

Separation of an Intact Monoclonal Antibody and Fractionation of Monoclonal Antibody Papain Digest Fragments Using Immobilized Metal Affinity Chromatography (IMAC)

INTRODUCTION

Monoclonal antibodies (MAbs) have been indispensable for the development of a wide range of products in the clinical and biological fields. For example, MAbs can be used as diagnostic agents or therapeutic treatment for many diseases.^{1,2} The most basic antibody structure has a Y shape and contains two heavy and two light polypeptide chains. The four chains are linked together by disulfide bonds.¹ Proteolytic cleavage with enzymes such as papain, pepsin, and ficin results in defined antibody fragments. The analysis of antibody fragments offers several advantages over intact antibodies. For example, the separated antigen-binding fragment (i.e., Fab region) can be used for antigen-antibody binding studies without interference from the non-antigen binding fragment (i.e., Fc region) and simplifies the characterization of MAb variants.

Antibody fragments can be fractionated using various methods, including cation-exchange chromatography, isoelectric focusing, protein A affinity chromatography, and immobilized metal affinity chromatography (IMAC).²⁻⁴ Protein A has been the most common technique used for the separation and purification of MAb fragments. Protein A is a cell wall component produced from several strains of *Staphylococcus aureus* that specifically binds the Fc region of IgG. The disadvantages of protein A fractionation are: protein A substrates often contaminate MAb samples with biological leachates and extreme eluent conditions can result in degradation of the sample during fractionation. Therefore, there is

increasing demand for new separation media that are nonbiological and use mild eluents.

IMAC has emerged as a powerful technique for fractionating MAb Fc and Fab due to the high affinity of the Fc region for immobilized copper. However, common limitations of commercially available IMAC columns include low chromatographic efficiency and resolution, excessive nonspecific binding, difficulty automating and monitoring separations, and low backpressure limits that limit flow rates.

The ProPac® IMAC-10 substrate was developed to address the limitations of the currently available IMAC columns. The performance of this IMAC column was improved by using a high-resolution 10- μm nonporous polystyrene-divinylbenzene substrate bead. The dense, hydrophilic inner layer shields the polymeric beads and inhibits hydrophobic interaction between the analyte and substrate. This hydrophilic layer contains grafted iminodiacetate (IDA) groups via controlled radical polymerization that collapse into surface-bound nanoparticles upon loading the column with the copper metal. The chain length and, therefore, nanoparticle size were optimized to correspond with the approximate size of the MAb Fc region. This application note describes a method for fractionating IgG1 MAb Fc and Fab fragments with the ProPac IMAC-10 column preloaded with copper and analyzing the fractions using the ProPac WCX-10 column.

The experimental procedure is broken into three stages, each having its own reagents, sample preparation, experimental conditions, and procedures for the application of the mobile phases:

- Stage 1:** Pretreatment of the ProPac IMAC-10 column to convert the column to the copper form
- Stage 2:** Separation of the intact and papain digested MAbs with the ProPac IMAC-10 column (copper form)
- Stage 3:** Separation and analysis of the intact and papain digested MAb fragments on the ProPac WCX-10 column.

EQUIPMENT

A Dionex ICS-3000 Chromatography system consisting of:

- DP Dual Pump module
- DC Detector/Chromatography module
- AD25 UV/Vis Absorbance Detector with 10-mm cell
- AS Autosampler
- Chromeleon® Chromatography Management Software

REAGENTS

- Deionized water, Type I reagent grade, 18.2 MΩ-cm resistivity or better
- Sodium Phosphate, anhydrous dibasic powder (Na₂HPO₄) (J.T. Baker, VWR P/N JT4062-1)
- Sodium Phosphate, monobasic (NaH₂PO₄·H₂O) (EM Science, VWR P/N EM-SX0710-1)
- Sodium Chloride, NaCl (J.T. Baker, VWR P/N JT3625-1)
- 2-(*N*-Morpholino)ethanesulfonic acid, (MES) (Sigma-Aldrich M-5287)
- Imidazole (Fluka Biochemika 56749)
- Ethylenediaminetetraacetic acid, disodium salt dihydrate, EDTA (Sigma-Aldrich E4884)
- L-cysteine (Sigma-Aldrich C7352)
- Copper (II) Sulfate, pentahydrate (CuSO₄·5H₂O) (J.T. Baker, VWR P/N JT1841-1)
- HEPES (Aldrich 233889)
- Papain (Roche 1010814001)
- Monoclonal IgG1 antibody (a generous gift from a biotechnology company)

EXPERIMENTAL

STAGE 1: PRETREATMENT OF THE ProPac IMAC-10 COLUMN (CONVERSION TO COPPER FORM)

Mobile Phases Required for Loading the ProPac IMAC-10 Column with Cu²⁺

The following four mobile phases are required for loading the column with copper:

Mobile Phase A: 20 mM MES + 200 mM NaCl (pH 5.5)

Mobile Phase B: 20 mM HEPES + 500 mM NaCl + 500 mM imidazole (pH 7.5)

Mobile Phase C: 50 mM CuSO₄ + 500 mM NaCl (pH 4.0)

Mobile Phase D: 50 mM EDTA + 500 mM NaCl (pH 7.0)

Preparation of Mobile Phase Solutions

Mobile Phase A: 20 mM MES + 200 mM NaCl (pH 5.5)

Combine 3.904 g MES and 11.688 g NaCl in a 1-L volumetric flask containing about 500 mL of degassed deionized water. Bring the volume to about 975 mL with deionized water, stir to dissolve, adjust the pH to 5.5 with 1 M NaOH, and then fill to a final volume of 1 L.

Mobile Phase B: 20 mM HEPES + 500 mM NaCl + 500 mM imidazole (pH 7.5)

Combine 5.206 g HEPES, 29.22 g NaCl, and 34.04 g imidazole in a 1-L volumetric flask containing about 500 mL of degassed deionized water. Bring the volume to about 975 mL, stir to dissolve the mixture, adjust the pH to 7.5 with concentrated HCl, and bring the total volume to 1 L.

Mobile Phase C: 50 mM CuSO₄ + 500 mM NaCl (pH 4.0)

Combine 12.484 g CuSO₄ and 29.22 g NaCl in a 1 L volumetric flask containing about 500 mL of degassed deionized water. Bring the volume to about 975 mL and adjust the pH to 4.0 with 1 M HCl.

Mobile Phase D: 50 mM EDTA + 500 mM NaCl (pH 7.0)

Combine 18.61 g EDTA and 29.22 g NaCl in a 1-L volumetric flask containing about 500 mL of degassed deionized water. Bring the volume to about 950 mL and adjust the pH to 7.0 with 1 M NaOH.

Note: Mobile phase D is not required if the column is converted to the copper form without exposure of the pump to copper metal (see the ProPac IMAC-10 column manual for further details).

Loading the ProPac IMAC-10 Column in the Copper Form

Option 1: Loading with an empty column body

To convert the IMAC column to the copper form without exposing the system to copper metal, fill a 9 x 50-mm (P/N 063710) empty column body with mobile phase C and place it between the pump and the IMAC column. Push the metal through the IMAC column using mobile phase B at 1 mL/min for at least 10 column volumes followed by rinsing with 5 column volumes of mobile phase A.

Option 2: Loading with the analytical pump

Although it is strongly recommended that an empty column body be used to load the column with copper, the analytical pump may be used as an alternative. Flush the lines with DI water prior to rinsing with metal solution. Begin by charging the column with three column volumes of mobile phase C. After charging the column, remove the column and rinse the lines with DI water followed by mobile phase D. **Warning:** do not pump 50 mM EDTA through the column as this will strip the column of the Cu²⁺ metal. Reinstall the column and rinse the column with 30 column volumes of mobile phase B. Equilibrate the column with 5 column volumes of mobile phase A. Once the column is loaded with copper, **do not** reload the column with a different metal.

STAGE 2: SEPARATION OF THE INTACT (METHOD 1) AND PAPAINE DIGESTED (METHOD 2) MABs WITH THE ProPac IMAC-10 COLUMN (COPPER FORM)

Method 1: Separation of Intact IgG1 MAb

Sample Preparation

5 mg/mL Intact IgG1 MAb

MABs are often stored in highly concentrated solutions (e.g. 50 mg/mL). Dilute the concentrated antibody to 5 mg/mL with deionized water.

Mobile Phases 1 and 2 Required for Separation of the Intact IgG1 MAb

The following two mobile phases are required for the separation of the intact IgG1 MAb with the ProPac IMAC-10 column in the copper form:

Mobile Phase 1: 20 mM MES + 500 mM NaCl + 1 mM imidazole (pH 5.5)

Mobile Phase 2: 20 mM MES + 500 mM NaCl + 100 mM imidazole (pH 5.5)

Preparation of Mobile Phases 1 and 2

Mobile Phase 1: 20 mM MES + 500 mM NaCl + 1 mM imidazole (pH 5.5)

Combine 3.904 g MES, 29.22 g NaCl, and 0.0681 g imidazole in a 1-L volumetric flask containing about 500 mL of degassed deionized water. Bring the volume to about 975 mL with deionized water, stir to dissolve, and adjust the pH to 5.5 with 1 M NaOH.

Mobile Phase 2: 20 mM MES + 500 mM NaCl + 100 mM imidazole (pH 5.5)

Combine 3.904 g MES, 29.22 g NaCl, and 6.808 g imidazole in a 1-L volumetric flask containing about 500 mL of degassed deionized water. Bring the volume to about 975 mL with deionized water, stir to dissolve, and adjust the pH to 5.5 with 1 M NaOH.

Experimental Conditions

| | |
|-----------------|---|
| Column: | ProPac IMAC-10, 4 x 250 mm (P/N 063278) |
| Mobile Phase 1: | 20 mM MES, 500 mM NaCl, 1 mM imidazole (pH 5.5) |
| Mobile Phase 2: | 20 mM MES, 500 mM NaCl, 100 mM imidazole (pH 5.5) |
| Gradient: | 4–50 min, 0–100% B |
| Flow Rate: | 0.5 mL/min |
| Injection: | 10 µL |
| Detection: | Absorbance, 280 nm |

Procedure

Separation of intact IgG1 MAb

| Time | A (%) | B (%) | Comments |
|-------|-------|-------|---------------|
| -5.00 | 100 | 0 | Equilibration |
| 0.00 | 100 | 0 | Injection |
| 4.00 | 100 | 0 | |
| 50.00 | 0 | 100 | End gradient |
| 60.00 | 0 | 100 | |

Method 2: Separation of Papain Digested IgG1 MAb

Reagent Preparation

200 mM Sodium Phosphate, Dibasic

Dissolve 14.196 g anhydrous dibasic sodium phosphate (Na_2HPO_4) in 500 mL of deionized water.

200 mM Sodium Phosphate, Monobasic

Dissolve 13.799 g monobasic sodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) in 500 mL of deionized water.

25 mM Sodium Phosphate Buffer, pH 7.6

Combine 108.8 mL of 200 mM dibasic sodium phosphate, 16.2 mL of 200 mM monobasic sodium phosphate, and 875 mL of deionized water. Verify that the pH is 7.6.

20 mM L-Cysteine

Dissolve 48.46 mg L-cysteine in 20 mL of phosphate buffer.

Sample Preparation

Papain Digested IgG1 MAb

Perform a papain digest of the intact IgG1 MAb by adding the components in the following order: (1) 70 μL phosphate buffer, (2) 10 μL of 50 mg/mL intact IgG1 MAb, (3) 10 μL L-cysteine, and (4) 10 μL of 0.50 mg/mL papain. The final MAb to papain concentration should be 100:1. Heat the digest at 37 °C in a heating block or bath for 140 min. Store the unused digest at -40 °C.

Mobile Phases 3 and 4 Required for Separation of the Papain Digested MAb

The following two mobile phases are required for the separation of the papain digested MAb with the ProPac IMAC-10 column in the copper form:

Mobile Phase 3: 20 mM MES + 200 mM NaCl (pH 5.5)

Mobile Phase 4: 20 mM MES + 200 mM NaCl + 100 mM imidazole (pH 5.5)

Preparation of Mobile Phases 3 and 4

Method 2: Separation of Papain Digested MAb with the ProPac IMAC-10 Column

Mobile Phase 3: 20 mM MES + 200 mM NaCl (pH 5.5)

Combine 3.904 g MES and 11.688 g NaCl in a 1-L volumetric flask containing about 500 mL of degassed deionized water. Bring the volume to about 975 mL with deionized water, stir to dissolve, adjust the pH to 5.5 with 1 M NaOH, and then fill to a final volume of 1 L.

Mobile Phase 4: 20 mM MES + 200 mM NaCl + 100 mM imidazole (pH 5.5)

Combine 3.904 g MES, 11.688 g NaCl, and 6.808 g imidazole in a 1-L volumetric flask containing about 500 mL of degassed deionized water. Bring the volume to about 975 mL with deionized water, stir to dissolve, adjust the pH to 5.5 with 1 M NaOH, and then fill to a final volume of 1 L.

Experimental Conditions

| | |
|-----------------|---|
| Column: | ProPac IMAC-10, 4 x 250 mm (P/N 063278) |
| Mobile Phase 3: | 20 mM MES, 200 mM NaCl (pH 5.5) |
| Mobile Phase 4: | 20 mM MES, 200 mM NaCl, 100 mM imidazole (pH 5.5) |
| Gradient: | 1–20 min, 1–100% B |
| Flow Rate: | 0.5 mL/min |
| Injection: | 100 μL |
| Detection: | Absorbance, 280 nm |

Procedure

| Time | A (%) | B (%) | Comments |
|-------|-------|-------|---------------|
| -2.00 | 99 | 1.00 | Equilibration |
| 0.00 | 99 | 1.00 | Injection |
| 1.00 | 99 | 1.00 | |
| 20.00 | 0 | 100 | End gradient |
| 40.00 | 0 | 100 | |

STAGE 3: SEPARATION AND ANALYSIS OF INTACT AND PAPAINE DIGESTED MAb FRAGMENTS ON THE ProPac WCX-10 COLUMN

Mobile Phases 5 and 6 Required for the Separation and Analysis of Intact and Papsine Digested MAb Fragments

The following two mobile phases are required for the separation and analysis of intact and papsine digested MAb fragments on the ProPac WCX-10 Column:

Mobile Phase 5: 20 mM MES + 40 mM NaCl + 1 mM EDTA (pH 5.5)

Mobile Phase 6: 20 mM MES + 180 mM NaCl + 1 mM EDTA (pH 5.5)

Preparation of Mobile Phases 5 and 6

Mobile Phase 5: 20 mM MES + 40 mM NaCl + 1 mM EDTA (pH 5.5)

Combine 3.904 g MES, 2.338 g NaCl, and 0.3722 g EDTA in a 1-L volumetric flask containing about 800 mL of degassed deionized water. Because EDTA dissolves very slowly, it is helpful to add 10–20 drops of 1 M NaOH to the mixture prior to stirring. Check the pH often and add more base as necessary. Bring the solution to a volume of approximately 950 mL, adjust to a final pH of 5.5 with 1 M NaOH, and bring the total volume to 1 L.

Mobile Phase 6: 20 mM MES + 180 mM NaCl + 1 mM EDTA (pH 5.5)

Combine 3.904 g MES, 10.519 g NaCl, and 0.3722 g EDTA in a 1-L volumetric flask. Follow the same procedure for adjusting the pH as described for mobile phase 5.

Experimental Conditions

Column: ProPac WCX-10, 4 x 250 mm (P/N 054993)

Mobile Phase 5: 20 mM MES, 40 mM NaCl, 1 mM EDTA (pH 5.5)

Mobile Phase 6: 20 mM MES, 180 mM NaCl, 1 mM EDTA (pH 5.5)

Gradient: 1–35 min, 0–100% B

Flow Rate: 1 mL/min

Injection: 10–15 µL

Detection: Absorbance, 280 nm

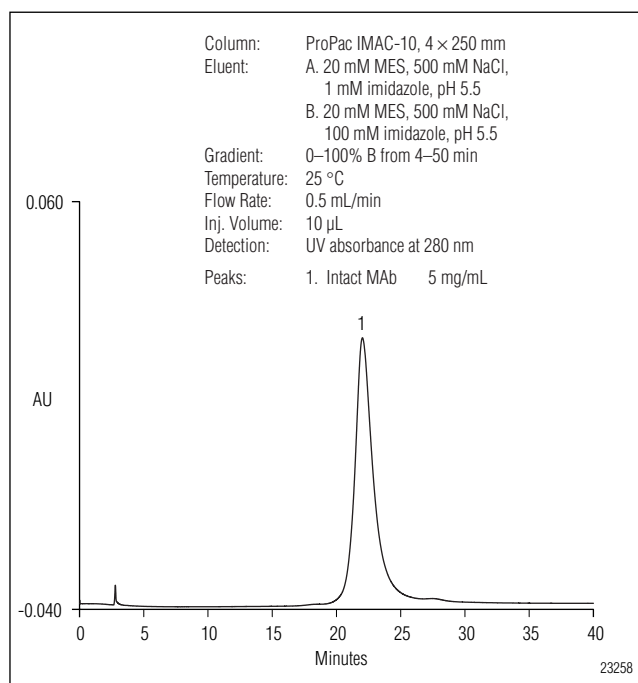


Figure 1. Separation of pure intact MAb on the ProPac IMAC-10 column.

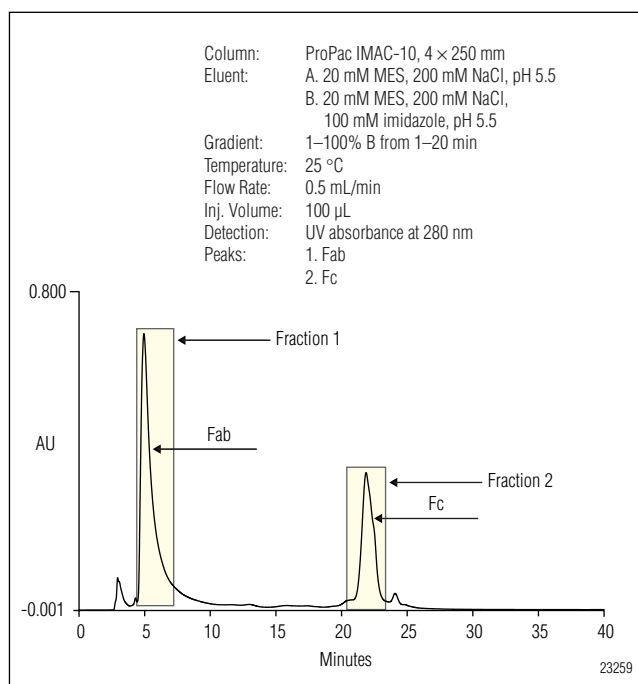


Figure 2. Separation of papain digested MAb fragments on the ProPac IMAC-10 column.

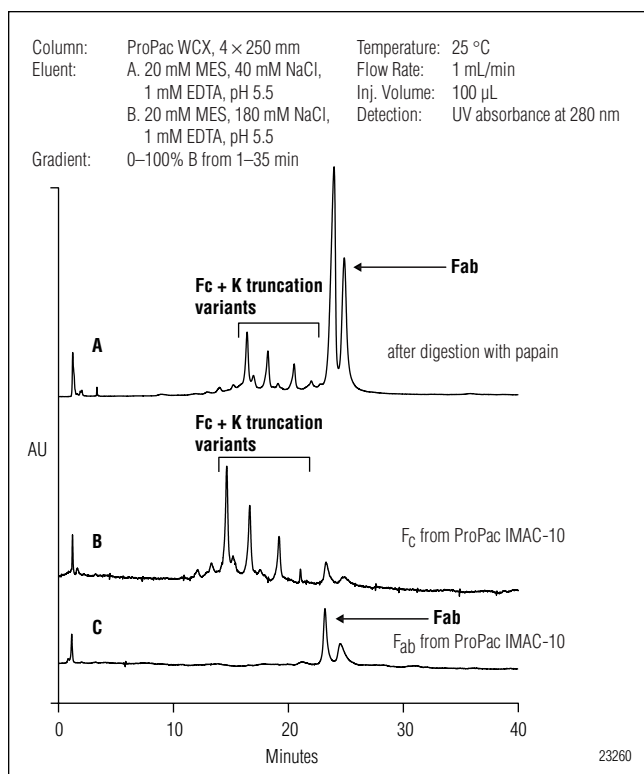


Figure 3. Separation of (A) total papain digest on the WCX-10 column, (B) IMAC-10 retained fraction and (C) IMAC-10 flow-through fraction on the WCX-10 column.

Procedure

| Time | A (%) | B (%) | Comments |
|-------|-------|-------|---------------|
| -5.00 | 100 | 0 | Equilibration |
| 0.00 | 100 | 0 | Injection |
| 1.00 | 100 | 0 | |
| 35.00 | 0 | 100 | End gradient |
| 40.00 | 0 | 100 | |

RESULTS AND DISCUSSION

The performance of IMAC-10 column was assessed for its ability to separate intact and papain digested monoclonal IgG1 fragments. Figure 1 shows a chromatogram of an intact MAb separated on the ProPac IMAC-10 column. The humanized MAb was then digested with papain at 37 °C for 140 min to yield Fc and Fab fragments. Papain is a non-specific thiol protease that cleaves IgG antibodies at the hinge region of the Y-shaped molecule into three fragments of similar size:

two Fab fragments and one Fc fragment.³ Figure 2 shows a separation of the papain digest on the ProPac IMAC-10 column. Only the Fc fragment was retained on the column due to the interaction of the surface exposed histidine residues on the Fc fragment with the immobilized Cu²⁺ on the IMAC resin.

Figure 3A shows a separation of papain digested IgG1 MAb fragments on the ProPac WCX-10 column. The Fc fragments elute in the order of increasing lysine content of the truncation variants followed by the separation of the Fab fragment using a NaCl gradient (40–180 mM over 35 min) at pH 5.5. The peak identification shown is supported by published data.⁴ The presence of the lysine truncation peaks of the Fc fragment was previously verified by performing a carboxypeptidase digest.⁵ To confirm that the non-retained and retained peaks on the IMAC were the Fab and Fc fragments, respectively each peak was collected and reinjected on the WCX column. Figure 3B and 3C show the separation of each ProPac IMAC fraction on the ProPac WCX column. This separation confirms that the Fab elutes in the flow-through fraction and the Fc elutes in the retained fraction

A commercially available HPLC protein A column was evaluated and compared against the ProPac IMAC-10 column. The papain digest fragments were injected on the Protein A column and the Fab and Fc fragments were also collected and reinjected on the WCX columns. Some degradation of the Fab fragment was observed due to the harsh elution buffer conditions (100 mM sodium citrate, pH 3.3) required for the protein A separation. Correlation of the peaks confirmed that the ProPac IMAC-10 separates Fab and Fc fragments.

CONCLUSION

The results shown in this application note demonstrate that the ProPac IMAC-10 separates papain digested IgG1 MAb fragments. The IMAC column uses mild eluent conditions and therefore minimizes the potential for sample degradation. The ProPac IMAC-10 column does not contain any biological materials that can leach off the column and contaminate the target protein.

REFERENCES

1. Josic, D., Lim, Y. P. *Food Tech. Biotechnol.* **2001**, 39(3), 215.
2. Todorova-Balvay, D., Pitiot, O., Bourhim, M., Srikrishnan, T., Vijayalakshmi, M. J. *Chromatogr. B*, **2004**, 808, 57.
3. Weitzhandler, M., Farnan, D., Avdalovic, N. *Am. Biotech. Lab.* September **2000**, 36.
4. Moorhouse, K. G., Nashabeh, W., Deveney, J., Bjork, N. S., Mulkerrin, M. G., Ryskamp, T. J. *Pharm. Biomed. Anal.* **1997**, 16, 593.
5. *Analysis of Monoclonal Antibody Heterogeneity by C-Terminal Lysine Variants*. Application Note 127, Dionex Corporation, Sunnyvale, CA.

Chromleon and ProPac are registered trademarks of Dionex Corporation.

Passion. Power. Productivity.



Dionex Corporation

1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America

U.S. (847) 295-7500
Canada (905) 844-9650

South America

Brazil (55) 11 3731 5140

Europe

Austria (43) 1 616 51 25 Belgium (32) 3 353 4294 Denmark (45) 36 36 90 90
France (33) 1 39 30 01 10 Germany (49) 6126 991 0 Italy (39) 02 51 62 1267
The Netherlands (31) 20 683 9768 Switzerland (41) 62 205 9966
U.K. and Ireland (44) 1276 691722

Asia Pacific

Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 28475235
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190



www.dionex.com

LPN 1840 12/06
©2006 Dionex Corporation