Antibody Solution Kit

Zenix™ SEC-300 &
Antibodix™ WCX NP5

Sepax Technologies, Inc.

5 Innovation Way Newark,
Delaware, USA
Tel: (302) 366-1101
Fax: (302) 366-1151
Toll free: 1-877-SEPAX-US
www.sepax-tech.com

Sepax Technologies

Content

Introduction

Zenix™ SEC phase

Developed based on innovative surface coating technology comprised of a uniform, hydrophilic, and neutral nanometer thick film chemically bonded on high purity and mechanically stabilized silica. The coating chemistry of Zenix™ SEC, with its stand-up monolayer bonded on porous silica, offers an ideal phase chemistry for high performance sized based separations. The 3 µm based Zenix™ provides a powerful solution for robust, reproducible, and highest resolution size based separations of biological molecules on the market.

Antibodix™ WCX phase

Comprised of rigid, spherical, highly cross-linked non-porous poly(styrene divinylbenzene) (PS/DVB) beads. The PS/DVB particle surface is grafted with a highly hydrophilic, neutral polymer layer with a thickness in the range of nanometers. The hydrophobic PS/DVB resin surface is totally covered by a hydrophilic coating which eliminates non-specific bindings with antibody proteins, leading to high efficiency and high recovery separations. On top of the hydrophilic layer, weak cation-exchange functional groups are attached via a proprietary chemistry, resulting in a high capacity ion-exchange layer.

Stationary Phase Structures

Figure 1. Phase structures for both the surface of Zenix™ SEC and Antibodix™ WCX.

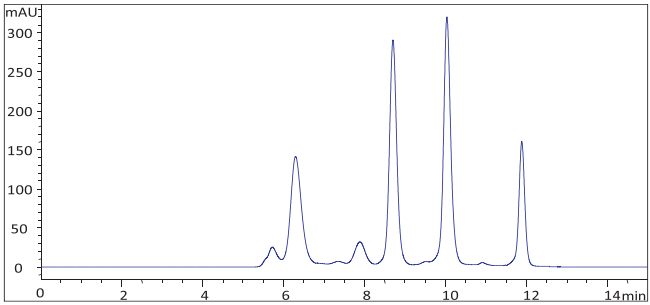
Key features of Zenix and Antibodix phases

Characteristics	Zenix™	Antibodix™
Particle size	3 µm	5 µm
Pore size (Å)	300	Non-porous
Surface structure	Chemically bonded stand-up monolayer	Weak cation exchange functional groups

Technical specifications of Zenix™ SEC-300 and Antibodix™ WCX NP5

Phase	Zenix™ SEC-300	Antibodix™ WCX NP5
Dimensions	4.6 x 300 mm	4.6 x 250 mm
Material	Neutral, hydrophilic film bonded silica	Weak cation exchange groups bonded to a hydrophilic film grafted on PS/DVB.
Particle size	3 µm	5 µm
Pore size (Å)	~ 300	Non-porous
pH stability	2 – 8.5 (pH 8.5-9.5 can be tolerated temporarily.)	2-12
Flow rate	0.35 mL/min	0.80 mL/min
Backpressure	~ 1,100 psi	~2,500 psi
Maximum backpressure	~ 3,500 psi	~6,000 psi
Maximum temperature (°C)	~ 80	~ 80
Mobile phase compatibility	Aqueous and organic	Aqueous or a mixture of water and acetonitrile, acetone, or methanol

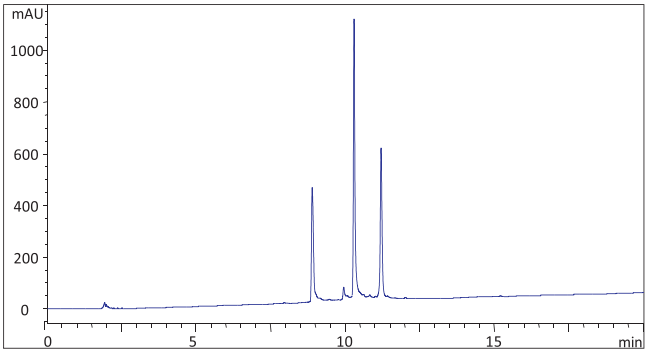
Quality Control Test for Zenix™ SEC-300 4.6 x 300 mm



Compound Name	RT (min)	Area	Plates	Tailing	Resolution
Thyroglobulin Aggr.	5.72	407	2783	0.92	—
Thyroglobulin	6.30	2643	2656	1.08	1.26
BSA Dimer	7.89	477	4558	1.00	3.33
BSA	8.71	3668	11546	1.06	2.08
Ribonuclease A	10.03	3865	17198	1.08	4.17
Uracil	11.89	1628	33656	0.94	6.56

Figure 2. A standard quality control test on a Zenix™ SEC-300 4.6 x 300 mm. Mobile phase was 150 mM sodium phosphate buffer, pH 7.0. Flow rate was 0.35 mL/min. UV detection was set at 214 nm. 3 µL of sample was injected and the sample is a mixture of Thyroglobulin, BSA, Ribonuclease A and Uracil (1 mg/mL each).

Quality Control Test for Antibodix™ WCX NP5 4.6 x 250 mm



Compound Name	RT (min)	Area	Plates	Tailing	Resolution
Aprotinin	8.91	1820	87584	1.30	—
Lysozyme	10.29	3811	234544	1.34	13.43
Ribonuclease A	11.20	2277	192966	1.12	9.72

Figure 3. A standard quality control test on an Antibodix™ WCX NP5 4.6 x 250 mm. Mobile phases were A: 10 mM sodium phosphate buffer, pH 6.0 and B: A + 1.0 M NaCl. Flow rate was 0.8 mL/min and the gradient went from 10-100% B in 25 minutes. UV detection was set at 214 nm. 5 µL of sample was injected and the sample is a mixture of Aprotinin, Lysozyme and Ribonuclease A (1 mg/mL each).

Zenix™ SEC-300 4.6 x 300 mm Monoclonal Antibody and Fragments Separation

Intact MAb analysis on Zenix™ SEC-300 4.6 x 300 mm

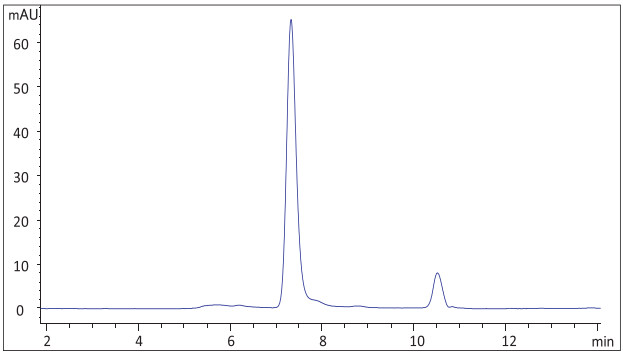


Figure 4. Intact MAb 321 analysis on Zenix™ SEC-300, 4.6 x 300 mm. Mobile phase was 150 mM sodium phosphate buffer, pH 7.0. Flow rate was 0.35 mL/min. UV detection was set at 280 nm. 2 µg of intact MAb 321 was injected.

Reduced MAb on Zenix™ SEC-300 4.6 x 300 mm

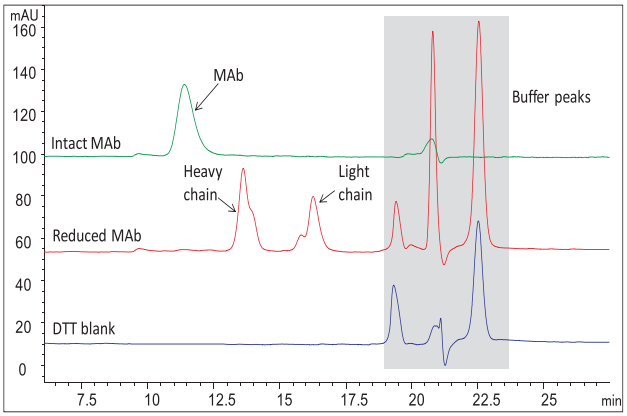


Figure 5. Reduced MAb 321 heavy and light chain separation on Zenix™ SEC-300, 4.6 x 300 mm. Mobile phase was 0.1% TFA, 0.1% formic acid with 20% acetonitrile. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. 5 µg of intact MAb 321 and 20 µg of DTT reduced MAb 321 were injected.

Reduced MAb heavy and light chain separation

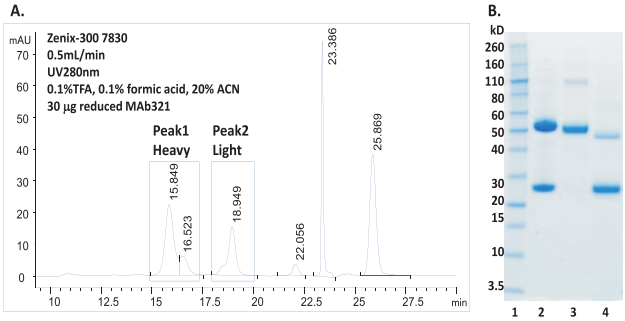


Figure 6. Panel A shows a separation profile of reduced MAb 321 on Zenix™ SEC-300, 7.8 x 300 mm. Peak 1 and Peak 2 were each collected and speed-vac dried. Dried samples were then dissolved in Invitrogen LDS sample buffer. Panel B. shows the 4-12% Bis-Tris gel image of reduced MAb sample and Peak 1, 2 fractions. Lane 1: Protein marker; Lane 2: Reduced MAb sample mixture; Lane 3: Peak 1 heavy chain; Lane 4: Peak 2 light chain.

Effect of TFA concentration on reduced MAb separation on a Zenix™ SEC-300 4.6 x 300 mm

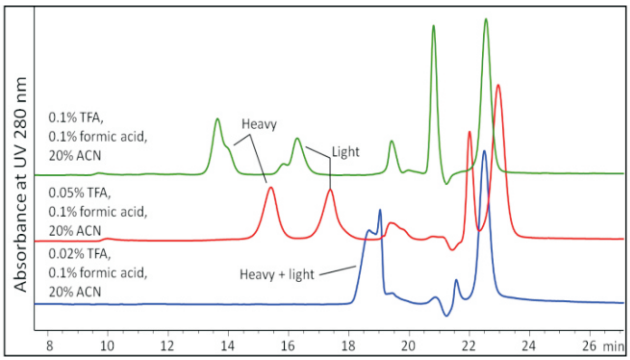


Figure 7. Effect of different TFA concentrations on the separation of heavy and light chains on a Zenix™ SEC-300, 4.6 x 300 mm column. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. 20 µg of reduced MAb 321 was injected. At 0.05% TFA, heavy and light chains are baseline separated. However, at 0.02% TFA, heavy and light chains are not separated.

Effect of TFA and Formic Acid concentration on the separation of reduced MAb

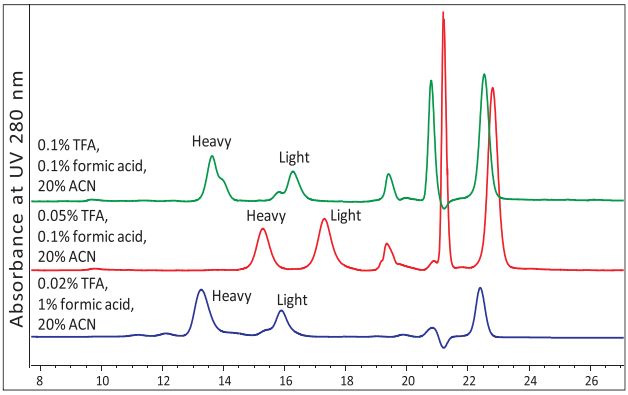


Figure 8. Effect of different TFA and formic acid concentrations on the separation of heavy and light chains on a Zenix™ SEC-300, 4.6 x 300 mm column. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. The chromatogram shows the overlays of 20 µg reduced MAb 321 with the mobile phases indicated.

Fab/Fc separation on Zenix™ SEC-300 4.6 x 300 mm

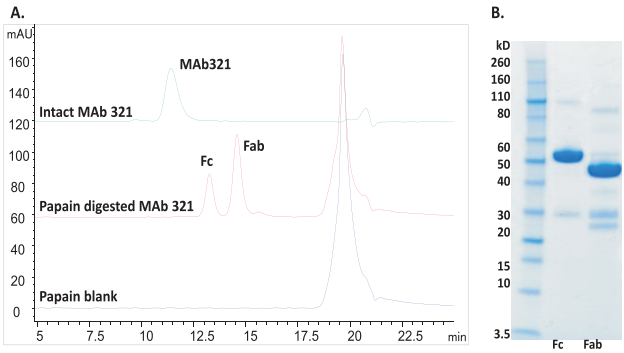


Figure 9. Panel A shows the papain digested MAb 321 (3.5 hour incubation time) -Fab/Fc separation on Zenix™ SEC-300, 4.6 x 300 mm. Mobile phase was 0.1% TFA, 0.1% formic acid with 20% acetonitrile. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. 5 µg of intact MAb 321 and 5 µg of papain digested MAb 321 were injected. Panel B shows the 4-12% Bis-Tris gel image of collected Fc and Fab fractions separated on a Zenix™ SEC-300, 7.8 x 300 mm column at 0.5 mL/min (LC profile not shown).

Effect of TFA and Formic Acid concentration on the separation of Fab/Fc

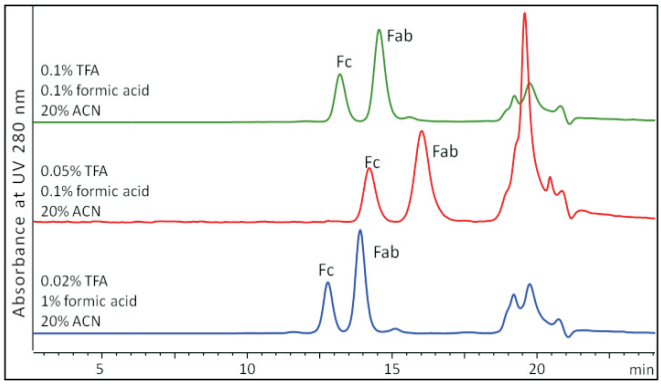


Figure 10. Effect of different TFA and formic acid concentrations on the separation of Fab/ Fc on a Zenix™ SEC-300, 4.6 x 300 mm column. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. The chromatogram shows the overlays of 5 µg of papain digested MAb 321 with the mobile phases indicated.

Fab/Fc separation using different mobile phases

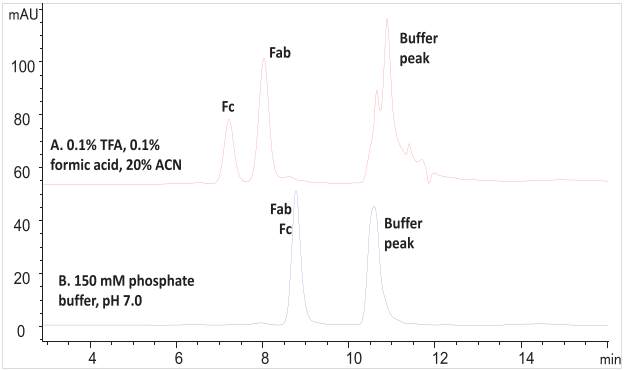


Figure 11. Organic mobile phase vs. salt mobile phase for Fab/Fc separations on Zenix™ SEC-300, 4.6 x 300 mm. Flow rate was 0.35 mL/min, 5 µg of papain digested MAb 321 was injected for both runs. LC profile A was obtained with 0.1% TFA, 0.1% formic acid in 20% acetonitrile, while profile B was generated with 150 mM sodium phosphate buffer, pH 7.0.

Effect of Acetonitrile concentration on Fab/Fc separation on Zenix™ SEC-300 4.6 x 300 mm

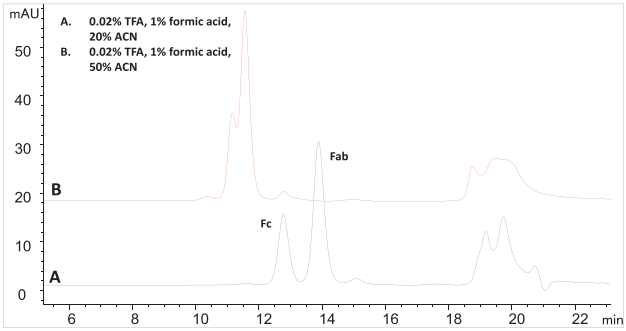


Figure 12. Acetonitrile concentration effect on Fab/Fc separations on Zenix™ SEC-300, 4.6 x 300 mm. Flow rate was 0.2 mL/min, 5 µg of papain digested MAb 321 was injected for both runs. LC profile A was obtained with 20% acetonitrile, while profile B was generated with 50% acetonitrile.

Sample loadings on Zenix™ SEC-300 4.6 x 300 mm

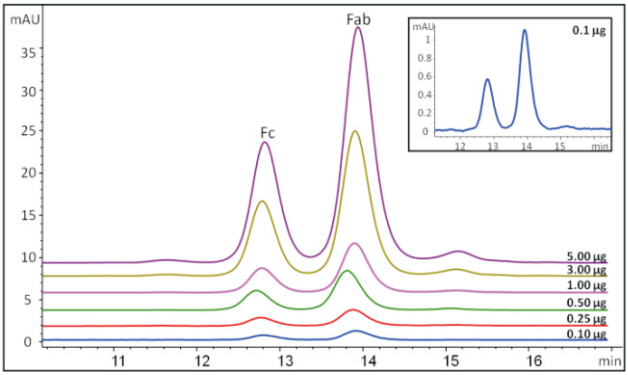


Figure 13. Overlays of different sample loadings of papain digested MAb from 0.1 µg to 5 µg on Zenix™ SEC-300, 4.6 x 300 mm. HPLC mobile phase was 0.02% TFA in 1% formic acid and 20% acetonitrile. Flow rate was 0.2 mL/min with UV 280 nm detection. The inset chromatogram shows the separation profile with 0.1 µg loading. Fab and Fc maintained a baseline separation at 0.1 µg loading.

MAb 321-F(ab')$_2$ separation on Zenix™ SEC-300 4.6 x 300 mm

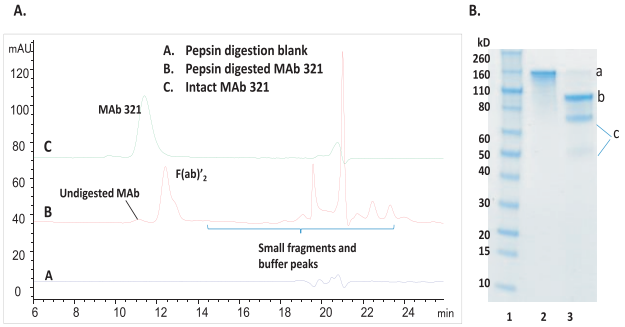


Figure 14. Panel A showed the pepsin digested MAb 321-F(ab')$_2$ separation on Zenix™ SEC-300, 4.6 x 300 mm. Mobile phase was 0.1% TFA, 0.1% formic acid with 20% acetonitrile. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. 5 µg of intact MAb 321 and 15 µg of pepsin digested MAb 321 were injected. Panel B shows 4-12% Bis-Tris gel image. 5 µg of each sample were loaded. Lane 1: Protein markers; Lane 2: Undigested MAb 321; Lane 3: Pepsin digested MAb 321. Band (a) is undigested MAb, band (b) is F(ab')$_2$, and bands (c) are smaller fragments from the digestion.

Effect of TFA and Formic Acid concentration on the separation of F(ab')$_2$

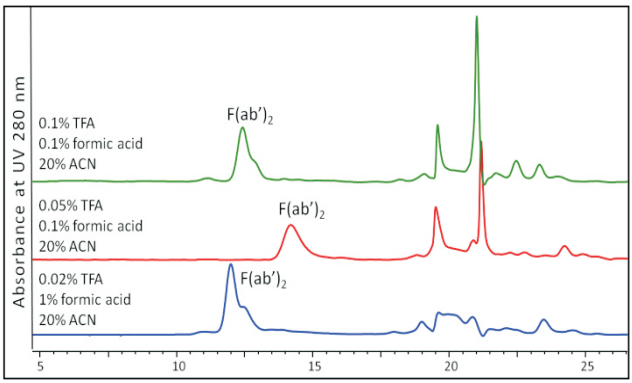


Figure 15. Effect of different TFA and formic acid concentrations in 20% acetonitrile on the separation of F(ab')$_2$ on a Zenix™ SEC-300, 4.6 x 300 mm column. Flow rate was 0.2 mL/min. UV detection was set at 280 nm. The chromatogram shows the overlays of 15 µg pepsin digested MAb 321 with the mobile phases indicated.

Antibodix™ WCX NP5 4.6 x 250 mm
Monoclonal Antibody and Fragments
Separation Applications

MAb separation on Antibodix™ WCX NP5 with a LiCl salt gradient

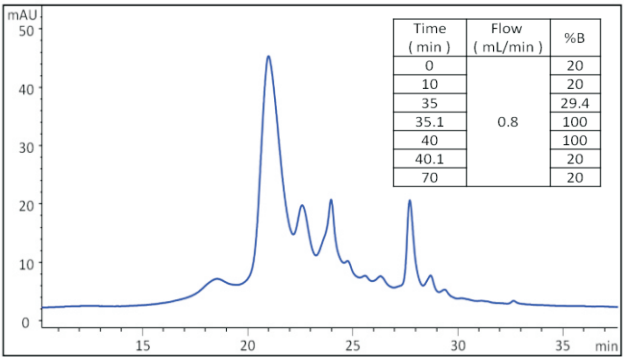


Figure 16. Monoclonal Antibody separation on an Antibodix™ WCX NP5 4.6 x 250 mm column, using a lithium chloride salt gradient. The mobile phases were A: 20 mM sodium acetate pH 5.15 and B: A + 1 M LiCl. Flow rate was 0.8 mL/min with UV 280 nm detection. The column temperature was set at 30 ˚C and 20 µL of a 5 mg/mL MAb sample was injected for analysis.

MAb separation on Antibodix™ WCX NP5 with a NaCl salt and pH gradient

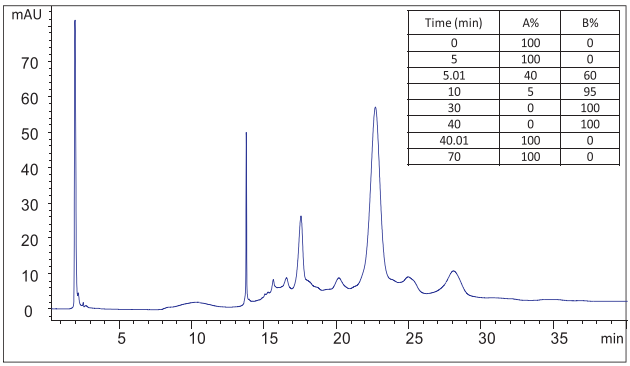


Figure 17. Monoclonal Antibody separation on an Antibodix™ WCX NP5 4.6 x 250 mm column, using a sodium chloride salt and pH gradient. The mobile phases were A: 20 mM Phosphate buffer, pH 5 and B: A + 10 mM NaCl, pH 7.5. Flow rate was 0.8 mL/min with UV 280 nm detection. The column temperature was set at 30 ˚C and 20 µL of a 5 mg/mL MAb sample was injected for analysis.

MAb stability test on Antibodix™ WCX NP5

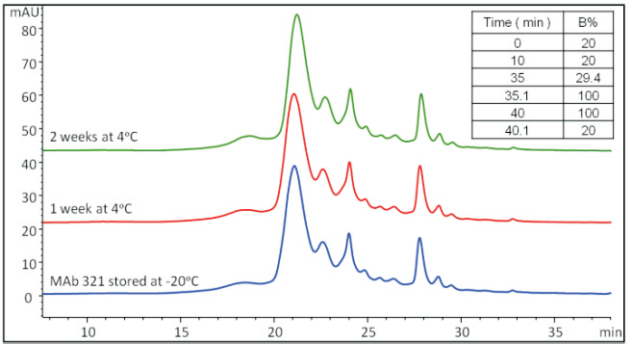


Figure 18. Monoclonal Antibody stability test on an Antibodix™ WCX NP5 4.6 x 250 mm column. The mobile phases were A: 20 mM sodium acetate, pH 5.15 and B: A + 1 M LiCl. Flow rate was 0.8 mL/min with detection at UV 280 nm. 20 µL of each sample was injected and the column temperature was 30 ℃. Samples were 5.0 mg/mL MAb in complex Tris buffer (sample freshly defrosted from -20 °C, one week old and two weeks old at 4 °C).

Papain digested MAb separation on Antibodix™ WCX NP5

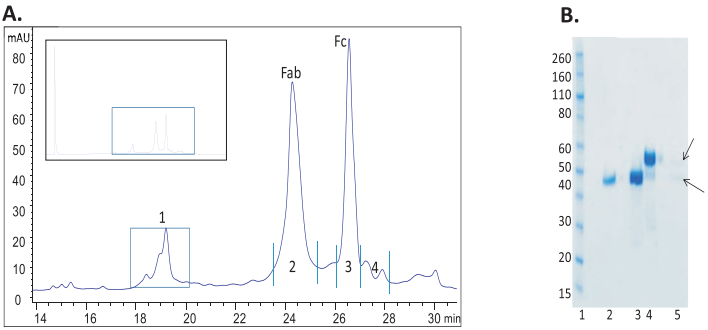


Figure 19. Panel A shows papain digested MAb separation on an Antibodix™ WCX NP5 4.6 x 250 mm column. The mobile phases were A: 20 mM acetic acid + 50 mM NaCl, pH 3.5 and B: 20 mM sodium succinate + 50 mM NaCl, pH 6.0. The gradient was: 5 min 30% B, 0.8 mL/min; 25 min 85%-100% B, 0.65 mL/min. 100 µg of digested MAb was injected for analysis. The papain digestion was 2 mM EDTA, 5 mM Cysteine and 100 mM Tris-HCl at pH 7.6 for 3.5 hour at 37 °C, with MAb at 1 mg/mL and protein : papain = 100 : 1. Detection at UV 280 nm. Panel B shows the 4-12% Bis-Tris gel of Fab and Fc fractions. Lane 1: Markers; Lane 2: Peak 1/Fab; Lane 3: Peak 2/Fab; Lane 4: Peak 3/Fc; Lane 5: Peak 4/Fc.

Fab/Fc different loadings on Antibodix™ WCX NP5

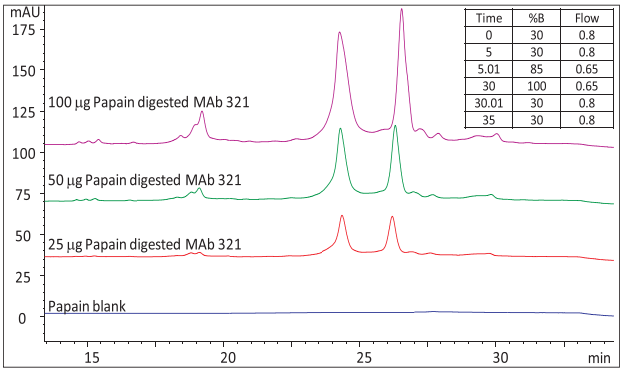


Figure 20. Papain digested MAb separation on an Antibodix™ WCX NP5 4.6 x 250 mm column. The mobile phases were A: 20 mM acetic acid + 50 mM NaCl, pH 3.5 and B: 20 mM sodium succinate + 50 mM NaCl, pH 6.0. The papain digestion was 2 mM EDTA, 5 mM Cysteine and 100 mM Tris-HCl at pH 7.6 for 3.5 hour at 37 °C, with MAb at 1 mg/mL and protein : papain = 100 : 1. Detection at UV 280 nm.

Fab/Fc separation on Antibodix™ WCX NP5

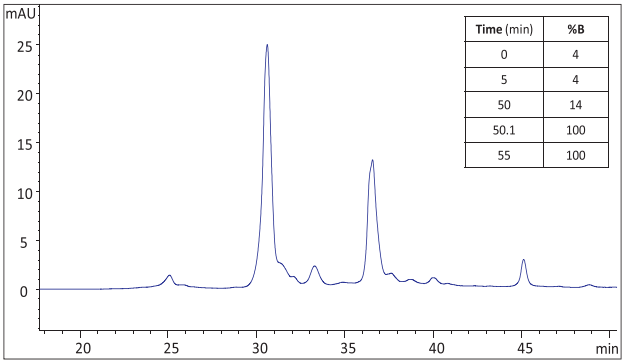


Figure 21. Papain digested MAb separation on an Antibodix™ WCX NP5 4.6 x 250 mm column. The mobile phases were A: 20 mM Phosphate Buffer, pH 5.5 and B: A + 1 M NaCl. 25 µg of sample was injected for analysis. The papain digestion was 2 mM EDTA, 5 mM Cysteine and 100 mM Tris-HCl at pH 7.6 for 3.5 hour at 37 °C, with MAb at 1 mg/mL and protein : papain = 100 : 1. Flow rate was 0.8 mL/min with UV 280 nm detection.

F(ab')$_2$ separation on Antibodix™ WCX NP5

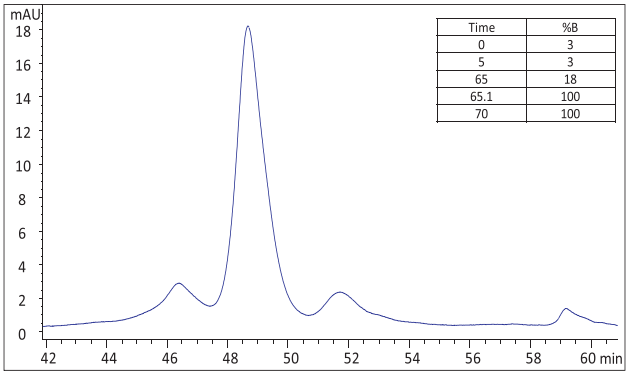


Figure 22. Pepsin digested MAb separation on an Antibodix™ WCX NP5 4.6 x 250 mm column. The mobile phases were A: 20 mM Phosphate Buffer pH 5.5 and B: A+ 1 M NaCl. 50 µg of sample was injected for analysis. The pepsin digestion used 1 mg/mL MAb with a ratio of MAb : pepsin= 40 : 1 in 20 mM sodium acetate, pH 4.0 for 15.5 hours at 37 °C; then quenched with 25 µL of 2 M Tris. Flow rate was 0.8 mL/min with UV 280 nm detection.

Column Installation and Operation for Zenix™ SEC-300 4.6 x 300 mm

1. Filter all samples and mobile phases through 0.45 μm or 0.2 μm filters before use.
2. Attach the column to your HPLC system following the flow direction as marked.
3. New columns are shipped in 150 mM sodium phosphate buffer, pH 7.0. Run 10-20 column volumes of 50 mM sodium phosphate buffer at pH 7.0 to activate the column. Equilibrate the column with desired mobile phase until detection signal reaches baseline.
4. Inject desired amount of sample and run the column with desired flow rate.
5. Store columns in 50 mM sodium phosphate buffer, pH 7.0, w/ 0.02% sodium azide for long term storage.

Note: Solvent compatibility

Zenix™ SEC columns are compatible with aqueous buffers, such as phosphate, acetate, Tris, etc; and water miscible organic solvents, such as MeOH, ethanol, isopropanol, acetonitrile, THF, etc. When switching from an aqueous buffer to an organic solvent, the column should be washed with nanopure water for at least 30-column volume, then ethanol for 20-column volume. When switching from an organic solvent to an aqueous buffer, the column should be washed with ethanol for at least 30-column volume, then nanopure water for 20-column volume, and finally 20-column volume aqueous buffer. After washing, it is recommended that the column be stored in the aqueous buffer for 48 hours to get well equilibrated for satisfactory performance.

Column Installation and Operation for Antibodix™ WCX NP5 4.6 x 250 mm

1. Filter all samples and mobile phases through 0.45 μm or 0.2 μm filters before use.
2. Attach the column to your HPLC system following the flow direction as marked.
3. New columns are shipped in 20 mM sodium phosphate buffer, pH 6.0. Run 10-20 column volumes of 20 mM sodium phosphate buffer at pH 6.0 to activate the column. Equilibrate the column with desired mobile phase until detection signal reaches baseline.
4. Inject desired amount of sample and run the column with desired flow rate.
5. Store columns in 20 mM sodium phosphate buffer, pH 6.0 for long term storage.

Note: Solvent compatibility

Antibodix™ columns are compatible with aqueous mobile phases or a mixture of organic and water, such as methanol or acetonitrile and water. Typical eluants contain sodium, potassium salts of phosphate, chloride, acetate, or Tris. Always use an inline degasser or degas the mobile phase prior to use. A simple way for degassing is to sonicate it for 5 minutes under water pumped vacuum. Antibodix columns are compatible with nonionic and zwitterionic detergents. Antibodix columns are incompatible with cationic detergents.

Ion exchange chromatography operates under the consideration that for every protein there is a unique relationship between net surface charge and pH. A protein with no net charge at a pH equal to its pI will exhibit no interactions with a charged stationary phase. At a pH below its pI, a protein will behind to a negatively charged stationary phase; a cation exchanger. When using a cation exchanger it is a good idea to begin with a starting buffer which is 0.5-1 pH unit less than the sample's pI and an elution buffer which is either at a higher pH or at a higher salt concentration.

Troubleshooting for Zenix™ SEC-300

It is the user's responsibility to determine the optimum sample loading and running conditions to best utilize Zenix™ SEC-300 columns. The following information is provided for reference to troubleshoot your experiments.

High back pressure

A sudden increase in backpressure suggests that the column inlet frit might be blocked. In this case it is recommended that the column be flushed in reverse flow with an appropriate solvent. To prevent the clogging, remove the particulates from samples and mobile phases with filtration.

Poor resolution

1. Column may be overloaded. Reduce sample injection.
2. Make sure the sample's molecular weight range falls in the separation range of the columns. Both columns have a pore size of 300Å with a separation range between 5,000 Da and 1,250,000 Da.
3. Two of the same columns in tandem may improve the resolution of close molecular weight separations.

Peak tailing

This may indicate secondary hydrophobic interaction between the sample and column matrix. To minimize the interaction, increase ionic strength of the mobile phase or add organic solvents (low percentage that do not change the protein conformation).

Samples with surfactants

Surfactants may irreversibly bind to the column matrix, which changes the matrix surface. This can result in column performance changes, such as retention time shift and altered peak shape for proteins with non-detergent mobile phases. Columns should be dedicated to the same surfactant application.

Column cleaning and regeneration

Zenix™ SEC-300 columns may be contaminated by strongly adsorbed samples, which results in decreasing column performance. It is usually indicated by an increase in backpressure and a broader peak. When this happens, the general procedure for column cleaning is as follows:

1. Disconnect the column from the detector.
2. Clean your column in the reverse flow direction.
3. Run the column at less than 50% of the maximum recommended flow rate. Monitor the backpressure.
4. 10-15 column volumes of cleaning solution are sufficient. Run 3-5 column volumes of nanopure water between each solution.

The following cleaning solutions are recommended:

1. Concentrated neutral salt (e.g., 0.5 M Na_2SO_4) at low pH (e.g., pH 3.0) to remove basic proteins.
2. Water soluble organic (MeOH, ACN, EtOH, 10%-20%) in aqueous buffer (e.g., 50 mM phosphate, pH 7.0) to remove hydrophobic proteins.
3. If both solutions fail to clean the column, use 6 M Urea (filter before use).
    a. 2cv 6 M urea at 0.5 mL/min
    b. 20cv nanopure water at 0.5 mL/min
    c. 7-10cv mobile phase at 1 mL/min

For information regarding mobile phase optimization, please visit the FAQ section of our website.

Column Protection

In addition to filtering the sample and the mobile phase, the best way to protect the separation column is to install a guard column or a pre-column filter in front of it. In most cases a pre-column filter helps to remove the residual particulates that are in the sample, the mobile phase, or leached from the HPLC system, such as pump and injector seals. However, a guard column is highly recommended because it is more effective in trapping highly adsorptive sample components and residual particulates in the sample, the mobile phase or from the HPLC system.

Troubleshooting for Antibodix™ WCX NP5

It is the user's responsibility to determine the optimum sample loading and running conditions to best utilize Antibodix™ WCX NP5 columns. The following information is provided for reference to troubleshoot your experiments.

High back pressure

A sudden increase in backpressure suggests that the column inlet frit might be blocked. In this case it is recommended that the column be flushed in reverse flow with an appropriate solvent. To prevent the clogging, remove the particulates from samples and mobile phases with filtration.

Poor resolution

1. Column may be overloaded. Reduce sample injection.
2. Try using different mobile phases in order to optimize you running conditions. Vary buffers, concentrations and pHs.

Peak tailing

This indicates that a different starting mobile phase should be used. To promote sample binding to the column try starting conditions at different pHs and at different salt concentrations.

Column cleaning and regeneration

Antibodix™ WCX columns may be contaminated by strongly adsorbed samples, which results in decreasing column performance. It is usually indicated by an increase in backpressure and a broader peak. When this happens, the general procedure for column cleaning is as follows:

1. Disconnect the column from the detector.
2. Clean your column in the reverse flow direction.
3. Run the column at less than 50% of the maximum recommended flow rate. Monitor the backpressure.
4. 10-15 column volumes of cleaning solution are sufficient. Run 3-5 column volumes of nanopure water between each solution.

In general, the recommended cleaning solution is 50 mM phosphate buffer with 1.0 M NaCl at pH 10.

Note: Separations on exchange columns are sensitive to the pH changes in the mobile phases. In order to have good reproducibility of the separations, make sure the pHs of the same buffer in different lots are the same. pH meters need to be calibrated correctly each time for buffer making.

Column Protection

In addition to filtering the sample and the mobile phase, the best way to protect the separation column is to install a guard column or a pre-column filter in front of it. In most cases a pre-column filter helps to remove the residual particulates that are in the sample, the mobile phase, or leached from the HPLC system, such as pump and injector seals. However, a guard column is highly recommended because it is more effective in trapping highly adsorptive sample components and residual particulates in the sample, the mobile phase or from the HPLC system.

Ordering Information

Antibody Solution Kit (P/N MABKIT-0000) Includes:

Zenix™ SEC-300

P/N	ID x Length (mm)	Pore Size (Å)
213300-4630	4.6 x 300	300

Antibodix™ WCX NP5

P/N	ID x Length (mm)	Column Material
602NP5-4625	4.6 x 250	Stainless Steel / PEEK

Other Available Dimensions:

Zenix™ SEC-300

P/N	ID x Length (mm)	Pore Size (Å)
213300-2130	2.1 x 300	300
213300-4630	4.6 x 300	300
213300-7830	7.8 x 300	300
213300-10030	10.0 x 300	300
213300-21230	21.2 x 300	300

Antibodix™ WCX NP5

P/N	ID x Length (mm)	Column Material
602NP5-4605	4.6 x 50 (Guard)	Stainless Steel / PEEK
602NP5-4625	4.6 x 250	Stainless Steel / PEEK