaction sites.

This structure has excellent mass-transfer kinetics compared to traditional gels, unhindered by diffusion through gel. Therefore binding is rapid and release is complete. Furthermore, pH-induced changes to the metal centers are rapidly reversible, making the material reusable.

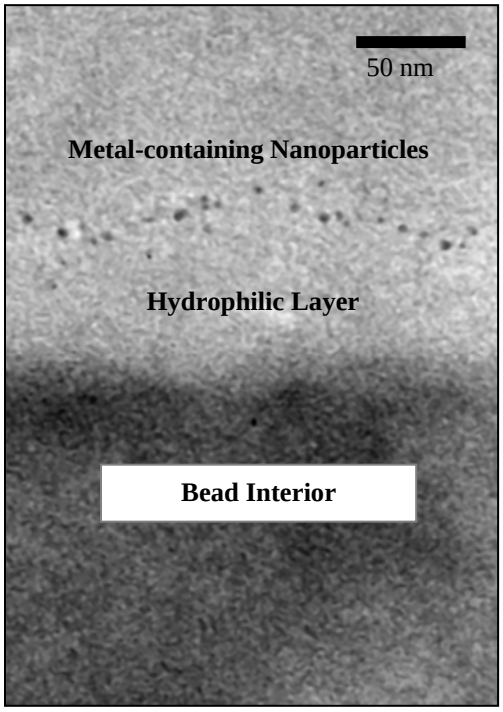


Figure 1
TEM of Cross-section of a Metal Charged IMAC Phase

The copper nanoparticles are covalently bound to the exterior of the hydrophilic layer of the 10µm nonporous, polymeric beads. Since the average diameter of proteins is similar to the average diameter of the surface bound nanoparticles, we believe that a single protein interacts with a single nanoparticle, as shown in Figure 2. This kind of interaction should be optimal for column performance, on-column refolding applications, and site-specific biotinylation experiments.

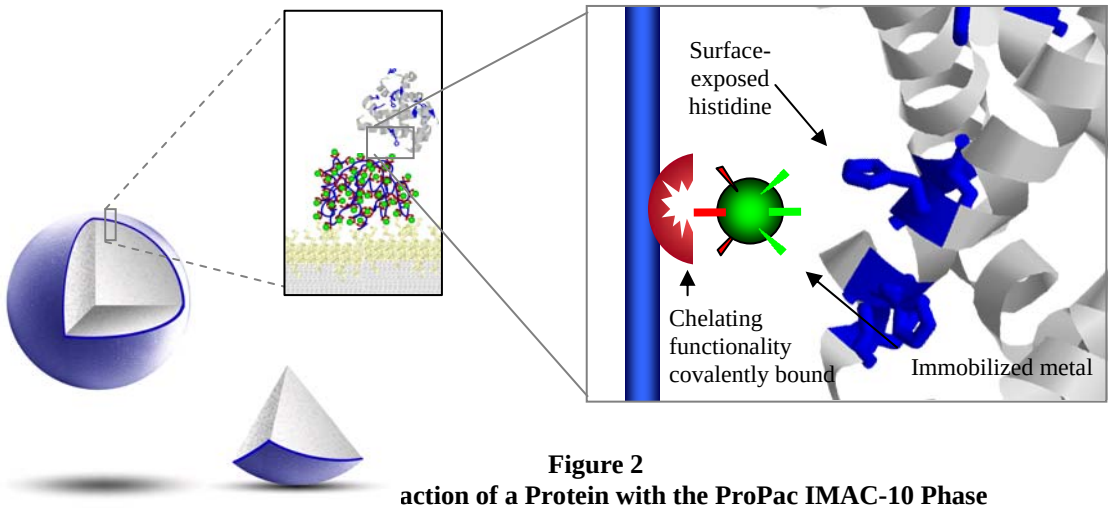


Figure 2
action of a Protein with the ProPac IMAC-10 Phase

1.3. ProPac IMAC-10 Operating Limits and Specifications

PARAMETER	RECOMMENDATION
Flow Rate Range:	0.1 – 10.0 mL/minute
Shipping Solution:	20 mM MES + 141 mM NaCl + 0.1% Sodium Azide, pH 6.1
Storage Solution:	20 mM MES + 141 mM NaCl + 0.1% Sodium Azide, pH 6.1
Buffers:	MES, HEPES, NEM, Formic Acid, Acetic Acid
Solvents:	Acetonitrile; Methanol
Temperature Range:	Ambient – 60 °C
Pressure Limit:	3,000 psi

1.4. Physical Characteristics

FEATURE	SPECIFICATION
Substrate Particle Size	10 μm
Substrate Pore Size	Non-porous
Substrate monomers	Polystyrene-divinylbenzene
Substrate Cross-linking	55%
Mode of Interaction	Coordination / Chelation
Functional Group	Iminodiacetate (IDA)
Acrylate Chain length	120
Size of Metal-Cross Linked Nanoparticles	2-5 nm

1.5. Formats of the ProPac IMAC-10 Column

ProPac IMAC-10 columns are available in a variety of formats to suit the need of the application. The smallest formats, recommended for phosphopeptide applications, are the 1 x 50mm and the 2 x 50 mm columns. The 4 x 50 mm column can be used as a column for phosphopeptide applications, or as a guard column for the analytical 4 x 250 mm column. The 9 x 50 mm column is designed for use as a guard for the 9 x 250 mm semi-prep column, or to enable users of Fast Protein Liquid Chromatography (FPLC) systems to take advantage of this technique. The 22 x 250 mm column is for semi-prep applications.

1.6. ProPac IMAC-10 Operating Conditions

ProPac IMAC-10 columns are stable between pH 2 and 12, and are compatible with purely aqueous eluents or those containing solvents, such as acetonitrile or methanol. The ProPac IMAC-10 column can be operated at any flow rate, as long as the backpressure remains below 3,000 psi. When setting up a system for use with this column, check the special precautions listed in Section 3 "Installation". PEEK™ (polyetheretherketone) is used to make the column hardware. PEEK has excellent resistance to most organic solvents and inorganic solutions.

1.7. Key Applications of the ProPac IMAC-10 Column

The ProPac IMAC-10 provides excellent peak efficiencies and fast run times for separating metal-binding proteins from non-metal binding proteins, and often for separating metal-binding proteins from each other. In addition, since it relies on HPLC, it is automatable, which results in considerably less hands-on time and less risk of operator error. Thus, the ProPac IMAC-10 column is a valuable tool for the purification of a variety of metal-binding proteins, using different metals, as listed in the table below.

Recommended Metals	Format	Recommended Applications	2D Companion column
Fe, Ga, or Zr	1 mm x 50 mm	Phosphopeptides/ optimal for 2-D/ MS compatible eluents	Acclaim 120 C18 2.1 x 150 mm
Fe, Ga, or Zr	2 mm x 50 mm	Phosphopeptides/ optimal for 2-D/ MS compatible eluents	Acclaim 120 C18 4.6 x 150 mm
Cu, Ni, or Zn	4 mm x 50 mm	His-tag proteins/ monoclonal antibodies/ prions/ FPLC compatible	N/A
Cu, Ni, or Zn	9 mm x 50 mm	His-tag proteins/ monoclonal antibodies/ prions/ FPLC compatible	N/A
Cu, Ni, or Zn	4 mm x 250 mm	Analytical separations/ His/tag proteins/ monoclonal antibodies/ prions	N/A
Cu, Ni, or Zn	9 mm x 250 mm	Semi-prep/his-tag proteins/ monoclonal antibodies/ prions	N/A
Cu, Ni, or Zn	22 mm x 250 mm	Semi-prep/his-tag	N/A

SECTION 2. CHROMATOGRAPHY SYSTEM

2.1. System Requirements

The ProPac IMAC-10 columns are designed to be used with a standard bore or microbore non-metallic HPLC system with a gradient pump module, injection valve, and a UV detector. A metal-free pump system is recommended for halide-salt eluents which may cause corrosion of metallic components leading to decreased column performance from metal contamination. A metal- free pump is recommended to avoid denaturation of the protein samples. Use of stainless steel tubing, ferrule, and bolt assemblies is not recommended because they may damage the threads of the PEEK end fittings.

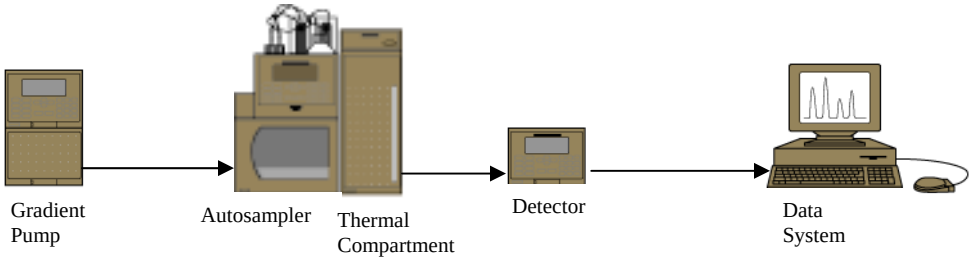


Figure 3
HPLC System Configuration

pH Range:	pH = 2 - 12
Temperature limit:	60 ºC
Pressure limit:	3,000 psi

2.2. System Void Volume

Tubing between the injection valve and detector should be ≤ 0.010 inch I.D. PEEK tubing. Minimize the length of all liquid lines, but especially the tubing between the column and the detector. The use of larger diameter and/or longer tubing may decrease peak efficiency and peak resolution.

2.3. Mobile Phase Limitations

The IMAC columns are compatible with typical mobile phase constituents, sodium, or chloride buffers, up to their limit of solubility. Use of organic solvents in the mobile phase is usually unnecessary. If you decide to use one, test the solubility limit of eluents in the presence of the chosen organic solvents. Some combinations of eluent salts and organic solvents are not miscible. Care should be taken when selecting mobile phase constituents to avoid stripping the metal from the column or inadvertently denaturing the protein.

2.4. Purity Requirements for Chemicals

Obtaining reliable, consistent and accurate results requires the use of mobile phases that are free of ionic and spectrophotometric impurities. Chemicals, solvents and de-ionized water used to prepare the mobile phase should be of the highest purity available. Maintaining low trace impurities and low particle levels in mobile phases helps to protect your columns and system components. Dionex cannot guarantee proper column performance when the quality of the chemicals, solvents and water used to prepare the mobile phase has been compromised.

2.4.1. Buffers, Salts, and Additives

Reagent Grade inorganic chemicals should always be used to prepare buffers. Whenever possible, inorganic chemicals that meet or surpass the latest American Chemical Society standard for purity should be used. These inorganic chemicals will detail the purity by having an actual lot analysis on each label. Some reagents are HPLC grade and are recommended. The quality of imidazole is especially important. Use of imidazole from Fluka (#56749) has been found to give satisfactory results and is recommended; some other sources have been found to give unacceptable results.

CAUTION ***When making mobile phases, the level of background absorbance depends on the purity of the imidazole used. Use imidazole from Fluka (#56749). When running an imidazole gradient, always add a small amount of imidazole to the Eluent 1 buffer to avoid imidazole breakthrough artifacts.***

2.4.2. De-ionized Water

The de-ionized water used to prepare the mobile phase should be Type I Reagent Grade Water with a specific resistance of 18.2 megohm-cm. The de-ionized water should be free from ionized impurities, organics, microorganisms and particulate matter larger than 0.2 μm. Many commercial water purifiers are designed for HPLC applications and are suitable for these applications.

2.4.3. Solvents

The use of solvents is usually unnecessary. If solvents are used, they must be free from ionic and UV-absorbing impurities. However, since most manufacturers of solvents do not test for ionic impurities, it is important that the highest grade of solvents available be used. Currently, several manufacturers manufacture ultrahigh purity solvents that are compatible with HPLC and spectrophotometric applications. Use of these ultrahigh purity solvents will usually ensure that your chromatography is not affected by ionic impurities in the solvent. Currently at Dionex, we have obtained consistent results using High Purity Solvents manufactured by Burdick and Jackson and Optima® Solvents by Fischer Scientific.

When using a solvent in an ionic mobile phase, column-generated backpressure will depend on the solvent used, concentration of the solvent, the ionic strength of the buffer, and the flow rate used. The column back pressure will vary as the composition or the water-methanol or water-acetonitrile mixture varies. The practical backpressure limit for the ProPac IMAC-10 columns is 3,000 psi.

SECTION 3. CHARGING THE COLUMN

3.1. Mobile Phase Preparation Precautions

3.1.1. Adjusting the pH of the Mobile Phase

The mobile phase should contain all the electrolytes before adjusting the pH. To ensure that the pH reading is correct, the pH meter needs to be calibrated at least once a day. Stirring and temperature correction should be employed.

3.1.2. Filtering the Mobile Phase

To extend the lifetime of your column as well as your HPLC pump, the high salt concentration buffers should be filtered using a 0.45 µm membrane filter to remove insoluble contaminants.

3.1.3. Degassing the Mobile Phase

The mobile phases should be degassed before use; either by using the Dionex pump degas function as described in the manual, or by using a vacuum pump. Vacuum degas the solvent by placing the mobile phase reservoir in a sonicator, and drawing vacuum on the filled reservoir with a vacuum pump for 5-10 minutes while sonicating.

3.2. Charging the Column with COPPER, NICKEL, or ZINC

3.2.1. Selecting Metal of Choice

Once the column is loaded with your metal of choice, we do not recommend reloading the column with a different metal.

3.2.2. Testing the Column

Once the column is loaded with metal, the isocratic Quality Assurance test cannot be performed. Do not run the Lot Validation Quality Assurance test unless you plan on using the column in the copper form.

3.2.3. Pumping Metal Solution

If you plan on running the metal solution through your pump, we recommend flushing the lines with DI water prior to rinsing with metal solution. Also, rinse lines with DI water, then EDTA solution, following metal loading.

3.2.4. Mobile Phase Preparation

Solution A: 20 mM HEPES + 0.5 M NaCl, + 2mM Imidazole pH 7.5
Solution B: 20 mM HEPES + 0.5 M NaCl + 0.5 M imidazole, pH 7.5
Solution C: 50 mM copper sulphate + 0.5 M NaCl, pH 4.0
Solution D: 50 mM EDTA + 0.5 M NaCl, pH 7.0

Strip: Strip the column with 10 column volumes of solution D or 10 mL, whatever is greater
Rinse: Rinse the column with 10 column volumes of solution B or 10 ml whatever is greater.
Charge: Charge the column with 3 column volumes of solution C or 10 ml or whatever is greater.
Rinse: Rinse the column with 30 column volumes of solution B or 10 ml or whatever is greater.
Equilibrate: Finally, equilibrate with 5 column volumes of solution A or 10 ml or whatever is greater.

Once the column is charged, it can be used without subsequent re-charging. Columns may be used for up to three months without re-charging. If the column performance is compromised then the column should be cleaned, according to the method described in Section 4.4 Before cleaning the column, use the STRIP protocol to strip the column of metal. After cleaning, re-charge the column with the above protocol.

3.2.5. Stripping the Metal Off the Column

Strip the column with 10 column volumes of solution D (see section 3.2.4) or 10 mL, whatever is greater
Flush the system and column with 10 column volumes of Solution A (of section 3.2.4) or 10 mL whatever is greater to remove residual EDTA.

3.3. Charging the Column: IRON, GALLIUM, ZIRCONIUM

3.3.1. Selecting Metal of Choice

Once the column is loaded with your metal of choice, we do not recommend reloading the column with a different metal.

3.3.2. Testing the Column

Once the column is loaded with metal, the isocratic Quality Assurance test cannot be performed. Do not run the Lot Validation Quality Assurance test unless you plan on using the column in the copper form.

3.3.3. Pumping Metal Solution

If you plan on running the metal solution through your pump, we recommend flushing the lines with DI water prior to rinsing with metal solution. Also, rinse lines with DI water, then EDTA solution following metal loading.

3.3.4. Mobile Phase Preparation

Solution A: 20 mM formic acid
Solution B: 20 mM formic acid titrated to pH 10 with ammonium hydroxide
Solution C: 25 mM ferric chloride + 100mM acetic acid
Solution D 50 mM EDTA+0.5M NaCl pH 4.0

RINSE: Rinse the column with mobile phase A for 3 column volumes.
CHARGE: Charge the column with 3 column volumes of mobile phase C.
RINSE: Rinse the column with 20 column volumes of mobile phase A.

Prepare the metal solution by dissolving the metal chloride in glacial acetic acid then diluting to volume.
Once the column is charged, it can be used without subsequent re-charging. Columns may be used for up to three months without re-charging. If column performance is compromised then the column should be cleaned, according to the method described in Section 4.4. Before cleaning the column, use 30 column volumes of solution D to strip the column of metal. After cleaning, re-charge the column with the above protocol. Equilibrate the column with solution A until the UV baseline stabilizes (approximately one hour).

3.3.5. Stripping the Metal Off the Column

Strip the column with 30 column volumes of solution D (see section 3.3.4)
Flush the system and column with 10 column volumes of solution A (of section 3.3.4) or 10 mL whatever is greater to remove residual EDTA.

3.4. Charging the Column without Exposing the System to Metal

To avoid contact of the metal solution with your system, fill an empty column body with the metal solution. After the first rinse place it between the pump and the IMAC column. Push the metal solution through the IMAC column with mobile phase A at a flow rate for the column dimension and metal application. After charging the IMAC column, remove the empty column body. Proceed to condition the column. Column body size can be adjusted to fit the IMAC column to be loaded.

3.4.1. Recommended Empty Column Body Size

IMAC COLUMN SIZE	RECOMMENDED EMPTY COLUMN BODY SIZE (FOR LOADING WITH 50 mM METAL SOLUTION)	VOLUME OF METAL SOLUTION
1 x 50 mm	4 x 50 mm (P/N 063667)	2ml
2 x 50 mm	4 x 50 mm (P/N 063667)	2ml
4 x 50 mm	4 x 50 mm (P/N 063667)	2ml
9 x 50 mm	9 x 50 mm (P/N 063710)	20ml
4 x 250 mm	9 x 50 mm (P/N 063710)	20ml
9 x 250 mm	9 x 250 mm (P/N 063718)	100ml
22 x 250 mm	9 x 250 mm (P/N 063718)	100ml

SECTION 4. METAL FREE VALIDATION COLUMN PERFORMANCE

WARNING

Once the column is loaded with metal, the isocratic Quality Assurance test cannot be performed. Do not run the Lot Validation Quality Assurance test unless you plan on using the column in the copper form.

Dionex recommends that you perform an efficiency test on your ProPac IMAC-10 column before you use it. The purpose of column performance validation is to make sure that no damage has been done to the column during shipping. Test the column using the conditions described on the isocratic Quality Assurance Report enclosed in the column box, and also included in the appendix of this manual. Note that slight variations may be obtained on two different HPLC systems due to system electronic, plumbing, operating environment, reagent quality, column conditioning, and operator technique.

Please see the example Quality Assurance Report in Appendix A.

NOTE

Note: As the analytical column ages it is normal to see some decrease in retention time or change in operating parameters. If the analytical column is older than one year it is recommended to run the column under test chromatogram conditions and confirm that the column is meeting the QAR specifications to ensure optimum performance of the column. It is recommended to repeat this performance test once a year (for columns in the copper form).

4.1. Procedure for Validating Column Performance

1) Connect the column to the LC system.
2) Purge the column with the mobile phase listed on the QA report for 20 to 40 column volumes.
3) Inject the test mix shown in the QA report, and also in Appendix A, and collect the data.
4) Compare your result with the QA report provided in the column box.
5) If the chromatograms look similar, you can use the column for your application work.

4.2. Equilibrating the Column

Equilibrate the column after installing it for the first time. Always re-equilibrate the column prior to use following periods of storage.

Purge the column with the shipping or storage solvent until the baseline is stable. Purge the column with enough mobile phase to remove the shipping solution. Equilibrate the column with at least 15 column volumes of the mobile phase until a stable baseline is achieved.

WARNING

Please exercise caution when handling sodium azide in the presence of metals. Sodium azide can potentially react with copper and other heavy metals to form a potentially explosive mixture that when dried, can be sensitive to both shock and friction. Heavy metal azides are very explosive when heated or shaken and appear on the Bureau of Tobacco and Firearms explosive list. Always wash the column thoroughly to prevent mobile phase mixing. Always separate waste containing sodium azide from waste containing metal ions to prevent any hazards. Sodium azide could be replaced with 0.1% phenol for column storage.

4.3. Caring for the Column

To ensure the high performance of the ProPac IMAC-10 column, the following guidelines should be followed:

1) Protect the column from contamination using a ProPac IMAC-10 guard column, where appropriate.
2) Ensure that solvents are miscible when changing mobile phases.
3) Always degas and filter mobile phases through a 0.22-µm membrane filter.
4) When switching to a new mobile phase, the column should be equilibrated for at least 30 column volumes before injecting the sample.
5) The recommended pH range is from pH 2 to 12. However, it is preferred that the column is used between pH 3 and pH 10 for compatibility with the protein separation.
6) The column can be stored in mobile phase for short term storage (e.g. overnight). However, it is highly recommended that the column be stored in 20 µm MES and 14 µm NaCl + 0.1% Sodium Azide, pH 6.1 for long term storage (more than 2 days).
7) The recommended operating maximum temperature is below 50 ˚C. In most cases, temperature control between ambient and 30 ˚C gives good results.
8) The recommended maximum back pressure is 3,000 psi.

4.4. Cleaning and Regenerating the Column

4.4.1. Decreased Retention or Peak Resolution

A decrease in retention or resolution may indicate contamination of the column. First, strip the metal from the column using the appropriate stripping procedure in Section 3.2.5 or 3.3.5. Flush with 20 column volumes of DI water, then 20 column volumes of 80% acetonitrile to remove the contaminant. If the mobile phase contained a buffer, first flush the column with 10 column volumes of 18 megohm-cm reagent water before changing to the pure solvent.

4.4.2. High Backpressure

If the system backpressure increases with age, replace the guard cartridge. If you are not using a guard cartridge, replace the column frit.

4.5. Storing the Column

Leaving the column unused for a short period of time does not require special storage procedures. For long-term storage, store the column in 20 µm MES and 14 µm NaCl + 0.1% Sodium Azide, pH 6.1, and be sure that the end plugs are firmly in place. Never let the column dry out.

If the mobile phase contained a buffer, first flush the column with 10 column volumes of 18 megohm-cm reagent water before changing over to storage solution.

WARNING

Please exercise caution when handling sodium azide in the presence of metals. Sodium azide can potentially react with copper and other heavy metals to form a potentially explosive mixture that when dried, can be sensitive to both shock and friction. Heavy metal azides are very explosive when heated or shaken and appear on the Bureau of Tobacco and Firearms explosive list. Always wash the column thoroughly to prevent mobile phase mixing. Always separate waste containing sodium azide from waste containing metal ions to prevent any hazards. Sodium azide could be replaced with 0.1% phenol for column storage.

SECTION 5. METHODS DEVELOPMENT

IMAC separations are typically carried out using an increasing imidazole gradient or a decreasing pH gradient. The mechanism of interaction between the analyte and the stationary phase is metal coordination. Whether you choose an imidazole gradient or a pH gradient, the ionic strength should always be at least that provided by a 0.5 M NaCl solution. At concentrations below 0.25 M NaCl, ion exchange interactions severely affect the retention time of analytes. The following description is intended to give you an understanding of the nature of IMAC chromatography and the tools needed to carry out efficient method development.

NOTE

In the copper mode, after loading with copper, always condition the column by pumping 30 column volumes of 20 mM HEPES + 0.5 M NaCl + 0.5 M imidazole, pH 7.0 over column prior to running the gradient.

5.1. Mobile Phase Selection

The constituents of the mobile phase will depend upon the nature of the sample and the goal of the application. Table 5.1.1. below lists some of the more common chemicals and their function. Be sure to choose chemicals that are compatible with the goal of the analysis.

5.1.1. Table of Buffers and Conditions

ADDITIVE	RECOMMENDATION
Buffers	Acetic acid, MES, HEPES, NEM (n-ethylmorpholine)
pH range for imidazole gradients	(5.5 - 10.0) recommend pH = 7.0
Additives:	imidazole, glycine
Chaotropic agents:	urea, guanidine
Salt species:	NaCl, KCl, sodium acetate (primary amine salts cannot be used with copper loaded columns)
Salt concentration range:	(0.25 M – 2.0 M) recommended [NaCl] = 0.5 M

5.2. Imidazole Gradient Method Development

The recommended starting place for method development is an imidazole gradient. When developing an imidazole gradient first choose the pH, followed by the buffer, salt, and imidazole concentrations.

5.2.1. Choosing a pH and a Buffer

When developing an IMAC method on the ProPac IMAC-10 column, a variety of buffers covering a wide pH range can be used. We recommend starting with a buffer system containing HEPES at pH 7.0 because this system provides the greatest selectivity for our applications. Many applications have narrow pH requirements. The list below contains buffers compatible with IMAC over a wide pH range. It is important to note that some buffers, such as Tris and phosphate, coordinate with metals and therefore are only suitable for analytes that bind very tightly to the IMAC phase, such as his-tagged-proteins and prion proteins.

5.2.2. Non-coordinating Buffers:

BUFFER SPECIES	pH RANGE
Acetic Acid	4.2 – 5.2
MES	5.5 - 6.9
HEPES	6.8 - 8.2
NEM (n-ethylmorpholine)	7.2 - 8.5

5.2.3. Effect of pH on Separation

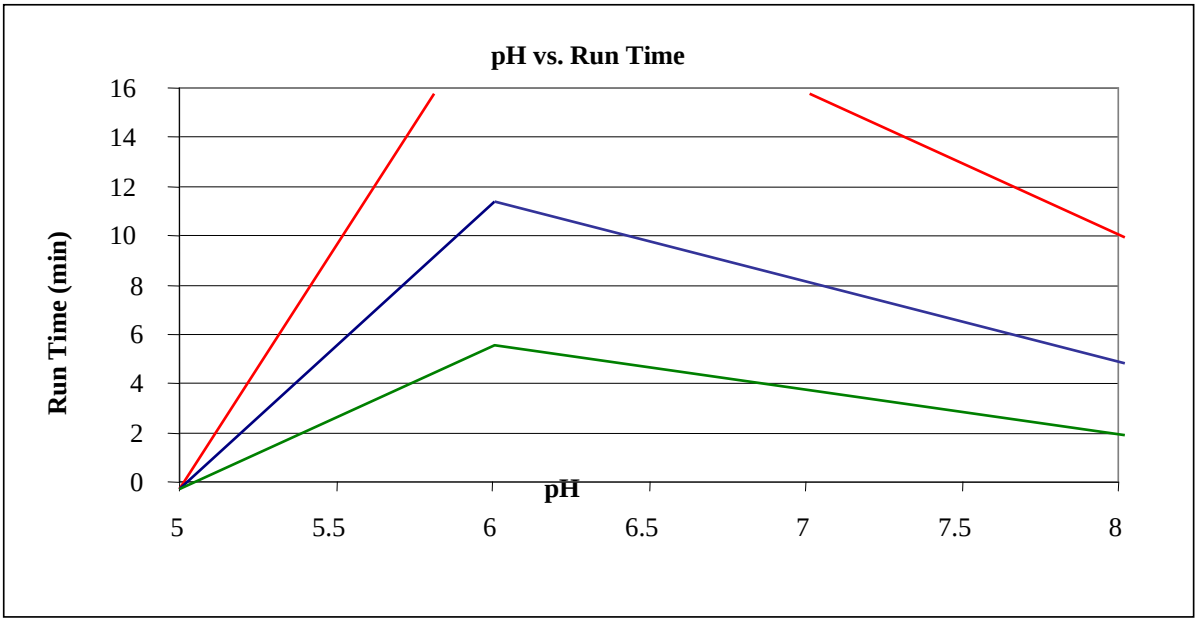
Optimization of the imidazole gradient method may involve varying pH. Varying the pH is likely to result in a shift in analyte retention time. There is a general trend of increasing retention time with decreasing pH for the same imidazole gradient. As the pH of the system approaches pH = 5.5, more imidazole may be needed to elute the analyte of interest. The experiment below describes this phenomenon, using a 4 x 50mm ProPac IMAC-10 column.

Eluent A: 20 mM Acetic acid/MES/HEPES + 0.5 M NaCl (pH varied)
Eluent B: 20 mM Acetic acid/MES/HEPES + 0.5 M NaCl + 40 mM imidazole (pH varied)

Gradient:

t (min)	%A	%B
0	100	0
15	0	100
20	0	100

Sample: Ribonuclease A (green), Myoglobin (blue), Carbonic Anhydrase (red)



5.2.4. Effect of Ionic Strength

As previously mentioned, the mechanism of retention in IMAC is coordination interaction, but secondary interactions are observed under certain buffer conditions. For example, strong ion exchange secondary interactions are observed when the ionic strength of the buffer is low and weak hydrophobic secondary interactions are observed when the ionic strength of the buffer is very high. Regardless, it is possible to achieve separations at as high as 2 M NaCl, indicating that the phase is very hydrophilic. The recommended ionic strength range for the ProPac IMAC-10 column is between 0.25 M NaCl and 2 M NaCl. However, the optimal ionic strength for most of our separations is 0.5 M NaCl.

SECTION 6. APPLICATIONS

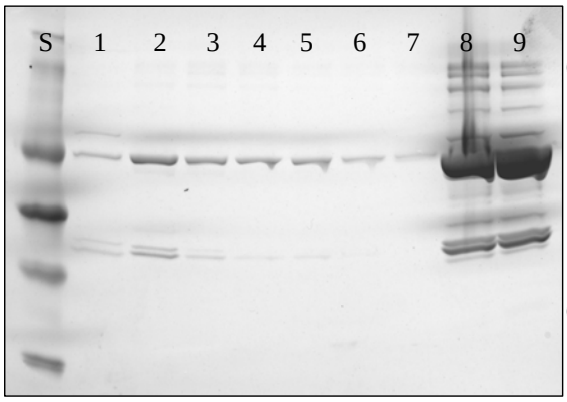
6.1. His-Tagged Proteins / Aggregation Variants

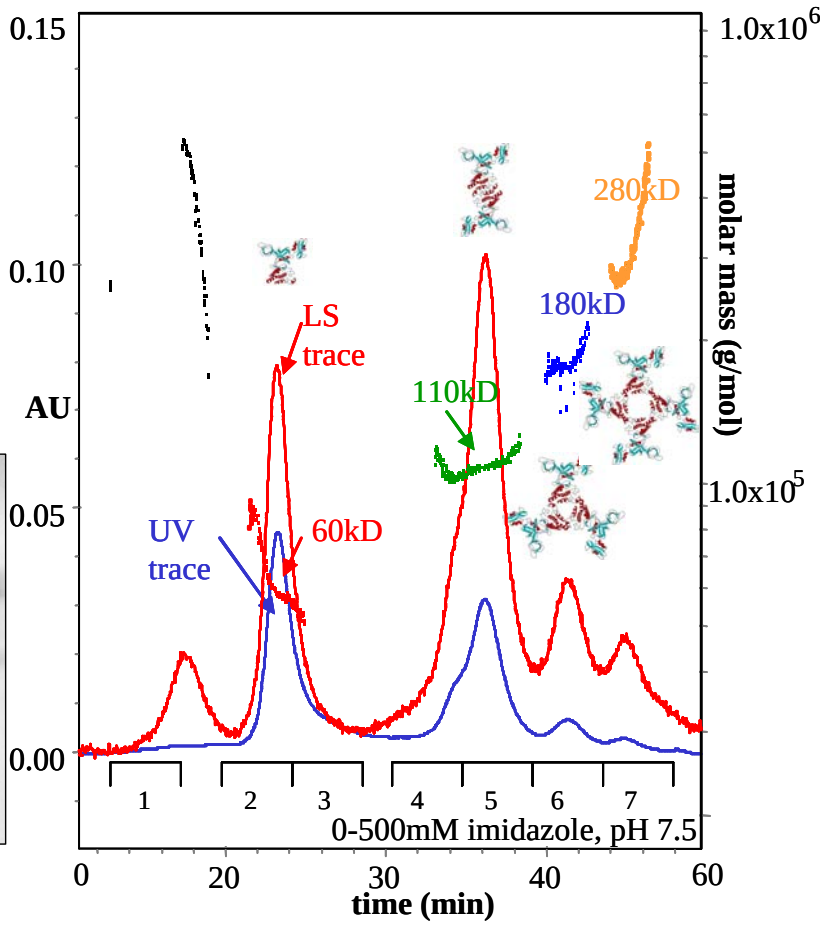
This application demonstrates the use of this column for identification of protein aggregation, his-tagged protein purification, QA/QC of IMAC cartridge purifications, and on-column refolding experiments.

His-tagged proteins are usually purified with an IMAC cartridge by applying a capture/release protocol, and the purity of the released fraction assessed by SDS-Page. SDS-Page often reveals the presence of protein impurities from the host system (i.e. e-coli) after IMAC cartridge purification (Lane 1 on the right). The amount of impurity can be reduced by optimization of rinse conditions, but impurities with affinity similar to, or greater than, his-tagged protein can not be excluded by this approach. To achieve high purity IMAC fractions, the IMAC analytical column allows you to carry out an imidazole gradient over the entire separation space. Monitoring the separation with a UV detector allows you to collect the fraction containing his-tagged protein and pass the impurities on to waste.

The chromatogram below shows an imidazole gradient separation of a 55 kD his-tagged protein previously purified by IMAC cartridge capture/release. The trace shows multiple peaks. Since this protein is known to aggregate and the separation was carried out using non-denaturing conditions, we expected the peaks to correspond to monomer, dimer, trimer, tetramer, etc. Each peak was collected and run on SDS-Page. An image of the gel is shown below.

Column:	IMAC (4 x 250 mm)
Eluent A:	20 mM HEPES + 0.5 M NaCl, 25 mM imidazole, pH = 7.5
Eluent B:	20 mM HEPES + 0.5 M NaCl, 500 mM imidazole, pH = 7.5
Gradient:	t (min) %A %B
	0 100 0
	80 0 100
	100 0 100
Inject vol:	100 µL
Sample:	His-Tagged Protein
Flow Rate:	0.5 mL/min
Detection:	UV @280 nm



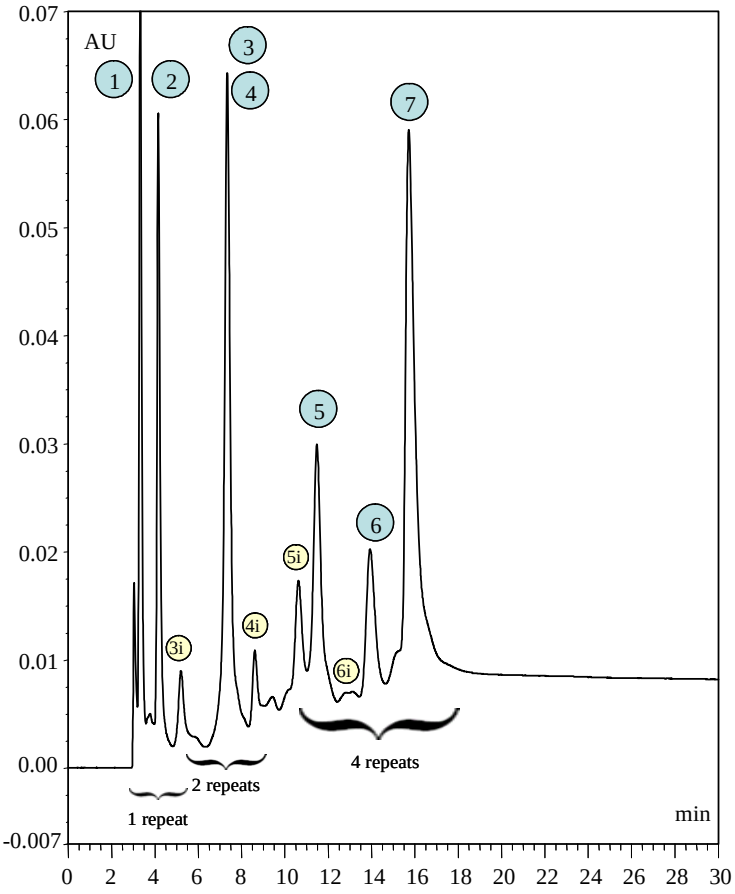


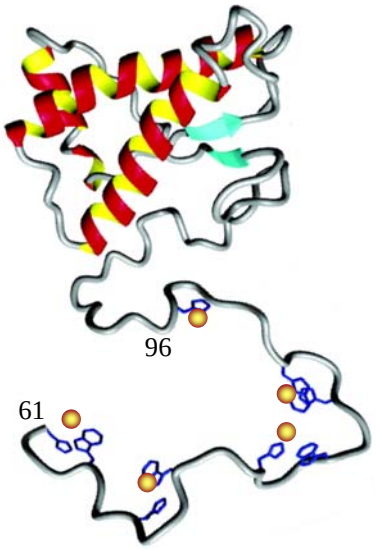
6.2. Histidine Containing Peptides

6.2.1. Prion Peptides

The proteomics approach involves identification of large numbers of proteins in very complex mixtures. Typically, the mixtures are simplified by class separation on an affinity column. IMAC can be used to select histidine containing proteins or peptides in the copper form, or phosphopeptides in the iron form. Prion proteins are commonly purified from tissue samples by IMAC. The affinity of prion protein for the IMAC phase is similar to a 6x-his-tagged protein.

Column:	IMAC (4 mm x 250 mm)			
Eluent A:	20 mM HEPES, 0.5 M NaCl, 25 mM imidazole, pH 7.5			
Eluent B:	20 mM HEPES, 0.5 M NaCl, 500 mM imidazole, pH 7.5			
Gradient:	t (min)	%A	%B	
	0	100	0	
	15	0	100	curve 7
	30	0	100	
Inject vol:	15 µL			
Sample:	Prion-derived peptides			
Flow Rate:	0.5 mL/min			
Detection:	UV @280 nm			





The chromatogram shows a separation of 6 synthetically prepared prion peptide derivatives. The peptides contain octapeptide repeats where each repeat has been found to bind one copper atom. The column is capable of separating prion-related peptides differing in number of octapeptide repeat units (PHGGGWGQ) (peak 1), (PHGGGWGQ)2 (peak 3), and (PHGGGWGQ)4 (peak 5). The column is also able to separate several prion-related peptide mixtures where the peptides contained the same number of copper binding sites but differed in the presence of a hydrophilic tail (peak 5 and peak 6) or Q→A mutation (peak 6 and peak 7)

Peak #:	Sequence
1	PHGGGWGQ
2	PHGGGWGQ (G1 methylated)
3 KKRPKP	PHGGGWGQPHGGGWGQ
4 KKRP	PHGGGWGQPHGGGWGQ (G1,G4 methylated)
5 KKRPKP	WGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQ
6	WGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQ
7	WGAPHGGGWGAPHGGGWGAPHGGGWGAPHGGGWGA

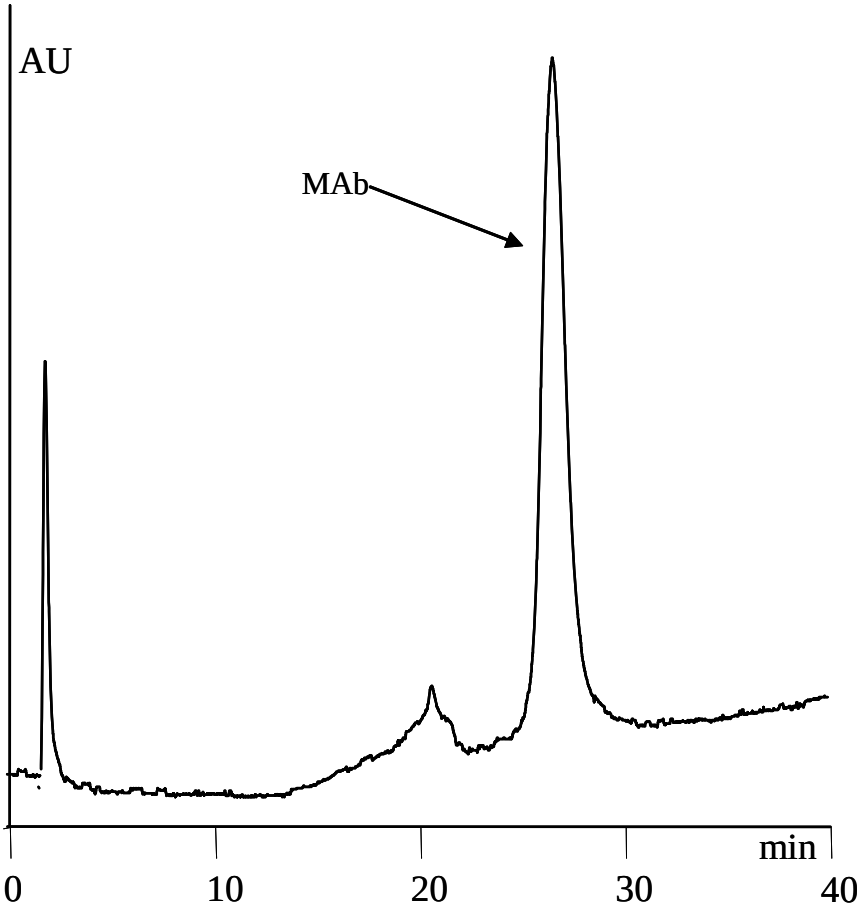
6.2.2. Monoclonal Antibodies

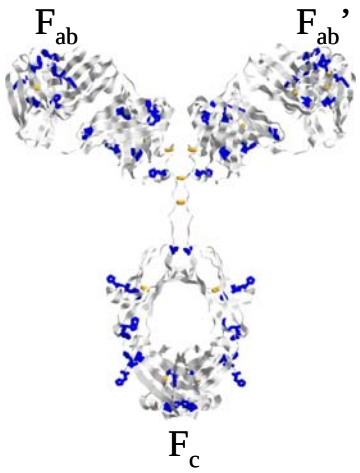
Monoclonal antibodies are used in a wide variety of applications. Each application has its own set of purity requirements. The chromatogram shows a trace of a highly pure monoclonal antibody. Furthermore, isolating individual proteins on individual nanoparticles is expected to result in higher purity fractions.

Column: IMAC (4 mm x 250 mm)
Eluent A: 20 mM MES, 0.5 M NaCl, 1 mM imidazole, pH 5.5
Eluent B: 20 mM MES, 0.5 M NaCl, 100 mM imidazole, pH 5.5
Gradient:

t (min)	%A	%B
0	100	0
80	0	100
100	0	100

Inject vol: 10 µL
Sample: MAb (5 mg/mL)
Detection: UV @280 nm





Surface-exposed Histidine and Tryptophan Clusters

6.2.3. Protein and Enzyme Purification

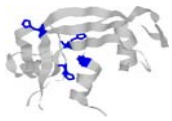
Analytical IMAC is capable of separating proteins based on the extent of surface exposed histidine residues. The chromatogram below shows resolution of three standard proteins.

Column: IMAC (4 mm x 250 mm)
Eluent A: 20 mM MES + 0.5 M NaCl + 4 mM imidazole, pH = 6.0
Eluent B: 20 mM MES + 0.5 M NaCl + 100 mM imidazole, pH = 6.0

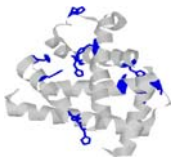
Gradient:	t (min)	%A	%B	
	0	100	0	
	15	0	100	curve 7
	40	0	100	

Inject volume: 15 µL
Sample: 1 - 0.25 mg/mL ribonuclease A
2 - 1.00 mg/mL myoglobin
3 - 1.50 mg/mL carbonic anhydrase
Flow Rate: 0.5 mL/min
Detection: UV @ 280 nm

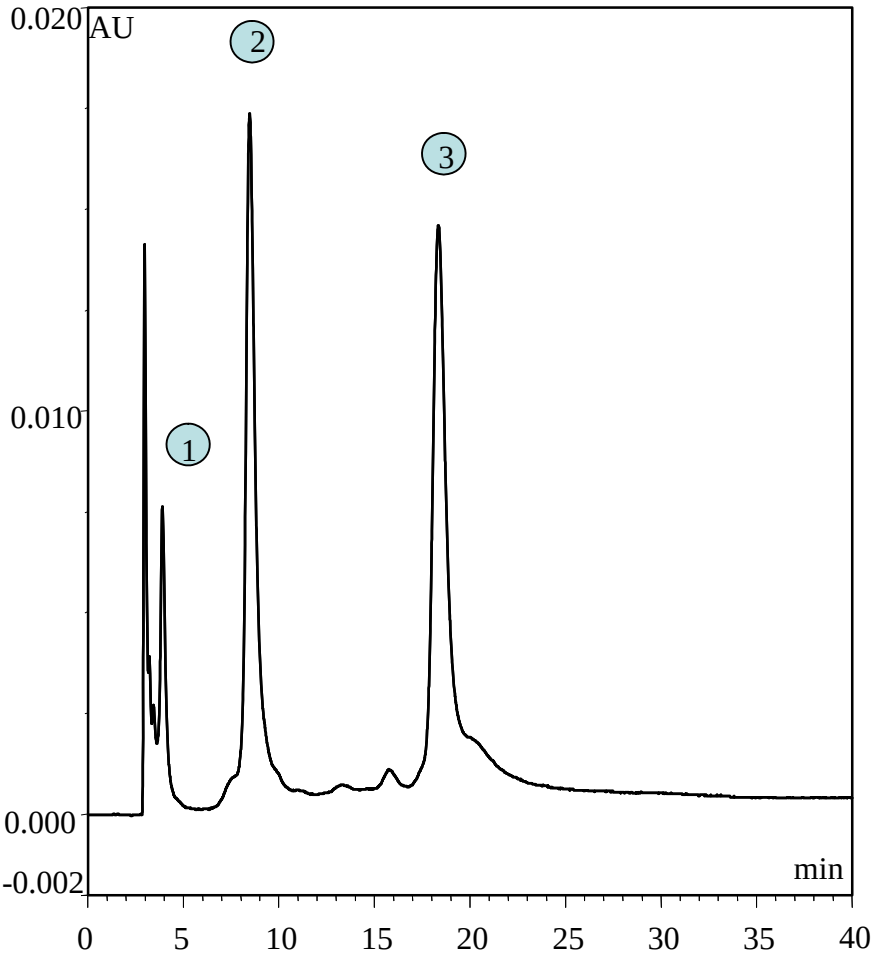
Proteins with Surface Exposed Histidines



Ribonuclease A



Myoglobin



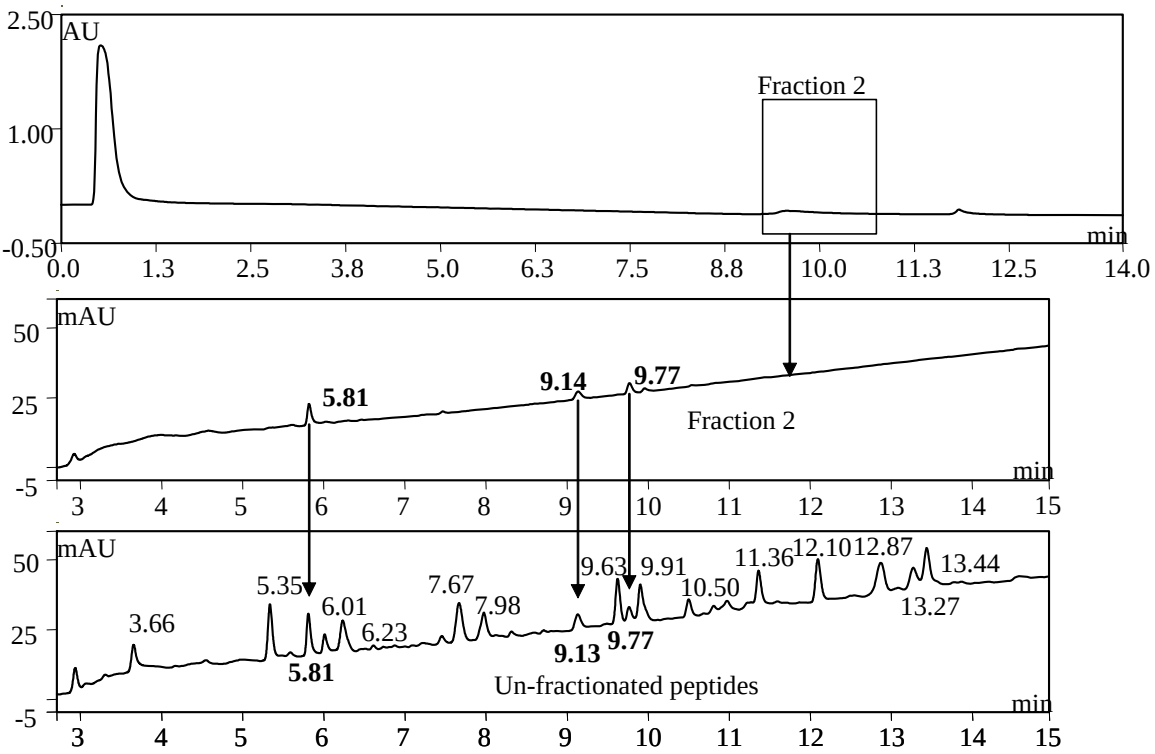
6.3. Iron Phosphopeptide Capture and Release

Detection of phosphorylation in proteins is necessary to the understanding of their biological functions, but the abundances of these proteins are usually low, making this a challenging analytical problem. Immobilized metal-affinity chromatography (IMAC) in the ferric form has been used to fractionate phosphopeptides and non-phosphopeptides.

The usual approach to the analysis of phosphoproteins is to first digest and then fractionate. Chelated ferric ions have an affinity for phosphorylated peptides, and the affinity can be controlled by pH, ionic strength and organic solvents. In a typical experiment, one binds the phosphopeptides to an iron-loaded stationary phase at low pH where most nonphosphorylated peptides bind weakly or not at all. After washing, the captured phosphopeptides are released into an alkaline buffer. This enriched fraction is then further characterized by reversed-phase LC or LC/MS using standard peptide protocols. See Dionex Technical Note 705 for more details and techniques for automation.

The model system for this work is beta-casein, a well-studied phosphoprotein. The complete tryptic digest has one monophosphorylated peptide (FQSEEQQQTEDELQDK) and one tetraphosphorylated peptide (ELEELNVPGEIVESLSSSEESITR), with all phosphorylations at serine.

Column:	IMAC (2 mm x 50 mm)			
Eluent A:	20 mM formic acid			
Eluent B:	20 mM formic acid titrated to pH 10 with ammonium hydroxide			
Gradient:	t (min)	%A	%B	
	0	100	0	
	6	0	90	curve 7
	15	0	90	
Inject vol:	15 µL			
Sample:	Beta Casein Digest			
Flow Rate:	0.21 mL/min			
Detection:	UV @214 nm			



SECTION 7. TROUBLESHOOTING GUIDE

7.1. Baseline Drift during Imidazole Gradient Elution

A. All imidazole has some background absorbance at 280 nm. Use high purity imidazole from Fluka (#56749) to minimize baseline drift.

B. Baseline drift can also be caused by weakly bound copper atoms leaking from the column. To avoid this, condition the column by pumping 30 column volumes of 20 mM HEPES + 0.5 M NaCl + 0.5 M imidazole, pH 7.0 over column prior to running the gradient.

7.2. Low Efficiency Peaks

A. In an imidazole gradient, low efficiency peaks are often caused by not properly conditioning the column prior to use. The column can be conditioned by pumping 30 column volumes of 20 mM HEPES + 0.5 M NaCl + 0.5 M imidazole, pH 7.0 over column prior to running the gradient.

7.3. Finding the Source of High System Backpressure

A. A significant increase in the system backpressure may be caused by a plugged inlet frit (bed support).

B. Before replacing the inlet bed support assembly of the column, be sure that the column is the cause of the excessive back pressure.

C. Check for pinched tubing or obstructed fittings from the pump outlet, throughout the eluent flow path to the detector cell outlet. To do this, disconnect the eluent line at the pump outlet and observe the backpressure at the usual flow rate. It should not exceed 50 psi (0.3 MPa). Continue adding components (injection valve, column, and detector) one by one while monitoring the system backpressure. The 4 x 250 mm ProPac IMAC-10 column should add no more than 1000 psi backpressure at 1.0 mL/min. The 4x50 mm ProPac column should add no more than 300 psi backpressure at 1.0 mL/min. No other component should add more than 100 psi (0.7 mPa) to the system backpressure.

D. If the high backpressure is due to the column, first try cleaning the column. If the high backpressure persists, replace the column bed support at the inlet of the column.

7.4. Column Performance is Deteriorated

7.4.1. Peak Efficiency and Resolution is Decreasing; Loss of Efficiency

A. If changes to the system plumbing have been made, check for excess lengths of tubing, tubing diameters larger than 0.010 inch ID, badly-fitted connections, and for leaks.

B. Check the flow rate and the gradient profile to be sure your gradient pump is working correctly.

C. The column may be fouled. Clean the column using the recommended cleaning conditions in Section 4.4.

D. If there seems to be a permanent loss of efficiency, check to see if headspace has developed in the column. This is usually due to improper use of the column such as submitting it to high backpressure. If the resin does not fill the column body all the way to the top, the resin bed has collapsed, creating a headspace. The column must be replaced.

E. If the peak shape looks good, but the efficiency number is low, check and optimize the integration parameters. If necessary, correct the integration manually, so the start, maximum, and end of the peak are correctly identified.

7.4.2. Unidentified Peaks Appear as well as the Expected Analyte Peaks

A. The sample may be degrading. Proteins tend to degrade faster in solutions; therefore, store your protein samples appropriately, and prepare only a small amount of solution/mixture for analysis.

B. The eluent may be contaminated. Prepare fresh, filtered eluent.

C. Run a blank gradient to determine if the column is contaminated. If ghost peaks appear, clean the column.

7.4.3. Peak Efficiency and Resolution is Poor

A. Try to use different eluents (buffer, pH, concentration etc.) to be sure you are using the optimum conditions for your separation problem.

B. The column may be overloaded. Dilute the sample and/or inject smaller volumes.

7.4.4. Peak Retention time Varies from Run-to-Run

The column may not be adequately equilibrated or washed.

A. Be sure that the equilibration time between runs is adequate and remains constant after every gradient run. Re-equilibration should be part of the method.
B. Column washing is usually not necessary between every run, unless your sample is extremely "dirty". If you need to use a wash, a consistent and adequate method for washing and equilibrating should be part of the method.

7.5. No Peaks, Small Peaks, Noisy Baseline

7.5.1. Detection Problem

Be sure that you are using the correct wavelength for your sample/buffer system. Adjust the selected detector range (AU) according to your injected sample amount. Check your lamp: aged UV lamps tend to give noisier response. Replace the lamp if necessary.

7.5.2. Chromatography Problem

Be sure that your sample can be eluted with the buffers and conditions you are using. Before trying a gradient separation, try isocratic elution with 100% B (high additive) buffer: the sample should elute at or near the void. If not, try a higher additive concentration or different pH.

7.5.3. Pump Problem

A noisy baseline can be caused by an improperly working pump. Be sure the pump is primed. Test the flow rate and also the pump head pressure fluctuation according to your pump manual.

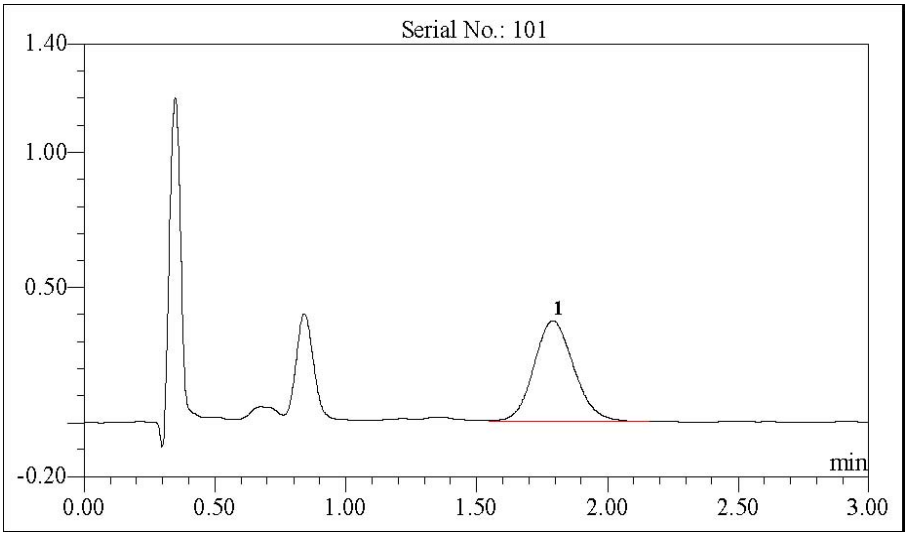
ATTACHMENT A QUALITY ASSURANCE REPORTS

A.1 ProPac IMAC-10 (1 x 50 mm)

ProPac® IMAC-10
Guard (1 x 50 mm)
Product No. 063617

Eluent: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10
Eluent Flow Rate: 0.06 mL/min
Temperature: 25 ºC
Detection: Absorbance Detection using AD25
UV at 280 nm
Injection Volume: 2.5 µL

Storage Solution: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10 + 0.1% NaN3



No.	Peak Name	Ret.Time (min)	Asymmetry (EP/USP)	Efficiency (EP)	Concentration (mg/mL)
1	Cytochrome C	1.79	1.12	681	0.025

QA Results:

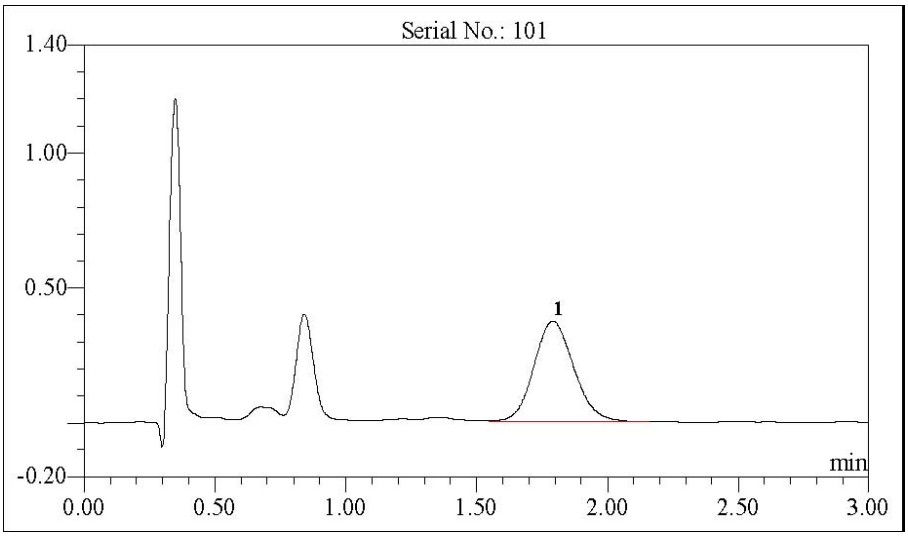
Analyte	Parameter	Specification	Results
Cytochrome C	Efficiency	>=450	Passed
Cytochrome C	Asymmetry	1.00-2.20	Passed
Cytochrome C	Retention Time	1.48-1.97	Passed
	Pressure	<=220	Passed

A.2 ProPac IMAC-10 (2 x 50 mm)

ProPac® IMAC-10
Guard (2 x 50 mm)
Product No. 063272

Eluent: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10
Eluent Flow Rate: 0.25 mL/min
Temperature: 25 ºC
Detection: Absorbance Detection using AD25
UV at 280 nm
Injection Volume: 2.5 µL

Storage Solution: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10 + 0.1% NaN3



No.	Peak Name	Ret.Time (min)	Asymmetry (EP/USP)	Efficiency (EP)	Concentration (mg/mL)
1	Cytochrome C	1.79	1.12	681	0.1

QA Results:

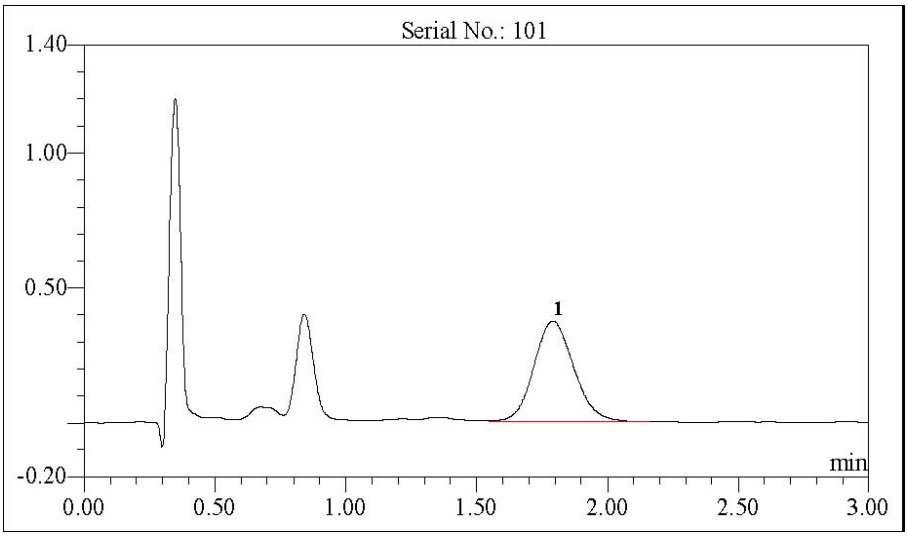
Analyte	Parameter	Specification	Results
Cytochrome C	Efficiency	>=450	Passed
Cytochrome C	Asymmetry	1.00-2.20	Passed
Cytochrome C	Retention Time	1.48-1.97	Passed
	Pressure	<=220	Passed

A.3 ProPac IMAC-10 (4 x 50 mm)

ProPac® IMAC-10
Guard (4 x 50 mm)
Product No. 063276

Eluent: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10
Eluent Flow Rate: 1.0 mL/min
Temperature: 25 ºC
Detection: Absorbance Detection using AD25
UV at 280 nm
Injection Volume: 10 µL

Storage Solution: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10 + 0.1% NaN3



No.	Peak Name	Ret.Time (min)	Asymmetry (EP/USP)	Efficiency (EP)	Concentration (mg/mL)
1	Cytochrome C	1.79	1.12	681	0.1

QA Results:

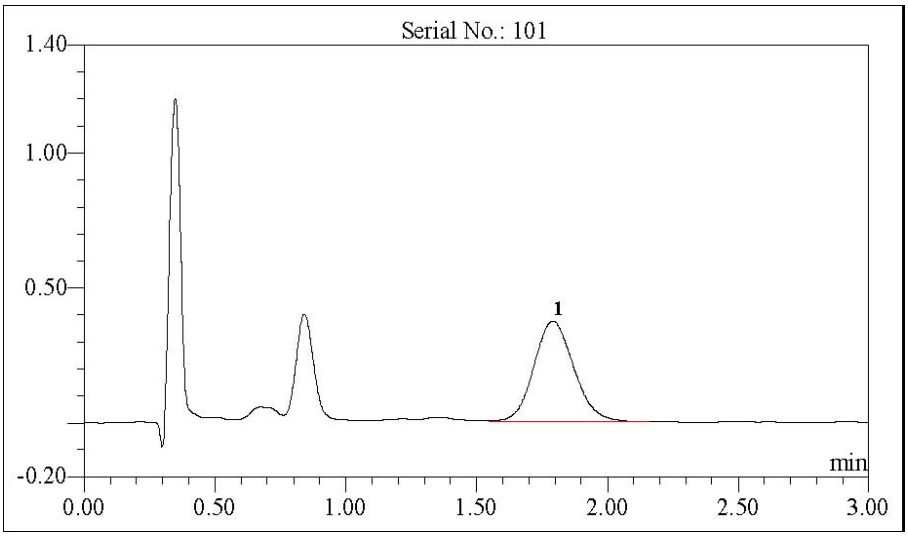
Analyte	Parameter	Specification	Results
Cytochrome C	Efficiency	>=450	Passed
Cytochrome C	Asymmetry	1.00-2.20	Passed
Cytochrome C	Retention Time	1.48-1.97	Passed
	Pressure	<=220	Passed

A.4 ProPac IMAC-10 (9 x 50 mm)

ProPac® IMAC-10
Guard (9 x 50 mm)
Product No. 063615

Eluent: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10
Eluent Flow Rate: 5.0 mL/min
Temperature: 25 ºC
Detection: Absorbance Detection using AD25
UV at 280 nm
Injection Volume: 50 µL

Storage Solution: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10 + 0.1% NaN3



No.	Peak Name	Ret.Time (min)	Asymmetry (EP/USP)	Efficiency (EP)	Concentration (mg/mL)
1	Cytochrome C	1.79	1.12	681	0.5

QA Results:

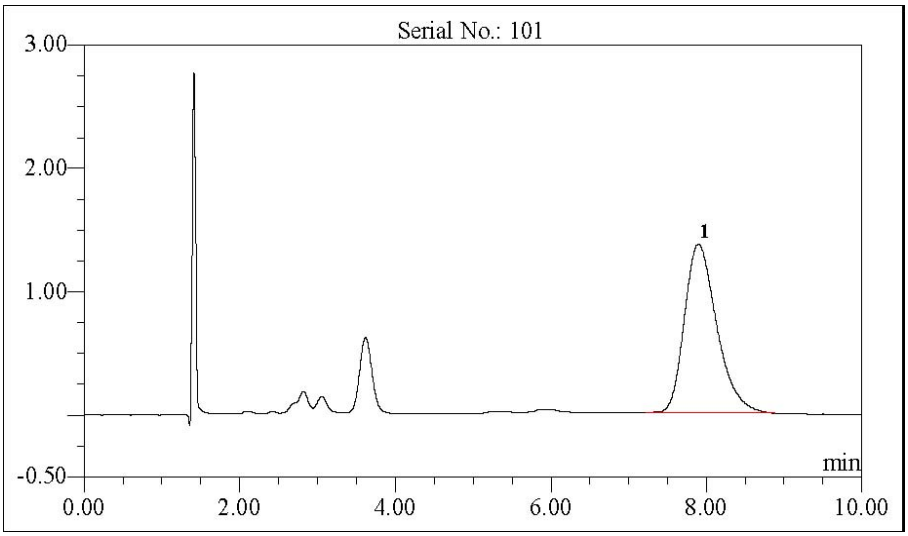
Analyte	Parameter	Specification	Results
Cytochrome C	Efficiency	>=450	Passed
Cytochrome C	Asymmetry	1.00-2.20	Passed
Cytochrome C	Retention Time	1.48-1.97	Passed
	Pressure	<=220	Passed

A.5 ProPac IMAC-10 (4 x 250 mm)

ProPac® IMAC-10
Analytical (4 x 250 mm)
Product No. 063278

Eluent: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10
Eluent Flow Rate: 1.0 mL/min
Temperature: 25 ºC
Detection: Absorbance Detection using AD25
UV at 280 nm
Injection Volume: 10 µL

Storage Solution: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10 + 0.1% NaN3



No.	Peak Name	Ret.Time (min)	Asymmetry (EP/USP)	Efficiency (EP)	Concentration (mg/mL)
1	Cytochrome C	7.90	1.33	1794	0.5

QA Results:

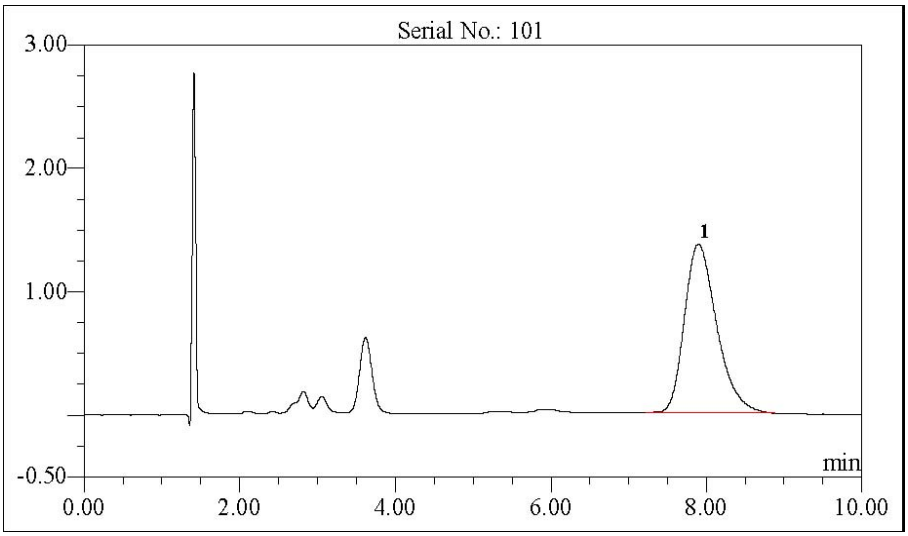
Analyte	Parameter	Specification	Results
Cytochrome C	Efficiency	>=1530	Passed
Cytochrome C	Asymmetry	1.00-1.98	Passed
Cytochrome C	Retention Time	7.18-8.83	Passed
	Pressure	<=825	Passed

A.6 ProPac IMAC-10 (9 x 250 mm)

ProPac® IMAC-10
Semi Prep (9 x 250 mm)
Product No. 063280

Eluent: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10
Eluent Flow Rate: 5.0 mL/min
Temperature: 25 ºC
Detection: Absorbance Detection using AD25
UV at 280 nm
Injection Volume: 50 µL

Storage Solution: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10 + 0.1% NaN3



No.	Peak Name	Ret.Time (min)	Asymmetry (EP/USP)	Efficiency (EP)	Concentration (mg/mL)
1	Cytochrome C	7.90	1.33	1794	0.5

QA Results:

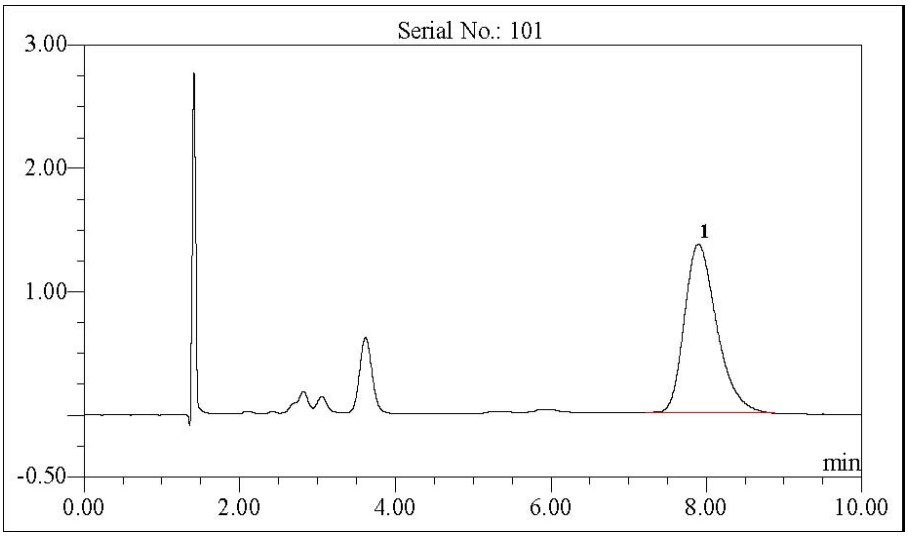
Analyte	Parameter	Specification	Results
Cytochrome C	Efficiency	>=1530	Passed
Cytochrome C	Asymmetry	1.00-1.98	Passed
Cytochrome C	Retention Time	7.18-8.83	Passed
	Pressure	<=825	Passed

A.7 ProPac IMAC-10 (22 x 250 mm)

ProPac® IMAC-10
Prep Scale (22 x 250 mm)
Product No. 063282

Eluent: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10
Eluent Flow Rate: 10.0 mL/min
Temperature: 25 ºC
Detection: Absorbance Detection using AD25
UV at 280 nm
Injection Volume: 250 µL

Storage Solution: 20 mM MES + 141.8 mM NaCl + 1 mM EDTA pH = 6.10 + 0.1% NaN3



No.	Peak Name	Ret.Time (min)	Asymmetry (EP/USP)	Efficiency (EP)	Concentration (mg/mL)
1	Cytochrome C	7.90	1.33	1794	0.5

QA Results:

Analyte	Parameter	Specification	Results
Cytochrome C	Efficiency	>=1530	Passed
Cytochrome C	Asymmetry	1.00-1.98	Passed
Cytochrome C	Retention Time	7.18-8.83	Passed
	Pressure	<=825	Passed

A.8 ProPac IMAC-10 Lot Validation (4 x 250 mm)

Lot Validation
ProPac® IMAC-10
(Using 4 x 250 mm)

Eluent Flow Rate:	0.5 mL/min
Temperature:	25 ºC
Detection:	Absorbance Detection using AD25
Detector Setting:	280 nm
Standard:	Myoglobin: 0.5 mg/mL, Carbonic anhydrase: 1.0 mg/mL
Injection Volume:	10 µL
Storage Solution:	N/A

Buffers:

%A: 20 mM HEPES + 0.5 M NaCl + 2 mM imidazole pH 7.20

%B: 20 mM HEPES + 0.5 M NaCl + 100 mM imidazole pH 7.20

Buffer Gradient

%A	%B	Time	
-5.00	100	0	Equilibration
-0.50	100	0	Autozero
0.00	100	0	Inject
20.00	0	100	Gradient
30.00	0	100	Back to weak eluent
30.10	100	0	Equilibration
35.00	100	0	End

The ProPac IMAC-10 (4 x 250 mm) undergoes strict quality control testing of column backpressure, ion exchange capacity, peak efficiency, selectivity, and asymmetry to ensure high quality and reproducible column to column performance. In addition, column production lots packed with the same stationary phase lot are batch tested to ensure the reliable performance of the user test outlined below.

The chromatogram demonstrates the performance which may be expected of your new ProPac IMAC-10 (4 x 250 mm), when charged with copper, for the separation of myoglobin and carbonic anhy drase using the conditions given below.

Because of slight system performance variations, the performance of a new ProPac IMAC-10 (4 x 250 mm) on your system may vary slightly from the accompanying chromatogram. If significant variations from the test chromatogram are observed, please review the operation of your system to ensure proper operation.

Caution:

Once a column is charge with any metal, Dionex does not recommend charging the column with a different metal. Dionex does not recommend reproducing this test unless your application requires the ProPac IMAC-10 column to be in the copper mode.